Investigation of Trace Amine Receptors in the Cardiovascular System

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Presented by

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SUMMARY

Trace amines (TAs), including β-phenylethylamine (β-PEA), tyramine and octopamine are structurally and functionally related to biogenic amines such as catecholamines and serotonin and to amphetamines. They are present in trace levels in the nervous system and in chocolate, cheese and wine. TAs are usually regarded as indirectly-acting sympathomimetic amines (ISAs) exerting vasoconstriction *via* α_1-adrenoceptors. However, they also stimulate trace amine-associated receptors (TAARs), of which only TAAR1 and TAAR4 are sensitive to TAs. The aim of the thesis was to examine whether vasoconstriction by TAs in blood vessels is *via* ISA or TA mechanisms.

TAs caused concentration-related and endothelium-independent contractions in rat isolated aortic rings in the presence of prazosin (α_1-adrenoceptor antagonist), cocaine (catecholamine uptake inhibitor), ICI-118,551 (β_2-adrenoceptor antagonist) and pargyline (MAO A and B inhibitor). The persistent and inhibitor-independent contractions suggest that mechanisms other than ISA and α- and β- adrenoceptor stimulation are involved, possibly TAARs. Differences in the profile of vasoconstrictor activities to a range of TAs were identified in rat and guinea-pig aorta, suggesting species variations in receptor distribution. Tyramine was identified as a partial agonist in isolated rat aorta and an antagonist of other TAs in this tissue. Finally, the presence of TAAR1 mRNA and protein was demonstrated for the first time in rat aorta by RT-PCR and Western blotting, respectively.

Most information about TAs relates to studies which have been done on the brain, or cloned receptors expressed in transfected cells. This study of different TAs and structurally related derivatives in aortic tissues has expanded the knowledge of the vasoconstrictor effects of TAs in isolated tissues. The molecular biological confirmation of the presence of TAAR1 and the pharmacological findings regarding the effects of TAs in rat aortic rings might explain their hypertensive effects and their role in coronary heart disease and migraine headache.

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INVESTIGATION OF TRACE AMINE RECEPTORS IN THE CARDIOVASCULAR SYSTEM

Chapter I: General Introduction

ABBREVIATIONS

A:	Adrenaline
AA:	Arachidonic acid
L-AADC:	Aromatic L-amino acid decarboxylase
AB:	Antibody
Ach:	Acetylcholine
AC:	Adenylate cyclase
ADHD:	Attention-deficit hyperactivity disorder
ANOVA:	One-way analysis of variance
AMP:	Adenosine monophosphate
APS:	Ammonium persulphate
L-arg:	L-arginine
ATP:	Adenosine triphosphate
BCA:	Bicinchoninic acid assay
BSA:	Bovine serum albumin
cAMP:	Cyclic adenosine 3′, 5′-monophosphate
CNS:	Central nervous system
CRC:	Concentration-response curve
DAG:	Diacylglycerol
kDa:	Kilodalton
DBH:	Dopamine-β-hydroxylase
DEPC:	Diethyl pyrocarbonate
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
cDNA:	Complementary deoxyribonucleic acid
IP_3:	Inositol 1,4,5-trisphosphate
EC_{50}:	Effective concentration 50%

ECL:	Enhanced chemiluminescence
EDHF:	Endothelium-derived hyperpolarizing factor
EDRF:	Endothelium-derived relaxing factor
EDTA:	Ethylene-diamine-tetraacetic acid
EtBr:	Ethidium bromide
Fab:	Fragment antigen binding
Fc:	Fragment crystallisable
GDP:	Guanosine diphosphate
GIT:	Gastrointestinal tract
GP:	Guinea-pig
GPCR:	G protein-coupled receptor
cGMP:	Cyclic guanosine monophosphate
GTP:	Guanosine triphosphate
HEK:	Human embryonic kidney
HRP:	Horse-radish peroxidase
5-HT:	Serotonin or 5-hydroxytryptamine
IgG:	Immunoglobulin G
IP_3:	Inositol 1,4,5-trisphosphate
ISA:	Indirectly-acting sympathomimetic amine
ISO:	Isoprenaline
KCl:	Potassium chloride
MAO:	Monoamine oxidase
MDMA:	Methylenedioxymethamphetamine or `Ecstasy`
MLCK:	Myosin light chain kinase
MMLV:	Moloney murine leukemia virus
NA:	Noradrenaline
NO:	Nitric oxide

eNOS:	Endothelial constitutive nitric oxide synthase
dNTPs:	Deoxyribonucleotide triphosphate
OD:	Optical density
P_{450}:	Cytochrome P450
PCR:	Polymerase chain reaction
PDE:	Phosphodiesterase
PEA:	Phenylethylamine
PGI:	Prostacyclin
PIP_2:	Phosphatidylinosital bisphosphate
PKA:	Protein kinase A
PLC:	Phospholipase C
PNS:	Peripheral nervous system
Rf:	Retardation factor
RNA:	Ribonucleic acid
mRNA:	Messenger ribonucleic acid
RT-PCR:	Reverse transcriptase polymerase chain reaction
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M:	Standard error of the mean
SR:	Sarcoplasmic reticulum
TA:	Trace amine
TAE:	Tris acetate EDTA
TAR:	Trace amine receptor
TAAR:	Trace amine-associated receptor
TAQ:	Thermus aquaticus
TBS:	Tris buffered saline
TBST:	Tris buffered saline Tween 20

TE:	Tris EDTA
TEMED:	Tetramethylethylenediamine
VSM:	Vascular smooth muscle
VTA:	Vental legmental area
WR:	Working reagent

Chapter 1

GENERAL INTRODUCTION

Chapter I

General Introduction

1.1 Catecholamines

The catecholamines, noradrenaline, dopamine and adrenaline are chemical compounds derived from the amino acid tyrosine. Most of the catecholamines are classical biogenic and endogenous amines and are 50% bound to plasma proteins.

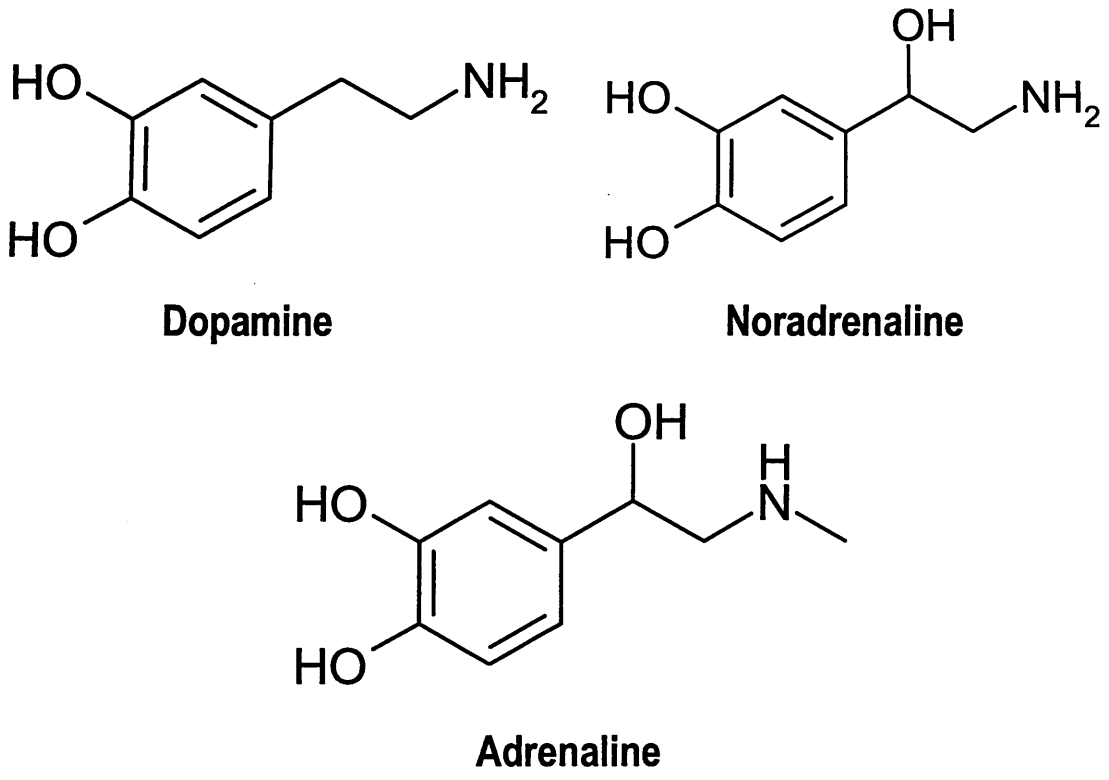


Figure 1.1: The catecholamines dopamine, noradrenaline and adrenaline are derived from the amino acid tyrosine.

1.1.1 Synthesis, release and function of catecholamines

The catecholamines, dopamine, noradrenaline and adrenaline, are synthesized from tyrosine in selected central and peripheral neurons and in the adrenal medulla by the sequential action of enzymes in a synthetic pathway (Rios *et al.*, 1999). The synthetic pathway was first postulated in 1939 by Blaschko (1939) and further developed by Nagatsu (1964; 1991). Tyrosine hydroxylase catalyzes the conversion of tyrosine to

L-dihydroxyphenylalanine (L-Dopa), a substrate for Dopa decarboxylase which converts L-Dopa to dopamine (Rios *et al.*, 1999) *(Figure 1.2)*. In noradrenergic neurons in the sympathetic nervous system, in the brainstem, and in adrenal chromaffin cells, dopamine is converted to noradrenaline by dopamine β-hydroxylase (Joh and Hwang, 1987). Noradrenaline is converted to adrenaline by phenylethanolamine-N-methyl transferase present in adrenergic cells in the adrenal medulla and in a few neuronal groups in the lower brainstem (Rios *et al.*, 1999) *(Figure 1.2)*.

Catecholamines are hormones that are released by the adrenal glands in situations of physiological stress or low blood sugar levels. They are produced mainly by the chromaffin cells of the adrenal medulla and the postganglionic fibers of the sympathetic nervous system (Goodman *et al.*, 1996). Dopamine is largely produced in neuronal cell bodies in two areas of the brainstem, the substantia nigra and the ventral tegmental area. Noradrenaline and dopamine act as neurotransmitters in the central nervous system and as hormones in the blood circulation. Noradrenaline is also a neurotransmitter of the peripheral sympathetic nervous system. Catecholamines cause general physiological changes that prepare the body for physical activity (fight-or-flight response). Some typical effects are increases in heart rate, blood pressure, blood glucose levels. The general activation of the sympathetic nervous system results in noradrenaline and adrenaline release which stimulate α and β-adrenoceptors in the membrane of postsynaptic effects cells to cause these physiological responses. An important mechanism for the termination of action of noradrenaline in the junctional space is active re-uptake into the nerve (Uptake1) and storage vesicles. Noradrenaline can also activate pre-synaptic α_2-adrenoceptors and enters postsynaptic effector cells *via* Uptake2 to modulate the release of neurotransmitter *(Figure 1.3)* (Goodman *et al.*, 1996). Adrenaline is a hormone when carried in the blood and a neurotransmitter when it is released across a neuronal synapse. When adrenaline is secreted into the bloodstream, it rapidly prepares the body for action in emergency situations. The hormone boosts the supply of oxygen and glucose to the brain and muscles, while suppressing other non-emergency bodily processes. Adrenaline increases heart rate and stroke volume, dilates the pupils and constricts aterioles in the skin. Furthermore, adrenaline dilates aterioles in skeletal muscles.

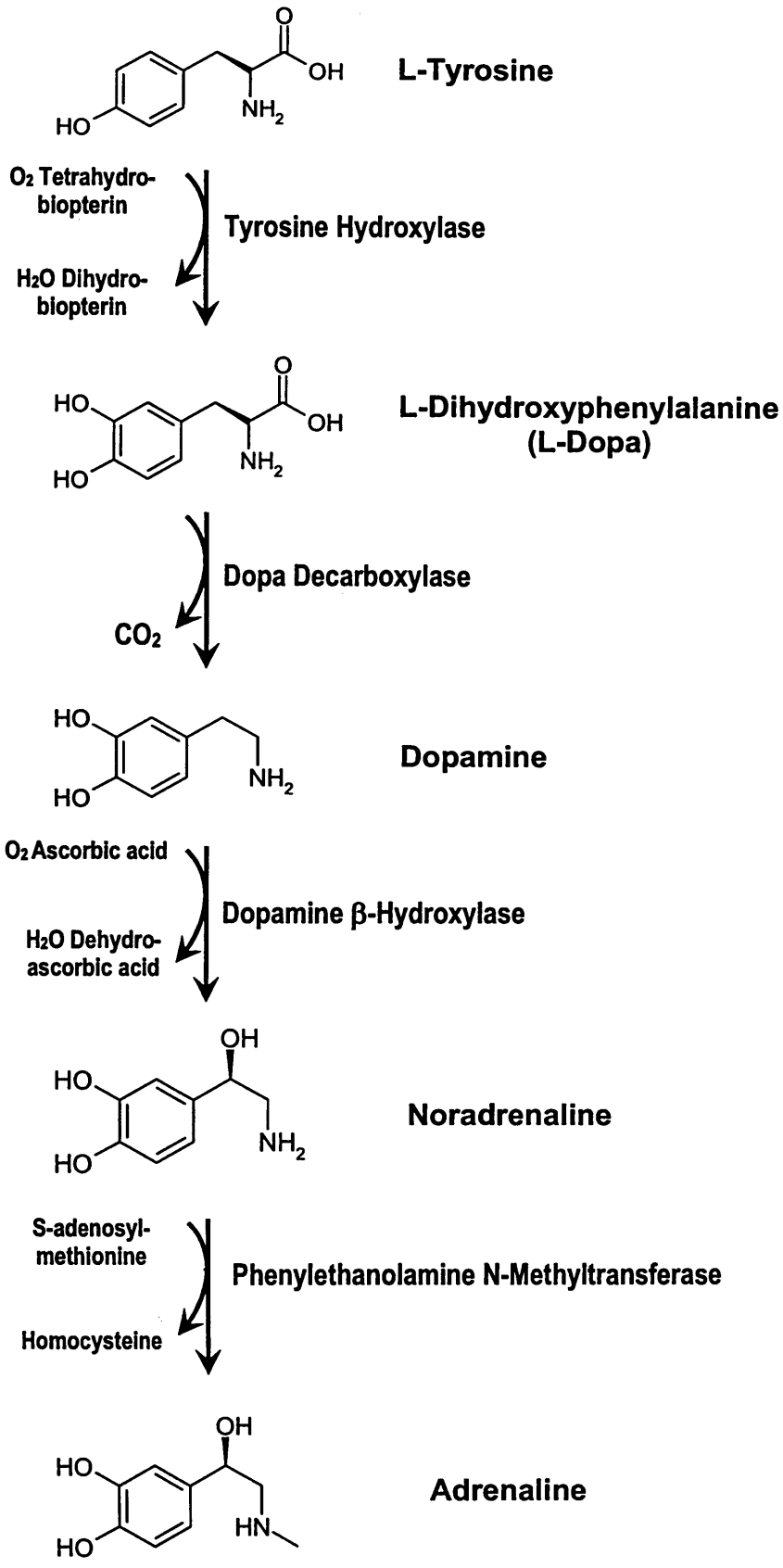


Figure 1.2:
The synthetic pathway of the catecholamines, including dopamine, noradrenaline and adrenaline from tyrosine with the sequentional action of different enzymes.

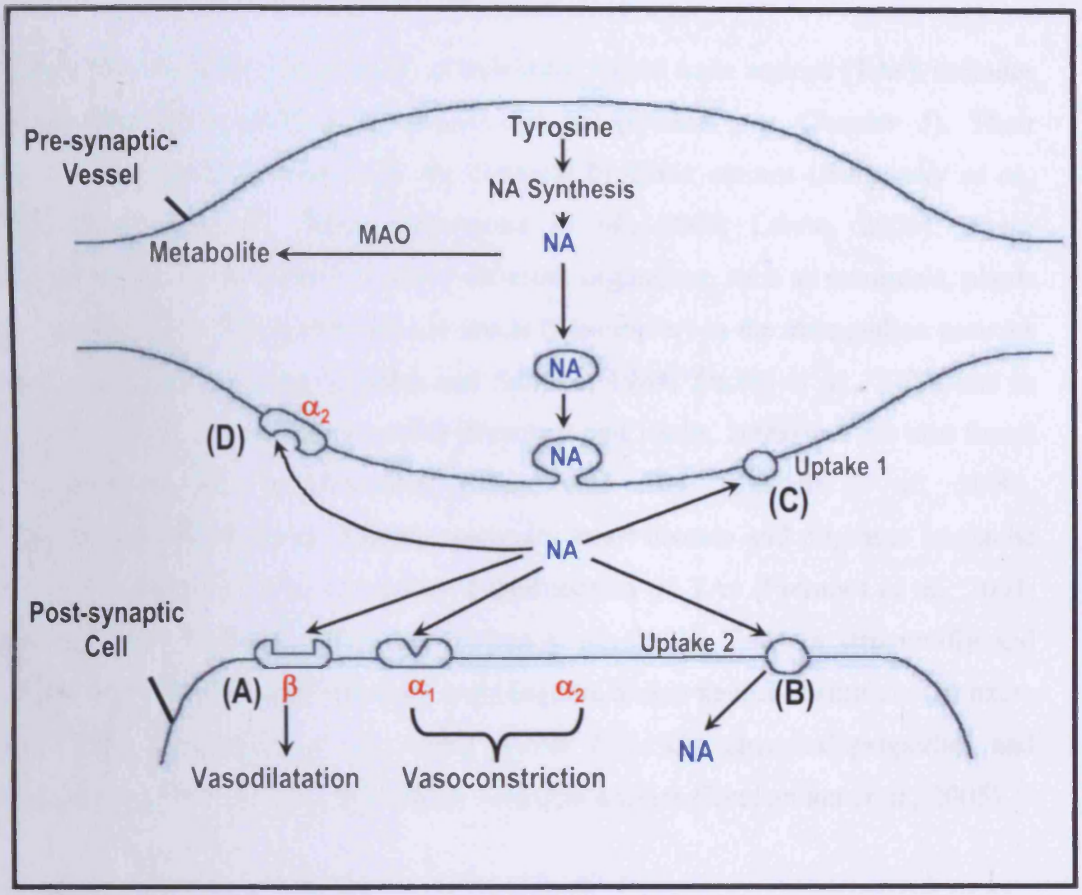


Figure 1.3: The synthesis, action and fate of noradrenaline at sympathetic neuroeffector junctions. The transmitter noradrenaline activates α- and β-adrenoceptors **(A)** in the membrane of post-synaptic effector cells. Noradrenaline also enters into post-synaptic effector cells (Uptake2) **(B)**. A important mechanism for the termination of action of noradrenaline in the junctional space is active re-uptake into the nerve (Uptake1) **(C)** (Iversen, 1975) and storage vesicles. Noradrenaline can also activate pre-synaptic α_2-adrenoceptors **(D)**. *Adapted from Goodman et al., (1990).*

1.2 Trace Amines (TAs)

Another class of endogenous amine compounds, called trace amines (TAs), includes β-phenylethylamine (β-PEA), tyramine and octopamine (*see Chapter 5*). Their actions significantly overlap with the classical biogenic amines (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001; Lindemann *et al.*, 2005; Lewin, 2006). These endogenous amines are found in many different organisms, such as mammals, plants and bacteria. They are present in trace levels (nanomolar) in the mammalian nervous system (neuronal synapses) (Usidin and Sandler, 1984; Zucchi *et al.*, 2006) and in peripheral tissues (0.1-100ng/g tissue) (Burchett and Hicks, 2006) and are also found in substances such as chocolate, cheese and wine (Skerritt *et al.*, 2000). Hypertension, hyperactivity disorder, coronary heart disease and migraine headache have been suggested to be caused by a dysfunction of TAs (Premont *et al.*, 2001; Branchek and Blackburn, 2003; Lindemann *et al.*, 2005). TAs are structurally and functionally related to amphetamines and biogenic amine neurotransmitters (Bunzow *et al.*, 2001; Lindemann *et al.*, 2005) *(Table 1.1)*. The chemical properties and biosynthesis of the TAs overlap with the classical amines (Lindemann *et al.*, 2005).

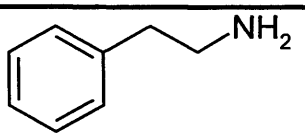
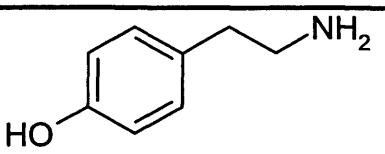
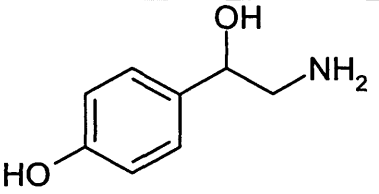
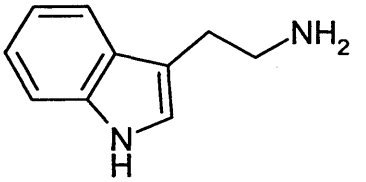
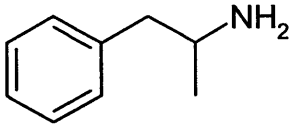
Drug Name	Systematic Name	Structure
β-Phenylethylamine	2-Phenylethylamine	
Tyramine	4-Hydroxy-phenylethylamine	
Octopamine	(4-(2-Amino-1-hydroxy-ethyl) phenol)	
Tryptamine	3- (2-Aminoethyl) indole	
Amphetamine	α-Methyl-phenylethylamine	

Table 1.1: Various trace amines (TAs) and their systematic names and chemical structures. TAs are structurally and functionally related to amphetamines and biogenic amines neurotransmitters, including adrenaline and noradrenaline.

1.2.1 Synthesis, metabolism and endogenous levels of trace amines

TAs are all primary amines (Lindemann and Hoener, 2005) generated directly by decarboxylation of their respective precursor amino acids by aromatic L-amino acid decarboxylase (L-AADC). β-PEA, para and- meta tyramine are synthesised from L-phenylalanine and L-tyrosine, respectively (Berry, 2004). Octopamine is the exception as it is metabolised from dopamine-β-hydroxylase (DBH) to *para-* and *meta*-octopamine (Durden *et al.*, 1973; Boulton and Dyck, 1974; Boulton *et al.*, 1974; Dyck and Boulton, 1975; Boulton, 1976; Bowsher and Henry, 1983; Berry, 2004).

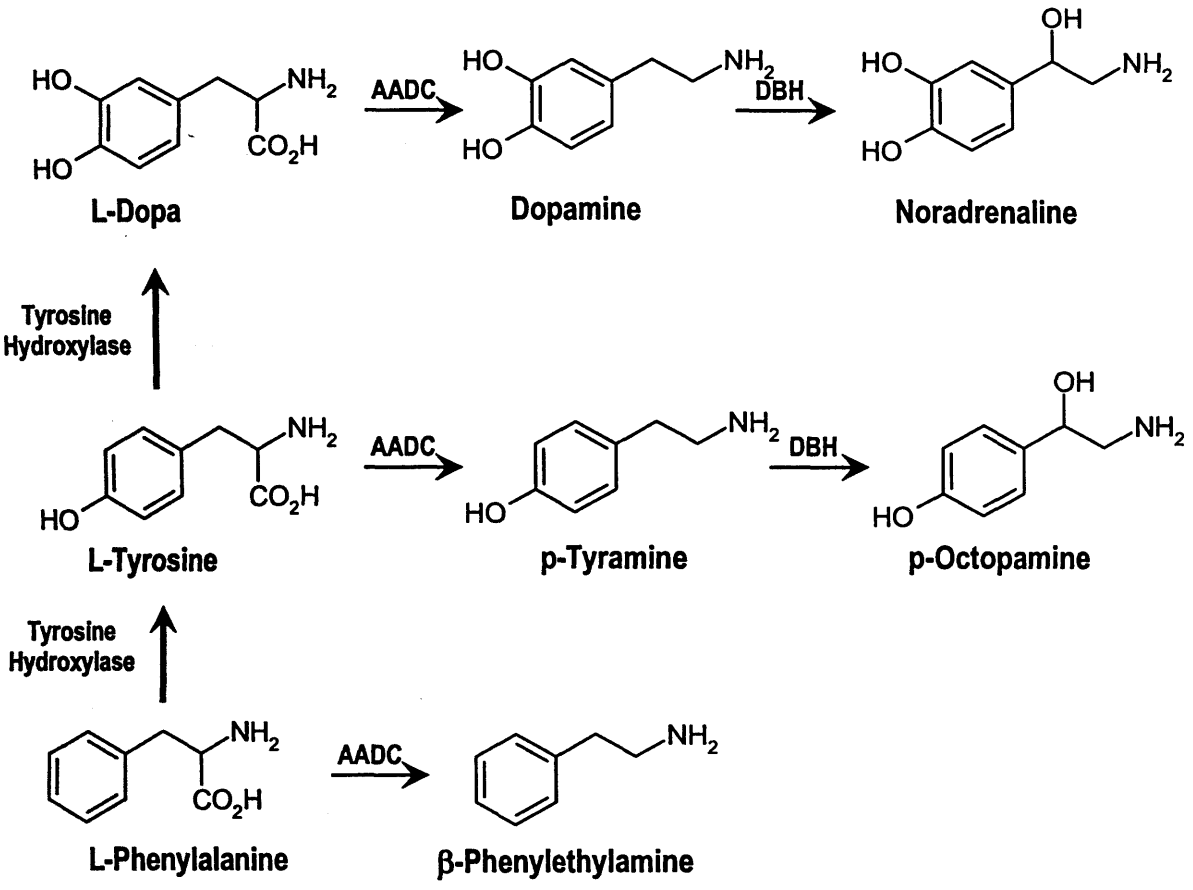


Figure 1.4: Synthetic routes of trace amines and monoamine neurotransmitters L-AADC = aromatic L-amino acid decarboxylase, DBH = Dopamine β-hydroxylase (Berry, 2004)

TAs are found throughout the central nervous system. As a result of the rapid turnover rate of TAs (Wu and Boulton, 1973; Wu and Boulton, 1974; Wu and Boulton, 1975), the endogenous extracellular levels of TAs in brain tissues are in the low nanomolar range (Boulton, 1976) *(Table 1.2)*. Therefore, the level of TAs in the brain is several hundred-fold below those of the classical biogenic amine neurotransmitters, such as dopamine, noradrenaline and 5-hydroxytryptamine (5-HT) (Durden *et al.*, 1973; Durden and Philips, 1980; Henry *et al.*, 1988; Durden and Davis, 1993). In addition, their rate of synthesis is equivalent to that of dopamine and noradrenaline (Durden and Philips, 1980; Paterson *et al.*, 1990). TAs show a heterogeneous distribution within the mammalian CNS, both regionally and at the sub-cellular level (Boulton and Baker, 1974; Boulton and Baker, 1975; Boulton, 1976; Boulton *et al.*, 1977; Juorio and Sloley, 1988; Juorio, 1988b). Further, variations are present in the distribution of individual TAs (Berry, 2004).

Tissue	β-PEA	Tyramine	Dopamine	Noradrenaline
Rat (whole brain) (ng/g)	1.8	1.1	600	490

Table 1.2: CNS levels in rats of β-phenylethylamine (β-PEA), tyramine, dopamine and noradrenaline. Values taken from *Boulton and Juorio (1982)* and *Juorio (1988)* are in ng/g fresh tissue.

1.2.2 Release of trace amines by neural stimulation

TAs are potential neuromodulators rather than neurotransmitters essentially because most of them, such as β-PEA and tryptamine are not released in an activity-dependent manner (Berry, 2004). However, the *para*- and *meta*-isomers of tyramine, which are highly concentrated in the rat striatum, show some activity-dependent release (Berry, 2004). Both isomers are released from the striatum by a veratridine-induced depolarization (Dyck, 1989) and their levels are moderately decreased by pre-treatment with reserpine (Boulton *et al.*, 1977). Therefore, both isomers appear to be stored in a reserpine-sensitive mechanism (Boulton *et al.*, 1977). Reserpine is a drug that interferes with biogenic amine packaging in synaptic vesicles and depletes dopamine, noradrenaline and serotonin from the nerve terminals. It caused depressive states in some patients who took in the past the substance as an anti-hypertensive agent (Carlsson *et al.*, 1963). Reserpine administration reduces the ability of intra-neuronal granules to store catecholamines (Bertler, 1961). Whilst treatment with reserpine reduced rat striatal tyramine and dopamine levels, reserpine had no effect on the concentration of β-PEA in the rat striatum (Boulton *et al.*, 1977; Juorio and Sloley, 1988). β-PEA appears to be released by diffusion from a "reserpine-insensetive pool" and is not stored in specific granules like the tyramine isomers (Juorio, 1988a; Berry, 2004).

β-Phenylethylamine

β-phenylethylamine (β-PEA) is the simplest phenylalkylamine endogenously present in the body (Boulton *et al.*, 1990). The existence of β-PEA in the CNS is well established (Ishida *et al.*, 2005). The monoamine has been found in low concentrations in the nervous tissue of all vertebrate and invertebrate species investigated so far (Durden *et al.*, 1973; Juorio, 1976; Philips *et al.*, 1978; Juorio,

1988a). The highest brain levels of β-PEA have been found in the mesolimbic area, caudate-putamen structures and hypothalamus (Durden *et al.*, 1973; Philips *et al.*, 1978). β-PEA is present in tyrosine hydroxylase-containing neurons and co-exists with dopamine in the nigrostriatal pathways (Juorio *et al.*, 1991). β-PEA has effects in different areas of the brain. Post-synaptically, the TA potentiated cortical neuronal responses to iontophoretically applied noradrenaline (Jones and Boulton, 1980; Jones, 1982; Paterson and Boulton, 1988). Furthermore, caudate nucleus neuronal responses to iontophoretically applied dopamine were increased by β-PEA (Jones, 1981; Paterson *et al.*, 1990). Moreover, systemic administration of β-PEA inhibited dopamine neurons in the rat substantia nigra pars compacta (Rodriguez and Barroso, 1995). However, the release of dopamine in the striatum (Dyck *et al.*, 1983; Philips, 1986; Bailey *et al.*, 1987) and postsynaptic dopamine receptors were stimulated by systemic β-PEA (Antelman *et al.*, 1977). It is well known that β-PEA can produce both psychostimulant-like and dopamine-releasing effects (Boulton, 1982; Dourish, 1982; Janssen *et al.*, 1999; Bergman *et al.*, 2001). An intact and functional dopamine transporter is required for these effects of β-PEA (Sotnikova *et al.*, 2004). β-PEA regulates catecholaminergic neurotransmitter release and this regulation is especially predominant in dopaminergic neurons (Kato *et al.*, 2001). Therefore, dopaminergic neurons could be involved in β-PEA-induced behaviours (Barroso and Rodriguez, 1996). However, the activity of dopaminergic neurons in the substantia nigra and ventral tegmental area (VTA) is inhibited by β-PEA (Geracitano *et al.*, 2004). The VTA (Oades and Halliday, 1987) is a group of neurons which activates the mesolimbic and mesocortical areas of the brain, which are important regions in schizophrenia (Shimazu and Miklya, 2004; Sotnikova *et al.*, 2004; Ishida *et al.*, 2005). β-PEA has been implicated in the neuropathology of schizophrenia (Sandler and Reynolds, 1976; O'Reilly and Davis, 1994). Altered levels of endogenous β-PEA have been found in the plasma of schizophrenic patients (Szymanski *et al.*, 1987; Shirkande *et al.*, 1995). Furthermore, patients suffering from schizophrenia showed increased urinary β-PEA levels (Yoshimoto *et al.*, 1987; Myojin *et al.*, 1989). β-PEA has been shown to be present in relatively small quantities in human urine (Oates *et al.*, 1963; Fischer *et al.*, 1968; Boulton and Milward, 1971). Patients suffering with phenylketonuria exhibited an increased excretion (Oates *et al.*, 1963) whereas patients with depression have impaired excretion (Fischer *et al.*, 1968; Boulton and

Milward, 1971). Stress also increased the urinary excretion rate of β-PEA in man (Paulos and Tessel, 1982) and also the plasma level of β-PEA (Paulos and Tessel, 1982). β-PEA and its metabolites have also been postulated to be modulators of affective behaviours, such as wakefulness, arousal and excitement (Sabelli and Mosnaim, 1974; Sabelli and Borison, 1976). As it was unclear whether the behavioral effects of β-PEA resulted from direct or indirect interactions in the brain, the binding of [^{3}H]-β-PEA to brain membranes was examined and high affinity and saturable binding sites for [^{3}H]-β-PEA in rat brain were found (Hauger *et al.*, 1982). Therefore, the effects of β-PEA in the CNS could be *via* direct stimulation of receptors.

Tyramine

Tyramine is an endogenous constituent naturally found in peripheral tissues as well as in the CNS of vertebrates (Spector *et al.*, 1963; Boulton and Majer, 1970; Tallman *et al.*, 1976; Paterson *et al.*, 1990) In the past, interest in tyramine was focused on its adverse hypertensive effects (Bieck and Antonin, 1988; Bieck, 1989; Brown *et al.*, 1989) when tyrosine- or tyramine- rich foods, such as certain aged cheeses, aged meat, some fruits and vegetables or wines (Hoffmann, 2001) were ingested by patients undergoing antidepressant therapy with MAO inhibitors ("Cheese Effect" and "Chianti Effect") (Bieck and Antonin, 1988; Bieck, 1989; Brown *et al.*, 1989; Zimmer, 1990; Anderson *et al.*, 1993; Tipton, 1997). However, using sensitive analytical methods, tiny amounts of tyramine were measured in the brain (Boulton, 1984) and it has been suggested that it may be involved in the pathogenesis of affective disorders (Murphy *et al.*, 1984; Sandler *et al.*, 1984). A possible correlation between the levels in urine and certain diseases states, such as Parkinson's disease and schizophrenia has been reported (Boulton *et al.*, 1967; Marjerrison *et al.*, 1972). Furthermore, tyramine acts as a neuromodulator (Kopin, 1968; Juorio, 1979) by regulating dopamine and phenylethylamine availability in selected brain areas or modulating their actions at receptor sites (Silkaitis and Mosnaim, 1976; Mosnaim and Wolf, 1977). The peripheral effects of tyramine are usually attributed to its action on promoting efflux of catecholamines from sympathetic neurons and the adrenal medulla (Schonfeld and Trendelenburg, 1989; Mundorf *et al.*, 1999). This results in the indirect stimulation of adrenergic receptors (Black *et al.*, 1980).

In the past it was reported that some of the neuronal effects of TAs might be related to actions on a specific receptor and, as such, they might possess a transmitter role (Jones, 1984). Further studies were also undertaken to investigate the possible existence of specific receptors for tyramine in the brain (Stoof *et al.*, 1976). In addition, binding of [^{3}H]-p-tyramine to membranes isolated from the rat brain was investigated (Vaccari, 1986; Vaccari, 1993). The first study reported high affinity of p-tyramine for a dopaminergic site, most probably involved in the vesicular transport mechanism of dopamine (Vaccari, 1985). Further work could not confirm the existence of a central tyramine receptor. Instead it was suggested that [^{3}H]-p-tyramine associates with a dopamine receptor (Vaccari, 1986). The cardiovascular profile of tyramine has been little studied (Khwanchuea *et al.*, 2008). In clinical studies, tyramine has been shown to increase blood pressure but not heart rate, while decreasing total peripheral resistance (Meck *et al.*, 2003). The vasodilatory action of tyramine has, however, been disputed (Jacob *et al.*, 2003).

1.2.3 Trace amines – effect as indirectly acting sympathomimetic amines

In the periphery, the TAs β-PEA and tyramine are usually regarded as indirectly acting sympathomimetic amines (ISA) (Aviado and Micozzi, 1981). The main action of both amines is to promote the efflux of catecholamines, such as noradrenaline, from sympathetic neurons *(Figure 1.5)*. Thus, noradrenaline is able to interact with receptors to produce sympathomimetic effects (Burn and Rand, 1958; Burn, 1960; Hawthorn *et al.*, 1985; Broadley, 1996). One of the pharmacological responses of the ISAs is to exert coronary vasoconstriction through release of noradrenaline from sympathetic neurons onto α-adrenoceptors after entering the sympathetic neuron *via* cocaine-sensitive pathways *(Figure 1.5)*. Therefore, the TAs stimulate adrenoceptors indirectly (Hawthorn *et al.*, 1985; Broadley, 1996). The actions of TAs are analogous to those of amphetamine to which β-PEA is structurally related. The release of noradrenaline may also cause vasodilatation *via* β-adrenoceptors *(Table 1.1)*.

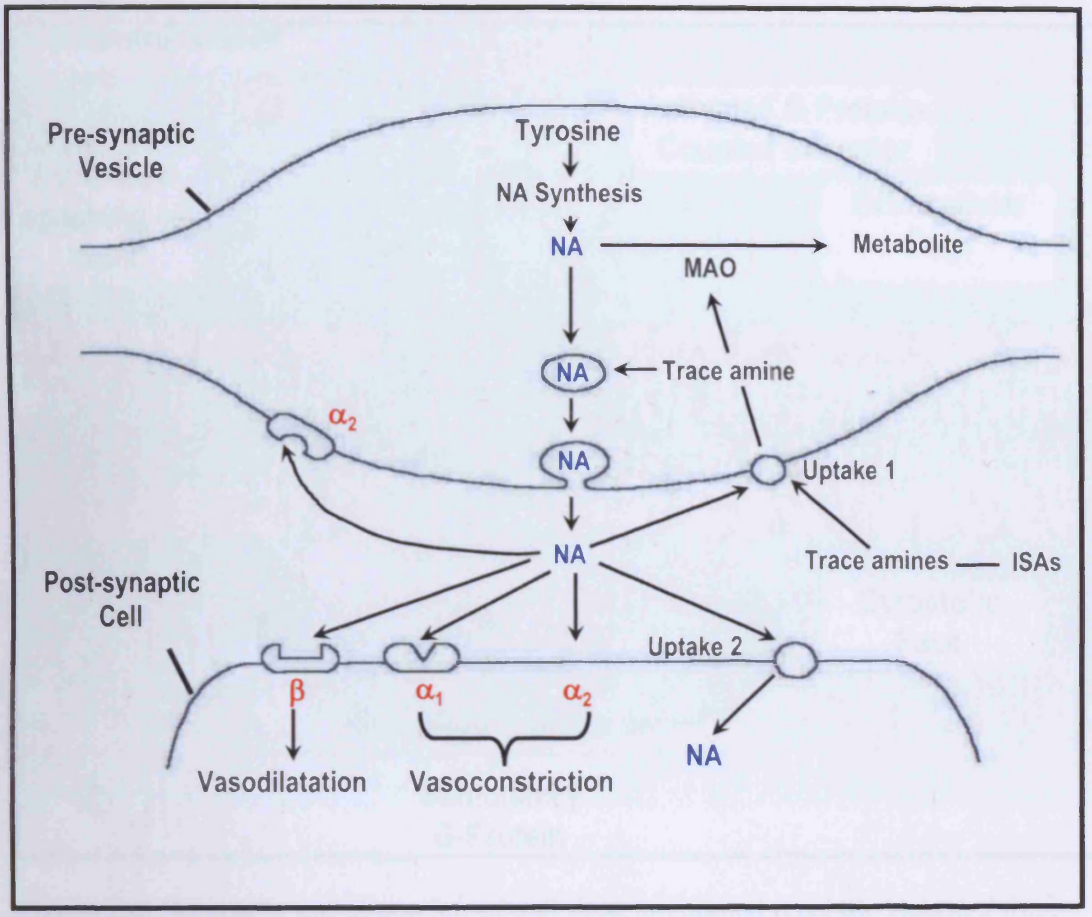


Figure 1.5: The effect of trace amines (TAs) as indirectly acting sympathomimetic amines (ISA). TAs enter the sympathetic neuron *via* cocaine-sensitive uptake pathways (Uptake1) and exert vasoconstriction through the release of noradrenaline (NA) from sympathetic neurones onto α-adrenoceptors. The release of NA may also cause vasodilatation *via* β-adrenoceptors.

1.3 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) form a large and diverse family of proteins in the mammalian genome (Fredriksson *et al.*, 2003) whose major function is to transfer extracellular stimuli into intracellular signals through interaction of their intracellular domains with heterotrimeric G proteins (Kroeze *et al.*, 2003) *(Figure 1.6)*. The crystal structure of one member of this group, bovine rhodopsin, has recently been solved (Palczewski *et al.*, 2000). Therefore, synonyms for the GPCRs are rhodopsin-like seven transmembrane (7TM) spanning receptors, heptahelical receptors or metabotropic receptors (Premont *et al.*, 2001; Davenport, 2003).

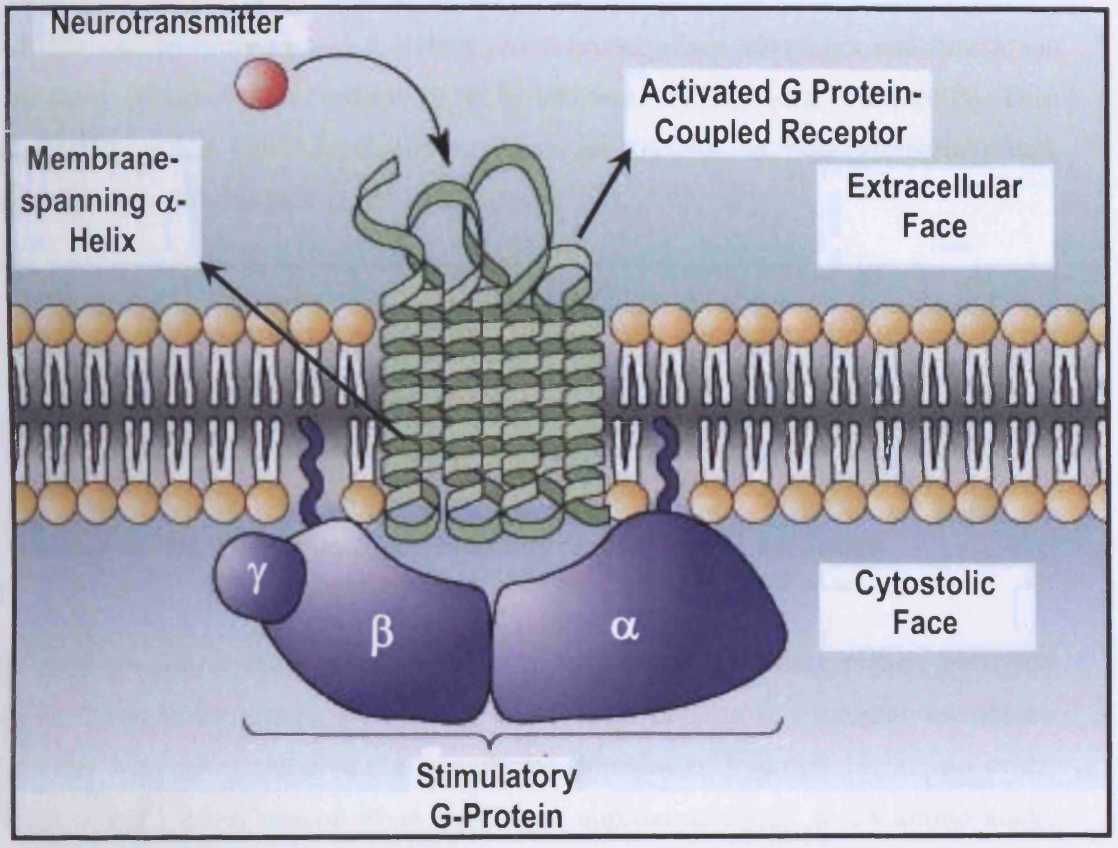


Figure 1.6: Schematic representation of a G protein-coupled receptor. *Adapted from Alzheimer's disease Science (2005)*

GPCRs can be grouped into 6 classes based on sequence homology and functional similarity (Attwook and Findlay, 1994; Kolakowski, 1994; Foord *et al.*, 2005). This classification has been developed to cover all GPCRs, in both vertebrates and invertebrates (Fredriksson *et al.*, 2003) *(Table 1.3)*.

Class A	Class B	Class C	Class D	Class E	Class F
Rhodopsin-like	Secretin-like	Metabotropic	Fungal	cAMP Receptors	Frizzled / Smoothened

Table 1.3: The six classes of GPCRs. The groupings of GPCRs are based on sequence homology and functional similarity (Fredriksson *et al.*, 2003)

GPCRs are only present in the genomes of eukaryotes, including plants, yeast and other invertebrate groups (Kroeze *et al.*, 2003). GPCRs are integral membrane proteins with seven putative transmembrane domains of hydrophobic amino acids. Each is believed to consist of an α-helix of approximately 21 to 28 amino acids, which are connected by three extracellular and three intracellular hydrophilic loops (Ralevic and Burnstock, 1998). The N-terminal of the protein lies on the extracellular side and the C-terminal on the cytoplasmic side of the membrane (Ralevic and Burnstock, 1998; Kroeze *et al.*, 2003). The ligand binding site is exposed outside the surface of the cell and the effector site extends into the cytosol *(Figure 1.5)*. The binding site for some agonists can be in the extracellular loop of the receptors, while others can penetrate into the transmembrane regions. Agonists, such as neurotransmitters and hormones, which activate GPCRs are called first messengers. Second messengers are signalling molecules produced by the stimulation of cell-surface receptors, such as cyclic 3',5'-adenosine monophosphate (cAMP), diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Kroeze *et al.*, 2003).

1.3.1 G proteins

Many receptors in the plasma membrane regulate distinct effector proteins through signalling *via* a group of proteins, known as guanosine triphosphate (GTP) binding proteins (Ross, 1992; Biology, 2006). These proteins are so-called because the G protein binds GTP in its active state and GDP in its inactive state *(Figure 1.7)* (Rang *et al.*, 1999; Biology, 2006; Strosberg and Nahmias, 2007). G proteins are divided

into two types, heterotrimeric and monomeric G proteins. The heterotrimeric G proteins are the proteins to which GPCRs are coupled. G proteins are bound to the inner face of the plasma membrane. The heterotrimeric G protein consists of three subunits, α, β and γ (Hurowitz *et al.*, 2000). At least 18 different human Gα subunits have been found (Hermans, 2003; Wong, 2003; Strosberg and Nahmias, 2007). The known mammalian β and γ subunits are encoded by 5 different β subunit genes (Watson *et al.*, 1994; Strosberg and Nahmias, 2007) and up to 11 different γ subunit genes (Morishita *et al.*, 1995; Ray *et al.*, 1995; Strosberg and Nahmias, 2007). The subunits differ in their molecular weight, α subunit: 39 - 46 kDa, β subunit: 35 - 39 kDa and γ subunit: ~ 8 kDa (Goodman *et al.*, 1990; Rang *et al.*, 1999). Their classification is based on the identity of their distinct α subunit. These polypeptides have highly homologous guanine nucleotide binding domains and have distinct domains for interactions with receptors and effectors (Goodman *et al.*, 1996; Hurowitz *et al.*, 2000). The β subunits showed very high sequence identity and exhibit a more or less ubiquitous expression pattern (Watson *et al.*, 1994). The functional specificity of the βγ subunit is therefore thought to rest with the γ subunits since they display much greater variation in sequence and tissue expression (Kleuss *et al.*, 1992).

1.3.2 G protein-mediated signal transduction

When the system is inactive, GDP is bound to the α-subunit *(Figure 1.7 a)*. Agonist binding to the receptor leads to a conformational change in the α subunit allowing GTP to bind *(Figure 1.7 b)* (Biology, 2006; Strosberg and Nahmias, 2007). The α-subunit is then activated and dissociates from the βγ-subunits as the affinity of α-GTP for the βγ-subunit is lower compared to α-GDP *(Figure 1.7 c)* (Strosberg and Nahmias, 2007). The activated α-subunit then interacts with a membrane-bound effector (Mixon *et al.*, 1995; Wall *et al.*, 1995; Lambright *et al.*, 1996; Strosberg and Nahmias, 2007) and the β- and γ-subunits are free to interact with their effectors (Clapham and Neer, 1993; Strosberg and Nahmias, 2007). Signal transmission is terminated by the hydrolysis of GTP to GDP by the intrinsic GTPase activity of the α-subunit (*Figure 1.7 d)* and the α- and βγ subunits re-associate (Doupnik *et al.*, 1997; Strosberg and Nahmias, 2007).

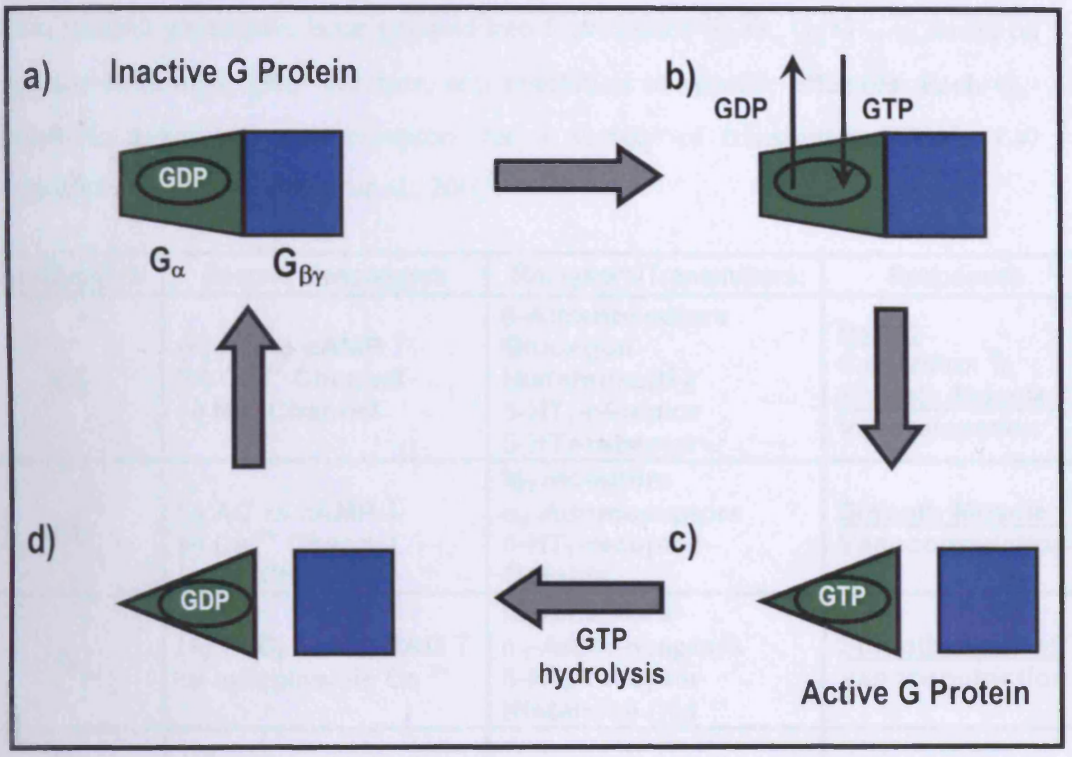


Figure 1.7: Cycling of a G protein between the active and inactive states
(a) In the inactive state, G_α binds to GDP (guanosine diphosphate). The G_α and $G_{\beta\gamma}$ subunits associate together.
(b) Interaction between G_α and the agonist-stimulated receptor causes the release of GDP. GTP (guanosine triphosphate) then binds to the empty site because its concentration in the cell is higher than GDP
(c) The GTP-bound G_α subunit has low affinity for the $G_{\beta\gamma}$ subunits, resulting in their dissociation. The separated G_α and $G_{\beta\gamma}$ subunits can then interact with their effectors.
(d) GTP is hydrolyzed to GDP because G_α has intrinsic GTPase activity

The α subunit genes have been grouped into four classes G_s, G_i, G_q $G_{11/12}$, based on sequence homology, gene structure, and regulation of specific effectors. Each G_α-subunit is associated with receptors for a variety of transmitters *(Table 1.4)* (Hurowitz *et al.*, 2000; Pierce *et al.*, 2002).

Gα-Subunits	Second Messengers	Receptors/Transmitters	Responses
G_s	(+) AC ⇒ cAMP ↑ (+) Ca^{2+} Channel (-) Na^+ Channel	β-Adrenoceptors Glucagon Histamine (H_2) 5-HT_1-receptor 5-HT_7-receptor	Heart: Ca^{2+} Influx ↑ Smooth Muscle: Vasorelaxation
G_i	(-) AC ⇒ cAMP ↓ (-) Ca^{2+} Channel (+) K^+ Channel	M_2 receptors α_2-Adrenoceptors 5-HT_1-receptor Opioids	Smooth Muscle: Vasoconstriction
G_q	(+) PLC_β ⇒ IP_3, DAG ↑ ⇒ cytoplasmic Ca^{2+}	M_3 receptors α_1-Adrenoceptors 5-HT_2-receptor Histamine (H_1)	Smooth Muscle: Vasoconstriction
$G_{11/12}$	(+) cGMP PDE ⇒ cGMP ↓	Photons (Rhodopsin and Color Opsins)	Detection of light

Table 1.4: Examples of receptors and transmitters and associated responses coupled to different G protein α subunits. Four classes of Gα proteins have been identified. (+) = activation and (-) = inhibition, PDE = Phosphodiesterase, AC = Adenylate cyclase, PLC_β = Phospholipase C_β.

1.4 Trace Amine-Associated Receptors

TAs and their possible receptors have been of interest for many years (Usidin and Sandler, 1976; Boulton, 1985; Boulton, 1988) particularly because substantial alterations in the amount of TAs present in neural tissue are associated with pathological conditions (O'Reilly and Davis, 1994). Following a dormant period, this research area regained prominence in 2001 with the discovery of a TA receptor family related to mammalian GPCRs . The detection of the first trace amine receptor (TAR) was made independently by two groups of investigators (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001), each using similar methods. Complex mixtures of oligonucleotides, whose sequences were based on GPCRs for serotonin (Borowsky *et al.*, 2001) or dopamine (Bunzow *et al.*, 2001), were used to amplify novel DNA

sequences by PCR using rat cDNA and genomic DNA as templates (Zucchi *et al.*, 2006). Borowsky et al., (2001) performed reduced stringency screening and phylogenetic analysis. In this study numerous degenerate oligonucleotide-primed PCRs were performed by using rat and human genomic DNAs as templates and 15 sequences with homology to each other were identified (Zucchi *et al.*, 2006). It was found that the TAR1 represents one member of three related subfamilies of mammalian GPCRs. Therefore, large subtype diversity may exist in this class of GPCRs. Bunzow et al., (2001) performed extensive pharmacological studies on TAR1 by cloning both rat and human TAR sequences and stably expressing them in human embryonic kidney (HEK) cells. Expression of the putative orphan receptor in either *Xenopus oocytes* or HEK cells showed that upon exposure to β-PEA or tyramine a stimulation of adenylate cyclase through $G\alpha_s$, led to the accumulation of cAMP (Zucchi *et al.*, 2006). In contrast, neither classical biogenic amines like dopamine, noradrenaline and adrenaline nor serotonin and histamine were shown to be effective agonists for this receptor (Kim and von Zastrow, 2001; Zucchi *et al.*, 2006). As a result of their pharmacological profile and their functional coupling to cAMP, both investigators assumed their orphan GPCR was a `bone fide` receptor for TAs (Zucchi *et al.*, 2006). This discovery of the TA-sensitive novel receptor family has led to speculations on the physiological and pathological role of these receptors (Lewin, 2006) and has provided a new perspective on the effects and mechanism of action of TAs. This interest has also led to both academic and pharmaceutical industry research into putative endogenous receptors for TAs and metabolic derivatives of classical biogenic amines (Scanlan *et al.*, 2004; Lindemann and Hoener, 2005; Hart *et al.*, 2006).

TARs belong to the superfamily of rhodopsin-like seven transmembrane GPCRs *(Table 1.3)*. The receptor family has been identified in human, chimpanzee, rat and mouse (Lindemann *et al.*, 2005; Lewin, 2006). In all species analyzed so far, three receptor subfamilies have been identified (Lindemann *et al.*, 2005). In contrast to subfamily 1, all other TARs failed to respond to TAs (Borowsky *et al.*, 2001). According to this insensitivity of many of the receptors to TAs, a novel receptor nomenclature was devised. The GPCR family of TAs are now called trace amine-associated receptors (TAARs).

Due to the new nomenclature, TAR1 is now called TAAR1 and TAR2 is called TAAR4 (Lindemann *et al.*, 2005; Lewin, 2006) *(Table 1.5)*.

Subfamily	Receptor	Sensitive to TAs
TAAR subfamily 1	TAAR1 - TAAR4	TAAR1 and TAAR4
TAAR subfamily 2	TAAR5	No
TAAR subfamily 3	TAAR6 – TAAR9	No

Table 1.5: Classification of trace amine-associated receptors (TAARs) into subfamilies. Three subfamilies for TAARs have been identified. Only TAAR1 and TAAR4 in subfamily 1 are considered to be sensitive to trace amines (TAs). All other TAARs failed to respond to TAs (Lindemann *et al.*, 2005; Lewin, 2006).

The human and rat genomes appear to have a different complement of TA-like GPCRs. In man there are fewer functional members. So far, 19 individual TAARs for rat, 9 TAARs each for human and chimpanzee, and 16 for mouse have been identified (Lewin, 2006). A considerable homology had been reported between the human (hTAARs) and rat (rTAARs) TAARs (Lewin, 2006). Based on sequence homology, the hTAAR1 has been called the human ortholog of rTAAR1 (Branchek and Blackburn, 2003; Lewin, 2006) *(Table 1.6)*.

Receptor	Species	Homology (%)
TAAR1	Human, Rat	79
TAAR6	Human, Rat	88
TAAR9	Human, Rat	87

Table 1.6: Sequence homology between human and rat trace amine-associated receptors (TAARs) (Lewin, 2006)

At present, putative, potential endogenous ligands for both TA-sensitive TAARs, TAAR1 and TAAR4 have been identified in transfected cell lines (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). In general, the potency of TAs, in particularly β-PEA and tyramine, to activate TAAR4 was lower compared to their potency at TAAR1 (Borowsky *et al.*, 2001; Davenport, 2003) *(Table 1.7)*.

Receptor	β-PEA	Tyramine	Tryptamine	Octopamine
h/r TAAR1	+ + +	+ + +	+	+
h TAAR4	+ + +	+ + +	?	?
r TAAR4	+ + +	?	?	?

Table 1.7: Comparison of the potency of potential endogenous ligands for trace amine-associated receptor 1 (TAAR1) and TAAR4 in human and rat. β-PEA activates TAAR1 and TAAR4 in human (h) and rat (r) with equal potency (+ + +). Tyramine is a potential ligand for human TAAR1 and TAAR4 and rat (r) TAAR1 (+ + +). Octopamine and tryptamine activate TAAR1 in both species with lower potency (+) compared to β-PEA and tyramine. ? = unknown (Borowsky *et al.*, 2001; Davenport, 2003; Lewin, 2006).

1.5 Signal Transduction Mechanisms of Trace Amines

In transfected cell lines, the most commonly recognized signal transduction pathway for TAARs is to stimulate the formation of cyclic AMP (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). TAAR1 stimulates adenylate cyclase through the α subunit and mediates an increase in cAMP *(Figure 1.8)* (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001; Lewin, 2006). In intact tissue, tyramine can also produce vasorelaxation by reduction of inositol 1,4,5-trisphosphate (IP_3) formation (Varma and Chemtob, 1993) *(Figure 1.8)*. But whether this response was mediated *via* TAAR was not determined.

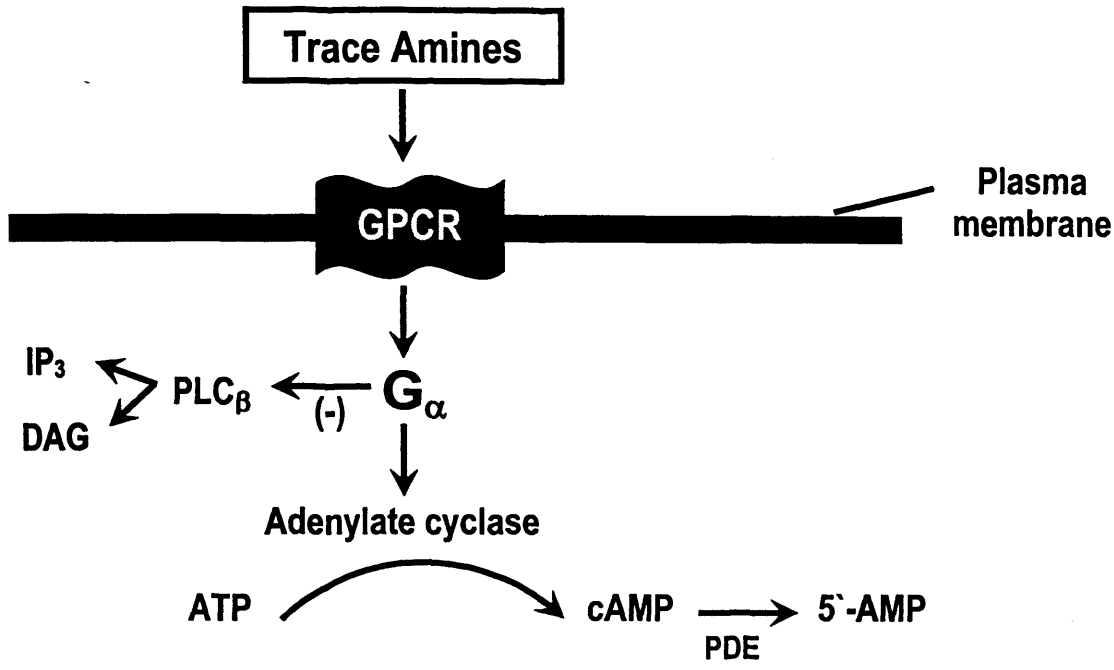


Figure 1.8: Known signal transduction pathways for trace amines (TAs). Primarily, TAARs stimulate the formation of cyclic AMP (cAMP) through the G_α-subunit when exposed to TAs. cAMP is inactivated by hydrolysis into 5`AMP by phosphodiesesterases (PDE). Also TAARs can mediate inhibition (-) of phospholipase C_β (PLC_β) and therefore a reduction of inositol 1,4,5-trisphosphate (IP_3).

Cyclic adenosine 3`, 5`- monophosphate (cAMP)

cAMP serves as a second messenger in many varied physiological events and is used for intracellular signal transduction by effectors which cannot cross the cell membrane, such as transferring the effects of hormones like glucagon and adrenaline. The main purpose of cAMP is the activation of various kinases, such as cAMP-dependent protein kinase (PKA) and myosin light chain kinase (MLCK). Kinases catalyse the phosphorylation of different amino acids, including serine and threonine. PKA is normally inactive as a tetrameric holoenzyme, consisting of two catalytic and two regulatory units (C_2R_2), with the regulatory units blocking the catalytic centers of the catalytic units. cAMP binds to specific locations on the regulatory units of the protein kinase, and causes dissociation between the regulatory and catalytic subunits, thus activating the catalytic units and enabling them to phosphorylate substrate proteins (Broadley, 1996). Adenylate cyclase, located at the cell membrane, catalyses

the conversion of ATP to 3',5'-cyclic AMP (cAMP) and pyrophosphate. The hydrolysis of cAMP is catalyzed by several phosphodiesterases (PDE) (Strada and Hidaka, 1992; Broadley, 1996). Adenylate cyclase is activated by effectors, such as β-adrenoceptors, H_2-receptors and 5-$HT_{1\ and\ 7}$-receptors *(Table 1.4)* through the activation of adenylate cyclase stimulatory G_s-subunit and inhibited by agonists of adenylate cyclase inhibitory G_i-subunit. cAMP accumulation from β_2-adrenoceptor stimulation is associated with smooth muscle relaxation, such as vasodilatation and bronchodilatation *(see Section 1.6)* (Goodman *et al.*, 1990; Broadley, 1996). PDEs convert cAMP to the inactive non-cyclic adenosine monophosphate (Strada and Hidaka, 1992; Broadley, 1996) (*see Section 1.7.3*).

Inositol 1,4,5-trisphosphate (IP_3)

Stimulation of muscarinic M_1- and M_3 receptors and α_1-adrenoceptors causes the G protein-mediated hydrolysis of the membrane phospholipid, phosphatidylinositol bisphosphate 4,5-bisphosphate (PIP_2) to IP_3 and 1,2-diacylglycerol (DAG) catalysed by phospholipase C (PLC). PLC is coupled to the receptor by G proteins of the Gq family *(Table 1.4)* (Broadley, 1996). IP_3 has been clearly identified as the second messenger for receptor-mediated release of intracellular Ca^{2+} from the sarcoplasmic reticulum (SR). The target is an IP_3 receptor which forms the Ca^{2+} channel spanning the membrane of the intracellular SR Ca^{2+} storage site. The release of intracellular Ca^{2+} then initiates contractile responses of smooth muscle resulting from α_1- or M_3 receptor stimulation (Broadley, 1996) (*see Section 1.7.3*).

1.6 Adrenoceptor Pharmacology

In view of the close structural similarity between TAs and noradrenaline (Lindemann and Hoener, 2005) the possibility must be considered that they could interact with both adrenoceptors and TAARs. Also, the indirect sympathomimetic activity of these TAs suggests that part of their pharmacological activity could be due to indirect stimulation of adrenoceptors (Hansen et al., 1980). TAs have been assumed to operate as potential neuromodulators rather than neurotransmitters *(see Section 1.2)* (Berry, 2004) or as vasoconstrictors (Branchek and Blackburn, 2003; Shimazu and Miklya, 2004). In order to understand the mechanism of action of TAs, the receptor pharmacology of α- and β-adrenoceptors must be considered. Adrenergic receptors

specifically bind and are activated by their endogenous ligands, the catecholamines adrenaline and noradrenaline *(Figure 1.9)*. They were originally divided into two major types, α- and β-adrenoceptors. However, based on pharmacological and molecular evidence, they are now divided into three major types, α_1, α_2, and β and further subdivided into three subtypes each (Bylund *et al.*, 1988; Bylund, 2005) *(Figure 1.9)*.

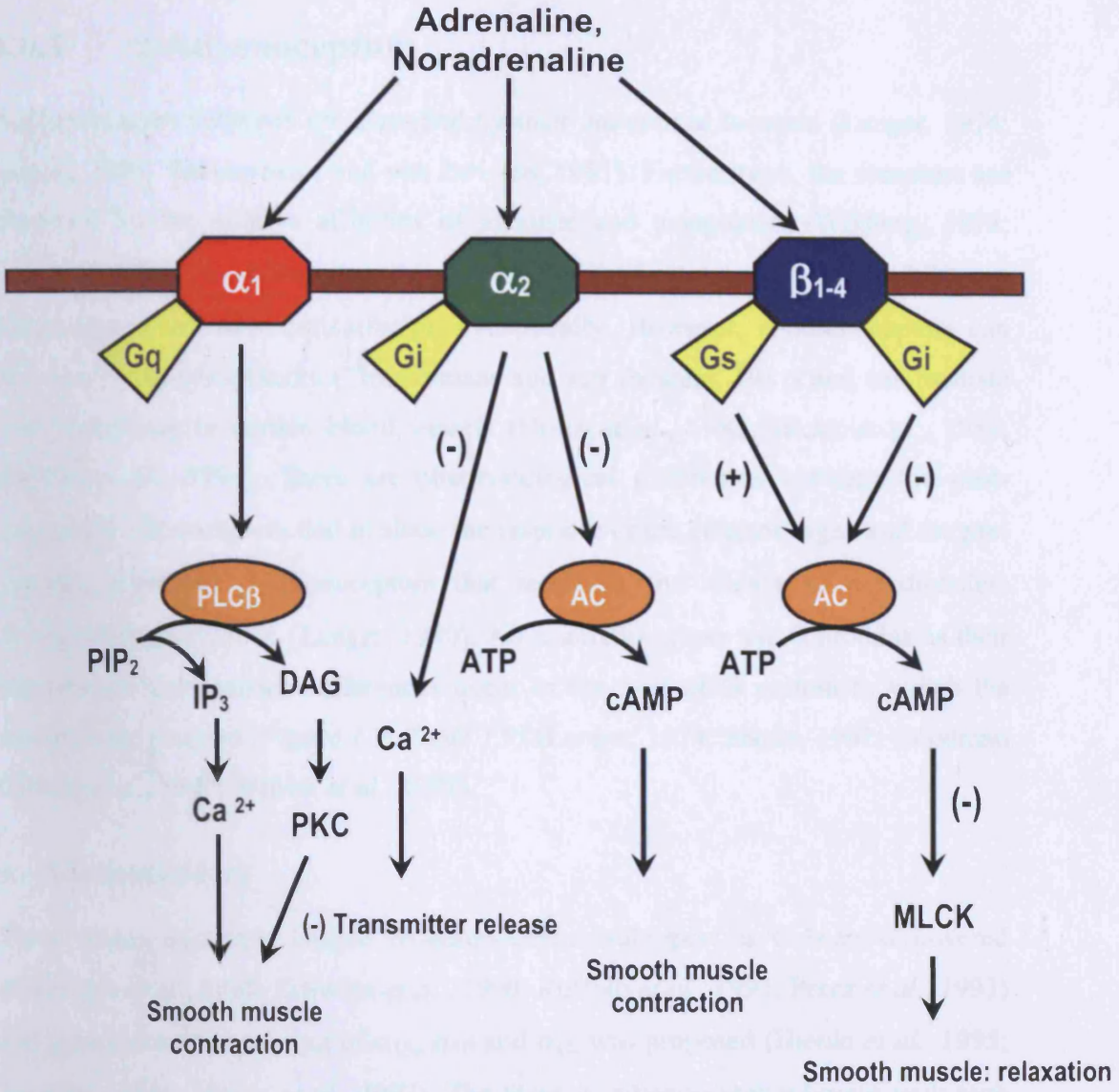


Figure 1.9: The mechanism of action of adrenergic receptors.
Adrenaline and noradrenaline are receptor ligands for α_1, α_2 or β-adrenoceptors. α_1-adrenoceptors couple to Gq, which results in increased intracellular Ca^{2+} and activation of PKC and therefore smooth muscle contraction. α_2-adrenoceptors couple to Gi and cause a decrease in cAMP and therefore smooth muscle contraction. The β-adrenoceptors couple either through Gi or Gs to adenylate cyclase in an inhibitory (-) or stimulatory (+) way, respectively resulting in either increased or decreased formation of cAMP. An increase of cAMP causes an inhibiton of the MLCK and therefore smooth muscle relaxation. PKC = Protein kinase C, PLC_β = Phospholipase C_β, MLCK = Myosin light chain kinase, AC = Adenylate cyclase, PIP_2 = Phosphatidyl inositol bisphosphate, DAG = Diacylglycerol, (-) = decrease/inhibition, (+) = activation. *Adapted from Answers.com (2007) (see Section 1.7.3).*

1.6.1 α-Adrenoceptors

α-adrenoceptors subtypes are classified by their anatomical location (Langer, 1974; Langer, 1980; Timmermans and van Zwieten, 1981). Furthermore, the receptors are classified by the relative affinities of agonists and antagonists (Wikberg, 1978; Wikberg, 1979). α_1-Adrenoceptors are mostly found post-synaptically, whilst α_2-adrenoceptors are sited primarily pre-synaptically. However, α-adrenoceptors can also occur post-synaptically (Timmermans and van Zwieten, 1981) and can mediate vasoconstriction in certain blood vessels (Holtz *et al.*, 1982; Hicks *et al.*, 1983; Ruffolo *et al.*, 1991). There are pharmacological differences between the post-synaptic α-adrenoceptors that mediate the response of the effector organ and the pre-synaptic inhibitory α-adrenoceptors that modulate the release of noradrenaline during nerve stimulation (Langer, 1980). All α-adrenoceptors use G proteins as their transduction mechanism. Differences occur in the type of G protein to which the receptors are coupled *(Figure 1.9, Table 1.9)* (Langer, 1974; Starke, 1981; Goodman Gilman *et al.*, 1990; Wilson *et al.*, 1991).

α_1-Adrenoceptors

Three genes encoding unique α_1-adrenoceptor subtypes have been discovered (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Ruffolo *et al.*, 1991; Perez *et al.*, 1993) and nomenclature consisting of α_{1A}, α_{1B} and α_{1D} was proposed (Hieble *et al.*, 1995; Broadley, 1996; Hague *et al.*, 2003). The three α_1-adrenoceptor subtypes with high affinity for prazosin (Martin *et al.*, 1997) have been cloned and the pharmacology of the cloned receptors has been characterised against that of their native forms (Michel and Insel, 1994; Blue *et al.*, 1995). The coupling of α_1-adrenoceptors to second messenger systems has been examined in detail (Hague *et al.*, 2003). These receptors are coupled to the activation of phospholipase C (Schwinn *et al.*, 1991; Perez *et al.*, 1993) *via* pertussis toxin-insensitive G proteins of the G_{q11} family (Wu *et al.*, 1992; Hague *et al.*, 2003). In addition it was reported that α_1-adrenoceptors utilize G_q to activate phosphoinositide hydrolysis and G_{11} to induce the release of calcium from intracellular stores (Macrez-Lepretre *et al.*, 1997) *(Figure 1.9, Table 1.9)*. In addition, to mobilize intracellular calcium, the α_1-adrenoceptors have also been shown to activate calcium influx *via* both voltage-dependent and- independent

calcium channels (Ljung and Kjellstedt, 1987; Han *et al.*, 1992; Lazou *et al.*, 1994; Minneman and Esbenshade, 1994). α_1-Adrenoceptors are found in both the central (CNS) and peripheral nervous system (PNS).

In the CNS they are found mostly post-synaptically and have an excitatory function. Although the density of α_1-adrenoceptors in the CNS is among the highest of any tissue in the body (Wilson and Minneman, 1989), it has been difficult to identify their functional role (Hague *et al.*, 2003). α_1-Adrenoceptors influence neuronal signalling throughout the brain (Marek and Aghajanian, 1999). However, their specific functional roles remain uncertain as many of α_1-adrenoceptor agonists and antagonists are unable to cross the blood-brain barrier (Pupo and Minneman, 2001). Peripherally they are responsible for smooth muscle contraction and are situated on vascular and non-vascular smooth muscle. α_1-Adrenoceptors on vascular smooth muscle are located intra-synaptically and function in response to neurotransmitter release (Langer, 1974; Starke, 1981). Previous publications suggested that all three α_1-adrenoceptor subtypes are expressed in the majority of blood vessels and mediate contractile responses *(Table 1.8)* (Han *et al.*, 1990; Broadley, 1996). Furthermore, all three α_1-adrenoceptor subtypes play an important role in the regulation of blood pressure. In knockout animals significant decreases in pressor responses to phenylephrine were found in α_{1A}- (Rokosh and Simpson, 2002) α_{1B}- (Cavalli *et al.*, 1997) and α_{1D}- adrenoceptor (Tanoue *et al.*, 2002). Furthermore, previous publications suggest that α_{1A}- and α_{1D}- adrenoceptors primarily control vasoconstriction *(Table 1.8)* with a minor contribution from α_{1B}- adrenoceptors (Broadley, 1996; Daly *et al.*, 2002).

Organ / Location	Response	α-Adrenoceptor
Blood vessel – rat mesentery	Vasoconstriction	α_{1A}
Blood vessel – rat aorta	Vasoconstriction	α_{1D}
Blood vessel – rat portal vein	Contraction	α_{1A}

Table 1.8: Locations of and responses mediated by α_1-adrenoceptor in rat blood vessels. *Adapted from Broadley (1996)*

α_2-Adrenoceptors

α_2-Adrenoceptors are found in both the central and peripheral nervous system. They are found both pre- and post-synaptically and are generally inhibitory. Pre-synaptic α_2-adrenoceptors inhibit the release of noradrenaline and thus serve as an important receptor in the negative feedback control of noradrenaline release. Post-synaptic α_2-adrenoceptors are located on liver cells, platelets, and the smooth muscle of blood vessels (Civantos Calzada and Aleixandre de Artinano, 2001). Activation of these receptors causes platelet aggregation, and blood vessel constriction. The evidence for three α_2-adrenoceptor subtypes was investigated by radioligands binding and functional studies in mammalian systems and their existence was confirmed by cloning in several species (Bylund *et al.*, 1994; Alexander *et al.*, 2004). The three subtypes are well characterised in mammalian species and identified as α_{2A}, α_{2B} and α_{2C}-adrenoceptors (Bylund *et al.,* 1994). The α_{2A}-adrenoceptor subtype is the main subtype in most brain regions in mammalian species. The α_{2C}-adrenoceptor subtype is found in high concentrations in the caudate nucleus, whilst the α_{2B} -adrenoceptor subtype has a more limited distribution (Bylund, 2005). The pharmacological characteristics of the three α_2-adrenoceptor subtypes are consistent in different species. However, the α_{2A}-adrenoceptor subtype has one pharmacological profile in man, dog, rabbit, pig and chicken and a different profile in rat, mouse and cow. Therefore, often investigators retain the α_{2D} nomenclature which was originally assigned to these α_{2A}-adrenoceptors (Bylund, 2005). The α_2-adrenoceptor has been shown to be negatively coupled to adenylate cyclase *via* G protein G_i/G_o. The activation of these receptors causes a reduction of cAMP which leads to a decreased influx of calcium during the action potential which is the ion responsible for transmitter release. Therefore, lowered levels of calcium will correspondingly lead to a decrease in transmitter release and smooth muscle contraction (Goodman *et al.*, 1990; Goodman Gilman *et al.*, 1990; Bylund *et al.*, 1994; Broadley, 1996; Roberts *et al.*, 1998) *(Figure 1.9, Table 1.9).*

Receptor	Agonists	Second Messenger	Antagonist
α_1	A=NA>>ISO	(+) PLC_β *via* $G_q \Rightarrow$ Vasoconstriction	Prazosin Doxazosin
α_2	A≥NA>>ISO	(-) cAMP *via* $G_i/G_o \Rightarrow$ Vasoconstriction	Yohimbine Idazoxan

Table 1.9: Characterisation of α-adrenoceptors.
Both α-adrenoceptor subtypes use G proteins as their transduction mechanism. Differences occur in the type of G protein the receptors are coupled to. Both adrenoceptor subtypes cause vasoconstriction. Representative agonists include adrenaline (A), noradrenaline (NA) and isoprenaline (ISO). PLC_β = phospholipase, (+) = increase and (-) = decrease (Broadley, 1996; Civantos Calzada and Aleixandre de Artinano, 2001)

1.6.2 β-Adrenoceptors

For many years, classical pharmacology suggested that there were two subtypes of β-adrenoceptor, β_1- and β_2-adrenoceptors (Lands *et al.*, 1967). β-Adrenoceptor classification was based initially on differing rank order potencies of β-adrenoceptor agonists (Alquist, 1948; Lands *et al.*, 1967) and, subsequently, on the discovery of appropriate specific antagonists (Black and Stephenson, 1962). The discovery of these receptor subtypes led to the development of selective agonists and antagonists for each subtype. Then, the discovery of agonists with a novel tissue selectivity backed up earlier evidence for a third subtype, an "atypical β-adrenoceptor" resistant to blockade by most β_1-adrenoceptor and β_2-adrenoceptor antagonists which was classified as β_3-adrenoceptor (Arch, 2002). The existence of a fourth type of β-adrenoceptor has also been suggested (Kaumann, 1997; Kaumann *et al.*, 1998; Cohen *et al.*, 2000). However, it seems that the pharmacology of the β_4-adrenoceptor is dependent upon the presence of β_1-adrenoceptors. Therefore, the putative β_4-adrenoceptor is now described as a low affinity state of the β_1-adrenoceptor (Konkar *et al.*, 2000; Kaumann *et al.*, 2001).

Stimulation of adenylate cyclase

All β-adrenoceptors were formerly considered to be associated with G_s. All β-adrenoceptors can positively couple to adenylate cyclase *via* activation of G_s protein *(Figure 1.9, Table 1.4)*. The resulting increase in intracellular cAMP level activates protein kinase A (PKA) *(see Section 1.5)*. An increase of cAMP is usually associated

with smooth muscle relaxation (β_2-adrenoceptors) *(Table 1.10)* (Broadley, 1996; Magocsi *et al.*, 2007; Strosberg and Nahmias, 2007).

β_1-Adrenoceptors

β_1-adrenoceptors are largely post-synaptic and are located primarily in the heart but are also found in platelets, the salivary glands and the non-sphincter part of the gastro-intestinal tract (GIT). However, they are also found pre-synaptically (Chang *et al.*, 1986; Heimburger *et al.*, 1989). Activation of β_1-adrenoceptors causes an increase in the rate (positive chronotropy) and contractile force (positive inotropy) of the heart due to the activation of cAMP-dependent protein kinase *(see Section 1.5)* (Broadley, 1996). Furthermore, the activation of β_1-adrenoceptors causes relaxation of the non-sphincter part of the GIT, aggregation of platelets and amylase secretion from the salivary glands. (Goodman Gilman *et al.*, 1990; Broadley, 1996; Rang *et al.*, 2003) *(Figure 1.9, Tables 1.10)*. Pre-synaptically, their activation causes an increase in noradrenaline release (Heimburger *et al.*, 1989).

β_2-Adrenoceptors

β_2-adrenoceptors are mainly post-synaptic and are located on a number of tissues including vascular smooth muscle (Lands *et al.*, 1967), airway smooth muscle, GIT, skeletal muscle, liver and mast cell. cAMP accumulation from β_2-adrenoceptors stimulation is associated with smooth muscle relaxation, such as vasodilatation and bronchodilatation. Furthermore, the activation of β_2-adrenoceptors causes relaxation of the GIT, glycogenolysis in the liver, tremor of skeletal muscle and inhibition of histamine release from mast cells (Goodman Gilman *et al.*, 1990; Broadley, 1996; Rang *et al.*, 2003) *(Figure 1.9, Tables 1.10)*.

β_3-Adrenoceptors

β_3-adrenoceptors are present in adipose tissue (Arch *et al.*, 1984; Wilson *et al.*, 1984) rat colon (Bianchetti and Manara, 1990) and guinea-pig ileum (Bond and Clarke, 1988). These adrenoceptors have a role in the regulation of lipolysis (Lafontan, 1994) and thermogenesis (Mizuno *et al.*, 2002). Moreover, β_3-adrenoceptors activation is associated with the relaxation of gastrointestinal (Manara *et al.*, 1995) and airway (Martin and Advenier, 1995) smooth muscle. In addition, β_3-

adrenoceptors have been characterised in human heart, activation of which induces a negative inotropic effect (Gauthier *et al.*, 1996). Furthermore, the existence of β_3-adrenoceptors in vascular smooth muscle has been reported where they cause vasorelaxation (Berlan *et al.*, 1994; Shen *et al.*, 1994). Previous workers suggest the presence of β_3-adrenoceptors in rat thoracic aorta mainly on endothelial cells. β_3-adrenoceptors act in conjunction with β_1- and β_2-adrenoceptors to mediate vasorelaxation through activation of the NO synthase pathway and a subsequent increase in cGMP levels (Trochu *et al.*, 1999; Arch, 2002).

Receptor	Agonists	Tissue	Response	Antagonists
β_1	ISO>NA>A	Heart	(+) Heart rate (+) Myocardial contractility	Atenonol Metoprolol
β_2	ISO>A>>NA	Smooth muscle	Vasodilatation Bronchodilatation	ICI-118,551 Butoxamine
β_3	ISO=NA>A	Adipose tissue	Lipolysis Thermogenesis	SR59230A

Table 1.10: Characterisation of β-adrenoceptors.
All β-adrenoceptor subtypes use Gs protein as their main transduction mechanism. Representative agonists include adrenaline (A), noradrenaline (NA) and isoprenaline (ISO). (+) = increase (Broadley, 1996; Goodman Gilman *et al.*, 1996).

1.7 Blood Vessels

1.7.1 Structure

Blood vessels are divided into four broad categories, arteries, such as aorta *(Figure 1.11)*, arterioles, capillaries and veins. The walls of the larger blood vessels consist of three layers including the *tunica intima*, the *tunica media* and the *tunica adventia*, whose thickness varies according to the type of vessel. The inner lining of all blood vessels is a thin layer of endothelium *(Figure 1.10)*.

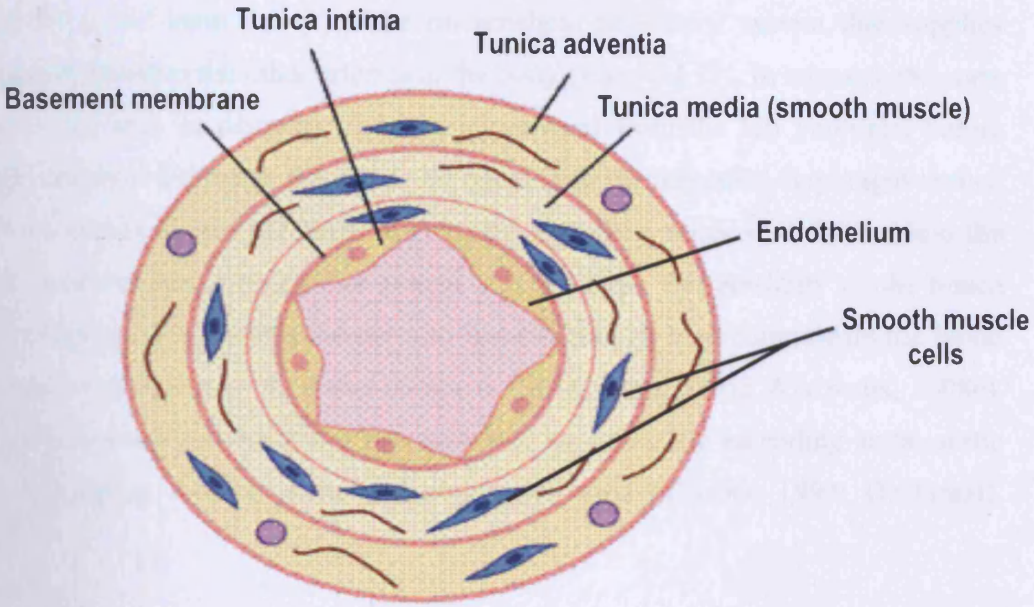


Figure 1.10: Blood vessel structure.
The arrangement of the principal layers of an artery. The walls of the larger blood vessels consist of three layers including the *tunica intima*, the *tunica media* and the *tunica adventia*, whose thickness varies according to the type of vessel. The inner lining of all blood vessels is a thin layer of endothelium. The smooth muscle of the *tunica media* is arranged in a circular manner. *Adapted from Lafleur et al., (2003)*

The *tunica intima* consists of a layer of flat endothelial cells overlying a thin layer of connective tissue and is separated from the *tunica media* by the internal elastic lamina. The endothelial cells of the tunica intima are in direct contact with the blood. The *tunica media* consists of circular layer of smooth muscle containing elastin and collagen. The smooth muscle of the *tunica media* is innervated by sympathetic nerve fibres. The *tunica media* provides the mechanical strength of the blood vessel. The *tunica adventitia* consists of a loosely formed layer of elastic and collagenous fibres orientated along the length of the vessel. It serves to anchor the blood vessel in place. The *tunica adventitia* is separated from the tunica media by the external elastic lamina (Pocock and Richards, 2004).

1.7.2 Aorta

The aorta is the main artery of the mammalian circulatory system that supplies oxygenated blood to the other arteries of the body *(Figure 1.11)*. In humans, the aorta is about one inch in diameter and extends upward from the left ventricle, before arching downward through the chest. An opening in the muscular diaphragm termed the aortic hiatus allows the aorta to enter the abdomen, whence it divides into the paired common iliac arteries that extend into the legs. The elasticity of the tunica media *(Figure 1.10)* enables the aorta to distend enough to accommodate the blood that surges through it as the heart contracts (Silverthorn, 2001; Wikipedia, 2008c). The aorta is usually divided into four sections, including the ascending aorta, aortic arch, descending thoracic aorta and abdominal aorta (Tortora, 1994; De Graaff, 1998).

