# The role of calcium-dependent potassium channels and mitochondria in the EDHF phenomenon

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*To my family and my supervisors Tudor and David*

# Abstract

**Background** Acetylcholine (ACh) and the calcium ionophore A23187 are both known to trigger EDHF-type responses in the rabbit iliac artery via endothelial cell hyperpolarization mediated by the opening of calcium-dependent potassium channels ($K_{Ca}$). In addition, ACh and A23187 also stimulate the release of hydrogen peroxide ($H_2O_2$) from the endothelium.

**Aims of study** 1) To determine the relative contribution of different $K_{Ca}$ channel subtypes to ACh- and A23187-evoked responses. 2) To determine whether there is a connection between the activation of endothelial $K_{Ca}$ channels and the release of $H_2O_2$. 3) To identify the source of endothelium-derived $H_2O_2$ in the rabbit iliac artery.

**Major findings** 1) Immunohistochemical investigations demonstrated expression of $SK_{Ca}$, $IK_{Ca}$ and $BK_{Ca}$ channels in the endothelium of rabbit iliac arteries. 2) Mechanical studies with the $SK_{Ca}$ inhibitor apamin, the $IK_{Ca}$ inhibitor 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) and the $BK_{Ca}$ inhibitor iberiotoxin demonstrated that all three $K_{Ca}$ channel subtypes participate in ACh- and A23187-evoked EDHF-type relaxations. 3) Mechanical investigations with catalase and the catalase/SOD-mimetic manganese porphyrin (MnTMPyP) demonstrated that responses to ACh and A23187 both included a significant $H_2O_2$-dependent component, that could be inhibited by combined $K_{Ca}$ channel blockade. 4) Investigations with the NADPH oxidase inhibitor apocynin, the xanthine oxidase inhibitor oxypurinol and the inhibitors of the mitochondrial electron transport chain rotenone and myxothiazol indicated that mitochondria are likely to be the main source of $H_2O_2$ in the endothelium of the rabbit iliac artery.

**Conclusions** The study has highlighted the concerted role of different $K_{Ca}$ channel subtypes in ACh- and A23187-evoked EDHF-type relaxations in rabbit iliac arteries. It has also demonstrated that both responses consist of an $H_2O_2$-dependent component which is attenuated when $K_{Ca}$ channels are inhibited. However, the evidence provided is not sufficient to prove that $H_2O_2$ release in the endothelium is coupled to $K_{Ca}$ activation Additional studies aimed to identify the intracellular compartment that produces $H_2O_2$ upon stimulation with ACh and A23187. It has been demonstrated that under the current experimental conditions the most likely source of $H_2O_2$ is the mitochondrial electron transport chain.

# Contents

**Chapter 3** The mitochondria and endothelial $H_2O_2$ generation in ACh- and
A23187-evoked EDHF-type relaxations.............................

# Abbreviations

| | |
|---|---|
| **ACh** | acetylcholine |
| **2-APB** | 2-aminoethoxydiphenyl borate |
| **4-AP** | 4-aminopyridine |
| **ATZ** | 3-amino-1,2,4-triazol |
| **ACEI** | angiotensin converting enzyme inhibitors |
| **ATP** | adenosine triphosphate |
| **BK** | bradykinin |
| **BK$_{Ca}$** | large conductance calcium-dependent potassium channel |
| **BH$_4$** | 6R-tetrahydrobiopterin |
| **COX** | cyclooxygenase |
| **CNP** | C-natriuretic peptide |
| **CCE** | capacitative calcium entry |
| **[Ca$^{2+}$]** | calcium concentration |
| **[Ca$^{2+}$]$_i$** | intracellular calcium concentration |
| **ChTX** | charybdotoxin |
| **Cu,ZnSOD** | cooper,zinc superoxide dismutase (cytosolic) |
| **CYP** | cytochrome P$_{450}$ |
| **ΔΨm** | difference in mitochondrial membrane potential |
| **ΔpH** | difference in mitochondrial membrane pH |
| **DCF** | 2,5-dihydrochlorofluoresceine |
| **EDHF** | endothelium-derived hyperpolarizing factor |
| **EDRF** | endothelium-derived relaxing factor |
| **eNOS** | endothelial nitric oxide synthase |
| **EETs** | epoxyeicosatrienoic acids |
| **ER** | endoplasmic reticulum |
| **1-EBIO** | 1-ethyl-2-benzimidazolinone |
| **EC-SOD** | extracellular superoxide dismutase |
| **ETC** | electron transport chain |
| **FAD** | flavin adenine dinucleotide |
| **FMN** | flavin mononucleotide |