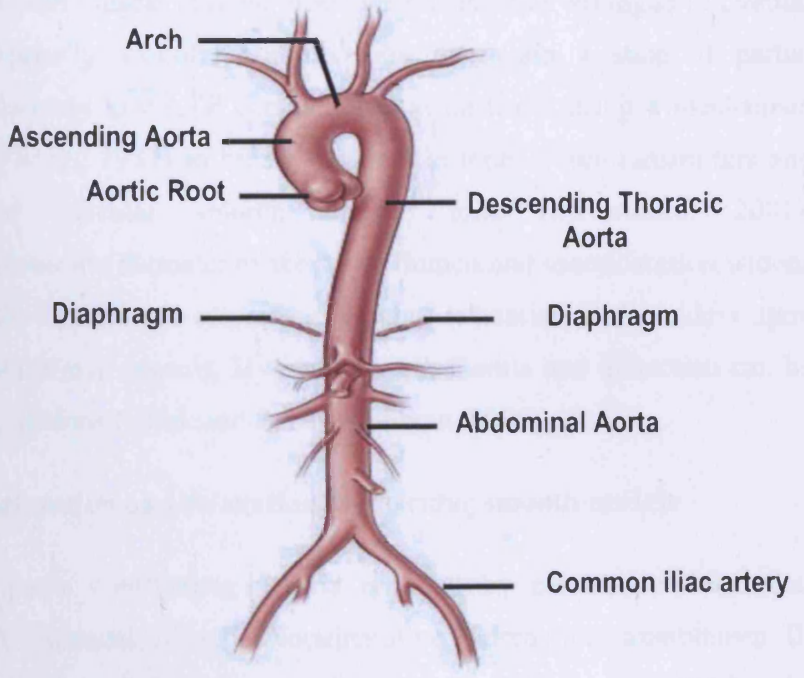


Figure 1.11: Picture of the aorta with the four main sections. *Adapted from Cleveland Clinic (2008)*

Extensive studies with rats and other animals have been performed to investigate the effects of various diseases on the aorta and to test the efficacy of potential treatments and preventive measures. Aortic tissues have been used to characterise α- and β-adrenoceptors (Digges and Summers, 1983b; Decker *et al.*, 1984; O'Donnell and Wanstall, 1984a). Furthermore, aortic tissues have been used to examine the influence of nitric oxide production on vascular smooth muscle contraction (Bang *et al.*, 1999) and vasodilatation (Chitaley and Webb, 2002) *(see Section for Endothelium).*

1.7.3 Vascular smooth muscle

Vascular smooth muscle (VSM) refers to the particular type of smooth muscle found within, and composing the majority of the wall of blood vessels (Somlyo and Somlyo, 1968). It is the muscular component of blood vessels (Clark and Pyne-Geithman, 2005). Smooth muscle in most blood vessels is either arranged in circular or spiral layers. Primarily vascular smooth muscle contain a state of partial contractions with relatively low ATP consumption at all times using a mechanism called Latch (Dillon *et al.*, 1981) to create the muscle tone. Neurotransmitters and hormones influence vascular smooth muscle tone (Silverthorn, 2001). Vasoconstriction narrows the diameter of the vessel lumen and vasodilatation widens it (Silverthorn, 2001). The control of contraction and relaxation is dependent upon intracellular and extracellular signals. Hypertension, ischemia and infarction can be due to abnormal contractions (Clark and Pyne-Geithman, 2005).

Mechanisms for contraction and relaxation in vascular smooth muscle

Vascular smooth muscle contraction can be initiated by mechanical, electrical (depolarization) and chemical stimuli. Noradrenaline, adrenaline, angiotensin II, vasopressin, endothelin-1 and thromboxane A_2 can cause contraction (Klabunde, 2007). Each vasoconstrictor binds to a specific receptor on the vascular smooth muscle cell or to receptors on the endothelium adjacent to the VSM which then leads to vascular smooth muscle contraction. The mechanism of contraction and relaxation involves different signal transduction pathways including Gs protein coupled pathway, phosphatidylinositol bisphosphate pathway (Gq protein coupled signal transduction) and nitric oxide-cGMP pathway.

➢ **Gs protein coupled pathway**

The Gs protein coupled pathway in smooth stimulates adenylate cyclase which catalyzes the formation of cAMP. Unlike the heart, however, an increase in cAMP in vascular smooth muscle causes relaxation. In the heart myosin light chain kinase (MLCK) phosphorylates myosin and causes contraction. However, in vascular smooth muscle MLCK is inhibited by cAMP and therefore causes relaxation *(see Section 1.5)*. Previous workers reported that a decrease in MLCK activity after phosphorylation by cAMP-dependent protein kinase and the subsequent dephosphorylation of myosin by phosphatases might be part of the mechanism of cAMP-mediated relaxation in smooth muscle (De Lanerolle *et al.*, 1984; Rang *et al.*, 1999). Furthermore, an increase in cAMP activates protein kinase A (PKA). PKA is a holoenzym (*see Section 1.5*).

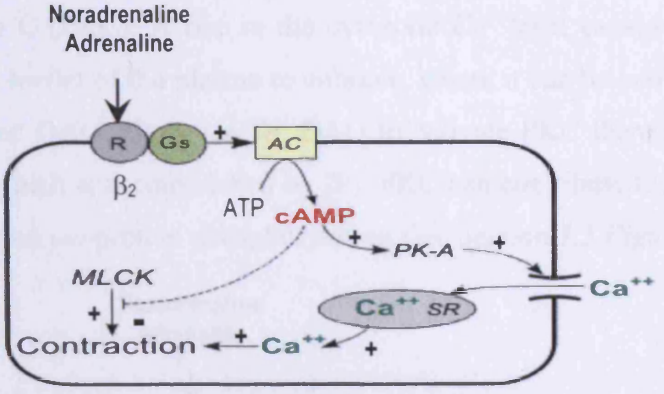


Figure 1.12: Gs protein coupled pathway in vascular smooth muscle.
R = receptor, Gs = stimulatory Gs protein, AC = adenylate cyclase, PKA = protein kinase, MLCK = myosin light chain kinase, SR = sarcoplasmic reticulum, β_2 = β-adrenoceptor, (+) = activation, (-) = inhibition. *Adapted from Klabunde (2008)*

➢ **Gq protein coupled pathway**

The phosphatidylinositol bisphosphate pathway in vascular smooth muscle is similar to that found in the heart. Adrenaline and noradrenaline acting *via* α_1-adrenoceptors activate phospholipase C (PLC) causing the formation of inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol bisphosphate (PIP_2). The IP_3 then stimulates the sarcoplasmic reticulum (SR) by binding to a specific IP_3 receptor, a Ca^{2+} channel

protein composed of four identical subunits, each containing an IP_3-binding site.to release calcium. The IP_3 binding induces opening of the channel and allowing Ca^{2+} to exit from the SR into the cytosol. An increase of intracellular calcium activates vascular smooth muscle. An increase in free intracellular calcium can result from either increased flux of calcium into the cell through calcium channels or by release of calcium from internal stores, such as from the sarcoplasmic reticulum. The free calcium binds to calmodulin, a specific calcium binding protein. The calcium-calmodulin complex then activates MLCK which phosphorylates myosin light chain (MLC). MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, smooth muscle contraction (Ruegg and Paul, 1982; Rasmussen *et al.*, 1987; Clark and Pyne-Geithman, 2005; Klabunde, 2007).

The formation of diacylglycerol (DAG) remains associated with the membrane and activates protein kinase C (PKC). A rise in the cytosolic Ca^{2+}level causes PKC to bind to the cytoplasmic leaflet of the plasma membrane, where it can be activated by the membrane associated DAG. However, for DAG to activate PKC there needs to be an increase of Ca^{2+} which is accomplished by IP_3. PKC can contribute to vascular smooth muscle contraction *via* protein phosphorylation *(see Section 1.5,Figure 1.9)*.

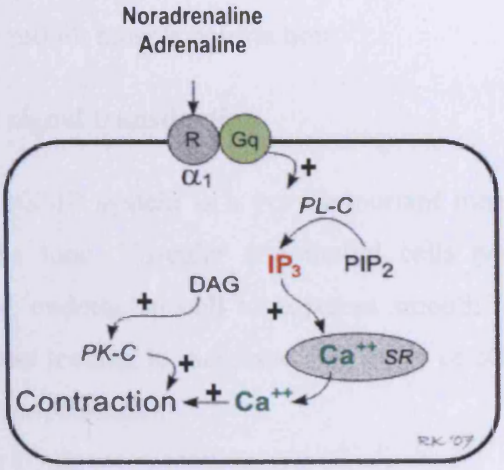


Figure 1.13: Gq protein coupled pathway in vascular smooth muscle.
R = receptor, Gq = phospholipase C-coupled Gq protein, AC = adenylate cyclase, PLC= Phospholipase C, PKC = protein kinase C, IP_3 = inositol 1,4,5-trisphosphate, DAG = diacylglycerol, PIP_2 = Phosphatidylinositol bisphosphate, SR = sarcoplasmic reticulum, β_2 = β-adrenoceptor, (+) = activation, (-) = inhibition. *Adapted from Klabunde (2008)*

Myosin light chain kinase

Myosin light chain kinase (MLCK) is a serine/threonine-specific protein kinase which phosphorylates myosin (Sweeney *et al.*, 1993; Kamm and Stull, 2001). In vertebrates there are two genes for MLCK (Stull *et al.*, 1998). The smooth muscle MLCK gene expresses three transcripts in a cell-specific manner to alternate promoters (Birukov *et al.*, 1998; Smith *et al.*, 1998; Watterson *et al.*, 1999). In skeletal and smooth muscle MLCK is activated by Ca^{2+}-calmodulin (Knighton *et al.*, 1992; Kamm and Stull, 2001) and the enzyme transfers the terminal phosphate group of ATP to serine or threonine-hydroxyl groups of phosphorylatable light chains. Myosin phosphorylation is important for the mechanism of contraction in smooth muscle (De Lanerolle *et al.*, 1984; Kamm and Stull, 1985; Woodrum and Brophy, 2001). Firstly, Ca^{2+} binds to the four Ca^{2+}- binding sites of calmodulin which then interacts with and activate Ca^{2+}/calmodulin-dependent MLCK and phosphorylate the regulatory light chain primarily on serine 19 and to lesser extent on threonine 18 (Poperechnaya *et al.*, 2000; Van Lierop *et al.*, 2002). This phosphorylation enables the myosin crossbridge to bind to the actin filament and allows contraction to begin (Gallagher *et al.*, 1997; Poperechnaya *et al.*, 2000). Since smooth muscle does not contain a troponin complex, unlike striated muscle, this mechanism is the main pathway for regulating smooth muscle contraction..

- **cGMP-coupled signal transduction**

The nitric oxide (NO)-cGMP system is a very important mechanism in regulating vascular smooth muscle tone. Vascular endothelial cells normally produce NO, which diffuses from the endothelial cell to adjacent smooth muscle cells where it activates guanylyl cyclase leading to increased formation of cGMP and vasodilation *(see Section below for Endothelium).*

1.7.4 Endothelium

The endothelium is the inner lining of all blood vessels *(see Section 1.7).* The endothelium mediates contractile and relaxant responses of isolated arteries and veins from animals and humans *(Figure 1.14).* The endothelium-dependent relaxation results from the release by the endothelial cells of potent non-prostanoid

vasodilator substances. Among these, the best characterized is endothelium-derived relaxing factor (EDRF), which most probably is nitric oxide. Nitric oxide is formed by the metabolism of L-arginine by the constitutive nitric oxide synthase of endothelial cells (Furchgott *et al.*, 1981; Collins *et al.*, 1986; Palmer *et al.*, 1988; Olesen *et al.*, 1998). In arterial smooth muscle, the relaxation evoked by nitric oxide is best explained by the stimulation by nitric oxide of soluble guanylate cyclase, which leads to the accumulation of cyclic guanosine monophosphate. The endothelial cells also release prostacyclin and a substance that causes hyperpolarization of the cell membrane (endothelium-derived hyperpolarizing factor, EDHF) (Waldron *et al.*, 1999; Chilian and Koshida, 2001). The release of relaxing factors can be initiated by circulating hormones, such as catecholamines, vasopressin, oxytocin, and estrogens. The release of EDRF from the endothelium can be mediated by either pertussis toxin-sensitive pathways, such as adrenergic activation, serotonin, aggregating platelets, leukotrienes or pertussis toxin-insensitive pathways, such as adenosine diphosphate, bradykinin G proteins (Vanhoutte, 2004).

The short-lived diffusible factor that underlies endothelium-dependent relaxation in response to acetylcholine (Furchgott and Zawadzki, 1980) has been identified as nitric oxide. Endothelial nitric oxide is formed from the guanidine-nitrogen terminal of L-arginine by the action of endothelial constitutive nitric oxide synthase (eNOS). The activation of eNOS depends on the intracellular concentration of calcium ions in the endothelial cells, and is Ca^{2+}-calmodulin-dependent *(Figure 1.14)*.

Nitric oxide diffuses to the underlying smooth muscle cells and, in them, stimulates cytosolic soluble guanylate cyclase, which accelerates the formation of cyclic guanosine monophosphate (cGMP). The cyclic nucleotide in turn inhibits the contractile process. Relaxation of vascular smooth msucle resutls from a decrease in cytosolic Ca^{2+} concentration or reduced Ca^{2+} sensitivity of the contractile apparatus (Sauzeau *et al.*, 2000). The cGMP-induced relaxation involves activation of the cGMP-dependent protein kinase (cGK) (Lohmann *et al.*, 1997; Pfeifer *et al.*, 1998). The cGMP/cGK pathway involves a decrease in cytosolic Ca^{2+} through activation of multiple Ca^{2+} lowering mechanisms (Lincoln and Cornwell, 1993) and Ca^{2+} desensitisation by stimulation of myosin light chain phosphatase (MLCP) activity through unknown mechansims (Wu *et al.*, 1996; Lee *et al.*, 1997).

Nitric oxide is the major contributor to endothelium-dependent relaxation in large arteries (Furchgott and Zawadzki, 1980; Moncada *et al.*, 1991). In the intact organism, both animal and human, the inhibitors of nitric oxide synthase cause vasoconstriction in most vascular beds and an increase in systemic arterial pressure, not only because they prevent the direct inhibitory action of nitric oxide on the vascular smooth muscle, but also because nitric oxide inhibits the production of renin and of endothelin1 (Vanhoutte, 2000) *(Figure 1.14)*.

Nitric oxide is also released in the lumen of the blood vessel. Because it is scavenged by the oxyhaemoglobin of the blood, it does not fulfil a hormonal role. However, at the interface between the blood and the blood vessel wall, it inhibits the adhesion of platelets and white cells to the endothelium. Furthermore, it acts in strong synergy with prostacyclin to inhibit platelet aggregation (Vanhoutte, 1988; Vanhoutte, 1989; Moncada *et al.*, 1991). It also inhibits the growth of the vascular smooth muscle cells and prevents the production of adhesion molecules (Scott-Burden and Vanhoutte, 1993)

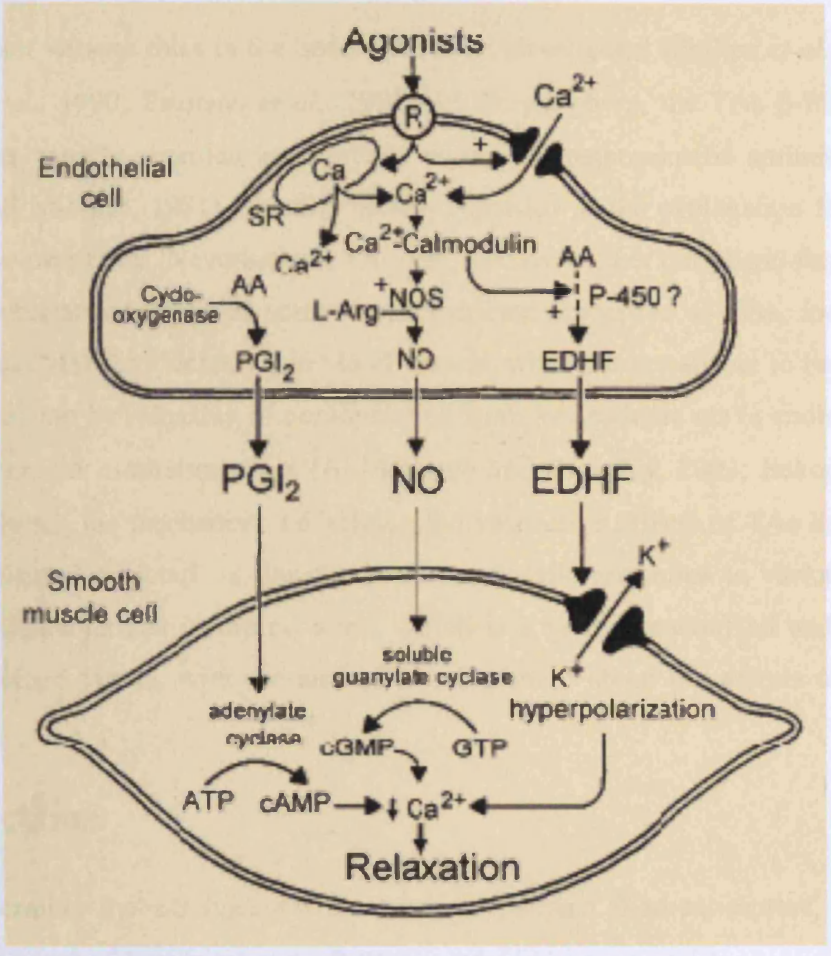


Figure 1.14: Role of the increase in cytosolic calcium concentration in the release of endothelium-derived relaxing factor (EDRF). Endothelial receptor activation induces an influx of calcium into the cytoplasm of the endothelial cell; after interaction with calmodulin, this activates nitric oxide synthase (NOS) and cyclooxygenase, and leads to the release of endothelium-derived hyperpolarizing factor (EDHF). Nitric oxide (NO) causes relaxation by activating the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). EDHF causes hyperpolarization and relaxation by opening potassium (K^+) channels. Prostacyclin (PGI_2) causes relaxation by activating adenylate cyclase, which leads to the formation of cyclic adenosine monophosphate (cAMP). Any increase in cytosolic calcium causes the release of relaxing factors. When agonists activate the endothelial cells, an increase in inositol phosphate may contribute to the increase in cytoplasmic Ca^{2+} by releasing it from the sarcoplasmic reticulum (SR). AA = arachidonic acid, L-Arg = L-arginine, P-450 = cytochrome P-450, R = membrane receptor. *Taken from Vanhoutte (2004)*

TAs and their various roles in the brain have been investigated (Philips *et al.*, 1978; Boulton *et al.*, 1990; Paterson *et al.*, 1990). In the periphery, the TAs β-PEA and tyramine are usually regarded as indirectly acting sympathomimetic amines (ISA) (Aviado and Micozzi, 1981) which is usually regarded as the explanation for their hypertensive properties. Nevertheless, TAs may also have other peripheral functions. Previous publications reported some vasoconstrictor properties of TAs, including cathinone and MDMA ('ecstasy') in blood vessels, which do not appear to be due to an indirect action by releasing of noradrenaline from sympathetic nerve endings nor a direct action on α-adrenoceptors (Al-Motarreb and Broadley, 2003; Baker *et al.*, 2007). However, the mechanism underlying the vasoactive effects of TAs have not been investigated in detail. In this thesis the contractile responses to various TAs have been characterized in the rat aorta, which is a well characterised and easily accessible blood vessel, with the aim of learning more about the effects of these amines.

1.8 Aims

a) Determine the adrenoceptor mechanisms (α- and β-adrenoceptors, uptake pathways) of TAs in rat aorta. β-PEA is used as a standard TA.

b) Investigate the effect of other TAs, including tyramine, octopamine, D-amphetamine and its derivatives in rat aorta.

c) Determine the contractile effect of various TAs in another species, such as guinea-pig aorta

d) Investigate the expression of TAARs in aortic tissues

1.9 Hypothesis

TAs exert vasoconstrictor effects *via* non-adrenergic mechanisms such as trace amine-associated receptors (TAARs).

Chapter 2

EFFECTS OF β-PHENYLETHYLAMINE IN RAT AORTA EXAMINED USING CUMULATIVE CONCENTRATION-RESPONSE CURVES

Chapter 2

Effects of β-phenylethylamine in rat aorta examined using cumulative concentration-response curves

2.1 Introduction

The catecholamine sympathomimetic amines include the classical biogenic amine neurotransmitters, adrenaline, noradrenaline and dopamine. They have important roles as neurotransmitters in the central and peripheral nervous system and their effects are mediated through interaction with G protein-coupled receptors (GPCRs) of the α- and β-adrenoceptor subtypes (Premont *et al.*, 2001).

A second class of endogenous amine compound, called trace amines (TAs), includes β-phenylethylamine (β-PEA), tyramine and octopamine, which also behave as sympathomimetic amines (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001; Lindemann *et al.*, 2005; Lewin, 2006). The TAs are unique, as they are present in very small (trace) concentrations in the mammalian brain (Borowsky *et al.*, 2001; Burchett and Hicks, 2006; Zucchi *et al.*, 2006). They are structurally and functionally related to the catecholamines and there are a large number of synthetic analogues, such as the amphetamines (Bunzow *et al.*, 2001; Branchek and Blackburn, 2003; Lindemann *et al.*, 2005). TAs are primary amines generated directly by enzymatic decarboxylation of their respective precursor amino acid (Boulton and Dyck, 1974; Tallman *et al.*, 1976; Borowsky *et al.*, 2001; Bunzow *et al.*, 2001; Lindemann *et al.*, 2005). They are produced by many organisms; invertebrates, mammals, plants, bacteria and insects. TAs are also found in dietary substances such as chocolate (β-PEA), cheese and wine (tyramine) (Branchek and Blackburn, 2003; Lindemann *et al.*, 2005).

In the periphery they cause an increase in blood pressure (BP) and tachycardia. The increase in BP is due to vasoconstriction usually regarded as arising from their indirectly acting sympathomimetic activity (ISA). Through indirect stimulation, TAs cause an increase of noradrenaline release from sympathetic neurons and therefore exert vasoconstriction *via* α_1-adrenoreceptors (Burchett and Hicks, 2006). TAs promote the efflux of noradrenaline from the synaptic vesicles of sympathetic

neurons following their uptake into the adrenergic nerve terminal. Noradrenaline interacts with adrenoceptors to produce sympathomimetic effects (Schonfeld and Trendelenburg, 1989; Broadley, 1996). However, there is some evidence to suggest that this is an oversimplification. For example, tachyphylaxis is a characteristic feature of ISAs in that repeated dosage results in a gradual decline of the response (Day, 1967). This is probably due to progressive exhaustion of stored noradrenaline, which is replaced in the vesicle by the less active TAs. ISAs show different degrees of tachyphylaxis and responses are generally not completely abolished. Also there is a notable lack of cross-tachyphylaxis between certain TAs (Day, 1967). No satisfactory explanation for these discrepancies has been put forward (Broadley, 1996). Therefore, the possibility exists that another mechanism may also be involved in these responses. For example, previous work from these laboratories has shown that the amphetamines, cathinone, the active constituent of khat leaves, and 3,4-methylenedioxymethamphetamine (MDMA) cause coronary vasoconstriction through a mechanism not involving α_1-adrenoceptors or cocaine-sensitive neuronal uptake (Al-Motarreb *et al.*, 2002b; Al-Motarreb and Broadley, 2003; Baker *et al.*, 2007). Furthermore, these responses are not prevented by antagonists of angiotensin, endothelin, 5-hydroxytryptamine, prostaglandin or leukotriene (Baker and Broadley, 2003). Thus, none of these vasoconstrictor autacoids or their receptors can explain the vasoconstriction. Therefore the possibility arises that the vasoconstriction is due to an as yet unidentified receptor. In this *Chapter,* these studies with MDMA and cathinone have been extended to a more classical TA, β-PEA, to investigate the mechanisms of its vasoconstrictor actions on rat aortic rings.

2.2 Aims

To study the effect of trace amines (TAs) employing cumulative concentration-response curves (CRCs) to investigate the contractile responses of rat isolated aortic rings to β-PEA in the presence of a range of different inhibitors.

a.) Determine whether β-PEA produces vasoconstriction in the rat isolated aortic tissue

b.) To investigate the involvement of endothelium to the contractile response to TAs in rat aortic rings

c.) Determine whether any vasoconstriction seen to β-PEA is mediated by sympathomimetic actions

d.) To investigate the possible receptor type mediating this response in rat aortic rings

2.3 Methods and Material

This *Chapter* details the materials and methods used for all the experimental work described in the thesis. Subsequent *Chapters* will provide a brief outline of the methods used together with any specific details of the protocols.

2.3.1 Animals

Male *Sprague-Dawley* rats (250-300g) were obtained through the Joint Animal Services department of Cardiff University from Harlan (Blackthorn, Bicester, Oxfordshire, U.K.). Rats were maintained under conventional animal housing conditions following a 12-hour light-dark cycle (8 a.m.-8 p.m.) and at an ambient room temperature of 21 ± 2°C and humidity of 55 ± 10%. Animals received food and drinking water *ad libitum*. The welfare of the animals was carried out by experienced technicians and all experiments described in this thesis observed strict compliance to the Animals (Scientific Procedures) Act 1986.

2.3.2 Main Methods

The male rats were killed by cervical dislocation and exsanguination. The thoracic and abdominal aorta was removed *(Figure 2.1)* and cut into ring sections, through which were passed a fixed hook and a mobile hook. The fixed hook was secured in a 50ml organ bath. The bath was filled with pre-warmed (37°C) Krebs-bicarbonate buffer gassed with CO_2/O_2 (5%/95%) *(Figure 2.2 a, b, c)*.

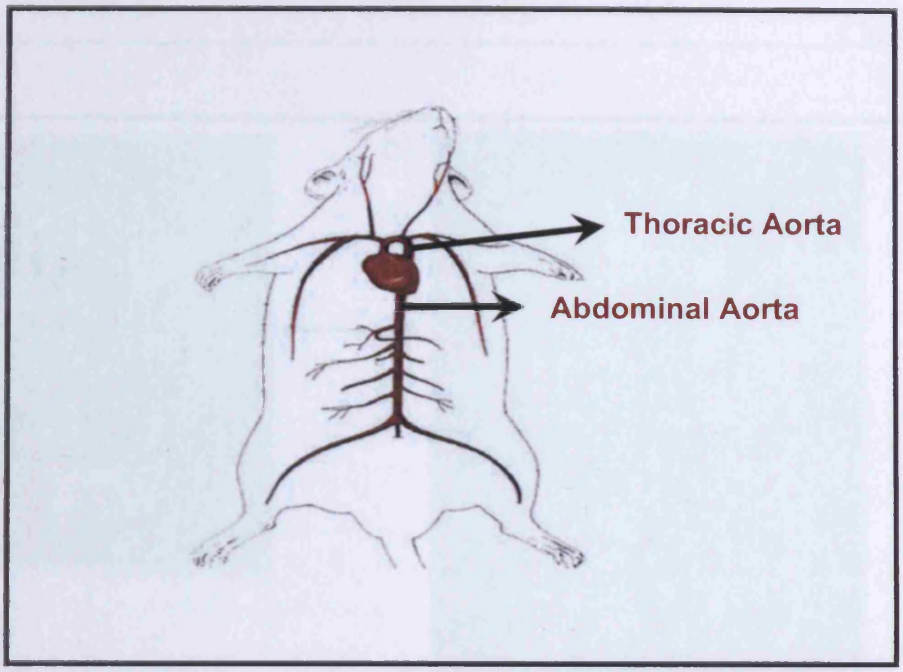


Figure 2.1: Rat circulatory systems.
The thoracic and abdominal parts of the aorta were used for the study. *Adapted from Biology corner (2005).*

Tissue Bath Set Up

A suture attached to the upper mobile hook was connected to an isometric transducer (Dynamometer UF1, 57g sensitivity range, Pioden Controls Ltd. Canterbury) and a resting tension of 1.5g was applied. Isometric tension was measured and displayed on a computer (Power Lab, Chart 5, AD Instruments) (Chalgrove, Oxfordshire, UK) (Fig. 2.2 c). The Krebs bicarbonate buffer was made up in distilled water and had the following composition (mM): NaCl (100), $NaHCO_3$ (20), glucose (10), $MgSO_4.7H_2O$ (1), KH_2PO_4 (1), KCl (5) and $CaCl_2.2H_2O$ (3). Organ baths were maintained at 37±0.5°C by a Grant Circulator (Grant Instruments, Cambridge, UK). To prepare 60mM isotonic Krebs solution the amount of NaCl was decreased to 43mM and the amount of KCl was increased to 60mM. The organ bath and reservoir were continuously aerated with a CO_2/O_2 (5%/95%) mixture (BOC Gases, Guildford, UK).

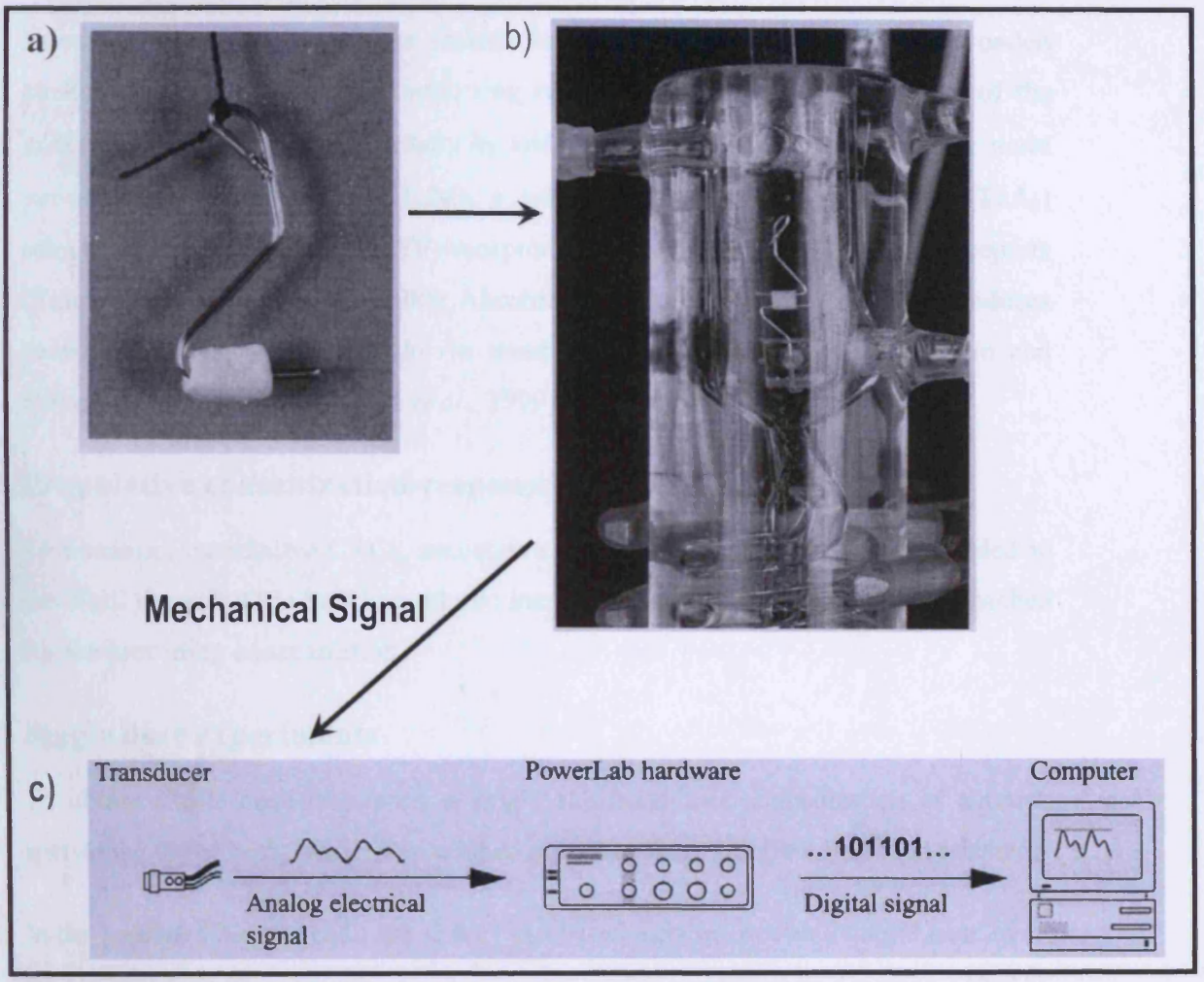


Figure 2.2: Tissue bath set up
A) Rat aorta ring on holder, before placing in organ bath. **B)** Organ bath with rat aorta ring in place, held on a fixed holder and the suspended one.**C)** Measurement and analysis of the isometric tension via an isometric transducer. A suture attached to the upper mobile hook was connected to an isometric transducer. The results were shown on a computer. *Adapted from Power Lab, Chart 5, AD Instruments.*

2.3.3 Experimental Protocol

After approximately 60 minutes equilibration time, Krebs solution containing KCl (60mM, isotonic solution) *(Section 2.3.2)* was added to contract the tissue and test for viability. After wash out and equilibration, cumulative concentration-response curves (CRCs) for β-PEA were obtained in the absence or presence of different inhibitors, which were incubated with the tissue for 15 minutes before commencing the CRC unless otherwise stated. At the end of each experiment, KCl (60mM, isotonic solution) was routinely added after washout of the final drug concentration.

In some of the experiments the endothelium was removed by inserting a wooden cocktail stick and rolling the aortic ring round it. The absence or presence of the endothelium was confirmed initially by adding acetylcholine (Ach, 100μM) to aorta pre-contracted with U46619 (1μM), a selective thromboxane A_2 agonist (TxA_2) selective for thromboxane A_2 (TP) receptors and prostaglandin H_2 (PGH_2) receptors (Trachte, 1986; Smith *et al.*, 1988; Alexander *et al.*, 2008). Acetylcholine produces vasodilatation in smooth muscle *via* muscarinic-receptors on the endothelium and release of nitric oxide (Waldron *et al.*, 1999) *(Figure 2.5 a, b)*.

Cumulative concentration-response curves (CRCs)

To construct cumulative CRCs, successive concentrations of agonist were added to the 50ml tissue bath in half logarithmic increments after the peak effect was reached for the preceding concentration.

Single dose experiments

To obtain single dose responses, a single sub-maximum concentration of agonist, was added to the bath, which was washed out after the peak effect had been reached.

In the present *Chapter*, each tissue was incubated only once with a single dose of β-PEA (30μM) in the absence, or presence of various antagonists (cocaine (10μM), prazosin (1μM) and propranolol (1μM)).

2.3.4 Analysis of Results

Contractions at the plateau response to each concentration of agonist were measured from the baseline before the CRC. These were then expressed as a percentage of the contraction to KCl in each experiment and the mean responses (± SEM) plotted. N values represent the number of rat aortas.

-log EC_{50} values of individual CRCs were calculated in *GraphPad Prism 4* and the mean (-) log molar EC_{50} value calculated together with the SEM. The EC_{50} value is the molar concentration of an agonist which produces 50% of the maximum possible response for that agonist. Mean (-) log molar EC_{50} values were compared by paired or unpaired Student's t-test or by Analysis of Variance (ANOVA) followed by a post-hoc test, such as Bonferoni and Dunnett, using *GraphPad Instat 3*.

2.3.5 Drugs Used

All chemicals were dissolved in distilled water, unless otherwise stated. Prazosin hydrochloride and U46619 were dissolved in DMSO.

- **Reagents obtained from Sigma Aldrich (Poole, Dorset, UK)**

Acetylcholine chloride, cocaine hydrochloride, prazosin hydrochloride, (±)-propranolol hydrochloride, (±) noradrenaline hydrochloride, β-phenylethylamine (β-PEA) and U46619 (9,11 dideoxy-11α, 9α epoxymethano-prostaglandine F2α) and indomethacin (prepared in water)

- **Reagents obtained from Fisher Scientific (Leicestershire, UK)**

All chemicals for the Krebs-bicarbonate buffer (analytical grade)

- **Reagents obtained from TOCRIS Biosciences (Northpoint, Avonmouth, UK)**

L- N^G- nitro-arginine methyl ester hydrochloride (L-NAME)

2.4 Results

2.4.1 Vasoconstriction in rat aortic rings to β-PEA (cumulative CRCs)

The addition of low concentrations of β-PEA in cumulative CRCs caused concentration-related contractions *(Figure 2.3)*.

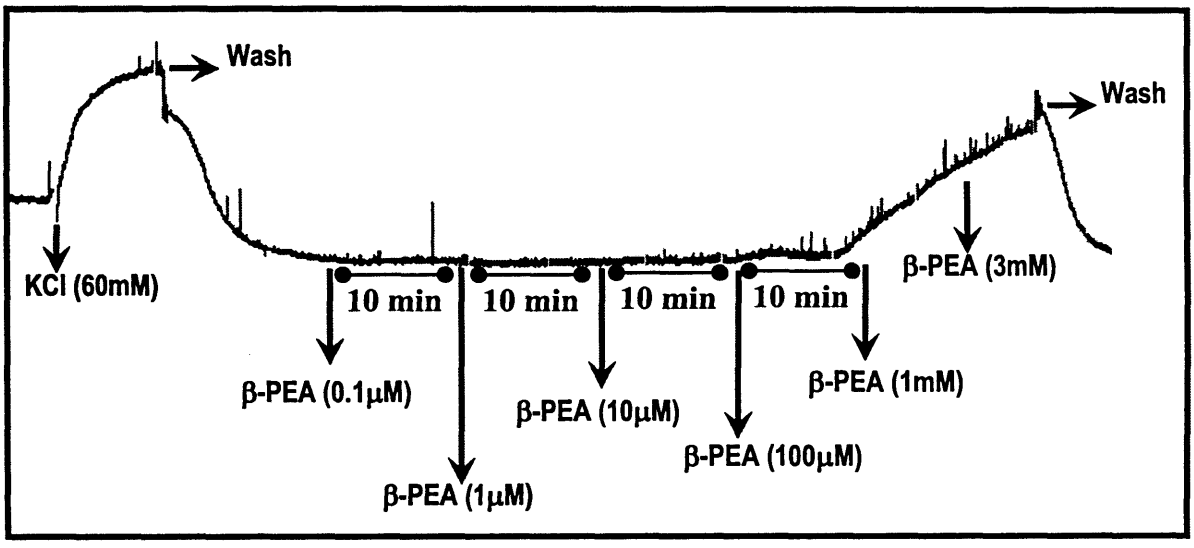


Figure 2.3: Representative chart recording showing a cumulative concentration-response curve (CRC) for the contractile response of rat aorta to β-phenylethylamine (β-PEA). At the beginning KCl (60mM, isotonic solution) was added to contract the tissue and test for viability. "Wash" = washout of tissue bath.

2.4.2 Contractile response to β-PEA - effect of different parts of the rat aorta and endothelium

β-PEA caused concentration-related contractions of the rat aorta *(Figure 2.3)*. Tissues taken from the abdominal or thoracic part of the aorta were shown to produce similar contraction to β-PEA. The contractile response at the maximum concentration of β-PEA of the abdominal part of the aorta (300μM, 58.7±13.1%, n=5) was not significantly different (P>0.05) from the corresponding response to β-PEA of the thoracic part of the aorta (300μM, 45.7±14.1%, n=4) *(Figure 2.4)*. All further experiments were therefore conducted using either abdominal or thoracic aorta. The contractile response of rat aorta to β-PEA was independent of the presence of endothelium *(Figure 2.5)*.The response at the maximum concentration of β-PEA (3mM, 197.9±21.1%, n=4) after endothelium removal was not significantly different

(P>0.05) from that in aorta with the intact endothelium (3mM, 145±44.8%, n=5). The -log EC_{50} values for the β-PEA of 4.17±0.27 in denuded and 3.94±0.27 in intact tissues were not significantly different (P>0.05) *(Figure 2.5)*. To test for the presence or absence of endothelium, responses to acetylcholine were examined in aorta pre-contracted with U46619 (1μM) *(Figure 2.6 a, b)*.

2.4.3 Contractile response to β-PEA - effect of L-NAME

The contractile response to β-PEA in the presence or absence of the endothelium was not affected by the presence of the nitric oxide synthase inhibitor, L-NAME (100μM). In endothelium-denuded tissues, the response to the maximum concentration of β-PEA in the presence of L-NAME (3mM, 224.7±97.4%, n=6) was not significantly different (P>0.05) from the response to β-PEA in endothelium-intact tissues (3mM, 161.1±49.1%, n=6) *(Figure 2.7)*. Furthermore, in the absence of the endothelium, the response at the maximum concentration of β-PEA in presence of L-NAME (3mM, 224.7±97.4%, n=6) was not significantly different (P>0.05) from the contractile response to β-PEA without L-NAME (3mM, 197.9±21.1%, n=4) *(Figure 2.8)*. Finally, in endothelium-intact tissues, the response at the maximum concentration of β-PEA in the presence of L-NAME (3mM, 161.1±49.1%, n=6) was also not significantly different (P>0.05) from the corresponding response to β-PEA without L-NAME (3mM, 145±44.8%, n=5) *(Figure 2.9)*.

2.4.4 Possible relaxation responses to β-PEA in the absence or presence of endothelium

Next, β-PEA was examined to determine if it could cause a relaxation in aortic rings pre-contracted with U46619 (1μM). β-PEA in the presence of U46619 (1μM) showed no relaxation in the presence or absence of endothelium. However, pre-contracted aortic rings showed a further contractile response to β-PEA *(Figure 2.10)*. The additional contractile response of endothelium-intact aortic rings to the maximum concentration of β-PEA (3mM, 120.7±21.9%, n=5) was significantly greater (P<0.05) than the response to β-PEA in the absence of the endothelium (3mM, 53.3±3.3%, n=5).

However, the -log EC_{50} values for β-PEA in endothelium-intact tissues (4.46±0.19) and in endothelium-denuded tissues (5.4±0.20) were not significantly different (P>0.05) *(Figure 2.10)*.

Pre-contracted and endothelium-denuded aortic rings showed a significantly (P<0.05) reduced contractile response to the maximum concentration of β-PEA (3mM, 53.3±3.3%, n=5) compared to the contractile response at the corresponding concentration in non pre-contracted, endothelium-denuded tissues (3mM, 197±21.1%, n=4) *(Figure 2.11)*. In endothelium-intact tissues, no significantly difference (P>0.05) of the contractile response to β-PEA was found between pre-contracted (3mM, 121±22%, n=4) and non pre-contracted tissues (3mM, 168.8±49%, n=4) *(Figure 2.12)*. In U46619 pre-contracted tissues to measure the reduced contraction (vasodilatation) the baseline was taken after U46619 contractions.

The contraction to U46619 (1μM, 164.6±43.6%, n=4) in endothelium-intact tissues was not significantly (P>0.05) different from the corresponding response to U46619 (1μM, 108.3±8.3%, n=4) in endothelium-denuded tissues *(Figure 2.13)*.

2.4.5 Contractile response to β-PEA - effect of indomethacin

The contractile response of rat aorta to β-PEA in the presence of indomethacin (10μM) was tested. β-PEA caused concentration-related contractions of the rat aortic rings which were not significantly (P>0.05) displaced by indomethacin (10μM). The contractile response at 100μM of β-PEA (64.2±9.4% n=3) was not significantly different (P>0.05) from the corresponding response to β-PEA with indomethacin (100μM, 52.7±15.1%, n=6). The -log EC_{50} values for β-PEA in the absence (4.62±0.13) and in the presence of indomethacin (4.88±0.15) were not significantly different (P>0.05) *(Figure 2.14)*.

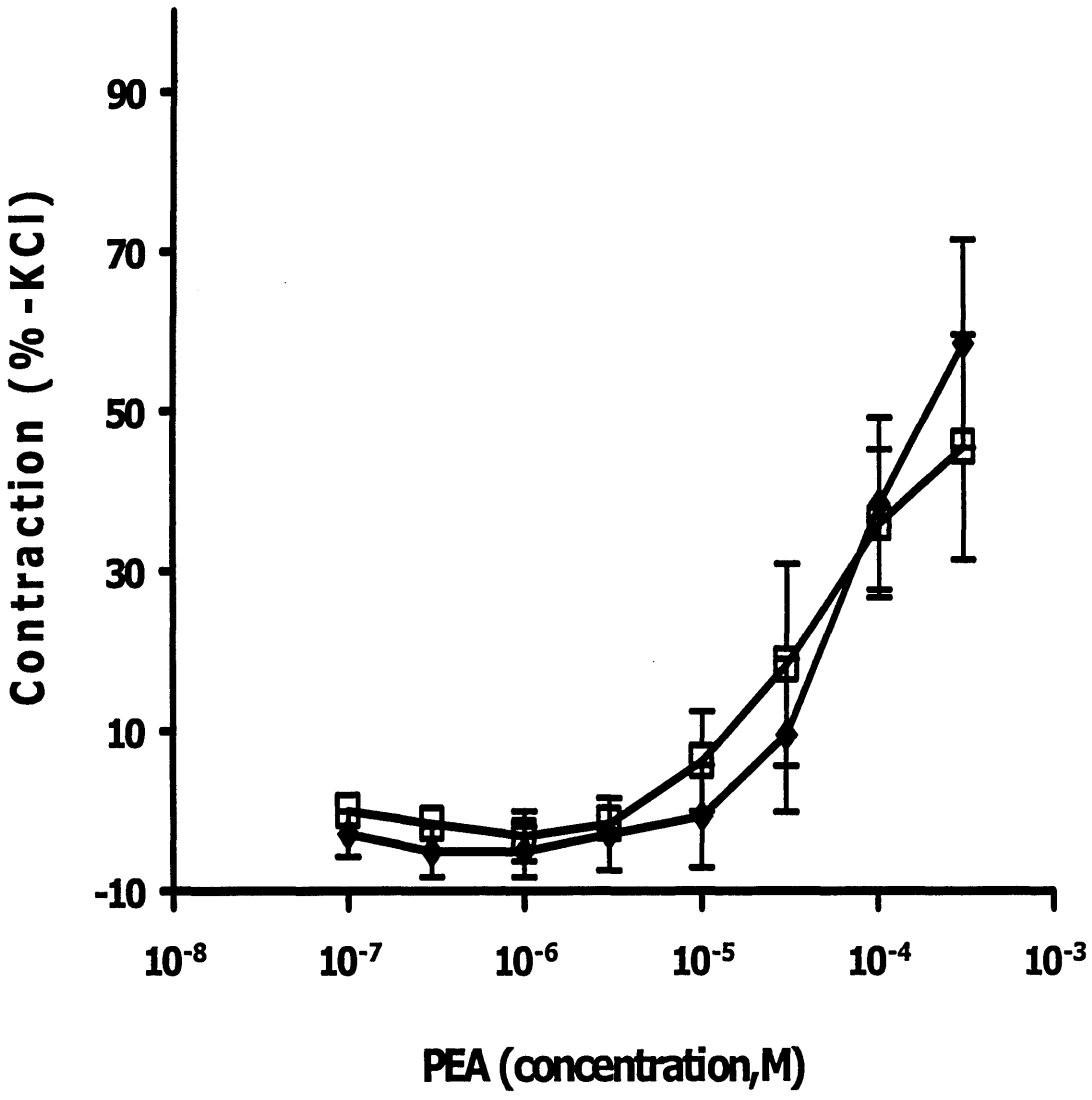


Figure 2.4: Mean cumulative CRCs for the contractile response of thoracic (□, n=4) and abdominal (◆, n=5) regions of rat aorta to β-PEA. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant (P>0.05) differences were seen at their maximum concentration (3mM).

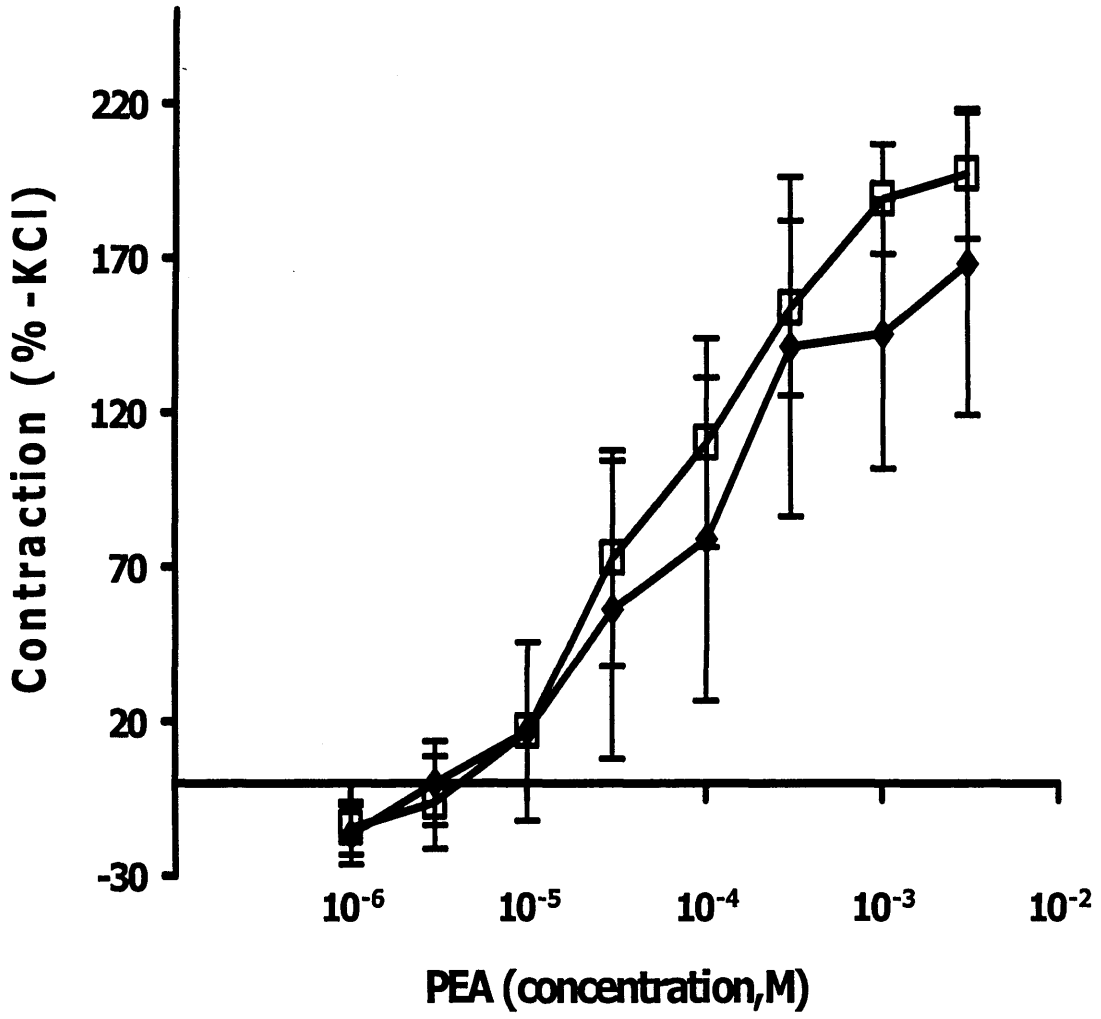


Figure 2.5: Contractile effects in rat aortic rings to β-PEA Cumulative CRCs for β-PEA in rat aorta were constructed in the presence (◆, n=4) or absence (□, n=4) of endothelium. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Endothelium was removed by inserting a cocktail stick and rolling the aortic ring round it. Mean responses (±SEM) were compared by Student's paired t-test. No significant ($P>0.05$) differences were seen at their maximum concentration (3mM).

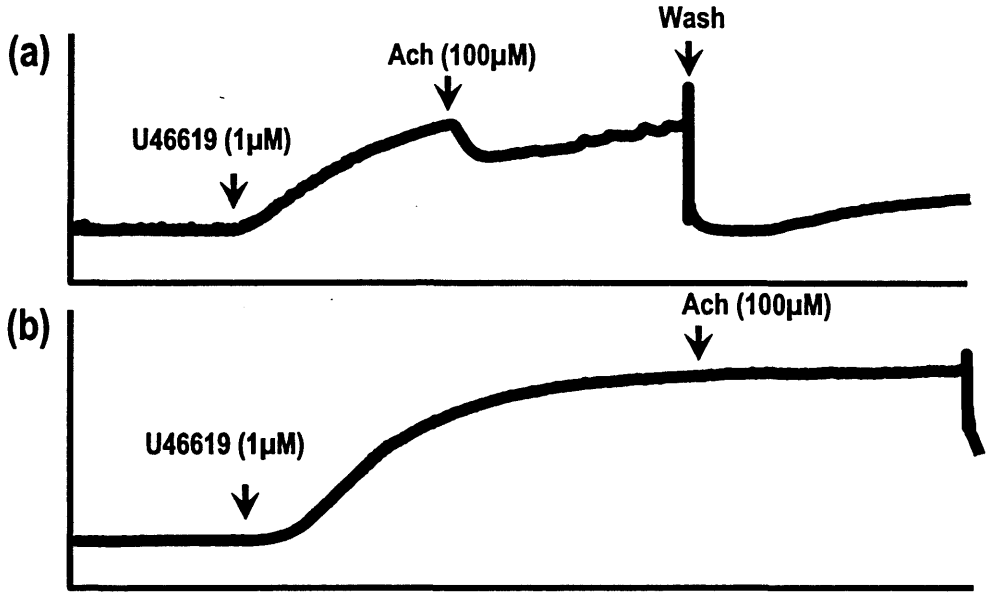


Figure 2.6: Typical traces showing the effect of acetylcholine (Ach, 100μM) added to rat aortic rings pre-contracted with U46619 (1μM), in the presence (a), or absence (b) of endothelium. The mechanical removal of endothelium was successfully shown by the absence of relaxation to Ach (100μM) in U46619-precontrated (1μM) aortic rings (b).

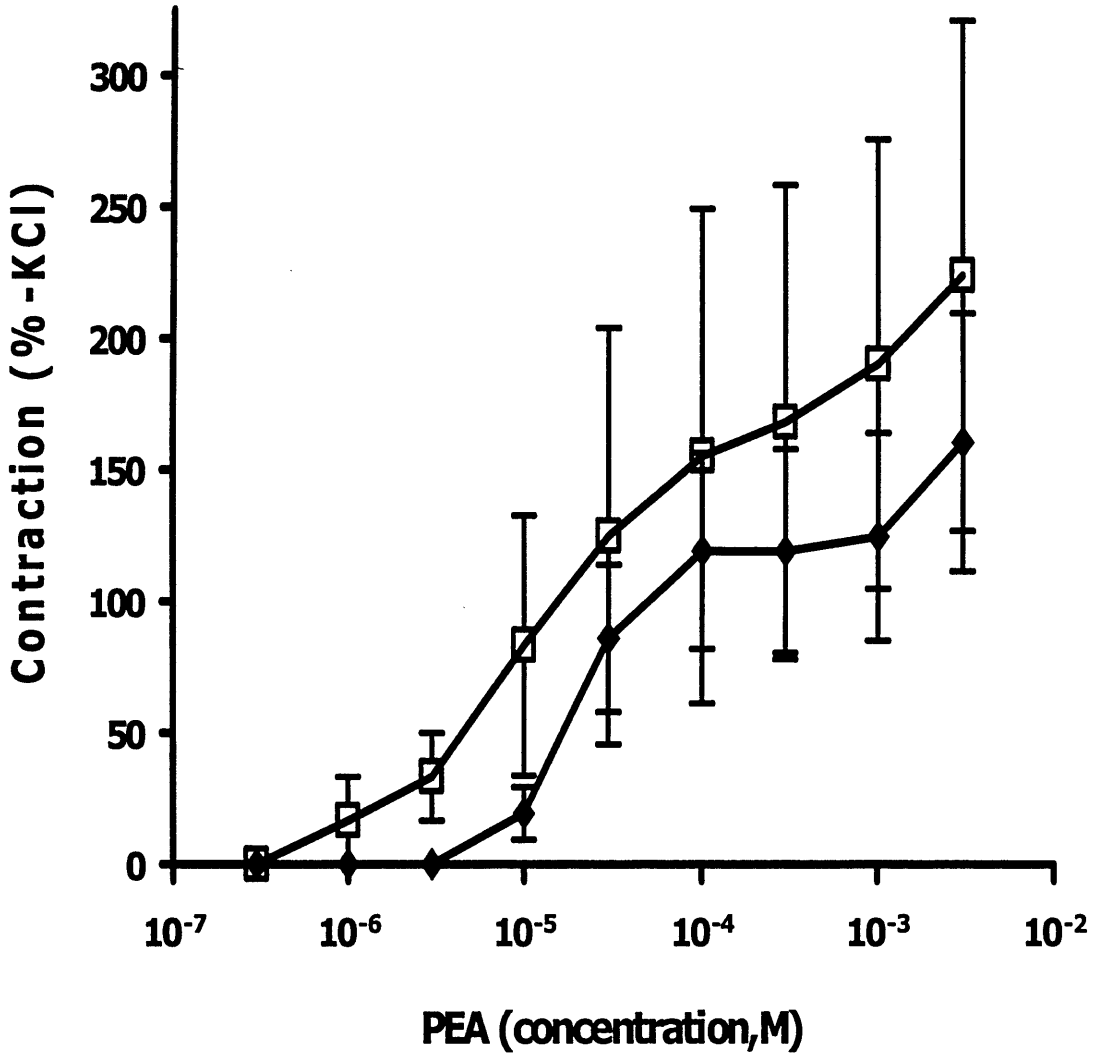


Figure 2.7: Effects of L-NAME (100μM) and endothelium removal on the mean CRCs for the contractile responses in rat aorta to β-PEA. Cumulative CRCs were constructed in the presence of L-NAME (100μM) and in endothelium-intact tissues (◆, n=6) or in the presence of L-NAME (100μM) and in endothelium-denuded tissues (□, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant ($P>0.05$) differences were seen.

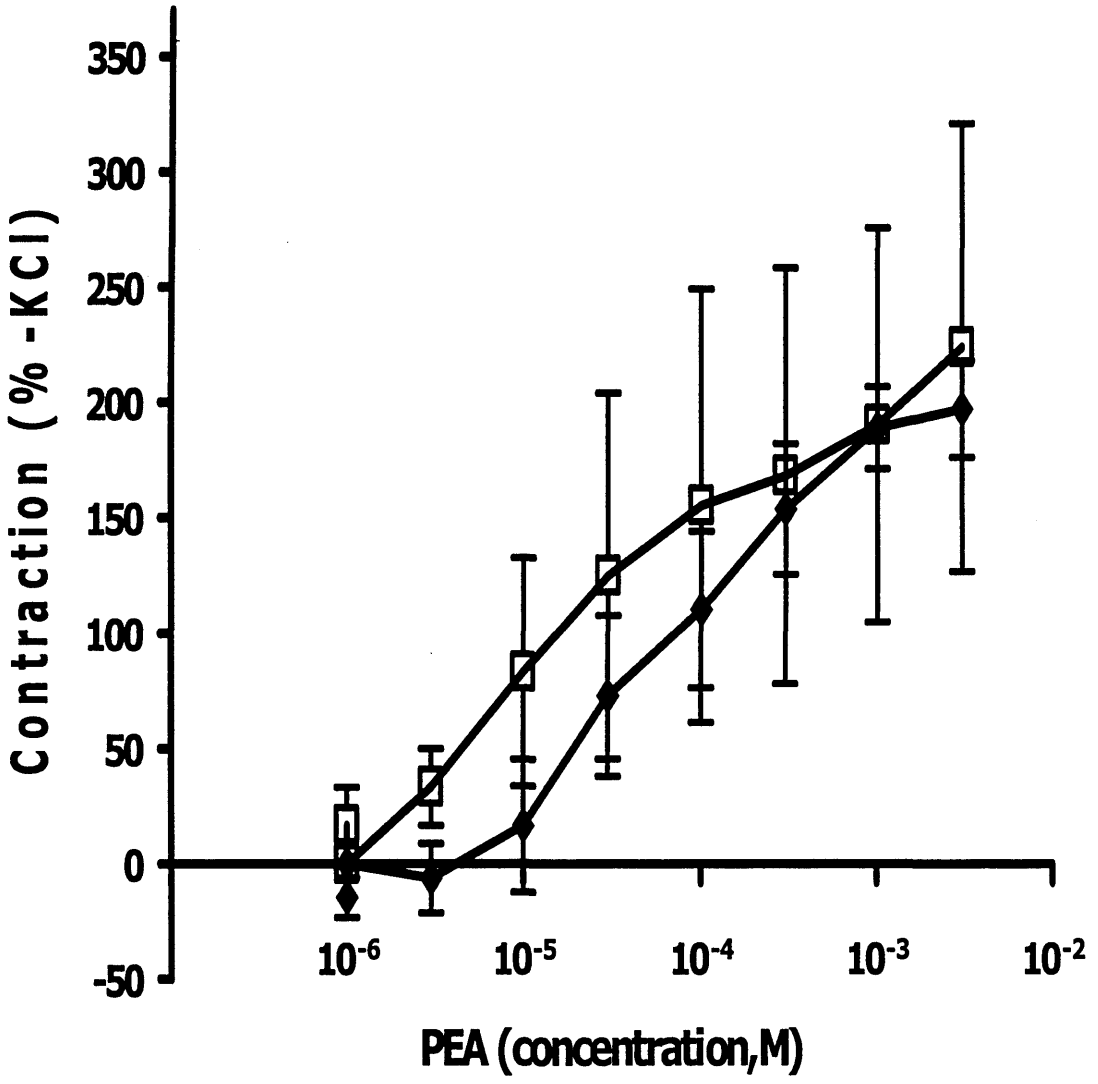


Figure 2.8: Mean CRCs for the contractile response in rat aorta to β-PEA in the absence of endothelium and in the presence or absence of L-NAME (100μM). Cumulative CRCs were constructed in the presence of L-NAME (100μM) and in the absence of endothelium (□, n=6) or in the absence of L-NAME and endothelium (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant (P>0.05) differences were seen.

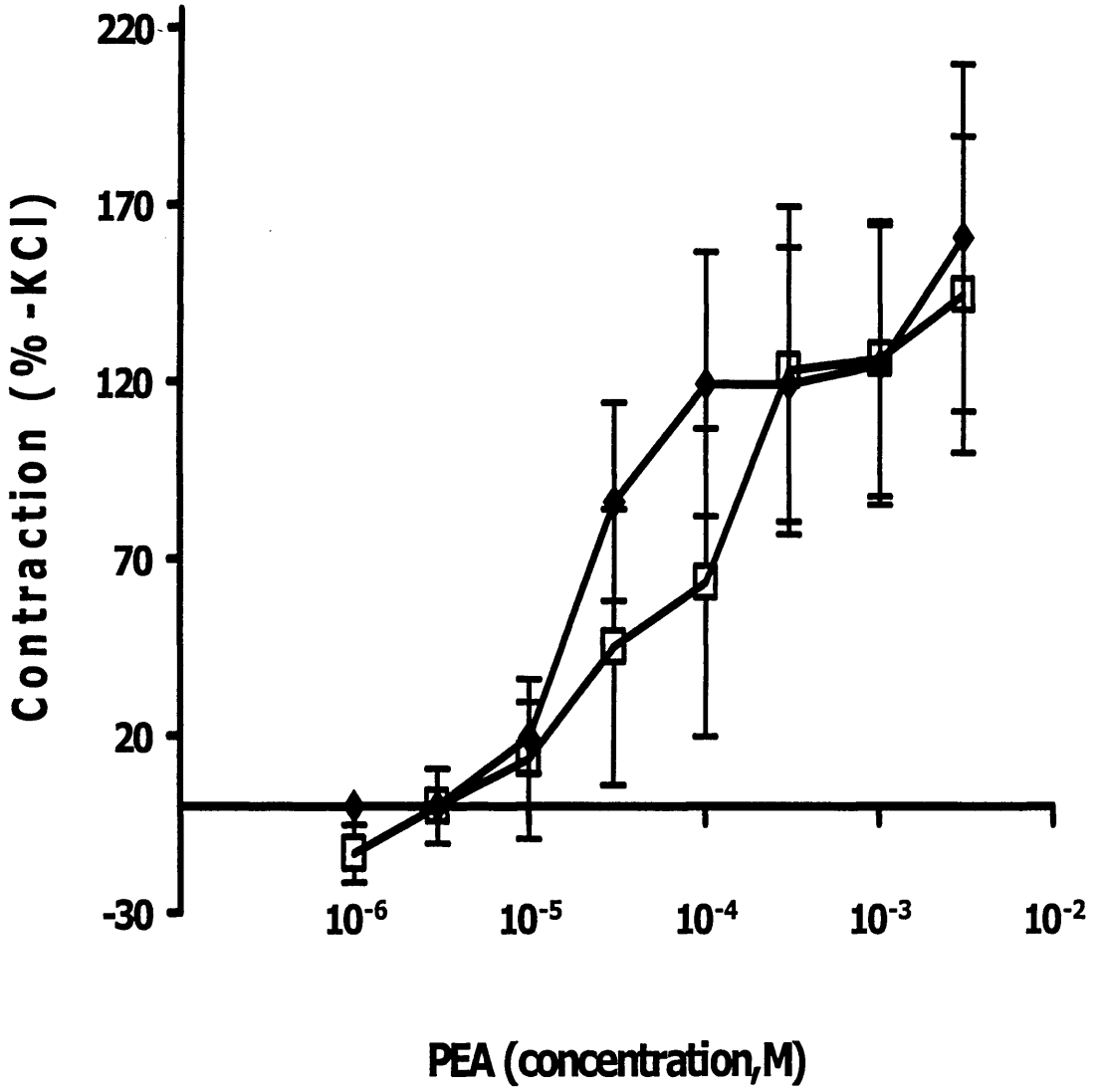


Figure 2.9: Effects of L-NAME (100μM) on the mean CRCs for the contractile response in rat aorta to β-PEA. Cumulative CRCs were constructed in the presence of L-NAME (100μM) and endothelium (◆, n=6) or in the absence of L-NAME and the presence of endothelium (□, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant (P>0.05) differences were seen at their maximum concentration (3mM).

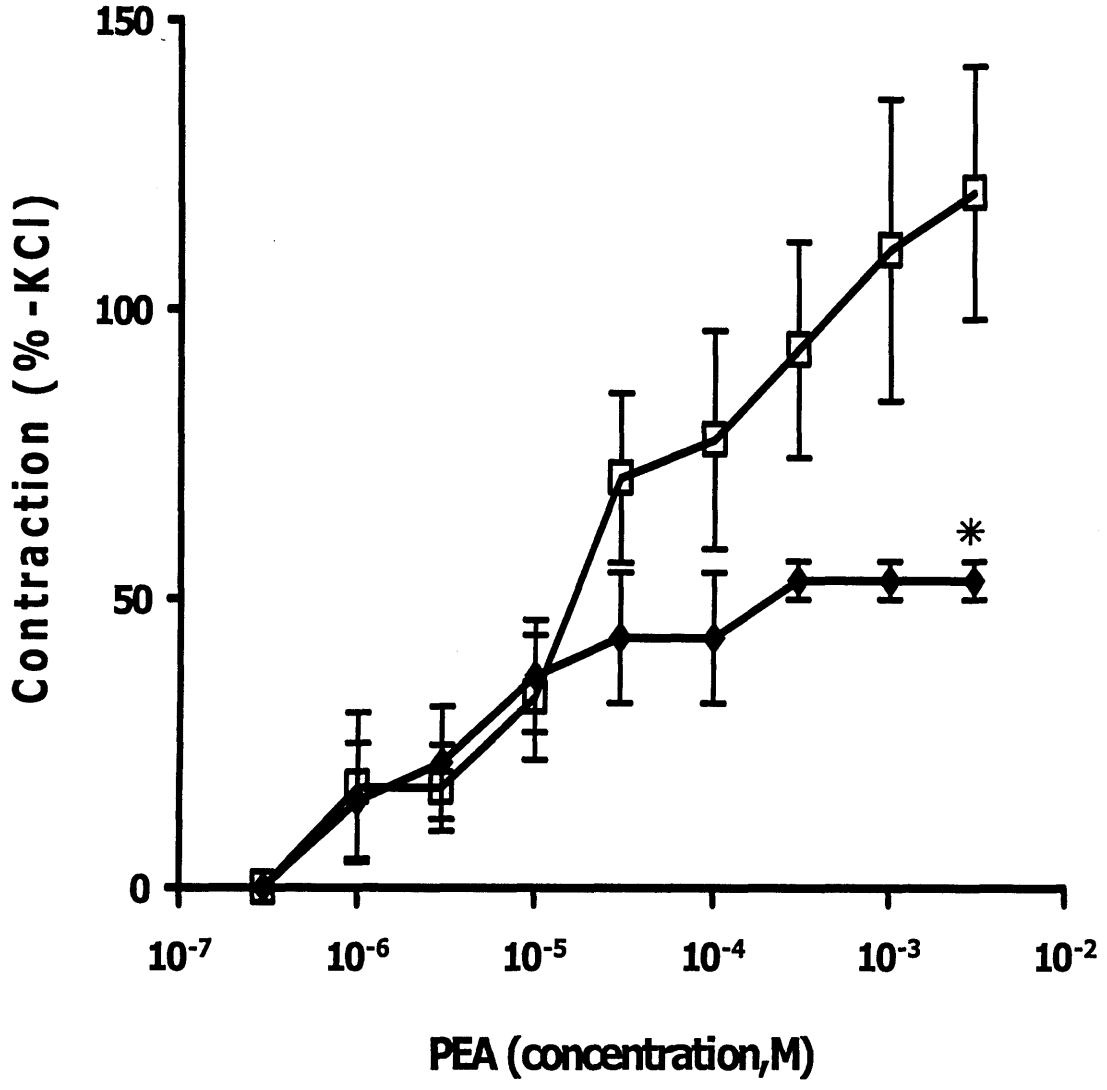


Figure 2.10: Contractile effect on β-PEA on pre-contracted (U46619, 1μM) rat aortic rings. Cumulative CRCs were constructed in the presence (□, n=5), or absence of endothelium (◆, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. A significant difference was seen at the maximum concentration (3mM).

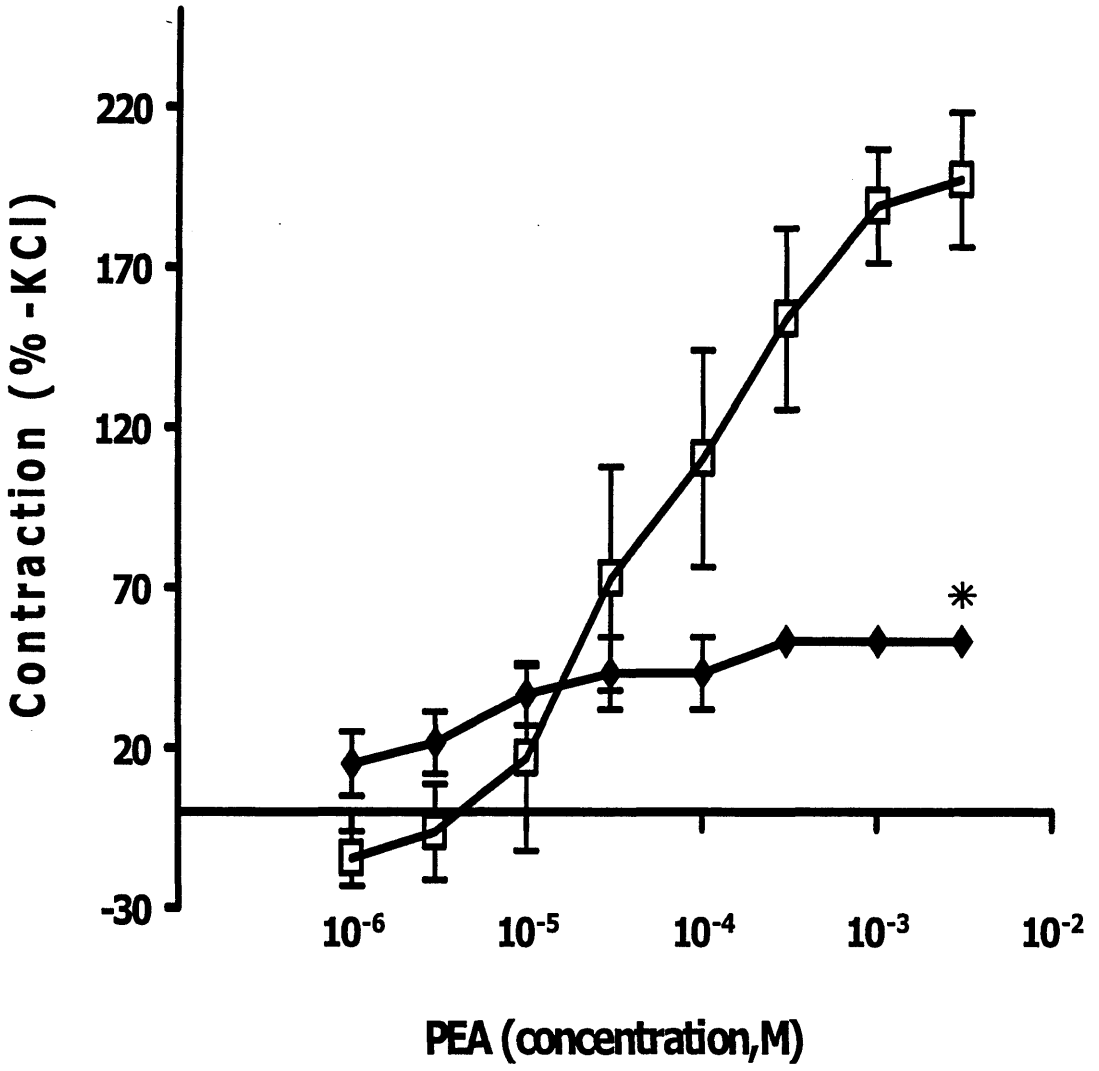


Figure 2.11: Contractile effect of β-PEA on pre-contracted (U46619, 1μM) and none pre-contracted rat aortic rings. Cumulative CRCs were constructed in pre-contracted and endothelium-denuded tissues (◆, n=5) or in non pre-contracted endothelium-denuded tissues (□, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. A significant difference was seen at the maximum concentration (3mM).

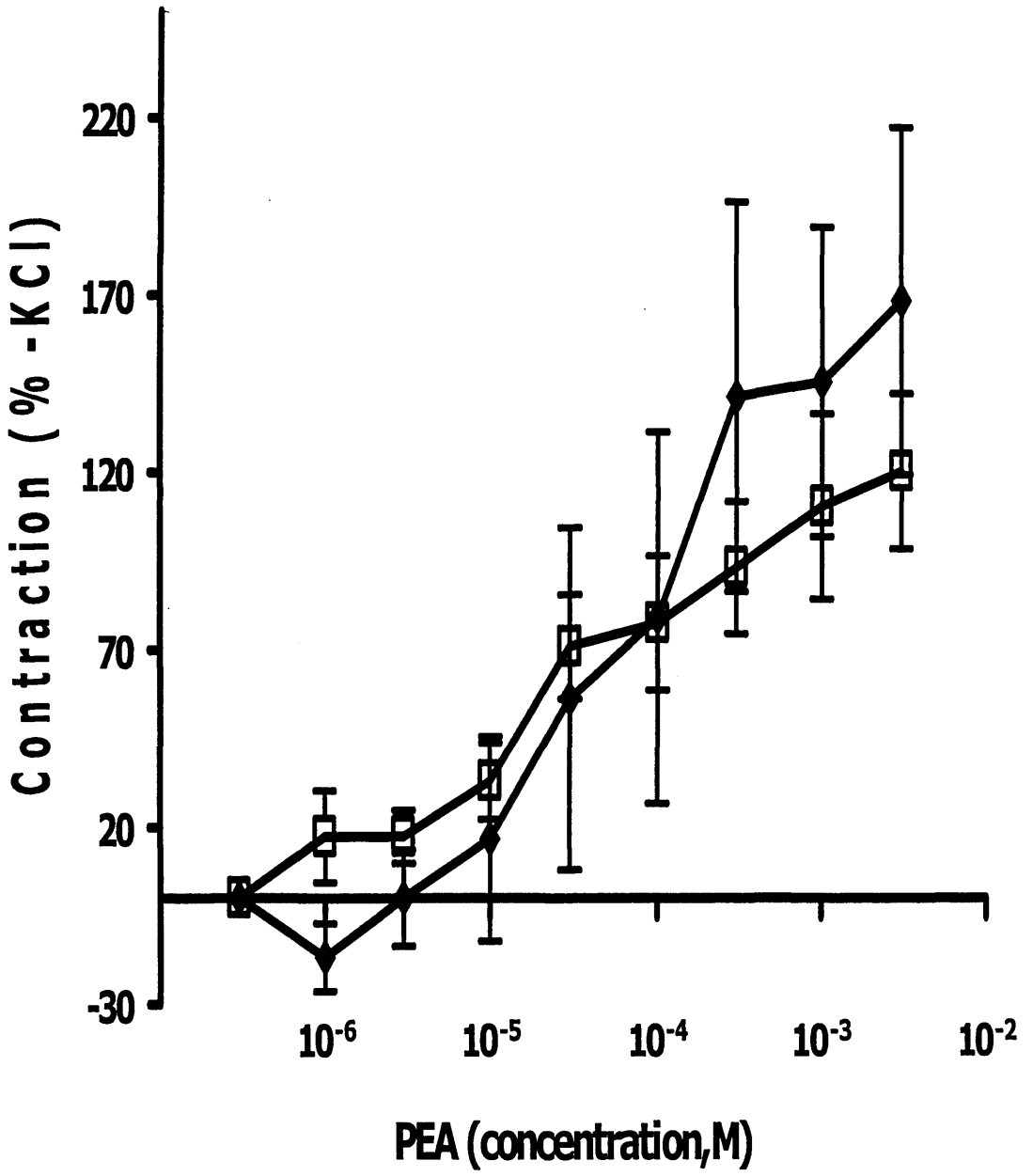


Figure 2.12: Contractile effect of β-PEA on pre-contracted (U46619, 1μM) and non-pre-contracted rat aortic rings. Cumulative CRCs were constructed in pre-contracted and endothelium-intact tissues (◆, n=5) or in non pre-contracted, endothelium-intact tissues (□, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant differences were seen at their maximum concentration (3mM).

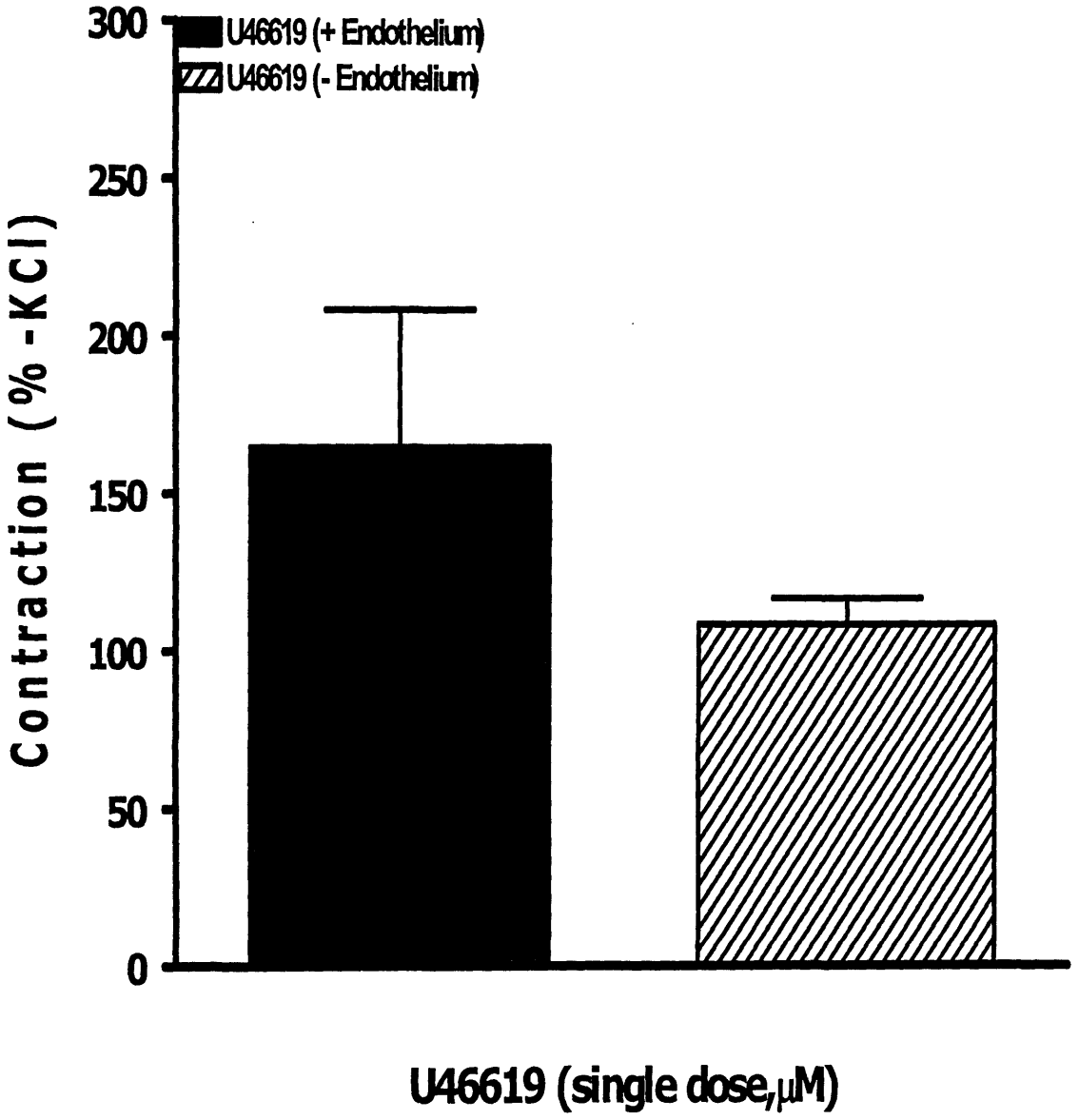


Figure 2.13: Contractile effect of U46619 (1μM) in rat aortic rings. U46619 (1μM) was tested in endothelium-denuded (-Endothelium, n=4) or endothelium-intact (+Endothelium, n=4) tissues. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant ($P>0.05$) difference was seen.

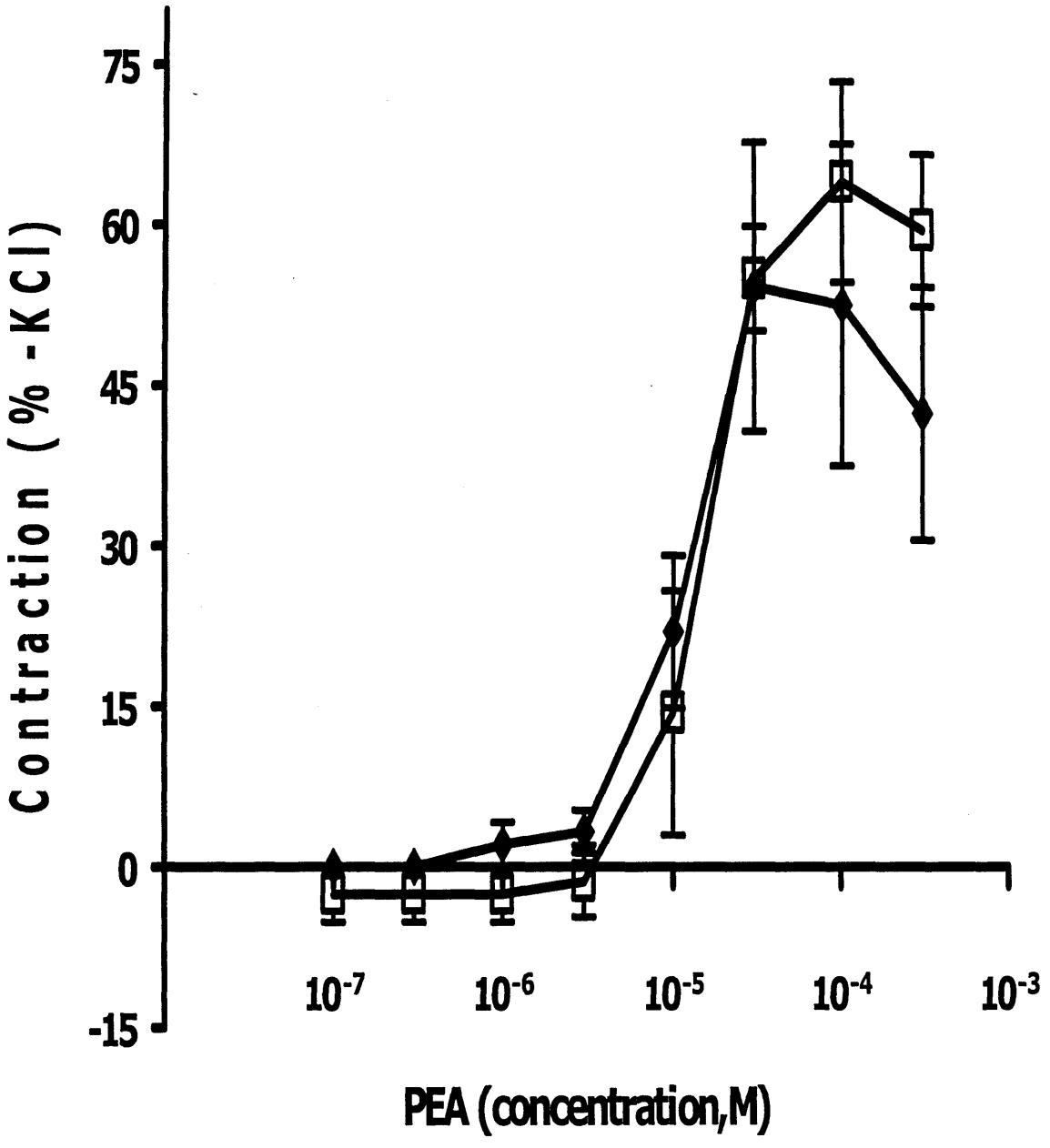


Figure 2.14: Effect of indomethacin on the contractile response to β-PEA on rat aortic rings. Cumulative CRCs were constructed in endothelium-denuded tissues in absence (□, n=3), or presence (◆, n=6) of indomethacin (10μM). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant ($P>0.05$) differences were seen at the maximum contraction (100μM).

2.4.6 Contractile response to noradrenaline - effect of adrenergic inhibitors

To establish suitable concentrations of cocaine (10μM), an Uptake1 inhibitor, prazosin (1μM), a selective α_1-adrenoceptor antagonist and propranolol (1μM), a non-selective β-adrenoceptor antagonist, they were examined against contractions to noradrenaline prior to evaluation against β-PEA.

Noradrenaline caused concentration-related contractions of rat aortic rings (30μM, 212.9±16.8%, n=14) *(Figure 2.15)*. The response at the maximum concentration of noradrenaline in the absence of blockers was significantly ($P<0.01$) reduced by prazosin (30μM, 33.3±33.3%, n=3). Propranolol (30μM, 235.4±52.9%, n=4) did not modify the corresponding response of noradrenaline significantly ($P>0.05$) *(Figure 2.15)*. Cocaine also failed to enhance the contractile response at the maximum concentration (30μM) of noradrenaline. The maximum contraction to noradrenaline was not significantly ($P>0.05$) inhibited by cocaine to 107.5±31% (n=4) *(Figure 2.15)*.