| | |
|---|---|
| **Fe-S** | iron-sulphur protein |
| **GSH$_{px}$** | glutathione peroxidase |
| **GSH** | glutathione; $\gamma$-L-glutamyl-L-cysteinylglycine |
| **GSSG** | glutathione disulphide |
| **GTP** | guanosine triphosphate |
| **HMEC-1** | human microvascular endothelial cells |
| **HUVECs** | human ambilical vein endothelial cells |
| **H$_2$O$_2$** | hydrogen peroxide |
| **IP$_3$** | inositol-1,4,5-trisphosphate |
| **IP$_3$R** | IP$_3$ receptor |
| **IK$_{Ca}$** | intermediate conductance calcium-dependent potassium channel |
| **IbTX** | iberiotoxin |
| **K$_{Ca}$** | calcium-dependent potassium channel |
| **K$_{ATP}$** | ATP-dependent potassium channel |
| **K$_{IR}$** | inward-rectifying potassium channel |
| **K$_v$** | voltage-dependent potassium channel |
| **LOX** | lipoxygenase |
| **MnSOD** | manganese superoxide dismutasae (mitochondrial) |
| **MnTMPyP** | manganese porphyrin |
| **NADH** | nicotinamide adenine dinucleotide (reduced form) |
| **NADPH** | nicotinamide adenine dinucleotide phosphate (reduced form) |
| **NO** | nitric oxide |
| **NS1619** | 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one |
| **Nox** | non-phagocytic NADPH oxidase |
| **ODQ** | 1H-[1,2,4]oxadiazolo[4,3,$\alpha$]quinoxalin-1-one |
| **O$_2^{\cdot-}$** | superoxide |
| **$\cdot$OH** | hydroxyl radical |
| **ONOO$^-$** | peroxynitrite |
| **PGI$_2$** | prostacyclin |
| **PLC** | phospholipase C |
| **PLA$_2$** | phospholipase A$_2$ |
| **PKA** | protein kinase A |

| | |
|---|---|
| **PKC** | protein kinase C |
| **PDGF** | platelet-derived growth factor |
| **PTP** | mitochondrial permeability trnasition pore |
| **Q** | ubiquinone |
| **QH$_2$** | ubiquinol |
| **RyR** | ryanodine receptor |
| **ROS** | reactive oxygen species |
| **SP** | substance P |
| **SERCA** | sarco/endoplasmic reticulum Ca$^{2+}$-ATPase |
| **SOCs** | store-operated calcium channels |
| **sGC** | soluble guanylyl cyclase |
| **SK$_{Ca}$** | small conductance calcium-dependent potassium channel |
| **SOD** | superoxide dismutase |
| **SPSHR** | stroke prone spontaneously hypertensive rat |
| **TF** | tissue factor |
| **TNF$_\alpha$** | tumor necrosis factor |
| **TRAM-34** | 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole |
| **TRAM-39** | (2-(-chlorophenyl)-2,2-diphenylacetonitrile |
| **TEA** | tetraethylammonium |
| **TBA** | tetrabutylammonium |
| **VEGF** | vascular endothelial growth factor |
| **XOR** | xanthine oxidoreductase |
| **XO** | xanthine oxidase |
| **XD** | xanthine dehydrogenase |