2.4.7 Contractile response to β-PEA - effect of adrenergic inhibitors

The contractile response of rat aorta to β-PEA was not altered in the presence of prazosin (1μM) or propranolol (1μM). β-PEA caused concentration-related contractions of the aortic rings (3mM, 175±61.9% n=4). The response at the maximum concentration of β-PEA in the absence of various inhibitors was not significantly different ($P>0.05$) from the response to β-PEA with prazosin (1μM) (3mM, 151.5±20.1%, n=5), or propranolol (1μM) (3mM, 138.6±34.4%, n=6) *(Figure 2.16)*. The experiments were then repeated in the presence of cocaine (10μM). Neither propranolol (1μM) alone, nor propranolol (1μM) with prazosin (1μM) altered the CRC compared with that in the presence of cocaine alone *(Figure 2.17)*. The contractile response in the presence of cocaine (300μM, 44.4±14.8%, n=6), cocaine and prazosin (300μM, 17.4±4.6%, n=4), cocaine and propranolol (300μM, 44.2±16.4%, n=5), and cocaine, propranolol and prazosin (300μM, 25.3±10.5%, n=7) were not significantly different ($P>0.05$) *(Figure 2.17)*.

It was possible that with a prolonged exposure to β-PEA during a cumulative CRC, there may have been tachyphylaxis or desensitization which could have led to underestimation of the maximum effect. To avoid this possibility, the contractile response to β-PEA (30μM) in the presence of various inhibitors was examined by using a single dose of β-PEA (30μM) in each tissue in the presence of various inhibitors. Neither cocaine (10μM) (30μM, 58.9±10.7%, n=4), propranolol (1μM) (30μM, 41.5±11.8%, n=7), propranolol (1μM) and prazosin (1μM) (30μM, 21.6±12.9%, n=4) nor propranolol (1μM) with cocaine (10μM) (30μM, 46.7±15.3%, n=7) significantly ($P>0.05$) altered the contractile response to β-PEA (30μM, 55.7±11.9%, n=10) *(Figure 2.18)*. However, the contractile response to β-PEA (30μM) was significantly inhibited ($P<0.05$) by prazosin (1μM) (30μM, 3.4±1.9%, n=5), and also in the presence of prazosin (1μM), propranolol (1μM) and cocaine (10μM) (30μM, 13.7±2.5%, n=6) *(Figure 2.18)*.

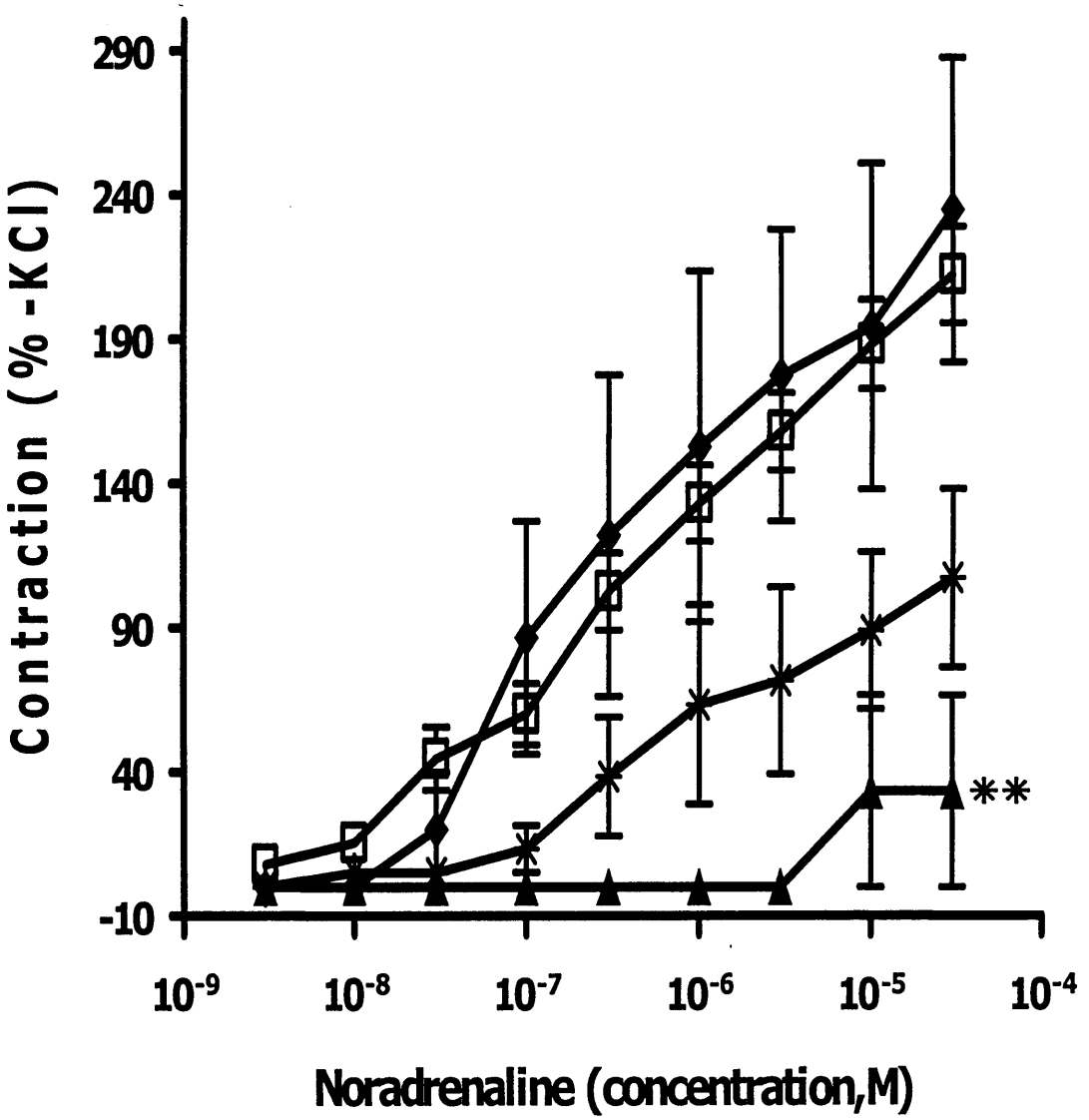


Figure 2.15: Contractile effect of noradrenaline (NA) in rat aortic rings in the presence of various inhibitors. Cumulative CRCs were constructed in absence of antagonists (□, n=14) or in the presence of prazosin (1μM,▲, n=3) or in the presence of cocaine (10μM, ✳, n=4) or in the presence of propranolol (1μM,◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Dunnett test. A significant difference was seen between the contractile response to noradrenaline in the absence, or presence of prazosin (1μM) ($P<0.01$,✳✳).

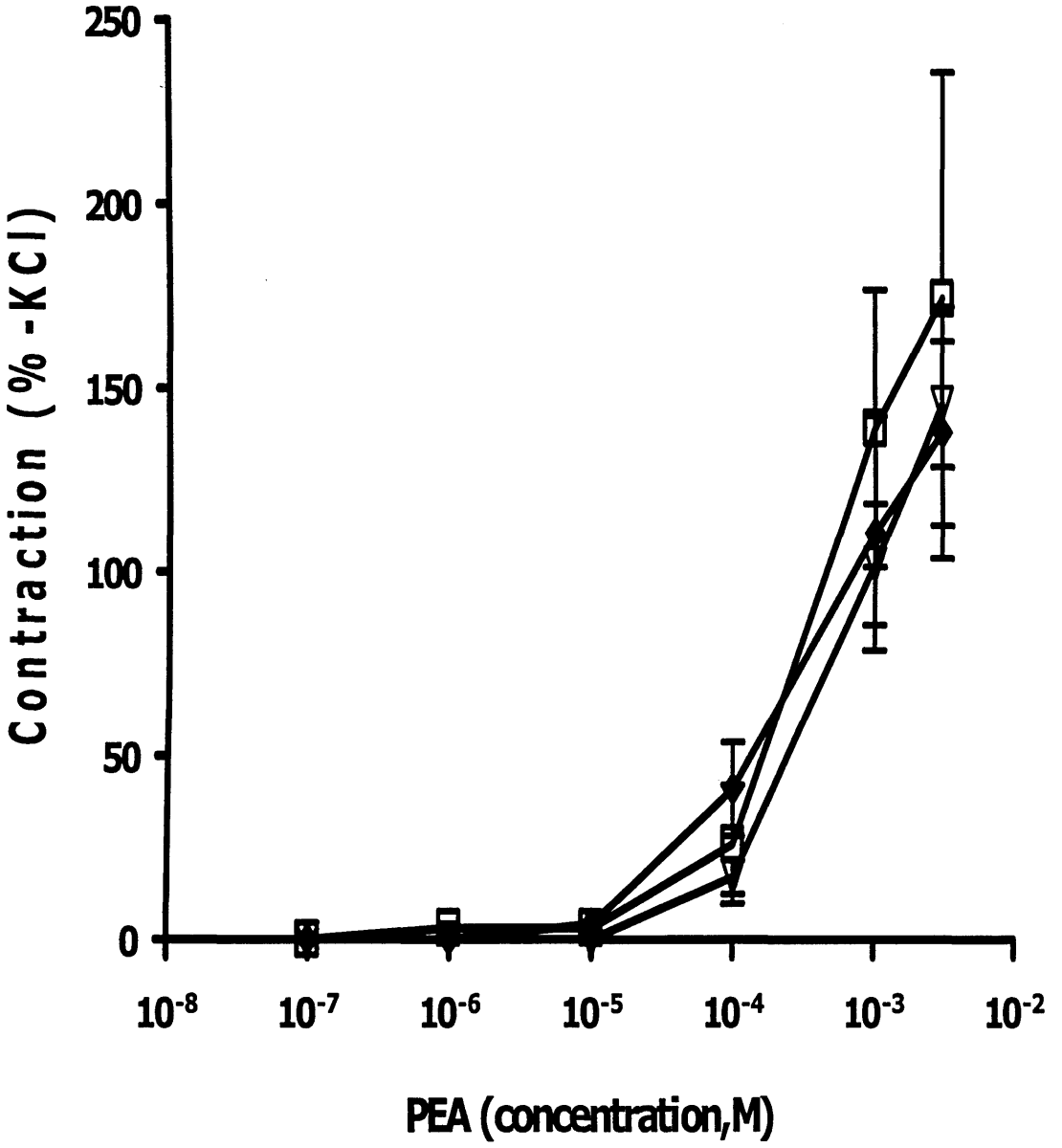


Figure 2.16: Contractile effect of β-PEA in rat aortic rings in the presence of prazosin (1μM) and propranolol (1μM). Cumulative CRCs were constructed in the absence of antagonists (□, n=4) or in the presence of prazosin (1μM,▽, n=5) or in the presence of propranolol (1μM, ◆, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant ($P>0.05$) differences were seen.

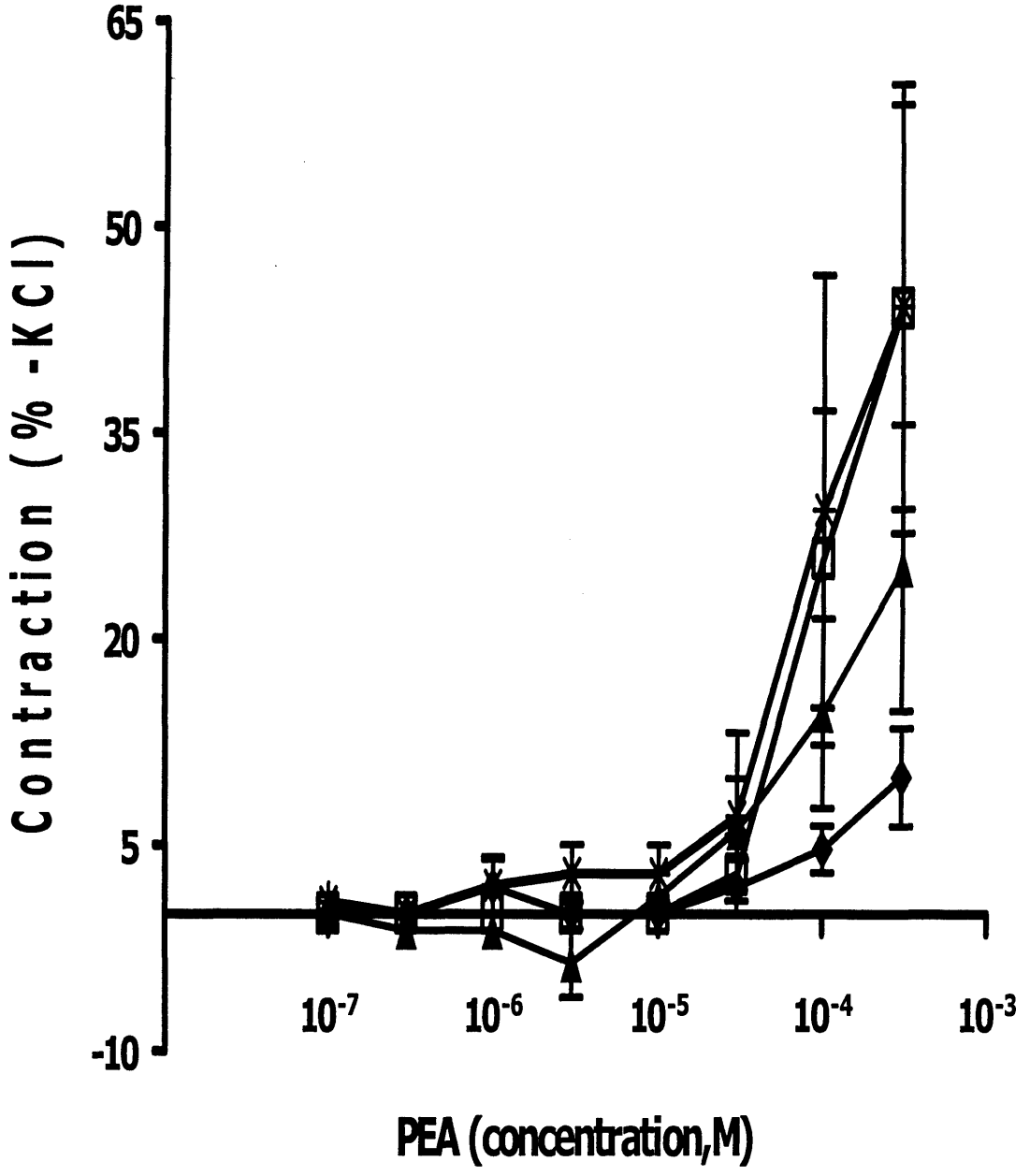


Figure 2.17: Contractile effect of β-PEA in rat aortic rings in the presence of various inhibitors. Cumulative CRCs were constructed in the presence of cocaine (10μM,✳, n=6) or in the presence of cocaine (10μM) and propranolol (1μM) (□, n=5) or in the presence of cocaine (10μM) and prazosin (10μM) (◆, n=4) or in the presence of cocaine (10μM), propranolol (10μM) and prazosin (10μM) (▲, n=7). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni and Dunnett test. No significant differences ($P>0.05$) were seen.

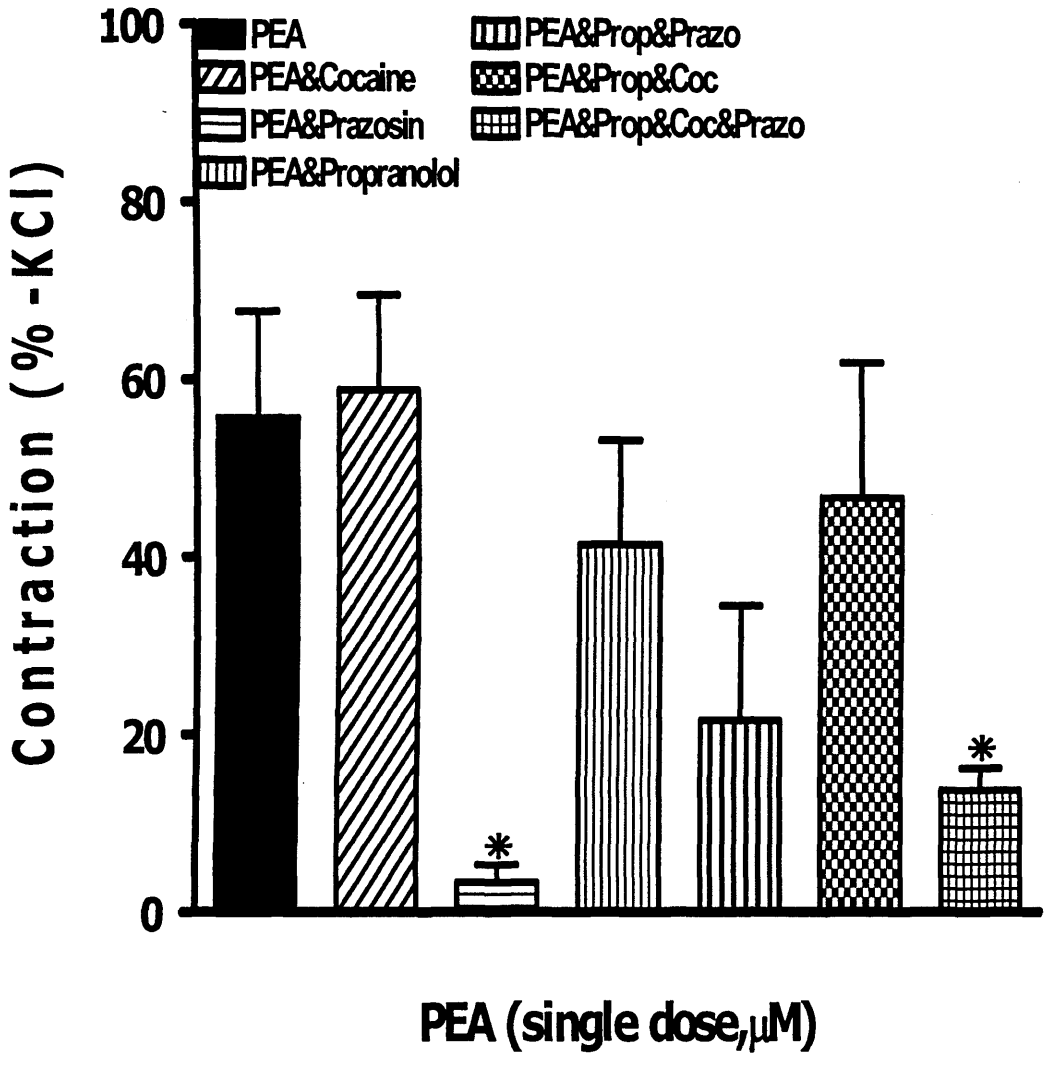


Figure 2.18: Single dose experiments for the contractile response in rat aortic rings to β-PEA (30μM) were constructed in the absence (n=10), or presence of various inhibitors. β-PEA (30μM) in the presence of prazosin (1μM, n=4), β-PEA (30μM) in the presence of propranolol (1μM, n=7), β-PEA (30μM) in the presence of cocaine (10μM, n=4), β-PEA (30μM) in the presence of cocaine (10μM) and propranolol (1μM, n=6). β-PEA (30μM) in the presence of propranolol (1μM) and prazosin (1μM, n=4) and β-PEA (30μM) in the presence of cocaine (10μM), prazosin (1μM) and propranolol (1μM, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Dunnett test. A significant difference ($P<0.05$,*) to the contractile response to β-PEA (30μM) was shown in the presence of prazosin (1μM) (n=5), and prazosin (1μM), cocaine (10μM) and propranolol (1μM) (n=6). Prop = Propranolol, Prazo = Prazosin, Coc = Cocaine

2.5 Discussion

2.5.1 Vasoconstriction in rat aortic rings to β-PEA

β-PEA caused concentration-related contractions of the rat aortic rings regardless of which part of the aorta was used (thoracic or abdominal aorta) *(Figure 2.3)*. Therefore, the mechanisms and receptor population upon which β-PEA acts in the rat aorta seems to be evenly distributed. So for future experiments either part of the rat aorta could be used.

2.5.2 Contractile response to β-PEA in rat aorta - effect of endothelium

The contractile response of rat aorta to β-PEA was shown to be independent of the presence of endothelium. The endothelium was not therefore responsible for the contractile response in rat aortic rings to β-PEA. There was also no opposing vasodilatator response to β-PEA mediated *via* the endothelium. If this had been the case endothelium removal would have potentiated the contractile response. The presence of an intact endothelium was demonstrated by the relaxation response to acetylcholine in the pre-contracted aorta, which is known to induce relaxation via endothelium-derived release of nitric oxide (Van de Voorde and Leusen, 1983; Furchgott, 1984; Waldron *et al.*, 1999).

Various experiments were then performed to verify this conclusion. Vascular tone is regulated by different factors which are produced by blood vessel endothelium. Amongst others, endothelium-derived relaxing factor (EDRF) is responsible for the vasodilator activity of acetylcholine (Pfister and Campbell, 1992; Das *et al.*, 1999), which has subsequently been identified to be nitric oxide (NO), synthesised from the terminal guanidino nitrogen atom of L-arginine, in the endothelial cell (Furchgott *et al.*, 1981; Gordon and Martin, 1983; Collins *et al.*, 1986; Palmer *et al.*, 1988; Olesen *et al.*, 1998). Also, it has been shown that endothelium-dependent hyperpolarizing factor (EDHF) causes endothelium-dependent relaxation (Waldron *et al.*, 1999). The results in the present study show conclusively, that β-PEA is not releasing these factors in rat aorta as the contractile response of rat aorta to β-PEA was unaffected by the presence of a nitric oxide synthase (NOS) inhibitor, L-NAME (100μM) (Cannell and Sage, 1989; Scheller *et al.*, 1998) in the absence, or presence of the endothelium.

The failure of endothelium removal to enhance the vasoconstriction indicates that β-PEA is not causing the release of endothelium-derived NO which would cause an opposing vasodilatation (Tschudi *et al.*, 1996). This was confirmed by the lack of effect of L-NAME in both endothelium-intact and denuded preparations. The latter result indicates that there was no endothelium-independent NO release by β-PEA.

2.5.3 Vasodilatator response to β-PEA

In previous publications, it was reported that TAs, such as tyramine and β-PEA showed reduced contractions (vasodilatation) at higher concentrations (Varma and Chemtob, 1993; Varma *et al.*, 1995). This observation was also seen in some of the results in this study *(e.g. Figure 2.13)*. Therefore, the effect of β-PEA in U46619 pre-contracted aortic tissues was examined. U46619 is a synthetic thromboxane/prostaglandin-H_2 (PGH_2) agonist and a selective thromboxane A_2 (TxA_2) mimetic that evokes vasoconstriction in smooth muscle (Coleman *et al.*, 1981; Trachte, 1986; Smith *et al.*, 1988). However, the effect of reduced vasoconstriction (vasodilatation) to β-PEA in rat aortic rings, at higher concentrations, cannot be confirmed with these experiments, as β-PEA showed further contractions in U46619-precontracted tissues rather than expected vasorelaxant effects in endothelium-intact and endothelium-denuded tissues. Furthermore, the presence of endothelium potentiated the contractile responses in pre-contracted tissues. Therefore the vasorelaxant factors of the endothelium, as previously discussed, were not affecting the contractile response to β-PEA in pre-contracted tissues. The possible vasodilator response to β-PEA will be examined in more detail in *Chapter 4*.

2.5.4 Contractile response to β-PEA - effect of indomethacin

Indomethacin also failed to affect the vasoconstrictor response to β-PEA. The failure of this cyclooxygenase (COX) inhibitor (Schachter and Sang, 1997; Fischer *et al.*, 2000) to attenuate the vasoconstriction confirms that the mechanism of action of β-PEA is not mediated through products of COX, such as prostaglandins and thromboxane. Thromboxane and its receptors (TBXR2R) cause constrictions of vascular and respiratory smooth muscles (Lefer *et al.*, 1980; Leung *et al.*, 2002).

2.5.5 Contractile responses to noradrenaline and β-PEA - effect of adrenergic inhibitors

In the periphery, β-PEA is regarded as an indirectly acting sympathomimetic amine (ISA) inducing pharmacological effects through the release of noradrenaline from sympathetic neurons (Burchett and Hicks, 2006). In rat isolated blood vessels this would be expected to induce vasoconstriction through stimulation of α-adrenoceptors with the possibility of some opposing vasodilatation *via* β-adrenoceptors. Therefore, the roles of neuronal uptake and α- and β adrenoceptors in β-PEA-induced vasoconstriction were examined. Cocaine was used as an inhibitor of noradrenaline neuronal uptake which should also interfere with the activity of indirectly acting sympathomimetic amines by inhibiting the carrier of the amine into the neuron and the outward transport of noradrenaline (Broadley, 1996). Propranolol was used to inhibit the smooth muscle relaxation of the rat aortic rings via β-adrenoceptors (Brawley et al., 2000) and prazosin was used as a selective α_1-adrenoceptor antagonist (Stanaszek *et al.*, 1983; Smit *et al.*, 1991). In a previous publication the concentrations of propranolol, cocaine and prazosin used here were shown to be effective in blocking β-adrenoceptors, the noradrenaline transporter and α_1-adrenoceptors, respectively (Baker *et al.*, 2007). In the present study there was no significant potentiation of the contractile response to noradrenaline by propranolol. Therefore, there is no β-adrenoceptor-mediated vasorelaxant role of noradrenaline in rat aortic rings. This confirms previous reports from this laboratory (Broadley and Penson, 2006) and by others (O'Donnell and Wanstall, 1984a; O'Donnell and Wanstall, 1984b) that noradrenaline does not stimulate β-adrenoceptors in this tissue. However, this does not exclude the possibility that β-PEA and other TAs do not have effects via β-adrenoceptors. The potency of prazosin as a selective α_1-adrenoceptor antagonist was confirmed by the effective suppression of the contractions over the noradrenaline concentration range used. The vasoconstriction by β-PEA was reduced by prazosin, but not significantly, suggesting that the response was only partially mediated *via* α-adrenoceptors. Propranolol failed to potentiate the contractile response to β-PEA. Therefore, a β-adrenoceptor-mediated vasorelaxation (Brawley *et al.*, 2000) does not contribute to the vascular response to β-PEA and does not oppose the vasoconstriction observed. A β-adrenoceptor mediated vasorelaxation

also does not explain the reduced contractions occasionally seen at high concentrations of β-PEA *(Figure 2.13)*. Therefore, the occasionally seen concentration-dependent vasorelaxant activity of β-PEA must be based on mechanisms other than β_2-adrenoceptors. It was suggested that various phenylethylamines produce endothelium- and β-adrenoceptor-independent relaxation of rat aorta which may involve novel vasorelaxant tyramine receptors (Varma and Chemtob, 1993; Varma *et al.*, 1995). A previous publication has shown that cocaine causes vasoconstriction in cardiovascular tissues (Egashira *et al.*, 1991) and others have shown that myocardial infarctions and various other ischemic heart diseases are often associated with cocaine use (Schachne *et al.*, 1984; Simpson and Edwards, 1986; Smith *et al.*, 1987).

In the present study, cocaine failed to enhance the contractions to noradrenaline in rat aortic rings compared to the effect of cocaine in other isolated tissues. This confirms others observations for cocaine in rat aorta (Maling *et al.*, 1971; Kuchii *et al.*, 1973; Al-Sahli *et al.*, 2001). This has been related to the sparse sympathetic interaction of the rat aorta and the fact that the neuronal endings are not located close to the α-adrenoceptors that mediated the vasoconstriction (Maling *et al.*, 1971; Kuchii *et al.*, 1973; Al-Sahli *et al.*, 2001). Cocaine also failed to potentiate the contractile response to β-PEA. Therefore, a cocaine-sensitive vasorelaxation (Li *et al.*, 2004) does not contribute to the vascular response to β-PEA and does not oppose the vasoconstriction observed. The failure to inhibit the vasoconstriction by cocaine suggests that β-PEA is not mediating its response *via* a cocaine-sensitive uptake pathway. This would suggest that it is not behaving as an ISA. Regarding other publications, the mechanism of action of cocaine in vascular tissues is complex and is not fully understood (Egashira *et al.*, 1991). Nevertheless, further experiments with β-PEA were conducted in the presence of cocaine to prevent any transport of β-PEA into sympathetic neurons and possible displacement of noradrenaline. The presence of cocaine, prazosin or prazosin in the additional presence of propranolol failed to inhibit the CRCs for vasoconstriction by β-PEA. This confirms that α-adrenoceptors play only a minor role in the response. Tachyphylaxis is a characteristic feature of ISAs, such as β-PEA and tyramine, in that repeated dosage results in a gradual decline of the response (Day and Rand, 1963; Day, 1967). The level of tachyphylaxis

between ISAs is inconsistent and the contractile responses are generally not completely abolished. Furthermore, the repeated exposure to a number of agonists at GPCRs is often associated with desensitisation of the receptor (Thomas *et al.*, 1992; Booker, 2002). Therefore, to avoid any possible tachyphylaxis or desensitisation, the contractile response to β-PEA was investigated in different tissues with a single dose (30μM) in the absence or presence of various inhibitors.

The effects of propranolol and cocaine indicate that the results from the single dose experiment are comparable to the previously discussed results from the CRCs experiment. Both inhibitors failed to potentiate the contractile response to β-PEA in rat aortic rings. Therefore, the results confirmed, that the cocaine and also the β-adrenoceptor-mediated vasorelaxation do not contribute to the vascular response to β-PEA and does not oppose the vasoconstriction observed (Brawley *et al.*, 2000; Li *et al.*, 2004). However, in contrast to the previous discussed results from the CRCs experiment *(Figure 2.15)* the contractile response to β-PEA in single dose experiment *(Figure 2.17)* was significantly reduced by prazosin. Moreover, in the presence of prazosin, cocaine and propranolol, the contractile response to β-PEA was also significantly inhibited. Therefore, in this case, the blocking effect of prazosin was stronger than expected and the contractile response was mainly mediated ***via*** α-adrenoceptors.

2.6 Conclusion

The mechanism by which β-PEA causes vasoconstriction cannot be determined from this study but the possibility must be considered that β-PEA is releasing other endogenous vasoconstrictors such as angiotensin or activating a novel receptor system. However, the endothelium cannot be a source of such vasoconstrictors, as the absence of the endothelium did not reduce the contractions to β-PEA. The results with prazosin were equivocal and in that with the CRCs there was no antagonism yet with single doses there appeared to be antagonism. Thus, to clarify the role of α-adrenoceptors and ISA in the vasoconstrictor action of β-PEA, further experiments were performed in the following *Chapters* using non-cumulative CRCs.

Chapter 3

EFFECTS OF ADRENERGIC INHIBITORS ON NON-CUMULATIVE CONCENTRATION-RESPONSE CURVES FOR β-PHENYLETHYLAMINE

Chapter 3

Effects of adrenergic inhibitors on non-cumulative concentration-response curves for β-PEA

3.1 Introduction

TAs are endogenous amine compounds closely related to classical monoamine neurotransmitters (Berry, 2004; Lindemann and Hoener, 2005; Zucchi *et al.*, 2006). Although the pathological and physiological importance (Kim and von Zastrow, 2001; Premont *et al.*, 2001; Berry, 2004) of TAs has been studied for a number of years, it was not until the discovery of the trace amine-associated receptor (TAAR) that interest was regenerated (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). The TA β-PEA is found in several mammalian tissues (Nakajima *et al.*, 1964) amongst them, the brain (Henry *et al.*, 1988; Paterson *et al.*, 1990). Moreover, β-PEA is involved in neuronal transmission (Panoutsopoulos *et al.*, 2004a). TAs have been found in certain foodstuffs, like chocolate, cheese and wine (Panoutsopoulos *et al.*, 2004a) and are thought to cause migraine attacks in susceptible individuals (Martin and Behbehani, 2001; Millichap and Yee, 2003). Although tyramine-containing foods are frequently implicated as headache precipitants, chocolate, the most common dietary trigger, does not contain tyramine, but does have large amounts of β-PEA (Martin and Behbehani, 2001; Millichap and Yee, 2003).

β-PEA is created by the decarboxylation of the amino acid L- phenylalanine (Dyck *et al.*, 1983). Monoamine oxidase (MAO), a flavin-containing enzyme, is widely distributed in the central and peripheral nervous systems (Waldmeier, 1987; Muller *et al.*, 1993) and has been found in the smooth muscle of guinea pig ileum (Banchelli *et al.*, 1985). MAOs are enzymes that catalyze the oxidative deamination of exogenous and endogenous monoamines including dietary amines and neurotransmitters, such as β-PEA, 5-HT, dopamine and noradrenaline (O'Brien *et al.*, 1993; Shih *et al.*, 1999). MAO-A and MAO-B are the two subtypes of the enzyme and both have been shown to be tightly bound to the outer mitochondrial membrane (Johnston, 1968; Shih *et al.*, 1999). β-PEA is mostly metabolised by MAO-B to form phenylacetalaldehyde initially (Yang and Neff, 1973; Banchelli *et al.*, 1985;

Wouters, 1998; Shih *et al.*, 1999), which is then transformed to phenylacetic acid by either aldehyde dehydrogenase or aldehyde oxidase (Klyosov, 1996; Panoutsopoulos *et al.*, 2004a; Panoutsopoulos *et al.*, 2004b).

Semicarbazide-sensitive amine oxidase (SSAO) is a copper-containing enzyme which deaminates (oxygen oxidoreductase) aromatic and aliphatic amines (Lyles, 1994; Lizcano *et al.*, 1998; El Hadri *et al.*, 2002). The enzyme is distinguished from MAO-A and MAO-B by substrate and inhibitor specificities (all SSAO are sensitive to inhibition by semicarbazide (Lyles, 1994; Lizcano *et al.*, 1998)), cofactors and cellular distribution (Lizcano *et al.*, 1998; El Hadri *et al.*, 2002). SSAO has been found in most mammalian tissues as either tissue-bound or soluble (plasma) isoforms (Lyles, 1994; Magyar *et al.*, 2001). Furthermore, SSAO has been detected in large amounts in the smooth muscle of rat aorta (Lyles and Singh, 1985; Yu, 1990; Yu *et al.*, 1992; Yu *et al.*, 1994).

In view of the close structural similarity between TAs and the noradrenergic transmitter, noradrenaline, the possibility exists that TAs could interact with adrenoceptors. Evidence suggests that the indirect sympathomimetic activity of TAs could be due to the indirect stimulation of adrenoceptors (Hansen *et al.*, 1980). The results contained in *Chapter 2* provide some evidence to suggest that another mechanism of action may also be involved in the contractile response to β-PEA. The work described in this *Chapter* was carried out to examine the receptor types mediating the contractile response to β-PEA by use of non-cumulative concentration-response curves

3.2 Aims

To study the effect of trace amines (TAs) employing non-cumulative concentration-response curves (CRCs) and single dose experiments to investigate the contractile responses of rat isolated aortic rings to β-PEA in the presence of a range of different inhibitors.

a.) Compare cumulative and non-cumulative CRCs for β-PEA to determine which are the more reproducible.

b.) Examine the contractile response to β-PEA in rat aortic rings in the absence, or presence of various inhibitors with non-cumulative CRCs.

c.) Compare the effects of propranolol and ICI-118,551 on the contractile response to β-PEA in rat aortic rings.

c.) Determine whether the presence of a MAO inhibitor (pargyline) or an SSAO inhibitor (semicarbazide) has any effect on the contractile response to β-PEA in rat aortic rings to determine whether its response might be limited by degradation by these enzymes.

3.3 Material and Methods

The main methods and experimental protocol described in *Chapter 2* were retained throughout the study unless otherwise stated.

3.3.1 Experimental Protocol

After equilibration, non-cumulative CRCs for β-PEA or single dose responses for β-PEA (30μM) were obtained, in the absence, or presence of different inhibitors, which were incubated with the tissue for 15 minutes before commencing the CRC or single dose administration unless otherwise stated. Endothelium removal was carried out in all experiments, its removal being confirmed as descried in *Chapter 2*.

Non-cumulative concentration-response curves (CRCs)

To obtain non-cumulative CRCs, successive concentrations of the agonist were added to the 50ml tissue bath after equilibration. The tissue bath was washed out (2 min, 2 min, and 6 min) after the peak effect had been reached for the preceding concentration.

3.3.2 Drugs Used

All chemicals were dissolved in distilled water, unless otherwise stated. Prazosin hydrochloride, ICI-118,551 hydrochloride and U46619 were dissolved in DMSO.

- **Reagents obtained from Sigma Aldrich**

Cocaine hydrochloride, pargyline hydrochloride (PG), prazosin hydrochloride, (±)-propranolol hydrochloride, β-phenylethylamine (β-PEA) and semicarbazide hydrochloride

- **Reagents obtained from TOCRIS Bioscience**

ICI-118,551 hydrochloride ((±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride)

- **Reagents obtained from Fisher Scientific**

All chemicals for the Krebs-bicarbonate buffer (analytical grade)

3.4 Results

3.4.1 Vasoconstriction in rat Aortic rings to β-PEA (non-cumulative CRCs)

The addition of low concentrations of β-PEA in non-cumulative concentration-response curves (CRCs) caused concentration-related contractions *(Figure 3.1)*.

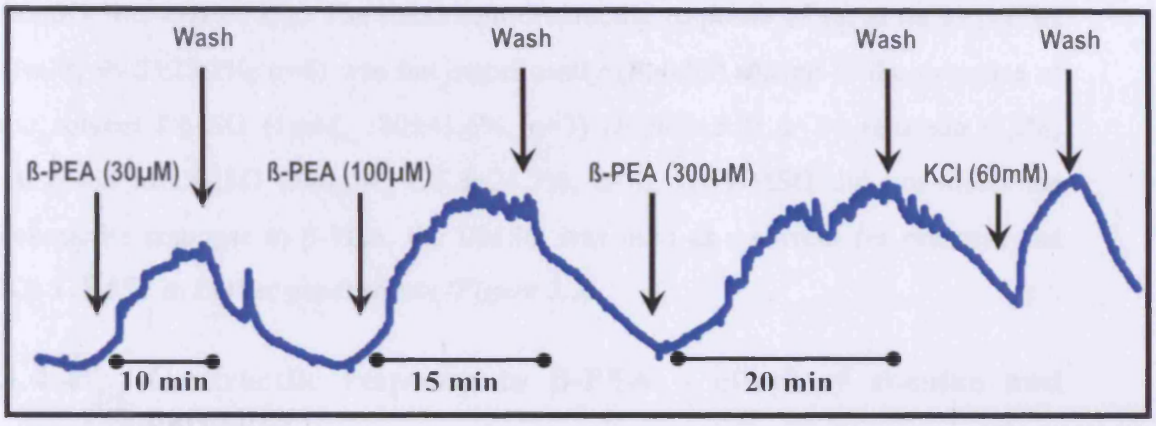


Figure 3.1: Chart recording showing a representative non-cumulative concentration-response curve (CRC) for the contractile response of rat aorta to β-phenylethylamine (β-PEA). To construct non-cumulative CRCs for β-PEA, successive concentrations of the drug were added. The 50ml tissue bath was washed out (2 min, 2 min, and 6 min) after the peak effect had been reached for the preceding concentration. At the end of each experiment, KCl (60mM, isotonic solution) was routinely added after washout of the final drug concentration. "Wash" = washout of tissue bath.

3.4.2 Comparison between cumulative and non-cumulative CRCs

Non-cumulative CRCs showed greater contractions than cumulative CRCs. The maximum response to β-PEA was significantly greater ($P<0.05$) (110±14.8%, n=6) compared to the maximum response to β-PEA achieved in cumulative CRCs (58.6%±13.3%, n=5) *(Figure 3.2)*. Furthermore, the –log EC_{50} values for cumulative (4.16±0.13, n=5) and non-cumulative CRCs (4.82±0.10, n=6) to β-PEA were significantly different ($P<0.01$). Thus, for the remaining thesis studies with β-PEA concentration-response curves were non-cumulative.

3.4.3 Contractile response to β-PEA – effect of the solvent DMSO and prazosin

Prazosin and ICI-118,551 are not soluble in deionised water, only in dimethyl sulfoxide (DMSO). DMSO is a versatile polar solvent which dissolves a wide range of organic compounds (Dishart and McKim, 2003). To determine the effect of DMSO on the contractile response to β-PEA, a control experiment in the presence of DMSO was carried out. The maximum contractile response of rat aorta to β-PEA (1mM, 99.2±25.2%, n=4) was not significantly (P>0.05) altered in the presence of the solvent DMSO (1mM, 180±41.6%, n=3) *(Figure 3.3)* or by prazosin (1μM) dissolved in DMSO (300μM, 125.8±24.2%, n=8). As DMSO did not affect the contractile response to β-PEA, the DMSO was used as a solvent for prazosin and ICI-118,551 in further experiments *(Figure 3.3)*.

3.4.4 Contractile response to β-PEA – effect of cocaine and pargyline

β-PEA caused concentration-related contractions of the rat aortic rings (1mM, 99.2±25.2% n=4). Cocaine (10μM) failed to alter the maximum contractile response to β-PEA (93.3±21.7%, n=6) significantly (P>0.05). Furthermore, the maximum contractile response to β-PEA in the presence of pargyline (PG, 10μM), a monoamine oxidase (MAO) inhibitor (187.5±34.3%, n=4) was not significantly greater (P>0.05) *(Figure3.4)*.

3.4.5 Contractile response to β-PEA - effect of propranolol and ICI-118,551

The contractile response to β-PEA was then examined in the presence of propranolol (1μM), a non-selective β-adrenoreceptor antagonist and ICI-118,551 (1μM), a selective β_2-adrenoceptor antagonist *(Figure 3.5)*. The maximum contractile responses to β-PEA in the absence of antagonists (1mM, 99.2±25.2&, n=4), β-PEA in the presence of propranolol (100μM, 147.7±29.7%, n=5) and β-PEA in the presence of ICI-118,551 (100μM, 130.5±29.3%, n=7) were not significantly different (P>0.05) *(Figure 3.5)*. Furthermore, the maximum contractile responses β-PEA in the absence of antagonists (1mM, 99.2±25.2&, n=4), β-PEA in the presence of

propranolol (100μM, 147.7±29.7%, n=5) and β-PEA in the presence of ICI-118,551 (100μM, 130.5±29.3%, n=7) were not significantly different (P>0.05) to β-PEA in the presence of DMSO only (1mM, 180±41.6%, n=3). A reduced contraction (vasodilatation) was revealed at the highest concentration of β-PEA by propranolol (1μM) and ICI-118,551 (1μM) *(Figure 3.5)*. The –log EC_{50} values of β-PEA in the absence of antagonist (4.37±15.2, n=3), in the presence of propranolol (3.89±34.9, n=5) and in the presence of ICI-118,551 (5.05±0.68, n=6) were not significantly different (P>0.05) *(Figure 3.5)*. The contractile response of the rat aorta to β-PEA was then investigated in the presence of prazosin (1μM) with either of the two β-adrenoceptor antagonists *(Figure 3.6)*. Neither propranolol (1μM), nor ICI-118,551 (1μM) in combination with prazosin, decreased the contractile response to β-PEA. The maximum contractile responses to β-PEA in the presence of prazosin (300μM, 125.8±24.2%, n=8), in the presence of prazosin and propranolol (100μM, 126.5±36.9%, n=5), and in the presence of prazosin and ICI-118,551 (300μM, 170.3±15.7%, n=8) were not significantly different *(Figure 3.6)*. Moreover, the –log EC_{50} values for β-PEA in the presence of prazosin (4.07±0.10, n=8), β-PEA in the presence of prazosin and ICI-118,551 (3.94±0.15, n=6) and β-PEA in the presence of prazosin and propranolol (4.23±0.13, n=4) were not significantly different (P>0.05) *(Figure 3.6)*. In the next set of experiments, the contractile response of rat aorta to β-PEA in the presence of ICI-118,551 (1μM) and cocaine (10μM) was examined *(Figure 3.7)*. The CRC to β-PEA in the presence of ICI-118,551 and cocaine (4.34±0.5, n=4) was not significantly (P>0.05) shifted to the right compared to the CRCs to β-PEA in the presence of either cocaine (4.06±0.36, n=4) or ICI-118,551 (5.05±0.68, n=6) *(Figure 3.7)*. The maximum contractile responses to β-PEA in the presence of cocaine (104.4±24.2%, n=6), ICI 118,551 (130±29.3%, n=7) and cocaine and ICI-118,551 (152±52.1%, n=5) were not significantly different (P>0.05) *(Figure 3.7)*.

3.4.6 Contractile response to β-PEA - effects of different combinations of prazosin, ICI-118,551, cocaine and pargyline

Finally, responses to β-PEA were studied in the presence of different combinations of the inhibitors. The biggest maximum contractile response to β-PEA was shown in the presence of prazosin (1μM), ICI-118,551 (1μM), cocaine (10μM) and PG (10μM) *(Figure 3.8)* of 138.2±23.1% at 300μM. β-PEA, in the presence of prazosin (1μM), ICI-118,551 (1μM) and cocaine (10μM), showed the smallest response, 83.3± 11.2% at 300μM. However, none of the responses were significantly different ($P>0.05$). Since the combination of prazosin, pargyline, cocaine and ICI-118,551 ("inhibitors") gave the largest and most consistent responses to β-PEA this combination was used for all future studies *(Figure 3.8)*.

3.4.7 Contractile response to β-PEA - effect of semicarbazide

In smooth muscle, β-PEA is metabolised by MAO-B (Panoutsopoulos *et al.*, 2004b) and also by semicarbazide-sensitive amine oxidase (SSAO) (Yu *et al.*, 1992; Yu *et al.*, 1994). Therefore, the contractile response to β-PEA in rat aortic rings in the presence of semicarbazide, a selective SSAO inhibitor, (1mM) was examined. Semicarbazide (1mM) did not alter the contractile response to a single dose of β-PEA (30μM). The contractile response to β-PEA (30μM, 20±15.3%, n=3) in the absence of semicarbazide (1mM) was not significantly different ($P>0.05$) in comparison to β-PEA in the presence of semicarbazide (1mM) (30μM, 19.4±10%, n=3) *(Figure 3.9)*.

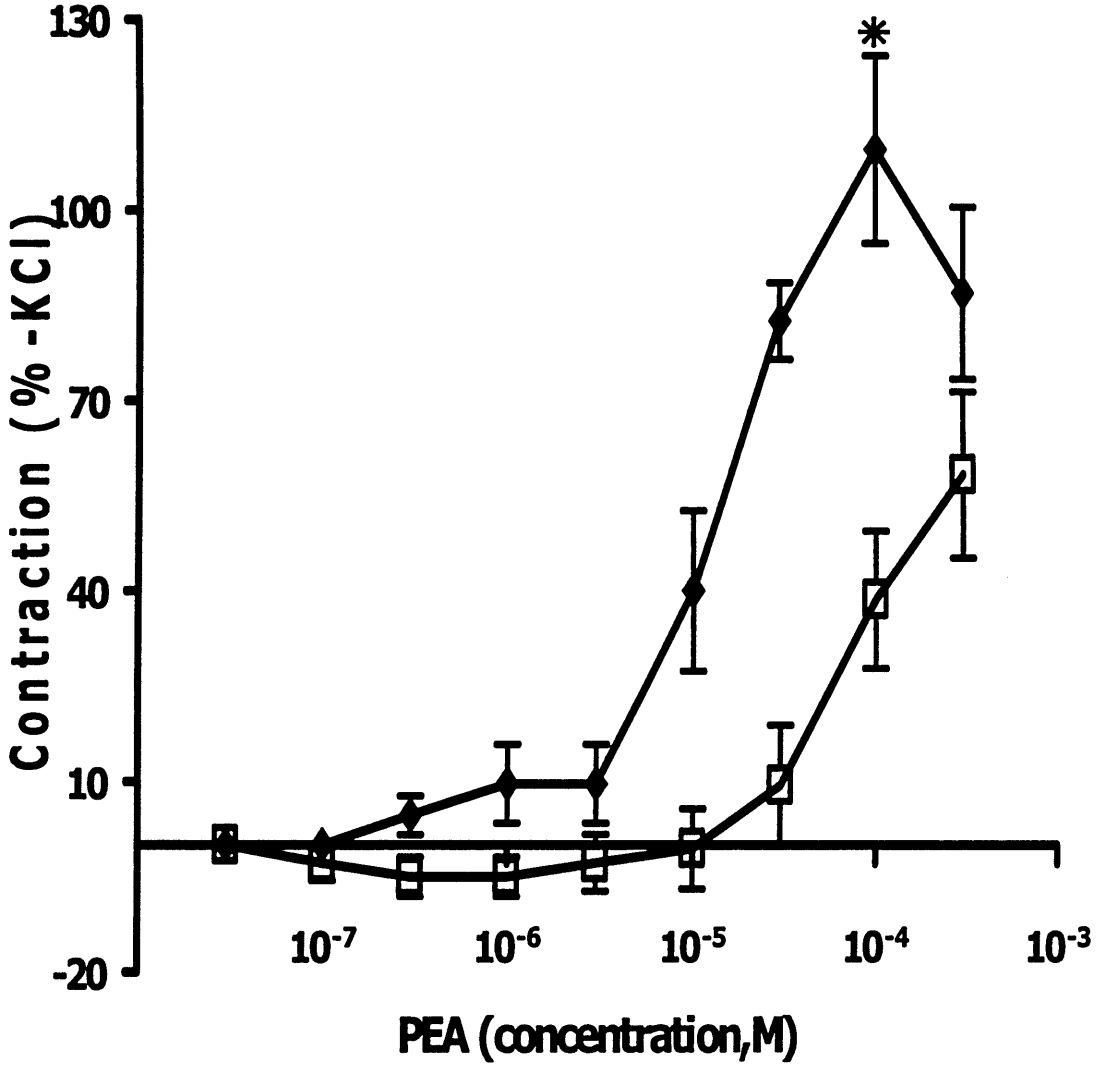


Figure 3.2: Mean CRCs for the contractile response of rat aorta to β-PEA. Cumulative CRCs (□, n=5) and non-cumulative CRCs (◆, n=6) to β-PEA were constructed. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant ($P>0.05$) differences were seen.

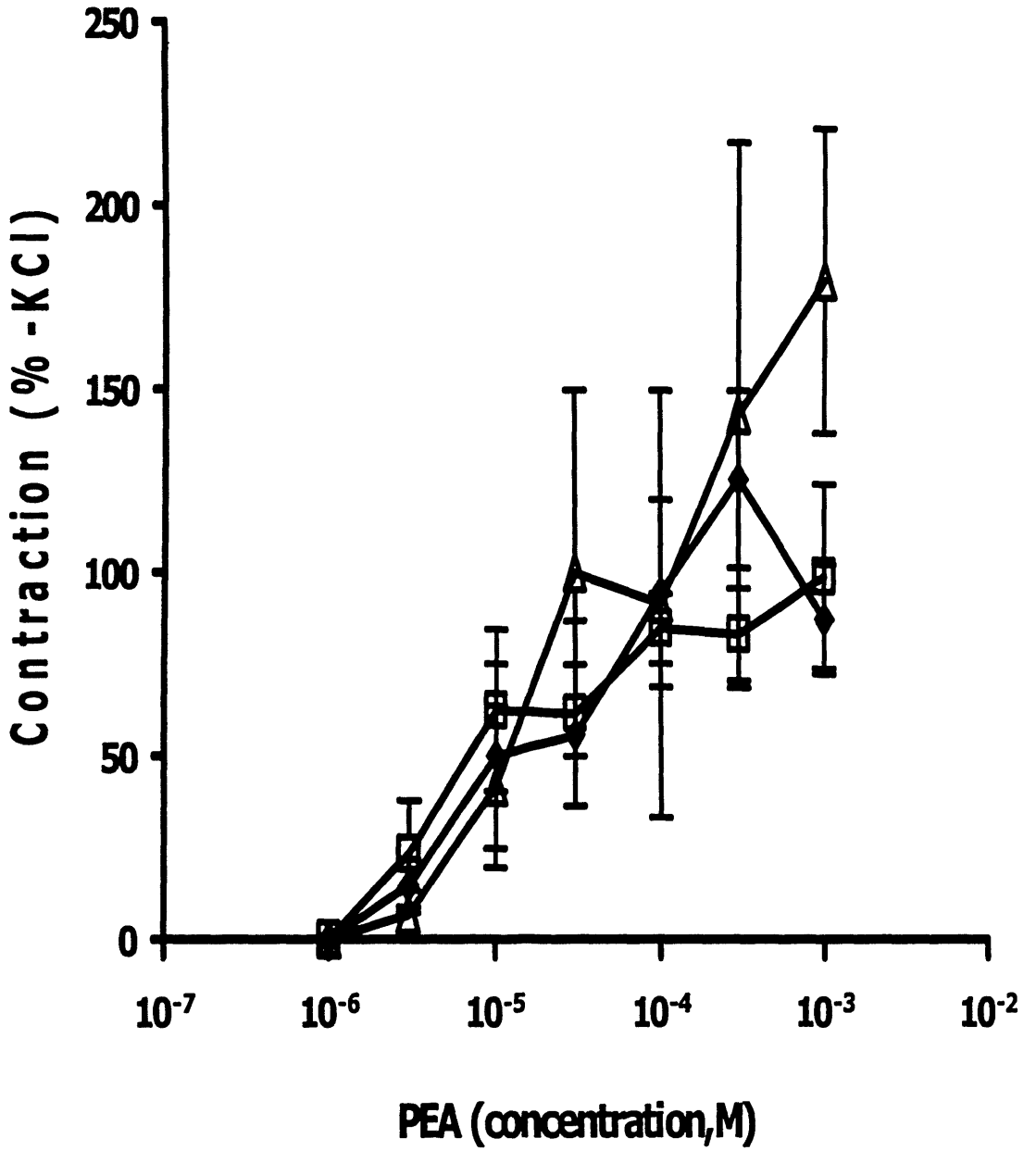


Figure 3.3: Effect of dimethyl sulfoxide (DMSO) on the mean non-cumulative CRCs for the contractile response of rat aorta to β-PEA. Non-cumulative CRCs were constructed in the absence of antagonist and DMSO (□, n=4) or in the presence of DMSO (△, n= 3) or in the presence of prazosin (1μM,◆, n=8). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant (P>0.05) differences were seen at their maximum responses.

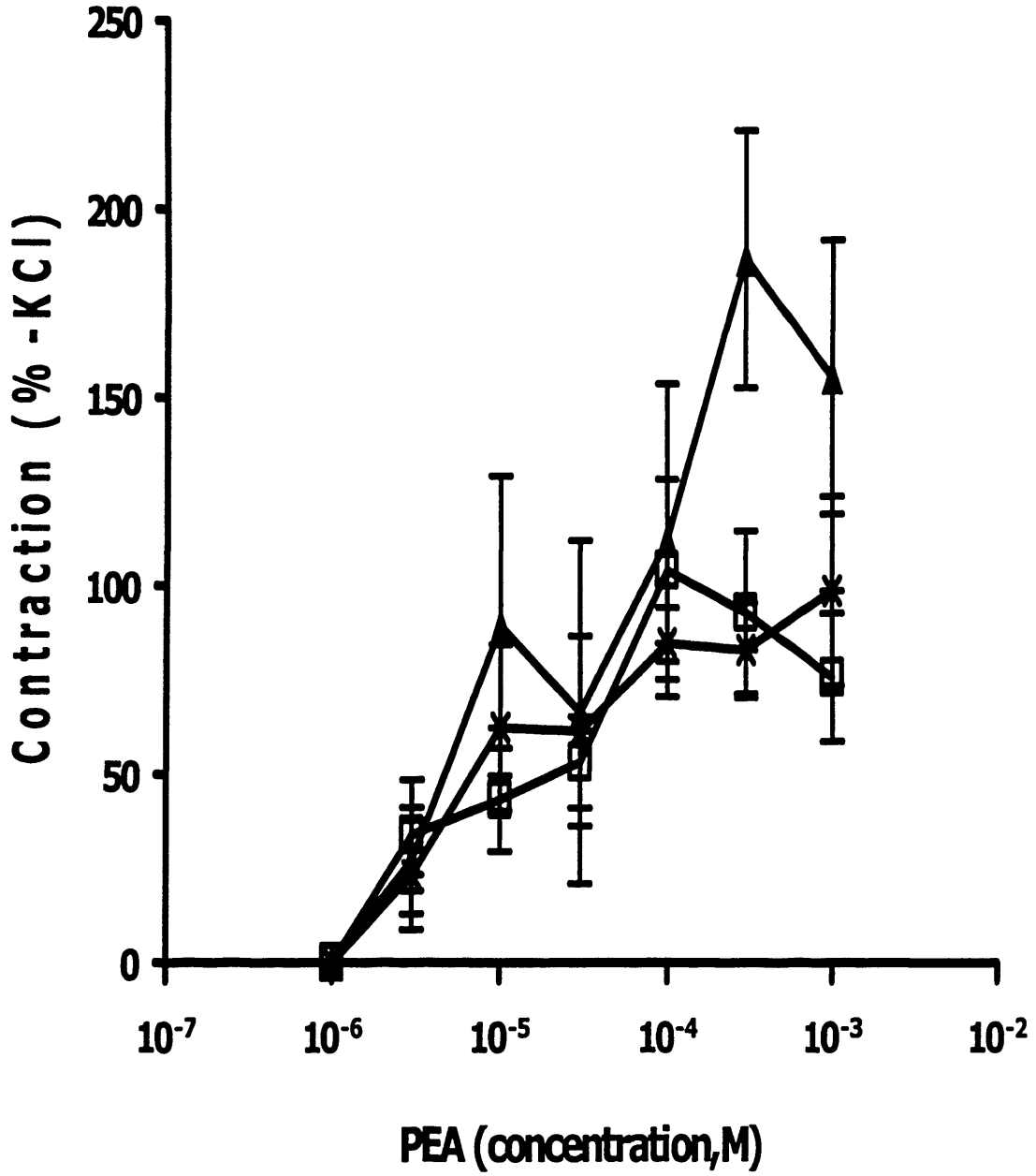


Figure 3.4: Effects of pargyline (10μM) and cocaine (10μM), on the mean non-cumulative CRCs for the contractile response of rat aorta to β-PEA. Non-cumulative CRCs to β-PEA were constructed in the absence of antagonists (✳, n=4) or in the presence of PG (10μM, ▲, n=4) or in the presence of cocaine (10μM, □, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant ($P>0.05$) differences were seen.

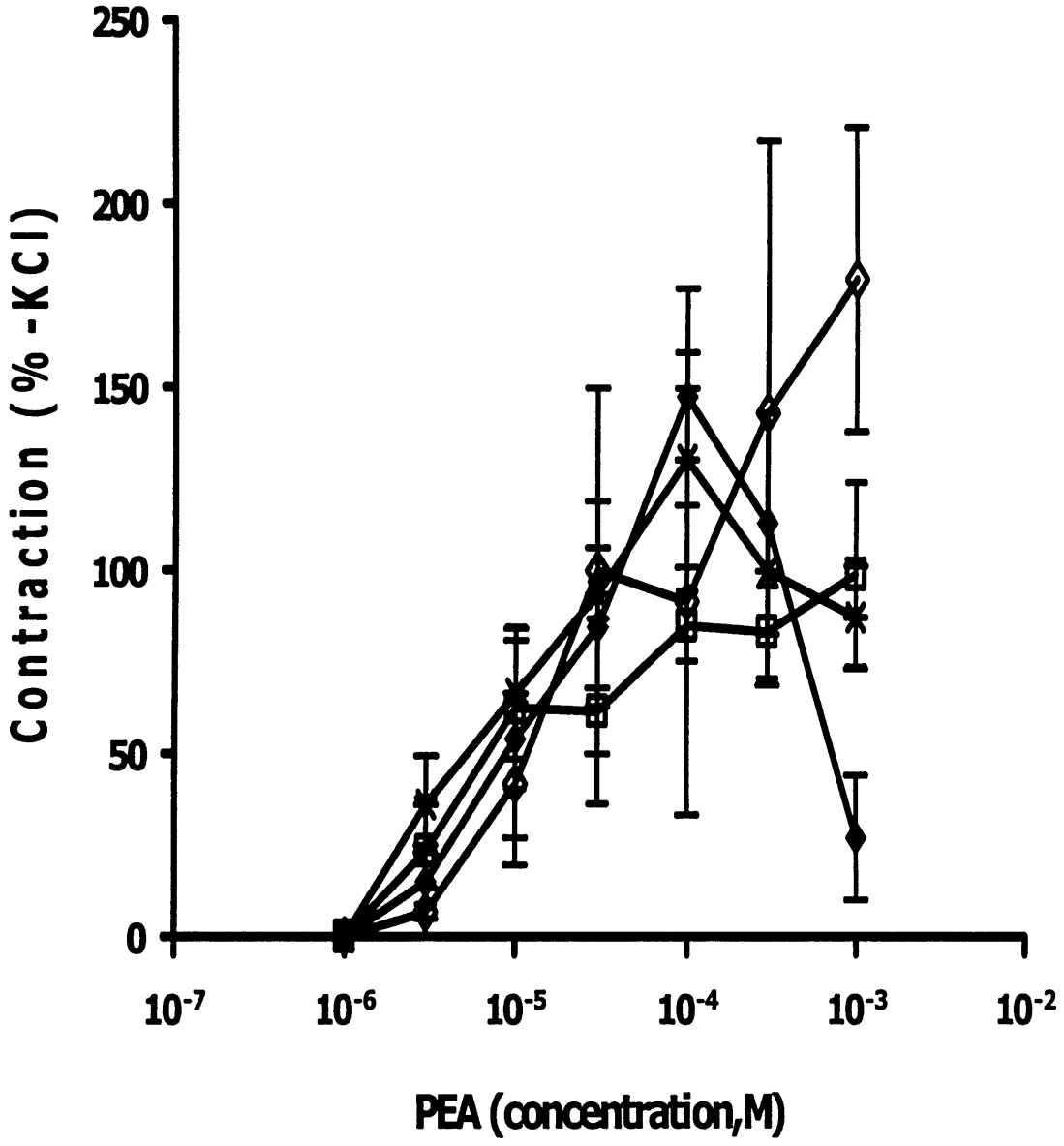


Figure 3.5: Effects of ICI-118,551 (1μM) and propranolol (1μM) on the mean non-cumulative CRCs for the contractile response of rat aorta to β-PEA. Non-cumulative CRCs were constructed in the absence of antagonists (□, n=4) or in the presence of solvent DMSO (◊, n=3) or in the presence of ICI-118,551 (1μM,✳, n=7) or in the presence of propranolol (1μM, ◆, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant (P>0.05) differences were seen.

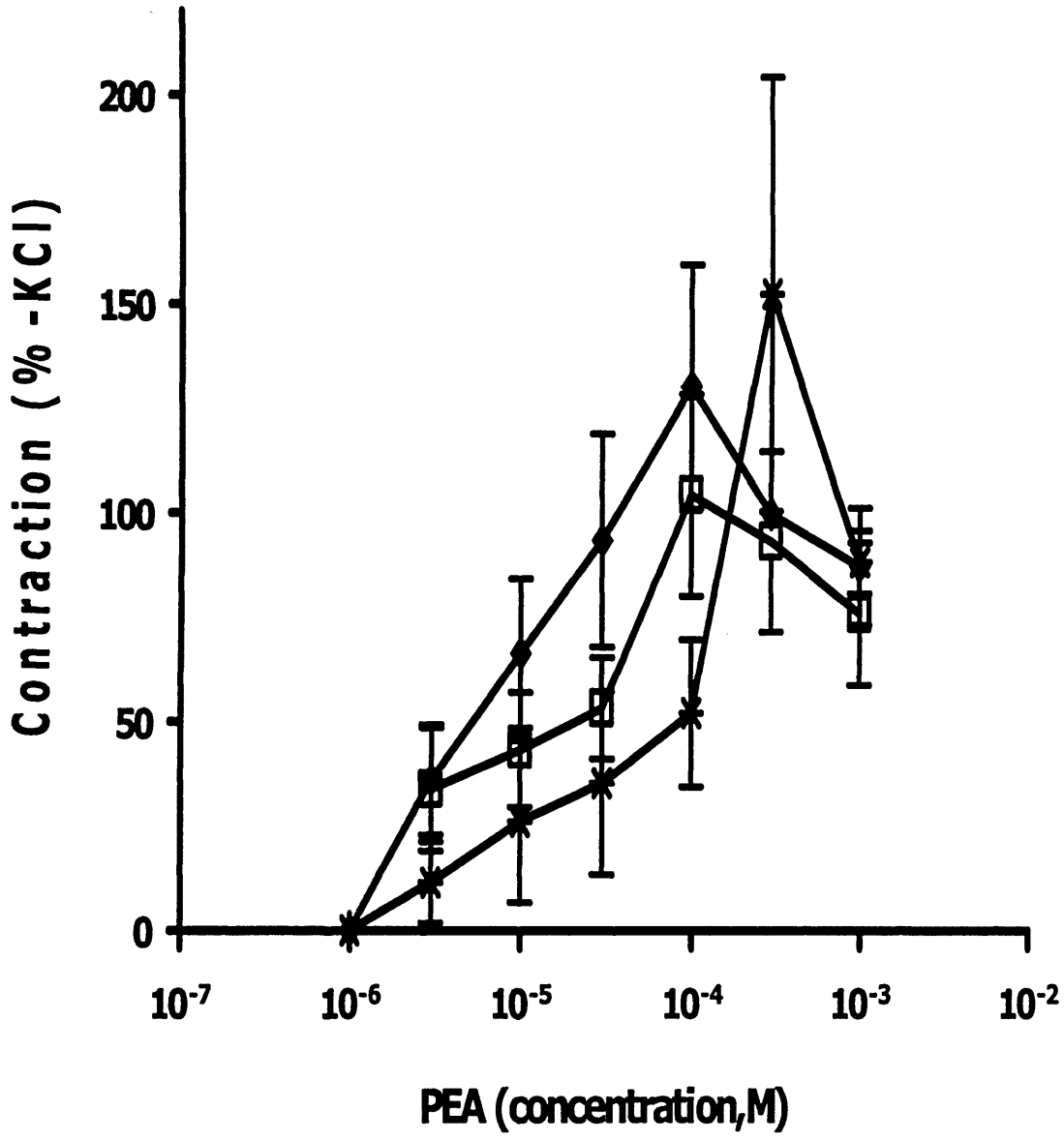


Figure 3.6: Effects of ICI-118,551 (1μM) and propranolol (1μM) and prazosin (1μM) on the mean non-cumulative CRCs for the contractile response of rat aorta to β-PEA. Non-cumulative CRCs were constructed in the presence of prazosin (□, n=8), ICI-118,551 and prazosin (◆, n=8) or in the presence of propranolol and prazosin (✳, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant (P>0.05) differences were seen.

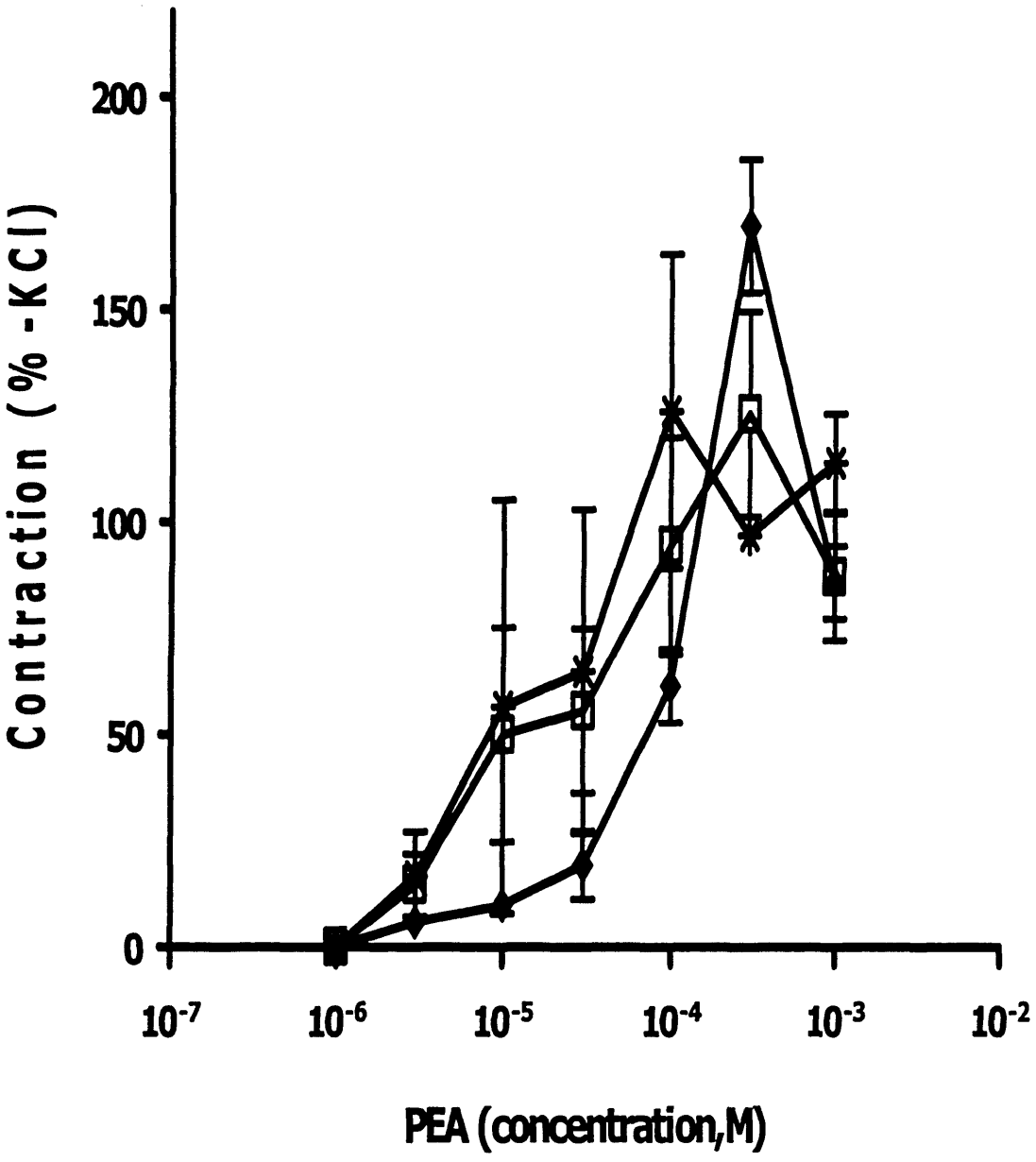


Figure 3.7: Effects of ICI-118,551 (1μM) and cocaine (1μM) on the mean non-cumulative CRCs for the contractile response of rat aorta to β-PEA. Non-cumulative CRCs were constructed in the presence of ICI-118,551 (◆, n=7), or in the presence of cocaine (□, n=6), or in the presence of ICI-118,551 and cocaine (✳, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant ($P>0.05$) differences were seen.

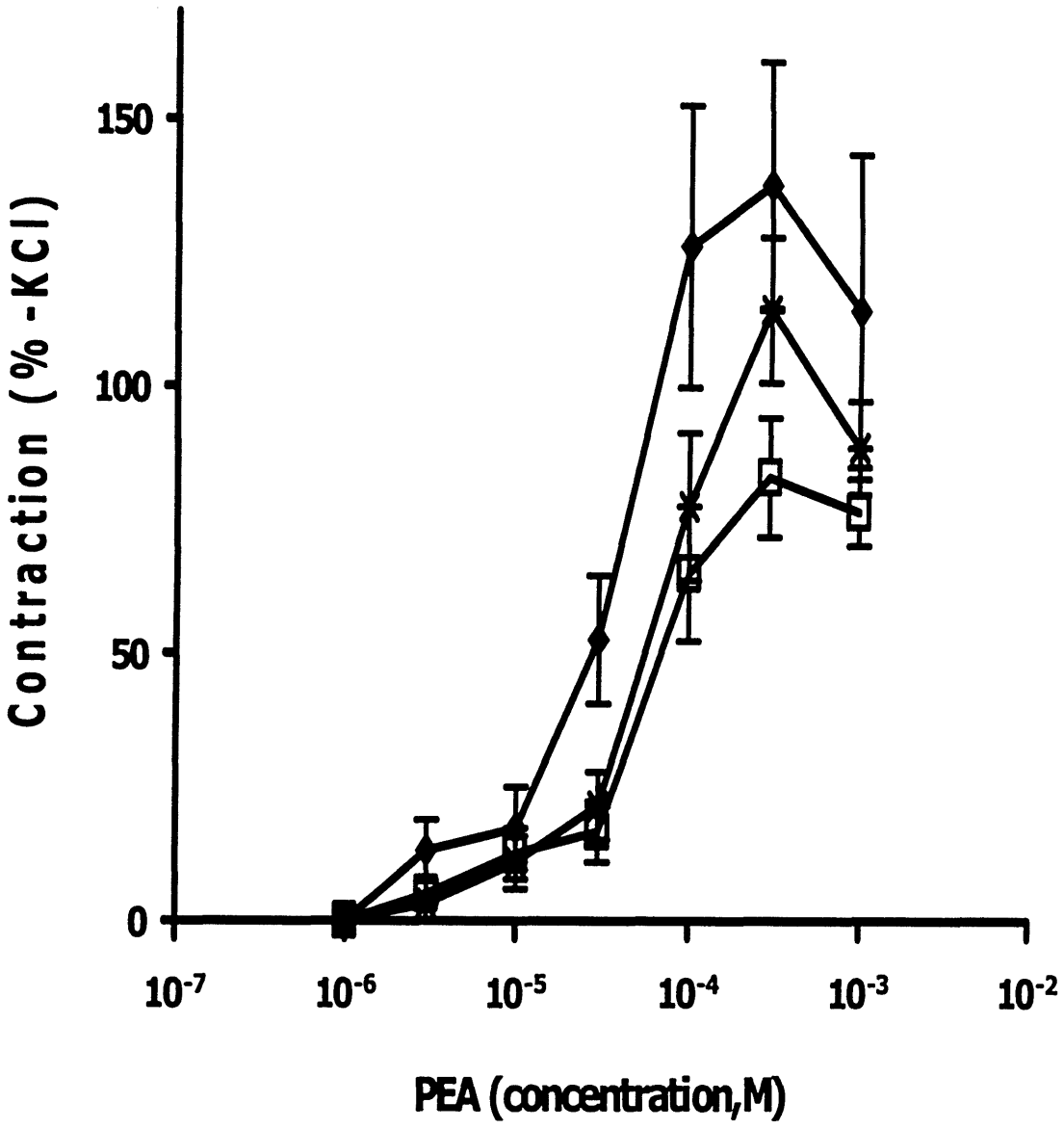


Figure 3.8: Effects of ICI-118,551 (1μM), prazosin (1μM), cocaine (10μM) and PG (1μM) on the mean non-cumulative CRCs for the contractile response of rat aorta to β-PEA. Non-cumulative CRCs were constructed in the presence of ICI-118,551, prazosin and PG (✳, n=10), ICI-118,551, prazosin and cocaine (□, n=5) or in the presence of ICI-118,551, prazosin, PG and cocaine ("inhibitors",◆, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant ($P>0.05$) differences were seen at their maximum contractile responses.

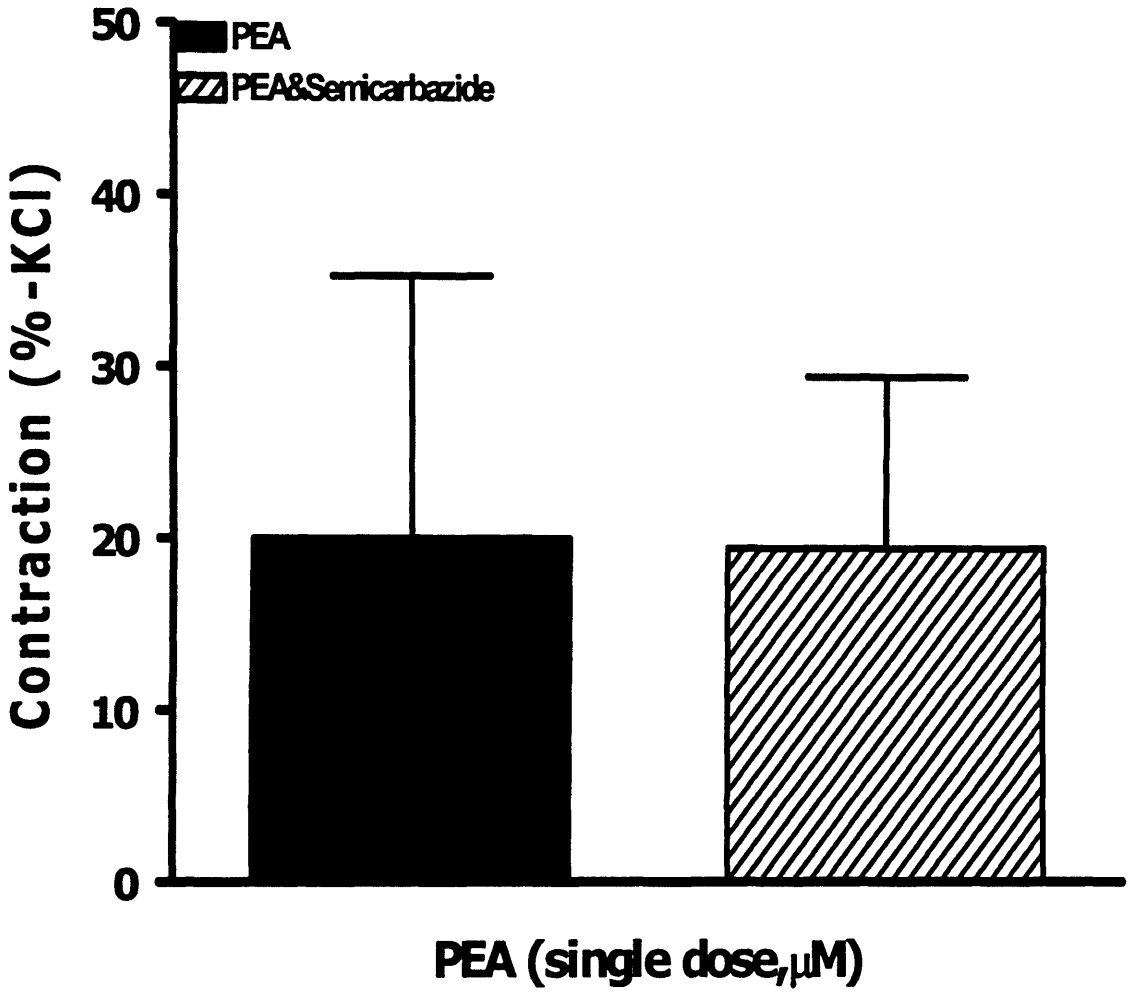


Figure 3.9: Effect of semicarbazide (1mM) on β-PEA contractions in rat aortic rings. A single dose of β-PEA (30μM) was tested in the absence, or presence of semicarbazide (1mM, n=3). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (+SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen.

3.5 Discussion

3.5.1 Vasoconstriction in rat aortic rings to β-PEA (non-cumulative CRCs)

Comparable to the results in *Chapter 2*, β-PEA caused concentration-related contractions of the rat aortic rings when given non-cumulatively. Previously, the minimum effective concentration for contraction to β- PEA upon isolated aortic strips was found to be 1μM and a maximum response was seen at a concentration of 10 μM (Hansen et al., 1980). In the present study, concentrations between 3μM-1mM of β-PEA caused contractions in isolated aortic rings in cumulative as well as in non-cumulative CRCs. Non-cumulative CRCs always showed a stronger contraction by β-PEA than cumulative CRCs. The fact that the cumulative CRC *(Figure 3.2)* was shifted to the right also suggests desensitisation or tachyphylaxis. ISAs are notorious for giving tachyphylaxis (Day, 1967). So this might suggest an ISA mechanism. However, lack of effect of prazosin indicates that this is not the case. This question will be examined in more detail in *Chapter* 4.

3.5.2 Contractile response to β-PEA - effect of prazosin, cocaine and pargyline

This study examined whether the constrictions of rat isolated aortic rings by β-PEA were mediated *via* an indirect sympathomimetic action through α-adrenoceptors. In support of the previous results with cumulative CRCs *(Chapter 2)* prazosin did not significantly reduce the vasoconstriction to β- PEA. Therefore, these results confirm the earlier observations, suggesting that the contractile response was not mediated *via* α-adrenoceptors. Furthermore, cocaine failed to potentiate or reduce the contractile response to β-PEA. It should be noted that the mechanism of action of cocaine in vascular tissues is not fully understood (Egashira *et al.*, 1991; Li *et al.*, 2004). However, the lack of block of β-PEA by cocaine suggests no transport of the β-PEA into the neurone, including no ISA mechanism. In spite of the lack of effect of cocaine, future experiments with β-PEA were performed in the presence of cocaine to prevent any transport of β-PEA into the sympathetic neurons and possible displacement of noradrenaline. In a previous publication the concentrations of

cocaine and prazosin were shown to be effective in blocking the noradrenaline transporter and α_1-adrenoceptors, respectively (Al-Motarreb and Broadley, 2003; Baker *et al.*, 2007). In *Chapter 2* prazosin was shown to block the vasoconstrictor effect of noradrenaline.

In the present study , although pargyline (PG), a MAO-inhibitor (MAO-B>MAO-A) (Murphy *et al.*, 1998) increased the contraction to β-PEA, this was not significant. Only a small amount of β-PEA may be metabolised *via* MAO, as MAO is localized intracellulary and the extracellular concentration of the substrate is high. Accordingly, further experiments with β-PEA were performed in the presence of PG to eliminate metabolism and to maximise the contractions seen. The contractile response in rat aortic rings to β-PEA was not altered by semicarbazide, a SSAO inhibitor (Lizcano *et al.*, 1998). Therefore, unlike MAO, this enzyme does not appear to be important for β-PEA degradation.

3.5.4 Contractile response to β-PEA – role of β-adrenoceptors

Previous workers have reported the presence of β_1- and β_2 -adrenoceptor subtypes in rat aorta (Kobayashi *et al.*, 1992). β-adrenoceptor-mediated vasorelaxation (Varma and Chemtob, 1993; Varma *et al.*, 1995) plays an important function in the regulation of vascular tone (Brawley *et al.*, 2000). Originally, in the vasculature of the rat, vasodilator β-adrenoceptors have been reported to be mainly of the β_2-adrenoceptor subtypes (Lands *et al.*, 1967; Wilffert *et al.*, 1982; Kazanietz *et al.*, 1989). Nevertheless, β_1–adrenoceptors can also contribute to vasodilatation (O'Donnell and Wanstall, 1984a; O'Donnell and Wanstall, 1985). Furthermore, the presence of atypical β-adrenoceptors (β_3–adrenoceptors) has been reported in rat aorta (Shafiei and Mahmoudian, 1999; Brawley *et al.*, 2000). In the present study, the effects of two different β-adrenoceptor antagonists propranolol, a non-selective β-adrenoceptor antagonist, and ICI-118,551, on the contractile response to β-PEA in isolated rat aortic rings, were examined. ICI-118,551 is the only β_2–adrenoceptor antagonist with high selectivity and specificity for β_2–subtypes (Bilski *et al.*, 1980; Bilski *et al.*, 1983). In contrast, various antagonists for β_1–adrenoceptors have already been well investigated, such as atenolol and betaxolol (Bilski *et al.*, 1983). The affinity of ICI-118,551 for β_2-adrenoceptors is much higher compared to

propranolol. In contrast, propranolol has a higher affinity for β_1-adrenoceptors compared to ICI-118,551 *(Table 3.1)*. Propranolol and ICI-118,551 both failed to potentiate significantly the contractile response to β-PEA. Therefore, the contraction in rat aortic rings to β-PEA is not affected by an opposing vascular β-adrenoceptor mechanism. In previous publications the concentrations of propranolol (Baker *et al.*, 2007)and ICI-118,551 (Molenaar *et al.*, 2006) were shown to be effective in blocking β_1- and β_2-adrenoceptors, respectively.

Accordingly, this result suggests that β-PEA does not stimulate β-adrenoceptors and cause a β-adrenoceptor-mediated vasorelaxation that occurs with other agonists, such as isoprenaline (Brawley *et al.*, 2000) *(see also Chapter 2.5.5)*.

β-Adrenoceptor Antagonist	β_1-Adrenoceptor Affinity (mean pA_2 values ±SEM)	β_2-Adrenoceptor Affinity (mean pA_2 values ±SEM)
ICI-118,551	7.17±0.11	9.26±0.99
Propranolol	8.30±0.05	8.64±0.11

Table 3.1: Affinity of propranolol and ICI-118,551 for β_1-and β_2-adrenoceptors (Bilski *et al.*, 1983)

Previous workers reported an endothelium- and β_2-adrenoceptor-independent vasodilatation effect of various phenylethylamines at higher concentrations in rat aorta in the presence of the unselective β-adrenoceptor antagonist propranolol (Varma and Chemtob, 1993; Varma *et al.*, 1995). Indeed, in the present study a relaxation response was revealed by propranolol and ICI-118,551 at the highest concentration of β-PEA. This was less with ICI-118,551. Therefore, the reduced contraction (vasodilatation) at the highest dose is probably not β-adrenoceptor mediated. Further investigations of the possible roles of β_1- and β_2 adrenoceptors will be made in *Chapter 4*.

In summary, the selective β_2–adrenoceptor antagonist ICI-118,551 did not inhibit or potentiate the contractile response to β-PEA in rat aortic rings. A benefit of using ICI-118,551 instead of propranolol was that other effects of the unselective β-adrenoceptor antagonist, such as membrane stabilization were avoided (local anaesthetic effects) (Anderson *et al.*, 1996). Therefore, future experiments were constructed in the presence of ICI-118,551. Further investigation showed that

prazosin in the presence of propranolol, or ICI-118,551 failed to eliminate the contractile response to β-PEA. Therefore, the remaining contractile response to β-PEA, in the presence of these inhibitors, is not caused by an α_1, or β-adrenoceptor effect. Moreover, the contractile response to the maximum concentration of β-PEA was also not affected by ICI-118,551 in the presence of cocaine. Although ICI-118,551, pargyline and cocaine had little effect on the contractile response to β-PEA all future experiments will be conducted in their presence along with prazosin. The rationale for this is that the influences of uptake in the neurone, MAO and β_2-adrenoceptors may be marginal but need to be eliminated if the mechanism for the vasoconstrictor effect of β-PEA is to be identified without their influence. Secondly, although these effects may be marginal, it cannot be excluded that when other conditions are applied to the tissues, such as introduction of other antagonists, then they may become more important. Thus, it was decided to avoid any complications from these mechanisms by abolishing them with appropriate antagonists.

To eliminate sympathomimetic responses involved in the contractile response of β-PEA, combinations of the inhibitors mentioned above were investigated. The largest response was found in the presence of prazosin, ICI-118,551, cocaine, and PG. This combination will be referred to as "inhibitors" in remainder of this thesis. This result suggests that the contraction to β-PEA in the presence of "inhibitors" is not caused by an uptake of β-PEA and subsequent release of noradrenaline onto α_1-adrenoceptors. The remaining contraction to β-PEA in the presence of "inhibitors" suggests that a mechanism other than ISA and α- and β-adrenoceptor stimulation is responsible for the contraction. The response to β-PEA is a direct effect and not the result of its uptake into noradrenergic neurones, because it is not affected by cocaine. The mechanism underlying the remaining contractile response to β-PEA in rat aortic rings will be investigated further in *subsequent chapters*.

3.6 Conclusion

As in *Chapter 2* the mechanism by which TAs cause vasoconstriction in rat aorta cannot be established from this study. In *Chapter 2* cumulative CRCs were used to show that various factors, such as the presence, or absence of endothelium or α_1- and β-adrenoceptors, do not affect the contractile response to TAs in rat aortic rings.

However, upon changing the experimental protocol to non-cumulative CRCs in the present *Chapter* and closer investigations of the effect of β-adrenoceptor antagonists on the contractile response to β-PEA, a better understanding of the mechanism of action of TAs has been gained. Within *Chapter 3,* findings from *Chapter 2* have been confirmed and developed. Therefore, as neither α- and β-adrenoceptors, nor ISA stimulation affect the vasoconstriction in rat aorta, it is likely that β-PEA and other TAs exert vasoconstriction by activation of a novel receptor system. This will be investigated in *subsequent Chapters.*

Chapter 4

INVESTIGATION OF THE CONTRACTILE RESPONSES TO β-PHENYLETHYLAMINE AND TRYPTAMINE IN RAT AORTA IN THE PRESENCE OF VARIOUS INHIBITORS

Chapter 4

Investigation of the contractile responses to β-PEA and tryptamine in the presence of various inhibitors

4.1 Introduction

In the past, the technique of cumulative addition of doses of agonists in isolated tissues has been well investigated (Ariens, 1954; Van Rossum and Ariens, 1959; Van Rossum, 1963). Both cumulative and non-cumulative addition of doses of inhibitors to isolated tissues have been used frequently to investigate drug-receptor relationships (Pennefather, 1973; Garcia-Sevilla and Erill, 1974; Abdelmawla *et al.*, 1996). Concentration-response curves (CRCs) with agonists and antagonists are the accepted standard for the characterization of receptor function and subtypes (Przyborski *et al.*, 1991). A previous publication compared cumulative and non-cumulative dose-response curves to noradrenaline in isolated tissues (Pennefather, 1973). The major effect observed in this study found that by increasing the concentration of an agonist cumulatively rather than non-cumulatively a reduction in the magnitude of the resultant response maximum resulted. These findings contrast with the observations of other studies in which cumulative addition of agonists showed identical results compared with non-cumulative CRCs of agonists (Garcia-Sevilla and Erill, 1974; Przyborski *et al.*, 1991; Kahonen *et al.*, 1993).

For a number of decades, TA-specific receptors had only been assumed to be located as anatomically distinct, high affinity TA binding sites in the CNS of humans and other mammals (Hauger *et al.*, 1982; McCormack *et al.*, 1986; Mousseau and Butterworth, 1995). For that reason, it was assumed that TAs can exert pronounced pharmacological effects in many species, such as affect the uptake or release of catecholamines (Black *et al.*, 1980; Parker and Cubeddu, 1988; Dyck, 1989; Premont *et al.*, 2001). Also, it was thought that TAs might affect the 5-HT activity at nerve endings (Baker *et al.*, 1977; Raiteri *et al.*, 1977), or act as neuromodulators through direct actions on receptors for catecholamines, or 5-HT (Jones and Boulton, 1980; Paterson and Boulton, 1988). These assumptions have to be reconsidered following the discovery of members of a novel family of related mammalian G protein-coupled receptors (GPCRs) called the TA-associate receptors (TAAR) (Borowsky *et al.*,

2001; Bunzow *et al.*, 2001; Lindemann *et al.*, 2005; Lindemann and Hoener, 2005) *(Chapter 1)*. Most work on TAs and TAARs has been done on the brain (Philips *et al.*, 1978; Vaccari, 1986; Boulton *et al.*, 1990; Paterson *et al.*, 1990) and cloned receptors expressed in transfected cells (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). However, TAs are present throughout the body (Premont *et al.*, 2001; Zucchi *et al.*, 2006). Indeed, the effects of TAs, such as MDMA (Gowing *et al.*, 2002; Bexis and Docherty, 2006) and cathinone (Al-Motarreb *et al.*, 2002b; Al-Motarreb and Broadley, 2003; Al-Motarreb, 2004; Al-Motarreb *et al.*, 2005) on the heart are known to be deleterious. However, very little is known about the distribution and function of these receptors and their endogenous ligands in the cardiovascular system (Davenport, 2003; Gloriam *et al.*, 2005a). TAs are related to classical biogenic amines (serotonin, noradrenaline, dopamine and histamine) (Lindemann and Hoener, 2005; Burchett and Hicks, 2006). The TAARs belong to a subfamily of rhodopsin-like seven transmembrane-spanning GPCRs and are related to the classical biogenic amine receptors such as muscarinic, adrenergic and serotonin receptors (Kim and von Zastrow, 2001; Davenport, 2003; Fredriksson *et al.*, 2003). In cell lines, the most commonly recognized signal transduction mechanism for TAARs is to activate the formation of cAMP when exposed to TAs. TAAR1 stimulates the Gs protein and mediates an increase in cAMP (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). However, the coupling with different G proteins by different TAAR subtypes is likely. It has been suggested that TAAR 1 might be able to couple with different G proteins in different cell lines (Lewin, 2006; Zucchi *et al.*, 2006).

The structural similarity between TAs and classical biogenic amines has led to the suggestion that the vasoconstriction in rat aortic tissues observed upon addition of TAs could be caused by an indirect sympathomimetic action releasing noradrenaline for adrenoceptors. The work described in this *Chapter* extends that in the previous two *Chapters* by examining whether β-PEA and tryptamine exert vasoconstriction of rat aorta through serotonin receptors.

4.2 Aims

To study the contractile response of rat isolated aortic rings to TAs

a.) Determine whether vasoconstriction to β-PEA is mediated *via* other mechanisms beside sympathomimetic actions.

b.) Determine whether tryptamine produces vasoconstriction in the isolated rat aortic tissues

c.) Determine whether any vasoconstriction to tryptamine is mediated *via* 5-HT serotonergic receptors.

4.3 Material and Methods

The main methods and experimental protocol described in *Chapter 2* were retained throughout the study unless otherwise stated.

4.3.1 Experimental Protocol

After equilibration cumulative and non-cumulative CRCs for β-PEA, clonidine, serotonin, tryptamine and dopamine were obtained in the absence, or presence of different inhibitors, which were incubated with the tissue for 15 minutes before commencing the CRC. In all experiments, the endothelium was removed by inserting a wooden cocktail stick and rolling the aortic ring round it.

4.3.2 Analysis of Results

$EC_{KCl\ 25}$ values

Contractions at the plateau response to each concentration of agonist were measured from the baseline before the CRC. These were then expressed as a percentage of the contraction to KCl in each experiment and the mean responses (± SEM) plotted. -log $EC_{KCl\ 25}$ values of individual CRCs were calculated together with the SEM. The $EC_{KCl\ 25}$ value is the molar concentration of an agonist which produces 25% of the contraction to KCl. Mean -log molar $EC_{KCl\ 25}$ values were compared by paired or unpaired Student's t-test using *GraphPad Instat 3.*

Dose-ratio

In the present *Chapter* dose-ratios were calculated for some of the agonist, including β-PEA and tryptamine in the presence of different antagonists. Dose-ratios were calculated for individual tissues and mean ± SEM values are presented in the text. The presence of an antagonist increases the EC_{50} value by a factor equal to $1+[B]/K_b$ which is called the dose-ratio. The graph below illustrates the dose-ratio *(Figure 4.1)*.

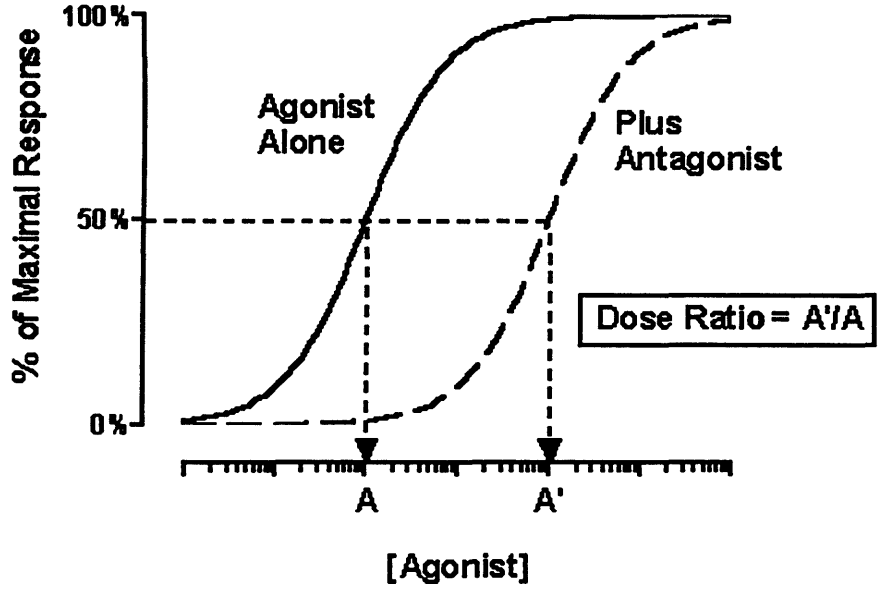


Figure 4.1: The dose-ratio
If concentration A gives a certain response in the absence of antagonist, but concentration A′ is needed to achieve the same response in the presence of a certain concentration of antagonist, then the dose-ratio equals **A′/A.** The dose-ratio varies with different concentration of antagonist. *Taken from GraphPad Software (1999)*

4.3.3 Drugs Used

All chemicals were dissolved in distilled water, unless otherwise stated. Prazosin hydrochloride, ICI-118,551 hydrochloride and U46619 were dissolved in DMSO (dimethyl sulfoxide).

- **Reagents obtained from Sigma Aldrich (Poole, Dorset, UK)**

Atenolol hydrochloride, clonidine hydrochloride, cocaine hydrochloride, (3,4-dihydroxyphenethylamine), haloperidol, indomethacin, pargyline hydrochloride (PG), β-phenylethylamine (β-PEA), prazosin hydrochloride, (±)-propranolol hydrochloride, yohimbine hydrochloride, serotonin (5-hydroxytryptamine (5-HT)) hydrochloride, SR 59230A oxalate salt, tryptamine hydrochloride, and U46619 (9,11 dideoxy-11α, 9α epoxymethano-prostaglandine F2α)

- **Reagents obtained from TOCRIS Bioscience (Northpoint, Avonmouth, UK)**

ICI-118,551 hydrochloride ((±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl) oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), ketanserin tartrate and methiothepin maleate

- **Reagents obtained from Fisher Scientific (Leicestershire, UK)**

All chemicals for the Krebs-bicarbonate buffer (analytical grade)

4.4 Results

4.4.1 Contractile response to β-PEA – effect of endothelium and U46619

The contractile response of rat aorta to β-PEA in the presence of "inhibitors" was independent of the presence of the endothelium *(Figure 4.2)*. The response at the maximum concentration of β-PEA (3mM, 113.3±8.2%, n=4) after endothelium removal was not significantly different (P>0.05) from that in aorta with intact endothelium (3mM, 90±36.7%, n=4). The -log EC_{50} values for β-PEA in endothelium-denuded (3.21±0.11, n=4) and in endothelium-intact tissues (2.93±0.15, n=4) were not significantly different (P>0.05). In tissues pre-contracted with U46619, β-PEA in the presence of "inhibitors" showed no relaxation in the presence, or the absence of endothelium. Indeed, pre-contracted aortic rings showed a further contractile response to β-PEA with "inhibitors". At the maximum concentration, the contractile response in aortic rings to β-PEA in endothelium-intact tissues (3mM, 141.2 ±30%, n=3) was not significantly different (P>0.05) compared to the response in endothelium-denuded tissues (3mM, 88.9±11.1%, n=3) *(Figure 4.3)*. Furthermore, the –log EC_{50} values for β-PEA in endothelium-intact (4.62±0.24, n=4) and in endothelium-denuded tissues (5.22±0.34, n=4) were not significantly different (P>0.05). In pre-contracted and endothelium-intact tissues no significant differences (P>0.05) in the contractile response to β-PEA were found between pre-contracted, endothelium-intact tissues in the presence of "inhibitors" (3mM, 141.2±30%, n=3) and pre-contracted, endothelium-intact tissues in the absence of "inhibitors" (120.7±21.9%, n=5) *(Figure 4.4)*. Endothelium-denuded, pre-contracted aortic rings showed, in the presence of "inhibitors", a significantly (P<0.01) greater contractile response at the maximum concentration of β-PEA (3mM, 88.8±11.1%, n=3) compared to endothelium-denuded, pre-contracted tissues without "inhibitors" (3mM, 53.3±3.3%, n=5) *(Figure 4.5)*. However the –log EC_{50} values of endothelium-denuded, pre-contracted tissues in the presence of "inhibitors" (5.22±0.34, n=3) and pre-contracted tissues in the absence of endothelium and "inhibitors" (5.44±0.20, n=4) were not significantly different (P>0.05) *(Figure 4.5)*.

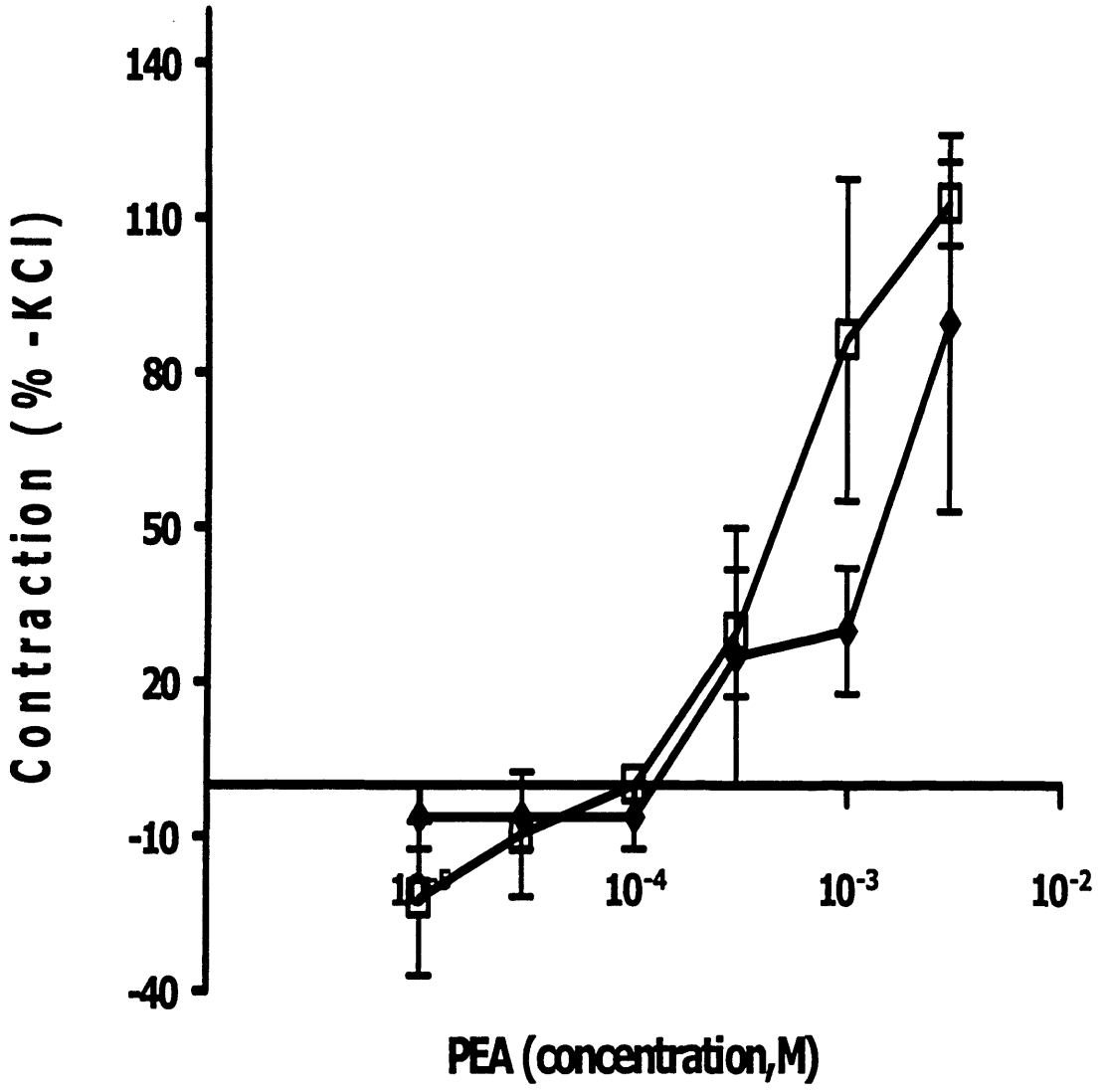


Figure 4.2: Effect of endothelium removal and "inhibitors" on the mean CRCs for the contractile response of rat aorta to β-PEA. Cumulative CRCs for β-PEA in rat aorta were constructed in the absence of endothelium and in the presence of "inhibitors" (□, n=4) or in the presence of endothelium and "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences (P>0.05) were seen at the maximum concentration (3mM).

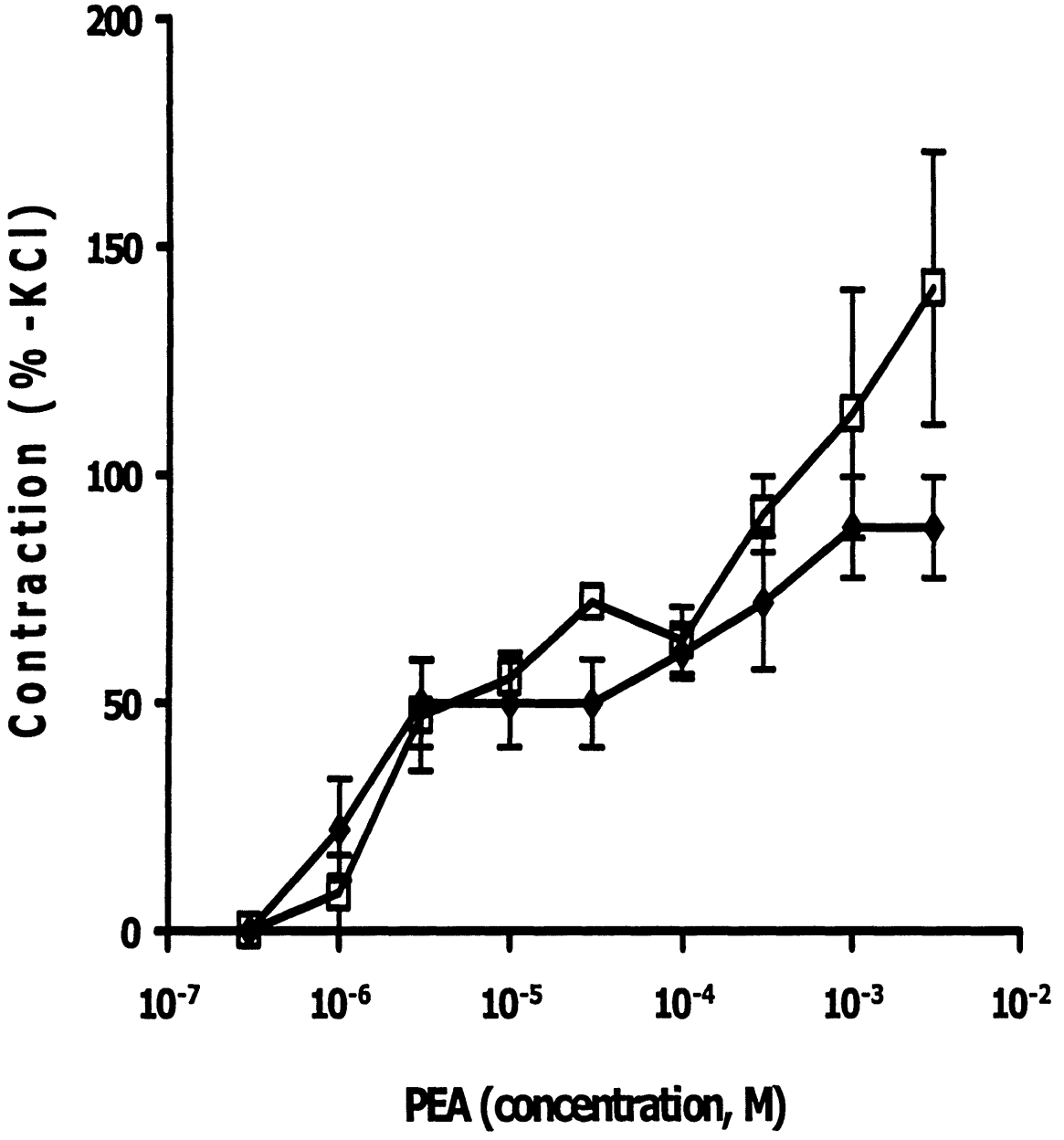


Figure 4.3: Contractile effects of β-PEA on rat aortic rings pre-contracted with U46619 (1μM) – Effect of endothelium. Cumulative CRCs for β-PEA in rat aorta were constructed in the presence of "inhibitors" and in the presence (□, n=3), or in the absence (◆, n=3) of endothelium above the U46619-induced contraction. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences (P>0.05) were seen at the maximum concentration (3mM).

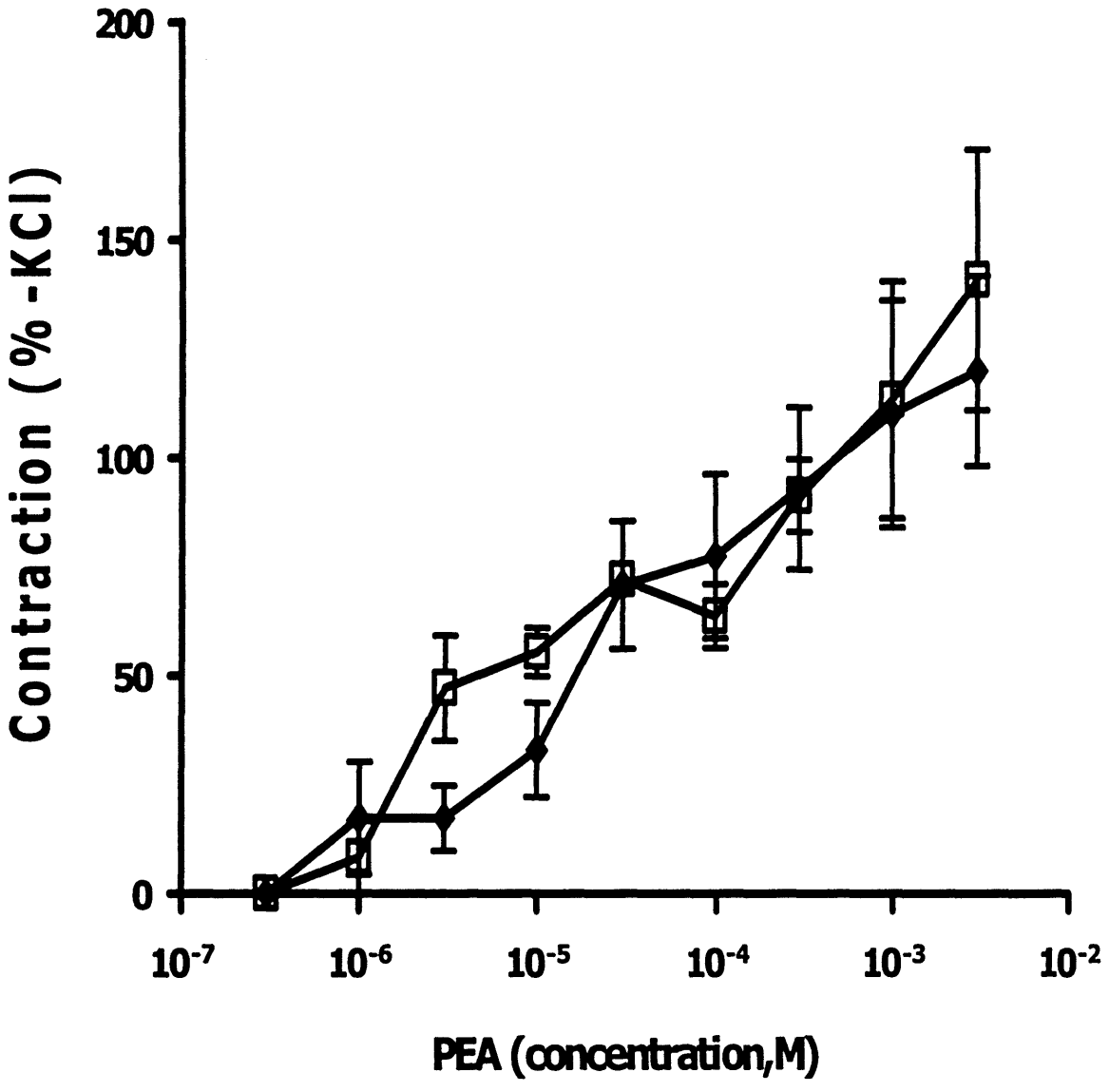


Figure 4.4: Contractile effects of β-PEA on rat aortic rings pre-contracted with U46619 (1μM). Cumulative CRCs for β-PEA in rat aorta were constructed in the presence of endothelium and in the presence (□, n=3) or in the absence (◆, n=5) of "inhibitors" above the U46619-induced contraction. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen at the maximum concentration (3mM).

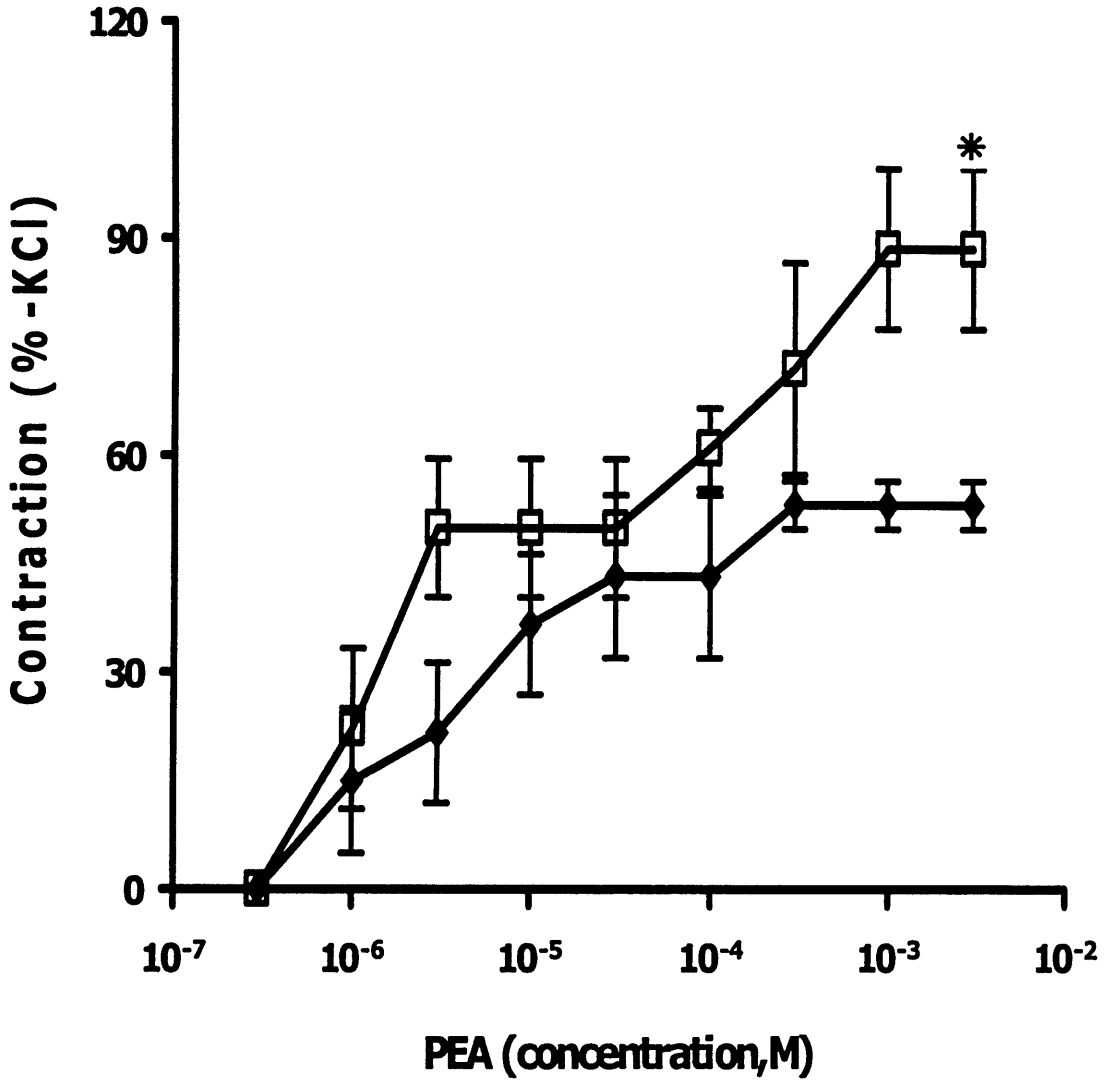


Figure 4.5: Contractile effects of β-PEA on rat aortic rings pre-contracted with U46619 (1μM) – Effect of endothelium removal. Cumulative CRCs for β-PEA in rat aorta were constructed in the absence of endothelium and in the presence (□, n=3), or above the U46619-induced contraction in the absence (◆, n=5) of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. A significant difference ($P<0.05$) was shown at the concentration of 3mM between both CRCs (*).

4.4.2 Contractile response to β-PEA and tryptamine – effect of indomethacin

The contractile response of rat aorta to β-PEA was not significantly (P>0.05) altered in the presence of indomethacin (10μM). The –log EC_{50} value for β-PEA in the presence of indomethacin (10μM) (4.69±0.42, n=4) was not significantly different from that in the absence of indomethacin (10μM) (4.37±0.15, n=4). Furthermore, the contractile response at the maximum concentration to β-PEA in the absence (1mM, 99.2±25.2%, n=4), or in the presence (1mM, 126.25±44%, n=4) of indomethacin were not significantly different (P>0.05) *(Figure 4.6)*. In addition, the contractile response of rat aorta to β-PEA in the presence of "inhibitors" was not significantly altered (P>0.05) in the presence of indomethacin (10μM) *(Figure 4.7)*. The maximum contractile response of β-PEA in the presence of "inhibitors" (300μM, 138.2±23.1%, n=6) was not significantly different (P>0.05) from that of β-PEA with "inhibitors" and indomethacin (10μM) (300μM, 123±32%, n=6) *(Figure 4.7)*. It is worth noting that more typical sigmoidal CRCs were obtained in the presence of "inhibitors".

After the effect of indomethacin (10μM) on the contractile response to β-PEA in rat aortic rings was investigated, the response to tryptamine in the presence of "inhibitors" and indomethacin (10μM) was examined. Tryptamine caused concentration-related contraction of the rat aorta. The -log EC_{50} value (5.5±0.29, n=4) was not altered by the presence of "inhibitors" (5.07±0.31, n=7). There was not further shift of the CRC and in the presence of "inhibitors" and indomethacin (10μM) (-log EC_{50} 5.3±0.62, n=4). Neither the maximum contractile response to tryptamine (10μM, 163.9±28.1%, n=4) nor to tryptamine with "inhibitors" (3μM, 144.4 ±34.9%, n=8) were significantly (P>0.05) altered by indomethacin (10μM) (3μM, 277.8±27.8%, n=3) *(Figure 4.8)*.

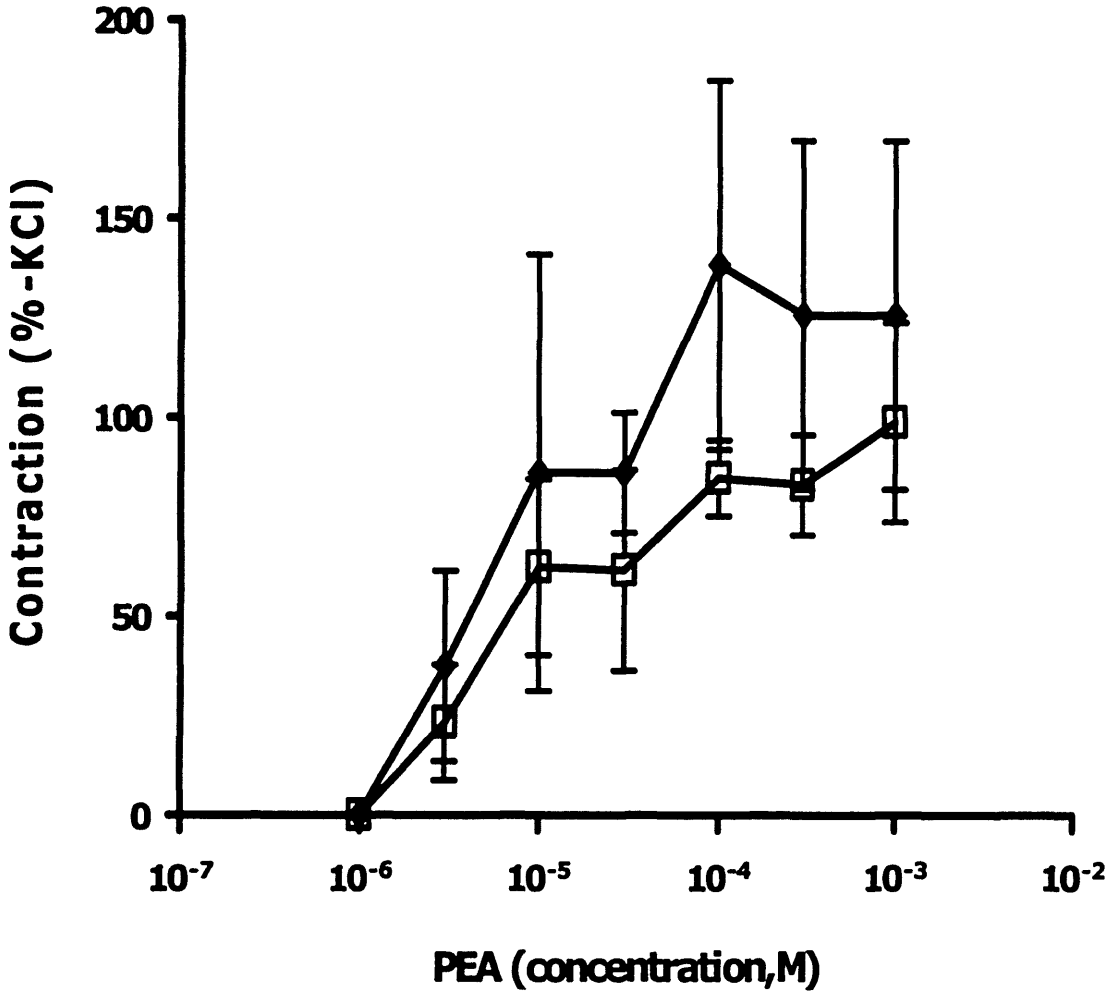


Figure 4.6: Mean non-cumulative CRCs for the contractile responses of rat aorta to β-PEA in the absence (□, n=4) and presence of indomethacin (10μM) (◆, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences were seen (P>0.05) at the concentration 100μM and 1mM.

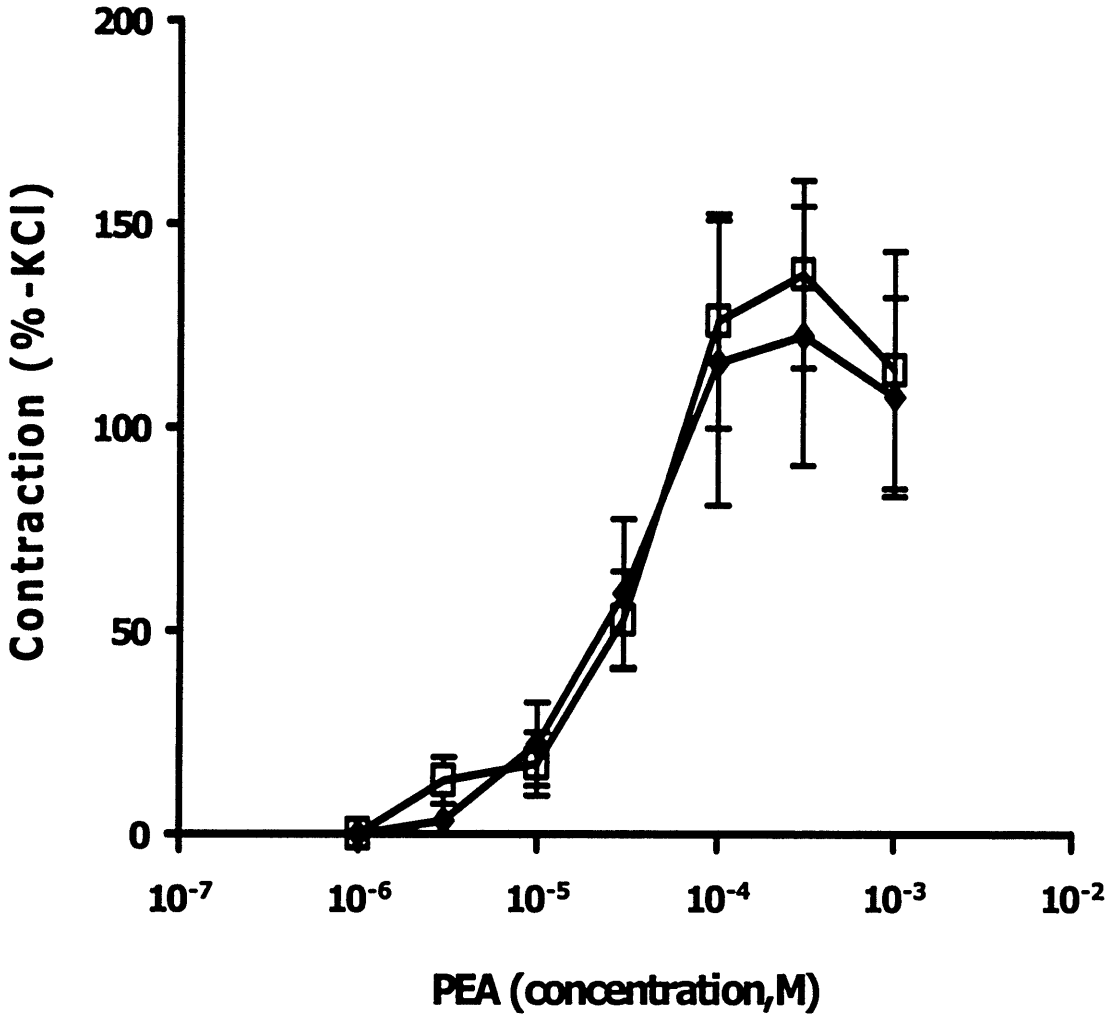


Figure 4.7: Mean non-cumulative CRCs for the contractile responses of rat aortic rings to β-PEA in the absence (□, n=6) and presence of indomethacin (10μM) and "inhibitors" (◆, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen at the maximum concentration (300μM).

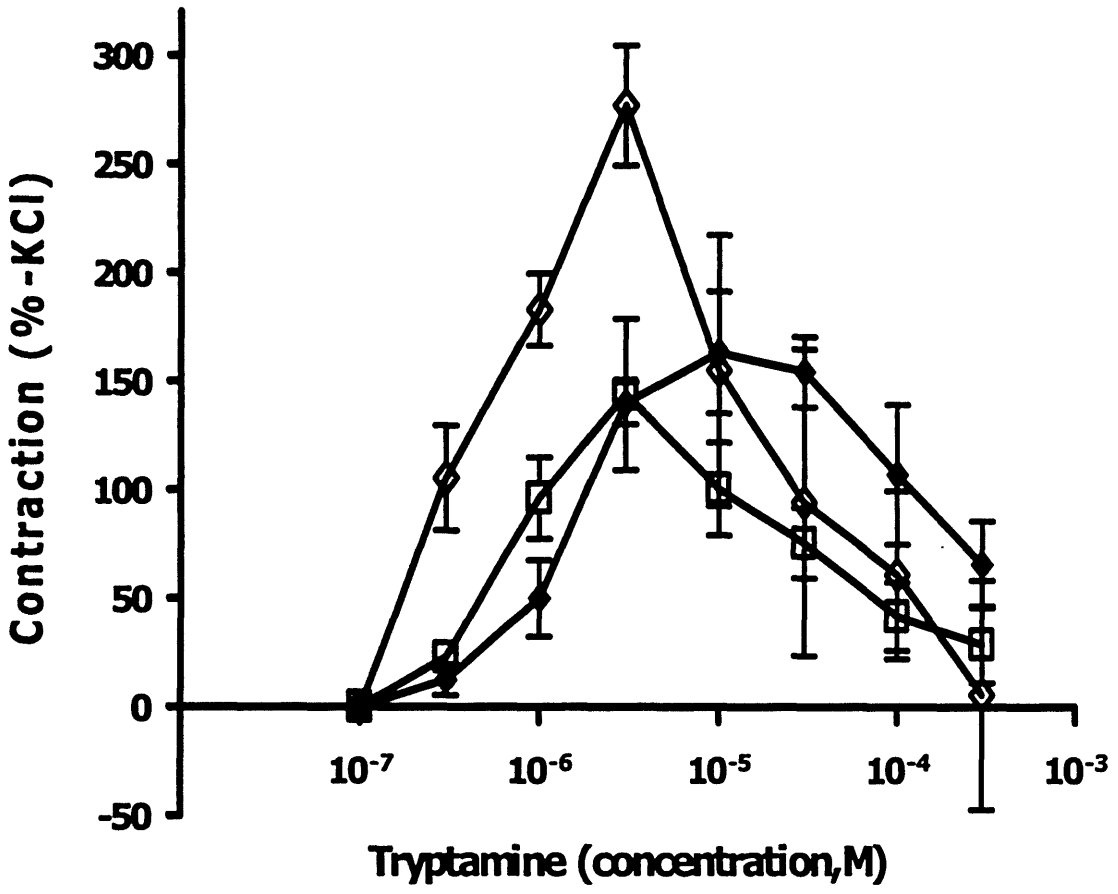


Figure 4.8: Effects of "inhibitors" and indomethacin (10μM) on the contractile response of rat aortic rings to tryptamine. Non-cumulative CRCs for tryptamine were constructed in the absence of antagonists (◆, n=5) or in the presence of "inhibitors" (□, n=8) or in the presence of "inhibitors" and indomethacin (10μM) (◇, n=3). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences (P>0.05) were seen at their maximum contractions.

4.4.3 Contractile response to β-PEA - effect of α_2, β_2 and β_3-adrenoceptor antagonists

In *Chapter 2*, the effect of the "inhibitors" prazosin (1μM), cocaine (10μM), ICI-118,551 (1μM), and pargyline (PG, 10μM) against β-PEA were examined. In this *Chapter*, the effects of antagonists of other adrenoceptor subtypes were examined.

Yohimbine, a selective, competitive α_2-adrenoceptor antagonist, was tested against β-PEA at two different concentrations (5μM and 50μM). The lower concentration of yohimbine (5μM) was used in a previous publication to examine its effect on contractions of rat aortic rings to different α_1- and α_2- adrenoceptor agonists, including noradrenaline, phenylephrine and guanfacine (Digges and Summers, 1983a). The maximum contractile response of β-PEA in the presence of "inhibitors" (300μM, 138±23.1%, n=6) was not significantly (P>0.05) inhibited by yohimbine (5μM) (300μM, 132±34.4%, n=4) *(Figure 4.9)*. Furthermore, yohimbine (50μM) in the presence of "inhibitors" did not potentiate the contractile response to β-PEA significantly (P>0.05) (300μM, 169±43.7% n=3) *(Figure 4.9)*. To confirm that yohimbine was used at a suitable concentration, the antagonist (50μM) was tested against clonidine, an α_2- adrenoceptor agonist. Clonidine caused concentration-related contractions of rat aortic rings with a maximum response at 0.3μM (130.4±28.3% n=3). The maximum contractile response of clonidine was greatly inhibited by yohimbine (50μM). Furthermore, the $-\log EC_{KCl\ 25}$ values of clonidine in the absence (7.42±0.17, n=4) and presence of yohimbine (50μM) (5.60±0.19, n=4) were significantly different (P<0.01) *(Figure 4.10)*. However, the $-\log EC_{KCl\ 25}$ values of β-PEA in the presence of "inhibitors" (4.92±0.17, n=6) and β-PEA plus "inhibitors" and yohimbine (50μM) (5.02±0.41, n=6) were not significantly (P>0.05) different *(Figure 4.9)*.

Previous results in *Chapter 3* showed the effects of propranolol and ICI-118,551 in isolated aortic rings against β-PEA *(non-cumulative CRCs)*. To complete the β_1- and β_2-adrenoceptor data, the effect of atenolol, a selective β_1-adrenoceptor antagonist, was examined. The maximum contractile response to β-PEA (1mM, 99.2±25.2%, n=4) was not significantly (P>0.05) potentiated by atenolol (300μM, 163.1±23.8%,

n=5) *(Figure 4.11)*. Furthermore, the contractile response to β-PEA in the presence of atenolol (1μM) alone, atenolol (1μM) with prazosin (1μM) and atenolol (1μM) with cocaine (10μM) was examined *(Figure 4.12)*. The maximum contractile responses of β-PEA in the presence of atenolol (300μM, 163.1±23.8, n=5), atenolol and prazosin (300μM, 263.6±0.50%, n=4) and atenolol and cocaine (300μM, 173±31.8%, n=4) were not significantly different (P>0.05) *(Figure 4.12)*.

Previous publications have studied atypical β-adrenoceptors in rat aorta, including the β_3-adrenoceptor (Shafiei and Mahmoudian, 1999; Matsushita *et al.*, 2003). The contractile response in rat aorta to β-PEA in the presence of SR59230A (10μM), a selective β_3-adrenoceptor antagonist, was examined on cumulative CRCs in endothelium-denuded tissues *(Figure 4.13)*. The maximum contractile response to β-PEA (100μM, 55.3±17.9%, n=4) was not inhibited by SR59230A (10μM) (300μM, 51.5±9.6%, n=4) *(Figure 4.13)*.

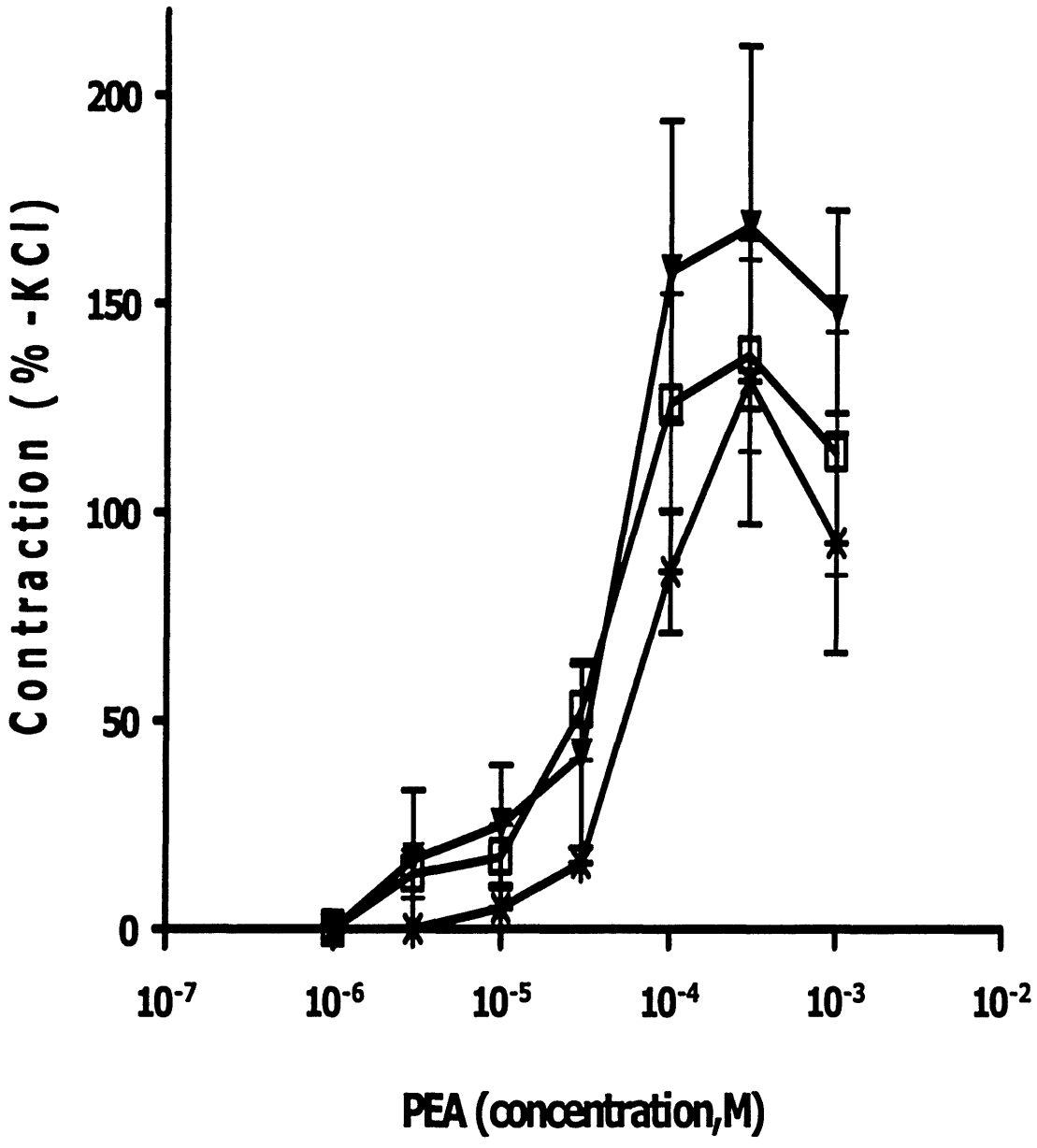


Figure 4.9: Effects of "inhibitors" and yohimbine (5μM and 50μM) on the contractile response of rat aortic rings to β-PEA. Non-cumulative concentration-response curves (CRCs) for β-PEA in rat aorta were constructed in the presence of "inhibitors" (□, n=5) or in the presence of "inhibitors" and yohimbine (5μM) (✳, n=4) or in presence of "inhibitors" and yohimbine (50μM) (▼, n=3). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences (P>0.05) were seen at their maximum contractile responses (300μM).

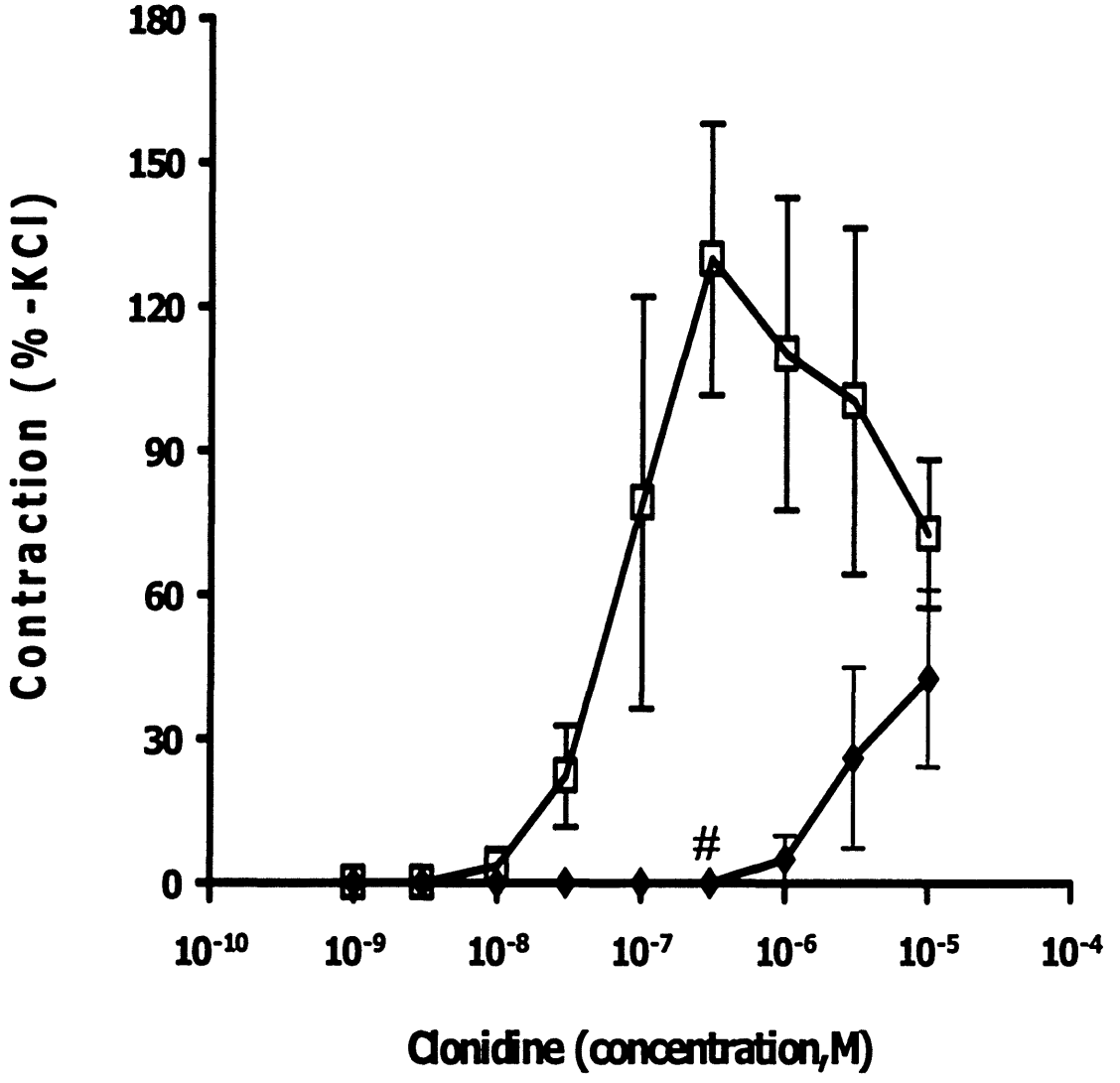


Figure 4.10: Effect of yohimbine (50μM) on the contractile response of rat aortic rings to clonidine. Non-cumulative CRCs for clonidine in rat aorta were constructed in the absence of antagonists (□, n=4), or presence of yohimbine (50μM) (◆, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) and –log EC_{50} values (±SEM) were compared by Student's paired t-test. The maximum contractile response of clonidine (0.3μM) was greatly inhibited (#, $P<0.05$) by yohimbine (50μM). The –log $EC_{KCl\ 25}$ values in the absence (7.42±0.0.17, n=4) and presence of yohimbine (50μM) (5.60±0.19, n=3) were significantly different ($P<0.01$).

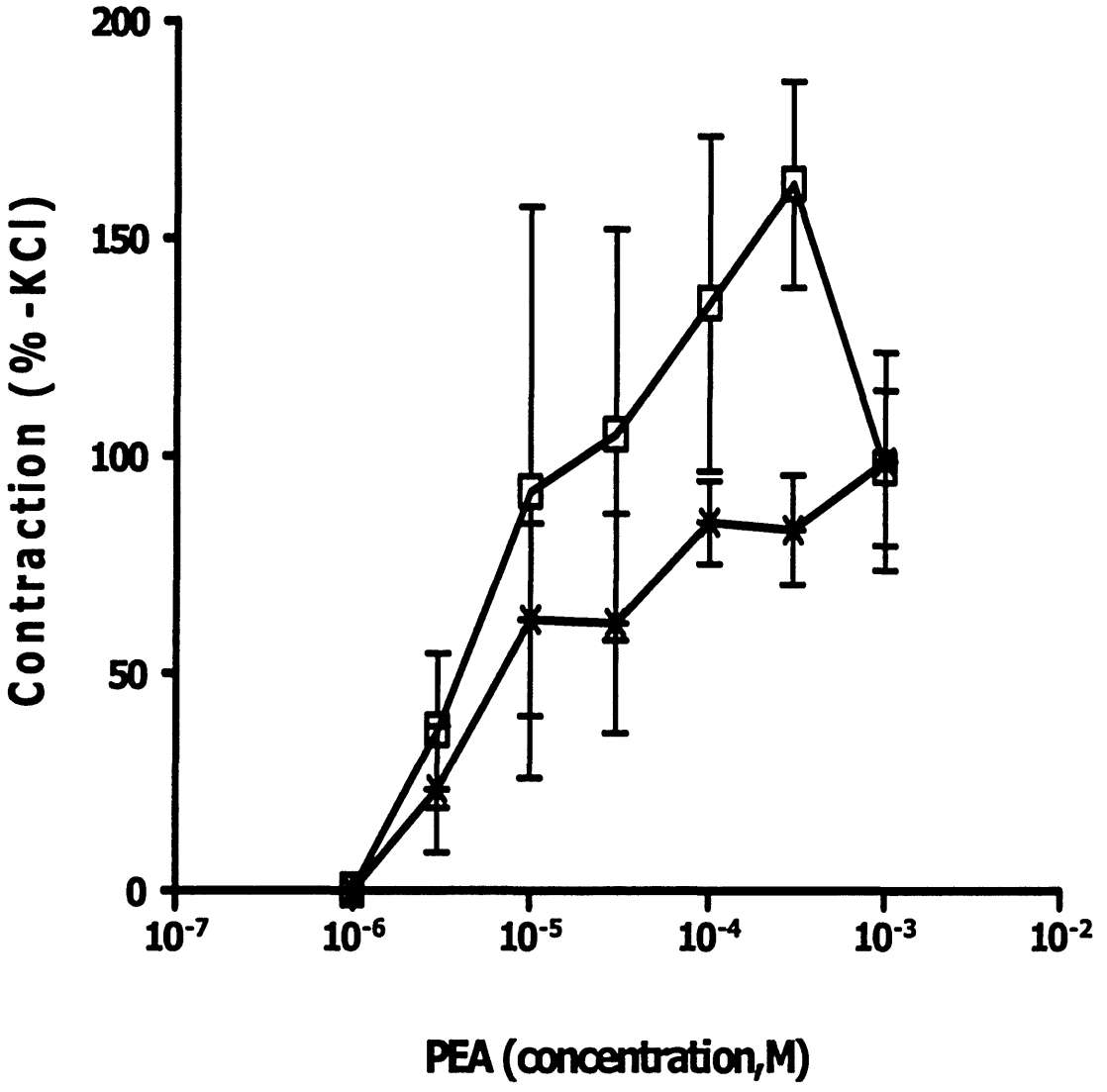


Figure 4.11: Effects atenolol (1μM) on the contractile response of rat aortic rings to β-PEA. Non-cumulative CRCs for β-PEA in rat aorta were constructed in the absence of antagonists (▼, n=4) or in the presence of atenolol (1μM) (□, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences (P>0.05) were seen between their maximum contractile responses.

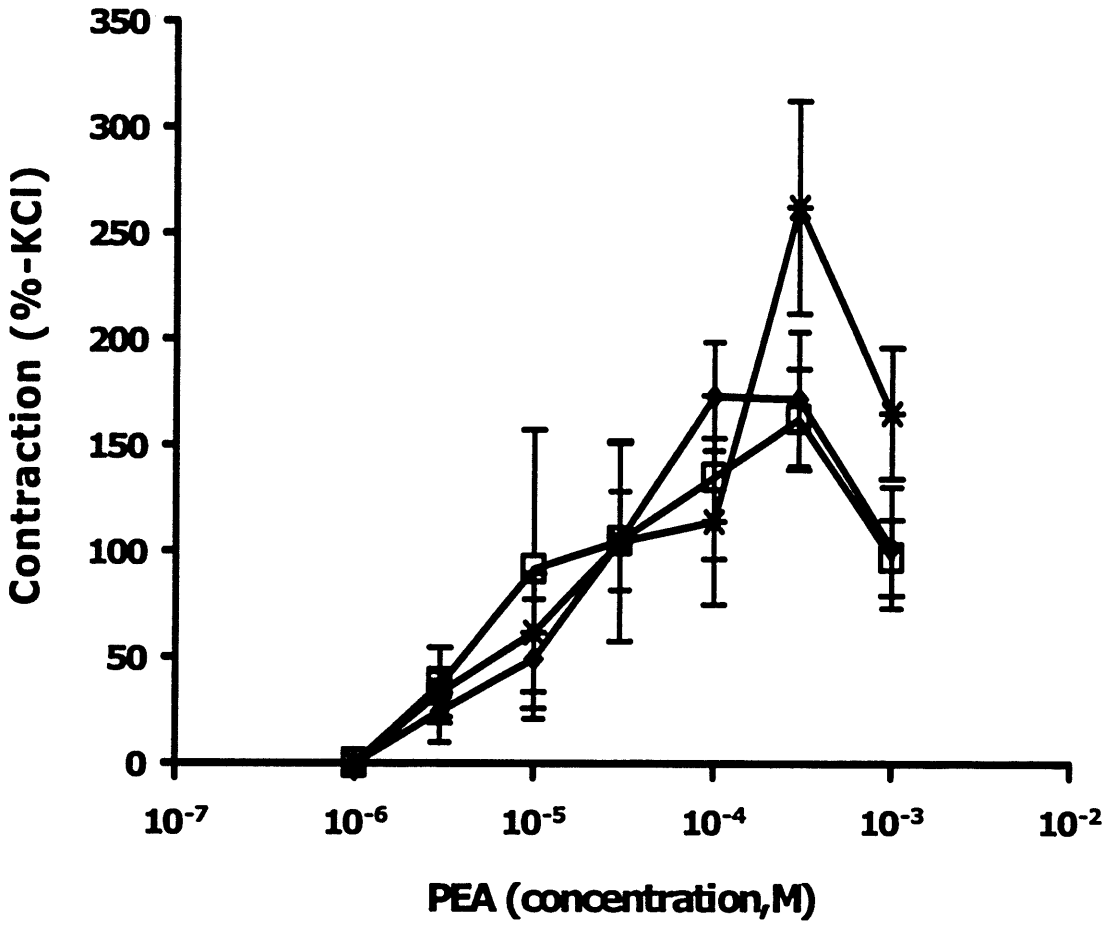


Figure 4.12: Effect of atenolol (1μM), prazosin (1μM) and cocaine (10μM) on the contractile response of rat aortic rings to β-PEA. Non-cumulative CRCs for β-PEA in rat aorta were constructed in the presence of atenolol alone (1μM) (□, n=5), atenolol (1μM) and prazosin (1μM) (✱, n=4) or atenolol (1μM) and cocaine (10μM) (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences ($P>0.05$) were seen between their maximum contractile responses (300μM).

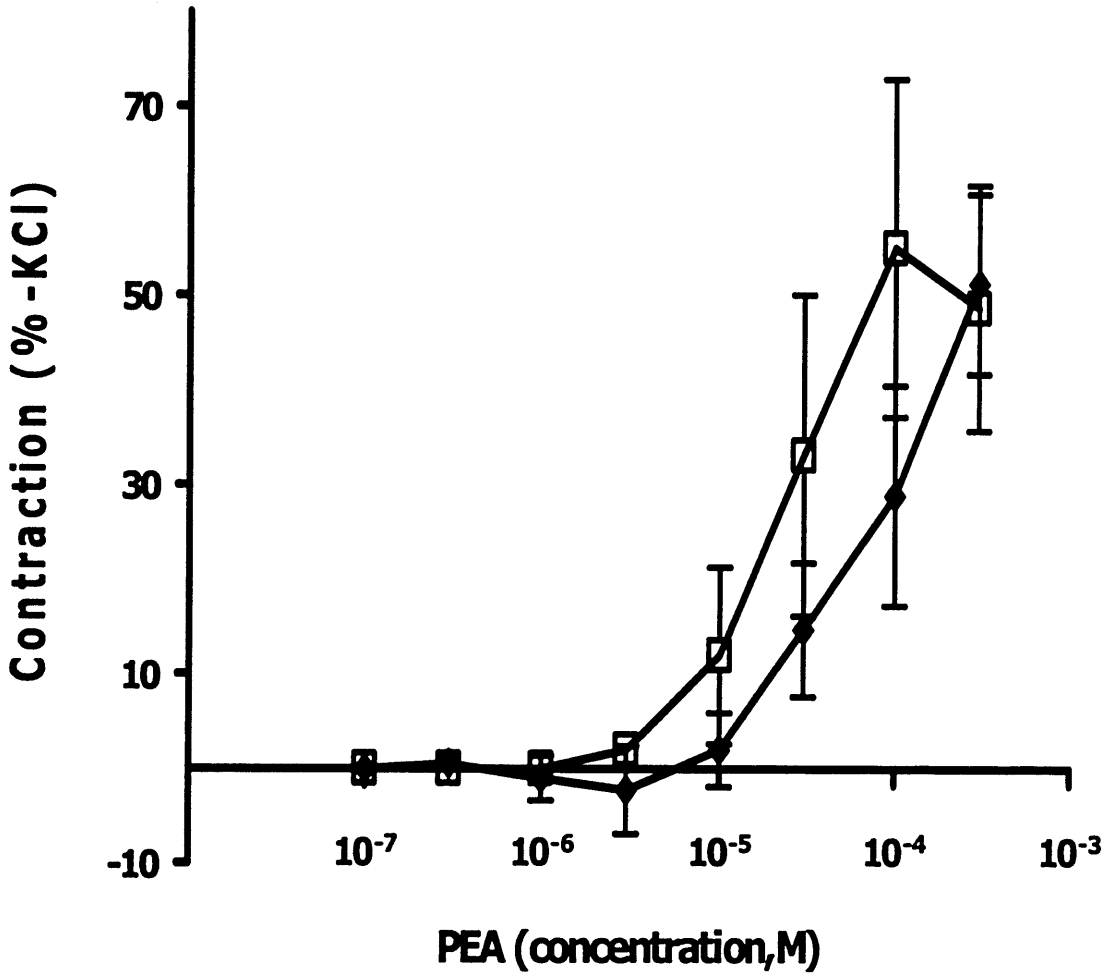


Figure 4.13: Effect of SR59230A (10μM) on the contractile response of rat aortic rings to β-PEA. Cumulative CRCs for β-PEA in rat aorta were constructed in the absence (□, n=4) or in the presence of SR 59230A (10μM) (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (+SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen at the maximum concentration (300μM).

4.4.4 Contractile responses to serotonin and tryptamine effect of various inhibitors

The contractile response of rat aortic rings to the TA tryptamine in the presence of the "inhibitors", ketanserin, a 5-HT_{2A}-serotonin receptor antagonist, and methiothepin, a non-selective 5-$HT_{1,2,7}$ serotonin receptor antagonist, was examined. To establish suitable conditions for the tryptamine experiment, the contractile responses in rat aorta to serotonin in the presence of "inhibitors", methiothepin (50nM), ketanserin (3nM and 30nM), and methiothepin (50nM) with ketanserin (3nM) were first investigated.

CRCs to serotonin in the presence, or absence of different inhibitors had different shapes compared with the CRCs for β-PEA. The CRC for the contractile responses in rat aorta to serotonin can be described as biphasic. The first peak of the contractile response to serotonin occurred at a concentration of 0.03μM (100±0%, n=6) and the second peak appeared at the maximum concentration of 3μM (186.1±24.8%, n=6) *(Figure 4.14)*. In the presence of "inhibitors", the biphasic CRC for the contractile response to serotonin was retained, but shifted upwards and to the left. The first peak of the CRC to serotonin now appeared at a concentration of 0.01μM (182.7±50.7%, n=4), the second peak was then reached at a concentration of 0.1μM (170.2±41.9%, n=4) *(Figure 4.14)*. The –log EC_{50} values of serotonin calculated for the first phase of the CRC in the absence (8.22±0.27, n=3), or presence of "inhibitors" (8.54±0.09, n=3) were not significant different (P>0.05) *(Figure 4.14)*.

These experiments were next repeated in the presence of methiothepin (50nM) and ketanserin (3nM). The contractile response to serotonin in the presence of "inhibitors", methiothepin (50nM) and ketanserin (3nM) also showed a biphasic CRC *(Figure 4.15)*. The first peak of the CRC to serotonin in the presence of "inhibitors" (-log EC_{50} 8.54±0.09, n=3) was significantly shifted to the right in the presence of methiothepin (50nM) alone (-log EC_{50} 7.99±0.01, n=3) (P<0.01), ketanserin (3nM) alone (-log EC_{50} 8.09±0.17, n=3) (P<0.05) and with the combination of both antagonists (-log EC_{50} 7.98±0.02, n=4) (P<0.01) *(Figure 4.15)*. Moreover, the contractile responses at 0.01μM in the presence of "inhibitors" (0.01μM, 182.7±50.7%, n=4), in the presence of "inhibitors" and methiothepin

(0.01μM, 21.9±12.9%, n=4), in the presence of "inhibitors" and ketanserin (0.01μM, 36.2±24.5%, n=4) and in the presence "inhibitors" plus both antagonists (0.01μM, 15.4±8.11%, n=4) were significantly different (P<0.01) *(Figure 4.15)*. Furthermore, the second peak of the CRC to serotonin (0.1μM, 170.2±41.9%, n=4) was significantly (P<0.01) reduced in the presence of methiothepin (0.1μM, 24.4±11.7%, n=4), and methiothepin (50nM) with ketanserin (3nM) (0.1μM, 9.8±4.44%, n=4) *(Figure 4.15)*. The contractile response in rat aorta to serotonin was next examined in the presence of a higher concentration of ketanserin (30nM) and "inhibitors" *(Figure 4.16)*. The biphasic CRC to serotonin in the presence of "inhibitors" was no longer obvious in the presence of ketanserin (30nM). However, the CRC of serotonin in the presence of "inhibitors" measured at the first peak of the CRC (-log EC_{50} 8.54±0.09, n=3) was significantly (P<0.001) shifted to the right by ketanserin (30nM) with "inhibitors" (-log EC_{50} 6.64±0.12, n=4) *(Figure 4.16)*.

After investigating the effect of serotonin antagonists in rat aortic rings, their effects on the contractile response to tryptamine were examined. Firstly, the effects of "inhibitors" on tryptamine were examined. Neither ICI-118,551 (1μM), prazosin (1μM), cocaine (10μM), pargyline (10μM) in different combinations, nor "inhibitors" significantly altered (P>0.05) the maximum response to tryptamine (10μM, 163.9±28.1%, n=4) *(Figure 4.17)*. The tryptamine CRC (-log EC_{50} 5.5±0.29, n=4) was not significantly (P>0.05) shifted by ICI-118,551 with prazosin (-log EC_{50} 5.34±0.21, n=5). The maximum contraction of tryptamine (10μM, 163.9±28.1%, n=4) was not significantly different from that in the presence of ICI-118,551 and prazosin (30μm, 203±47.2%, n=5), which was not significantly different (P>0.05) from that in the presence of ICI-118,551 prazosin and cocaine (30μM, 130±21%, n=6) *(Figure 4.17)*. In the presence of all four "inhibitors" neither the –log EC_{50} (-log EC_{50} 5.1±0.31, n=7) nor the maximum response (3μM, 144.4±34.9%, n=8) to tryptamine was significantly (P>0.05) altered (-log EC_{50} 4.97±0.59, n=5, 30μM, 210.1±57.1%, n=5) *(Figure 4.17)*. Ketanserin at the lower concentration (3nM) and methiothepin (50nM) produced a small inhibition of the response to tryptamine in the presence of "inhibitors" *(Figure 4.18)*. The maximum contraction to tryptamine in the presence of "inhibitors" (3μM, 144.4±34.9%, n=8) was not significantly altered by the combination of both serotonin antagonists (10μM, 138.9±65.4%, n=6).

Furthermore, the –log EC_{50} values for tryptamine in the presence of "inhibitors" (6.23±0.08, n=5) and in the presence of "inhibitors" plus both serotonin antagonists (5.93±0.18, n=4) were not significantly different (P>0.05) *(Figure 4.18)*.

The effect of ketanserin alone at a higher concentration (30nM) was next examined against tryptamine. The contractile response to tryptamine in the presence of "inhibitors" and ketanserin (30nM) showed a biphasic CRC *(Figure 4.19)*. The –log EC_{50} values measured for the first peak of tryptamine in the presence of "inhibitors" (6.23±0.08, n=5) and in the presence of "inhibitors" plus ketanserin (30nM) (5.51±0.12, n=3) were significantly (P<0.001) different. However, at the first contractile response peak, the maximum contractile responses to tryptamine (10μM, 163.9±28.1%, n=4), to tryptamine in the presence of "inhibitors" (3μM, 144.4±34.9%, n=8) and to tryptamine in the presence of "inhibitors" and ketanserin (30nM) (10μM, 164.8±36.1%, n=3) were not significantly different (P>0.05) *(Figure 4.19)*.

The dose-ratio *(see Figure 4.1)* was calculated for ketanserin (30nM) at the EC_{50} values between tryptamine and "inhibitors" and tryptamine plus "inhibitors" and ketanserin (30nM) (dose-ratio of 5.7±1.7, n=3) and statistically compared with the dose-ratio for ketanserin (30nM) against serotonin. The dose-ratio for ketanserin (30nM) at the EC_{50} values between serotonin and "inhibitors" and serotonin plus "inhibitors" and ketanserin (30nM) (dose-ratio of 90.9±23.3, n=4) was significantly higher (P<0.01) compared to the dose-ratio for ketanserin against tryptamine (dose-ratio of 5.7±1.7, n=3).

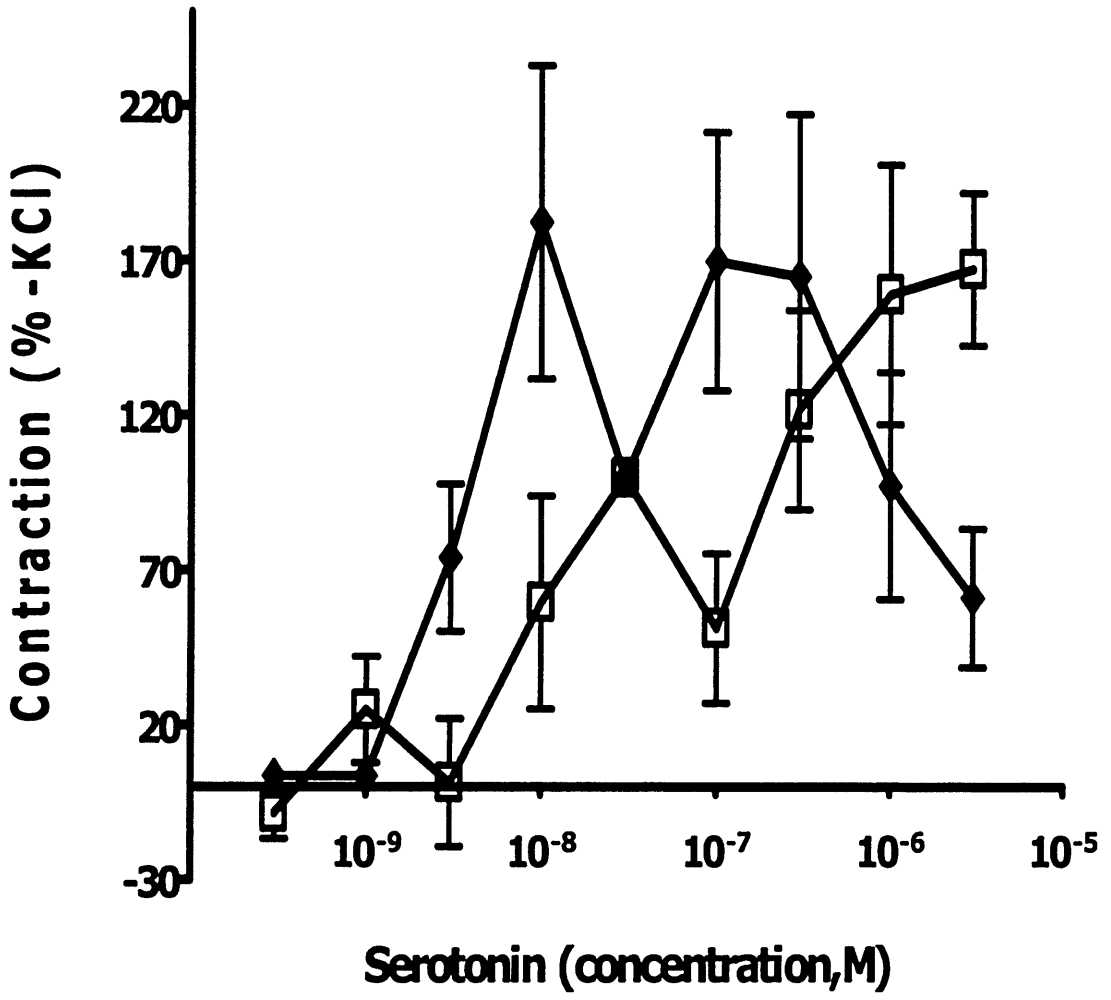


Figure 4.14: Effect of "inhibitors" on the contractile response of rat aortic rings to serotonin. Non-cumulative CRCs for serotonin in rat aortic rings were constructed in the absence (□, n=6) or in the presence of "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean –log EC50 values (±SEM) were compared by Student's paired t-test. No significant differences (P>0.05) were seen.

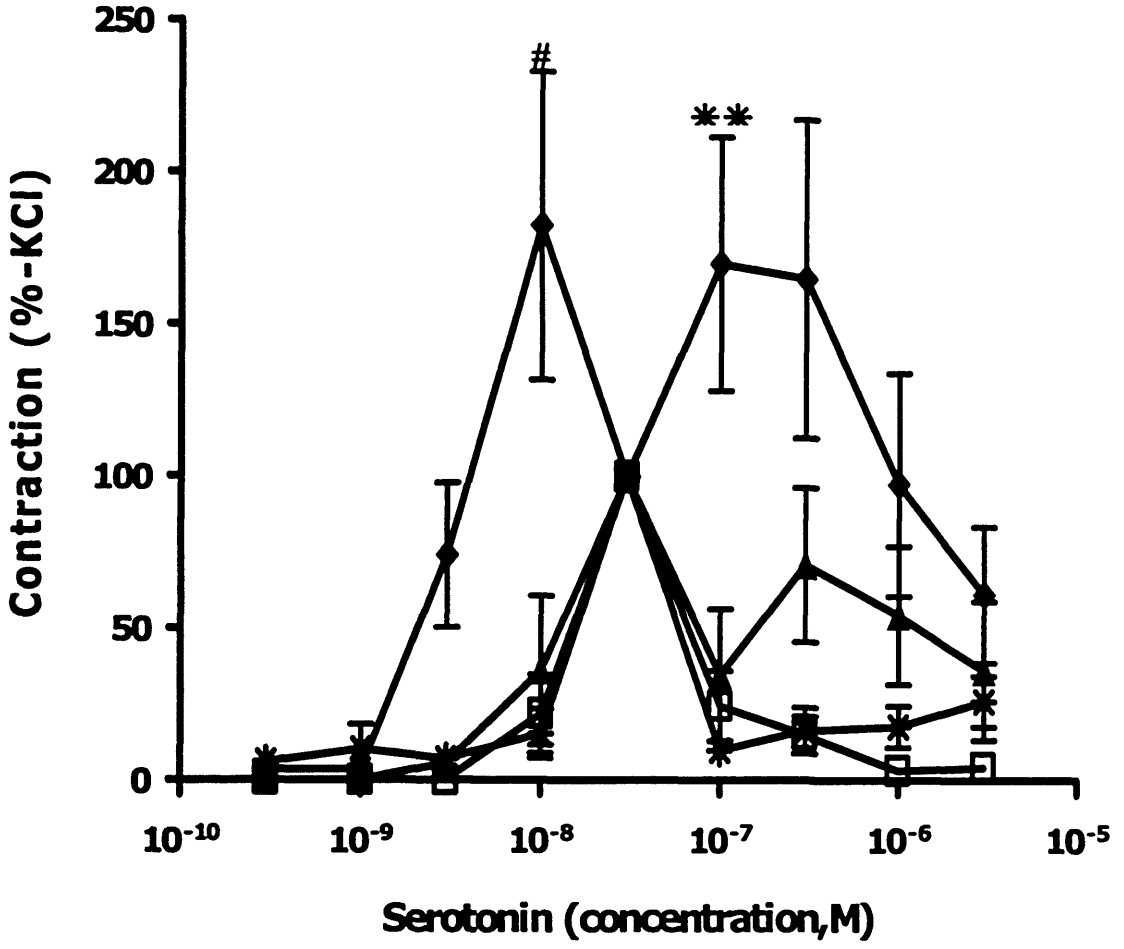


Figure 4.15: Effects of "inhibitors", methiothepin (50nM) and ketanserin (3nM) on the contractile response of rat aortic rings to serotonin. Non-cumulative CRCs for serotonin in rat aortic rings were constructed in the presence of "inhibitors" (◆, n=4), in the presence of "inhibitors" with methiothepin (50nM) (□, n=4), in the presence of "inhibitors" with ketanserin (3nM) (▲, n=5) or in the presence of "inhibitors" with methiothepin (50nM) and ketanserin (3nM) (✳, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (+SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. Mean –log EC_{50} values (+SEM) were compared by ANOVA followed by the "post hoc" Dunnett test. The first peak of the CRC to serotonin in the presence of "inhibitors" was significantly shifted to the right in the presence of methiothepin ($P<0.01$), ketanserin ($P<0.05$) and methiothepin in the combination with ketanserin ($P<0.01$). The first peak of the contractile response to serotonin at the concentration of 0.01μM was significantly inhibited ($P<0.01$,#) by methiothepin (50μM), ketanserin (3nM) and methiothepin in combination with ketanserin. The second peak of the contractile response to serotonin in the presence of "inhibitors" at the concentration of 0.1μM was also significantly inhibited ($P<0.01$,✳✳) by methiothepin (50nM) and methiothepin in the combination with ketanserin (3nM).

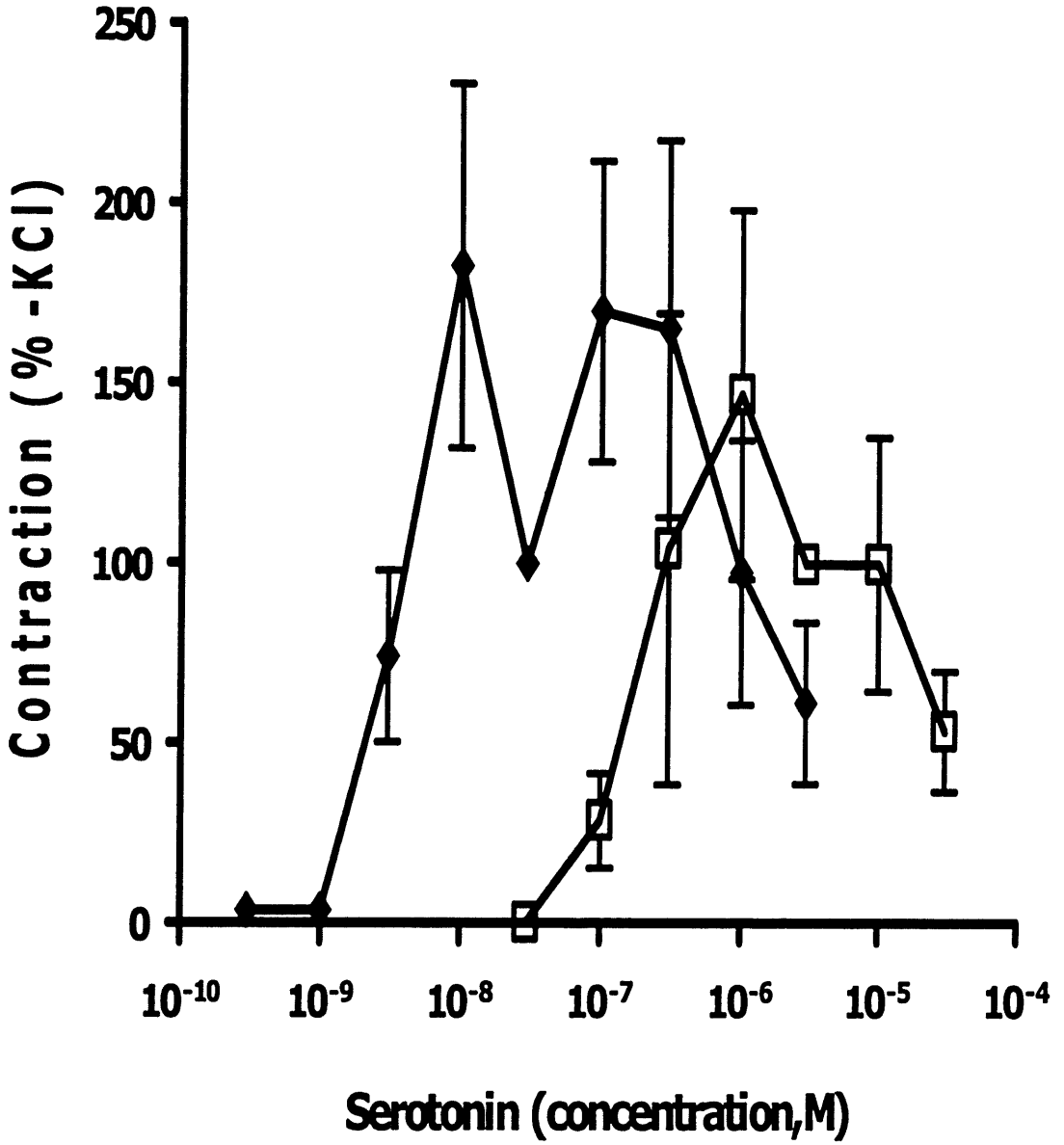


Figure 4.16: Effects of "inhibitors" and ketanserin (30nM) on the contractile response of rat aortic rings to serotonin. Non-cumulative CRCs for serotonin in rat aortic rings were constructed in the presence of "inhibitors" (✳, n=4), or in presence of "inhibitors" and ketanserin (30nM) (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) and mean –log EC_{50} values were compared by ANOVA followed by the "post hoc" Bonferroni test. The –log EC_{50} values of the first peak of the CRC to serotonin in the presence of "inhibitors" and of the CRC to serotonin in the presence of "inhibitors" and ketanserin (30nM) were significantly different ($P<0.001$,✳✳). No significant differences ($P>0.05$) were seen regarding the maximum contractile responses.

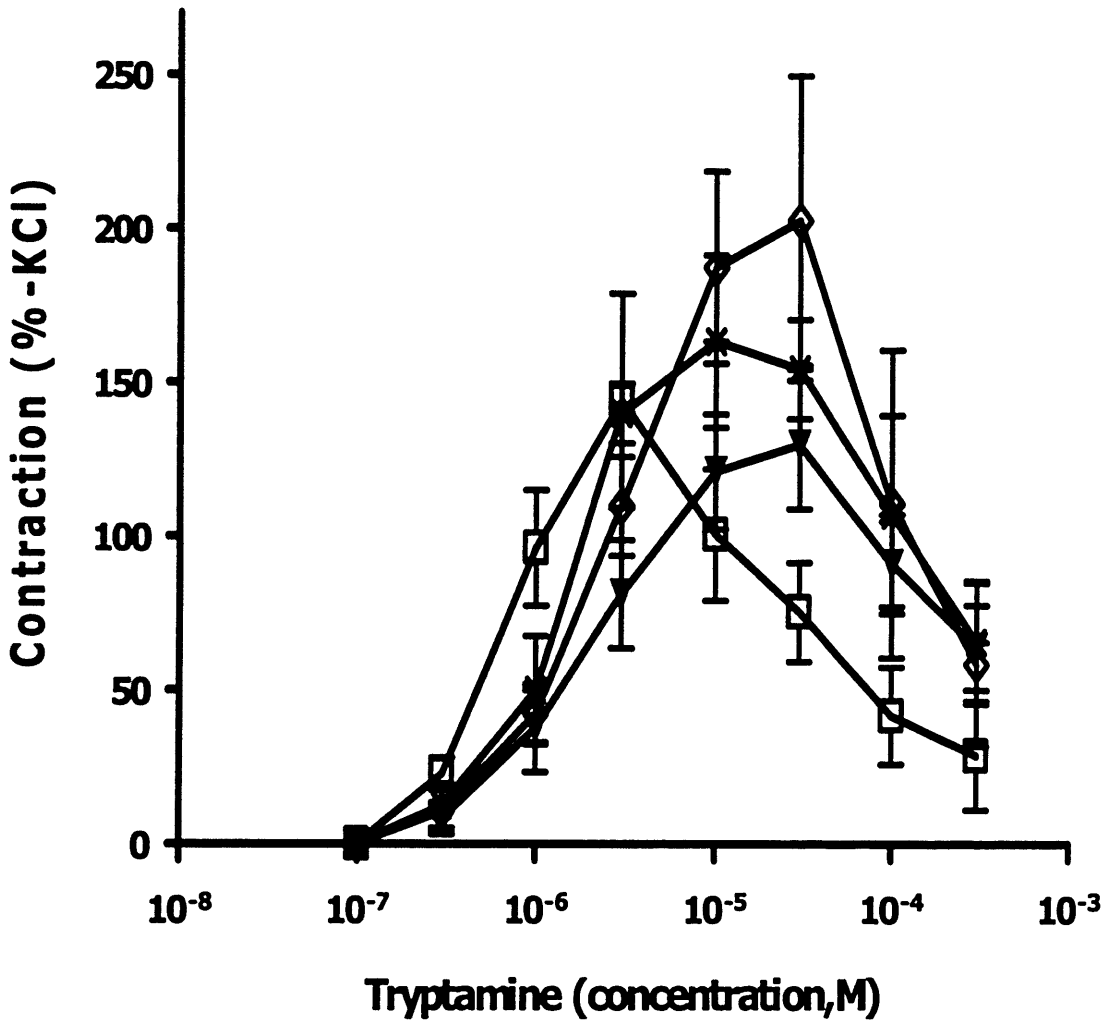


Figure 4.17: Effects of prazosin (1μM), ICI-118,551 (1μM), cocaine (10μM) and pargyline (1μM) on the contractile response of rat aortic rings to tryptamine. Non-cumulative CRCs for tryptamine were constructed in the absence (✳, n=4), in the presence of ICI-118,551 (1μM) and prazosin (1μM) (◊, n=5), ICI-118,551 (1μM), prazosin (1μM) and cocaine (10μM) (▼, n=6) or ICI-118,551 (1μM), prazosin (1μM) and cocaine (10μM) and pargyline (10μM) ("inhibitors", □, n=8). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Dunnett test. No significant differences ($P>0.05$) were seen.