*Chapter One*

# General Introduction

## 1.1 Function of the endothelium

The blood vessels are a closed system of conduits that transfer blood from the heart to the tissues and vice versa from the tissues to the heart. Blood flow takes place primarily due to the forward motion imparted to it by the pumping of the heart, although other factors such as diastolic recoil of the arterial walls and compression of the veins play a major role. Indeed, vascular contractility is responsible for resistance to blood flow and is regulated by chemical, neuronal and humoral mechanisms. As the size of the vessels decreases their contribution to the overall resistance to blood flow increases, which could be partially attributed to differences in the structure of these vessels. The walls of all arteries consist of an outer layer of connective tissues, known as adventitia, a middle layer of variable thickness which consists of smooth muscle cells, known as media and the innermost layer which is the endothelium or else intima (Fig. 1.1).
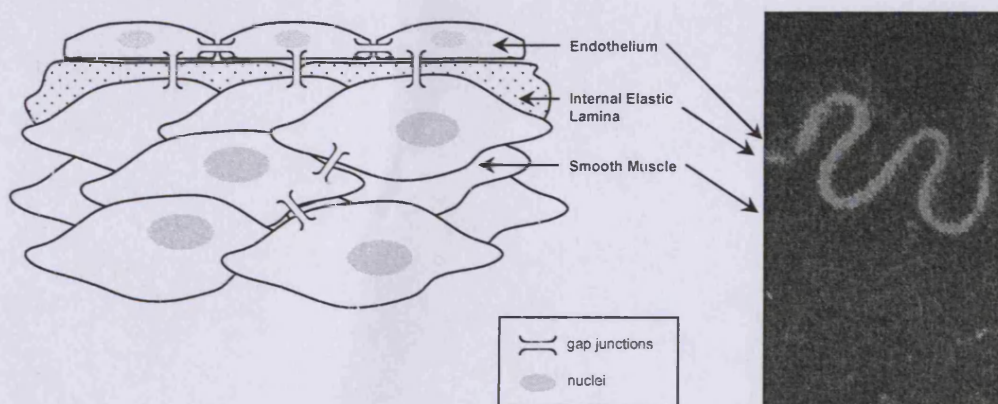


**Fig. 1.1** The endothelial monolayer and the smooth muscle layer, including the internal elastic lamina and gap junctions. The left side of the panel is a schematic representation of the three structural components of the vascular wall. The right side of the panel shows an image of these layers from a segment of a rabbit iliac artery obtained using confocal microscopy.

The vascular endothelium is a monolayer of cells, which is in direct contact with the blood and an approximately 80 nm thick basal layer, also known as the internal elastic lamina (Fig. 1.1). In response to various stimuli endothelial cells synthesize and release factors, which determine vascular contractility, blood coagulability, angiogenesis and vascular permeability. The contribution of the endothelium in vascular contractility is largely dependent on the communication of this layer with the underlying smooth

muscle, a process which can take place via the diffusion of molecules through the intracellular space or directly though myoendothelial gap junctions (Fig. 1.1).

*Vascular relaxation*

The modulation of the tone of the underlying smooth muscle is the most significant function of the endothelium. In many vessels, agonists such as acetylcholine (ACh) and calcium ionophore A23187 induce an endothelium-dependent relaxation which is known to depend on 1] nitric oxide (NO) synthesized by the endothelial nitric oxide synthase (eNOS), 2] prostanoids and 3] the 'EDHF phenomenon', a mechanism controversially associated with the propagation of hyperpolarizations from the endothelium to smooth muscle via myoendothelial gap junctions and the production of freely transferable factors that may directly hyperpolarize vascular smooth muscle (Griffith, 2004). Although the nature and the role of NO and prostanoids in vascular relaxation are now undisputed, there is still controversy about the nature of the EDHF phenomenon. As it will be described in a following section of this chapter, several candidates have been proposed to have EDHF-type properties, but thus far none of them fulfils all the criteria to be considered the universal factor. By contrast, the propagation of endothelial hyperpolarizations to smooth muscle via gap junctions is an alternative mechanism which has gained much recognition in recent years, and it is responsible for the mediation of EDHF-type responses in many vessels and species. This electrotonic mechanism comprises the basis for the investigations presented in this thesis, and its physiological role will be emphasized extensively throughout the entire manuscript.