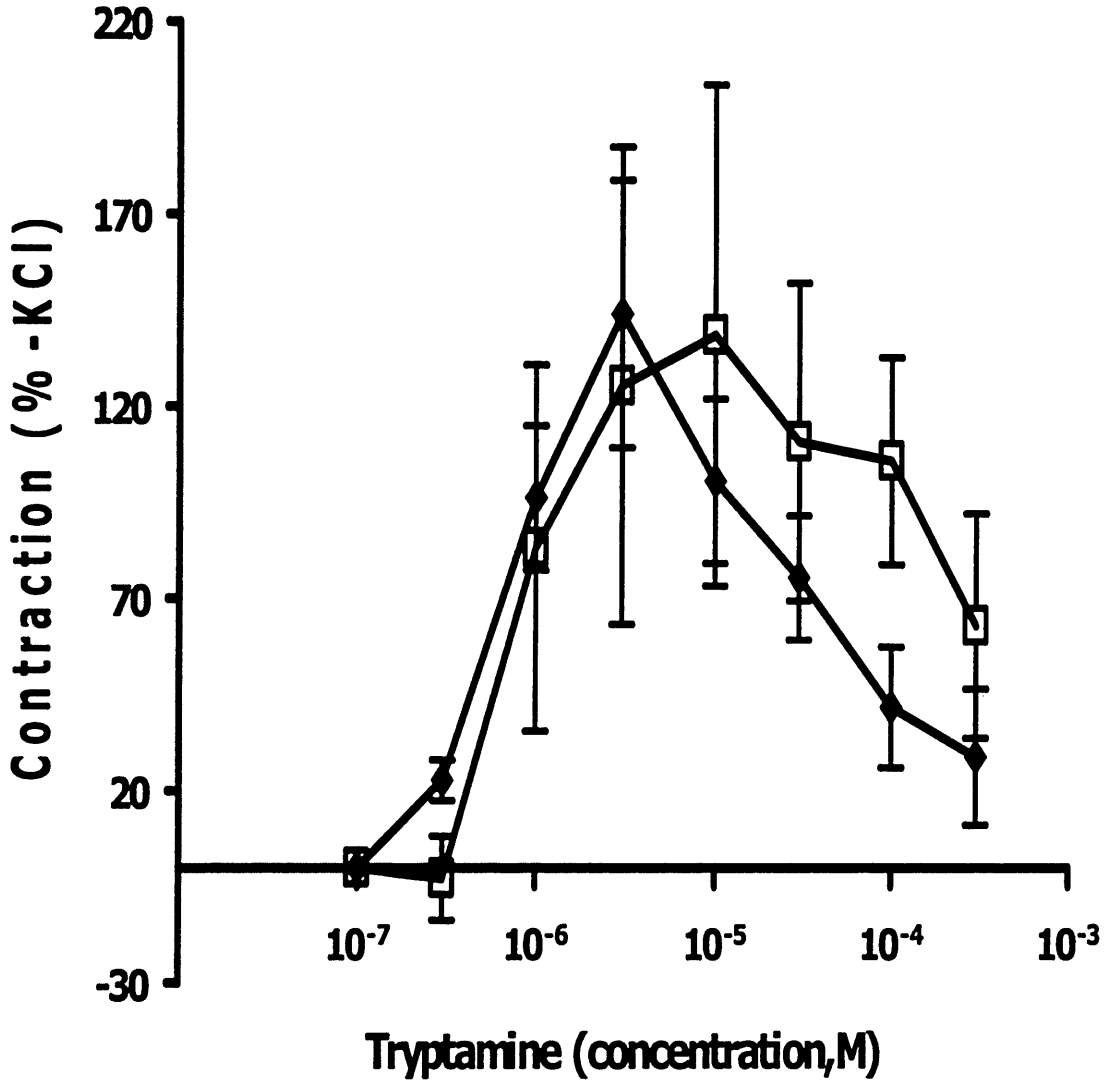


Figure 4.18: Effects of "inhibitors", methiothepin (50nM) and ketanserin (3nM) on the contractile response of rat aortic rings to tryptamine. Non-cumulative CRCs for tryptamine were constructed in the presence of "inhibitors" (◆, n=8) or in the presence of "inhibitors", methiothepin and ketanserin (3nM) (□, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant differences (P>0.05) were seen.

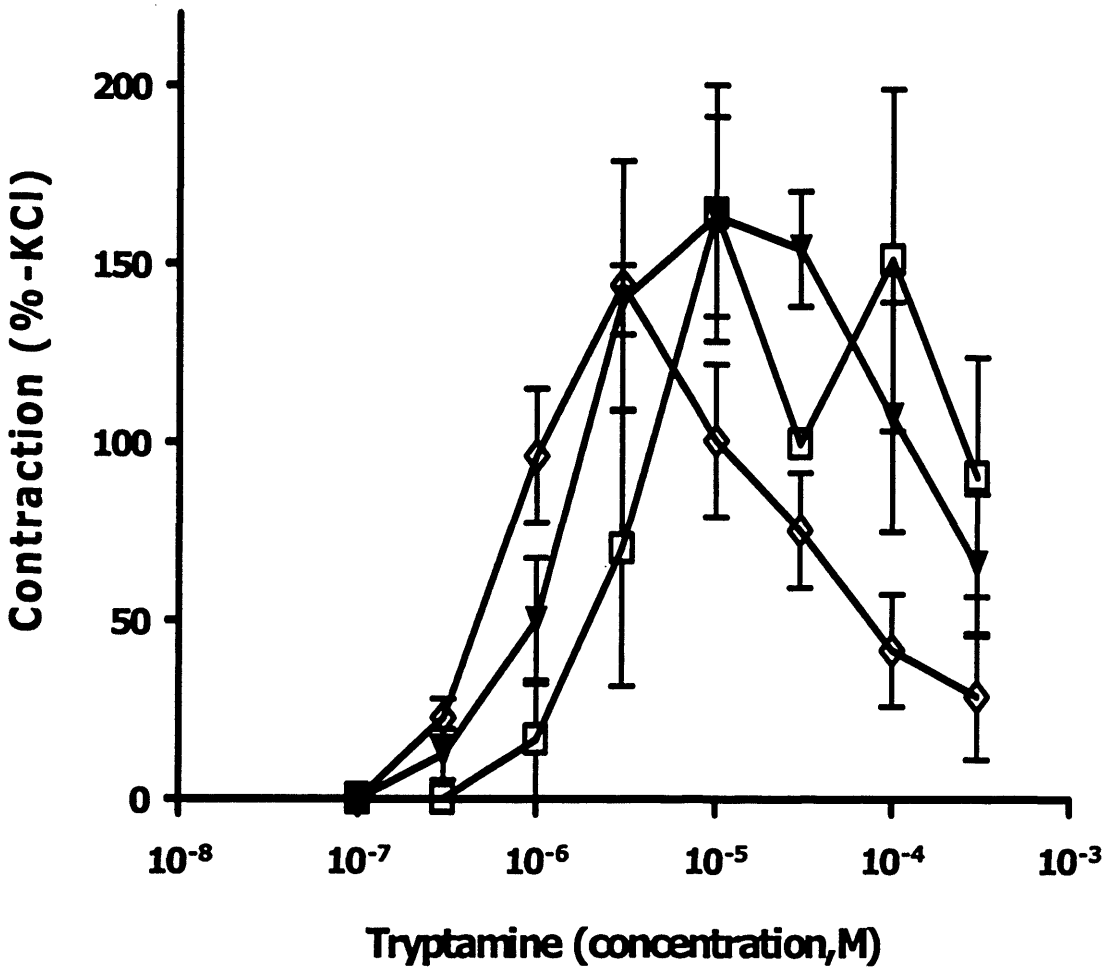


Figure 4.19: Effects of "inhibitors", ketanserin (30nM) on the contractile response of rat aortic rings to tryptamine. Non-cumulative CRCs for tryptamine were constructed in the absence of antagonists (▼, n=5) in the presence of "inhibitors" (◇, n=8) or in the presence of "inhibitors", with ketanserin (30nM) (□, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. Mean $-\log EC_{50}$ values (±SEM) were compared by unpaired student's t-test. The $-\log EC_{50}$ values of the first peak of the CRC to tryptamine in the presence of "inhibitors" and of the CRC to tryptamine in the presence of "inhibitors" and ketanserin (30nM) were significantly different ($P<0.001$). No significant differences ($P>0.05$) were seen at their maximum contractions.

4.5 Discussion

All studies in this *Chapter* were generally conducted in the presence of "inhibitors".

4.5.1 Contractile response to β-PEA - effect of endothelium and U46619

The contractile response in rat aorta to β-PEA in the presence of "inhibitors" was independent of the presence of endothelium, which is comparable with the results in *Chapter 2*. Therefore, the endothelium is not responsible for the contractile response in rat aorta to β-PEA. The presence of an intact endothelium was demonstrated by the relaxation response to acetylcholine in the pre-contracted aorta, which is known to induce relaxation *via* endothelium-derived release of nitric oxide (Van de Voorde and Leusen, 1983; Furchgott, 1984; Waldron *et al.*, 1999) *(data not shown, see Chapter 2)*. This also means there is no opposing vasodilator effect from release of NO. This also eliminates an endothelium component for the relaxation at high doses.

Analogous to *Chapter 2*, the effect of β-PEA on tissue pre-contracted with U46619 in the presence or absence of endothelium was investigated. However, in contrast to previous experiments, the contractile response was also measured in the presence of "inhibitors". As with the results in *Chapter 2* the reduced vasoconstriction (vasodilatation) to β-PEA in rat aortic rings at higher concentrations (Varma and Chemtob, 1993; Varma *et al.*, 1995) could not be confirmed with these experiments. β-PEA showed further contractions in U46619-precontracted tissues rather than vasorelaxant effects in endothelium-intact and endothelium-denuded tissues in the presence of "inhibitors". Furthermore, the presence of endothelium enhanced the contractile responses in pre-contracted tissues in the presence of "inhibitors". Therefore, the vasorelaxant factors released by the endothelium (NO, EDHF), as discussed in *Chapter 2* do not affect the contractile response to β-PEA in pre-contracted tissues in the presence of "inhibitors". If they were released, the contraction would be increased by the removal of endothelium. In fact, the contraction was reduced by endothelium removal, suggesting some release of an endothelium derived vasoconstrictor under these conditions. As the contractile response in U46619 pre-contracted tissues was not affected by "inhibitors", it cannot

be caused through an α- or β-adrenoceptor-mediated mechanism.

4.5.2 Contractile response to β-PEA and tryptamine – effect of indomethacin

As with the results for cumulative CRCs in *Chapter 2* indomethacin, a non-selective inhibitor of COX 1 and COX 2 (Schachter and Sang, 1997; Fischer *et al.*, 2000; Stanke-Labesque *et al.*, 2004) did not affect the vasoconstrictor response to β-PEA in the absence, or presence of "inhibitors" in non-cumulative CRCs. As indomethacin did not have any effect on the contractile response to β-PEA, the vasoconstriction is not mediated *via* prostaglandins or thromboxane (Schachter and Sang, 1997; Fischer *et al.*, 2000). Furthermore, indomethacin potentiated not significantly ($P>0.05$) the contractile response to tryptamine in the presence of "inhibitors" at lower concentrations.Therefore, the contractile response to tryptamine in rat aortic rings is also not mediated *via* prostaglandins and thromboxane (Schachter and Sang, 1997; Fischer *et al.*, 2000). The reason for the potentiation of the contractile response to tryptamine in the presence of indomethacin is unclear. However, previous publications reported that indomethacin caused an enhanced vasoconstriction to different vasoconstrictors (Gordon *et al.*, 1986; Tiritilli, 2000). The potentiation by indomethacin may result from inhibition of the vasodilator effect of prostaglandins (Kochar and Itskovitz, 1978; Abate *et al.*, 1979).

4.5.3 Contractile response to β-PEA – effect of α_2, β_2 and β_3-adrenoceptor antagonists

In *Chapters 2 and 3*, the roles of α- and β_2-adrenoceptors in the contractile responses of rat aortic rings to β-PEA were investigated by constructing cumulative and non-cumulative CRCs in the presence of various inhibitors. Therefore in this *Chapter*, the roles of adrenoceptor subtypes on the remaining contractile response to β-PEA in rat aortic rings was further investigated. α–Adrenoceptors can be classified into two different subtypes, α_1- and α_2–adrenoceptors (Starke, 1981; Digges and Summers, 1983b). Furthermore, α-adrenoceptors are separated in two distinct anatomical locations, post- and pre-junctional (Langer, 1974; Langer, 1980; Timmermans and van Zwieten, 1981) *(see Chapter 1)*. Post-junctional α–adrenoceptors are either α_1 or α_2 (Berthelsen and Pettinger, 1977). However, there seems to be some discrepancy

regarding α-adrenoceptors *in vitro*, between different species (Ruffolo and Waddell, 1982a; Ruffolo *et al.*, 1982c), as well as between the organs of the same species (Ruffolo *et al.*, 1980; Ruffolo *et al.*, 1981). Rat isolated aortic strips have been commonly used to investigate the existence of vascular α–adrenoceptors (Digges and Summers, 1983a). The presence of α_1–adrenoceptors in rat aortic tissues is general agreed as the selective α_1–adrenoceptor antagonist prazosin inhibits the responses to α_1–adrenoceptor agonists with high affinity (Randriantsoa *et al.*, 1981; Ruffolo *et al.*, 1982c). α_2–adrenoceptors were first identified on noradrenergic terminals where their activation leads to inhibition of transmitter release (Langer, 1974; Starke and Langer, 1979). They were subsequently demonstrated in vascular smooth muscle in which they subserve vasoconstriction, resulting in a pressor response in intact animals (Docherty *et al.*, 1979; Timmermans *et al.*, 1979; Timmermans and van Zwieten, 1980), and contraction in isolated preparations of certain blood vessels (Holtz *et al.*, 1982; Hicks *et al.*, 1983). However, in the past, the presence of post-synaptic α_2–adrenoceptors in isolated blood vessels, especially in rat aortic rings, has been difficult to prove (Digges and Summers, 1983a; Decker *et al.*, 1984). It has been suggested that either two types of adrenoceptor, one a classical α_1-adrenoceptor and the other not simply classifiable as either α_1 or α_2 are present (Randriantsoa *et al.*, 1981). As another possibility it was reported that the receptor is a variety of the α-adrenoceptor which has the properties of both the α_1 or α_2 subtypes (Ruffolo *et al.*, 1982; Ruffolo and Waddell, 1982b). However, a later publication has reported the presence of α_2-adrenoceptors in rat aorta smooth muscle cells (Fauaz *et al.*, 2000).

In *Chapter 2 and 3*, prazosin (1μM), a selective α_1–adrenoceptor antagonist, (Wood *et al.*, 1975; Cavero *et al.*, 1977) was shown to have little or no effect on the contractile response to β-PEA. It was therefore assumed that β-PEA did not cause contraction *via* α_1-adrenoceptors. In further experiments here, yohimbine, a selective, competitive α_2–adrenoceptor antagonist (5μM and 50μM) was used in combination with "inhibitors" to eliminate possible α_2–adrenoceptor effects in isolated rat aorta tissues. In previous publications, clonidine, a selective α_2–adrenoceptor agonist, was used to differentiate post-synaptic α-adrenoceptors (Ruffolo *et al.*, 1980). In rat aorta, clonidine has been shown to have a high affinity for α-adrenoceptors compared to that in vas deferens (Ruffolo *et al.*, 1980; Ruffolo

et al., 1981). Based on the known selectivity of clonidine for α_2–adrenoceptors (Berthelsen and Pettinger, 1977) and high affinity of the α–adrenoceptor for clonidine in the rat tissue, it was suggested that there are post-synaptic α_2–adrenoceptors in the rat aorta (Ruffolo *et al.*, 1980). Thus, post-synaptic α_2–adrenoceptors can mediate vasoconstrictor responses in the vasculature (Ruffolo *et al.*, 1981).

In the present study, clonidine in the presence, or absence of yohimbine (50μM) was used as a positive control to confirm the blocking effect of yohimbine. Yohimbine (50μM) greatly reduced the contractile response to clonidine in rat aortic tissues which confirms the selectivity of yohimbine as an α_2–adrenoceptor antagonist in rat aortic tissues (Digges and Summers, 1983a). In contrast, yohimbine at a concentration of 5μM used in a previous publication (Digges and Summers, 1983a) failed to inhibit the contractile response to β-PEA in the presence of "inhibitors". Moreover, with an increased concentration (50μM) of the α_2-adrenoceptor antagonist, the contractile response to β-PEA was not significantly potentiated in the presence of "inhibitors". Therefore, the remaining contractile response in rat aorta to β-PEA is not based on an α_2–adrenoreceptor effect.

In *Chapter 3*, the effects of ICI-118,551, a selective β_2–adrenoceptor antagonist (Bilski *et al.*, 1980; Bilski *et al.*, 1983) and propranolol, an non-selective β-adrenoceptor antagonist, were discussed. Neither propranolol nor ICI-118,551 altered the contractile response to β-PEA in rat aortic rings. Previous workers reported the presence of both β_1- and β_2- adrenoceptors in rat aortic tissues (O'Donnell and Wanstall, 1984a). Therefore, the use of atenolol, a selective β_1–adrenoceptor antagonist, should eliminate even more clearly the possibility that the contractile response in rat aorta to β-PEA could be affected by β-adrenoceptor effects. The effect of atenolol on β-PEA contraction in rat aortic rings was very similar to that of propranolol as both antagonists failed to modify the contractile response of β-PEA. Furthermore, the β-adrenoceptor antagonists failed to block the vasorelaxant component seen at higher concentrations of β-PEA. Therefore, the vasoconstriction and the vasorelaxation responses to β-PEA in rat aortic rings are not due to vascular β-adrenoceptor mechanisms. Moreover, previous publications (Varma and Chemtob,

1993; Varma *et al.*, 1995) have reported that tyramine, another TA agonist, caused a vasorelaxant effect in rat aortic tissues which was not blocked by β_1- and β_2-adrenoceptor antagonists. These findings in the present study suggest that this is also the case for the contractile response in rat aortic rings to β-PEA. Also, an opposing vasodilator action of β-PEA is eliminated as the β-adrenoceptor antagonists would cause potentiation of the contractile response. The cloning of a third β-adrenoceptor subtype (β_3-adrenoceptor) (Emorine *et al.*, 1989) has allowed an explanation of some of the effects of catecholamines which are not related to activation of β_1-and β_2-adrenoceptors. β_3-adrenoceptors (Manara *et al.*, 1996; Brawley *et al.*, 2000; Harada *et al.*, 2003) were found to mediate lipolysis in adipose tissues (Hollenga and Zaagsma, 1989; Lafontan, 1994), the relaxation of gastrointestinal (Manara *et al.*, 1995; Roberts *et al.*, 1999) and airway (Martin and Advenier, 1995) smooth muscle. Furthermore, β_3-adrenoceptors have been characterized in human heart, causing a negative inotropic effect (Gauthier *et al.*, 1996). In addition, β_3-adrenoceptors were found in rat colon (Bianchetti and Manara, 1990) and guinea pig ileum (Bond and Clarke, 1988). After the pindolol-elicited relaxation of canine mesenteric vessel (Clark and Bertholet, 1983) and rat aorta (Doggrell, 1990) was not antagonised by propranolol, the presence of β_3-adrenoceptors in vascular smooth muscles has been suggested (Matsushita *et al.*, 2003). Propranolol-resistant relaxations in response to the stimulation of putative β_3-adrenoceptors in vascular smooth muscle were found in rat carotid artery (Oriowo, 1994; MacDonald *et al.*, 1999) and rat thoracic aorta (Oriowo, 1995; Brawley *et al.*, 2000). All these findings have accumulated evidence for the presence of β_3-adrenoceptors in the vascular system. In the present study, the contractile response in rat aortic rings to β-PEA was therefore examined in the presence of the selective β_3–adrenoceptor antagonist SR59230A (Manara *et al.*, 1996; Nisoli *et al.*, 1996). The concentration-dependent vasorelaxant effect to β-PEA is construed as an effect of reduced contraction at the highest concentrations of β-PEA, rather than a vasodilatation effect. β-PEA caused inconsistent reduced contractions at high concentrations in rat aortic tissues. However, when comparing cumulative and non-cumulative CRCs to β-PEA, the reduced contraction effect occurred more frequently in cumulative rather than in non-cumulative CRCs. Therefore, to examine the effect of SR59230A (10μM) (Manara *et al.*, 1996) on the

contractile response to β-PEA, cumulative CRCs were constructed. It was difficult to conclude whether SR59230A (10μM) inhibited the reduced contraction to β-PEA. At the maximum concentration (300μM) the reduced contraction was absent but to confirm this, a higher concentration of β-PEA should have been added. The concentration-dependent vasodilatation effect to β-PEA did not always occur and when examined in rat aorta pre-contracted with U46619, there was no relaxation. Thus, it is questionable whether there was a significant vasorelaxant component to the response to β-PEA.

4.5.4 Contractile response to serotonin and tryptamine - effect of various inhibitors

To examine the possible role of serotonin (5-HT) receptors in the contractile response to TAs, experiments were performed with another TA, tryptamine. It was selected because of its close structural similarity to serotonin. Serotonin is the most well-known tryptamine derivative and previous work on serotonin as a vasoconstrictor (Vanhoutte, 1987; Mawatari *et al.*, 1997) was used to provide a basis to investigate the contractile response in rat aortic rings to tryptamine. There are seven main serotonin receptor types and several subtypes of serotonin receptors (Hoyer *et al.*, 1994; Hoyer *et al.*, 2002) *(Table 4.1, 4.2)*. Serotonin has both vasoconstrictor and vasodilator capabilities (Vanhoutte, 1987; Hoyer *et al.*, 2002). In smooth muscle, vasoconstriction can be caused through activation of 5-HT_1 and 5-HT_2 receptors (Peroutka *et al.*, 1983; Vanhoutte, 1987; Hoyer *et al.*, 2002) *(Table 4.1, 4.2)*. Therefore, the contractile effects of serotonin and tryptamine were investigated in the presence of methiothepin, a non-selective 5-$HT_{1A, 1B, 1D}$ antagonist and ketanserin, a selective 5-HT_{2A} receptor antagonist (Van Nueten *et al.*, 1985; Van Neuten *et al.*, 1986; Hoyer *et al.*, 2002) *(Table 4.2)*. Previous publications reported that serotonin might be an important mediator of coronary vasospasm in the variant form of ischaemic heart disease (Vanhoutte and Shimokawa, 1989) and augmented vasoconstrictor responses to serotonin have been reported in atherosclerotic blood vessels of animals and humans (Henry and Yokoyama, 1980; Ginsburg *et al.*, 1984). serotonin also possesses vasodilator properties.

The vasodilatator effects can be due to activation of endothelial cells which release endothelium-derived relaxing factors. The receptors mediating the release of endothelium-derived relaxing factors can be subtyped as 5-HT_1 receptors (Vanhoutte, 1987).

Receptor	Distribution	Function
5-HT_1	CNS Blood Vessels	Neuronal inhibition Vasoconstriction
5-HT_2	CNS Periphery (VSM, platelets)	Vasoconstriction Vasodilatation Smooth muscle contraction
5-HT_3	CNS, PNS	Neuronal Excitation
5-HT_4	CNS, PNS Atria Smooth muscle	Neuronal Excitation (+) Gastric motility altered cardiac rhythmus
5-HT_6	CNS Immune cells	Modulation of CNS Release of Ach and DA
5-HT_7	CNS Periphery (GIT, VSM)	Vasodilatation Smooth muscle relaxation (GIT)

Table 4.1: Serotonin (5-HT) receptor distribution and function
CNS = central nervous system, PNS = peripheral nervous system, DA = dopamine, GIT = gastrointestinal tract, Ach = acetylcholine, VSM = vascular smooth muscle, (+) = activation (Hoyer *et al.*, 2002)

Receptor	Transduction Mechanism	Antagonist
5-HT_{1A}	$G_i/G_o \Rightarrow$ (-) cAMP	Methiothepin
5-HT_{1B}	$G_i/G_o \Rightarrow$ (-) cAMP	Methiothepin
5-HT_{1D}	$G_i/G_o \Rightarrow$ (-) cAMP	Methiothepin
5-HT_{2A}	$G_q/G_{11} \Rightarrow$ (+) IP_3 and DAG	Ketanserin
5-HT_{2B}	$G_q/G_{11} \Rightarrow$ (+) IP_3 and DAG	SB204741
5-HT_{2C}	$G_q/G_{11} \Rightarrow$ (+) IP_3 and DAG	RS102221
5-HT_3	Ligand-gated ion channel (+) $[Ca^{2+}]_i$	Odansetron
5-HT_4	$G_s \Rightarrow$ (+) cAMP	GR113808
5-HT_6	$G_s \Rightarrow$ (+) cAMP	Ro046790
5-HT_7	$G_s \Rightarrow$ (+) cAMP	Methiothepin SB238719

Table 4.2: Serotonin (5-HT) receptor transduction mechanism and antagonists
(+) = Increase, (-) = Decrease, IP_3 = inositol 1,4,5-trisphosphate, DAG = diacylglycerol *(see also Chapter 1, Table 1.4)* (Hoyer *et al.*, 2002)

In the present study, the mechanism of action for vasoconstriction by serotonin in rat aortic rings seems to be complex and not fully understood. The first peak of the contractile response to serotonin seems to be distinguishing. The first peak of the CRC to serotonin seems to be potentiated by "inhibitors" suggesting the possibility of some opposing vasodilatation *via* β-adrenoceptors and no stimulation of α-adrenoceptors. The potentiation could also be due to the pargyline as serotonin is a substrate for monoamine oxidase (MAO) (Campbell *et al.*, 1979; Campbell *et al.*, 1985). Furthermore, the first peak of the contraction was progressively shifted to the right by ketanserin (3nM and 30nM) indicating that it was mediated *via* 5-HT_{2A} receptors. This finding confirms results in various isolated smooth muscle tissues of a previous publication (Van Neuten *et al.*, 1986) which showed that ketanserin is a potent antagonists of contractions of isolated blood vessels caused by serotonin due to 5-HT_2 receptors (Van Nueten *et al.*, 1981; Van Nueten *et al.*, 1982; Van Nueten *et al.*, 1984; Van Nueten *et al.*, 1985). The second peak of contraction to serotonin was largely blocked by methiothepin (50nM) and is therefore likely to be due to activation of a serotonin receptor, other than 5-HT_{2A} such as 5-HT_1 receptor. In the present study, tryptamine induced vasoconstriction in rat aortic rings in the absence and presence of various inhibitors. However, the shape of the CRC for tryptamine is unlike the CRCs for serotonin and β-PEA in the presence of "inhibitors". The large contractile response to tryptamine at lower concentrations, in the presence of "inhibitors", is not caused by stimulation of α- adrenoceptors, nor is it an indirect sympathomimetic action. Tryptamine is a partial 5-HT_3 receptor agonist (Van Hooft and Vijverberg, 1996) and also interacts on different sites of the allosteric 5-HT_2 receptor system (Frenken and Kaumann, 1988). In the present study, the combination of "inhibitors", methiothepin and ketanserin at the lower concentration (3nM) had no effect on the contractile response to tryptamine. However, ketanserin at a higher concentration (30nM) in the presence of "inhibitors" changed the shape of the CRC to tryptamine to a curve similar to the biphasic serotonin CRC. In addition, this concentration of ketanserin (30nM) acted as a competitive antagonist for tryptamine, as in the presence of the serotonin antagonist, the CRC was significantly shifted to the right. However, the dose-ratio for ketanserin (30nM) against tryptamine was significantly lower than against serotonin. Furthermore, peak contraction was not inhibited in the presence of "inhibitors" and ketanserin (30nM).

In conclusion, the remaining contractile response to tryptamine is not due to an interaction with 5-HT_1 or 5-HT_{2A} receptors, indirect sympathomimetic activity or direct stimulation of α- adrenoceptors or ISA stimulation. The failure of pargyline to potentiate suggests that tryptamine was also not being metabolised by MAO.

Previous workers investigating the effect of tryptamine vasoconstriction in human coronary arteries (Nilsson *et al.,* 1999) found that the contractile response to tryptamine was mainly mediated by 5-HT_{1B} and 5-HT_{2A} receptors. Furthermore, vasoconstriction to tryptamine in endothelium-denuded rabbit coronary arteries was inhibited by ketanserin (Ellwood and Curtis, 1997). Therefore the contractile response to tryptamine seems to be due to different mechanisms of action dependent on the species. Activation of 5-HT_3 receptors does not cause vasoconstriction in blood vessels *(Table 4.2)* (Peroutka *et al.*, 1983; Vanhoutte, 1987). Therefore, in the present study, the contractile response to tryptamine in the presence of a 5-HT_3 receptor antagonist was not investigated.

4.6 Conclusion

The conclusion from this *Chapter* is that the vasoconstriction by β-PEA is not mediated or influenced by α_2-and β-adrenoceptors, and also the vasoconstriction by tryptamine is not mediated *via* serotonin receptors. Antagonists for these receptors did not reduce the vasoconstriction to these TAs. In *Chapter 2 and 3* various factors, such as the presence, or absence of endothelium, α_1-and β_2-adrenoceptors have been already examined and had been shown not to affect the contractile response to TAs in rat aortic rings. As in *Chapters 2 and 3*, the mechanism by which TAs, such as β-PEA, causes vasoconstriction in rat aorta cannot be established from this study. However, it is likely that β-PEA and tryptamine exert vasoconstriction by activation of a novel receptor system. This will be investigated further in *Chapters 5 and 6.*

Chapter 5

INVESTIGATION OF THE CONTRACTILE RESPONSES TO RANGE OF TRACE AMINE AGONISTS IN THE PRESENCE OF VARIOUS INHIBITORS

Chapter 5

Investigation of the contractile responses to a range of trace amines agonists in the presence of various inhibitors

5.1 Introduction

In this Chapter a range of trace amines (TAs) in addition to β-PEA will be investigated. These will include octopamine and amphetamines. For most invertebrate species, certain TAs are the primary amines especially in insects (octopamine and tyramine) (Lewin, 2006). In invertebrates, the role of TAs acting as neurotransmitters *via* stimulation of a GPCR is well known. The GPCR was characterised as the octopamine receptor (Axelrod and Saavedra, 1977; David and Coulon, 1985; Evans and Robb, 1993; Roeder, 1999). Four classes of octopamine receptors have been studied. The signal transduction of these four GPCRs is caused through either activation or inhibition of adenylate cyclase or activation of phospholipase C (David and Coulon, 1985; Lewin, 2006). In vertebrates, the existence of functional receptors for TAs had not been closely investigated until the gene for TAAR, a mammalian receptor for TAs (GPCR), was discovered by amplification of novel DNA sequences by PCR using cDNA and genomic DNA as templates (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001; Lewin, 2006). In cell lines, in comparison to β-PEA and p-tyramine, octopamine activates TAAR1 with minor affinity (Borowsky *et al.*, 2001).

Octopamine *(see Chapter 1)* was first discovered in the posterior salivary gland of Octopus vulgaris (Erspamer and Boretti, 1951). Later, it was also found to be present in the urine of man and other mammals (Armstrong *et al.*, 1956). Octopamine is now known to be present in mammals and in invertebrates (David and Coulon, 1985; Roeder, 1999). It is highly concentrated in neurones of several invertebrate species and appears to serve as a major neurotransmitter and a neuromodulator (Axelrod and Saavedra, 1977; Roeder, 1999). Octopamine is a biogenic monoamine and is structurally related to noradrenaline. It is synthesised in nerves from tyrosine and tyramine *via* dopamine-β-hydroxylase *(Chapter 1)* and is metabolised primarily by monoamine oxidase (Axelrod and Saavedra, 1977; Roeder, 1999).

In mammals, it is found in generally low levels in nerves of peripheral tissues and the brain, where it exists in parallel with noradrenaline (Axelrod and Saavedra, 1977).

As amphetamine *(Table 5.1)* is structurally similar to β-PEA, amphetamine and also substituted derivates, such as MDMA ('ecstasy') *(Table 5.1)* can also directly activate TAAR (Kim and von Zastrow, 2001). Initially, amphetamine and its derivatives were sold as stimulants and appetite suppressants (Bunzow *et al.*, 2001). However, in modern times amphetamines are mostly used as a treatment of attention deficit hyperactivity disorders (ADHD) (Bradley, 1937; Seiden *et al.*, 1993; Seeman and Madras, 1998). Nevertheless, amphetamines are also listed as controlled drugs due to their ability to produce wakefulness and intense euphoria (Prinzmetal and Bloomberg, 1935). Amphetamine and the synthetic trace amine methamphetamine (METH) *(Table 5.1)* (Anglin *et al.*, 2000) are potent psychostimulants that can lead to abuse and often addiction (Reese *et al.*, 2007). METH is the most widely used illegal drug in the world according to the United Nations (Anglin *et al.*, 2000; Iversen, 2006). In North America, South-east Asia, Australia and Japan, methamphetamine abuse causes major public health problems (Iversen, 2006). Furthermore, substituted amphetamine-derivatives, such as MDMA and DOI (2,5-Dimethoxy-4-iodoamphetamine) are used for their hallucinogenic and 'empathogenic' effects (Shulgin and Shulgin, 1991; Eisner, 1994). Hyperthermia (Henry *et al.*, 1992; Coore, 1996; Byard *et al.*, 1998), neurotoxicity (Ricaurte and McCann, 1992), psychosis (Seiden *et al.*, 1993) and psychological dependence (Murray, 1998) are some of the consequences associated with use of these amphetamines (McCormack, 2006).

MDMA is a synthetic amphetamine derivative with strong effects on serotonergic neurotransmission (Mas *et al.*, 1999). MDMA is one of the most commonly used drugs of abuse in the U.K (Ramsay *et al.*, 2001). There is a marked worldwide increase in the popularity of 'ecstasy' use (Landry, 2002), which is attributed to its positive effects on mood, euphoria, friendliness, empathy after use and perceived safety (Peroutka *et al.*, 1988; Mas *et al.*, 1999). These symptoms are sometimes called "entactogen" (Cami and Farre, 1996). Undesirable effects associated with the recreational use of MDMA include loss of appetite, jaw clenching, trismus, headache, muscle aches and insomnia (Mas *et al.*, 1999).

Acute medical complications include malignant hyperthermia (Sprague *et al.*, 2004), hepatitis, intracerebral haemorrhage (Harries and De Silva, 1992) and acute renal failure (McCann *et al.*, 1994). Tachycardia (Gordon *et al.*, 1991), hypertension (Hayner and McKinney, 1986) and cardiovascular mortality (Dowling *et al.*, 1987) have been reported in man (McDaid and Docherty, 2001). The use of MDMA together with other serotonergic compounds may enhance the appearance of serotonin syndrome, including diarrhoea, diaphoresis and ataxia (Demirkiran *et al.*, 1996)

Within this *Chapter*, the effects of other amphetamine-related reagents, methylphenidate (MPH, Ritalin®) and cathinone *(Table 5.1)* will also be considered. Ritalin® (Maxwell *et al.*, 1957; Maxwell *et al.*, 1958) is the most common drug for the treatment of ADHD (Swanson *et al.*, 1991; Greenhill *et al.*, 2002; Wilens *et al.*, 2002). ADHD is the most common behavioral disorder of childhood (Volkow *et al.*, 2003). It is a condition which includes deficits in executive functions (working memory and attention) (Hunt *et al.*, 1984; Hunt, 1987). Moreover, Ritalin® is effective for the treatment of narcolepsy (Littner *et al.*, 2001) and as an antidepressant elderly patients (Kaufmann *et al.*, 1984; Rozans *et al.*, 2002). Unfortunately, Ritalin® also has reinforcing effects particularly when taken intravenously or when snorted, which can lead to abuse and addiction (Parran and Jasinski, 1991). As an analogue of D-amphetamine, Ritalin® enhances the release of noradrenaline and blocks the re-uptake of noradrenaline and dopamine in mammalian brain (Carlsson *et al.*, 1966; Axelrod, 1970; Hendley *et al.*, 1972; Raiteri *et al.*, 1974). However, D-amphetamine releases newly synthesised dopamine, while Ritalin® releases stored dopamine (Moore *et al.*, 1977).

In the last few years, the use of the khat leaf (*Catha edulis Forsk.*) has gained in importance in East Africa and parts of the Middle East, such as The Yemen (Brenneisen *et al.*, 1986; Brenneisen *et al.*, 1990; Al-Motarreb *et al.*, 2002b). The social and cultural function of chewing the khat leaf for its euphoric actions, has now spread to ethnic communities in Britain (Griffiths, 1998). Leaves of the khat bush are widely used as an amphetamine-like stimulant (Al-Motarreb *et al.*, 2002b). For many years the stimulant effects of khat were attributed to cathine a phenylethylamine derived compound in the plant (Alles *et al.*, 1961). However in a range of studies

cathinone has been shown to be more potent in its action than cathine (W.H.O. Advisory Group, 1980; Kalix, 1983b). S (-)-cathinone is the major active compound of khat leaves *(Table 5.1)* (Brenneisen *et al.*, 1984; Kalix, 1984a; Brenneisen and Geisshusler, 1985; Goudie, 1985). Chewing of the fresh khat leaves provides amphetamine-like CNS-stimulating effects. Like MDMA and amphetamine *(Table 5.1)*, cathinone causes euphoric effects, hyperactivity and restlessness with hyperthermia (Schorno, 1982; Kalix, 1984b). It has been reported that amphetamine and cathinone have similar actions, such as operant behaviours (Johanson and Schuster, 1981) and anorectic properties and also show anorectic cross-tolerance (Foltin and Schuster, 1982; Foltin and Schuster, 1983; Foltin *et al.*, 1983).

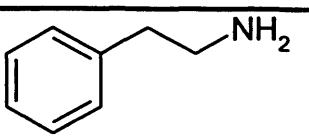
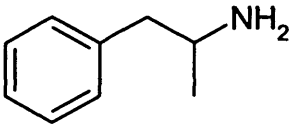
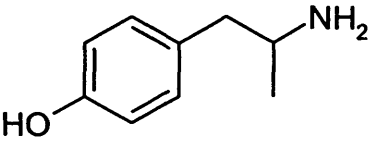
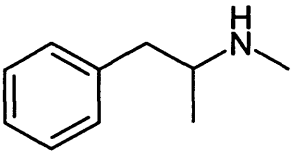
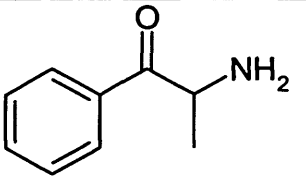
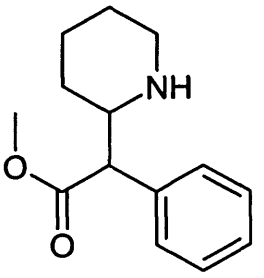
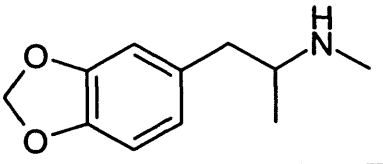
Drug Name	Systematic Name	Structure
β-Phenylethylamine	2-Phenylethylamine	
Amphetamine	α-Methyl-phenylethylamine, 1-Phenylpropane-2-amine	
p-Hydroxy-amphetamine (4-Hydroxyamphetamine)	(1*R*,2*S*)-2-(Methylamino)-1-phenylpropan-1-ol, α-Methyltyramine	
(+) Methylamphetamine (Methamphetamine) (-) Methylamphetamine (Desoxyephedrine)	N-Methyl-1-phenyl-propan-2-amine	
Cathinone (β-Ketoamphetamine)	(*S*)-2-Amino-1-phenyl-1-propanone	
Methylphenidate (MPH, Ritalin®)	Methyl 2-phenyl-2-(2-piperidyl)acetate	
Methylene-dioxymethylamphetamine, (MDMA, `Ecstasy`)	1-(benzo[*d*][1,3]dioxol-5-yl)-*N*-methylpropan-2-amine	

Table 5.1: Structure, drug and systematic names of β-PEA, amphetamine and their derivatives. β-PEA and other TAs including tyramine and octopamine *(Chapter 1)* are structurally and functionally related to amphetamines.

In *Chapters 2, 3 and 4* the effects of β-PEA and tryptamine were investigated. In the present *Chapter* other TAs, including tyramine, octopamine, amphetamine and various amphetamine-derivatives *(Table 5.1)* are investigated to see if they have similar effects. Moreover, the effect of TAs in guinea-pig aorta was studied to see if there were species differences as pig arteries are already know to be different (Baker *et al.*, 2007).

5.2 Aims

To study the contractile response of rat isolated aortic rings to a range of trace amine (TA) agonists employing cumulative and non-cumulative CRCs and single dose experiments

a.) Determine whether β-PEA produces vasoconstriction in isolated guinea-pig aortic tissues

b.) Determine whether the other TAs, tyramine, octopamine and D amphetamine, produce vasoconstriction in isolated guinea-pig and rat aortic tissues.

c.) Determine whether the contractile responses to various TAs differ between isolated guinea-pig and rat aortic tissues.

d.) Determine whether MDMA, Ritalin®, S-(-)-cathinone, (+)- and (-)-4-OH-amphetamine and (+)- and (-)-methamphetamine produce vasoconstriction in isolated rat aortic tissue.

5.3 Material and Methods

The main methods and experimental protocol described in *Chapter 2* were retained throughout the study unless otherwise stated.

5.3.1 Experimental Protocol

After equilibration cumulative CRCs for cathinone, MDMA and Ritalin®, non-cumulative CRCs for β-PEA, tyramine, octopamine, D-amphetamine, (+)- and (-)-methamphetamine and also single dose experiments for (+)- and (-)-4-OH-amphetamine (300μM), were obtained, in the absence, or presence of different inhibitors, which were incubated with the tissue for 15 minutes before commencing the CRC or single dose experiment unless otherwise stated. In all experiments, the endothelium was removed by inserting a cocktail stick and rolling the aortic ring round it.

5.3.2 Drugs Used

All chemicals were dissolved in distilled water, unless otherwise stated. Prazosin hydrochloride and U46619 were dissolved in DMSO. (+)-and (-)-4-OH-amphetamine hydrobromide were kindly donated by the National Institute of Drug Abuse (NIDA), Division of Neuroscience and Behavioural Research, Bethesda, MD,USA.

➢ **Reagents obtained from Sigma Aldrich**

D-amphetamine sulfate, S (-)-cathinone (β-ketoamphetamine), cocaine hydrochloride, (-)–deoxyephedrine hydrochloride ((-)-Methamphetamine hydrochloride), pargyline hydrochloride (PG), β-phenylethylamine (β-PEA), prazosin hydrochloride, (±)-propranolol hydrochloride, MDMA (3,4-methylenedioxy-N-methylamphetamine, 'Ecstasy'), methylphenidate (MPH, Ritalin®), (+)-Methamphetamine hydrochloride, octopamine

➢ **Reagents obtained from Fisher**

All chemicals for the Krebs-bicarbonate buffer (analytical grade)

➢ **Reagents obtained from TOCRIS**

ICI-118,551 hydrochloride ((±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride), ketanserin tartrate and methiothepin maleate

5.4 Results

5.4.1 Contractile response to β-PEA in rat and guinea-pig aorta - effect of "inhibitors"

A comparison was made between guinea-pig and rat regarding the contractile response of TAs in aortic rings. As the contractile response in rat aorta to β-PEA was investigated in *Chapters 2 and 3*, the contractile response in guinea-pig aorta to β-PEA was examined here. The maximum contractile responses of guinea-pig aorta to β-PEA in the absence of any antagonist (100μM, 125.8±32.5%, n=5), in the presence of prazosin (1μM) and ICI-118,551 (1μM) (100μM, 77.9±17.1%, n=4), and in the presence of prazosin (1μM), ICI-118,551 (1μM) and cocaine (10μM) (300μM, 162.1±65%, n=4) were not significantly different (P>0.05) *(Figure 5.1)*. Furthermore, the CRC to β-PEA in the absence (-log EC_{50} 4.13±0.28, n=3) or presence (-log EC_{50} 3.1±1.4, n=6) of "inhibitors" were not significantly different (P>0.05). Furthermore, the maximum contractile response to β-PEA (100μM, 125.8±32.5%, n=5) was not significantly (P>0.05) altered by "inhibitors" (300μM, 158.2±39.7%, n=5) *(Figure 5.1)*. Isolated aortic tissues from rat and guinea-pig showed similar maximum contractile responses to β-PEA in the presence of "inhibitors". The maximum responses to β-PEA in rat (300μM, 138.2±23.1%, n=6) and guinea-pig aortic tissues (300μM, 158.7±39.7%, n=5) and the –log EC_{50} values in rat (4.92±0.17, n=6) and guinea-pig (4.22±0.21, n=5) were not significantly different (P>0.05) *(Figure 5.2)*.

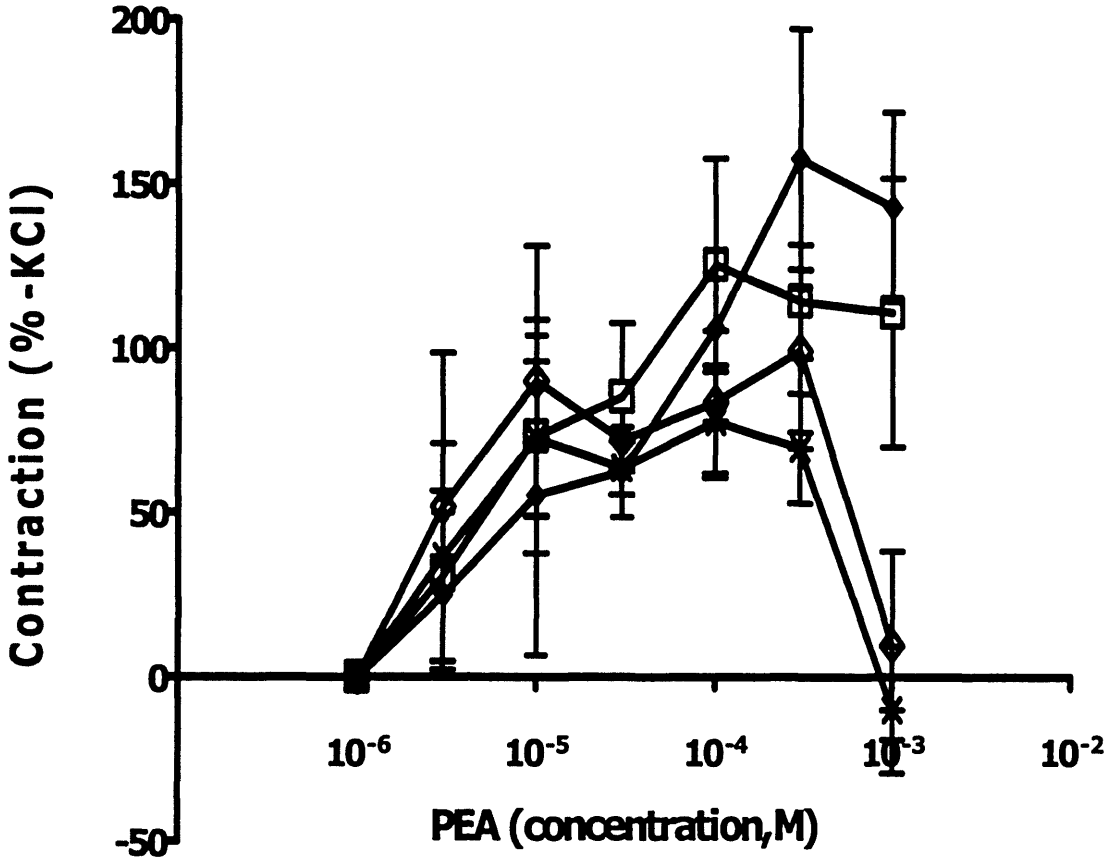


Figure 5.1: Effects of prazosin (1μM), ICI-118,551 (1μM), cocaine (10μM) and pargyline (1μM) on the contractile response of guinea-pig aortic rings to β-PEA. Non-cumulative CRCs for β-PEA were constructed in the absence of antagonists (□, n=5) or in the presence of ICI-118,551 and prazosin (✳, n=4) or in the presence of ICI-118,551, prazosin and cocaine (◇, n=3) or in the presence of ICI-118,551, prazosin, cocaine and pargyline ("inhibitors",◆, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences (P>0.05) were seen at their maximum contractions.

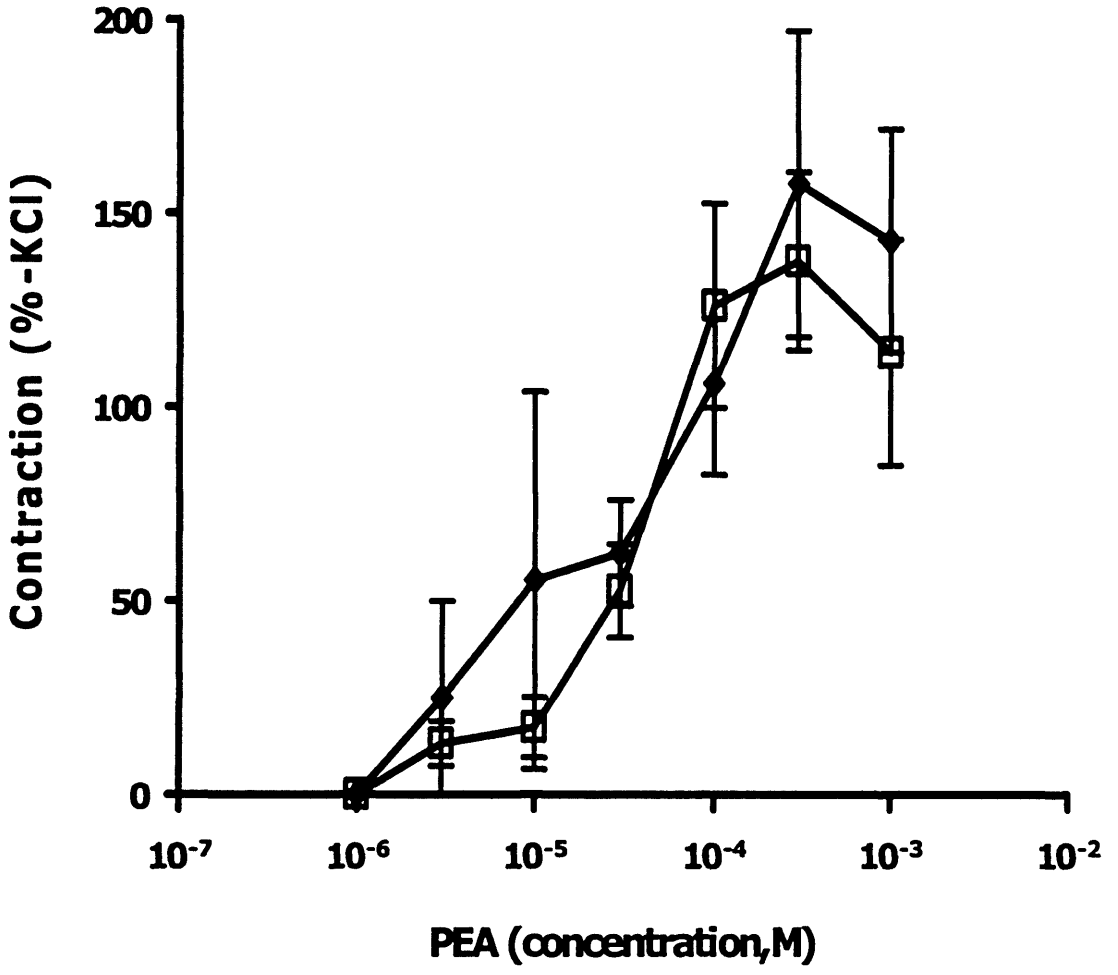


Figure 5.2: The contractile response of guinea-pig and rat aortic rings to β-PEA in the presence of "inhibitors". Non-cumulative CRCs for β-PEA in guinea-pig (◆, n=5) and in rat aortic rings were constructed in the presence of "inhibitors" (□, n=6). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) at the maximum contractile responses (300μM) were compared by Student's unpaired t-test. No significant differences (P>0.05) were seen.

5.4.2 Contractile response to tyramine in rat and guinea-pig aorta - effect of "inhibitors"

The contractile response to tyramine in guinea-pig aorta was examined. The presence of "inhibitors" (1mM, 95.1±23.6%, n=5) did not alter the contractile response to tyramine (1mM, 52.3±22.5%, n=5) significantly (P>0.05) *(Figure 5.3)*.

The contractile response in rat aortic rings to tyramine in the absence of "inhibitors" showed very inconsistent and variable results. In some experiments tyramine in the absence of "inhibitors" did not show any contractile responses whereas in others a contraction was visible but often only in high concentrations (data not shown). In general the contractile responses to tyramine in the absence and presence of "inhibitors" in rat aortic rings were least active compared to the contractile response to tyramine in guinea-pig aorta. When comparing the contractile response to tyramine in the presence of "inhibitors" in rat and in guinea-pig aortic rings, the contractile responses at the maximum concentration (1mM) in guinea-pig (1mM, 95.1±23.6%, n=5) was significantly greater (P<0.01) compared to that in rat aortic rings (1mM, 0.7±14%, n=5) *(Figure 5.4)*.

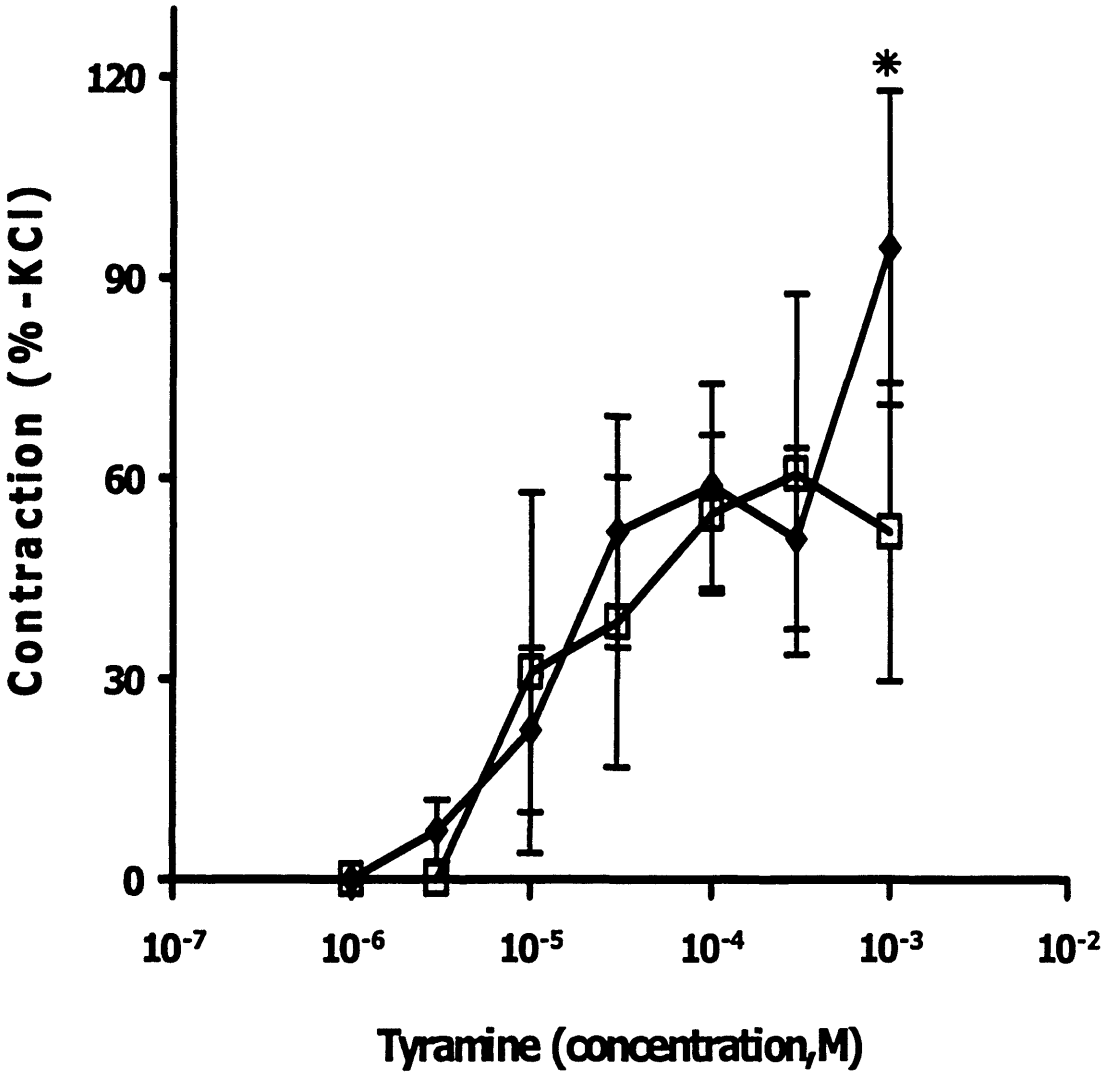


Figure 5.3: Effect of "inhibitors" on the contractile response of guinea-pig aortic rings to tyramine. Non-cumulative CRCs for tyramine in guinea-pig aortic rings were constructed in the absence (□, n=5), or in the presence of "inhibitors" (◆, n=5). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. A significant difference ($P<0.05$,*) was seen at the maximum concentration (1mM).

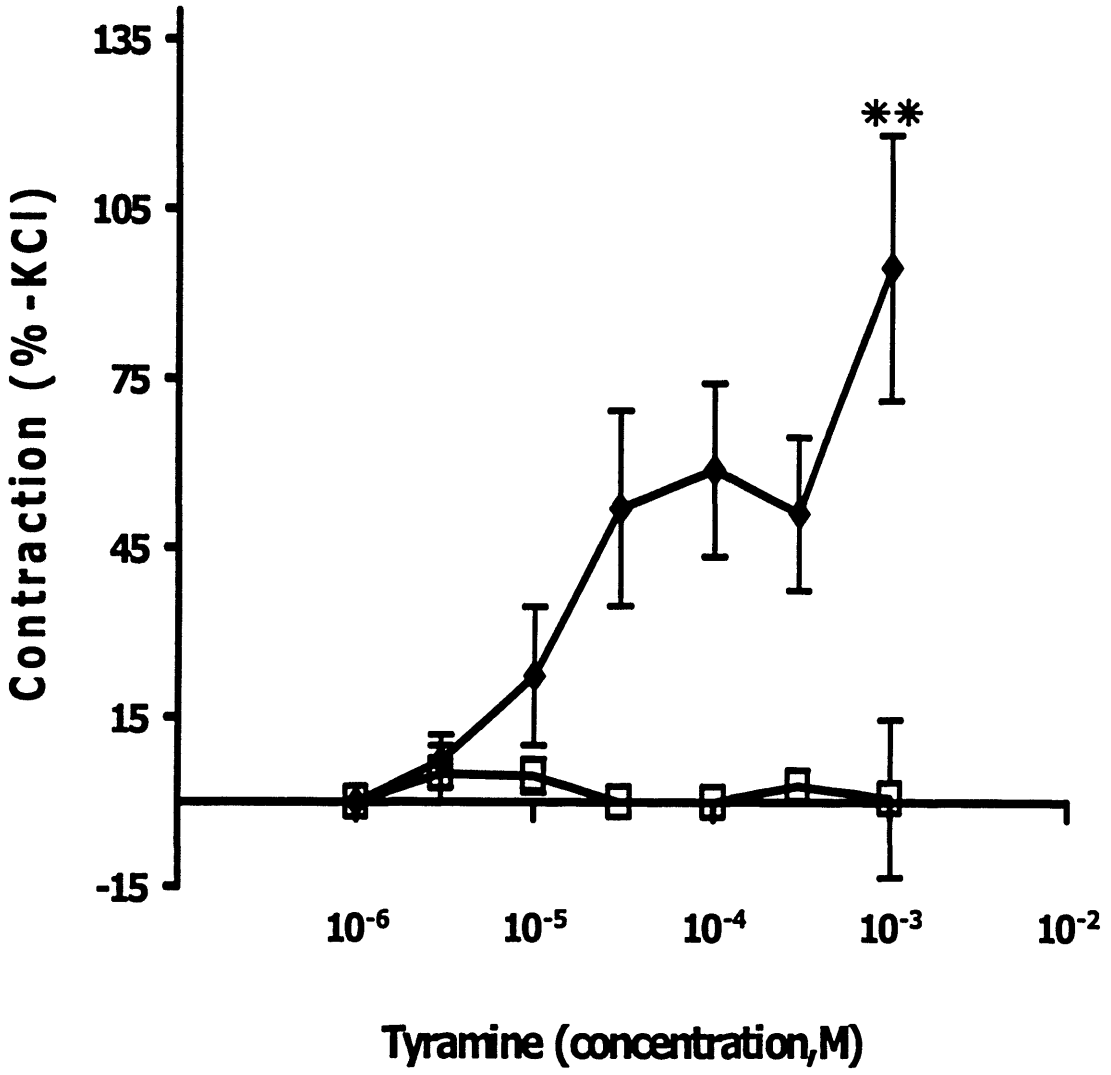


Figure 5.4: The contractile response of guinea-pig and rat aortic rings to tyramine in the presence of "inhibitors". Non-cumulative CRCs for tyramine in guinea-pig (◆, n=5) and in rat (□, n=5) aortic rings were constructed in the presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. At the maximum concentration (1mM) a significant difference between the two CRCs was shown ($P<0.01$,**).

5.4.3 Contractile response to octopamine in rat and guinea-pig aorta - effect of "inhibitors"

In the next set of experiments, the contractile responses in rat and in guinea-pig aorta to octopamine were examined.

In isolated rat aortic rings, the CRC for octopamine (-log EC_{50} 6.01±0.99, n=4) was displaced to the right but not significantly (P>0.05) by "inhibitors" (-log EC_{50} 4.22±0.13, n=4). However, the contractile response to octopamine at the concentration of 3μM (93.8±20.8%, n=4) and 10μM (79.2±7.2%, n=4) were significantly decreased in the presence of "inhibitors" (3μM, 6.3±6.3%, n=4) (P<0.05) and (10μM, 0±0%, n=4) (P<0.01) *(Figure 5.5)*. The contractile response to octopamine at the maximum concentration (300μM, 140.5±23.4%, n=4) was significantly (P<0.05) inhibited by "inhibitors" (300μM, 49.9±6.9%, n=4) *(Figure 5.6)*. In addition, the contractile responses to octopamine in the two different species in the presence of the "inhibitors" were compared. Neither the maximum contractile responses in rat (100μM, 73.6±35.3%, n=4), and in guinea-pig aorta (100μM, 55.9±10.7%, n=4) nor the contractile responses at the maximum concentration in rat (300μM, 72.6±19.4%, n=4) and in guinea-pig aorta (300μM, 49.9±6.9%, n=4) to octopamine were significantly different (P>0.05). Moreover, the –log EC_{50} values in rat (4.36±0.17, n=5) and in guinea-pig aorta (4.97±0.23, n=5) were not significantly different (P>0.05) *(Figure 5.7)*.

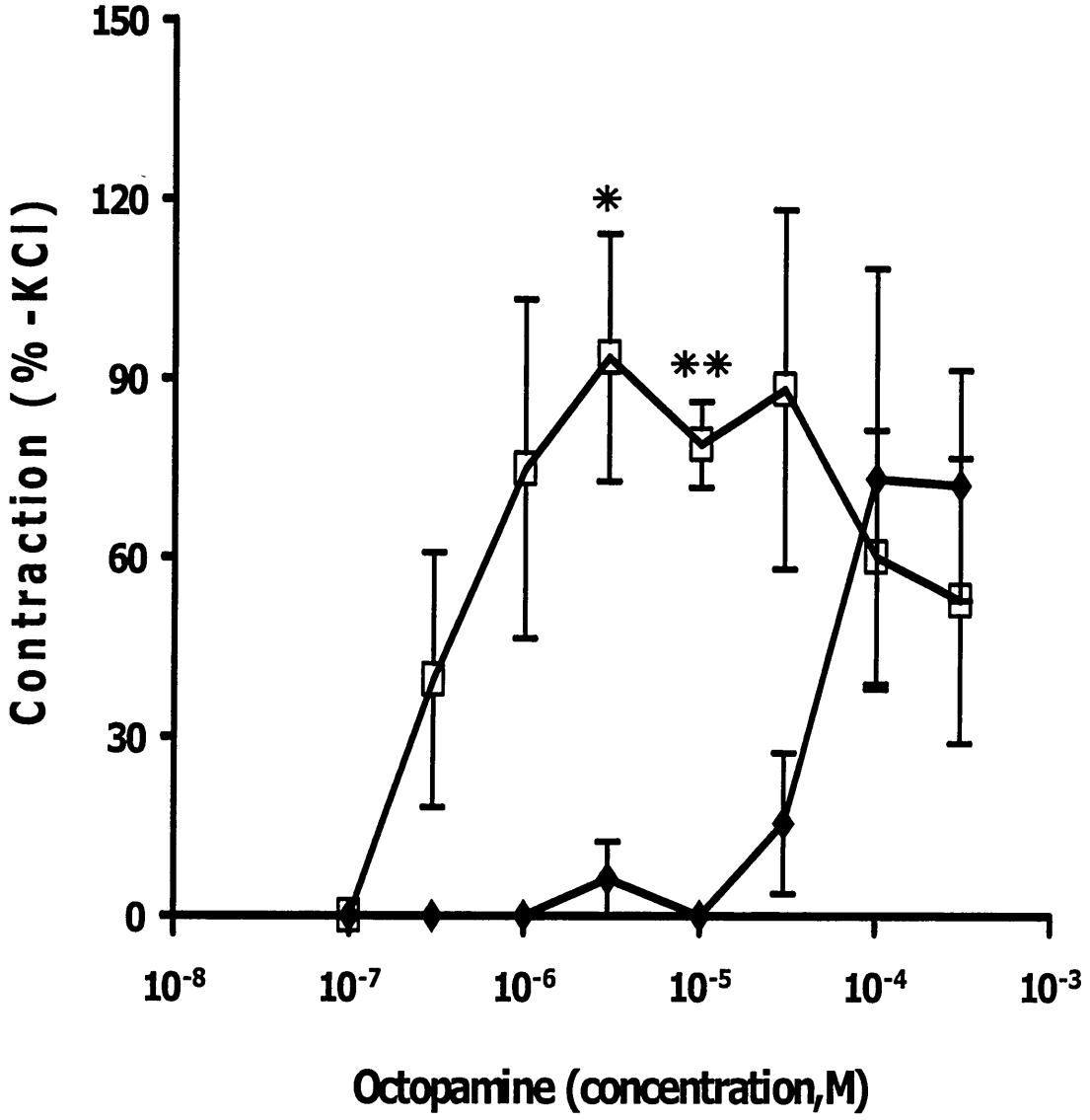


Figure 5.5: Effect of "inhibitors" on the contractile response of rat aortic rings to octopamine. Non-cumulative CRCs for octopamine in rat aortic rings were constructed in the absence (□, n=4), or in the presence of "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. Significant differences were seen at the concentrations 3μM ($P<0.05$,*) and 10μM ($P<0.01$,**).

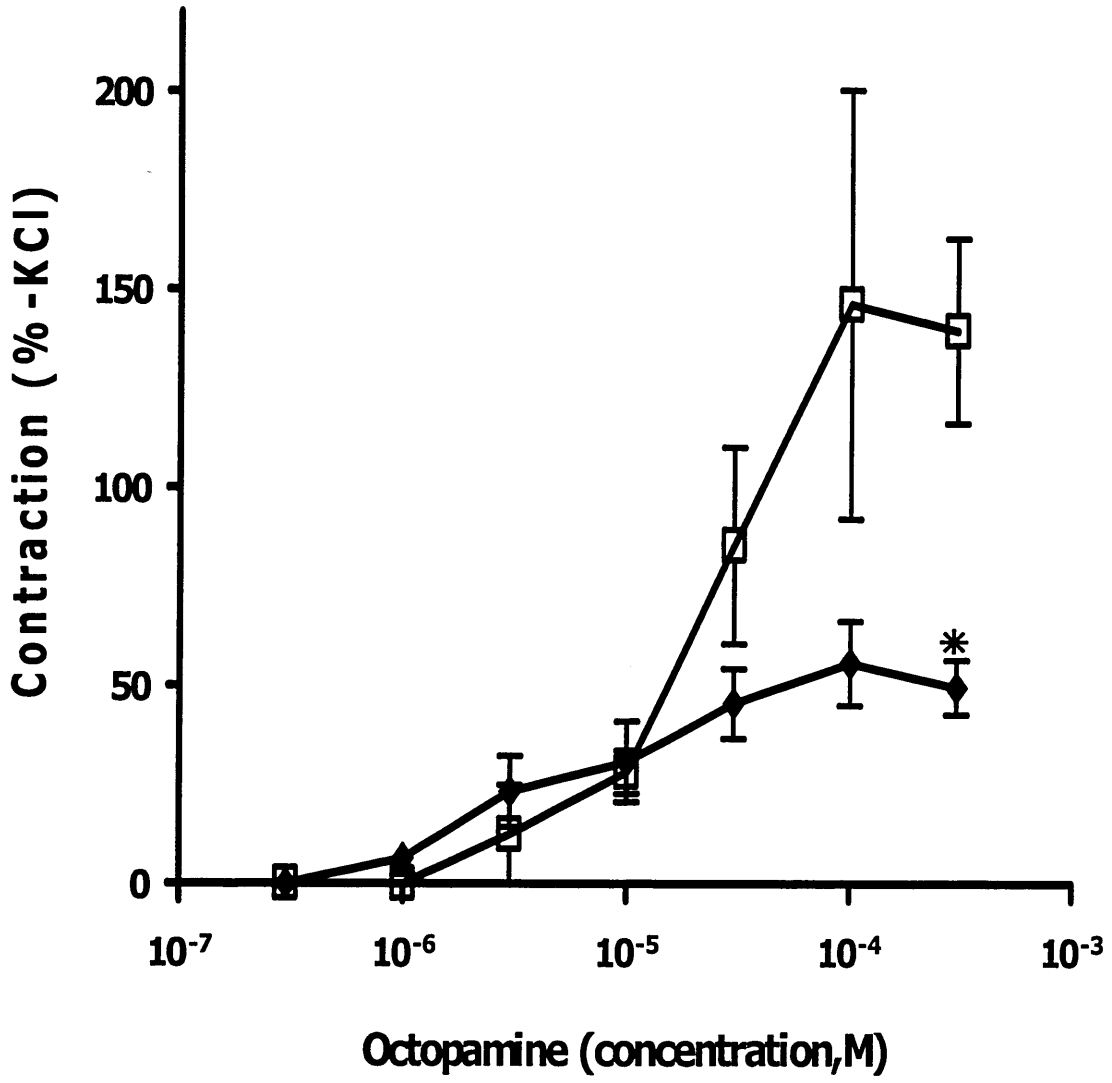


Figure 5.6: Effect of "inhibitors" on the contractile response of guinea-pig aortic rings to octopamine. Non-cumulative CRCs for octopamine in guinea-pig aortic rings were constructed in the absence (□, n=4), or in the presence of "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. A significant difference was seen at the maximum concentration in the absence or presence of "inhibitors" ($P<0.05$,*).

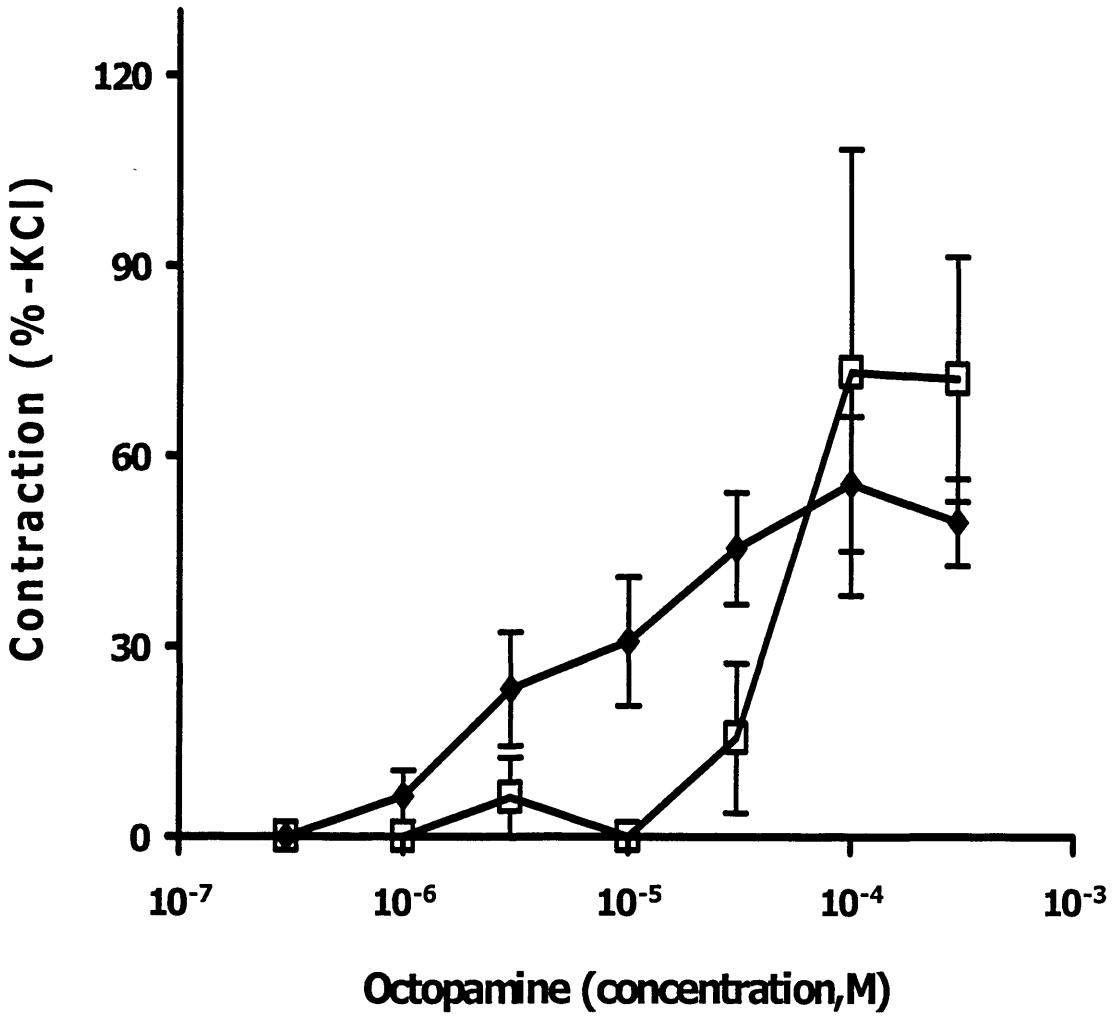


Figure 5.7: The contractile response of guinea-pig and rat aortic rings to octopamine in the presence of "inhibitors". Non-cumulative CRCs for octopamine in guinea-pig (◆, n=4) and in rat (□, n=4) aortic rings were constructed in the presence of "inhibitors" Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant differences ($P>0.05$) were seen.

5.4.4 Contractile response to D-amphetamine in rat and guinea-pig aorta - effect of "inhibitors"

The contractile response in rat aorta to D-amphetamine was modified by "inhibitors". The CRC to D-amphetamine (-log EC_{50} 6.15±0.78, n=4) was shifted to the right by "inhibitors" (-log EC_{50} 3.76±0.34, n=4), but not significantly (P>0.05). The maximum contractile response to D-amphetamine (10μM, 108.3±53.4%, n=4) was not significantly decreased by "inhibitors" (10μM, 25±25%, n=4) *(Figure 5.8)*.

The contractile response in guinea-pig aorta to D-amphetamine was then examined. The contractile response to D-amphetamine at the concentration of 10μM (98.9±14.5%, n=4) was significantly inhibited (P<0.05) by "inhibitors" (50±10.6%, n=4). However, the maximum contractions in the absence (30μM, 140.6±29.4%, n=4), and presence (30μM, 86.3±24.3%, n=4) of "inhibitors" were not significantly different (P>0.05) *(Figure 5.9)*.

In addition, the contractile responses in rat and guinea-pig aorta to D-amphetamine, in the presence of the "inhibitors" were compared. The –log EC_{50} values to D-amphetamine in rat (3.76±0.34, n=4) and guinea-pig (4.85±0.32, n=4) aortic tissues were not significantly (P>0.05) different. The maximum contractions to D-amphetamine in rat (100μM, 75.8±30.3%, n=4), and guinea-pig (30μM, 121±39.5%, n=4) to D-amphetamine were not significantly different (P>0.05) *(Figure 5.10)*.

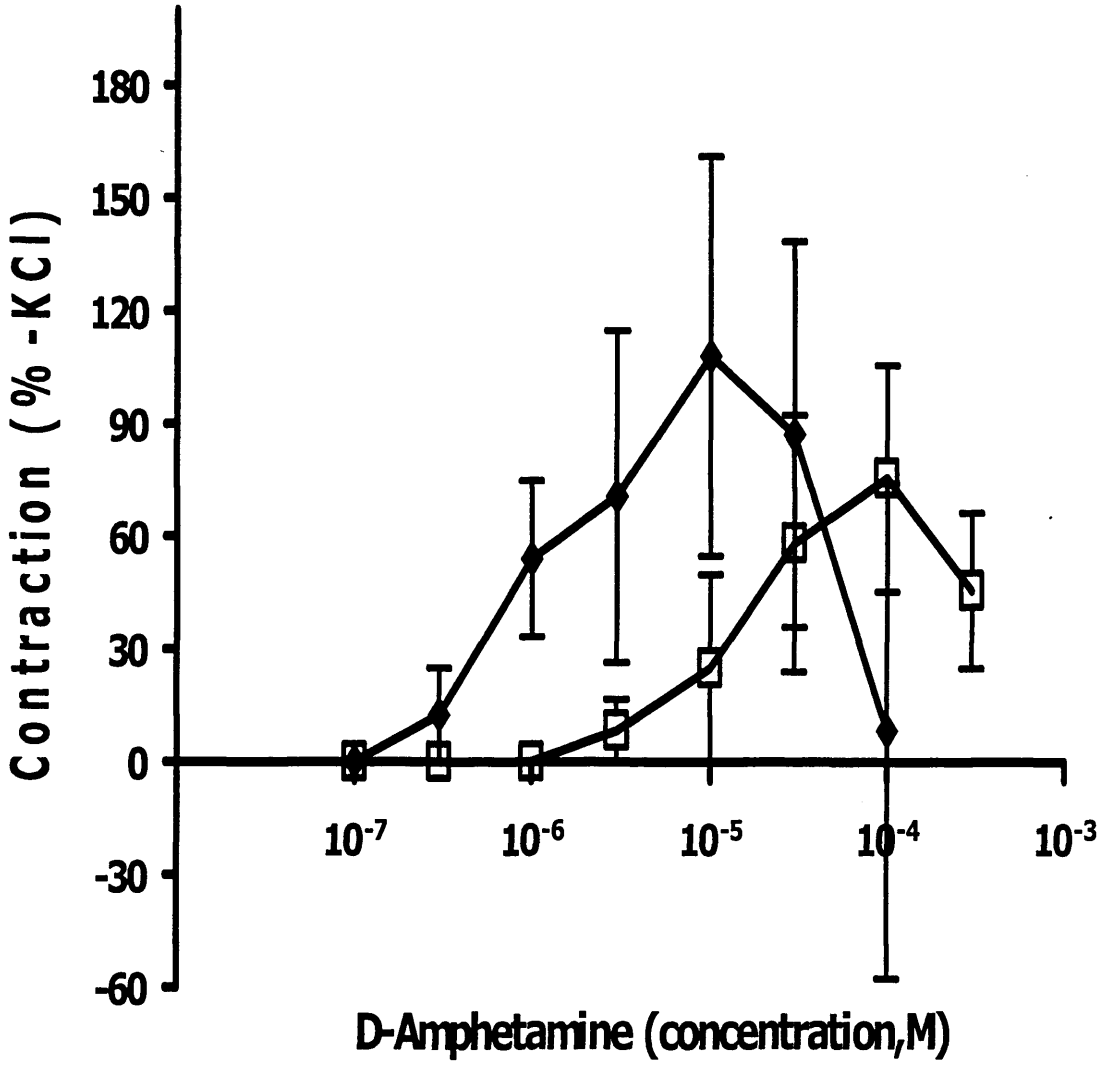


Figure 5.8: Effect of "inhibitors" on the contractile response of rat aortic rings to D-amphetamine. Non-cumulative CRCs for D-amphetamine in rat aortic rings were constructed in the absence (◆, n=4) or in the presence of "inhibitors" (□, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen.

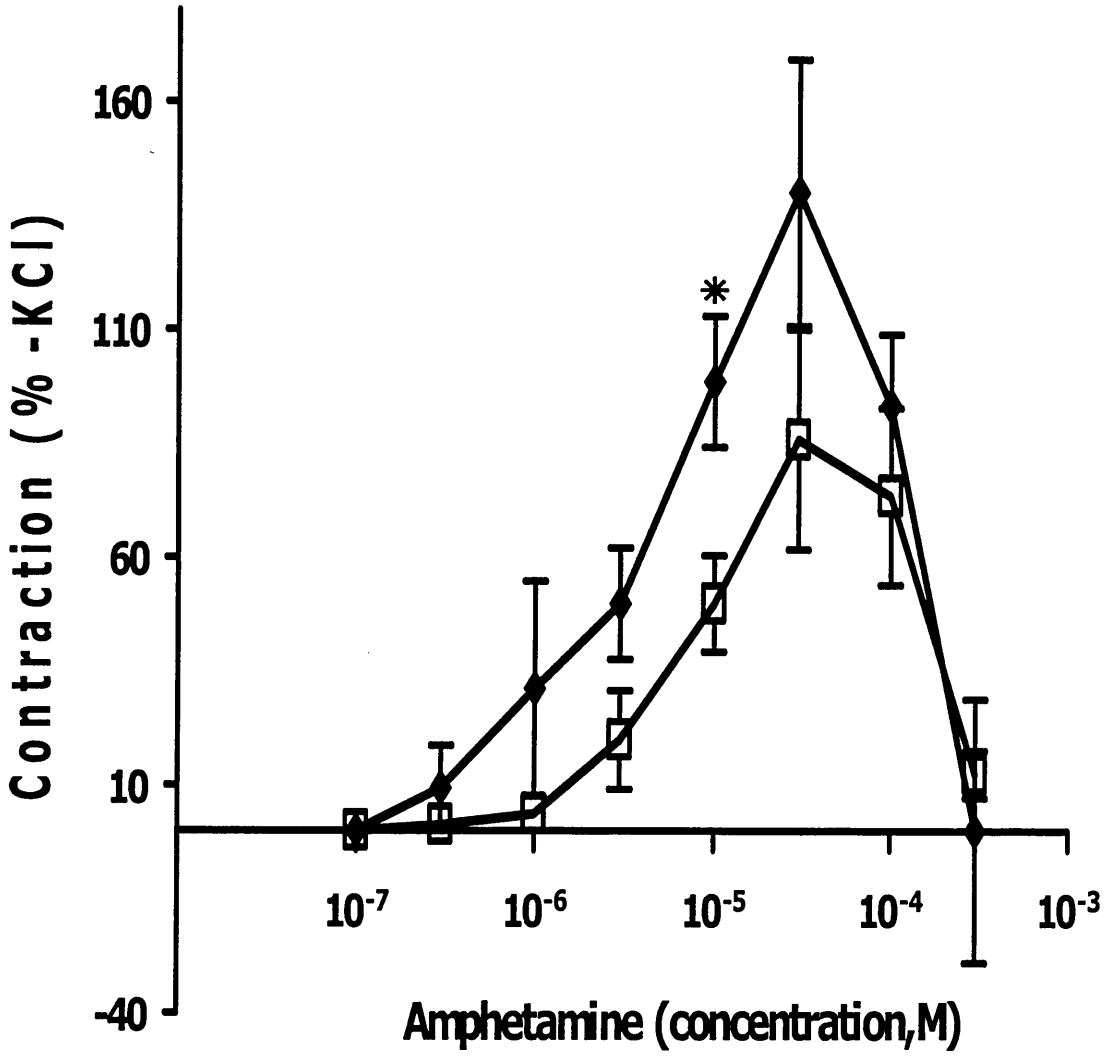


Figure 5.9: Effect of "inhibitors" on the contractile response of guinea-pig aortic rings to D-amphetamine. Non-cumulative CRCs for D-amphetamine in guinea-pig aortic rings were constructed in the absence (◆, n=4), or in the presence of "inhibitors" (□, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. A significant difference was seen at the concentration of 10μM (P>0.05,*).

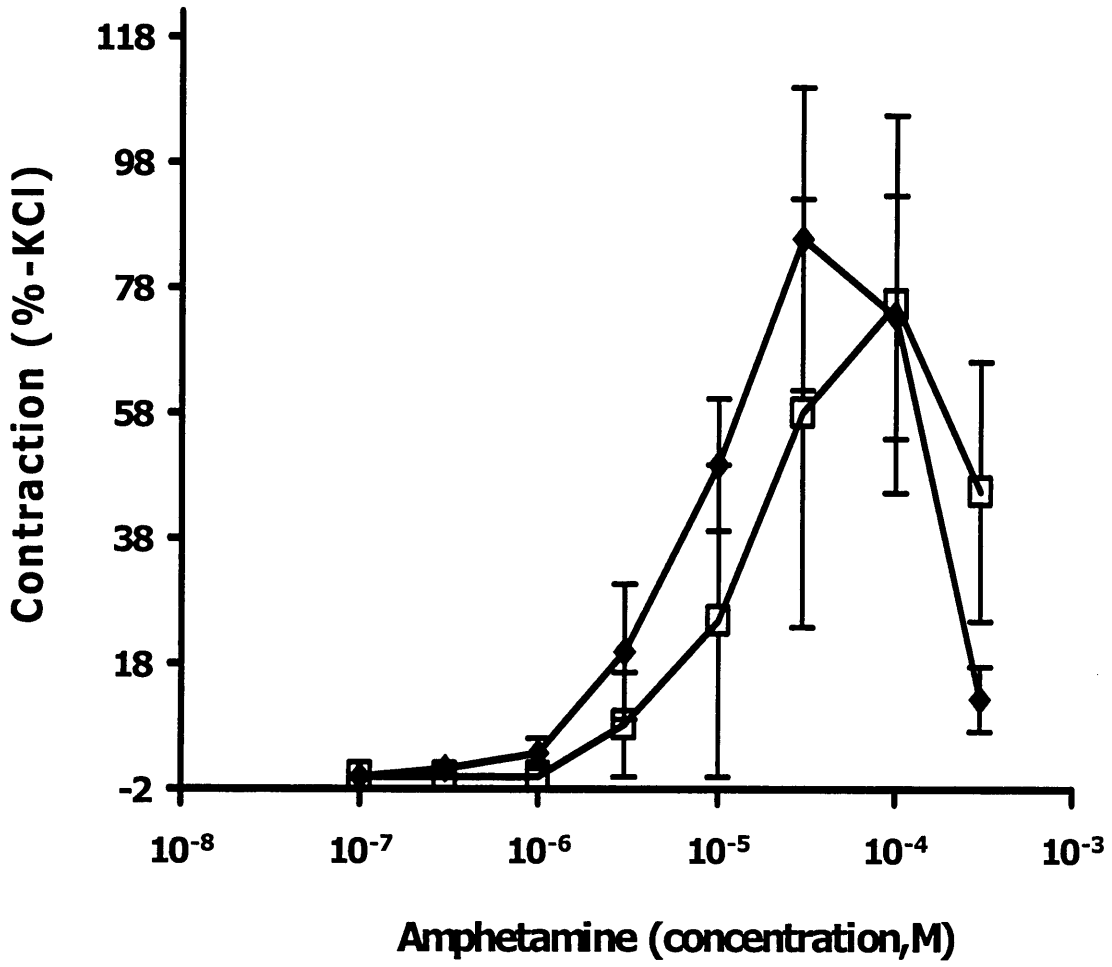


Figure 5.10: The contractile response of guinea-pig and rat aortic rings to D-amphetamine in the presence of "inhibitors". Non-cumulative CRCs for D-amphetamine in guinea-pig (◆, n=4) and in rat aortic rings were constructed in the presence of "inhibitors" (□, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's unpaired t-test. No significant differences ($P>0.05$) were seen.

5.4.5 Summary of contractile responses to different trace amines in rat aorta – effect of "inhibitors"

The contractile responses to β-PEA in rat, and guinea-pig aortic rings were used as a basis for the investigation of contractile responses to different TAs. The contractile responses of a range of TAs were therefore compared between the two species.

In rat aorta, tyramine in the presence of the "inhibitors" turned out to be the weakest TA agonist. There was virtually no contractile response in the presence of "inhibitors" up to 1mM (7.1±14%, n=5). β-PEA gave the largest response of the agonists (300μM, 138.2±23.1%, n=6) *(Figure 5.11, Table 5.1)*. In the presence of "inhibitors" both the contractile response to β-PEA at the maximum concentration (1mM, 114.6±29.3%, n=6) and the maximum contractile response (300μM, 138.2±23.1%, n=6) were significantly greater ($P<0.01$) than the contractile response to tyramine (1mM, 7.1±14%, n=5) *(Figure 5.11)*. Moreover, the maximum contractions of D-amphetamine (100μM, 75.8±30.3%, n=4) and octopamine (100μM, 73.6±35.3%, n=4) occurred at lower concentrations compared to β-PEA (300μM, 138.2±23.1%, n=6). Furthermore, the CRCs for D-amphetamine (-log EC_{50} 3.76±0.34, n=4), octopamine (-log EC_{50} 4.45±0.39, n=4) and β-PEA (-log EC_{50} 4.46±0.15, n=6) were not significantly different ($P>0.05$) *(Figure 5.11)*.

Trace amine	Mean Max. Response (%)	Concentration (μM)
β-PEA	138.2±23.1	300
D-amphetamine	75.8±30.3	100
Octopamine	73.6±35.3	100
Tyramine	7.1±14.0	1000

Table 5.1: Comparison of the maximum contractile response in rat isolated aortic rings of different trace amines (TAs). In the presence of "inhibitors" β-PEA is the most active and tyramine the least active agonist.

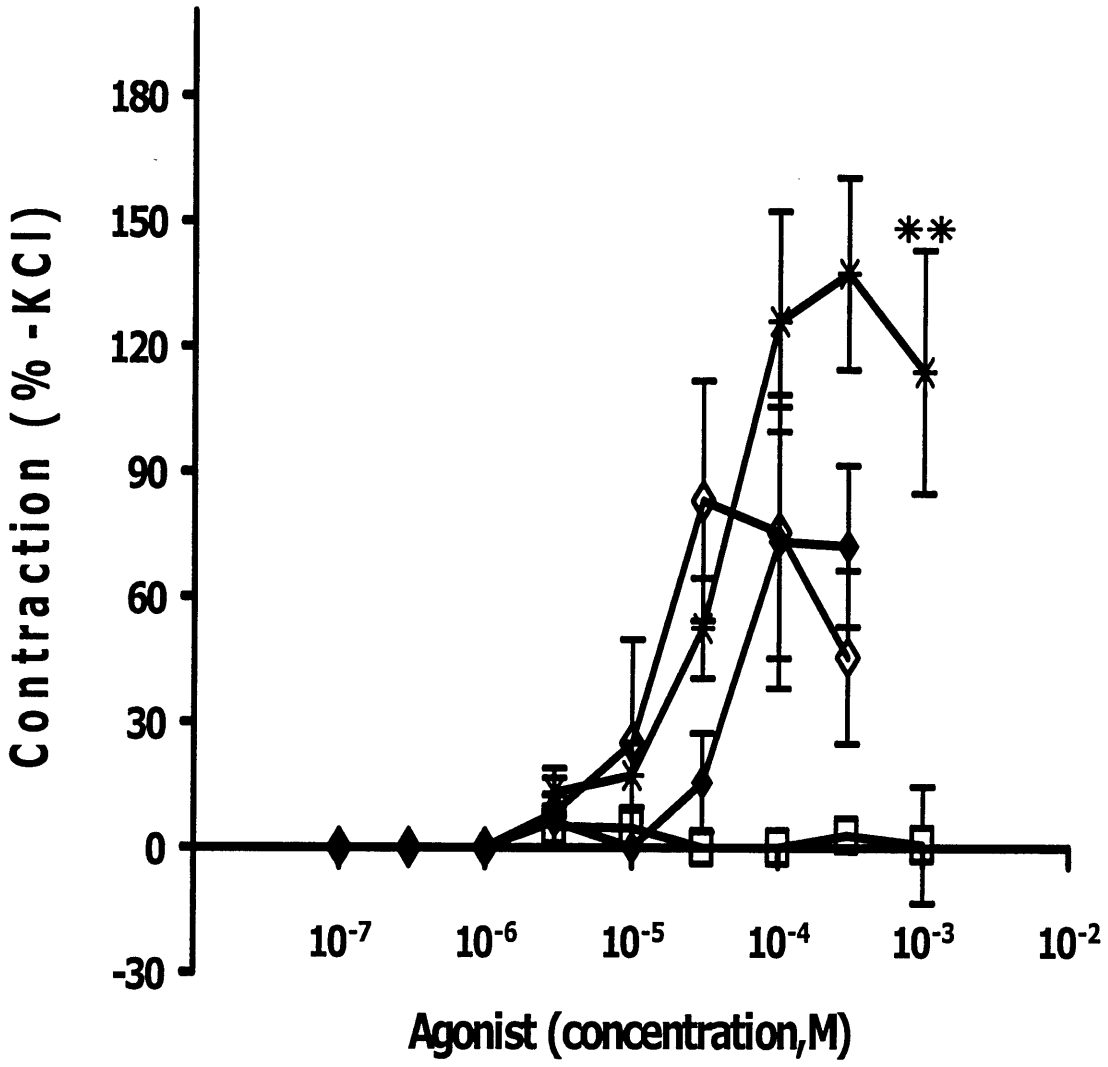


Figure 5.11: Comparison of the contractile responses of rat aortic to various trace amine agonists. Non-cumulative CRCs for β-PEA (✳, n=6), D-amphetamine (◇, n=4), octopamine (◆, n=4) and tyramine (□, n=5) in rat aortic rings were constructed in the presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Analysis of ANOVA followed by the "post hoc" Bonferroni test. A significant difference was seen between the responses to the maximum concentration of β-PEA and tyramine ($P<0.01$,✳✳).

5.4.6 Contractile response to tryptamine in guinea-pig- effect of adrenergic inhibitors

Analogous to previous experiments in rat aortic rings *(Chapter 4)*, the contractile responses to tryptamine in guinea-pig aortic rings in the absence, or presence of various inhibitors was examined. The contractile response in guinea-pig aorta to tryptamine was first tested in the presence of "inhibitors". The maximum contractile response to tryptamine (100μM, 138.5±31.1%, n=3) was not significantly altered (P>0.05) by "inhibitors" (100μM, 114.1±52.1%, n=3) *(Figure 5.12)*. In addition, these experiments were analogous to previous experiments in rat aortic rings *(Chapter 4)* repeated in the presence of methiothepin (50nM) and ketanserin (3nM). The maximum contractile response of tryptamine in the presence of "inhibitors" (100μM, 114.1±51.1%, n=3) was not significantly altered (P>0.05) by methiothepin and ketanserin (100μM, 99.6±35.1%, n=4) *(Figure 5.12)*.

In *Chapter 4* ketanserin (3nM) alone and in combination with methiothepin (50nM) caused a significantly right shift of the CRCs to serotonin *(Figure 4.15)*. However, the CRC to tryptamine was not shifted by the combination of both serotonin antagonists in rat isolated aorta *(Figure 4.18)*. Referring to the experiments in *Chapter 4* both serotonin antagonists did not shift the CRC to tryptamine to the right. The $-\log EC_{50}$ values of both CRCs to tryptamine in the presence of "inhibitors" alone (5.18±0.55, n=3) and in the presence of "inhibitors" plus methiothepin (50nM) and ketanserin (3nM) (4.42±0.07, n=4) were not significantly different (P>0.05) *(Figure 5.12)*.

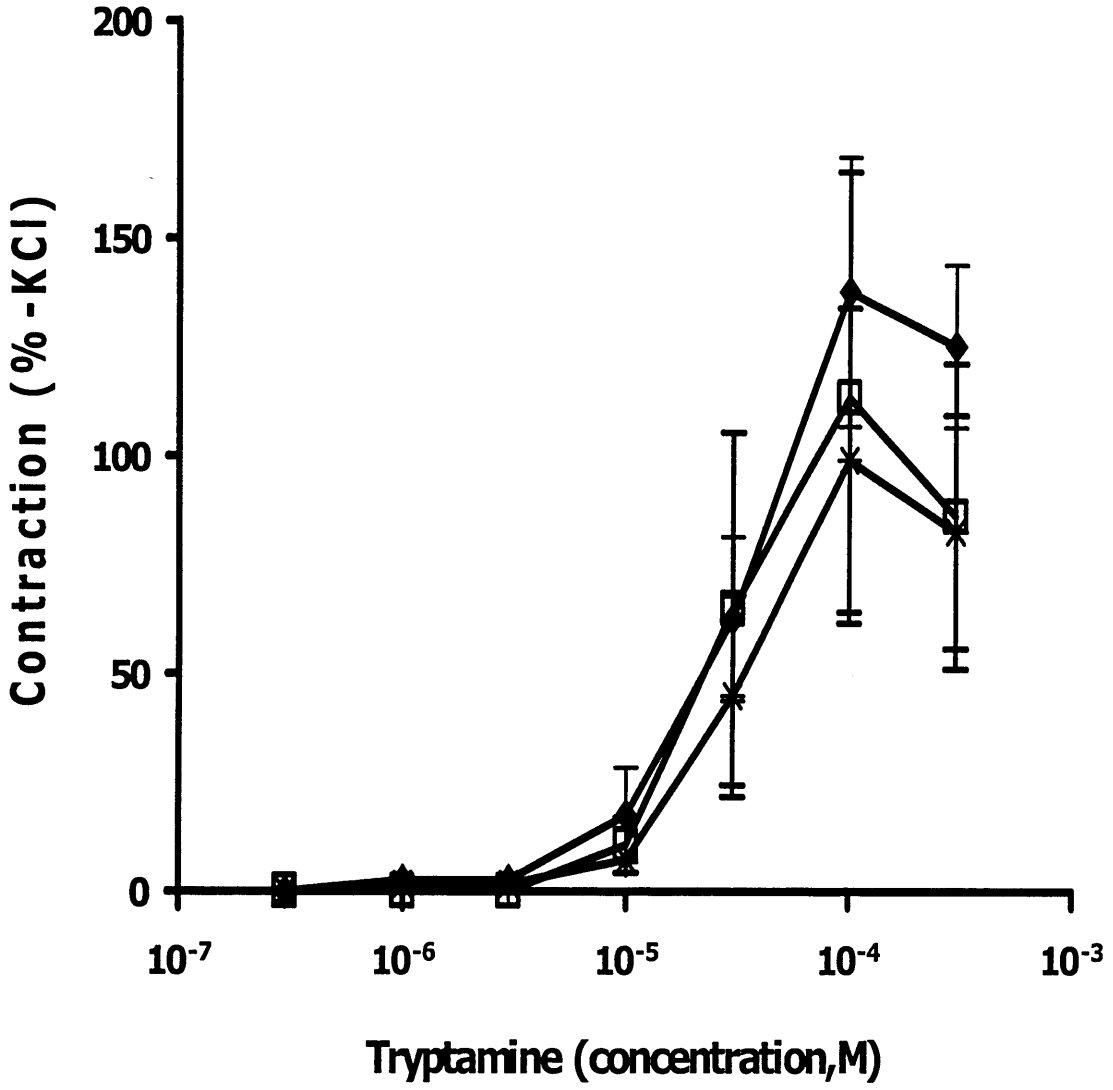


Figure 5.12: Effects of "inhibitors", methiothepin (50nM) and ketanserin (3nM) on the contractile response of guinea pig aortic rings to tryptamine. Non-cumulative CRCs for tryptamine in guinea pig aortic rings were constructed in the absence (◆, n=3), in the presence of "inhibitors" (□, n=3) or in the presence of "inhibitors", methiothepin (50nM) and ketanserin (3nM) (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (+SEM) were compared by ANOVA followed by "post hoc" test Bonferroni. No significant differences ($P>0.05$) at their maximum contractile responses were seen.

5.4.7 Summary of contractile responses to different trace amines in guinea-pig aorta – effect of "inhibitors"

Similar to previous results in rat aortic tissues *(Chapter 5.4.5)*, in the presence of "inhibitors" β-PEA also turned out to be the most active TA in guinea-pig aortic rings. However, in contrast to the results in rat aorta octopamine gave the smallest maximum response in the presence of "inhibitors" *(Figure 5.12, Table 5.2)*.

In the presence of "inhibitors", the maximum contractile responses in guinea-pig aorta to β-PEA (300μM, 158.7±39.7%, n=5), tyramine (1mM, 95.1±23.4%, n=5), D-amphetamine (30μM, 86.3±24.3%, n=4), octopamine (100μM, 55.9±10.7%, n=4) and tryptamine (100μM, 114.1±52.1%, n=3) were not significantly different (P>0.05). Moreover, the CRCs to D-amphetamine (-log EC_{50} 4.86±0.31, n=4), β-PEA (-log EC_{50} 4.26±0.41, n=4), octopamine (-log EC_{50} 4.98±0.23, n=5), tyramine (-log EC_{50} 4.37±0.36, n=5) and tryptamine (-log EC_{50} 5.18±0.55, n=3) were not significantly different (P>0.05) *(Figure 5.12)*.

Trace amines	Mean Max. Response (%)	Concentration (μM)
β-PEA	158.2±39.7	300
Tryptamine	114.1±51.1	100
Tyramine	95.1±23.4	300
D-amphetamine	86.3±24.3	30
Octopamine	55.9±10.7	100

Table 5.2: Comparison of the maximum contractile response in guinea-pig isolated aortic rings of different TAs. In the presence of "inhibitors", β-PEA is the most active and octopamine the least active agonist.

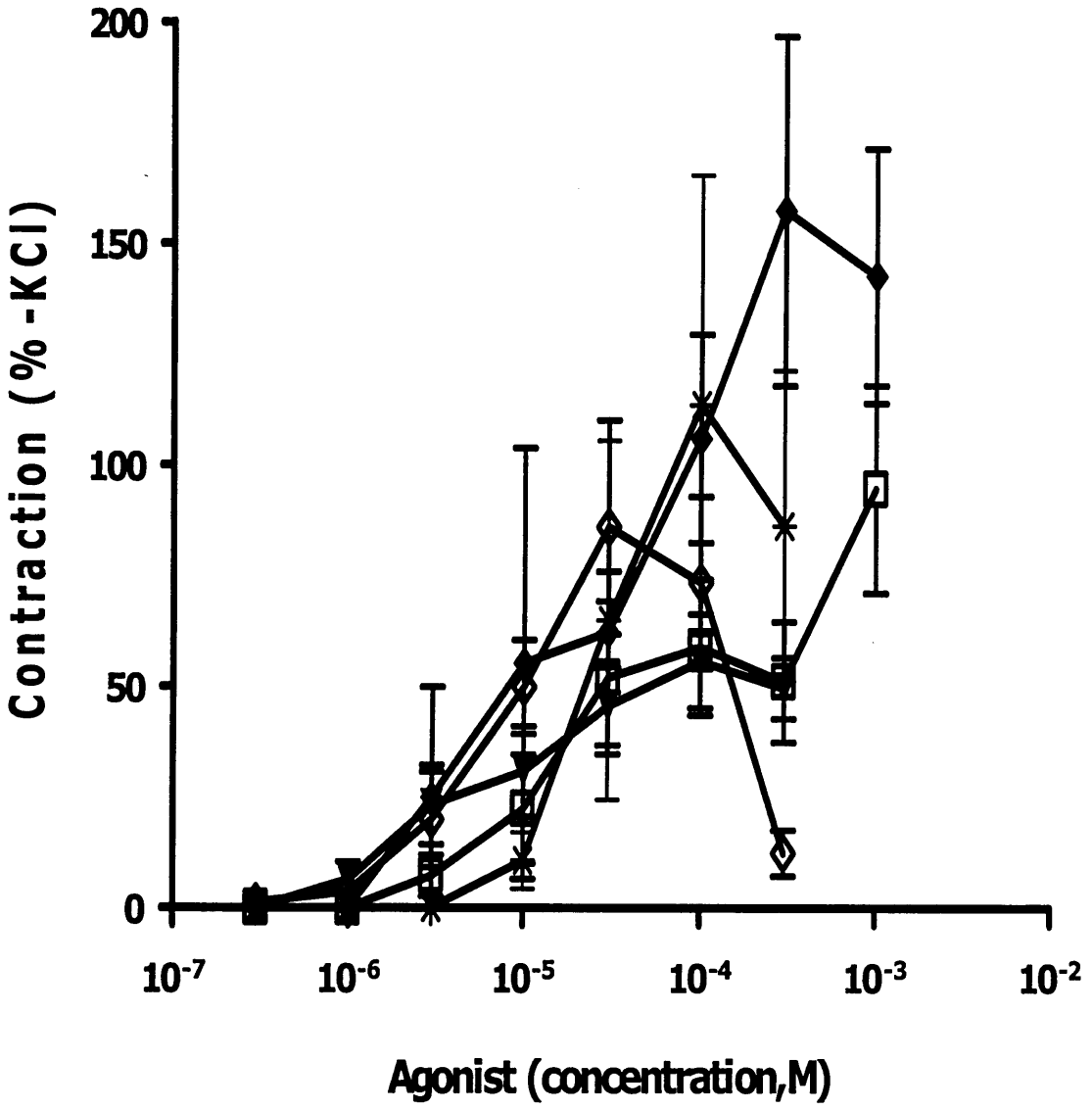


Figure 5.13: Comparison of the contractile responses of guinea-pig aortic to various trace amine agonists to various trace amine agonists. Non-cumulative CRCs for β-PEA (◆, n=5), D-amphetamine (◊, n=4), octopamine (▼, n=4), tyramine (□, n=5) and tryptamine (✳, n=3) in guinea pig aortic rings were constructed in the presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences were seen at their maximum contractile responses ($P>0.05$).

5.4.8 Contractile response to amphetamine-derivatives - effect of "inhibitors"

In the following experiments, the contractile response in rat aortic rings to further TA agonists, Ritalin®, MDMA ('ecstasy') and cathinone were investigated. Due to the high costs of these reagents only cumulative CRCs were constructed.

Ritalin® and MDMA alone both showed a small effect compared to other previously discussed agonists. The contractile response to Ritalin® at the maximum concentration (1mM, 44.4±5.6%, n=3) was not significantly modified (P>0.05) by "inhibitors" (1mM, 35±6.1%, n=5) *(Figure 5.14)*. The maximum contractile response in rat aortic rings to MDMA (300µM, 80±25.5%, n=5) was also not significantly (P>0.05) modified by "inhibitors" (300µM, 54.2±15.8%, n=4). Furthermore, at the maximum concentration (1mM), the presence of "inhibitors" (1mM, 37.5±14.2%, n=4) did not significantly (P>0.05) modify the reduced contraction (vasodilatation) (1mM, 70±20%, n=5) *(Figure 5.15)*. In addition, the contractile response in rat aortic rings to cathinone was investigated. For financial reasons, the experiment with cathinone in the absence of "inhibitors" was carried out only once to see the tendency of the contractile effect of cathinone in rat aortic rings. Cathinone seems to be very weakly active alone and compared to Ritalin® and MDMA, it was the weakest agonist. At the maximum concentration (3mM) the contractile response to cathinone seems to be potentiated by "inhibitors" (3mM, 45.2±12.9%, n=4) *(Figure 5.16)*. The most active of these three TA agonists, in the presence of "inhibitors" was MDMA (300µM, 54.2±15.8%, n=4), followed by Ritalin® (1mM, 35±6.1%, n=4) then cathinone (3mM, 45.2±12.9%, n=4) *(Figure 5.17)*. The maximum contractile responses were not significantly different (P>0.05) but significantly different (P<0.01) from β-PEA (300µM, 138.2±23.1, n=6). However, the CRC of Ritalin® was shifted to the left of the CRC of cathinone. The –log EC_{50} values of Ritalin® (4.53±0.27, n=4) and cathinone (3.23±0.05, n=4) were significantly different *(Figure 5.17)*. Neither the CRC to cathinone (-log EC_{50} 3.23±0.05, n=4) nor the CRC to Ritalin® (-log EC_{50} 4.53±0.27, n=4) was significantly different from the CRC to MDMA (-log EC_{50} 3.96±0.38, n=4) *(Figure 5.17)*.

At the maximum concentration of MDMA (1mM, 70±20%, n=5), a reduced contraction (vasodilatation) was observed. However, the contractile responses to MDMA (1mM, 70±20%, n=5), Ritalin® (1mM, 35±6.1%, n=4) and cathinone (3mM, 45.2±12.9%, n=4) at their maximum concentrations were not significantly different (P>0.05) *(Figure 5.17, Table 5.3).*

Trace amines	Mean Max. Response (%)	Concentration (μM)
β-PEA	138.2±23.1	300
MDMA	54.2±6.4	300
Cathinone	45.2±12.9	3000
Ritalin®	35.0±61.2	1000

Table 5.3: Comparison of activity of different TAs in rat isolated aorta in the presence of "inhibitors". MDMA, Ritalin® and cathinone are less active agonists compared to the maximum contractile response to β-PEA in the presence of "inhibitors".

5.4.9 Contractile response to various amphetamine enantiomers – effect of "inhibitors"

The final set of experiments compared stereoisomers of the different amphetamine derivates, D-amphetamine, (+)- and (-)-4-OH-amphetamine and (+)- and (-)-methamphetamine in rat isolated aortic rings. For their effects as trace amine (TA) agonists, the contractile responses to (+)- and (-)-methamphetamine in the presence of "inhibitors" were examined. Again, due to the high cost or limited amounts of these reagents, cumulative CRCs for (+)- and (-)-methamphetamine were constructed in the presence of "inhibitors" only. The maximum contractile response to (-)-methamphetamine in rat aortic rings (100μM, 83.3±16.7%, n=3) was significantly greater (P<0.05) compared to (+)-methamphetamine (300μM, 23.3±11.3%, n=4) *(Figure 5.18).* However, compared to the maximum contractile response to D-amphetamine (100μM, 75.8±30.3%, n=4) neither the contractile response to the (+) (300μM, 23.3±11.3%, n=4) nor to the (-) stereoisomer (100μM, 83.3±16.7%, n=3) of methamphetamine was significantly different (P>0.05) *(Figure 5.18).* In addition, the contractile responses in rat aorta to (+)- and (-)-4-OH-amphetamine in the presence of "inhibitors" were examined. Due to limited drug supply, only single dose experiments were performed.

In the presence of "inhibitors", the contractile response in rat aortic rings to (+)-4-OH-amphetamine (300μM, 22.2±6.4%, n=3) was not significantly different (P>0.05) compared to (-)-4-OH-amphetamine (300μM, 7.4±3.7%, n=3) *(Figure 5.19)*.

Previous work (Bunzow *et al.,* 2001) reported 4-OH-amphetamine to be the most potent TA agonist in cloned TAAR1 transfected cell lines. Therefore, the contractile responses to single doses of (+)-4-OH - and (-)-4-OH-amphetamine (300μM), were compared in separate experiments with a single dose of D-amphetamine at the same concentration (300μM). The contractile response to D-amphetamine (300μM, 46.7±16.2%, n=5) in rat aortic rings was not significantly different (P>0.05) compared to (-)-4-OH-amphetamine (300μM, 7.4±3.7%, n=3) and (+)-4-OH-amphetamine (300μM, 22.2±16.4%, n=3). However, they were all significantly less than the response to the single maximum dose of β-PEA (300μM, 138.2±23.1, n=6) *(Figure 5.20)*.

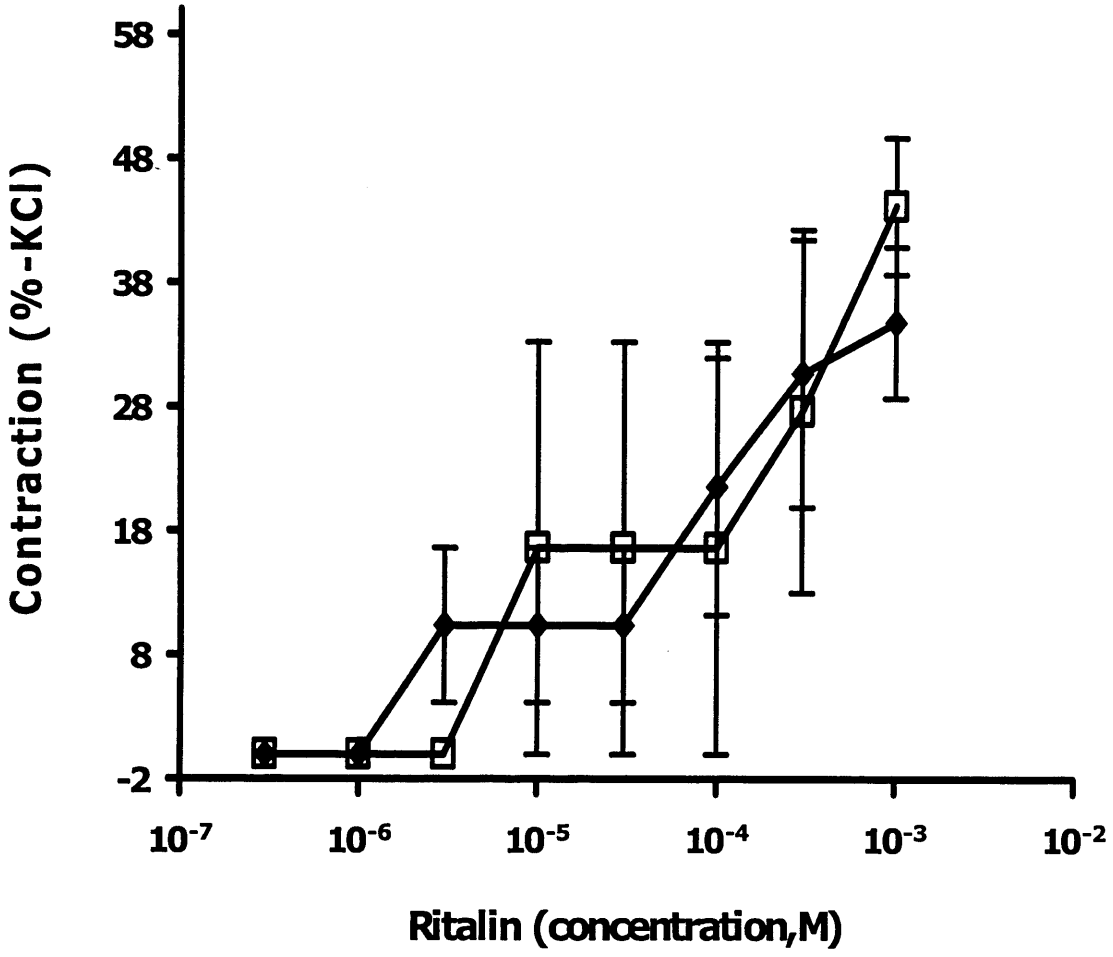


Figure 5.14: Effects of "inhibitors" on the contractile response of rat aortic rings to Ritalin®. Cumulative CRCs for Ritalin® in rat aortic rings were constructed in the absence (□, n=3) or in the presence of "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by student's paired t-test. No significant differences (P>0.05) were seen.

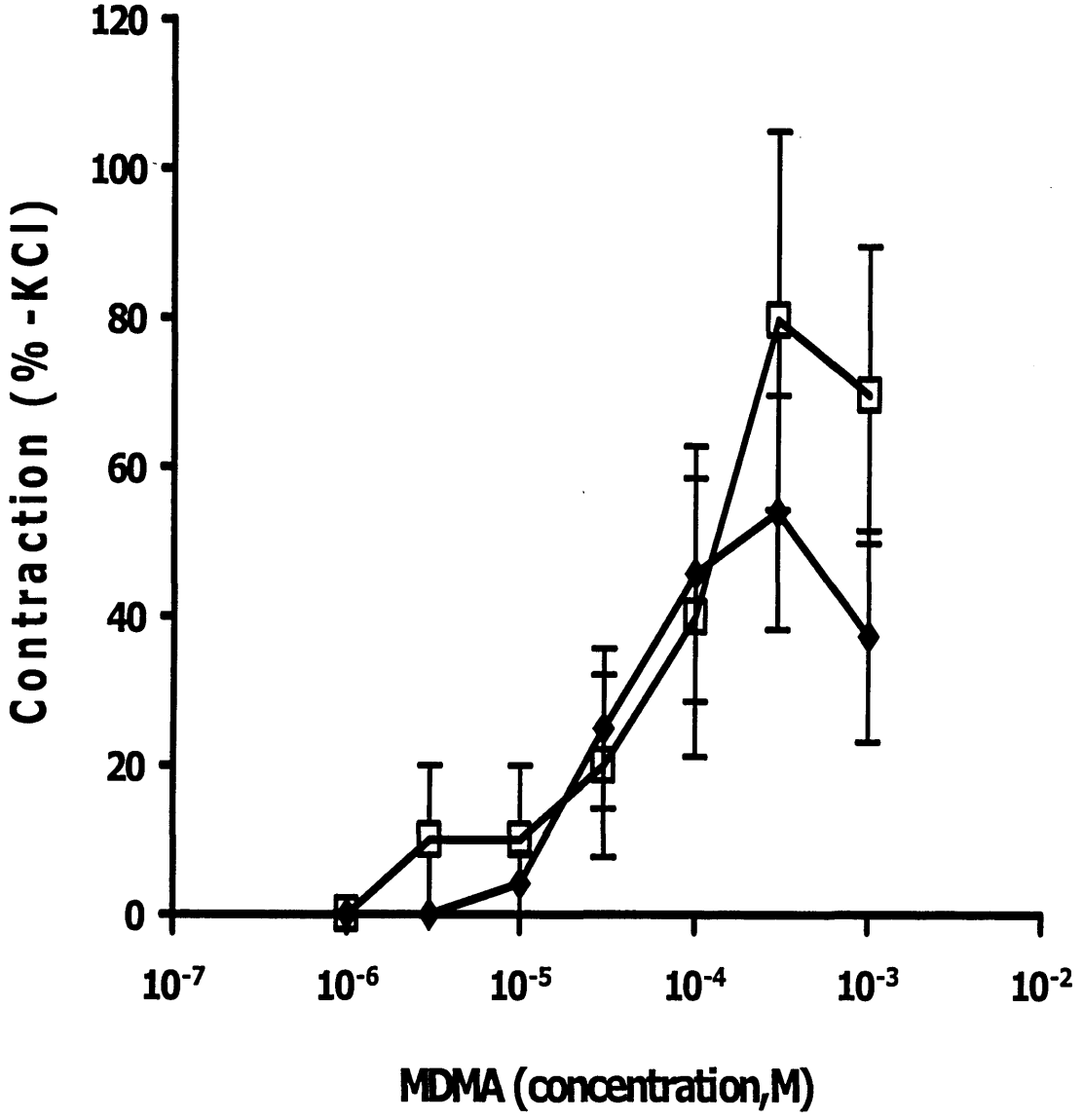


Figure 5.15: Effects of "inhibitors" on the contractile response of rat aortic rings to MDMA. Cumulative CRCs for MDMA in rat aortic rings were constructed in the absence of antagonists (□, n=5) or in the presence of "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by student's paired t-test. No significant differences (P>0.05) were seen.

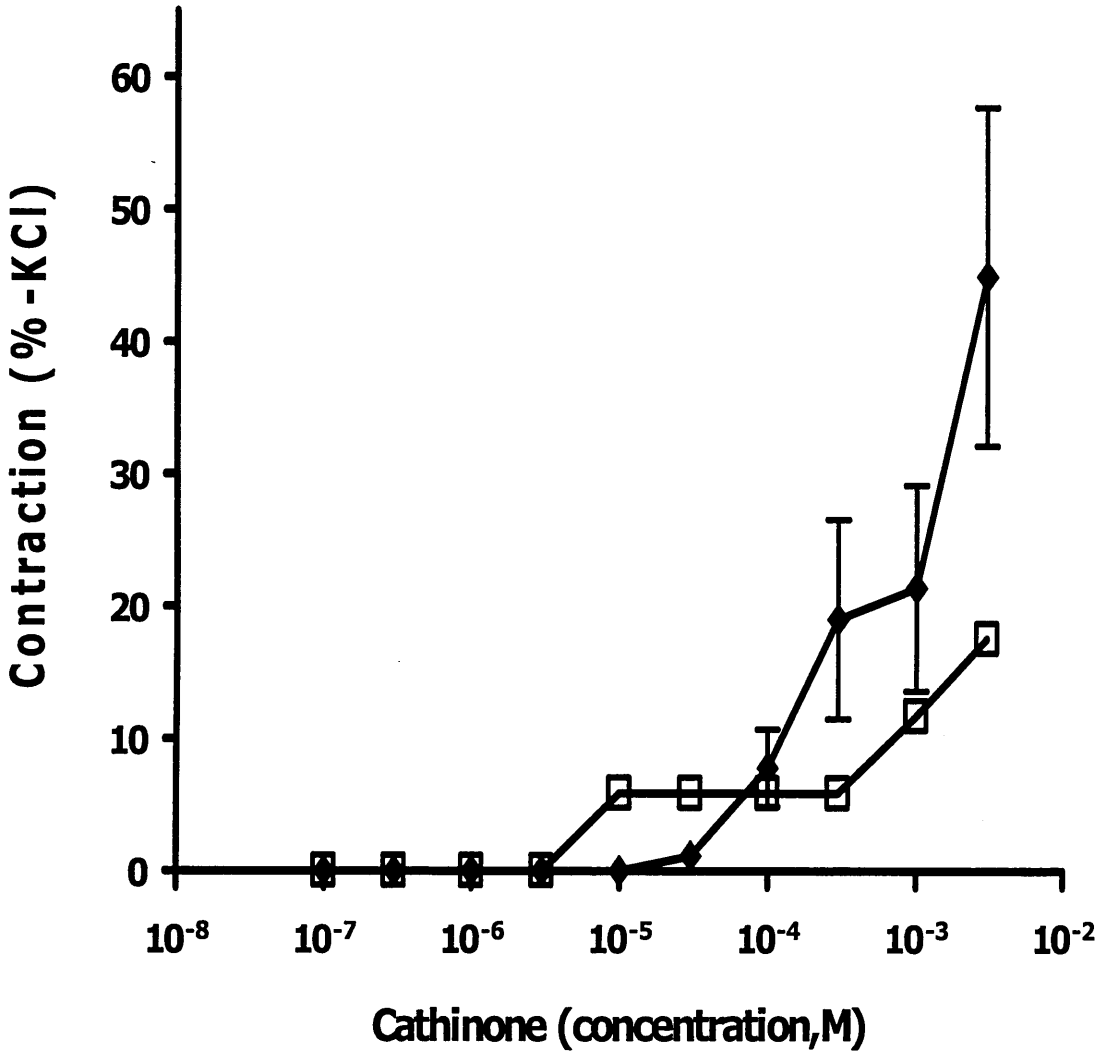


Figure 5.16: Effects of "inhibitors" on the contractile response of rat aortic rings to cathinone. Cumulative CRCs for cathinone in rat aortic rings were constructed in the absence of antagonists (□, n=1) or in the presence of "inhibitors" (◆, n=4). Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution).

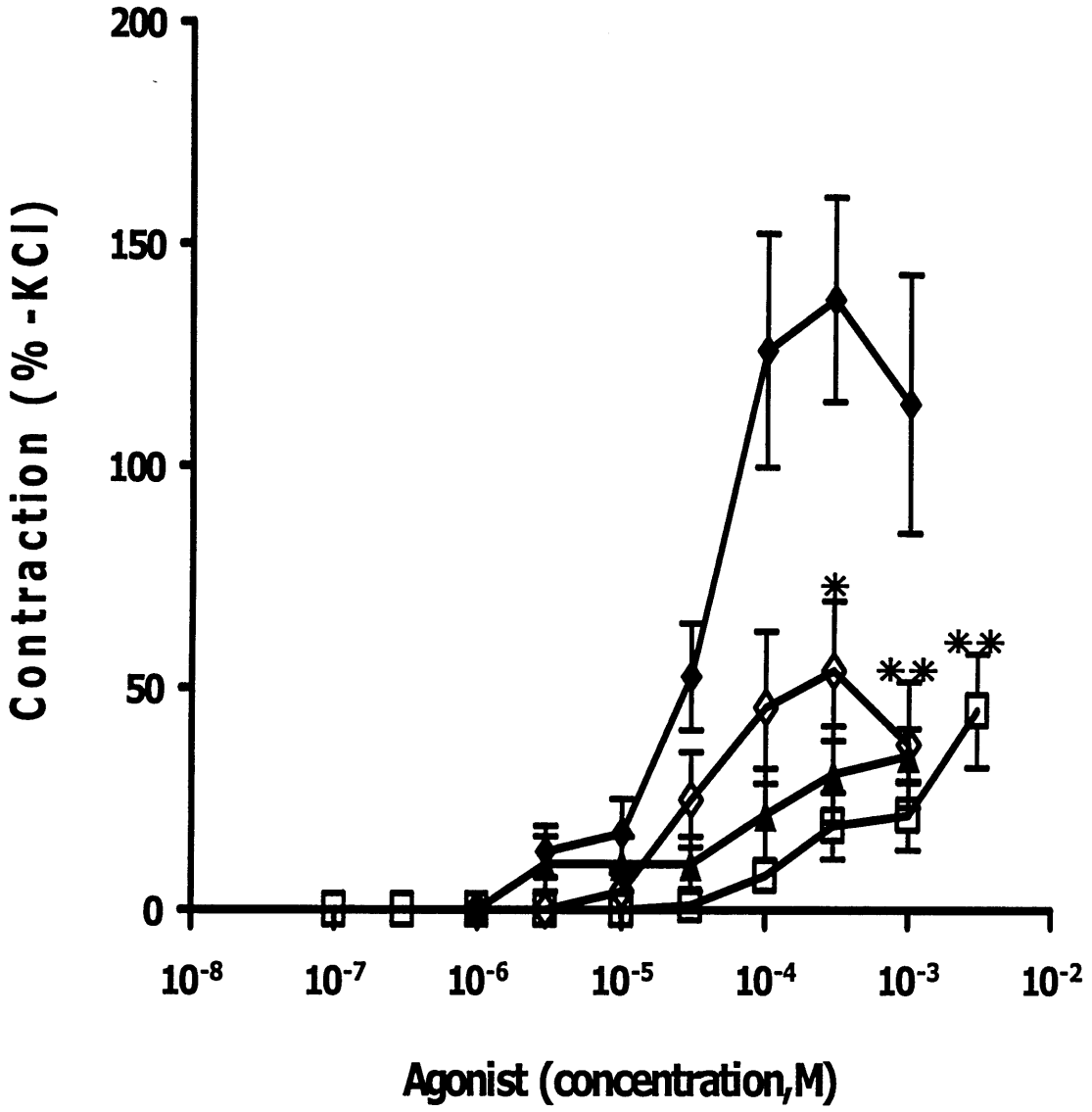


Figure 5.17: The contractile response of rat aortic rings to cathinone, MDMA and Ritalin® compared to β-PEA in the presence of "inhibitors". Cumulative CRCs for cathinone (□, n=4), MDMA (◊, n=4) and Ritalin® (▲, n=4) and non-cumulative CRC for β-PEA (♦, n=6) in rat aortic rings were constructed in the presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. The maximum contractile responses to Ritalin®, cathinone ($P<0.01$,**) and MDMA ($P<0.05$,*) were significantly different compared to the maximum contractile response to β-PEA.

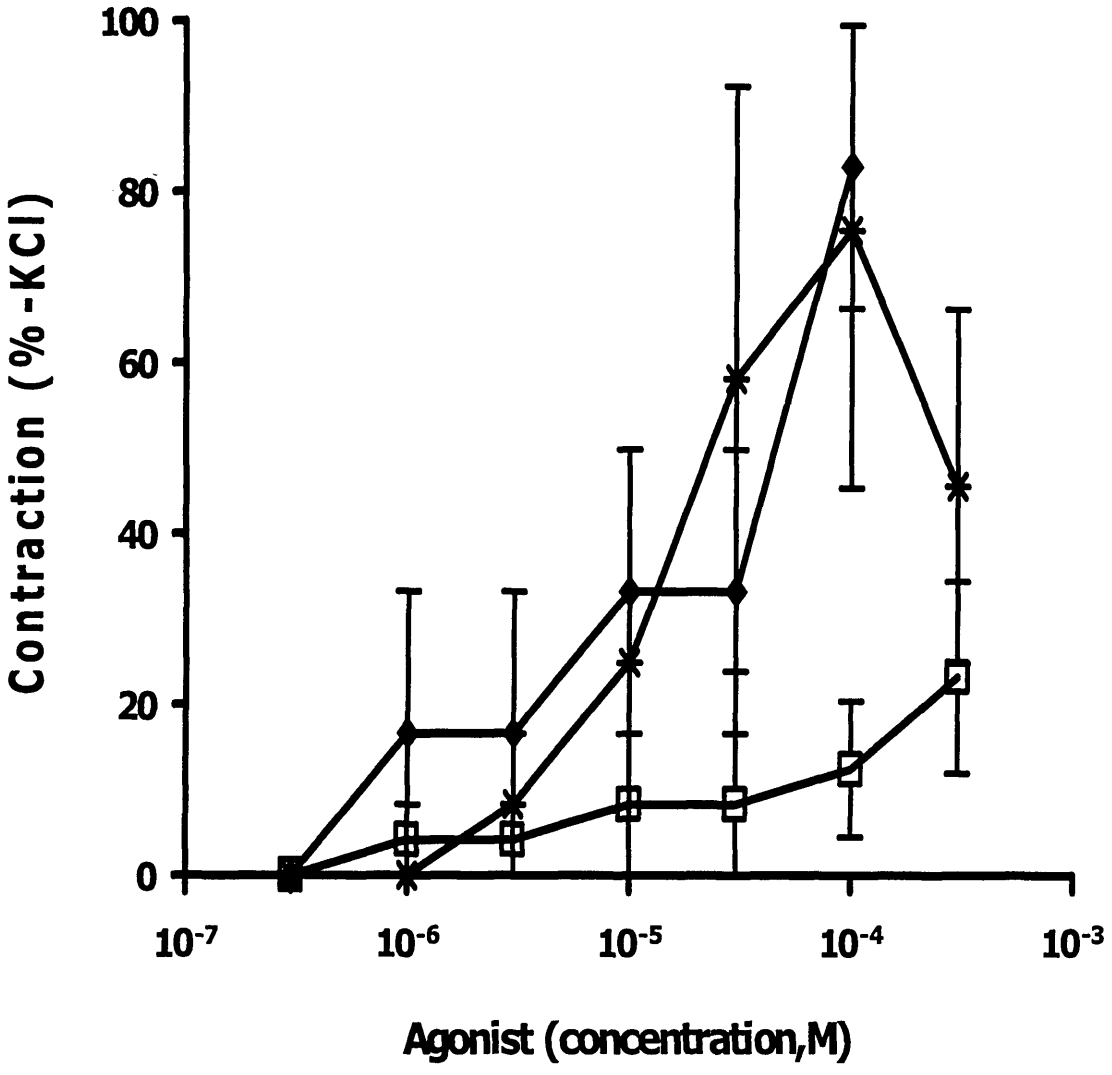


Figure 5.18: Comparison of the contractile response of rat aortic rings to (+)- and (-) methamphetamine and D-amphetamine. Cumulative CRCs for (+)-methamphetamine (□, n=4) and for (-)-methamphetamine (◆, n=3) and non-cumulative CRC to D-amphetamine (✳, n=4) in rat aortic rings were constructed in the presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences (P>0.05) were seen at their maximum contractile respons

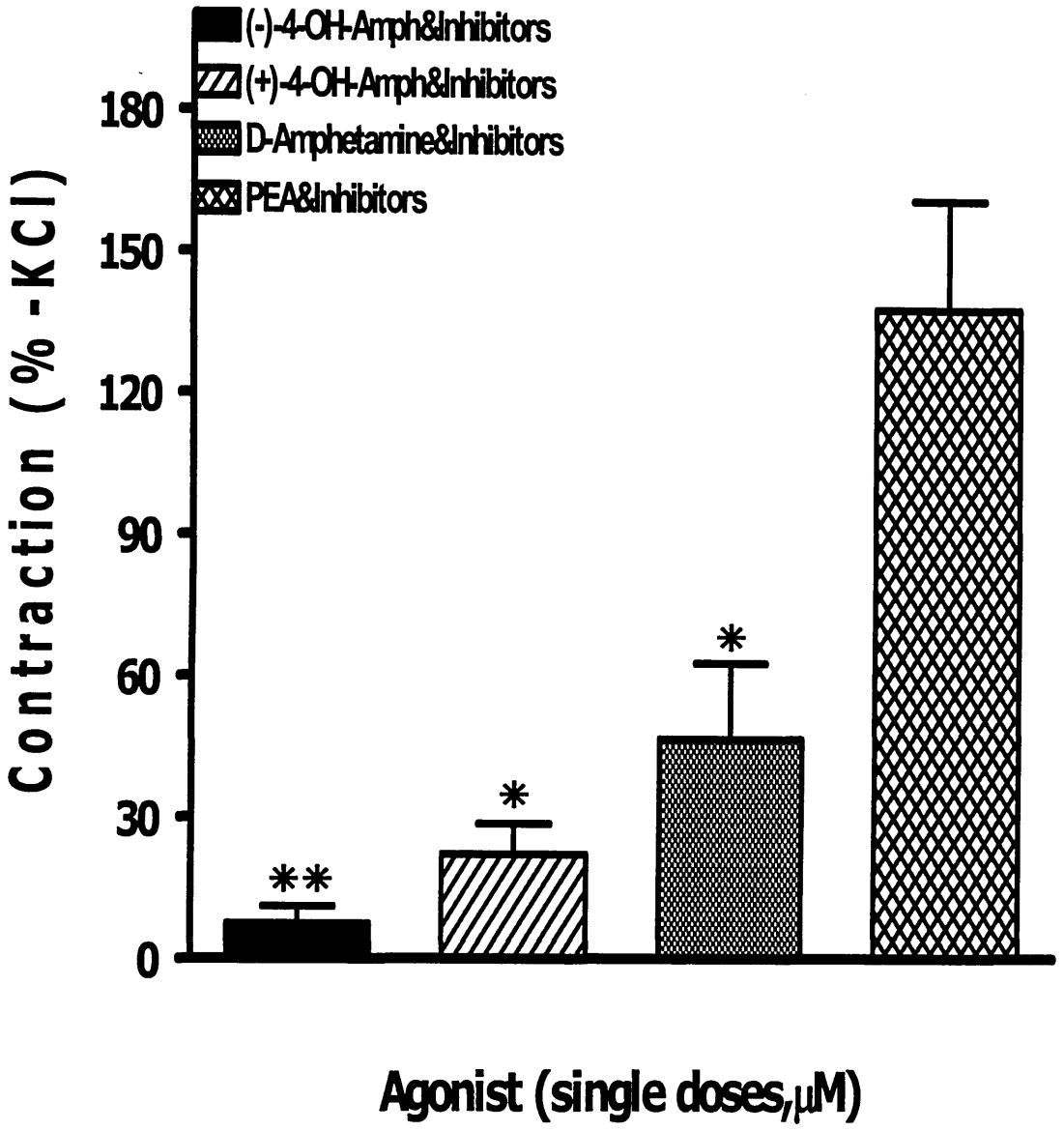


Figure 5.19: A comparison between (+)- and (-)-4-OH amphetamine (300μM), D-amphetamine (300μM) and β-PEA (300μM) in the presence of "inhibitors". Single dose experiments for (+)-4-OH (n=3) and (-)-4-OH amphetamine (n=3), for D-amphetamine (n=5), and for β-PEA in rat aortic rings were constructed in the presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. The contractile response to β-PEA (300μM) was significantly greater compared to (-)-4-OH amphetamine ((300μM, $P<0.01$,**), (-)-4-OH-amphetamine (300μM, $P<0.05$,*) and D-amphetamine (300μM, $P<0.05$,*).

5.5 Discussion

Very little is known about the relationship between vasoconstriction, TAs and smooth muscle in isolated tissues. Most information on TAs refers to studies which have been done on the brain (Philips *et al.*, 1978; Vaccari, 1986; Boulton *et al.*, 1990; Paterson *et al.*, 1990) or cloned receptors expressed in transfected cells (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). I am not aware of any studies where the effects of TAs on isolated aortic tissue have been investigated other than these from these laboratories. In previous *Chapters* the possible effect of α- and β-adrenoceptors and a possible ISA stimulation on the contractile response in rat aortic rings to β-PEA and tryptamine was investigated. The effects of other TAs on isolated aortic tissues and a comparison of their actions were examined in this *Chapter*. In addition, a comparison was made between the effects of several TAs at the aorta of rats and guinea-pigs. In previous *Chapters*, the contractile responses in isolated rat aortic rings to β-PEA and tryptamine were investigated.

5.5.1 Contractile response to β-PEA in rat and guinea-pig aorta - effect of "inhibitors"

In the presence of "inhibitors", aortic tissues from rat and guinea-pig aorta showed similar contractile responses to β-PEA. Therefore, the comparable remaining contractile responses to β-PEA in rat and guinea-pig aorta suggest that the mechanism of action in both tissues is *via* a similar mechanism. Moreover, based on earlier results *(Chapters 2 and 3)* a mechanism other than ISA and α- and β-adrenoceptor stimulation is responsible for the vasoconstriction in these two different aortic tissues.

5.5.2 Contractile response to tyramine in rat and guinea-pig aorta - effect of "inhibitors"

Tyramine has been characterised as a sympathomimetic amine (Barger and Walpole, 1909; Dale and Dixon, 1909; Barger and Dale, 1910). The sympathomimetic activity is mainly indirect and caused by a release of endogenous noradrenaline (Burn and Rand, 1958). The indirect action of tyramine has been confirmed by previous workers (Trendelenburg, 1961; Furchgott *et al.*, 1963; Trendelenburg *et al.*, 1963;

Varma *et al.*, 1964). Furthermore, a recent publication reported that tyramine caused vasoconstriction in endothelium-denuded thoracic aortic tissue (Khwanchuea *et al.*, 2008). However, since the discovery of a novel GPCR, called the TAAR, which is activated by TAs, such as tyramine and β-PEA *(Chapter 4)* (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001), in rats, the mechanism of action of tyramine has evoked new interest. In contrast to the results from previous study (Khwanchuea *et al.*, 2008) in the present study, tyramine in the absence of "inhibitors" has shown very inconsistent and variable contractile responses in endothelium-denuded aortic tissues. This inconsistent contractile response might be due to low tyramine sensitivity in rat aortic tissue, or may reflect the tissue distribution of TAARs. In the presence of "inhibitors" there was virtually no contractile response to tyramine in rat aortic tissues.

In *Chapters 2 and 3*, the best conditions to measure contractile responses to TAs in isolated aortic tissues were established and it was found, generally, that the responses to TAs improved in the presence of "inhibitors". In guinea-pig aorta, tyramine turned out to be an active agonist and its contractile response was not altered by "inhibitors". Furthermore, the contractile responses to tyramine were significantly different between the two species. These findings suggest that the small responses to tyramine in rat aortic rings were mainly mediated *via* α-adrenoceptors, or due to ISA stimulation. However, in the presence of "inhibitors", the vasoconstriction to tyramine in guinea-pig aorta is mediated *via* a mechanism other than ISA, or α- and β-adrenoceptor stimulation. Moreover, referring to previous publications regarding the TAARs (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001), it might be possible that tyramine in guinea-pig activates a TAAR which does not exist in rat aorta tissue.

5.5.3 Contractile response to octopamine in rat and guinea - pig aorta - effect of "inhibitors"

The effect of octopamine in invertebrates has been studied intensively (David and Coulon, 1985; Roeder, 1999). The mechanism of action, *via* a GPCR, called the octopamine receptor, is well understood (Axelrod and Saavedra, 1977; Roeder, 1999). However, since the recent discovery of TAARs (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001), it is clear that very little is known about the effect of octopamine in isolated aortic tissues of vertebrates.

In the present study the contractile responses to octopamine in rat and guinea-pig isolated aortic tissues were blocked by "inhibitors". Therefore, it looks like octopamine exerts vasoconstriction mainly *via* α-adrenoceptors stimulation. However, in the presence of "inhibitors" the remaining response could be a weak TAAR response which would fit with its activity at cloned TAARs (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001).

5.5.4 Contractile response to D-amphetamine in rat and guinea-pig aorta - effect of "inhibitors"

The TAs, β-PEA and tyramine, are structurally *(Tables 1.1 and 5.1)* and functionally related to the amphetamines (Sabelli and Mosnaim, 1974; Hauger *et al.*, 1982; Bunzow *et al.*, 2001). Recent reviews have investigated the pharmacological, amphetamine-like actions of TAs (Kim and von Zastrow, 2001; Branchek and Blackburn, 2003; Davenport, 2003). Given the close relationship between TAs and the amphetamines, the mechanism of action of D-amphetamine in isolated aortic tissues was investigated. Previous workers had reported a contractile response to D-amphetamine in rabbit aorta (Silvestrini *et al.*, 1991; Silvestrini *et al.*, 1994). In the present study, it was shown that D-amphetamine also causes contraction in rat and guinea-pig aorta in the absence and presence of "inhibitors". The remaining contractile response in the presence of "inhibitors" is not based on α- and β-adrenoceptor stimulation or as a result of an ISA effect. Therefore, D-amphetamine might activate the same TAAR in both tissues. The relationship between D-amphetamine and different amphetamine-derivatives *(Section 5.4.7)* on the contractile response in rat aortic rings will be discussed in *Sections 5.5.7 and 5.5.8.*

5.5.5 Contractile response to tryptamine in guinea-pig - effect of adrenergic inhibitors

Analogous to the experiments in rat aortic rings *(Chapter 4)* the contractile responses in guinea-pig aorta to tryptamine were examined. As with the responses in rat aortic rings, tryptamine induced vasoconstriction in guinea-pig aortic rings in the absence or presence of various inhibitors. Even though the shape of the CRCs to tryptamine was different in rat compared to guinea-pig aortic tissues, the remaining contractile response to tryptamine in both tissues in the presence of "inhibitors" is neither

caused through the stimulation of α- adrenoceptors, nor *via* ISA stimulation *(Chapter 4)*. The remaining contractile response in guinea-pig aortic rings to tryptamine was not altered in the presence of "inhibitors", ketanserin and methiothepin. Therefore, the remaining contractile response to tryptamine in guinea-pig aortic rings is also not evoked *via* 5-HT_1 or 5-HT_2 receptors. Adequacy of the concentration of methiothepin (50nM) and ketanserin (3nM) to block 5-HT_1- and 5-HT_2 receptors can be inferred from the experiments in rat aorta with 5-HT in *Chapter 4*. Previous workers reported that the TA tryptamine activates the recently discovered TAARs. Tryptamine is a weak agonist for TAAR1 and also a slightly more potent agonist for TAAR4 (Borowsky *et al.*, 2001; Lindemann *et al.*, 2005). Therefore the contraction to tryptamine in guinea-pig and rat aortic *(Chapter 4)* tissue might be due to an activation of TAARs.

5.5.6 Summary of contractile responses to different trace amines in rat and guinea-pig aorta - effect of "inhibitors"

The contractile responses to different TAs in rat *(section 5.4.5)* and guinea-pig *(section 5.4.6)* aortic tissues were examined. Overall, in both species β-PEA, octopamine and D-amphetamine proved to be potent agonists in the presence of "inhibitors" *(Table 5.4)* even in a slightly different potency order. However, tyramine in the presence of "inhibitors" behaved differently in rat and guinea-pig aortic tissue. Previous publications investigated the effect of TAs in cells transfected with human and rat cloned receptors (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). The mechanism of action for tyramine in rat and guinea-pig aortic tissues is unclear, so must be investigated in further studies for both species. Tryptamine also proved to be a potent agonist in both rat (-log EC_{50} 5.07±0.13, n=7) and guinea-pig (-log EC_{50} 5.17±0.55, n=3) aortic tissues in the presence of "inhibitors". The present study mainly focused on the investigation of the contractile response to β-PEA, D-amphetamine, octopamine and tyramine, therefore the possible effect of tryptamine on TAARs in aortic tissue was not investigated in further details.

Trace amines	RAT Mean -log EC_{50} values	GP Mean -log EC_{50} values
β-PEA	4.46±0.15	4.22±0.21
Octopamine	4.36±0.17	4.98±0.23
D-amphetamine	3.76±0.34	4.86±0.32
Tyramine	3.71±0.29	4.37±0.36

Table 5.4: Comparison of the potency (mean –log EC_{50} values) of TAs in rat and guinea-pig isolated aortic rings in the presence of "inhibitors"

Species	Vasoconstrictor activity (max. contraction)	Potency order (-log EC_{50} values)
Rat Aorta	β-PEA > D-amphetamine > octopamine > tyramine	β-PEA > octopamine > D-amphetamine > tyramine
Guinea-pig Aorta	β-PEA > tyramine > D-amphetamine > octopamine	Octopamine > D-amphetamine > tyramine > β-PEA

Table 5.5: Comparison of vasoconstriction activity (max. contraction) and potency orders (-log EC_{50} values) for TAs rat and guinea-pig isolated aortic rings in the presence of "inhibitors".

In summary, the investigated TAs all work *via* a similar mechanism therefore activating probably the same receptor. However, there must be more than one receptor present as tyramine is inactive in the rat aortic tissue and the vasoconstriction activity and potency orders to TAs in rat and in guinea-pig are not coincident *(Table 5.5)*.

5.5.7 Contractile response to different amphetamine-derivatives - effect of "inhibitors"

Ritalin® is a psychomotor stimulant with some sympathomimetic actions (Carlsson *et al.*, 1966; Brichard and Johnstone, 1970; Hendley *et al.*, 1972). Ritalin® is structurally related to amphetamine (Patrick and Markowitz, 1997). Previous studies showed that Ritalin® reduces pain by decreasing vasoconstriction as a result of blocking the α-adrenoceptors (Johnstone, 1974). Moreover, it was reported that Ritalin® eliminates tyramine activity and increases noradrenaline action in isolated rabbit aortic rings (Maxwell *et al.*, 1961). Among the side effects of Ritalin® are its cardiovascular effects. It increases heart rate and blood pressure (Ballard *et al.*, 1976; Brown *et al.*, 1984). Though these cardiovascular effects are considered clinically

insignificant at typical therapeutic doses (Findling *et al.,* 2001). However, a recent publication reported a significant increase of blood pressure with therapeutic doses of Ritalin® in children with ADHD (Stowe *et al.,* 2002). Furthermore, because of unintentional overdoses, medication errors and overdose caused by abuse or by suicide attempts, symptoms like hypertension and tachycardia were reported (Klein-Schwartz, 2002). It blocks the noradrenaline transporter and therefore increases the release of the neurotransmitter because of reduced reuptake after release (Kuczenski and Segal, 1997) *(Section 5.1).* It is suggested that the cardiovascular effects of Ritalin® are caused through noradrenaline stimulation, but the mechanism of action has not been fully investigated as previous work suggested that dopamine effects also may be involved (Volkow *et al.*, 2003).

In the present study, it was shown that Ritalin® caused contractions in isolated rat aortic tissues. The contractile response in rat aortic rings to Ritalin® was not altered in the presence of "inhibitors". Therefore, the remaining contractile response is not through stimulation of α-adrenoceptors with the possibility of some opposing vasodilatation *via* β-adrenoceptors, nor due to ISA stimulation. Furthermore, as Ritalin® is structurally related to amphetamine (Patrick and Markowitz, 1997), it is likely Ritalin® activates TAARs in rat aortic tissues with the same mechanism of action as D-amphetamine *(Chapter 5.5.4).* However, compared to D-amphetamine, Ritalin® is much less active as the maximum contractile effect in rat aortic rings was much smaller.

MDMA is a psychostimulant with structural similarities to amphetamine *(see Chapter 1 and Table 5.1).* and also has some pharmacological properties of the hallucinogenic phenethylamine mescaline (Lester *et al.*, 2000; Kalant, 2001). MDMA is reported to be widely abused resulting in fatalities, but MDMA has been much less studied than cocaine and classical amphetamines (Lavelle *et al.*, 1999; Al-Sahli *et al.*, 2001). MDMA is known to release monoamine transmitters in the CNS (Fitzgerald and Reid, 1990; Fitzgerald and Reid, 1993). However, there are also effects of MDMA in the peripheral nervous system (Fitzgerald and Reid, 1994). Given the major involvement of serotonergic nerves in the central actions of MDMA (Green *et al.,* 1995), possible effects of MDMA involving the noradrenergic system have been less investigated. In the peripheral autonomic nervous system, indirectly

acting sympathomimetic amines such as amphetamine, cause the release of noradrenaline (Burn and Rand, 1958; Trendelenburg *et al.*, 1962; Muscholl, 1966), inhibit the neuronal uptake carrier (Burgen and Iversen, 1965) and facilitate responses to neuronally released and exogenous noradrenaline (Furchgott *et al.*, 1963). The major metabolite of MDMA, methylenedioxyamphetamine (MDA) has sympathomimetic effects. MDA increases the heart rate, blood pressure and causes the release of [^{3}H] noradrenaline from rabbit isolated atria (Fitzgerald and Reid, 1994). In the past it was reported that MDMA has only relatively mild sympathomimetic effects (Shulgin, 1986). However, in 1994 the first evidence was provided that MDMA, like amphetamine, has sympathomimetic actions (Fitzgerald and Reid, 1994). MDMA induces vasoconstriction and facilitates vasoconstrictor responses to noradrenaline. MDMA acts to displace noradrenaline from adrenergic nerve terminals (Fitzgerald and Reid, 1994; Lavelle *et al.*, 1999). Therefore, MDMA might increase the level of noradrenaline (Paton, 1975) but due to another mechanism of action, such as displacement of noradrenaline rather than block of re-uptake (Al-Sahli *et al.*, 2001). These results might give a basis for understanding some of the cardiovascular complications associated with the use of MDMA. A few studies have evaluated the effects of MDMA on the cardiovascular system in either human or animal systems (Lester *et al.*, 2000; Al-Sahli *et al.*, 2001; McDaid and Docherty, 2001; Lai *et al.*, 2003; Baker *et al.*, 2007). It was reported that MDMA increases heart rate (Gordon *et al.*, 1991) and blood pressure in rats due to α_1 and α_2 –adrenoceptor stimulation (McDaid and Docherty, 2001). It is suggested that MDMA use in crowded conditions, high ambient temperature, and physical activity may be connected with a potential life-threatening increase in the cardiovascular toxicity of the drug (Mas *et al.*, 1999). One of the most life-threatening consequences of the abuse of MDMA is hyperthermia (Logan *et al.*, 1993) with maximum body temperature correlating with mortality (Gowing *et al.*, 2002). In the present study, the effect of MDMA was examined in rat aortic rings to determine whether MDMA causes vasoconstriction and whether this was an indirect sympathomimetic action. The contractile response to MDMA in isolated rat aortic tissues was not altered by "inhibitors". Therefore, MDMA-induced vasoconstriction in rat aortic tissues is *via* mechanisms other than indirect sympathomimetic activity or α- and β- adrenoceptor activation. The present results confirm earlier findings that MDMA caused

vasoconstriction in isolated guinea-pig aorta *via* a direct mechanism of action (Baker and Broadley, 2003). Moreover, MDMA is a synthetic amphetamine-derivatives with structural similarity to amphetamine (Mas *et al.*, 1999; Lester *et al.*, 2000; Kalant, 2001). Therefore it is likely that MDMA act with a similar mechanism of action like amphetamine, by activating the same TAARs in rat aortic tissues *(Section 5.5.4)*.

Cathinone is the major, active central nervous system stimulant constituent of khat and the increase in blood pressure following the chewing of khat leaves occur simultaneously with raised plasma levels of cathinone (Gugelmann *et al.*, 1985; Al-Motarreb and Broadley, 2003; Baker *et al.*, 2007). In the cardiovascular system, cathinone increases blood pressure and heart rate in anaesthetized rats (Kalix and Braenden, 1985) and dogs (Kohli and Goldberg, 1982) and has a positive inotropic and chronotropic action in isolated atria (Gugelmann *et al.*, 1985). Referring to a study in The Yemen, cathinone is a risk-factor for acute myocardial infarction and a suggested risk-factor for ischaemic heart disease (Al-Motarreb *et al.*, 2002b). *In vitro* studies have confirmed that cathinone causes vasoconstriction in guinea-pig aortic rings and coronary vasoconstriction in isolated Langendorff hearts. This vasoconstriction is unlike that of amphetamines as it does not appear to be due to an indirect action by release of noradrenaline from sympathetic nerve endings neither due to a direct action on α_1-adrenoceptors (Al-Motarreb and Broadley, 2003; Baker *et al.*, 2007).

In the present study, the contractile response to cathinone in the presence of "inhibitors" in rat aortic tissues was not inhibited. Therefore, the vasoconstriction in isolated rat aortic rings is caused *via* mechanisms other than indirect sympathomimetic activity or α- and β- adrenoceptor activation. The observation that cathinone is not behaving as an indirectly acting sympathomimetic amine in an isolated vasculature preparation contrasts with pharmacology at other sites where cathinone is reported to have indirect amphetamine-like actions by releasing noradrenaline (Kalix, 1983a; Kalix, 1983b). Nevertheless, the results confirm previous findings that cathinone caused vasoconstriction in isolated guinea-pig aorta *via* direct stimulation (Al-Motarreb *et al.*, 2000; Al-Motarreb *et al.*, 2002b; Al-Motarreb and Broadley, 2003; Baker and Broadley, 2003). Earlier studies in other laboratories had only shown that cathinone could enhance the electrically induced

constrictions of rabbit isolated ear and pulmonary arteries (Knoll, 1979) rather than vasoconstriction alone.

My study shows that there is also vasoconstriction by cathinone without any electrical pre-stimulation. Thus, the vasoconstrictor effects seen in the present study can explain the pressor effects after khat chewing. It remains to be established by what mechanism cathinone causes this vasoconstriction. Compared to the previous discussed amphetamine-derivatives, MDMA and Ritalin®, cathinone was the least potent agonist of all three with the lowest –log EC_{50} value. Therefore, it is likely that the mechanism of action of cathinone is similar to amphetamine, MDMA and Ritalin® by activating TAARs in isolated aortic rings but with lower potency *(Table 5.5)*.

Drug	Mean -log EC_{50} Values
Cathinone	3.23±0.05
MDMA	4.34±0.08
Ritalin®	4.53±0.27

Table 5.5: Comparison of the potency (mean –log EC_{50} values) of amphetamine-derivatives. Ritalin® is the most potent and cathinone the least potent agonist in between the amphetamine-derivatives in the presence of "inhibitors".

In summary, the contractile responses in the presence of "inhibitors" in rat aortic rings to MDMA, and cathinone were not significantly different. However, compared to the maximum contractile response to the TA β-PEA, all the amphetamine-derivates are less active TAs agonists. In isolated aortic rings, the order of vasoconstrictor activity was β-PEA > MDMA > Ritalin® > Cathinone *(Section 5.4.3)*. The mechanism of action cannot be identified within this study. However, it is likely that β-PEA and the amphetamine-derivatives all act *via* a direct stimulation of TAARs. It is possible that β-PEA and the amphetamine-derivatives activate different TAARs or the same receptor with different affinity. This could explain the different amount of vasoconstriction seen.

5.5.8 Contractile response to different amphetamine enantiomers - effect of "inhibitors"

A chemical and structural relationship exists between the catecholamine neurotransmitters, TAs (tyramine and β-PEA) and the synthetic amines, methamphetamine, amphetamine and their metabolite 4-OH-amphetamine (Reese *et al.*, 2007). Given the structural similarity of amphetamine to TAs, the amphetamine analogues methamphetamine, amphetamine and 4-OH-amphetamine (Bunzow *et al.*, 2001; Reese *et al.*, 2007) have been tested as agonists for the TAAR1 in transfected cell lines. It was found that amphetamine, methamphetamine and 4-OH-amphetamine are able to act as potent and efficacious agonist of TAAR1s heterologously expressed *in vitro*. Furthermore, these agonists display concentration-dependent and species-dependent stereospecific pharmacological profiles (Reese *et al.*, 2007). In addition, it was reported that 4-OH-amphetamine, the major amphetamine metabolite (Cho and Kumagai, 1994) was found to be the most potent (lowest EC_{50} value) TAAR1 agonist in transfected cells lines (Bunzow *et al.*, 2001). However, as far as I know the effects of TAs and related amphetamine-derivatives on isolated aortic tissue have not been investigated.

In the present study I have examined the effect of both stereoisomers (+) and (-) of methamphetamine and 4-OH-amphetamine in isolated rat aortic rings in the presence of "inhibitors". Stereoselectivity was not found to be a crucial factor for the contractile response. For methamphetamine, the (-) stereoisomer caused a significantly greater maximum contractile response compared to the (+) stereoisomer. In contrast the (+) stereoisomer of 4-OH-amphetamine caused the bigger, but not significantly different, contractile response compared to its (-) stereoisomer. Nevertheless, in the presence of "inhibitors" both stereoisomers of methamphetamine and 4-OH-amphetamine caused vasoconstriction in rat aortic tissue. Therefore, amphetamine-derivatives induced vasoconstriction in rat aortic tissues *via* mechanisms other than indirect sympathomimetic activity or α- and β-adrenoceptor activation. In the past it was shown that the physiological and behavioral responses of an animal to (+) isomers of both amphetamine and methamphetamine are more potent end efficacious than their optical antipodes at inducing motor hyperactivity (Angrist *et al.*, 1971; Segal, 1975). Although both isomers were full agonists at mouse and human TAAR1 the potencies of the (+)

isomers of methamphetamine and amphetamine were significantly greater than the potencies of the (-) isomers. In cells expressing rat TAAR1 both enantiomers of methamphetamine were approximately equipotent but only partial agonists. Regarding the isomers of 4-OH-amphetamine, the (+) isomer was the more potent of the two isomers (Reese *et al.*, 2007).

The fact that the activities of isomers in the present study were not very different suggests that the configuration of the molecule does not matter for receptor activation. This suggests that the methyl group is not a point of receptor attachment. It also suggests that the methyl group does not exert any steric inference with receptor binding. However, other studies reported that the stereoselectivity of the drug, such as thiopentone, has an important influence on the activation of the receptor, such as GABA receptor and the distribution in the CNS tissue (Cordato *et al.*, 1999). Primarily, D-amphetamine is the active isomer for CNS effects such as in for the treatment for attention-deficit/hyperactivity disorder (ADHD). D-Amphetamine was more potent than L-amphetamine at inhibiting accumulation of DA or NA in synaptosomes and vesicles (Easton et al., 2007).

Previous work (Bunzow *et al.*, 2001) reported a direct activation of TAAR by amphetamines in cloned receptor transfected cell lines. In the present study, even though vasoconstriction was examined in isolated aortic rings rather than in cloned TAAR1 expressed transfected cells, the direct stimulation of a receptor can be confirmed with the present study. However, the finding that 4-OH-amphetamine is the most potent TAAR1 agonist with the lowest EC_{50} value in transfected cell lines cannot be confirmed in the present study.

In isolated aortic rings, 4-OH-amphetamine proved to be the least active TA vasoconstrictor (maximum contractile response) amongst the group of amphetamine-derivatives. The contractile responses in rat aortic rings to (-) methamphetamine and D-amphetamine were comparable. However, in isolated rat aortic rings, all amphetamine-derivatives proved to be less active, compared to the contractile response to β-PEA. In summary, the investigated amphetamine enantiomers and all previous discussed TAs, including β-PEA, all work *via* a similar mechanism therefore, probably the same receptor.

However, there must be more than one receptor present as both amphetamine enantiomers are less active in the rat aortic tissue compared to β-PEA.

5.6 Conclusion

In the present *Chapter* it was established that the contractile responses to TAs in rat and guinea-pig aorta were similar except for tyramine. Therefore, referring to the variable contractile responses to TAs in aortic tissues, perhaps there are more than one TAAR present, or there are some species variations in the distribution of receptors. Furthermore, the similar contractile responses to amphetamine-derivatives and analogues all suggest the presence of TAARs in aortic tissues. The clear differences in contractile response to TAs in isolated aortic tissues suggest either more TAARs, or more than one receptor for which TAs have efferent affinities. Most information about TAs relates to studies which has been done on the brain (Philips *et al.*, 1978; Boulton *et al.*, 1990; Paterson *et al.*, 1990) or cloned TAAR1 expressed in transfected cells (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). My investigations of different TAs and structurally related derivatives in rat and guinea-pig aortic tissues have expanded the knowledge of the vasoconstriction effects of TAs in isolated tissues. The vasoconstrictor effect of all these TAs in aortic tissues might explain their hypertensive effects.

Chapter 6

TACHYPHYLAXIS AND CROSS-TACHYPHYLAXIS TO TRACE AMINES IN RAT ISOLATED AORTA USING CONCENTRATION-RESPONSE CURVES AND SINGLE DOSE EXPERIMENTS

Chapter 6

Effect of tachyphylaxis and cross tachyphylaxis to trace amines on concentration-responses curves and single dose experiments for a range of trace amines

6.1 Introduction

Tachyphylaxis is a characteristic feature of ISAs in that repeated dosage results in a gradual decline of the response (Day, 1967). This is probably due to progressive exhaustion of stored noradrenaline, which is replaced in the vesicle by less active TAs. ISAs show different degrees of tachyphylaxis and responses are generally not completely abolished. A previous publication reported that the pressor effects of β-PEA and tyramine displayed a slow and easily reversible tachyphylaxis on repeated dosages (Day and Rand, 1963). Also, there is a notable lack of cross-tachyphylaxis between certain TAs (Day, 1967). For example, the development of tachyphylaxis to the pressor effect of (+)-amphetamine is crossed to phenylephrine but not to tyramine. This effect could be explained by the conversion of tyramine to the more potent directly acting agonist, octopamine, by dopamine-β-hydroxylase (DBH) *(Chapter 1)* (Broadley, 1996). Tyramine is a precursor of noradrenaline (Chidsey *et al.,* 1964) and it has been suggested that it releases noradrenaline from a different storage site than other ISAs, such as dexamphetamine and phenylethylamine. However, it is possible that the two stores may be functionally connected (Zaimis, 1964). This could explain the persistence of tyramine pressor responses after other ISA tachyphylaxis and the lack of cross tachyphylaxis (Day, 1967).

Various hypotheses have been proposed to explain the lack of cross-tachyphylaxis between different ISAs. A previous publication suggested that tyramine might release noradrenaline from other storage sites than mephentermine (Fawaz and Simaan, 1965). In contrast, Bhagat et al., (1965) proposed that tyramine may accelerate noradrenaline synthesis possibly by acting as a noradrenaline precursor itself.

In previous *Chapters*, cumulative *(Chapter 2)* and non-cumulative CRCs *(Chapters*

3, 4, and 5) were discussed. In *Chapter 3*, the possible effect of desensitisation or tachyphylaxis of the TAs was discussed by comparing cumulative and non-cumulative CRCs. In this *Chapter*, repeated cumulative and non-cumulative CRCs and single doses experiments for the TAs, were used to determine whether tachyphylaxis or desensitisation was occurring and whether the dosing regimen makes a difference. These studies might throw some light on TA receptor mechanisms and explain the lack of tachyphylaxis observed for some ISAs.

6.2 Aims

To study the effect of TAs employing single dose experiments, cumulative and non-cumulative CRCs to investigate the contractile responses of rat isolated aortic rings to a range of agonists and inhibitors

a) To investigate further the effect of tachyphylaxis on the contractile response to β-PEA in rat aortic rings *via* repeated cumulative and non-cumulative CRCs and single dose experiments in the absence or presence of various inhibitors.

b) Comparison of the contractile responses in rat aortic tissues to β-PEA, D-amphetamine and octopamine in the presence of "inhibitors" *via* single dose experiments.

c) Determine whether β-PEA, D-amphetamine and octopamine activate the same receptor system in rat aortic rings.

d) Determine whether Ritalin®, D-amphetamine and tyramine react as partial agonists on the contractile response in rat aortic tissues and as antagonists of other TAs in the presence of "inhibitors"

6.3 Material and Methods

The main methods and experimental protocol described in *Chapter 2.3* were retained throughout the study unless otherwise stated.

6.3.1 Experimental protocol

After equilibration in the absence, or presence of different inhibitors, which were incubated with the tissue for 15 minutes before commencing the experiments, unless otherwise stated cumulative or non-cumulative CRCs for β-PEA were obtained. Further, single dose experiments to β-PEA, tyramine, D-amphetamine, octopamine and Ritalin® were performed. In all experiments, the endothelium was removed by inserting a cocktail stick and rolling the aortic ring round it.

Cumulative and non-cumulative CRCs

In the absence and presence of "inhibitors", repeated cumulative CRCs for β-PEA were obtained. After obtaining the first cumulative CRC, the tissue was left after washout (2 min, 2 min and 6 min) for approximately 45 minutes to equilibrate before the second cumulative CRC was constructed. After each CRC, KCl (60mM, isotonic solution) was added after washout of the final drug concentration.

Non-cumulative CRCs

In the absence and presence of "inhibitors" repeated non-cumulative CRCs for β-PEA were obtained. After obtaining the first non-cumulative CRC the tissue was left after washout (2 min, 2 min and 6 min) for approximately 45 minutes to equilibrate before the second non-cumulative CRC was performed. After each CRC, KCl (60mM, isotonic solution) was added after washout of the final drug concentration.

Single dose experiments

Four different kinds of single dose experiments were performed. At the end of the experiments, KCl (60mM, isotonic solution) was always added after washout of the final drug concentration.

Protocol 1: Tachyphylaxis

After equilibration *(see Chapter 2)* the tissue was incubated for 15 minutes with "inhibitors" before the first single dose of β-PEA (30μM or 100μM), D-amphetamine (100μM) or octopamine (100μM) was added to the bath. The tissue bath was washed out (2 min, 2 min, and 6 min) after the peak effect was reached. The tissue was left for approximately 20 minutes to equilibrate. Afterwards, the tissue was incubated for a further 15 minutes with "inhibitors" before the second single dose of the same TA was added. The tissue bath was washed out after the peak effect was reached. As a control experiment, single doses of β-PEA (30μM, 100μM) were repeated in the absence of "inhibitors".

Protocol 2: Cross-tachyphylaxis

Single doses of β-PEA (100μM), D-amphetamine (100μM) and octopamine (100μM) were tested against each other. After equilibration, the tissue was incubated for 15 minutes with "inhibitors" before the first single dose of any TA was added to the bath. The tissue bath was always washed out (2 min, 2 min, and 6 min) after the peak effect of the first single dose of any TA was reached. The tissue was left for approximately 20 minutes to equilibrate before it was incubated with "inhibitors" for a further 15 minutes and the second single dose of one of the other TAs was added to the tissue bath.

Protocol 3: Multiple dosing

For this set of experiments the incubation time with "inhibitors" (15 minutes), the wash out period (2 min, 2 min, and 6 min) and the equilibration time for approximately 20 minutes were kept as above. However, a single dose of either β-PEA (100μM) or octopamine (100μM) was added three times to the bath, instead of two single doses. Moreover, the second single dose of β-PEA (100μM) was left in the tissue bath for 60 minutes before washout. In the control experiment, the second single dose of β-PEA (100μM) was left out. However, the tissue was still incubated with "inhibitors" during the 60 minutes.

Protocol 4: Antagonism by a partial agonist

The first single dose of the first TA, either β-PEA (100μM), octopamine (100μM) or D-amphetamine (100μM) was added to the tissue bath after the tissue was incubated with "inhibitors" for 15 minutes. The tissue bath was washed out (2 min, 2 min, and 6 min) after the peak effect was reached and the tissue was then left to equilibrate for approximately 20 minutes. The "inhibitors" were then returned to the bath and approximately 5 minutes later a single dose of a second TA, either Ritalin® (1mM) or tyramine (1mM) was added to the bath and left for approximately 25 minutes. The second single dose of the first TA was then added to the bath **without washout**. In the control experiment the protocol was identical except that the single prolonged exposure to Ritalin® (1mM) or tyramine (1mM) was left out *(Figure 6.1)*.

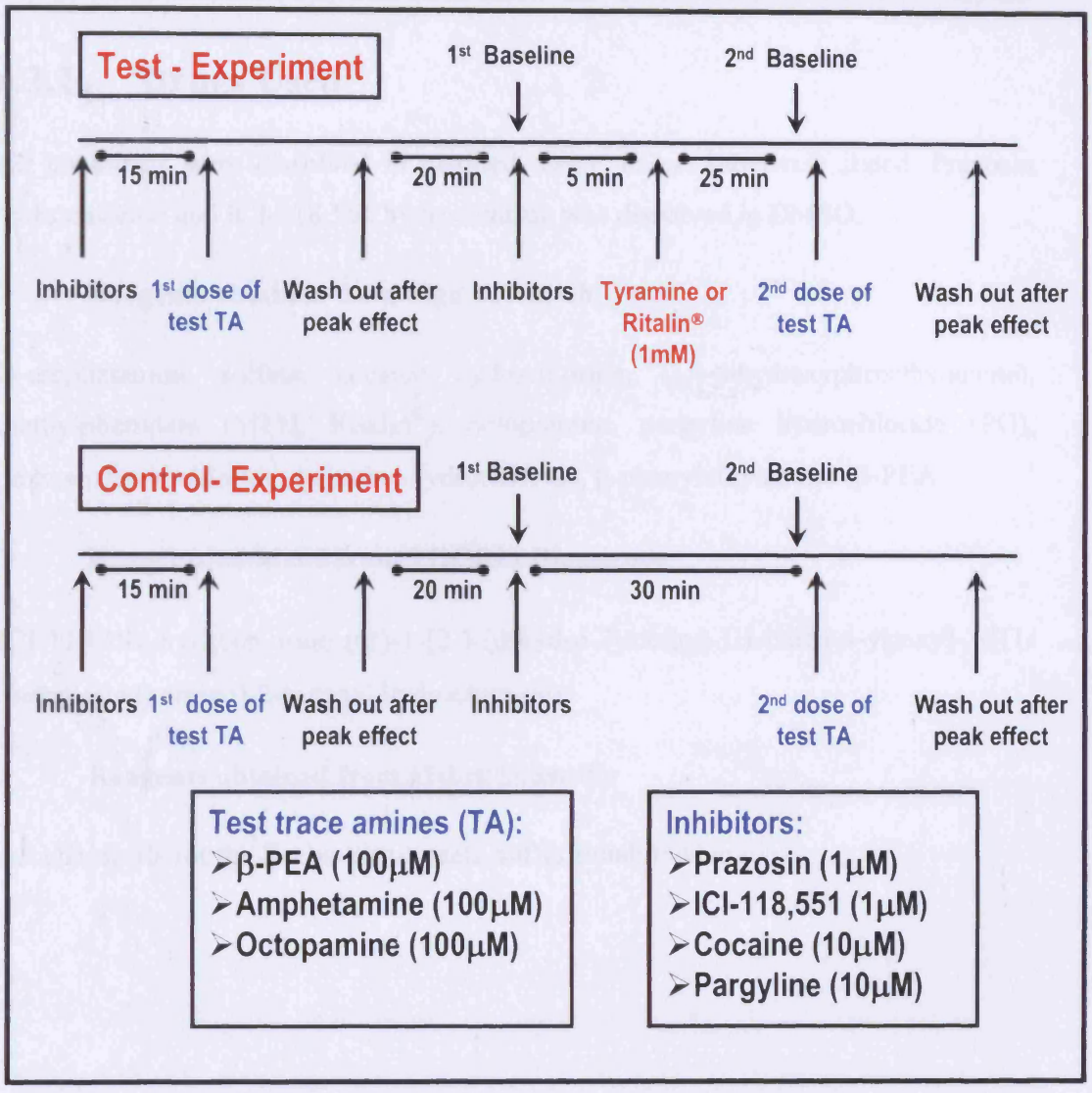


Figure 6.1: Experimental protocol for single dose studies to examine the effect of tyramine (1mM) or Ritalin® (1mM) on the contractile response to other TAs, β-PEA (100μM) D-amphetamine (100μM) and octopamine (100μM) in the presence of "inhibitors". Test- and control experiments were constructed. In the control experiment the protocol was identical to the test experiment except that the single prolonged exposure to tyramine (1mM) or Ritalin® (1mM) was left out *(Protocol 4)*.

6.3.2 Drugs Used

All chemicals were dissolved in distilled water, unless otherwise stated. Prazosin hydrochloride and ICI-118,551 hydrochloride was dissolved in DMSO.

- **Reagents obtained from Sigma Aldrich**

D-amphetamine sulfate, cocaine hydrochloride, (3,4-dihydroxyphenethylamine), methylphenidate (MPH, Ritalin®), octopamine, pargyline hydrochloride (PG), prazosin hydrochloride, tyramine hydrochloride, β-phenylethylamine (β-PEA)

- **Reagents obtained from TOCRIS Bioscience**

ICI-118,551 hydrochloride ((±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride)

- **Reagents obtained from Fisher Scientific**

All chemicals for the Krebs-bicarbonate buffer (analytical grade)

6.4 Results

6.4.1 Development of tachyphylaxis or cross-tachyphylaxis by repeated administration of trace amines (TAs)

6.4.1.1 Repeated cumulative and non-cumulative CRCs to β-PEA in the absence or presence of "inhibitors"

The contractile response in rat aortic rings to β-PEA was examined in repeated cumulative and non-cumulative CRCs.