Furthermore, it is noteworthy that the endothelium is one of the primary sites of oxygen metabolism in the vasculature and it is now known to generate reactive oxygen species (ROS), which play a major role in the regulation of endothelial function, vasodilatation and inflammation (Schiffrin, 2008). Indeed, arterial dilatation is largely dependent on the balance between mediators of vasodilatation such as NO, and ROS that could potentially scavenge the former when produced in excess (Tschudi *et al.,* 1996). The role of ROS as modulators of vascular contractility has also been demonstrated in the context of the EDHF phenomenon and there are reports which have suggested that in some species ROS such as hydrogen peroxide ($H_2O_2$) might be an EDHF (Matoba *et al.,* 2000). Nevertheless, in the majority of investigations ROS fail to trigger

hyperpolarizations of the magnitude that leads to EDHF-type relaxations, and their role as an EDHF is disputed (Griffith, 2004).

*Blood coagulability*

The endothelium of arteries, capillaries and veins is normally anti-thrombotic and forms a protective barrier, which prevents adherence of platelets and leukocytes to the surface of the vessel. Blood coagulation is controlled by several anti-coagulant factors, which under normal conditions prevail over pro-coagulant forces. For instance, it has been accepted that an enhanced production of endothelium-derived NO reduces the expression of tissue factor (TF), the primary activator of the blood coagulation cascade, and has direct effects on plasma coagulation factors such as Xa (Dusse *et al.*, 2007, Papapetropoulos *et al.*, 1998). Also, by regulating the function of anti-coagulant pathways such as the protein C / protein S pathway, the endothelium can maintain blood fluidity and thereby prevent thrombosis (Dahlbäck & Villoutreix, 2005). The interaction of thrombin with thrombomodulin in the endothelium activates protein C, a process which is facilitated by the subsequent formation of a complex with protein S. The activated complex of protein C and protein S in turn inactivates factors VIIIa and Va, which are essential for the coagulation of the blood. Because this pathway is entirely restricted to the endothelium of the vessels, immunohistochemical detection of factor VIII (also known as von Willebrand factor) has been widely used for the localization of the intimal layer.

*Angiogenesis*

The formation of new blood vessels from pre-existing is a pre-requisite for normal development and repair of the vasculature. The endothelial cells play a significant role in angiogenesis, a process which involves their rapid proliferation and the concomitant degradation of the underlying lamina by metalloproteinases, a group of matrix-degrading enzymes (Egeblad & Werb, 2002). Endothelial cells migrate and interact with cells, such as other endothelial cells, smooth muscle cells and fibroblasts, and establish tubular structures, which gradually evolve into the new vessel (Egeblad & Werb, 2002; Keane *et al.*, 2006).

*Vascular permeability*

The control of solute and macromolecule transfer across the blood vessel wall is another major function of the endothelium, especially in capillaries. Indeed, in most blood vessels the endothelium is continuous, while in others such as those of glands the monolayer possesses fenestrations, which facilitate the diffusion of solutes and molecules. Damage to the endothelium leads to abnormal extravasation of fluid which is particularly augmented in inflammation, atherosclerosis and diabetes (Knotkova & Pappagallo, 2007; Moreno *et al.*, 2006; Wright *et al.*, 1999). Increases in plasma extravasation have also been linked to the effects of growth factors such as the vascular endothelial growth factor (VEGF), which has been shown to increase vascular permeability by increasing NO release (Félétou *et al.*, 2001). Finally, the endothelium is also known to facilitate the diffusion of $H_2O_2$ to adjacent smooth muscle cells, where it interacts with signalling molecules and channels which regulate vascular contractility.