The second contractile response to β-PEA in cumulative CRCs at the maximum concentration (3mM, 186.1±86.1%, n=3) was not significantly different (P>0.05) from the first contractile response to β-PEA in the same tissue (3mM, 169.4±65.3%, n=3) *(Figure 6.2)*. These experiments were then repeated in the presence of "inhibitors". At the maximum concentration (3mM) the contractile response of the second cumulative CRC to β-PEA (3mM, 183.3±16.7%, n=3) was not significantly different (P>0.05) compared to the first CRC (3mM, 240±130.1%, n=3) in the same aortic tissue *(Figure 6.3)*. Furthermore, in the presence of "inhibitors" the $-\log EC_{50}$ values of the first (3.22±0.10, n=3) and second (2.96±0.09, n=3) cumulative CRCs were not significantly different (P>0.05). In addition to cumulative CRCs, the possible effect of tachyphylaxis in rat aortic rings to β-PEA in repeated non-cumulative CRCs was examined. The contractile response to β-PEA of the first CRC (300μM, 61.1±4.84%, n=3), was abolished when repeated for a second CRC *(Figure 6.4)*.

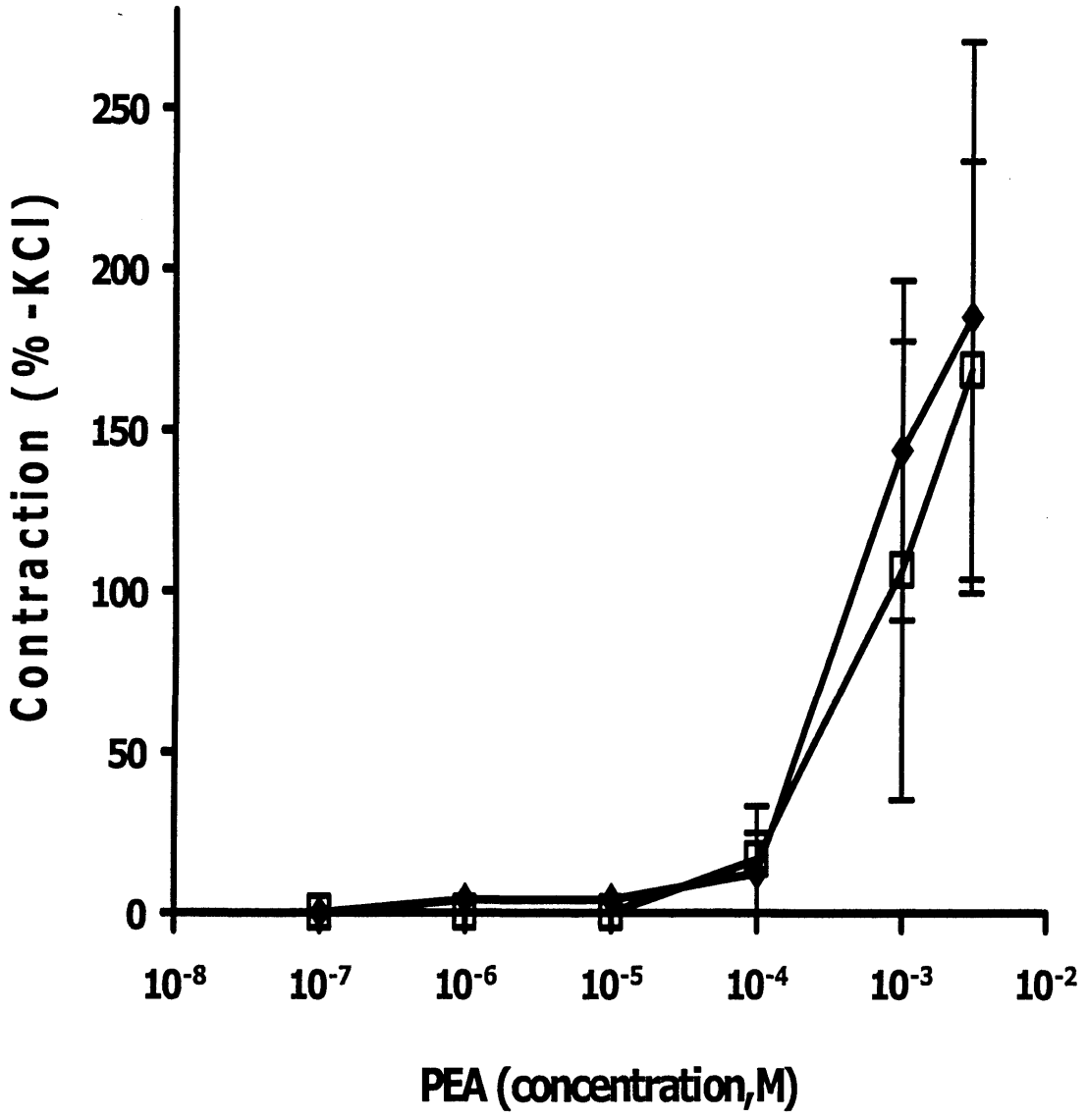


Figure 6.2: Effect of repeated cumulative CRCs to β-PEA on contractile responses generated in the absence of "inhibitors". Mean cumulative CRCs for the contractile response in rat aortic rings to β-PEA. Two cumulative CRCs for β-PEA in the same aortic tissue were constructed. The first CRC (□, n=3) was followed by the second CRC (◆, n=4) after approximately 45 minutes equilibration time. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen at their maximum contractile responses.

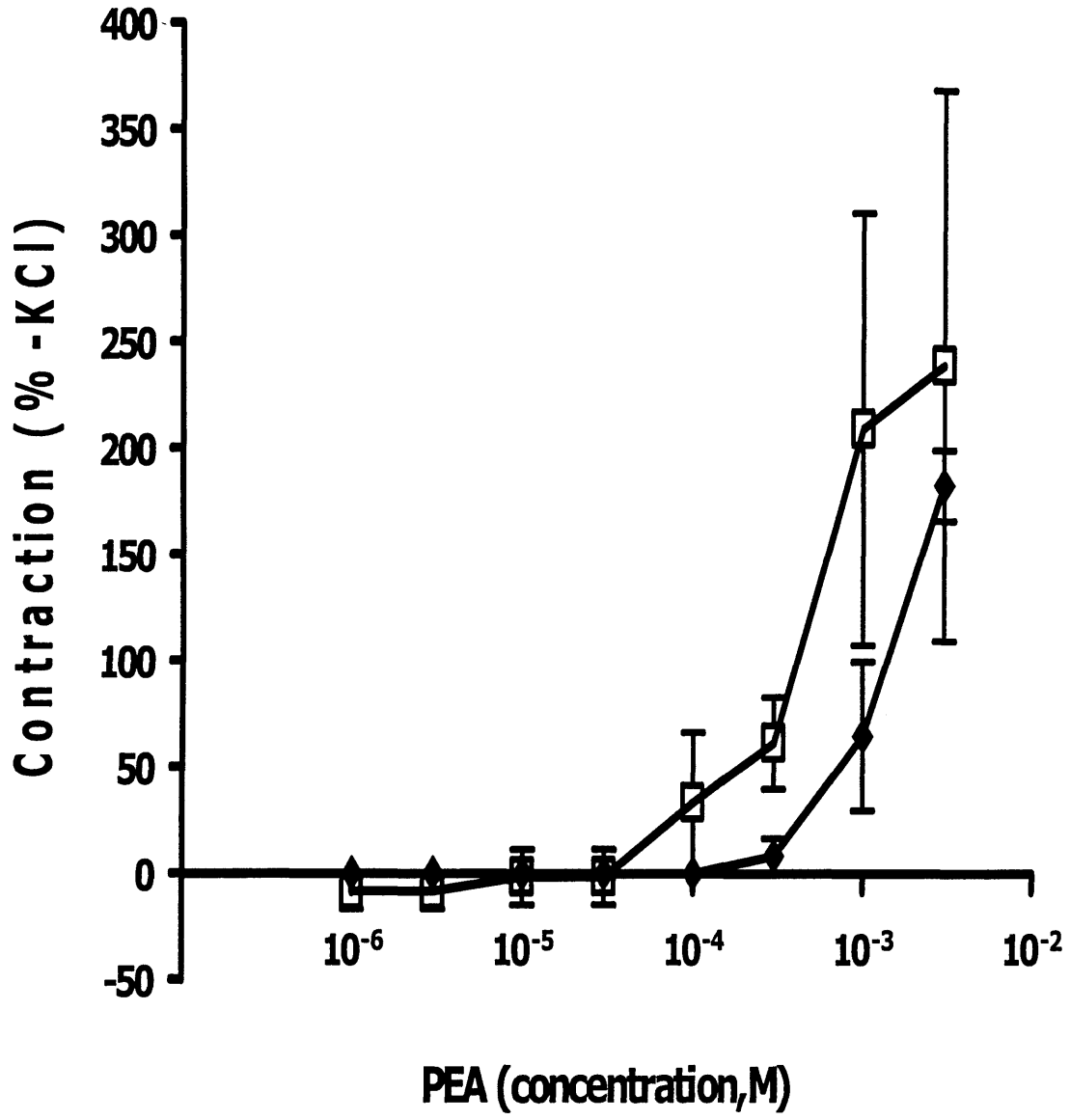


Figure 6.3: Effect of repeated cumulative CRCs to β-PEA on contractile responses generated in the presence of "inhibitors". Mean cumulative CRCs for the contractile response in rat aortic rings to β-PEA. Two cumulative CRCs for β-PEA in the same aortic tissue were constructed in the presence of "inhibitors". The first CRC (□, n=3) was followed by the second CRC (◆, n=3) after approximately 45 minutes equilibration time. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-test. No significant differences ($P>0.05$) were seen at their maximum contractile responses.

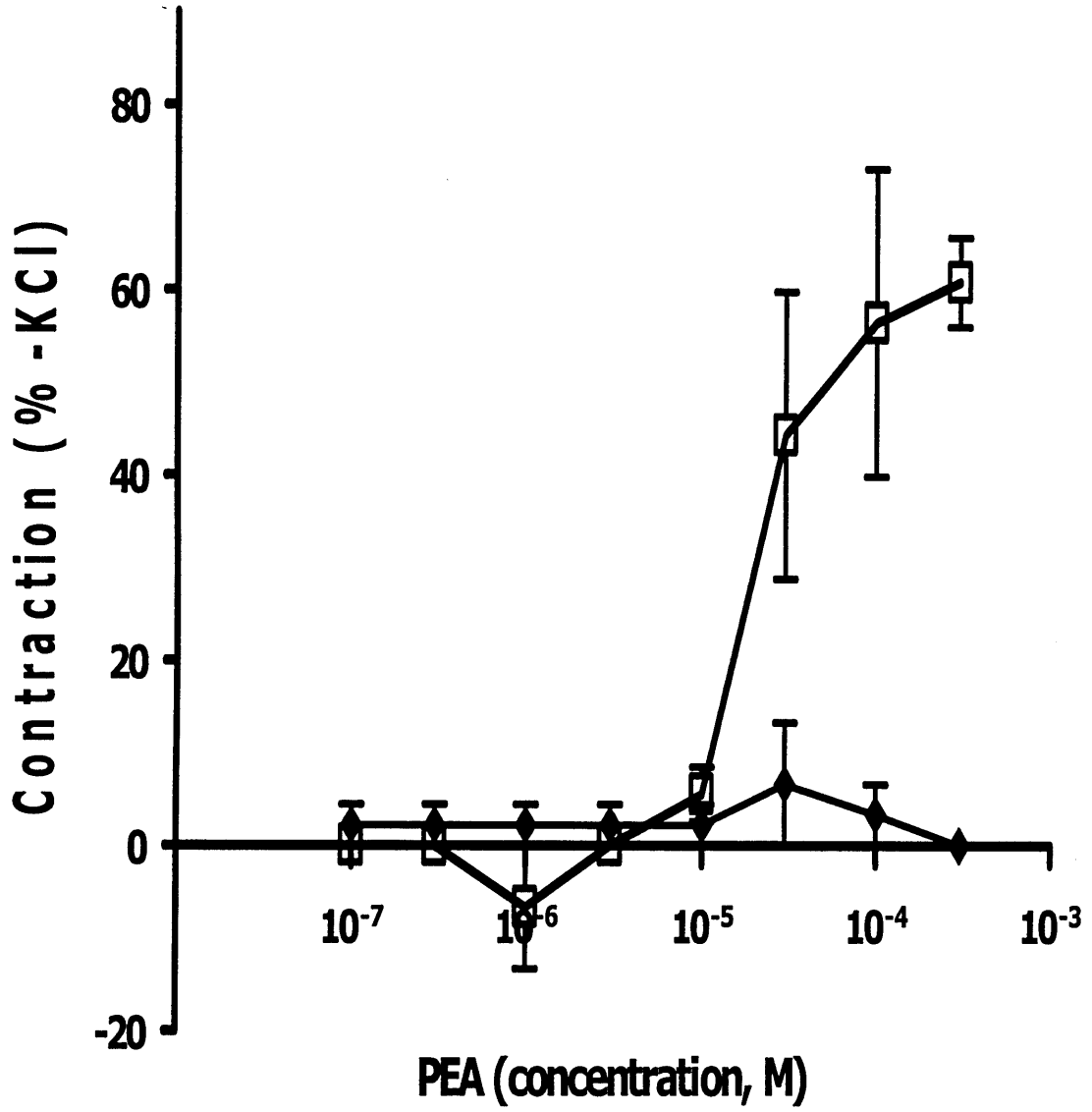


Figure 6.4: Effect of repeated non-cumulative CRCs to β-PEA on contractile responses generated in the absence of "inhibitors". Mean non-cumulative CRCs for the contractile response in rat aortic rings to β-PEA. Two non-cumulative CRCs to β-PEA in the same aortic tissue were constructed in the absence of "inhibitors". The first CRC (□, n=3) was followed by the second CRC (◆, n=3) after approximately 45 minutes equilibration time. The contractile response to β-PEA of the first CRC was abolished when repeated for a second CRC. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution).

6.4.1.2 Tachyphylaxis β-PEA in the absence and presence of "inhibitors" (Protocol 1)

The possible effect of tachyphylaxis in rat aortic rings in repeated exposures to β-PEA (30μM and 100μM) in the absence, or presence of "inhibitors" was examined. In the absence of "inhibitors", the contractile response of the second single dose of β-PEA (100μM, 145.8±20.8%, n=4) was significantly less ($P<0.05$) compared to the first single dose (100μM, 233.3±11.8%, n=4) *(Figure 6.5)*. However, the contractions for the first (100μM, 140±25.7%, n=5) and second (100μM, 89.3±16.6%, n=5) single doses of β-PEA in the presence of "inhibitors" were not significantly different ($P>0.05$). Furthermore, no significant difference ($P>0.05$) was shown between the lower first (30μM, 44±22.5%, n=3) and second (30μM, 16.2±25.8%, n=3) dose of β-PEA in the absence of "inhibitors" (30μM) *(Figure 6.5)*.

6.4.1.3 Cross-Tachyphylaxis between pairs of different TAs (Protocol 2)

The possible effect of cross-tachyphylaxis in rat aortic rings was investigated between pairs of different TAs, including β-PEA (100μM), D-amphetamine (100μM) and octopamine (100μM).

➢ **Effect of cross-tachyphylaxis between D-amphetamine and β-PEA**

In *tissue 1* in the presence of "inhibitors" a single dose of D-amphetamine (100μM) was added to the bath followed by a single dose of β-PEA (100μM). The contractile responses to D-amphetamine (100μM, 93.3±17.2%, n=5) and β-PEA (100μM, 137.3±30.5%, n=5) were not significantly different ($P>0.05$) *(Figure 6.6)*. In *tissue 2* in the presence of "inhibitors a single dose of β-PEA (100μM) was added to the bath followed by a single dose of D-amphetamine (100μM). Furthermore, neither the contractile responses to D-amphetamine in *tissue 1* (100μM, 93.3±17.2%, n=5) and *tissue 2* (100μM, 91.5±21.2%, n=5) nor the contractile response to β-PEA in *tissue 1* (100μM, 137.3±30.5%, n=5) and *tissue 2* (100μM, 152.3±14.6%, n=5) were significantly different ($P>0.05$) *(Figure 6.6)*.

➢ **Effect of cross-tachyphylaxis between D-amphetamine and octopamine**

In *tissue 1* in the presence of "inhibitors" a single dose of D-amphetamine (100μM) was added to the bath followed by a single dose of octopamine (100μM). The contractile responses to D-amphetamine (100μM, 54.2±20.8%, n=4) and octopamine (100μM, 91.7±31.6%, n=4) were not significantly different (P>0.05) *(Figure 6.7)*. In *tissue 2* in the presence of "inhibitors a single dose of octopamine (100μM) was added to the bath followed by a single dose of D-amphetamine (100μM). The contractile responses between octopamine (100μM, 100±50%, n=3) and D-amphetamine (100μM, 83.3±33.3%, n=3) were not significantly different (P>0.05) *(Figure 6.7)*. Furthermore, neither the contractile responses to D-amphetamine in *tissue 1* (100μM, 54.2±20.8%, n=4) and *tissue 2* (100μM, 83.3±33.3%, n=3) nor the contractile response to octopamine in *tissue 1* (100μM, 91.7±31.6%, n=4) and *tissue 2* (100μM, 100±50%, n=3) were significantly different (P>0.05) *(Figure 6.7)*.

➢ **Effect of cross-tachyphylaxis between D-amphetamine and octopamine**

D-amphetamine and octopamine cause equal effects. In *tissue 1* in the presence of "inhibitors" a single dose of octopamine (100μM) was added to the bath followed by a single dose of β-PEA (100μM). The contractile responses to octopamine (100μM, 133.3±16.7%, n=3) and β-PEA (100μM, 222.2±40.1%, n=3) were not significantly different (P>0.05) *(Figure 6.8)*. In *tissue 2* in the presence of "inhibitors a single dose of β-PEA (100μM) was added to the bath followed by a single dose of octopamine (100μM). The contractile responses between β-PEA (100μM, 75.0±6.5%, n=5) and octopamine (100μM, 55.0±13.8%, n=5) were not significantly different (P>0.05) *(Figure 6.8)*. Furthermore, the contractile responses to octopamine in *tissue 1* (first exposure, 100μM, 133.3±16.7%, n=3) and *tissue 2* (second exposure, 100μM, 35±13.8%, n=5) might be not significant (P>0.05) but quite a large difference was shown. However, the contractile responses to β-PEA in *tissue 1* (second exposure, 100μM, 222.2±40.1%, n=3) was significant larger (P<0.01) in *tissue 2* (first exposure, 100μM, 75.0±6.5%, n=5) *(Figure 6.8)*.

6.4.1.4 Tachyphylaxis of β-PEA, D-amphetamine and octopamine in the presence of "inhibitors" (Protocol 1)

The possible effect of tachyphylaxis in rat aortic rings was investigated between pairs of the same TA, either D-amphetamine (100μM), octopamine (100μM) or β-PEA (100μM).

No significant difference (P>0.05) was shown between the first (100μM, 120.1±31.5%, n=4) and second (100μM, 112.5±42.7%, n=4) single dose of D-amphetamine (100μM), the first (100μM, 140±25.7%, n=5) and second (100μM, 89.3±16.6%, n=5) single dose of β-PEA (100μM) (*see Figure 6.5*) or between the first (100μM, 77.8±27.8%, n=5) and second (100μM, 91.7±8.3%, n=5) single dose of octopamine (100μM) *(Figure 6.9)*.

6.4.1.5 Multiple dosing (Protocol 3)

In the following experiments, in the presence of "inhibitors" the relationship between the contractile responses and the incubation times in rat aortic rings to TAs were investigated. Also, the contractile responses of TAs were compared in relation to the order the agonists were added to the tissue bath.

A single dose of β-PEA (100μM) in the presence of "inhibitors", was added to the bath three times *(Figure 6.10)*. The maximum contractile responses for the first (100μM, 86.7±8.2%, n=4) and third dose (100μM, 92.9±12.7%, n=4) were both significantly different (P<0.05) to the second dose of β-PEA (100μM, 163.8±28.5%, n=4) *(Figure 6.10)*. The first and the third single doses were not significantly different (P>0.05) from each other. In the control experiment which received only two doses but separated by a 1hour incubation, the maximum contractile responses of the first (100μM, 89.6±34.9%, n=4) and second single doses (100μM, 145.8±20.8%, n=4) were not significantly different (P>0.05) *(Figure 6.10)*.

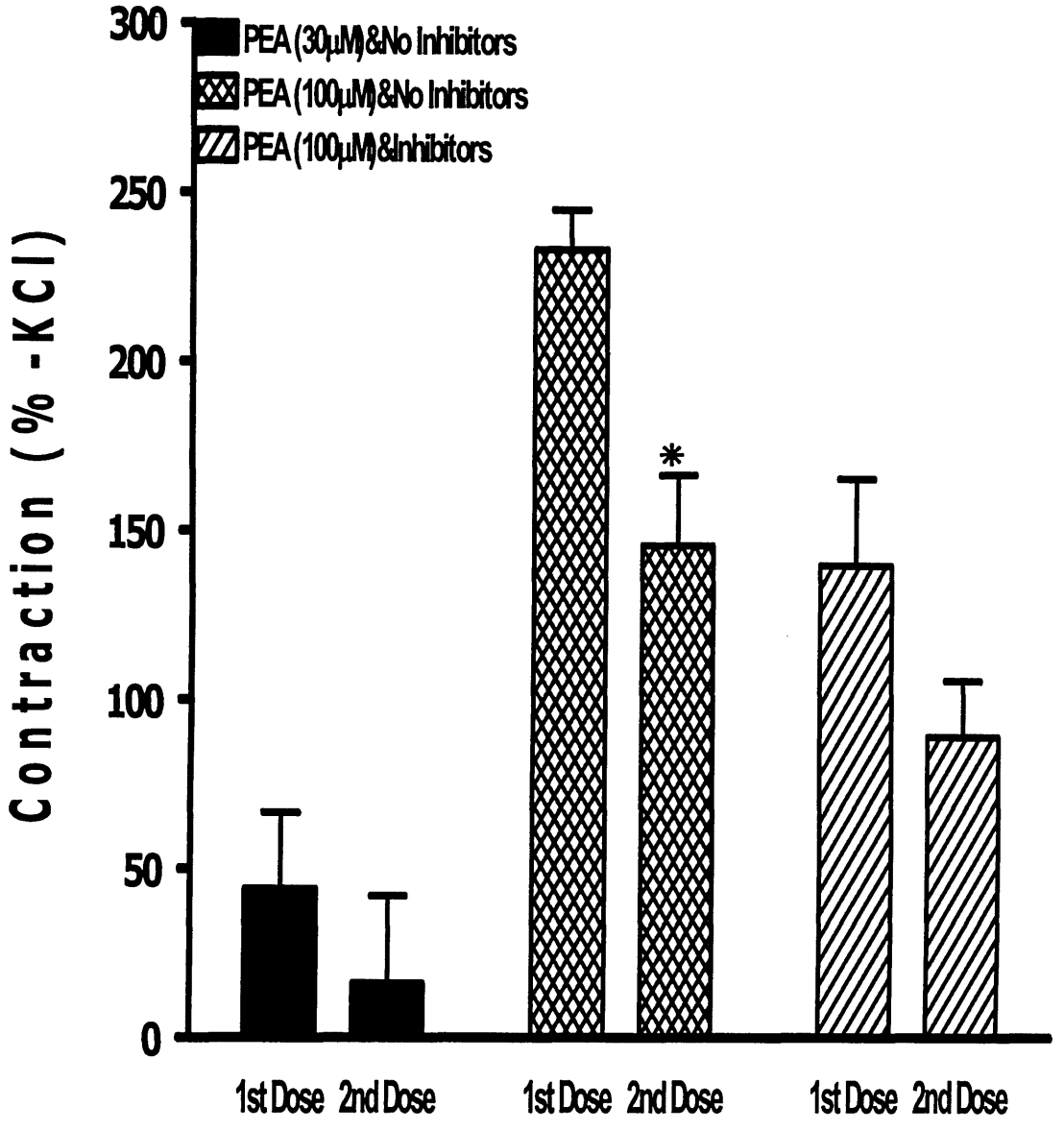


Figure 6.5: Single dose experiments for the contractile response in rat aortic rings to β-PEA (30μM and 100μM) were constructed in the absence, or presence of "inhibitors". Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-tests. A significant difference ($P<0.05$,*) was shown between the first (n=4) and second dose (n=4) of β-PEA (100μM) in the absence of "inhibitors".

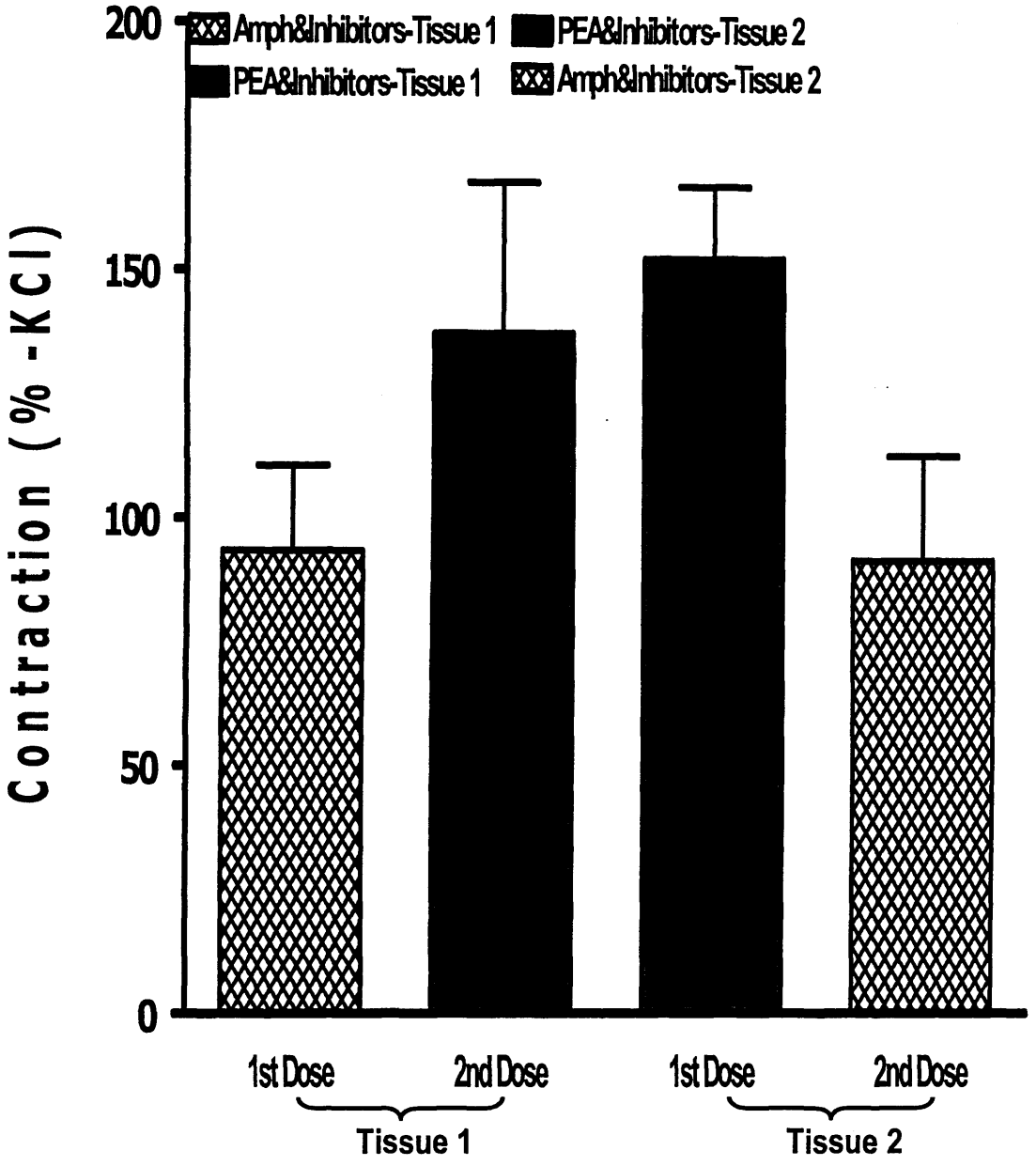


Figure 6.6: Single dose experiments for the contractile response in rat aortic rings to D-amphetamine (100μM) and β-PEA (100μM) were constructed in paired experiments *(tissue 1 and tissue 2)* in the presence of "inhibitors". The 1st and 2nd doses each of D-amphetamine (100μM, n=5) and β-PEA (100μM, n=5) were left in the bath until the maximum contractile response was reached. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences were seen ($P>0.05$).

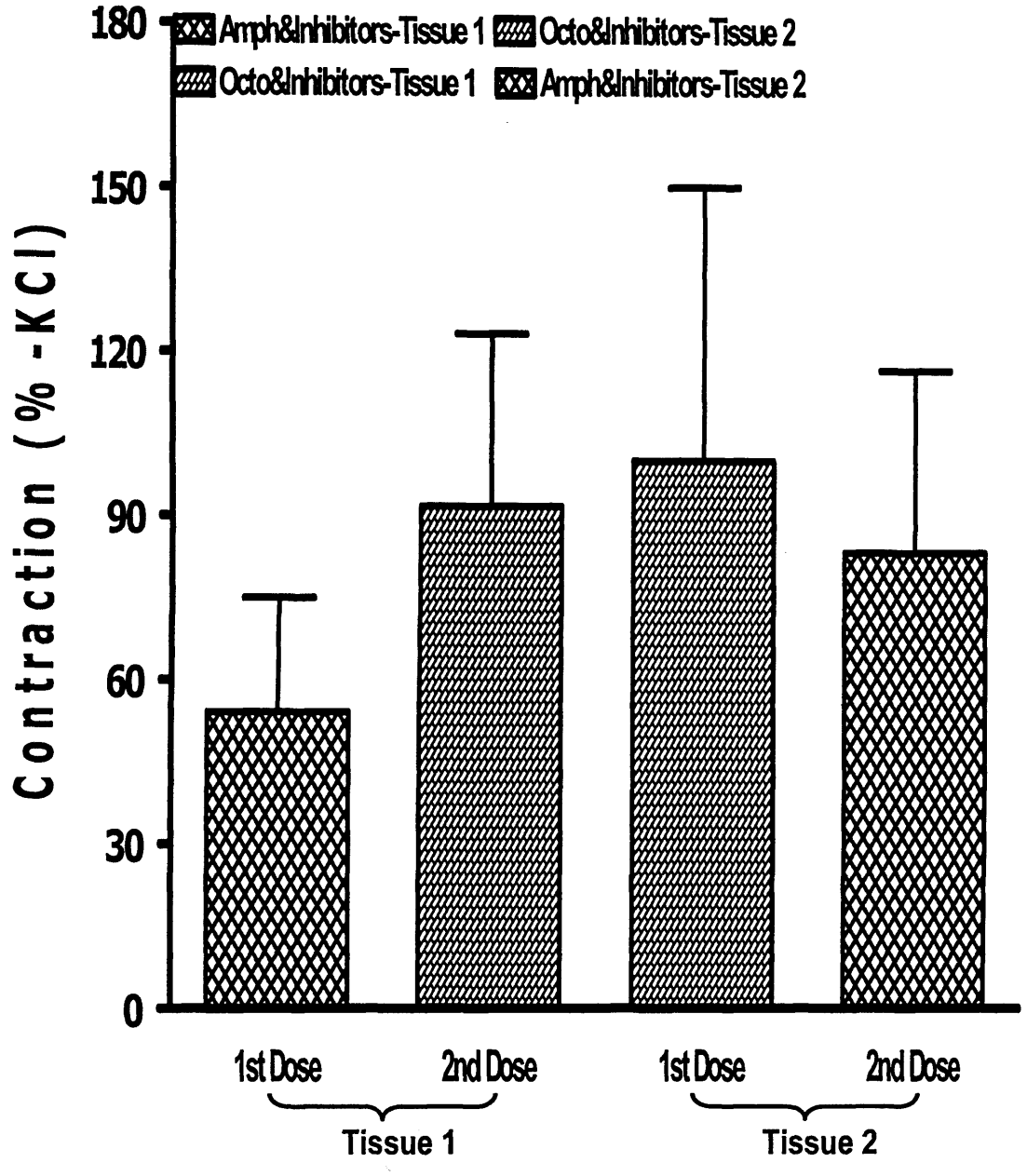


Figure 6.7: Single dose experiments for the contractile response in rat aortic rings to D-amphetamine (100μM) and octopamine (100μM) were constructed in paired experiments *(tissue 1 and tissue 2)* in the presence of "inhibitors". The 1st (n=3) and 2nd doses (n=4) each of octopamine (100μM, n=3) and the 1st (n=4) and 2nd doses (n=3) of D-amphetamine (100μM) were left in the bath until the maximum contractile response was reached. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. No significant differences were seen (P>0.05).

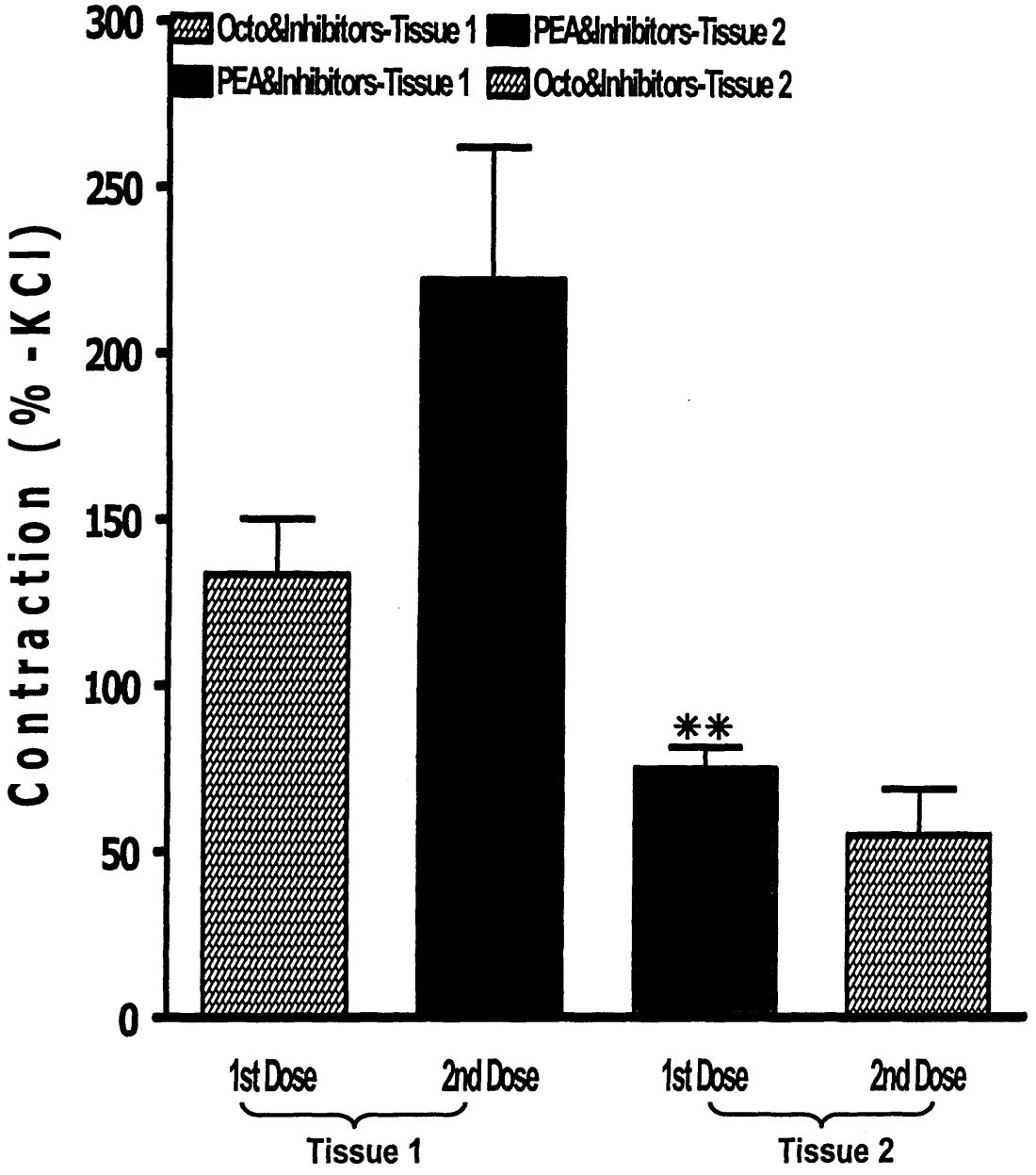


Figure 6.8: Single dose experiments for the contractile response in rat aortic rings to β-PEA (100μM) and octopamine (100μM) were constructed in paired experiments *(tissue 1 and tissue 2)* in the presence of "inhibitors". The 1st (n=3) and 2nd (n=5) doses of octopamine (100μM) and the 1st (n=5) and 2nd (n=3) doses of β-PEA (100μM) were left in the bath until the maximum contractile response was reached. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test. The contractile responses to β-PEA in *tissue 1* and *tissue 2* were significantly different ($P<0.01$, **).

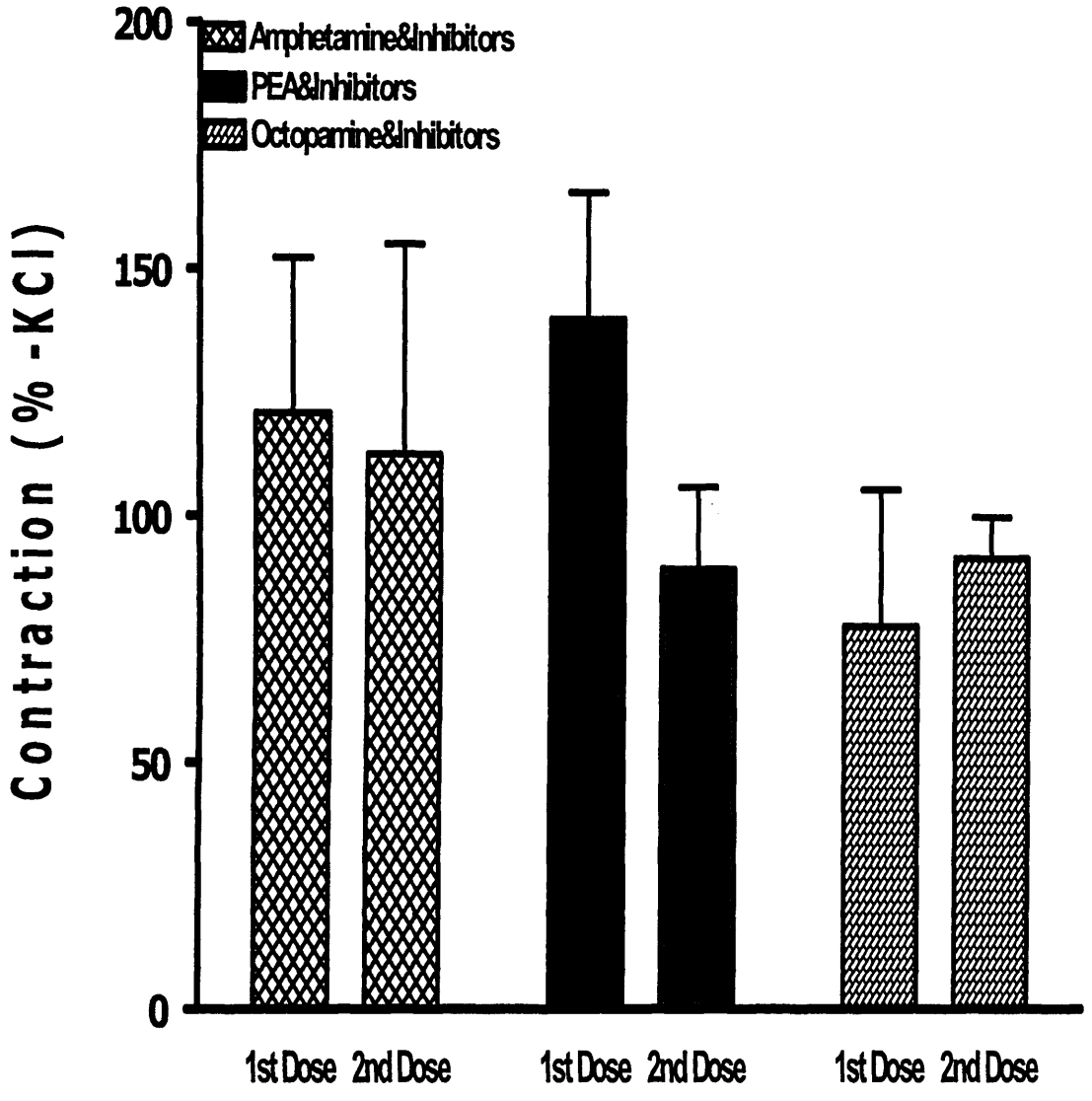


Figure 6.9: Single dose experiments for the contractile response in rat aortic rings to β-PEA (100μM) and octopamine (100μM) and D-amphetamine (100μM) were constructed in paired experiments *(1st and 2nd dose)* in the presence of "inhibitors". The 1st and 2nd doses each of octopamine (100μM, n=3), D-amphetamine (100μM, n=4) or β-PEA (100μM, n=5) (β-PEA data was taken from *Figure 6.5*) were left in the bath until the maximum contractile response was reached. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by Student's paired t-tests. No significant differences were seen (P>0.05).

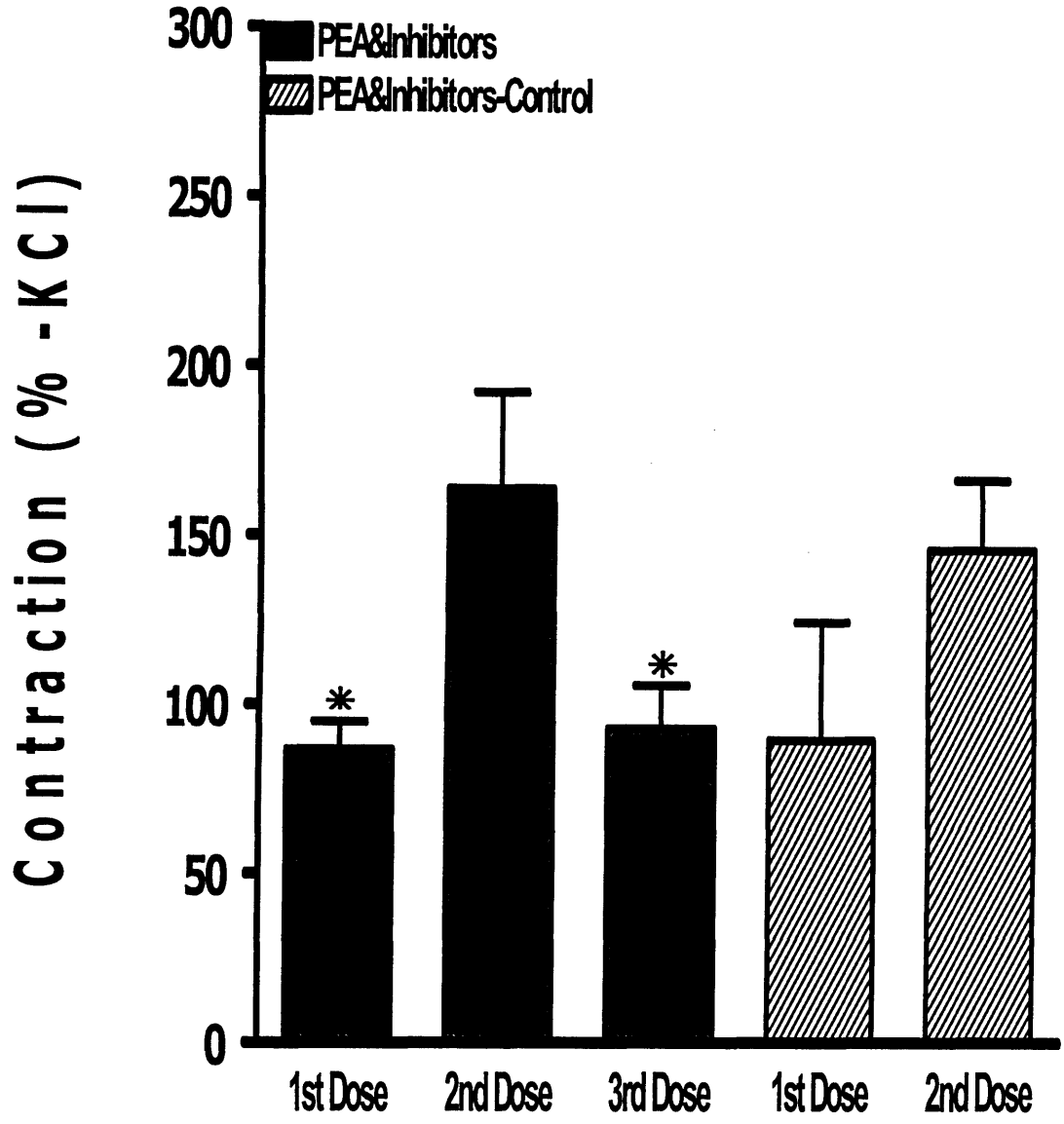


Figure 6.10: Single dose experiments for the contractile response in rat aortic rings to β-PEA (100μM) were constructed in the presence of "inhibitors". The first and third doses of β-PEA (100μM, n=5) was left in the bath until the maximum response was reached. The second dose of β-PEA (100μM, n=5) was kept in the bath for one hour. In the control experiment (n=4) the second dose of β-PEA was left out, but the tissue was still incubated with "inhibitors" during this time. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Dunnett test. The first and the third doses of β-PEA (100μM) were significantly different ($P<0.05$,*) compared to the second dose of β-PEA (100μM).

6.4.2 Effect of partial agonism of trace amines (Protocol 4)

The contractile responses in rat aortic rings to single doses of β-PEA (100μM), D-amphetamine (100μM) and octopamine (100μM) were examined in the presence of "inhibitors" and also in the presence of a second TA, either Ritalin® (1mM) or tyramine (1mM). These were selected because of their weak partial agonist activities *(Chapter 5)*.

The contractile responses of TAs were measured as a percentage of KCl (isotonic solution, 60mM) The responses to TAs were measured approximately 30 minutes after the tissue was incubated with "inhibitors" plus tyramine (1mM) or Ritalin® (1mM) *(Figures 6.1, 6.11)*. There are two different ways of interpreting the contractile response for the TA added in the presence of tyramine (1mM) or Ritalin® (1mM). Either from the first or second baseline (1st and 2nd baseline) *(Figures 6.1 and 6.11)*.

- **First Baseline**

Taken just before the single dose of the second TA (tyramine or Ritalin®) was added to the bath *(see arrow in Figures 6.1 and 6.11)*

- **Second Baseline**

Taken just before the second dose of the first TA was added to the bath *(see arrow in Figures 6.1 and 6.11)*

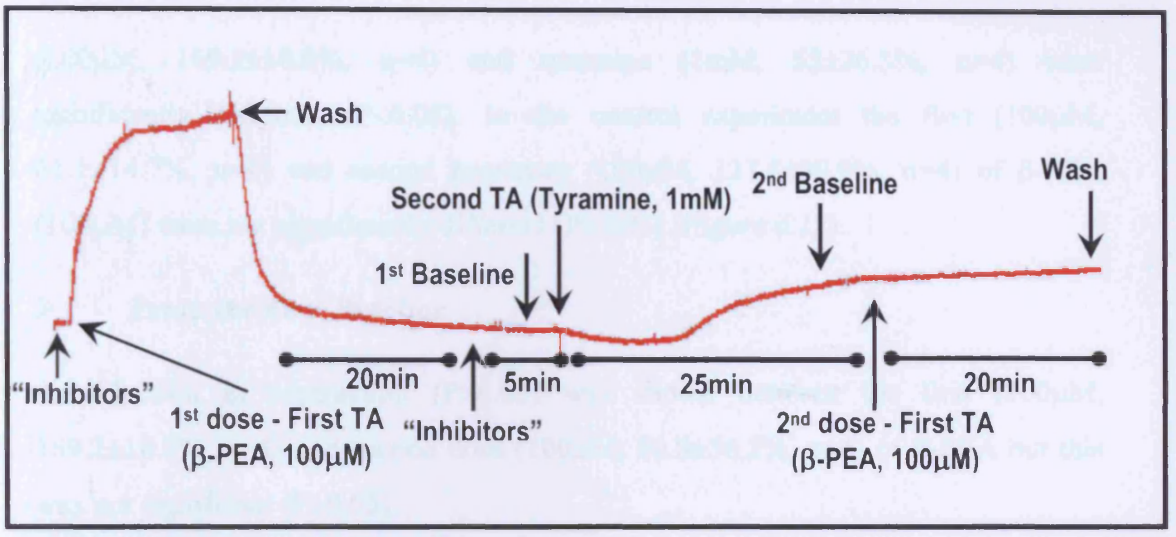


Figure 6.11: Chart recording showing a representative single dose experiment for the contractile response in rat aorta to β-PEA (100μM) and tyramine (1mM) in the presence of "inhibitors". To construct the single dose experiment, two single doses of β-PEA (100μM) were added to the bath, the 1st dose only in the presence of "inhibitors", the 2nd dose in the presence of "inhibitors" and tyramine (1mM), the second trace amine (TA). The 1st single dose of β-PEA (100μM) was added to the tissue bath after the tissue was incubated with "inhibitors" for 15 minutes. The tissue bath was washed out (2 min, 2 min, and 6 min) after the peak effect was reached. The tissue was then left to equilibrate for approximately 20 minutes. The "inhibitors" were then returned to the bath and approximately 5 minutes later, a single dose of tyramine (1mM), was added and left for approximately 25 minutes. The second single dose of the first TA (β-PEA, 100μM) was then added to the bath. The tissue bath was washed out after the peak effect was reached. In the control experiment the protocol was identical to the test experiment except that the single prolonged exposure of tyramine (1mM) was left out. At the end of each experiment, KCl (60mM, isotonic solution) was routinely added after washout of the final drug concentration (not shown). "Wash" = washout of 50ml tissue bath *(see Figure 6.1)*.

In the following set of experiments the effect of tyramine (1mM) was investigated on the contractile responses to β-PEA (100μM), D-amphetamine (100μM) and octopamine (100μM) in the presence of "inhibitors".

Effect of tyramine (1mM) on the contractile response to β-PEA

- **From the second baseline**

A significant difference ($P<0.01$) in the contractile response to β-PEA was shown between the first (100μM, 169.2±10.8%, n=4) and the second dose (100μM, 25.8±10.5%, n=4) *(Figure 6.12)*. Moreover, the response to the first dose of β-PEA

(100μM, 169.2±10.8%, n=4) and tyramine (1mM, 55±26.3%, n=4) were significantly different (P<0.05). In the control experiment the first (100μM, 61.1±14.7%, n=4) and second responses (100μM, 127.8±30.9%, n=4) of β-PEA (100μM) were not significantly different (P>0.05) *(Figure 6.12)*.

- **From the First Baseline**

A reduction in contraction (P>0.05) was shown between the first (100μM, 169.2±10.8%, n=4) and second dose (100μM, 80.0±36.2%, n=4) of β-PEA but this was not significant (P>0.05).

Effect of tyramine (1mM) on the contractile response to D-amphetamine

- **From the second baseline**

A significant reduction in contraction (P<0.01) was shown between the first (100μM, 99.3±9.5%, n=4) and the second dose (100μM, -32±16.9%, n=4) of D-amphetamine (100μM). However, the contractile response to tyramine (1mM, 43.9±26.2%, n=4) was not significantly different (P>0.05) compared to the first dose of D-amphetamine (100μM, 99.3±9.5%, n=4) *(Figure 6.13)*. In the control experiment the first (100μM, 109.9±24.4%, n=4) and second doses (100μM, 112.4±26.5%, n=4) of D-amphetamine in the presence of "inhibitors" were not significantly different (P>0.05) *(Figure 6.13)*.

- **From the first baseline**

A significant reduction in contraction (P<0.05) was shown between the first (100μM, 99.3±9.5%, n=4) and second dose (100μM, 11.9±13.9%, n=4) of D-amphetamine.

Effect of tyramine (1mM) on the contractile response to octopamine

➢ **From the second baseline**

A significant reduction (P<0.001) in contraction was seen between the first (100μM, 148.7±26.8%, n=5) and the second dose of octopamine (100μM, -20±20%, n=5) *(Figure 6.14)*. Furthermore, the single dose of tyramine (1mM, 45.3±23.7, n=5) was significantly less (P<0.05) than the first dose of octopamine (100μM, 148.7±26.8%, n=5) *(Figure 6.14)*. In the control experiment, the contractile response to the first (100μM, 96.4±14.1%, n=4) and second single doses (100μM, 55.9±7.9%, n=4) of octopamine were not significantly different (P>0.05) *(Figure 6.14)*.

➢ **From the first baseline**

The contractile response to the first dose of octopamine (100μM, 148.7±26.8%, n=5) was significantly greater (P<0.01) compared to the second dose of octopamine (100μM, 25.3±8.9%, n=5) and to tyramine (1mM, 45.3±23.7%, n=5) (P<0.05).

Effect of Ritalin® (1mM) on the contractile response to β-PEA

➢ **From the second baseline**

In the presence of "inhibitors" and Ritalin® (1mM) the contractile response of the second dose of β-PEA (100μM, 31.1±9.2%, n=5) was significantly lower (P<0.05) compared to the response of the first dose (100μM, 107.9±23.4%, n=5) in the presence of "inhibitors" *(Figure 6.15)*. In the control experiment a significant increase (P<0.05) in the contractile response was shown between the first (100μM, 74±10.8%, n=5) and the second single dose of β-PEA (100μM, 170.1±25.5%, n=5) in the presence of "inhibitors" *(Figure 6.15)*.

➢ **From the first baseline**

The contractile response to the first (100μM, 107.9±23.4%, n=5) and second (100μM, 94.6±14.9%, n=5) dose of β-PEA (100μM) were not significantly different (P>0.05).

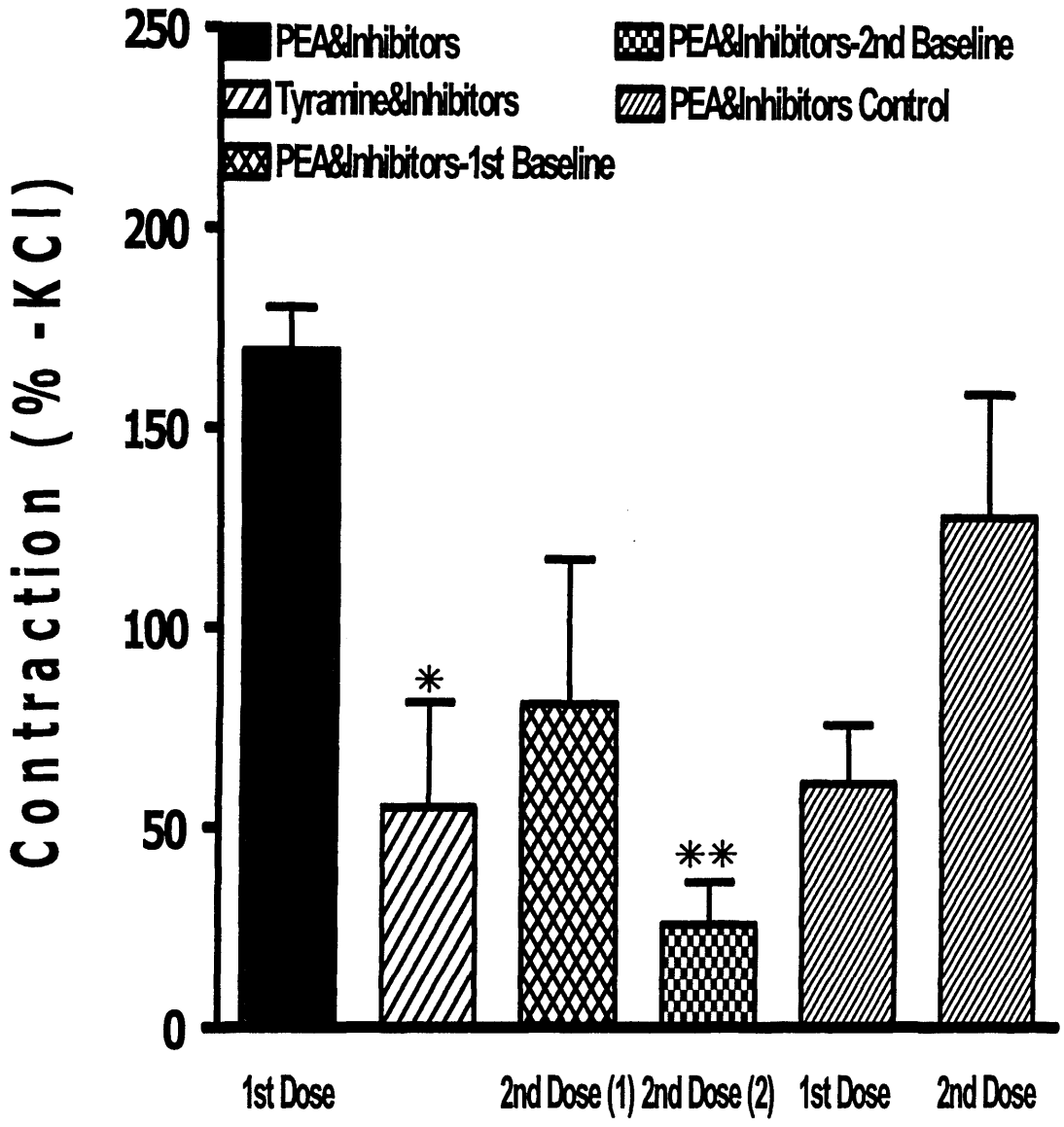


Figure 6.12: Single dose experiments for the contractile response in rat aortic rings to β-PEA (100μM, n=4) and tyramine (1mM, n=4) were constructed in the presence of "inhibitors". The second single dose of β-PEA (100μM) was in the presence of "inhibitors" and tyramine (1mM). To calculate the contractile response to β-PEA (100μM) (2^{nd} dose) two different baselines were used. The 1^{st} baseline was taken just before the tyramine was added, the 2^{nd} baseline was taken just before the 2^{nd} dose of β-PEA (100μM) was added to the bath *(see Figure 6.11)*. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test and Student's t-test (control experiment). A significant difference was shown between the 1^{st} and 2^{nd} dose (2^{nd} Baseline) of β-PEA (100μM, $P<0.01$,**) (2^{nd} Baseline) and between the first dose of β-PEA (100μM) and tyramine (1mM, $P<0.05$,*).

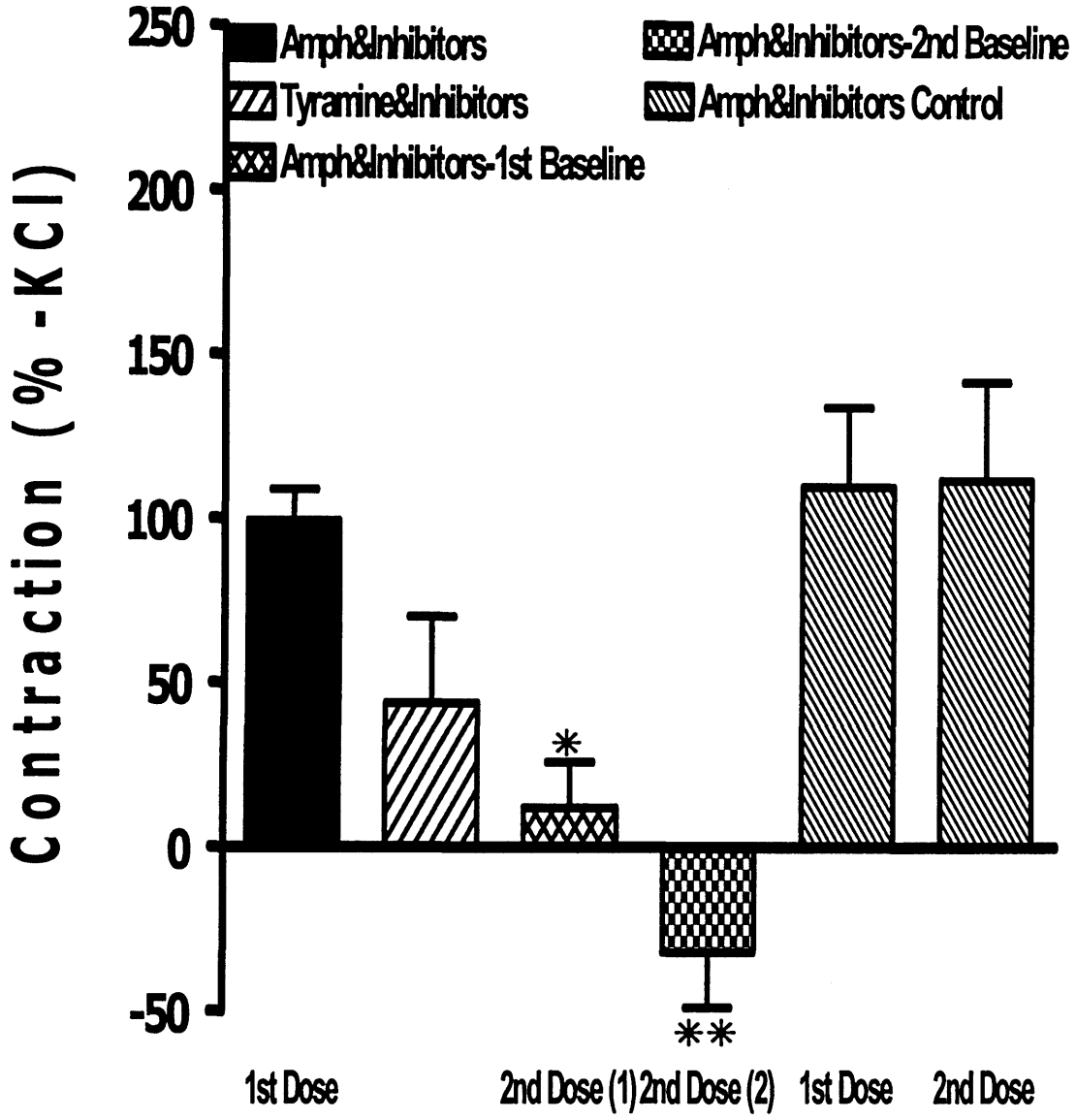


Figure 6.13: Single dose experiments for the contractile response in rat aortic rings to D-amphetamine (100μM, n=4) and tyramine (1mM, n=4) were constructed in the presence of "inhibitors". The second single dose of D-amphetamine (100μM) was in the presence of "inhibitors" and tyramine (1mM). To calculate the contractile response to D-amphetamine (100μM) (2^{nd} dose) two different baselines were used. The 1^{st} baseline was taken just before the tyramine was added, the 2^{nd} baseline was taken just before the 2^{nd} dose of D-amphetamine (100μM) was added to the bath *(see Figure 6.11)*. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test and by Student's paired t-test (control experiment). A significant difference was shown between the first and second dose of D-amphetamine (100μM, $P<0.05$,*) (1^{st} Baseline) and between the first and second dose of D-amphetamine (100μM, $P<0.01$,**) (2^{nd} Baseline).

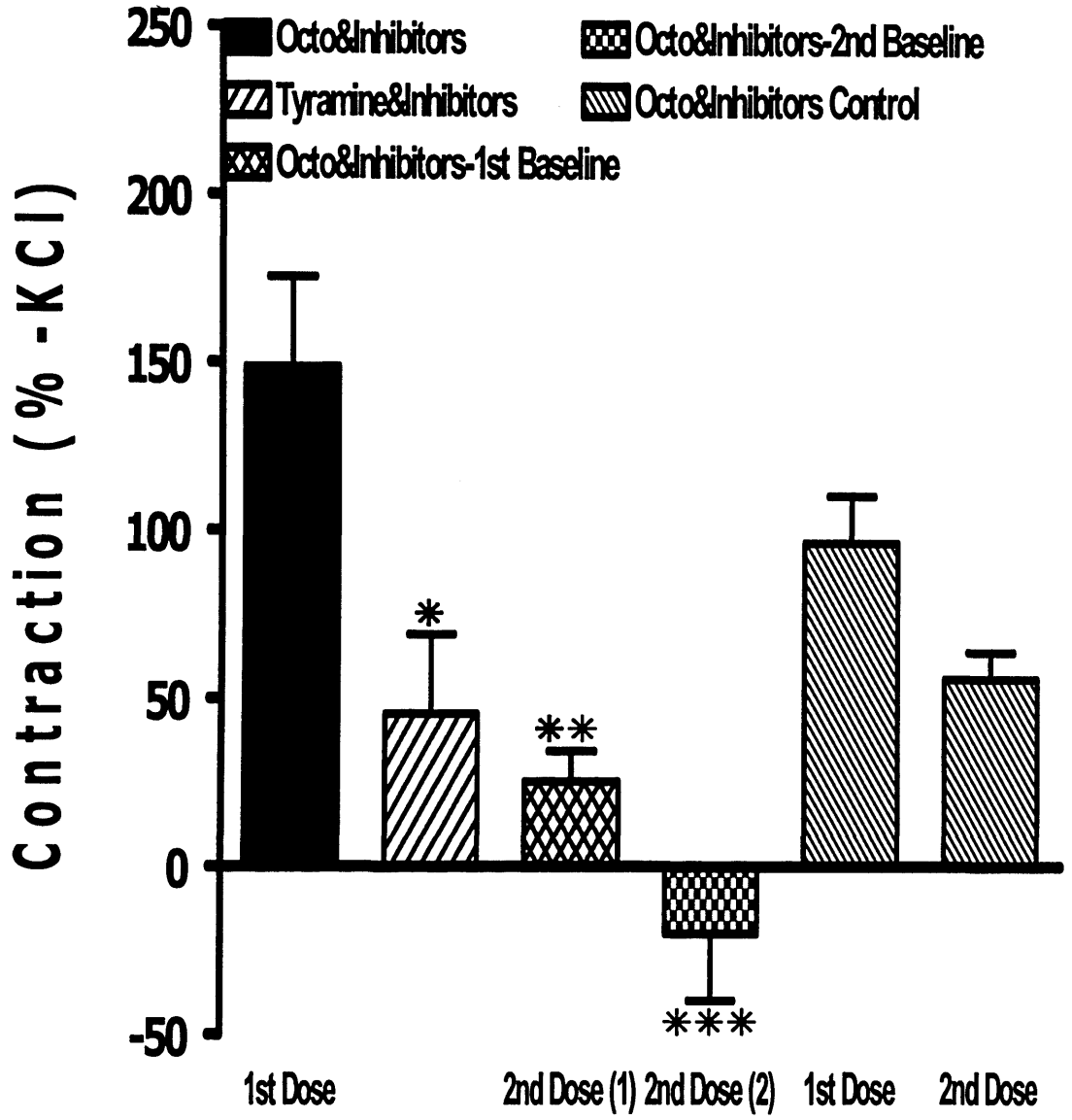


Figure 6.14: Single dose experiments for the contractile response in rat aortic rings to octopamine (100μM, n=5) and tyramine (1mM, n=5) were constructed in the presence of "inhibitors". The second single dose of octopamine (100μM) was in the presence of "inhibitors" and tyramine (1mM). To calculate the contractile response to octopamine (100μM) (2nd dose) two different baselines were used. The 1st baseline was taken just before the tyramine was added, the 2nd baseline was taken just before the 2nd dose of octopamine (100μM) was added to the bath *(see Figure 6.11)*. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test and by Student's paired t-test (control experiment). A significant difference was shown between the first and second dose of octopamine (100μM, $P<0.01$,**) (1st Baseline), between the first and second dose of octopamine (100μM, $P<0.001$,***) (2nd Baseline) and between the first dose of octopamine (100μM) and tyramine (1mM, $P<0.05$,*).

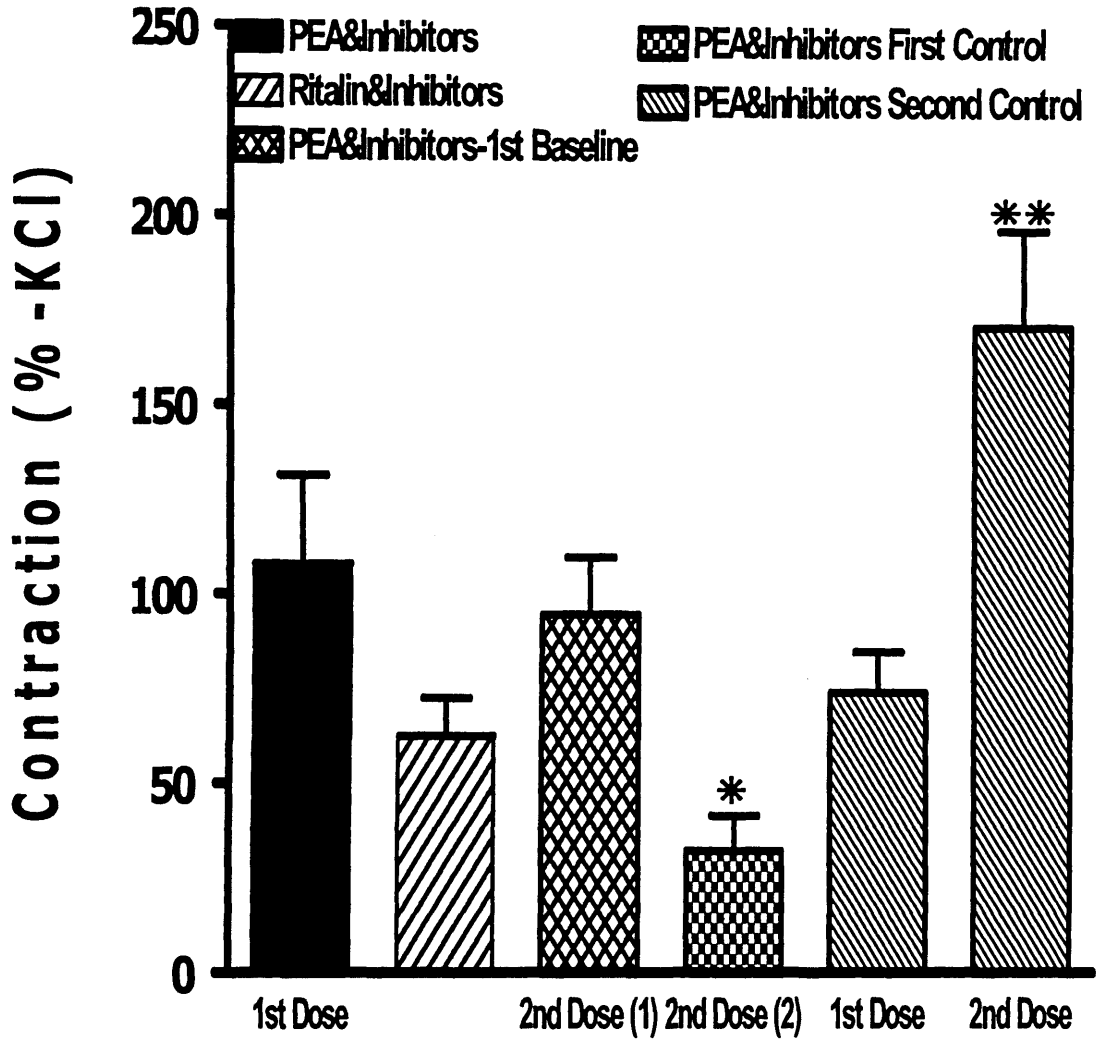


Figure 6.15: Single dose experiments for the contractile response in rat aortic rings to β-PEA (100μM, n=5) and Ritalin®(1mM, n=5) were constructed in the presence of "inhibitors". The second single dose of β-PEA (100μM) was in the presence of "inhibitors" and Ritalin® (1mM). To calculate the contractile response to β-PEA (100μM) (2nd dose) two different baselines were used. The 1st baseline was taken just before the Ritalin® was added, the 2nd baseline was taken just before the 2nd dose of β-PEA (100μM) was added to the bath *(see Figure 6.11)*. Responses are the mean (±S.E.M.) contractions expressed as a percentage of the contraction to KCl (60mM, isotonic solution). Mean responses (±SEM) were compared by ANOVA followed by the "post hoc" Bonferroni test and by paired Student's t-test (control experiment). A significant difference was shown between the first and second single dose of β-PEA (100μM, $P<0.05$,*) (2nd Baseline). The control experiment to β-PEA (100μM) also showed a significant difference between the first and second dose of β-PEA (100μM, $P<0.01$,**).

6.5 Discussion

6.5.1 Development of tachyphylaxis or cross-tachyphylaxis by repeated administration of TAs

6.5.1.1 Repeated cumulative and non-cumulative CRCs to β-PEA in the absence or presence of "inhibitors"

In *Chapter 3*, a comparison was made between cumulative and non-cumulative CRCs to the TA β-PEA. Only one CRC experiment per rat aortic tissue was obtained. The observation that the cumulative CRC was shifted to the right compared to that for the non-cumulative CRC suggested desensitisation or tachyphylaxis when the β-PEA was administered cumulatively. It has been suggested that ISAs are notorious for giving tachyphylaxis (Day, 1967), so this might suggest an ISA mechanism. However, the lack of effect of prazosin on the contractile response to β-PEA in rat aorta *(see Chapters 2 and 3)* indicates that the response does not involve ISAs. However, if the contractile response is due to activation of TAARs, then the possibility of desensitisation at these receptors must be considered. The possible effect of desensitisation in rat aortic rings to TAs, including β-PEA, was therefore further investigated by obtaining repeated cumulative and non-cumulative CRCs to β-PEA. Cumulative additions of β-PEA gave almost identical CRCs when repeated both in the absence, or presence of "inhibitors". In contrast, non-cumulative CRCs showed an obvious and significant decrease of the contractile responses in the repeated CRC in the absence of "inhibitors" *(Figure 6.4)*. My results are in agreement with those of Przyborski *et al.* (1991) even though the author worked with different isolated preparations (rat jejunum) and agonist (bethanechol). Thus, one obvious question is why in the present study, repeated non-cumulative CRCs to β-PEA gave such striking tachyphylaxis, while cumulative additions of β-PEA did not show the phenomenon. There are differences, however, between the cumulative and non-cumulative CRC experiments (Przyborski *et al.*, 1991). In the latter experiment, each single dose of β-PEA was allowed to act until the peak effect was achieved then the tissue bath was washed out and the tissue got the chance to equilibrate before the next single dose was added. In the former experiment, β-PEA was added repeatedly immediately after the peak effect of each response was acquired. Thus, by prolonged

exposure (cumulative), the impulse is constantly increasing, is never allowed to go to completion, and is generally of short duration. These may be the critical factors preventing tachyphylaxis (Przyborski *et al.,* 1991). The results in the present study indicate that β-PEA responses in the absence of "inhibitors" undergo desensitisation. Since these responses are not blocked by prazosin *(Chapters 2 and 3)*, they are not due to ISA. Therefore, the results suggest that the receptor under study is desensitised with repeated non-cumulative exposure, rather than prolonged exposure (cumulative). Nevertheless, throughout my whole study, only one non-cumulative CRC experiment at a time in each tissue was performed to investigate the effects of TAs in rat aortic rings. Therefore, the possible effect of tachyphylaxis with non-cumulative CRCs did not affect the results throughout the present study.

6.5.1.2 Effect of tachyphylaxis of β-PEA doses in the absence and presence of "inhibitors"

The results of repeated single dose experiments to β-PEA supported to some extent, these findings regarding the effect of tachyphylaxis to the contractile response to β-PEA in repeated non-cumulative CRCs. However, only β-PEA (100μM) in the absence of "inhibitors" showed a significant decrease of the contractile response between the first and the second dose. This effect did not occur with the lower dose of β-PEA (30μM) in the absence of "inhibitors", or with β-PEA (100μM) in the presence of "inhibitors". Therefore, the possible effect of tachyphylaxis to β-PEA in single dose experiments seems to be concentration-dependent. Also, the presence of "inhibitors" seems to avoid the possible effect of tachyphylaxis in repeated single dosage experiments.

6.5.1.3 Effect of tachyphylaxis and cross-tachyphylaxis of doses of different TAs

Previous studies have reported that amphetamine and other TAs might display the phenomenon of tachyphylaxis or tolerance (Zelger and Carlini, 1980; Foltin and Schuster, 1982). Tachyphylaxis to the peripheral effects of indirectly acting sympathomimetic amines has been demonstrated widely, the pressor effects become progressively diminished with repeated exposure (Day, 1967). In addition, there is cross-tachyphylaxis, whereby tachyphylaxis to one amine, such as amphetamine, is associated with the reduction in response to a second amine such as β-PEA.

In the present study, the results of repeated single dosage paired experiments with various TAs, including β-PEA, D-amphetamine and octopamine did not show any tachyphylaxis or cross-tachyphylaxis between these TAs. The contractile responses to β-PEA, D-amphetamine and octopamine were repeatable over two single dosage exposures and also the contractile response of each TA was consistently independent of which other TA agonist was incubated with the tissue before or after (first or second dose). This supports the contention that the vasoconstriction by these TAs is not the result of indirect sympathomimetic activity as such a mechanism would be expected to lead to tachyphylaxis. In *Figure 6.8* a surprising significant increase difference in the contractile response of β-PEA was seen on the second single dosage. However, this observation is unlikely to be based on any interactions between TAs. Since each response was obtained in a different tissues it is more likely to be based on a variable distribution of the receptor under study in rat aortic tissues (*see Chapter 5)* than to a sensitizing effect. Since no such evidence was obtained elsewhere in this thesis.

6.5.1.4 Multiple dosing

The contractile response to β-PEA in the presence of "inhibitors" was dependent on how long the tissue was incubated with the TA. The contraction of rat aortic rings to β-PEA in the presence of "inhibitors" was larger at the peak response with longer incubation times. In short exposure experiments it was often considered that the plateau of the response had been achieved and the bath washed out. However, longer exposure time allows the tissue to equilibrate more fully and therefore produce the "true" peak effect. There was no evidence of desensitisation by a one hour exposure to the TA.

6.4.2 Effect of partial agonism of trace amines

Currently, little is known about how TAs affect vascular tone in isolated rat aortic tissues. So far, no antagonist has been identified for TAs. Therefore, to investigate the contractile response to various TAs in isolated rat aortic tissue, the responses were examined in the presence of "inhibitors" to remove other mechanisms of action *(Chapters 2 and 3)*. In cloned receptors expressed in transfected cell lines, tyramine was identified as a potent TAAR1 agonist (Borowsky *et al.*, 2001; Bunzow *et al.*,

2001; Lindemann *et al.*, 2005). However, tyramine was a very weak vasoconstrictor in rat aortic tissues, as in the absence or presence of "inhibitors" the TA showed only very small contractions *(Chapter 5)*. Therefore, the possibility was considered that tyramine was a partial agonist for a TAAR and may therefore behave as an antagonist. With single dose studies of TAs *(Figures 6.1, 6.11)* it was determined whether tyramine could act as an antagonist of other TAs, including β-PEA, octopamine and D-amphetamine. The contractile responses of the TAs were measured as a percentage of KCl (isotonic solution, 60mM) and the effect of tyramine on responses to other TAs were interpreted in two different ways (first and second baseline) *(Figures 6.1 and 6.11)*. In the presence of "inhibitors", β-PEA, D-amphetamine and octopamine caused strong contractile responses in rat aortic rings *(Chapter 5)*. However, in the presence of "inhibitors" and tyramine, the contractile responses to β-PEA, D-amphetamine and octopamine were significantly inhibited irrespective of the plotting method. This finding suggested that tyramine acted at the same receptor as β-PEA, D-amphetamine and octopamine and was behaving as an antagonist. The inhibitory effects of tyramine on the contractile responses of β-PEA, D-amphetamine and octopamine were different. The contractile response to β-PEA was inhibited the least and the response to D-amphetamine was inhibited the most by tyramine (reduced vasoconstriction). That might be simply explained by the different size responses to start with. The contractile responses to D-amphetamine and octopamine in the presence of "inhibitors" and tyramine calculated from the second baseline *(Figures 6.1 and 6.11)* even fall below baseline. This observation could be explained by tyramine completely blocking the contractile response to both D-amphetamine and octopamine. The fall below the tyramine response may have been fade of the tyramine-induced contraction. Furthermore, only in the case of β-PEA did the contractile responses in the presence of "inhibitors" and tyramine not show a significant inhibition calculated from the first baseline. In experiments for D-amphetamine and octopamine both responses, calculated from the first and second baseline, were significantly inhibited. These results might be explained because of the inconsistent contractile response to tyramine. Tyramine might have caused a stronger contractile response with β-PEA compared to experiments with D-amphetamine and octopamine. The effect of tyramine on the contractile response to octopamine is similar to the effect of tyramine on D-amphetamine. These findings

confirmed previous results that β-PEA activates the same TAAR as D-amphetamine and octopamine, but with a higher activity *(Chapter 5)*. Another explanation for the smaller inhibition of β-PEA could be that β-PEA activates two different TAARs in rat aorta while D-amphetamine, octopamine and tyramine act at only one of these TAAR *(Chapter 5)*. Therefore, the contractions to β-PEA would be inhibited less. Furthermore, throughout the present study β-PEA always showed the largest contractions in comparison to the responses to octopamine and D-amphetamine. Therefore, the contractile responses to β-PEA is obviously more difficult to block than the response of other TAs.

It was interesting that compared to previous results *(Chapter 5)*, in the presence of "inhibitors", tyramine caused stronger contractile responses. This was attributed to the longer exposure time that allows the tissue to equilibrate more fully and produce stronger responses to the TA. However, the contractions to tyramine were still not very consistent and the responses were still significantly lower compared to the contractile responses to β-PEA, D-amphetamine and octopamine i.e. it sill behaved as a partial agonist. To investigate the contractile response to tyramine with longer exposure times in isolated aortic tissues, it might be of interest in further studies to analyse the time to peak effect (time courses) and also to look into the kinetics of drug-receptor interactions. The weak contractile activity of tyramine in the rat aorta and antagonism of the contractile response to different TAs suggests that it is a weak partial agonist of TAARs in this tissue. A previous publication showed in human, rat and mouse TAAR1 transfected cell lines that β-PEA act as a full agonist for all TAAR1s whilst tyramine was a full agonist for rat and mouse TAAR1 but not for human TAAR1. The human receptor was cloned in rat cell line and this receptor and cell combination showed tyramine to be a partial agonist (Reese *et al.*, 2007). However, I am not aware of any other studies in isolated tissues where the effect of tyramine or any other TAs as an antagonist of the other TAs has been investigated. These findings in the present study identified tyramine as the first potential antagonist of the other TAs, including β-PEA, D-amphetamine and octopamine in isolated rat aortic rings. In contrast to the low efficacy of tyramine in isolated rat aortic tissues, in guinea-pig aortic rings, tyramine was identified as a good vasoconstrictor in the absence and presence of "inhibitors" and therefore a full

agonist *(Chapter 5)*. Therefore, the effect of tyramine as an antagonist for TAs seems to be rat-specific.

The contractile response to Ritalin® in the presence of "inhibitors" in rat aortic rings was significantly smaller compared to β-PEA and other TAs and could also be regarded as a partial agonist *(Chapter 5)*. Therefore, as with tyramine above, the effect of Ritalin® on β-PEA responses was examined. Comparable to the effect of tyramine on β-PEA in the presence of "inhibitors", the contraction to β-PEA was significantly reduced by Ritalin® (second baseline). These results suggest that Ritalin® acts on the same receptor as β-PEA.

6.6 Conclusion

In order to continue the investigations on the potential mechanism of the contractile response of TAs in rat aorta, initial studies were carried out in rat aortic tissues. The effect of tachyphylaxis was seen for some TA agonists but not others. It appears to be concentration-dependent and also "inhibitors"-dependent. β-PEA, D-amphetamine and octopamine activate either one TAAR or two different ones with different affinities. Tyramine was identified as the first partial agonist in tissue and therefore antagonist of the other TAs in isolated rat aortic tissues. Ritalin® was also identified as a weak partial agonist compared to tyramine, capable of antagonising β-PEA. This observation might be of interest for further studies, as no antagonist for TAs has been described in the literature.

Chapter 7

DETERMINATION OF TRACE AMINE-ASSOCIATED RECEPTOR 1 EXPRESSION BY MOLECULAR BIOLOGY

Chapter 7

Determination of trace amine-associated receptor 1 expression by molecular biology

7.1 Introduction

The TAARs have been shown to be phylogenetically related to other biogenic amine binding receptors, such as serotonin, dopamine, adrenergic, histamine and muscarinic receptors. These together make up the larger part of the α-group of rhodopsin-like GPCRs (Borowsky *et al.*, 2001; Fredriksson *et al.*, 2003). From a number of invertebrates, such as the fruit fly (Saudou *et al.*, 1990), honey bee (Blenau *et al.*, 2000) and molluscs (Gerhardt *et al.*, 1997) GPCRs that bind TAs have been cloned and characterized pharmacologically. However, their relationship to mammalian TA receptors is unclear (Borowsky *et al.*, 2001). The superfamily of GPCRs constitutes one of the largest families of proteins in the mammalian genome (Lander *et al.*, 2001; Venter *et al.*, 2001; Kroeze *et al.*, 2003). It has been estimated that more than half of all modern drugs are targeted at these receptors (Flower, 1999). The cloning and sequencing of many unanticipated subtypes of receptors or "orphan receptors", as well as the identification of altogether unknown receptors, has greatly altered the landscape of the pharmacology of GPCRs (Horn *et al.*, 1998). Mostly, receptors are differentiated by the effect of selective drugs. Nevertheless, receptors can be also defined by their cDNA and amino acid sequences (Shi and Javitch, 2002).

Molecular biology describes the interactions between the various systems of a cell, including the inter-relationship of DNA, RNA and protein biosynthesis (Alberts *et al.*, 1994). Furthermore, molecular biology detects how these interactions are regulated (Astbury, 1961). Expression cloning, polymerase chain reaction (PCR), gel electrophoresis, Southern-, Northern- and Western blotting are commonly used techniques in molecular biology. PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA (Mullis and Faloona, 1987; Sambrook and Russel, 2001). This use of PCR enhances methods such as Southern and Northern blotting and DNA cloning. These techniques need relatively large amounts of DNA to represent a specific DNA region. PCR provides

these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material (Alberts *et al.*, 1994). PCR has many variations, such as reverse transcriptase PCR (RT-PCR) and quantitative PCR (Q-PCR). RT-PCR is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA. The PCR is preceded by a reaction using reverse transcriptase (RT) to convert RNA to cDNA (Alberts *et al.*, 1994; Wilson and Walker, 2000). RT-PCR is widely used to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites and, if the genomic DNA sequence of a gene is known, to map the location of exons and introns in the gene . Gel electrophoresis is a technique used for the separation of DNA, RNA or protein molecules using an electric current applied to a gel matrix (Bier, 1959; Berg *et al.*, 2002; Lodish *et al.*, 2004). Separation of proteins may be by isoelectric point, molecular weight, electric charge, or a combination of these factors. The gel greatly retards the mobility of all molecules present. It is usually performed for analytical purposes. By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS), such as for Western blotting. Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of an electric field that is space uniform (Reuss, 1809). Electrophoresis occurs because particles dispersed in a fluid almost always carry an electric surface charge. An electric field exerts electrostatic Coulomb force on the particles through these charges. In general, after separation of the molecules they can then be detected using a variety of methods. In the case of proteins, the separated molecules are incubated with antibodies. In *Section 7.3.1.2* more details are discussed.

Previous *Chapters* looked at the pharmacology of TAs in aortic tissues. The results suggested the presence of at least one TAAR. Since no specific antagonist for TAs is available, molecular biological methods were used to investigate the presence of a TAAR in rat aorta.

Aims

To determine the presence of TAAR1 in isolated aortic tissues from male rats

a) The presence of TAAR1 in rat aortic rings was determined *via* molecular biological methods, including RT-PCR and Western blotting.

7.3 Material and Methods

7.3.1 Western Blotting

7.3.1.1 Methods

Western blotting is a method to detect one protein in a mixture of any number of proteins and provides information about the size of the protein of interest. Furthermore, the method can also give semi-quantitative information as to how much of the protein is present in cells. The method is dependent on the use of a specific antibody that is directed against a desired protein (Laurell, 1965; Towbin *et al.*, 1979; Burnette, 1981).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl (lauryl) sulfate (SDS) is an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures and therefore linearises the protein (Rybicki and Maud Purves). SDS applies a negative charge to each protein in proportion to its length. In this way they can be separated by their molecular weight. The samples were separated by SDS-PAGE on a 10% polyacrylamide gel. SDS-PAGE is a method for the evaluation of molecular weights and analytical separation of proteins/peptides (Weber and Osborn, 1969; Laemmli, 1970; Aubertin *et al.*, 1983; Okajima *et al.*, 1993). In electrophoresis the protein molecules are moved across the polyacrylamide gel *via* an electric current *(Figure 7.1 a, b)*. The polyacrylamide gel is a three-dimensional cross-linked matrix. Due to the electric current, negatively charged protein molecules are transferred to the positive electrode. However, smaller molecules are transferred quicker to the positive electrode compared to bigger molecules. Therefore, a separation of protein molecules according to their size takes place. One way of making conditions suitable for

peptide analysis is by decreasing the pore size of the gel (Takagi and Kubo, 1979; Anderson *et al.*, 1983). In order to decrease the pore size of the gel the concentration of acrylamide is important. A very high concentration of acrylamide means the gel has smaller holes and therefore separates smaller molecules better. If the concentration of acrylamide is lower, there will be bigger holes in the gel and therefore larger proteins can be separated (Swank and Munkres, 1971).

Two different gels *(Appendix)* were used for the separation. In the lower part of the cassette, a separating gel (10%) was poured and, once it was set, the stacking gel (5%) was added on top of it.. The stacking gel is not like the separating gel which separates proteins according to their sizes but is used to ensure an even distribution of the protein mixture. Therefore all proteins start at the same point in the gel which is very important, because the recognisable end result is a band corresponding to a particular protein which can be identified by how far it has moved through the separating gel. If the proteins are not loaded evenly at the start it will affect how far they move relative to each other.

Electroblotting

Once the separation of protein molecules *via* SDS-PAGE electrophoresis is completed the separated protein molecules were transferred from the polyacrylamide gel onto a nitrocellulose membrane as a solid support *(Figure 7.1 c, d, Figure 7.2)*. A semi-dry blotting transfer system was set up. This technique is based upon an electronic current and a semi-dry blotting buffer *(Appendix)* to transfer the proteins on the nitrocellulose membrane. The membrane needs to be located between the gel and the anode ("sandwich") as the current and protein molecules will be moving in that direction. Moreover, before the transfer could take place it was important that all bubbles in the system were carefully removed to ensure even transfer and that the filter papers were cut to the same size as the membrane *(Figure 7.2)*.

Blocking

Once the transfer was completed steps had to be taken to avoid non-specific interactions between the membrane and the antibodies used for detection of the target protein. Blocking was achieved by placing the membrane in a dilute solution of non-fat dried milk and the detergent Tween 20. The protein in the dilute solution binds to the membrane in all places where the target protein molecules have not attached. Therefore the antibody (AB) cannot attach other than, to the binding sites of the specific target protein. Thus, clearer results can be achieved and false positive results are avoided.

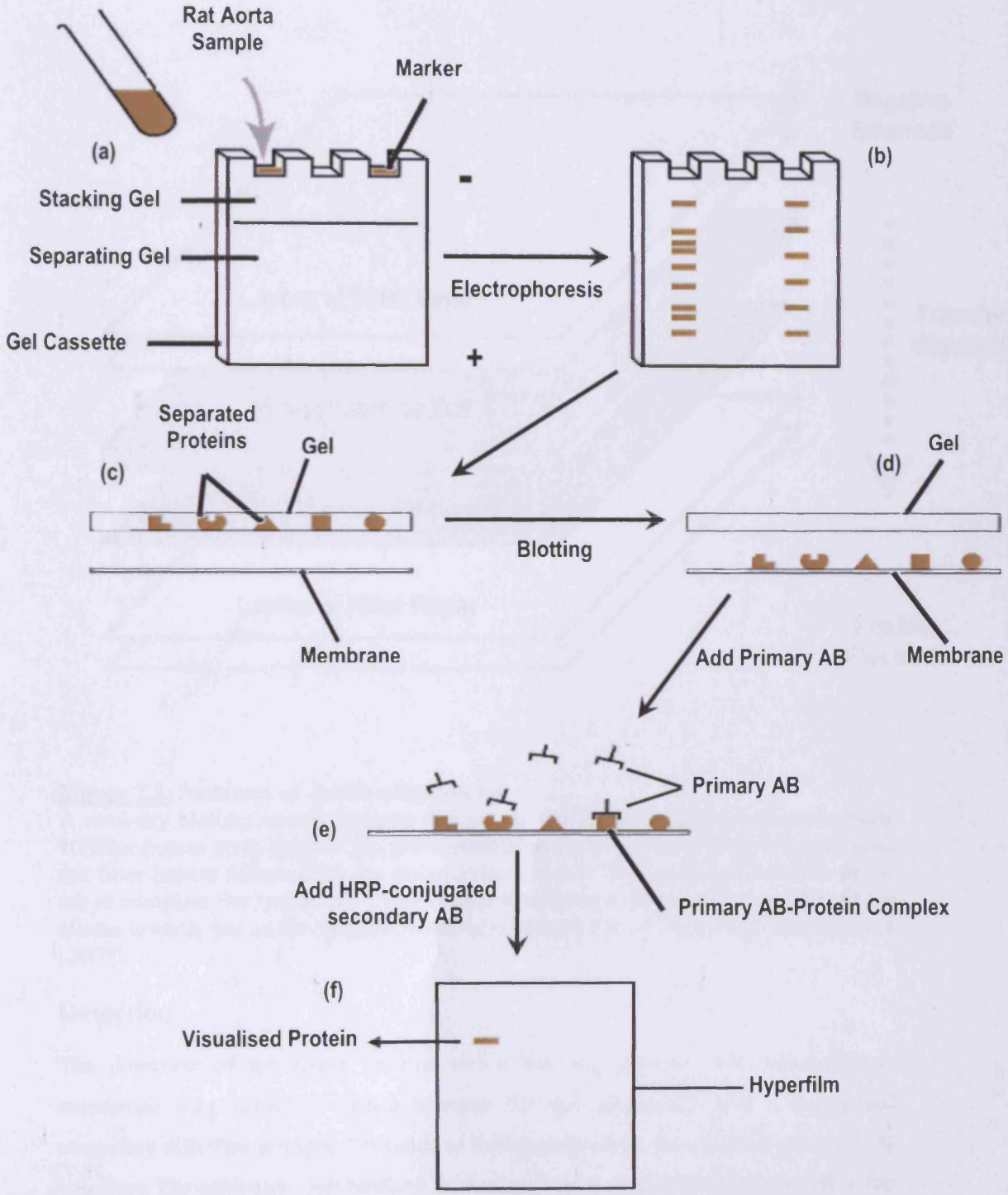


Figure 7.1: Summary of Western Blotting Process. *Adapted from The BSE Inquiry (2000)*

(a) Preparation of gel for protein separation
(b) Proteins separated on the basis of size and charge in the electrical field
(c) Preparation for Blotting
(d) Blotting: Separated proteins transferred from gel to membrane
(e) Incubation of separated proteins with primary and conjugated secondary ABs ⇒ protein-primary AB-secondary AB-enzyme complex (HRP=horse radish peroxidase)
(f) Membrane exposure. The protein of interest can be visualised

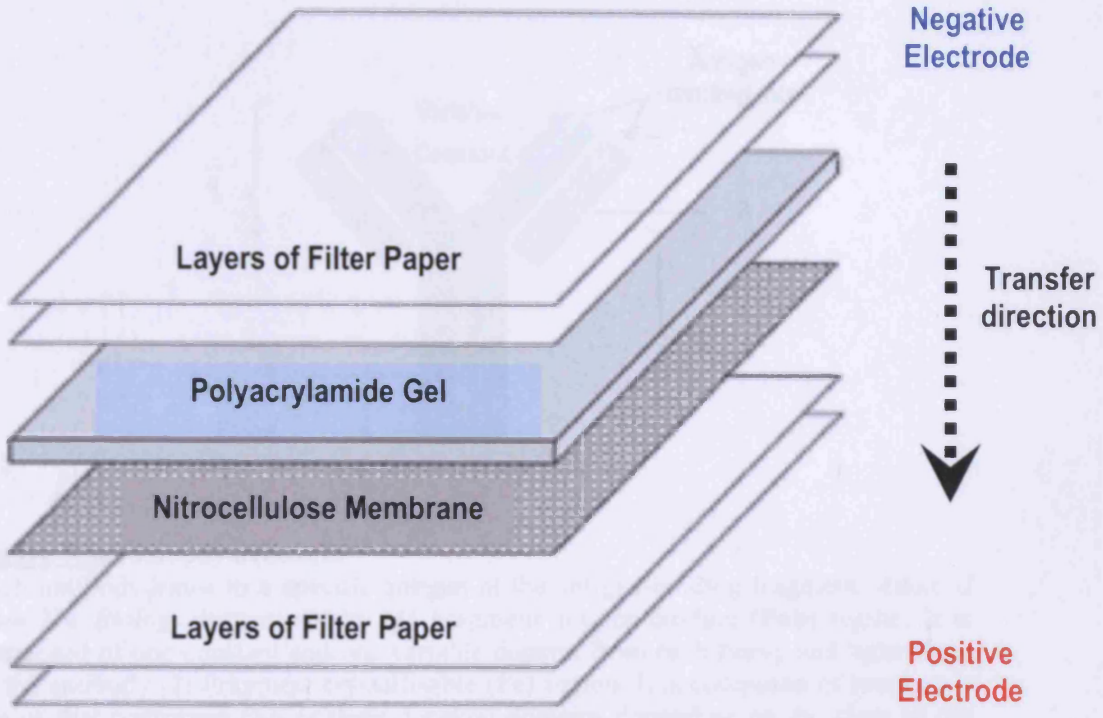


Figure 7.2: Summary of the Blotting process
A semi-dry blotting transfer system was set up. On the pre-wetted positive electrode 10 filter papers were placed. The pre-wetted nitro-cellulose membrane was placed on the filter papers followed by the polyacrylamide gel. 10 filter paper layers went on top to complete the "sandwich". The system was run at a constant current (39mA) for 60min towards the anode (positive electrode). *Adapted from Fermentas Life Sciences (2007).*

Detection

The detection of the target protein was a two-step process. The nitrocellulose membrane was incubated with a primary AB and afterwards with a conjugated secondary AB. The primary AB binds to the target protein *via* a protein-primary AB complex. The secondary AB binds to Fc region *(Figure 7.3)* of the primary AB in the primary AB-protein complexes. The enzyme conjugated to the secondary AB, horseradish peroxidase (HRP), allows visualisation of the antibodies, as it is used in conjunction with a chemiluminescent agent. The visualised bands are protein-primary AB-secondary AB-enzyme complexes. These complexes indicate the size of

the target protein, and produce luminescence in proportion to the amount of protein *(Figure 7.1 e, f)*.

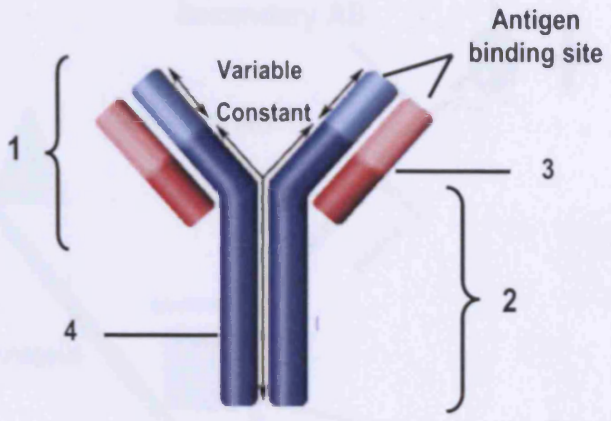


Figure 7.3: Antibody structure.
Each antibody binds to a specific antigen at the antigen-binding fragment. *Adapted from The Biology Project (2000)* **(1)** Fragment antigen-binding **(Fab)** region. It is composed of one constant and one variable domain from each heavy and light chain of the antibody **(2)** Fragment crystallisable **(Fc)** region. It is composed of two heavy chains that contribute two or three constant domains depending on the class of the antibody **(3) Light Chain**: consists of one constant and one variable domain. The variable region is important for binding antigen **(4) Heavy Chain**: consists of the constant and the variable region

Chemiluminescence

After incubation with both antibodies, the Western blot is ready for detection of the protein of interest. The membrane is incubated with "enhanced chemiluminescent" (ECL) reagents which are metabolised in the presence of HRP to release light which is then detected by X-ray films *(Figures 7.4 and 7.1 f)*.

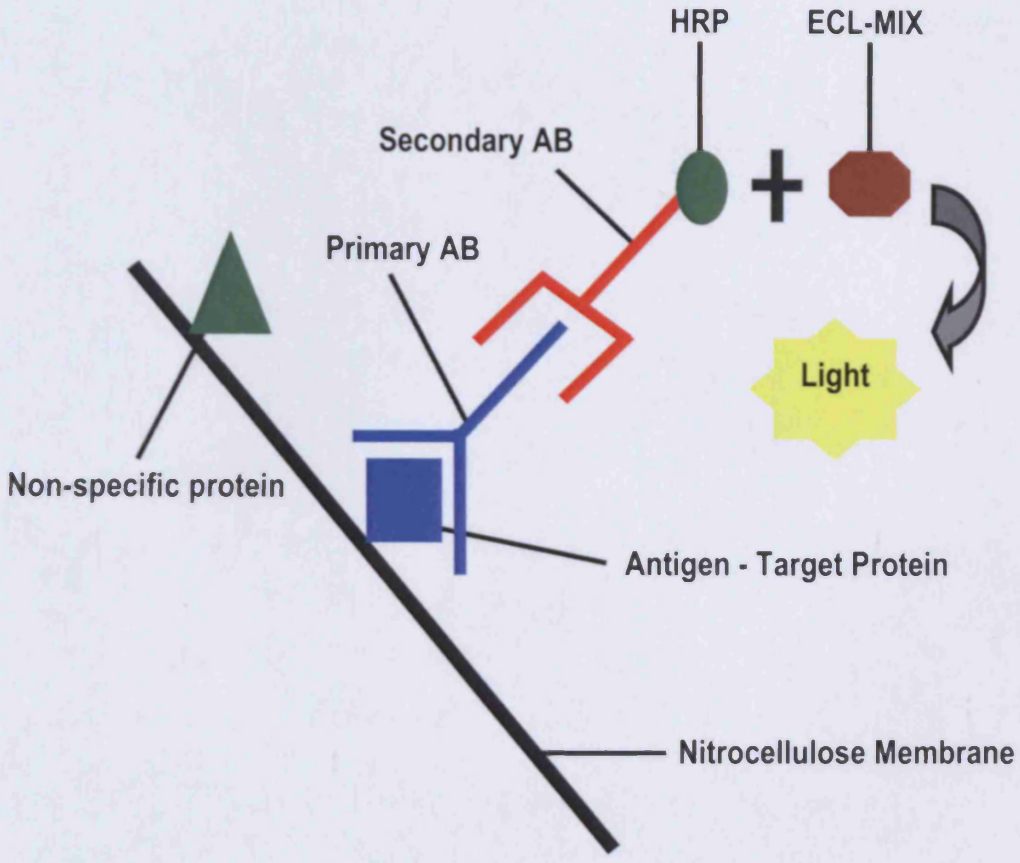


Figure 7.4: Western Blotting: Detection and Chemiluminescence process
The primary antibody (AB) detects the target protein. The secondary AB detects the Fc region of the primary AB and built up a protein-primary AB-secondary AB-enzyme complex. The secondary AB is conjugated to horse radish peroxidase (HRP) which converts a luminol substrate (ECL) to a light-releasing substance. The light was detected by specialised X-ray film. *Adapted from Molecular Station (2006)*

7.3.1.2 Experimental Protocol

Preparation of the polyacrylamide Gel

Two different polyacrylamide gels were used, the separating (10%) and stacking (5%) gel. The same reagents were used (acrylamide, Tris-HCl, SDS, APS, TEMED) for both polyacrylamide gels, but at different concentrations *(Appendix)*. Both gels were prepared in gel cassettes of the Novex gel system (Invitrogen, UK). A 10% polyacrylamide gel was chosen because of the size of the protein of interest, the TAAR protein. The TAAR1 belongs to the GPCR family and the average molecular weight of GPCRs is around 45-55kDa.

Rat aortic membrane preparation

Rat aortic membrane samples were prepared. Fresh rat aortic tissues were taken from male *Sprague-Dawley* rats (250-300g) *(Chapter 2)*. The aortic tissues were homogenised for 3x 10 seconds (Polytron, Ultra-Turrax, Fisher UK). Afterwards the homogenate was span at 500g for 10 minutes at 4°C. The supernatant was removed and kept on ice. The pellet was resuspended in Tris buffer (NaCl 150mM, Tris (pH 7.4) 50mM, EDTA 1mM) plus two different peptidase inhibitors, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF 1mM), an irreversible serine protease inhibitor and bacitracin (0.1mg/ml) (10ml) a mixture of related cyclic polypeptides produced by organisms of the licheniformis group of Bacillus subtilis var Tracy (Johnson *et al.*, 1945). After repeated homogenising (Polytron, 3x 10 seconds) the homogenate was spinned as above at 500g for 10 minutes at 4°C. The supernatant was removed and combined with the earlier one and span at 48000g for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in Tris buffer by vortexing. The homogenate was span as above at 48000g for 15 minutes at 4°C, before the pellet was resuspended in Tris buffer without peptidase inhibitors and homogenised with a syringe and a fine gauge needle.

BCA protein assay

The amount of protein in the sample was analysed using the BCA Protein Assay. The BCA™ Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein (Smith *et al.*, 1985).

a.) Preparation of diluted Bovine Serum Albumin (BSA) standards (Bovine Albumin fraction V)

Final BSA concentration (protein mg/ml)	Distilled Water (µl)	BSA stock (2mg/ml) (µl)	Dilution factor
1	500	500	1:2
0.5	500	500	1:2
0.25	500	500	1:2
0.2	175	750	1:1.25
0.1	500	500	1:2
0.05	500	500	1:2
0.025	500	500	1:2
0.01	750	500	1:2.5
BLANK	1000	0	-

b.) Preparation of BCA™ Working Reagent (WR):

To prepare the BCA™ WR 50 parts of BCA™ Reagent A with 1 part BCA™ Reagent B (50:1 Reagent A:B) were combined. Then 25µl of each standard and 0.5µl - 1µl of samples were added in duplicate to the 96 well plate. Afterwards 200µl of the WR was added to each well and the plate was mixed on a plate shaker for 30 seconds and then incubated at 37°C for 30 minutes. Afterwards the plate was cooled for 5 min at room temperature before the absorbance at 540nm was read by using a spectrophotometer. The optimal wavelength is 562nm, however, wavelengths from 540-590nm have been successfully with this method. The concentration of the protein was determined based on a standard curve with the *GraphPad Prism 4* program.

Preparation of samples for Western blotting

The rat aorta membrane sample was prepared in 3X Sample Buffer *(Appendix)* (Laemmli, 1970) plus 50mM Tris (pH 7.4). 50μg of rat aorta membrane sample was loaded into the cassette. A molecular weight marker (Precision Plus Protein marker) (5μl) was used to estimate the molecular weights of proteins *(Figure 7.1 a, b)*.

Proteins are generally folded into 2° and 3° structures. Unfolded proteins will not move well through the gel, therefore the protein structure has to be disrupted. This process is called protein denaturation. Therefore, 3X sample buffer (Laemmli, 1970) was used to prepare the samples. The buffer changes proteins from 2° and 3° structures into a linear 1° structure. It contains β-mercaptoethanol which binds to cysteine residues and breaks down S-S bonds, SDS detergent which solubilises the protein, and a blue colour indicator which allows visualisation of the bands in the gel. The samples are kept in SDS all the time to prevent proteins from reverting back to their 2° and 3° structures. To support the denaturation, the samples were boiled at 95°C before each use.

SDS-PAGE electrophoresis

Once the loading of the gel was completed, the separation of the protein samples was started *(Figure 7.1 b)*. The system was run at 50V for 30 minutes for ensure that all samples were equally distributed in the wells and then for approximately 2.5 hours at 110V (until the blue dye indicator reached the cassette slot).

Electroblotting

After separation, the protein molecules were electro-transferred to a nitrocellulose membrane (Hybond™-ECL™, pore size 0.2μm) using a vertical semi-dry blotting system (Multiphor II electrophoresis system) plus electrode paper 200x250mm (Novablot, GE Healthcare, UK) for 1hour at 39mA (0.8mA/cm^2 of gel) in blotting buffer *(Appendix, Figure 7.2)*.

Blocking

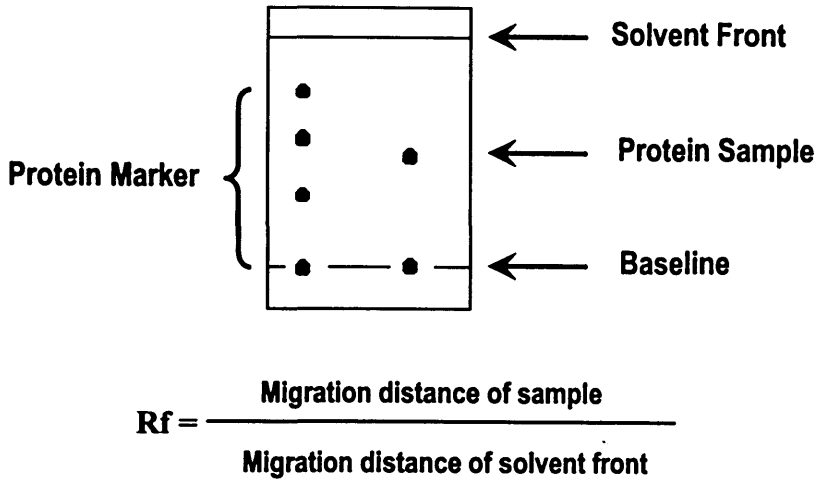
After transfer, membranes were washed briefly in Tris-buffered saline Tween-20 (TBST) *(Appendix)* to remove the blotting buffer. The nitrocellulose membrane was blocked for 1 hour at room temperature (22°C) in a solution of 5% non-fat dried milk in TBST (Blotto 5%) *(Appendix)*.

Detection

The membrane was incubated with the primary antibody (AB), a rabbit anti-human TAAR1 polyclonal antibody, LS-A2042 (stock 1mg/ml; 1:750 dilution) in 1% Blotto at 4°C overnight on a rocker to detect the proteins of interest. The next day, the membrane was washed with TBST for 2 x 5 minutes and 1 x 15 minutes and incubated with the secondary AB, an anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:10000 dilution) in 1% Blotto for 1 hour at room temperature (RT, 22°C) under constant agitation. After three washes with TBST as above, the membrane was incubated with the ECL reagents according to the manufacturers' instructions for 5 minutes at room temperature (22°C) and was afterwards removed. The ECL metabolised in the presence of HRP to release light which is then detected by X-ray films (Hyperfilm) *(Figure 7.4, Figure 7.1 f)*. Wiping all surfaces with 100% ethanol reduced contamination of the ECL reaction.

7.3.1.3 Analysis of Results

Values for the molecular weight (kDa) of protein bands were approximated by measuring retardation factor (Rf)-values for the bands using the protein standard markers as a reference. The Rf-value is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front (Coyne *et al.*, 1996) *(Figure 7.5)*. The molecular weight of the proteins were then estimated using the Rf-values (GraphPad, PRISM 4) as there is a linear relationship between the logarithm of the molecular weight of an SDS-denatured polypeptide and its log Rf-value.



$$Rf = \frac{\text{Migration distance of sample}}{\text{Migration distance of solvent front}}$$

Figure 7.5: Calculation of retardation (Rf)-values of the sample using the protein standard markers as a reference. *Adapted from University of Wisconsin (2000)*

7.3.1.4 Drugs Used

All other chemicals, unless otherwise specified, were obtained either from Sigma Chemicals, UK or Fisher Scientific, UK.

- **Reagents obtained from Sigma Aldrich, UK**

Beta-mercaptoethanol, Bromophenol Blue (Tetrabromophenolsulphonephthalein, BPB), Glycerol, Tetramethylethylenediamine (TEMED)

- **Reagents obtained from Fisher Scientific UK Ltd**

Tris (hydroxymethyl)-aminomethane (Tris Base), Tris (hydroxymethyl) aminomethane Hydrochloride

- **Reagents obtained from Bio-Rad Laboratories Headquarters (Hercules, CA, USA)**

Sodium dodecyl (lauryl) sulphate (SDS), Ammonium persulphate (APS), Polyacrylamide (Acrylamide/Bis 37, 5:1), Precision Plus Protein marker

- **Reagents obtained from Caltaq-Med System Ltd. (Buckingham, Botolph Claydon, UK)**

Primary anti-human TAAR1 antibody LS-A2042 (Rabbit/Polyclonal)

- **Reagents obtained from Vector Laboratories, Ltd. (Peterborough, UK)**

Horse-radish peroxidase (HRP) conjugated anti-rabbit IgG

- **Reagents obtained from GE-Healthcare (Uppsala, Sweden)**

Hybond™–ECL™ nitrocellulose membrane, Multiphor II (two graphite electrode-system, semi-dry transfer system), Electrode paper (Novablot), Amersham Hyperfilm

- **Reagents obtained from Perbio Science UK Ltd. (Cramlington, Northumberland, UK)**

Enhanced Chemiluminescence-mix (ECL Super Signal®, West Dura Extended Duration Substrate), BCA Protein Assay Kit

7.3.2 Reverse Transcriptase-Polymerase Chain Reaction (RT- PCR)

7.3.2.1 Methods

In molecular biology, RT-PCR is a technique for amplifying a defined section of an RNA molecule (Alberts *et al.*, 1994; Wilson and Walker, 2000). The RNA strand is first reverse-transcribed into its complementary DNA, followed by amplification of the resulting DNA using PCR. The exponential amplification *via* RT-PCR provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected. In the first step of RT-PCR (the first strand reaction) complementary DNA is made from a messenger RNA template using dNTPs and an RNA-dependent DNA polymerase (reverse transcriptase) through the process of reverse transcription (RT). After the RT-reaction is complete, and complementary DNA has been generated from the original single-stranded mRNA, standard PCR (second strand reaction) is initiated (Davidson College, 2000). PCR is a technique which is commonly used in molecular biology (Saiki *et al.*, 1988; Erlich and Arnheim, 1992) and is an in vitro enzymatic replication of DNA (target) by DNA polymerase. DNA

is a nucleic acid which contains genetic instructions (Mullis and Faloona, 1987). It is a long polymer made from repeating nucleotides and it is shaped as a double-stranded helix, as two strands of DNA wrap around each other (Watson and Crick, 1953). Both strands of DNA are copied during PCR. Therefore, PCR is used for an exponential amplification of specific regions of DNA strands (target DNA) *(Figure 7.6)*.

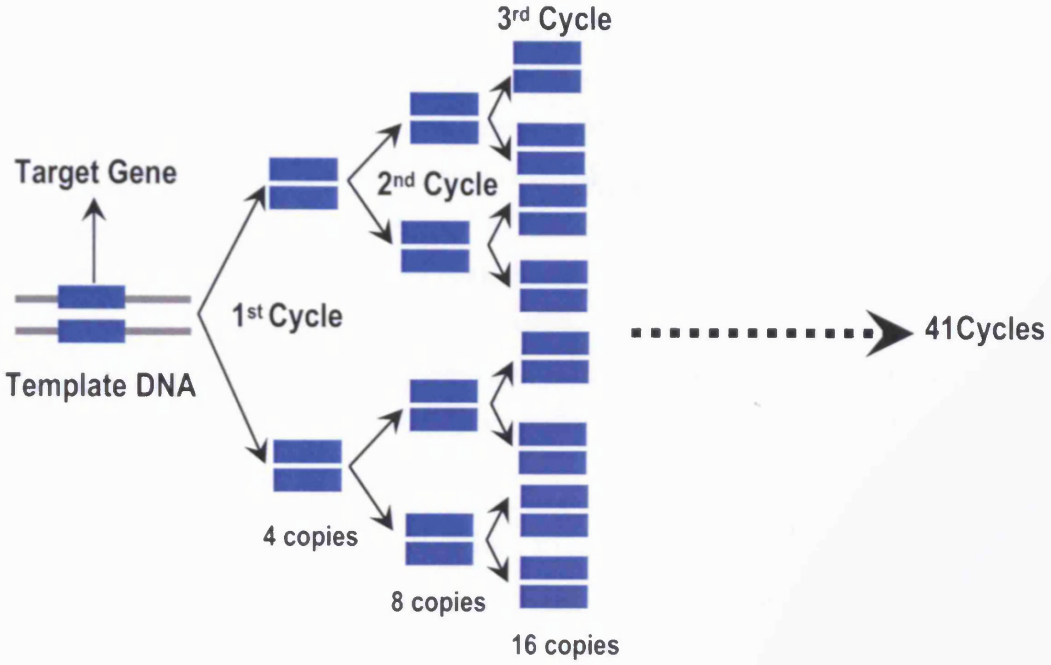


Figure 7.6: Exponential amplification
PCR requires a template DNA molecule (target gene). DNA generated in this replication is itself used as a template as the PCR progresses. There is one copy of the target gene before the chain reaction starts. After the first cycle there will be 4 copies and after the second cycle there will be 8 copies and so on.

PCR is based on the DNA polymerisation reaction. The chain reaction requires a heat-stable enzyme (TAQ polymerase) which synthesizes a new DNA strand, using a single-stranded DNA template, DNA oligonucleotides and DNA primers *(Figure 7.7)* (Sambrook and Russel, 2001). TAQ polymerase is a thermostable DNA polymerase named after the thermophilic bacterium Thermus aquaticus from which it was originally isolated (Chien *et al.*, 1976; Lawyer *et al.*, 1993). Polymerases are enzymes which catalyze the polymerization of deoxyribonucleotides alongside a DNA strand. The newly-polymerized molecule is complementary to the template DNA strand and identical to the template's partner strand (Burgers *et al.*, 2001; Hubscher *et al.*, 2002). Primers are single-stranded and consist of a string of nucleotides (oligonucleotides) in a specific order which bind to a specific complementary sequence of nucleotides of the single-stranded DNA. Primers are used to assign the DNA fragment to be amplified by the PCR process. The selectivity of PCR is primarily due to selecting primers that are complementary to the DNA region targeted for amplification. Primers must be duplicates of nucleotide sequences

on either side of the target DNA. A primer is required because most DNA polymerases cannot begin synthesizing a new DNA strand from scratch, but can add to an existing strand of nucleotides. The primers add here to the DNA template at these starting points (five (5′) prime) where DNA polymerase binds and begins the synthesis of the new DNA strand *(Figure 7.7)*. It is important to have the naming convention (5′) prime and (3′) prime as nucleic acids can only be synthesized in a 5′ to 3′ direction, as the polymerase used to assemble new strands must attach a new nucleotide to the 3′ hydroxyl (-OH) group *via* a phosphodiester bond (Eguchi *et al.*, 1991). By convention, single strands of DNA and RNA sequences are written in 5′ to 3′ direction.

Primer sequences need to be uniquely designed (Primer3, 2007) for a region of DNA, avoiding the possibility of mishybridization to a similar sequence nearby for an other section of DNA.

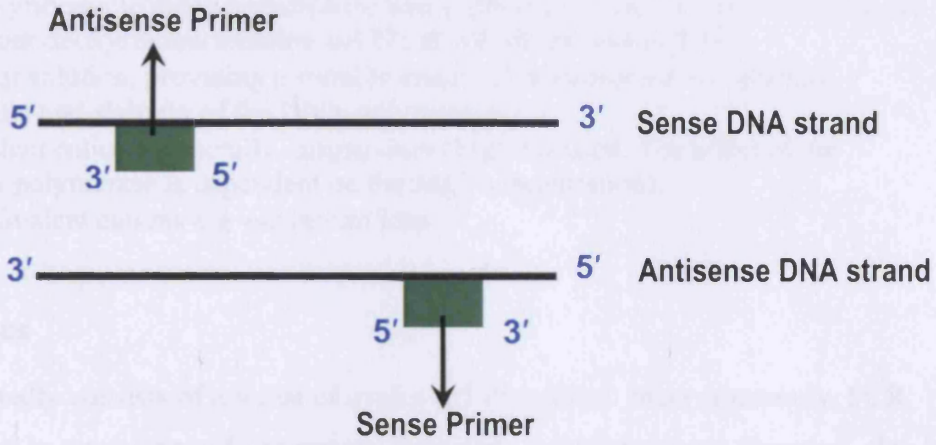


Figure 7.7: Directionality
End-to-end chemical orientation of a single strand of nucleic acid. Primers add here to the DNA template at these starting points (5′) prime where DNA polymerase binds and begins the synthesis of the new DNA strand. Nucleic acids can only be synthesized in a 5′ to 3′ direction. Primers interact with complementary strands of nucleic acids. The sense DNA strand is also the coding strand.

PCR-Set Up

PCR is commonly carried out in a reaction volume of 15-100µl in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler (PCR machine). The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step. Thin-walled reaction tubes allow suitable thermal conductivity to allow for rapid thermal equilibration. Thermal cyclers with heated lids prevent condensation at the top of the reaction tube (Sambrook and Russel, 2001).

➢ **PCR requires several components and reagents**

a) Components

- DNA template (contains the DNA region to be amplified)
- Primers (single nucleic acid strands)
- Thermostable DNA polymerase with an optimum temperature around 70°C

b) Reagents

- Deoxyribonucleotides triphosphate bases (dNTPs) (a generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP)
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase
- Divalent cations (generally, magnesium (Mg^{2+}) is used. The effect of the DNA polymerase is dependent on the Mg^{2+}concentration).
- Monovalent cations e.g. potassium ions

PCR Cycles

The PCR usually consists of a series of cycles (25-40 cycles). Most commonly, PCR is carried out in three steps (denaturation, annealing and elongation). However, the PCR procedure is often preceded by one temperature hold at the start (initialisation step) and followed by one hold at the end (final hold).

➢ **Initialization step**

This step consists of heating the reaction to a temperature of 94-98°C, which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR (Sharkey *et al.*, 1994).

➢ **Denaturation**

This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. During denaturation, the DNA double strand melts open to single stranded DNA by disruption of the hydrogen bonds between complementary bases of the DNA strands.

➢ **Annealing (hybridisation)**

The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Ionic bonds are constantly formed and broken between the single-stranded primer and the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-DNA template hybrid and begins DNA synthesis.

➢ **Elongation**

a) Extension/Elongation step

The temperature at this step depends on the DNA polymerase which is used. Taq polymerase has its optimum activity temperature at 75-80°C (Chien *et al.*, 1976; Lawyer *et al.*, 1993). Therefore, commonly a temperature of 72°C is used with this enzyme. During this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in the 5' to 3' direction *(Figure 7.7)*. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.

b) Final elongation step

This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

➢ **Final Hold**

This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

PCR Detection

➢ **Agarose gel electrophoresis**

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA, RNA, or protein molecules according to their sizes. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate further than longer ones (Sambrook and Russel, 2001) *(see also 7.3.1.1-SDS electrophoresis)*.

To check whether the PCR generated the anticipated DNA fragments agarose gel electrophoresis is employed for size separation of the PCR products. The PCR product size is determined by comparison with a DNA ladder, which contains DNA fragments of known size. The marker is run on the gel alongside the PCR products. Ethidium bromide (EtBr) is used as a nucleic acid stain for techniques such as agarose gel electrophoresis as it binds to nucleic acids. When exposed to ultraviolet light, it will fluoresce with a red-orange colour, intensifying almost 20-fold after binding to DNA. Ethidium bromide may be a very strong mutagen, and may possibly be a carcinogen or teratogen (Huang and Fu, 2005) therefore must be handled with great care.

7.3.2.2 Experimental Protocol

RNA Isolation

Fresh rat aortic tissues were taken from male Sprague-Dawley rats (250-300g) *(Chapter 2)* and frozen in liquid N_2 and stored at -80°C until use. The rat aortic tissues were homogenized (Polytron) in TRI®-Reagent™ (1ml per 5-100mg of tissue). TRI®-Reagent™ is an RNA/DNA and protein isolation reagent and is a mixture of guanidine thiocyanate and phenol in a mono-phase solution (Chomczynski and Sacchi, 1987). The sample was left for 5min at room temperature

(22°C). 0.2ml 1 Bromo-3-chloropropane per ml of TRI®-Reagent™ were added to the mixture for separation of the RNA from the DNA and protein and left for 15min at room temperature (22°C) after shaking for 15 seconds. After centrifugation (12000g, 15 minutes, 4°C) the mixture was separated into 3 phases. A red organic phase (contains protein), an interphase (contains DNA) and a colourless upper aqueous phase which contains RNA. The aqueous phase was transferred to a fresh tube and 0.5ml isopropanol per ml of TRI®-ReagentTM was added. The mixture was left for 5 minutes at room temperature (22°C). After centrifugation (12000g, 10 minutes, 4°C), the pellet was washed in 1ml of ethanol (75%) per 1ml TRI®-ReagentTM. After centrifugation (7500g, 4 minutes, 4°C) the pellet was left to dry air for approximately 10min. The pellet was resuspended in 100µl sterile water *(see Sigma TRI®-ReagentTM product information sheet)*. The absorbance of the rat aortic RNA sample was measured at A_{260} and A_{280} using a spectrophotometer.

A_{260} and A_{280} measurements

A_{260} = RNA
A_{280} = DNA / Protein

Total A_{260} = OD x dilution factor
Conc (µg/ml) = Total A_{260} x 40

Yield = Conc x Final Volume

$$\text{Purity} = \frac{A_{260}}{A_{280}} = 1.7 \rightarrow 2$$

↳ RNA purity (contamination with small amounts of DNA possible)

OD = optical density (absorbance)
40 = Standard factor (OD for 1mg/ml of a single stranded RNA)

DNase treatment of RNA samples

Prior to the RT-PCR, the rat aortic messenger RNA (mRNA) sample (0.63µl=1µg mRNA) was DNase-treated (RNase-Free DNase Kit) to remove any genomic DNA. For DNase treatment of the rat aortic RNA sample, RNA-Qualified (RQ) 1 RNase-Free DNase was used according to the manufacturers' instructions. RNase-Free DNase is a DNase 1 (endonuclease) that degrades both double-stranded and single-stranded DNA *(see Promega RQ1 Rnase-Free DNase Kit product information)*. To avoid any RNAse contamination during PCR sample preparation, laboratory gloves were worn and DNA/RNA-free water, sterile PCR tubes and pipette tips were used.

Reverse Transcriptase (RT)-Reaction

After DNase treatment of the rat aortic mRNA sample (0.63µl=1µg mRNA), complementary DNA was immediately made from the whole mRNA template using dNTPs (10mM each), 5x reaction buffer, recombinant RNase inhibitor (40units/µl), an oligo $(dT)_{18}$ primer (20µM) and an RNA-dependent DNA polymerase (Moloney murine leukaemia virus (MMLV) reverse transcriptase) (200units/µl) *via* reverse transcription (RT) according to the manufacturers' instructions. The Advantage RT-for-PCR Kit with the MMLV reverse transcriptase was used *(see BD Biosciences Advantage™ RT-for-PCR Kit user manual).*

Polymerase Chain Reaction (PCR)

cDNA was produced as detailed above from DNase-treated mRNA from isolated rat aortic tissues. PCR reactions were carried out using a Uno-Thermoblock™ in accordance with the manufacturer's instructions (Biometra biomedizinishe Analytic GmbH, Germany) using sense and antisense primers for *Rattus norvegicus* TAAR1, *Thermus aquaticus* (TAQ)- polymerase, dNTPs, 10xNH_4 buffer and $MgCl_2$.

Reagents	Concentrations per sample (in 24µl)
dNTPs	0.25µM
10x Buffer NH_4 Buffer $(NH4)_2$ SO_4 Tris-HCl	 16mM 70mM
$MgCl_2$ (50mM/ml)	1.5mM
TAAR1 sense and antisense primer (50nM/µl of sample)	2nM
TAQ-polymerase (5U/µl)	3U
Sterile Water	make up to volume

β-actin was used as a control to normalize for differences in the amount of mRNA present in each sample. β-actin is ubiquitously expressed in all cells and serves as a positive control for mRNA (cDNA) quantitative expression studies (Gene Link, 2008). Furthermore, control reactions where the MMLV enzyme was left out of in the RT reaction or where sterile water was used in the PCR reaction instead of the RT reaction were performed.

PCR procedure

Rat aortic cDNA (1µl) obtained from DNAse pre-treated RT reactions as explained above was amplified for 40 cycles using the following cycling parameters.

Initialization:	94°C for 120 sec	
Denaturation:	94°C for 60 sec	40 Cycles
Annealing:	61°C for 30 sec	
Elongation:	72°C for 60 sec	
Elongation:	72°C for 600 sec	1 Cycle
Final Hold:	4°C (for an indefinite time)	

➢ **Primer Design**

Specific sense and antisense primer pairs for rat β-actin and rat TAAR1 were designed using two different databases, *Primer3* and *BLAST*. The primers did not recognize any other sequences in the database (data not shown).

Primer	Sequences (5'- 3')
Rat TAAR1 sense primer	ATT GGA AGG GGA AAG CAG AG
Rat TAAR1 antisense primer	GGT TAG AGG CAG AGT TCA GG
Rat β-actin sense primer	GAG AGG CAT CCT GAC CCT GA
Rat β-actin antisense primer	ATC ACA ATG CCA GTG GTA CG

PCR Detection

After completing the PCR procedure, the samples were studied to determine whether the PCR had generated the anticipated DNA fragment. The PCR products were separated according to their sizes *via* agarose gel (2%) electrophoresis. To each PCR sample an appropriate volume of Blue/Orange 6X Loading Dye was added before 20µl of each sample was loaded on the gel. To determine the size of the anticipated DNA fragment, a DNA ladder (4µl), which contains DNA fragments of known sizes was run on the gel parallel to the PCR products.

The gel, containing 100nM ethidium bromide, was placed in TAE-buffer (see Appendix) in a tank and an electrical current was applied. The electrophoresis process was completed after approximately 45 minutes. Afterwards, the gel was

analysed on a UV transilluminator, photographed and scanned *(TyphoonTM 9410 Phosphoimager, GE Healthcare, Amersham, UK).*

7.3.2.3 Analysis of Results

Sequencing

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. To find out the exact nucleotide sequence of the DNA fragments obtained the band were excised from the agarose gel kept in 30µl distilled water overnight and then sequenced. The sequencing progress was done by the service at the Heath Hospital (Cardiff, UK) *(Figure 7.10).*

7.3.2.4 Drugs Used

- **Reagents obtained from Bioline GmbH (Luckenwalde, Germany)**

BIOTAQ Core Kit (500U)

- **Reagents obtained from Sigma Aldrich**

TRI®-Reagent™, 2-Propanol (Isopropanol), sterile RNA/DNAse free water, sterile mineral oil, ethidium bromide (10mg/tablette), 1-Bromo-3-Chlorpropane

- **Reagents obtained from Clontech-Takara Bio Europe (Saint-Germain-en-Laye, France)**

Advantage™ RT-for-PCR Kit

- **Reagents obtained from Promega Corporation (Southampton Science Park Southampton, UK)**

RQ1 RNase-Free DNase, Blue/Orange 6X Loading Dye, Bench Top 1kb DNA Ladder, Ethylenediaminetetraacetic acid (EDTA) solution (0.5M, pH to 8.0)

- **Reagents obtained from Invitrogen Ltd. (Paisley, UK)**

TAAR1 sense and antisense primers, β-actin sense and antisense primers

- **Reagents obtained from Fisher Scientific UK Ltd**

Agarose (for routine electrophoresis), Glacial acetic acid

- **Reagents obtained from Elkay Laboratory Products (Basingstoke, Hampshire, UK)**

PCR tubes (0.2ml, thin wall, DOMED CAP)

7.4 Results

7.4.1 Western Blotting

TAAR1 was identified in rat aortic tissues *via* Western Blotting. The mean molecular weight of the band was 40.3±1.1kDa (n=3) *(Figure 7.8)*. In some of the experiments weak bands in the rat aorta were seen but with lower molecular weights (not all shown in Figure 7.8)

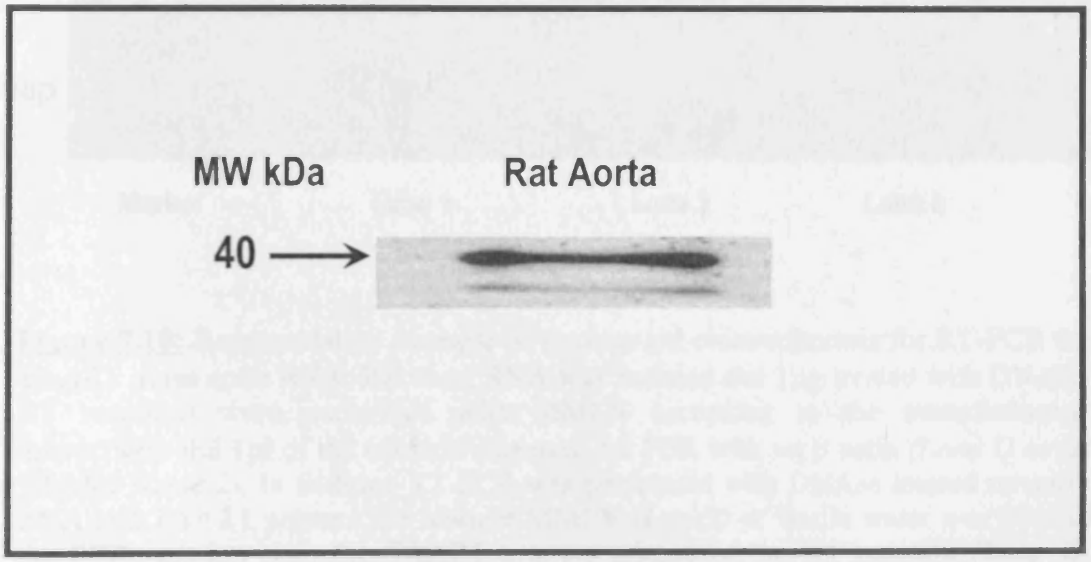


Figure 7.8: Western Blot
Representative example of a Western blot for TAAR1 in rat aorta. 50μg of rat aorta membranes was separated on a 10% polyacrylamide gel and TAAR1 was detected using a rabbit polyclonal anti-human antibody, LS-A2041. The band was visualised using ECL. In the rat aorta sample a band appeared with a molecular weight of 40kDa (n=3).

7.4.2 RT-PCR

Agarose gel electrophoresis

The PCR products were separated according to their sizes *via* agarose gel electrophoresis *(Figure 7.9)*. PCR of rat aortic samples with TAAR1 primers produced a band of the expected size (200bp) *(Figure 7.9, Lane 2)*. The integrity of the samples was confirmed using β actin primers which also produced a band of the expected size (250bp) *(Figure 7.9, Lane 1)*. Furthermore, control reactions where the MMLVenzyme was left out of the RT reaction *(Figure 7.9, Lane 3)* or where sterile water was used in the PCR reaction instead of the RT reaction *(Figure 7.9, Lane 4)* did not produce any bands with the TAAR1 primers.

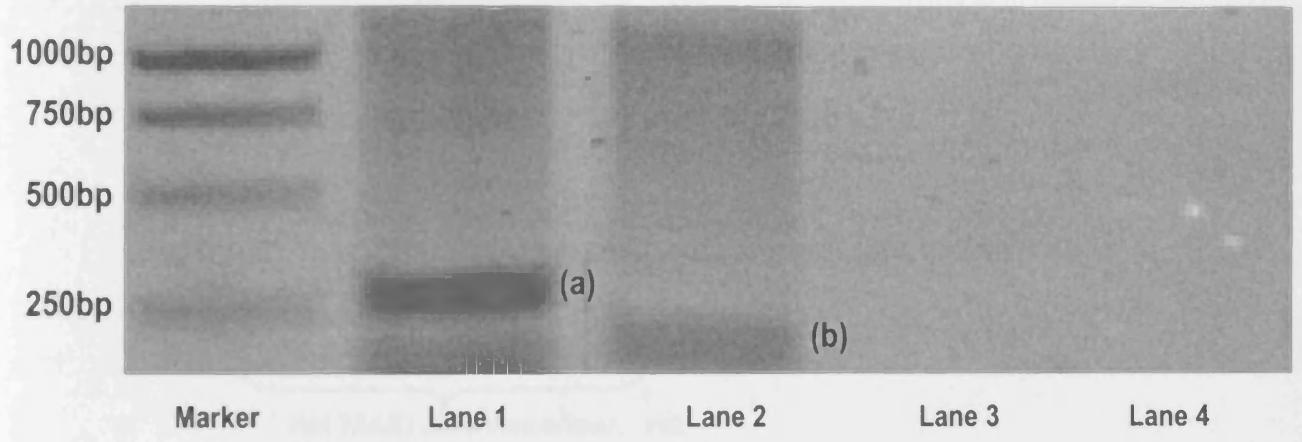


Figure 7.10: Representative example of agarose gel electrophoresis for RT-PCR for TAAR1 in rat aorta RNA. Rat aorta RNA was isolated and 1μg treated with DNAse. RT reactions were performed with MMLV according to the manufacturers' instructions and 1μl of the reaction was used for PCR with rat β actin *(Lane 1)* or rat TAAR1 *(Lane 2)*. In addition RT-PCR was performed with DNAse-treated rat aorta RNA and TAAR1 primers but without MMLV *(Lane3)* or sterile water was used in the PCR reaction with the TAAR1 primers instead of the RT reaction *(Lane 4)*. Bands of the expected sizes for β actin (250bp) **(a)** and TAAR1 (200bp) **(b)** were seen (n=3).

The sequence obtained was compared using BLAST to the database. The sequence obtained for TAAR1 were compared using BLAST to the database and found to be identical to the expected sequence for *Rattus norvegicus*. Below the sequence for rat TAAR1 obtained from the PCR band *(Figure 7.10, Lane 2)* is shown from base 693-892 (*Figure 7.8*). The missing and non-identical bases are probably due to the low level of cDNA for the TAAR1 and sequencing errors.

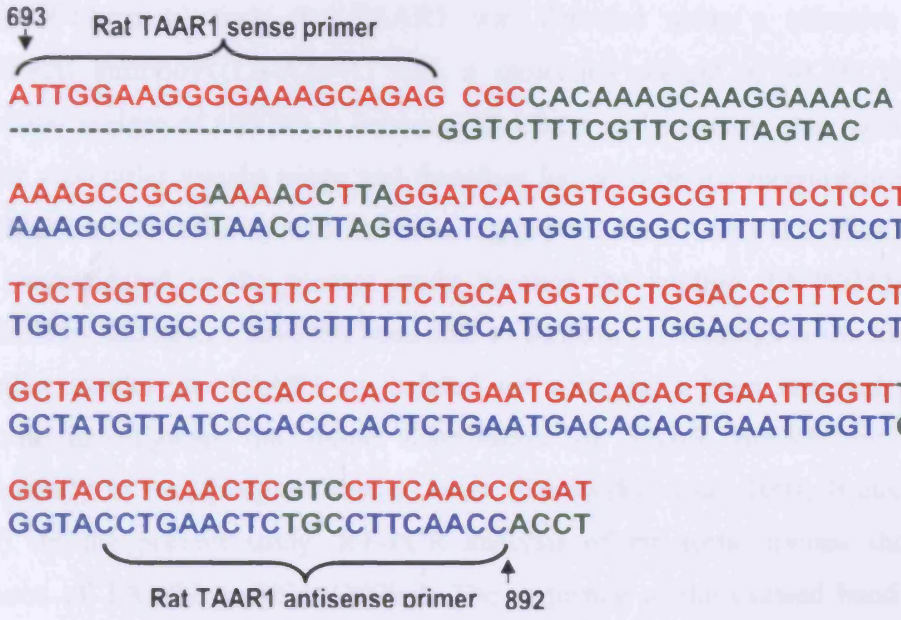


Figure 7.10: Sequencing data
Part of the cDNA sequence of *Rattus norvegicus* TAAR1 is shown. The sequence of the band excised from the gel (Blue) *(Figure 7.9, Lane 2)* matched with the majority of the sequence of the region from bases 693 to 892 (Red, Primer3 output, BLAST database). Green = non-identical bases; ---- no sequencing data available. n = 1.

7.5 Discussion

Currently, little is known about the existence of TAARs in smooth muscle tissues. The pharmacological effect of TAs on TAARs has mostly been investigated on cloned receptors in transfected cell lines (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). I am not aware of any studies where the presence of TAARs in rat aortic tissues has been determined.

In the present study, the presence of TAAR1 in rat aortic tissues was investigated. Using Western blotting, the TAAR1 was detected using a selective TAAR1 polyclonal antibody (LS-A2041) with a molecular weight of 40.3±1.1kDa. The molecular weight of GPCRs is between 35-45kDa and therefore this band is in the correct molecular weight range and therefore likely to be the receptor protein. The specificity of the antibody with the blocking peptide for TAAR1 (LS-P2042) has not been investigated in the present study because the peptide (LS-P2042) was no available by the time. I have not been able to confirm my findings in the literature as no other studies on TAAR1 in isolated rat aorta have been reported. Previous workers investigated the tissue distribution of TAAR mRNA by RT-PCR successfully in rat *(Sprague Dawley)* brain (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). In the present study, RT-PCR analysis of rat aortic tissues showed the presence of TAAR1 m-RNA (200bp). The sequence of the excised band matched with the sequence of TAAR1 in the database. However, it is necessary to repeat the sequencing to confirm the data as sequence errors seen. Thus, the receptor mRNA is clearly present in the tissue.

To discount the possibility of experimental error, different control experiments were performed to validate the results. A β-actin band (250bp) was detected and its relative position to TAAR1 used to prove that the product with smaller size the (200bp) was isolated. β-actin, a housekeeping gene, is widely accepted in the literature as a loading control in molecular biology techniques including PCR and Western blotting and also shows the integrity of RNA sample (Gilliland *et al.*, 1990). Two control experiments showed that the RT-PCR process was performed correctly and validated the results. Firstly, the exclusion of the MMLV enzyme from the RT reaction showed that no contaminating DNA was present after the DNAse treatment.

Therefore, the band seen can only have been amplified from cDNA from the RT reaction. Secondly the use of sterile water in the PCR reaction demonstrated that no DNA contamination was present in any of the components used.

7.6 Conclusion

In the present *Chapter*, the presence of TAAR1 was successfully demonstrated for the first time in rat aorta by two methods, Western blotting (receptor protein) and RT-PCR (mRNA). These results show that at least one TAAR is found in rat aorta and therefore may explain vasoconstriction seen to TAs in aortic tissues. The molecular biology confirmation of the presence of TAAR1 and the pharmacological findings regarding the effect of TAs in rat aortic rings can be used in further studies to investigate the mechanism of action of TAs in rat smooth muscle.

Chapter 8

GENERAL DISCUSSION

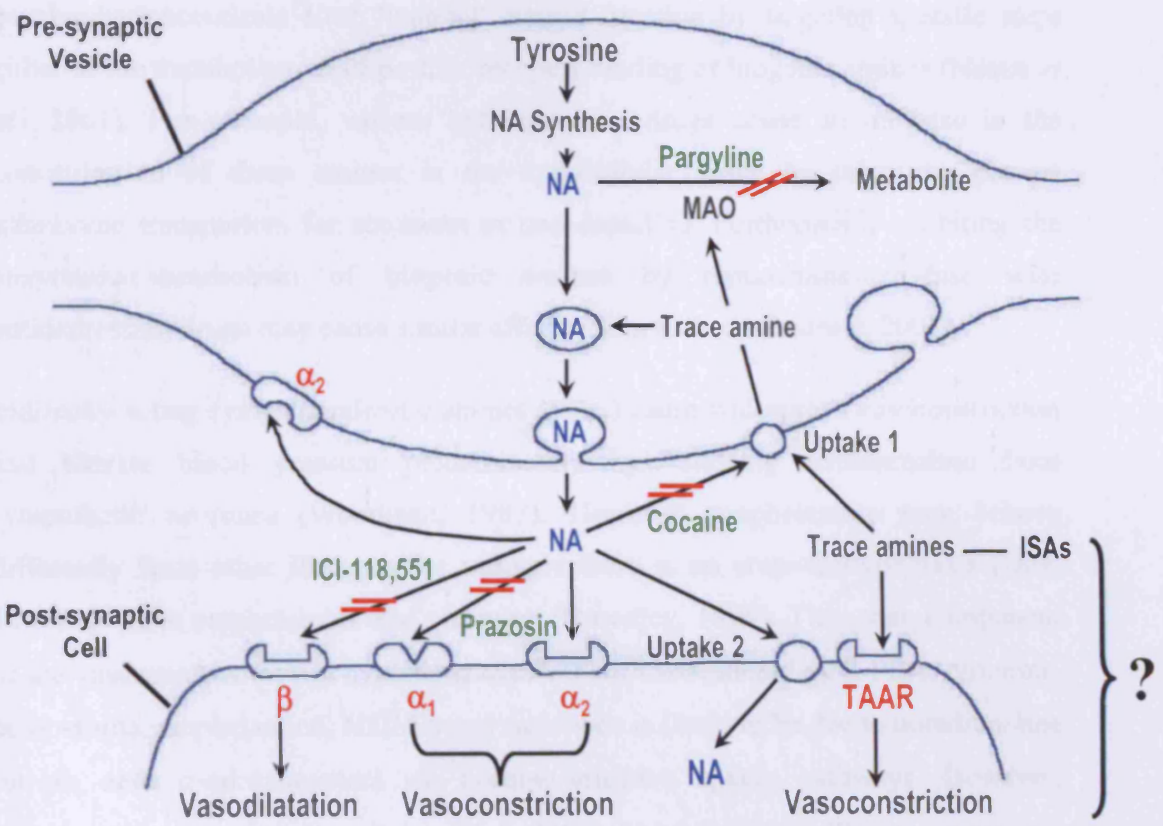
Chapter 8

8.1 General Discussion

The aim of my thesis was to provide a wider understanding of the mechanisms and roles of TAs in the cardiovascular system. This research is particularly relevant because of the increasing exposure to TAs from the consumption of recreational drugs, such as MDMA and cathinone, biosynthesis from endogenous sources, such as octopamine and dietary sources, like tyramine from cheese and β-PEA in chocolate.

Very little is known about the relationship between TAs and vasoconstriction in isolated tissues. Therefore, work in *Chapters 2 and 3* used β-PEA as a standard TA to clarify the mechanism of vasoconstriction of TAs in aortic tissues. In *Chapter 2* it was shown that various factors, such as the presence, or absence of endothelium, blocking α_1- and β-adrenoceptors or blocking Uptake1 did not affect the contractile response to β-PEA in rat aortic rings. The endothelium therefore cannot be a source of vasoconstrictors responsible for the response to β-PEA as the absence of the endothelium did not reduce the contractions to β-PEA. Thus, to clarify the role of α- and β- adrenoceptors and ISA in the vasoconstrictor action of β-PEA, the findings in *Chapter* 2 were then developed in *Chapters 3 and 4*. To eliminate possible the sympathomimetic responses involved in the contractile response to β-PEA, a combination of "inhibitors" (prazosin, ICI-118,551, pargyline and cocaine) was employed *(Figure 8.1, Chapter 4)*. These did not inhibit the vasoconstriction by β-PEA. Therefore, neither α- and β-adrenoceptors, nor ISA stimulation were responsible for the vasoconstriction in rat aorta and therefore TAs might cause vasoconstriction *via* direct stimulation. These "inhibitors" were also used to investigate the contractile responses of other TAs, including tyramine, octopamine, amphetamine and its derivatives, such as MDMA and cathinone *(Chapter5)*. As neither α- and β-adrenoceptors, nor ISA stimulation could explain the vasoconstriction seen with TAs in rat aorta, it is likely that β-PEA and other TAs exert this effect directly by activation of a novel receptor system, probably the TAARs *(Figure 8.1)*.

The identity of the TAARs involved, TAAR1 or other subtypes, remains to be established. In the present work in rat aortic tissue, TAAR1 was successfully identified *via* Western blotting and polymerase chain reaction (PCR) *(Chapter 7)*. So this receptor could be involved in the vasoconstrictor response to TAs.



Trace amines – Effect as indirectly acting sympathomimetic amines

It is thought that TAs, including β-PEA and tyramine, stimulate adrenergic receptors indirectly (Black *et al.*, 1980). Thus, TAs promote the efflux of catecholamines from sympathetic neurons and adrenal glands. β-PEA and tyramine are able to deplete neurotransmitters from their storage vesicles and therefore compete with neurotransmitters for uptake (Black *et al.*, 1980). Furthermore, these TAs stimulate outward neurotransmitter flux through plasma membrane carriers in a manner similar to amphetamines (Seiden *et al.*, 1993; Amara and Sonders, 1998). A wide range of psychopharmaceuticals alter "indirect" neural function by targeting specific steps either in the metabolism, transport, or receptor binding of biogenic amines (Nester *et al.*, 2001). For example, various antidepressant drugs cause an increase in the concentration of these amines in the extracellular space by inhibiting plasma membrane transporters for serotonin or noradrenaline. Furthermore, inhibiting the enzymatic metabolism of biogenic amines by monoamine oxidase with antidepressant drugs may cause similar effects (Kim and von Zastrow, 2001).

Indirectly acting sympathomimetic amines (ISAs) cause widespread vasoconstriction and elevate blood pressure predominately by releasing noradrenaline from sympathetic neurones (Woodman, 1987). However, amphetamine may behave differently from other ISAs, as for example there is no cross-tachyphylaxis (Day, 1967) between amphetamine and tyramine (Broadley, 1996). Thus, one component of the vasoconstriction and hypertensive effects of ISAs, including β-PEA, tyramine, octopamine, amphetamine, MDMA and cathinone is likely to be due to noradrenaline release onto α-adrenoceptors *via* cocaine-sensitive uptake pathways. However, referring to previous studies (Baker *et al.*, 2007) TAs including β-PEA and tyramine, amphetamine and other psychostimulant drugs, such as MDMA and cathinone might also act as direct agonists on TAARs and therefore cause vasoconstriction in coronary and peripheral blood vessels *(Figure 8.1)* .

Trace amines – effect on TAARs

MDMA and cathinone are recreational psychotropic agents for which cardiovascular actions may pose serious health problems for individuals using them on a regular basis or who abuse them. The use of MDMA has increased over the past decade (Ramsay *et al.*, 2001; Landry, 2002) and the chewing of the cathinone-containing leaf, khat, has spread from East Africa to the UK (Brenneisen *et al.*, 1986; Brenneisen *et al.*, 1990; Al-Motarreb *et al.*, 2002b). Until recently, there has been no information on the coronary vascular actions of MDMA and cathinone, but studies have now demonstrated coronary and peripheral vasoconstrictor effects in guinea-pig hearts and aorta and pig coronary vessels (Al-Motarreb *et al.*, 2000; Al-Motarreb *et al.*, 2002b; Baker and Broadley, 2003). These contractile responses are not mediated *via* any known receptor. In view of the literature on putative receptors for TAs and the structural similarity between tyramine, β-PEA, MDMA, cathinone and amphetamine, these TAARs may be their site of action.

In the present work, different TAs produced varying contractile responses in isolated aortic tissues in the presence of "inhibitors" *(Chapter 5, Table 8.1)*. In rat aortic tissues β-PEA produced much larger responses than tyramine while the responses to D-amphetamine and octopamine were intermediate *(Table 8.1)*. In guinea-pig aorta the contractile responses in general seemed to be stronger to TAs compared to rat aorta. β-PEA produced the largest response followed by tyramine and D-amphetamine. Octopamine produced the lowest contractile response *(Table 8.1)*. These findings suggest that in isolated aortic tissues there may be more than one TAAR present and that the distribution of different TAARs in rat and guinea-pig aorta differs. Alternatively there may be one receptor for which various TAs have different affinities and efficacies and the efficacy of tyramine in the rat is particularly weak *(Tables 8.1 and 8.2)*. These findings suggest species variations in the distribution of receptors or efficacy *(Chapter 5)*. Furthermore, in rat aortic tissues the activity and potency orders for D-amphetamine and octopamine were not analogous *(Tables 8.1 and 8.2)*. D-amphetamine is more active than octopamine *(Table 8.1)* but octopamine was more potent than D-amphetamine *(Table 8.2)*. In guinea-pig aortic tissues, the activity and potency orders for D-amphetamine and β-PEA were also not analogous. D-amphetamine was the most potent and β-PEA the least potent agonist

but β-PEA was the most active agonist. In addition, tyramine was significantly more active in guinea-pig compared to the rat tissue but not significantly more potent.

Agonist	Rat Aorta – Mean maximum contraction (M)	GP Aorta – Mean maximum contraction (M)
β-PEA	138.2±23.1	158.2±39.7
D-Amphetamine	75.8±30.3	86.3±24.3
Octopamine	73.6±35.3	55.9±10.7
Tyramine	0.7±14.0	95.1±23.6

Table 8.1: The contractile effects (mean maximum contraction (±SEM) %-KCl) of various TAs in rat and guinea-pig aortic tissues. In rat aorta, β-PEA produced the largest response and tyramine the smallest. In GP aorta, β-PEA produced the largest response followed by tyramine and D-amphetamine. Octopamine produced the smallest contractile response but this was not significantly different. tyramine was significantly more active in GP compared to the rat tissue ($P<0.01$). Mean responses (±SEM) were compared unpaired Student's t-test.

Agonist	Rat Aorta – Mean vasoconstriction –log EC_{50} values (M)	GP Aorta – Mean vasoconstriction –log EC_{50} values (M)
β-PEA	4.46±0.15	4.22±0.21
Octopamine	4.36±0.17	4.98±0.23
D-amphetamine	3.76±0.34	4.86±0.32
Tyramine	3.71±0.29	4.37±0.36

Table 8.2: The potency order (mean –log EC_{50} values (±SEM)) of various TAs in rat and guinea-pig (GP) aortic tissue. In rat aorta, β-PEA was the most potent agonist and tyramine the least potent. In GP aorta, D-amphetamine was the most potent agonist. tyramine was not significantly ($P>0.05$) more potent in GP aorta compared to the rat tissue. Mean responses (±SEM) were compared by the unpaired Student's t-test.

Comparison of the effect of TAs in aortic tissue and cloned TAAR1 transfected cell lines

The information available in the literature about TAARs is predominately based on their discovery and functional expression in transfected cell lines. The stimulation of cloned rat TAAR1 by TAs resulted in the generation of cAMP *via* activation of $G\alpha_s$ (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001). The cloned rat TAAR1 is potently activated by tyramine and β-PEA and displays low affinity for tryptamine,

octopamine and dopamine *(Table 8.3)* (Borowsky *et al.*, 2001).

In contrast, the TAAR subtypes by which TAs cause vasoconstriction in aortic tissues cannot be determined from the present study. Although the presence of TAAR1 was identified by molecular biological techniques in rat aorta *(Chapter 7)*, the possibility must be considered that other TAAR subtypes are also present in the aortic tissue. Furthermore, apart from the possible activation of TAARs in aortic tissues, it must be considered that other factors could be involved in the vasoconstriction as TAs also release other endogenous vasoconstrictors, such as angiotensin or endothelin. In TAAR1-transfected cell lines, only rat TAAR1 was cloned and the cAMP level was measured. Therefore, a direct comparison between the studies in cloned receptor-transfected cell lines and the present work in isolated aortic tissue is not possible. However, the results from the previous study (Bunzow *et al.*, 2001) can be used to suggest a potency order for different TAs to activate the novel transfected receptor system *(Table 8.3)*.

Agonist	Cloned rat TAAR1 transfected cell lines - cAMP accumulation -log EC_{50} values (M) (Bunzow *et al.*, 2001)	Rat Aorta – Mean vasoconstriction -log EC_{50} values (M)
Tyramine	7.16	3.71±0.29
β-PEA	6.62	4.46±0.15
D-amphetamine	6.36	3.76±0.34
Octopamine	5.89	4.36±0.17

Table 8.3: The potency order (mean –log EC_{50} values) of various trace amines (TAs) in cloned TAAR1 transfected cell lines and in isolated rat aorta. In the TAAR1 transfected cell lines the accumulation of cAMP upon exposure to TAs was measured and in isolated rat aorta the contractile responses to various TAs were determined. In rat aorta, in the presence of "inhibitors" β-PEA was the most potent agonist and tyramine the least potent. In the TAAR1 transfected cell lines, tyramine was the most potent agonist while octopamine was the least potent (Bunzow *et al.*, 2001; Lewin, 2006).

The potency orders for TAs, including β-PEA, tyramine, D-amphetamine and octopamine in rat aorta and TAAR1 transfected cell lines are different. The (-) log EC_{50} values show at least 2 log difference between cloned TAAR1 and rat vasoconstriction. Therefore, there must be a weak amplification of signal or reduced receptor numbers in rat *(Table 8.3)*. In rat aorta β-PEA was the most potent agonist

and tyramine the least potent. In TAAR1 transfected cell lines, tyramine was the most potent agonist (Bunzow *et al.*, 2001) *(Table 8.3)*. When comparing the potent order within cloned TAAR1 transfected cell lines and rat aorta *(Table 8.3)*, the data suggest that octopamine is a less potent agonist in the TAAR1 transfected cell lines than in rat aorta. In rat aorta more than one TAAR subtype might be present, so octopamine could be activating several receptors. As a consequence, the potency order for octopamine in rat aorta is higher than in the cloned TAAR1 transfected cell lines *(Table 8.3)*. The cloned TAAR1 in transfected cell lines was strongly stimulated by tyramine, which suggests a high sensitivity for tyramine for this receptor. In contrast, in rat aortic tissues, tyramine in the presence of "inhibitors" showed a minor contractile response *(Table 8.3)*. This result suggests a low tyramine sensitivity in rat aortic tissues. Until now, no antagonists for TAs were reported and also the effects of tyramine or any other TAs as an antagonist of the other TAs have not been investigated. In the present study tyramine was identified as the first potential partial agonist of TAs in isolated rat aortic tissues as tyramine antagonizes the contractile effects of other TAs, including D-amphetamine and octopamine, at TAARs *(Chapter 6)*.

Amphetamine and its derivatives

Amphetamine and its derivatives were found to be potent agonists of cloned TAAR1 in transfected cell lines. The amphetamine-derivative MDMA was only slightly less potent than amphetamine (Bunzow *et al.*, 2001; Zucchi *et al.*, 2006). In TAAR1 transfected cell lines (-) 4-OH-amphetamine was tested and found to be the most potent TAAR1 agonist *(Table 8.4)* (Bunzow *et al.*, 2001).

In the present study the contractile response to both stereoisomers of 4-OH-amphetamine were tested but EC_{50} values were not obtained for (-) or (+) 4-OH amphetamine as both stereoisomers were only used in a single dose due to very limited supply *(Table 8.4)*. Compared to the studies in receptor-transfected cell lines in the present study a different potency order was seen with MDMA being more potent than D-amphetamine *(Table 8.4)*. As the contractile responses to (+) and (-) 4-OH-amphetamine (300μM) were not significantly different ($P>0.05$) from the same dose of D-amphetamine (300μM) *(Figure 5.20)* this suggests an equivalent potency for both stereoisomers of 4-OH-amphetamine and D-amphetamine *(Table 8.4)*.

Agonist	Cloned rat TAAR1 transfected cell lines - cAMP accumulation -log EC_{50} values (M) (Bunzow *et al.*, 2001)	Rat Aorta – Mean vasoconstriction -log EC_{50} values (M)
4-OH-amphetamine	7.29	~3.8
β-PEA	6.61	4.46±0.15
D-amphetamine	6.36	3.76±0.34
(±)-MDMA	5.77	4.34±0.08

Table 8.4: The potency order (mean –log EC_{50} values) of amphetamine and its derivatives including (-) 4-OH-amphetamine and MDMA, compared to β-PEA in cloned rat TAAR1-transfected cell lines (Bunzow *et al.*, 2001) and in isolated rat aorta. In TAAR1-transfected cell lines the accumulation of cAMP upon exposure to TAs was measured and in rat aorta the contractile responses to various TAs were calculated. In TAAR1-transfected cell lines (-) 4-OH-amphetamine was the most potent amphetamine derivative agonist and (±) MDMA was the least potent (Bunzow *et al.*, 2001). In rat aorta amphetamine and MDMA were not significantly ($P>0.05$) less potent compared to β-PEA. Mean responses (±SEM) were compared by Analysis of ANOVA followed by the "post hoc" Dunnett test. EC_{50} values for (+) and (-) 4-OH-amphetamine were not obtained as a single dose experiment was performed to investigate the vasoconstriction in the tissue. However, as the contractile responses of both stereoisomers of 4-OH-amphetamine were not significantly different ($P>0.05$) from the contractile response of D-amphetamine an equivalent potency is suggested.

Agonist	Rat Aorta - Vasoconstriction (%-KCl) for 300μM
β-PEA	138.3±23.1
MDMA	54.2±15.8
D-amphetamine	45.8±20.8
(-) Methamphetamine	33.3±145.3
(+) 4-OH-amphetamine	22.2±6.4

Table 8.5: Comparison of the contractile effects (mean maximum contraction (±SEM)) of amphetamine and its derivatives, including MDMA, (-) methamphetamine and (+) 4-OH-amphetamine at 300μM compared to β-PEA. (+) 4-OH-amphetamine (300μM) was not significantly ($P<0.05$) less active as a vasoconstrictor than the other amphetamine-derivatives. Mean responses (±SEM) were compared by Analysis of ANOVA followed by the "post hoc" Bonferroni test.

The effect of vasoconstriction in aortic tissue of two further amphetamine-derivatives, cathinone and Ritalin®, were studied alongside the (+) and (-) stereoisomers of methamphetamine *(Chapter 5)*. Ritalin® is the most common drug for the treatment of ADHD (Wilson *et al.*, 1984; Greenhill *et al.*, 2002). However, among the side-effects of Ritalin® are its cardiovascular effects, such as increased heart rate and blood pressure. These side-effects become more serious because of unintentional overdoses, medication errors and overdose caused by abuse or suicide (Ballard *et al.*, 1976; Brown *et al.*, 1984). Cathinone, which is the major active compound of khat leaves (Brenneisen and Geisshusler, 1985; Kalix and Braenden, 1985), has been found to cause cardiovascular effects such as increasing heart rate and blood pressure (Gugelmann *et al.*, 1985; Kalix and Braenden, 1985). It was also reported that cathinone causes vasoconstriction in isolated guinea-pig aortic tissues and coronary artery (Al-Motarreb *et al.*, 2000; Al-Motarreb and Broadley, 2003; Al-Motarreb, 2004).

The binding affinities of Ritalin®and cathinone in cloned TAAR1 transfected cell lines were not investigated by Borowsky et al., (2001) and Bunzow et al., (2001). In isolated aortic tissues, both amphetamine-derivatives caused vasoconstriction in the presence of "inhibitors". Therefore, the contractile responses in aortic tissues are likely to be based on activation of TAARs *(Figure 8.1)*. Methamphetamine is the most widely used illegal drug in the world according to the United Nations (Anglin *et al.*, 2000; Iversen, 2006). Given the structural similarity of amphetamine to TAs, this amphetamine analogue was tested and found to be a potent agonist for TAAR1 in transfected cell lines (Zucchi *et al.*, 2006; Reese *et al.*, 2007). In isolated aortic tissues, both the (+) and (-) enantiomers of methamphetamine were tested in the presence of "inhibitors". The contractile responses to (-)-methamphetamine was comparable with the contractile response to D-amphetamine. Therefore, the contractile response to (-)-methamphetamine in aortic tissues is likely to be based on activation of TAARs *(Figure 8.1)*.

In summary, compared to β-PEA in the presence of "inhibitors", amphetamine, (-)-methamphetamine, MDMA, cathinone and Ritalin® showed a weak contractile response in isolated aortic tissues *(Table 8.6)*. Ritalin® produced the lowest response followed by cathinone and MDMA. Furthermore, the synthetic amphetamine, (-)-

methamphetamine and D-amphetamine were the most active vasoconstrictors of the group of amphetamine-derivatives *(Table 8.6)*. The varying contractile responses in rat aortic tissues to amphetamine-derivatives confirm my earlier theory that aortic tissues may either contain more then one TAAR or more than one receptor for which various TAs have different affinities.

Agonist	Rat Aorta – Mean Maximum Contraction (M)
β-PEA	138.2±23.1
(-)-Methamphetamine	83.3±16.6
D-amphetamine	75.8±30.3
(±) MDMA	54.2±15.8
Cathinone	45.2±12.9
Ritalin®	35.0±61.2

Table 7.6: The contractile effects (mean maximum contraction (±SEM)) of amphetamine and its derivatives compared to β-PEA and in isolated rat aorta. Amphetamine and its derivatives were all less active compared to β-PEA. Ritalin® is the least active vasoconstrictor followed by cathinone and MDMA. (-)-Methamphetamine and D-amphetamine are the most active vasoconstrictors of the group of amphetamine-derivatives compared to β-PEA.

Trace amines - signal transduction mechanism

In TAAR1 transfected cell lines, the most commonly recognized signal transduction pathway for TAARs is to stimulate the formation of cAMP *(Chapter 1)*. TAAR1 stimulates adenylate cyclase through the α subunit and mediates an increase in cAMP (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001; Lewin, 2006). In the present study, the responses to TAs in isolated rat aorta do neither support the definition of TAAR1 and TAAR4 (Borowsky *et al.*, 2001; Bunzow *et al.*, 2001) or the signal transduction pathway *via* activation of $G\alpha_s$. The Gs protein coupled pathway in vascular smooth muscle stimulates adenylyl cyclase which catalyzes the formation of cAMP. However, an increase in cAMP in vascular smooth muscle causes relaxation as the myosin light chain kinase (MLCK) is inhibited by cAMP and therefore causes relaxation *(Chapter 1)*

TAs caused vasoconstriction in aortic tissues with different activities, especially tyramine which acted as a very weak vasoconstrictor. The contractile response of rat

and guinea-pig aorta is not consistent with an increase in cAMP. Contractile responses are usually associated with activation of $G\alpha_q$ to stimulate phospholipase C_β and therefore cause an increase in IP_3 and DAG *(Chapter 1)*. Thus, the coupling of the TAAR in rat aorta differs from the cloned receptor.

Trace amines – cardiovascular effects

Hyperactivity disorder, malignant hyperthermia and migraine headache have been suggested to be caused by a dysfunction of TAs (Premont *et al.*, 2001; Branchek and Blackburn, 2003; Lindemann *et al.*, 2005). Furthermore, among the side-effects of amphetamine and its derivatives, including methamphetamine, MDMA, cathinone and Ritalin® are their cardiovascular effects, such as tachycardia, hypertension and cardiovascular mortality *(Chapters 1 and 5)*.These effects become more serious because of unintentional overdoses, medication errors and overdose caused by abuse or suicide (Cleary *et al.*, 2002; Klein-Schwartz, 2002).

The present study has demonstrated that TAs such as β-PEA, tyramine, octopamine, amphetamine and its derivatives, including cathinone, MDMA methamphetamine and Ritalin® have direct vasoconstrictor properties in a major conduction vessel, the aorta. Although the mechanism of vasoconstriction cannot be definitively determined from this study, activation of TAARs in rat aortic tissues are likely to be an explanation for the effect. Historically, the cardiovascular actions of TAs have been attributed to an indirectly-acting sympathomimetic effect. Therefore, the results in the present study are highly relevant regarding the cardiovascular effects of TAs. The vasoconstrictor effect of TAs in aortic tissues might explain their cardiovascular effects, including hypertension and tachycardia. Furthermore, the direct contractile responses of amphetamine-derivatives, cathinone and MDMA could explain the increased incidence of myocardial infarction in khat chewers (Al-Motarreb *et al.*, 2002a; Al-Motarreb *et al.*, 2005) and the occurrence of cardiovascular collapse and sudden death associated with ecstasy use (Dowling *et al.*, 1987; Bedford Russell *et al.*, 1992; Milroy *et al.*, 1996). In the present study Ritalin® produced weaker vasoconstriction in aortic tissues compared to other TAs. However recent reports linking cardiac arrest, tachycardia and cardiac arrhythmia with Ritalin® use might be explained due to the vasoconstrictor properties of Ritalin® in aortic tissues (Lucas *et al.*, 1986; Gracious, 1999; Scientist, 2006).

8.2 Future Work

The mechanism by which trace amines (TAs) cause vasoconstriction in aortic tissue cannot be fully established from the present study. However, it is likely that TAs cause direct vasoconstriction due to activation of TAARs were excluded. Therefore, to determine if the functional responses are *via* TAARs in blood vessels, further studies are needed to evaluate the presence of additional members of the TAAR family in different blood vessels in the rat.

In the present study, tyramine was found to be the first partial agonist of TAs in isolated rat aortic tissues. Tyramine antagonizes the contractile effects of TAs, including β-PEA, D-amphetamine and octopamine, probably at TAARs. It is likely that TAs with a weak contractile response act as partial agonists of the other TAs. Therefore, further studies could focus on the investigation of other partial agonists, as antagonists of the contractile effects of TAs.

In the present study, the presence of TAAR1 in rat aortic tissues was detected. Therefore it is of interest in further studies to detect possible further TAARs in aorta and also other blood vessels.

In the present study, the responses in isolated rat aorta do not support the definitions of the cloned TAAR1 and TAAR4 (Bunzow *et al.*, 2001) because tyramine is a very weak vasoconstrictor in the tissue. Therefore, it is of interest to investigate in further studies, the signal transduction mechanism of TAs that causes vasoconstriction in isolated aortic tissues. Furthermore, it is of interest why various TAs cause different amounts of vasoconstriction in aortic tissues.

The β-PEA skeleton is an essential molecular feature that all TAAR1 agonists share and is present in the decarboxylated skeleton of thyroid hormone molecular derivatives known as thyronamines (Scanlan *et al.*, 2004; Hart *et al.*, 2006). Given the structural similarity between iodothyronamines and other biogenic amines, it follows that iodothyronamines could be agonists of TAARs (Scanlan *et al.*, 2004). In this study nine different thyronamines were synthesized and evaluated in transfected HEK cells stably expressing either the rat or mouse TAAR1. It was found that thyronamine derivatives, including 3-iodothyronamine, thyroxine and

triiodothyronine, induced a concentration-dependent increase in cAMP levels (Scanlan *et al.*, 2004; Hart *et al.*, 2006). Therefore, it would be of interest to evaluate the role of iodothyronamines in the control of vascular tone in different blood vessels.

In vitro studies have shown that hypertension, hyperactivity disorder, coronary heart disease and migraine headache could be caused by a dysfunction of TAs including β-PEA and tyramine. Therefore, it is of interest to compare my findings with the effects of TAs in *in vivo* studies in the rat.

8.3 General Conclusion

TAs, including β-PEA, tyramine, octopamine, amphetamine and its derivatives, cause vasoconstriction in rat aorta. The contractile responses to TAs in isolated aortic tissues are not mediated by α- and β-adrenoceptors, or ISA stimulation. Therefore, the contractile responses to TAs are probably based on activation of TAARs.

In the present work in rat aortic tissue, TAAR1 was successfully identified *via* Western blotting and polymerase chain reaction (PCR). However, it might be possible that more than one receptor for which TAs have different affinities are present in rat aortic tissues. In the present study the functional responses were different to the cloned TAAR1 in transfected cell lines. Tyramine produced weak vasoconstriction in rat aortic tissues and acted as a partial agonist of the other TAs. Therefore, the responses in aortic tissues do not support the definitions of TAAR1 and TAAR4. The signal transduction mechanism for cloned TAAR1 in transfected cell lines is likely to be different to the one coupled to functional receptors in isolated aortic tissues. The vasoconstriction in rat aortic tissues cannot be caused *via* activation of $G\alpha_s$. It is possible that the contractile responses to TAs are due to activation of $G\alpha_q$.

My investigations on different TAs and structurally related derivatives in rat and guinea-pig aortic tissues have expanded the knowledge of the vasoconstrictor effects of TAs in isolated tissues. Increasing knowledge about the underlying mechanisms and receptors for the functional responses to TAs in the vasculature will provide insights into their roles in the cardiovascular system in health and disease.

Chapter 9

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Chapter 9: Bibliography

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APPENDIX

Solutions for Western Blotting

Sample Buffer

Reagents	Sample Buffer (1X)	Sample Buffer (3X)
Tris base	0.063M	0.19M
SDS	4% (w/v)	12% (w/v)
Glycerol	10% (v/v)	30% (v/v)
β-Mercaptoethanol	5% (v/v)	15% (v/v)
Bromophenol blue	0.33mg/ml	1mg/ml
Distilled Water	make up to volume	make up to volume

Tank Buffer

Reagents	1X Tank Buffer	10X Tank Buffer
Tris Base	2.5mM	25mM
Glycerine	19mM	190mM
SDS	0.005%	0.05%
Distilled Water (pH 8.3)	make up to volume	make up to volume

Semi-dry Blotting Buffer

Reagents	Semi-dry Blotting Buffer
Tris Base	42.9mM
Glycerine	38.9mM
SDS	0.028%
Methanol (MeOH)	20% (v/v)
Distilled Water	make up to volume

Methanol is important to avoid gel swelling and to keep proteins adsorbed to the membrane.

Tris-buffered saline (TBS)

Reagents	1X TBS (pH 7.5)	10X TBS (pH 7.5)
Tris Base	20mM	200mM
NaCl	0.15M	15M
Distilled Water	make up to volume	make up to volume

Tris-buffered saline Tween-20 (TBST)

Reagents	1X TBST (pH 7.4)
Tris Base	20mM
NaCl	0.15M
Distilled Water	make up to volume
Tween-20	0.1% (v/v)

Blotto (5%)

Reagents	Blotto (5%)
TBS	1X
Non-fat dried milk	5%
Tween-20	0.1%
Distilled water	make up to volume

Western Blotting-Polyacrylamide Gels

Reagents	Concentrations
Acrylamide	30% acrylamide monomer / 0.8% bisacrylamide
Tris	3M Tris-HCl (pH to 8.8)
SDS	10% (w/v) SDS
SDS/Tris	0.4% (w/v) SDS/0.5M Tris-HCl (pH to 6.8)
APS	10% (w/v) APS

Separating Gel (10%) and Stacking Gel (5%)

Reagents	Separating Gel (10%)	Stacking Gel (5%)
Acrylamide	10%	5%
Tris-HCl	2.5mM	n/a
SDS	0.1%	n/a
Tris-HCl/ SDS	n/a	125μM/0.1%
APS	0.005%	0.005%
TEMED	0.05%	0.08%

Solution for PCR

Tris Acetate EDTA (TAE) – Buffer

Reagents	Stock Solution (50X)
Tris base	2M
Glacial Acetic Acid	5.7%
EDTA (0.5M, pH to 8.0)	0.05M
DEPC-treated water	make up to volume

Reagents	Working Solution (1X)
Tris-acetate	0.04M
Glacial Acetic Acid	0.114%
EDTA	0.001M

DEPC-treated water

Reagents	Concentrations
DEPC	0.1%
Distilled water	make up to volume

Leave lid on loosely and stir for at least 30 minutes until DEPC has dissolved. Decant into 500ml glass bottles, shake to treat the lid and autoclave for 90 minutes to ensure smell has gone (wear protective clothing as DEPC is a carcinogen).

Tris EDTA (TE)-Buffer

Reagents	Concentrations
Tris-HCl (pH to 7.5)	10mM
EDTA	1mM
DEPC-treated water	make up to volume (pH to 8.0)

dNTPs

	Concentrations
Stock solution	40mM
	4mM (1:10 Dilution)
Working solution	1mM (1:4 Dilution)

PCR Agarose Gel

Reagents	Concentrations
Agarose	2%
TAE-Buffer	make up to volume