This chapter is mainly concerned with the electrotonic mechanism that underpins the EDHF phenomenon and aims to review the major findings of the available literature concerning the contribution of potassium channels in the development of endothelial hyperpolarizations. The second part of this chapter will focus on reactive oxygen species and their role as modulators of vascular contractility. Due to the importance of nitric oxide and prostanoids in vascular relaxation a brief description of their mechanism of action is also given in the following paragraphs.

## 1.1.1 Nitric Oxide

Following the princeps paper by Furchgott & Zawadzki (1980), it has been established that the endothelium generates a diffusible factor that influences vascular contractility, and which in some vessels is associated with decreases in intracellular calcium concentrations ($[Ca^{2+}]_i$)(Bolz *et al.*, 1999; Hashitani *et al.*, 2002; Soloviev *et al.*, 2004). Further investigations demonstrated that this endothelium-derived relaxing factor (EDRF) is NO, a gaseous transmitter, which activates soluble guanylyl cyclase (sGC) and increases cGMP levels (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Furchgott *et al.*, 1991). Increases in cGMP lead to phosphorylation of protein kinase G (PKG), and subsequently inhibition of actin-myosin filament sliding (Pelligrino & Wang, 1998). NO is synthesized in endothelial cells by the type III isoform of eNOS, and in addition to the maintainance of vascular tone, it also regulates blood coagulability and the anti-

adhesive properties of the endothelium (Rees *et al.,* 1989; Radomski *et al.,* 1993)(Fig. 1.2). Endothelial-derived NO is synthesized and released continuously under basal conditions (Griffith *et al.,* 1984), and provides a constant vasodilator influence that opposes sympathetic vasoconstriction through its direct action on subjacent smooth muscle cells, and through a direct effect on sympathetic innervation (Tesfamariam & Cohen, 1995). Indeed, stimulation of the endothelial layer with ACh, bradykinin (BK) or substance P is known to cause vasodilation through a mechanism that involves the acceleration of $Ca^{2+}$ uptake by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) in smooth muscle, and the extrusion of $Ca^{2+}$ via the plasma membrane $Ca^{2+}$-ATPase and the $Na^{+}$-$Ca^{2+}$ exchanger (Cohen *et al.,* 1999; Popescu *et al.,* 1985; Seip *et al.,* 2001).

Furthermore, it has been established that NO affects vascular contractility through a direct action on potassium channels. For instance, patch-clamp techniques have demonstrated that NO activates $BK_{Ca}$ channels (for channel classification see section 1.2.5) in isolated smooth muscle cells of cerebral and carotid arteries and aorta of the rabbit (Taniguchi *et al.,* 1993; Li *et al.,* 1998; Tanaka *et al.,* 2004; Waldron & Cole, 1999; Waldron *et al.,* 1999). In most blood vessels, the activation of $BK_{Ca}$ is mediated by the cGMP-dependent protein kinase phosphorylation of the channel, although studies carried out in rat mesenteric arteries have also suggested that it is possible for a direct cGMP-independent activation to take place (Mistry & Garland, 1998). This activation may possibly involve the binding of NO to thiol groups of the channel with the concomitant formation of S-nitrosothiols (Lang *et al.,* 2003). Indeed, NO can act as a weak thiol oxidant, which affects cell function by interacting with cysteine thiol groups of proteins through oxidation reactions, such as S-glutathiolation. Notably, in addition to the binding of NO to thiol groups of $BK_{Ca}$, SERCA is S-glutathiolated by NO, leading to an increase in $Ca^{2+}$ uptake, and finally to the development of cGMP-independent arterial relaxation (Adachi *et al.,* 2004). The effects of NO on potassium channels have also been demonstrated on $SK_{Ca}$, $K_V$ and $K_{ATP}$ channels (Waldron & Cole, 1999). For instance, in rat pulmonary arteries, NO promotes the opening of $K_V$ channels by a mechanism that is independent of sGC (Yuan *et al.,* 1996), while in the canine femoral vein, the relaxation to NO involves both $K_{ATP}$ and $BK_{Ca}$ channels, with the former depending on a cGMP-independent pathway, and the latter on a cGMP-dependent mechanism (Bracamonte *et al.,* 1999).
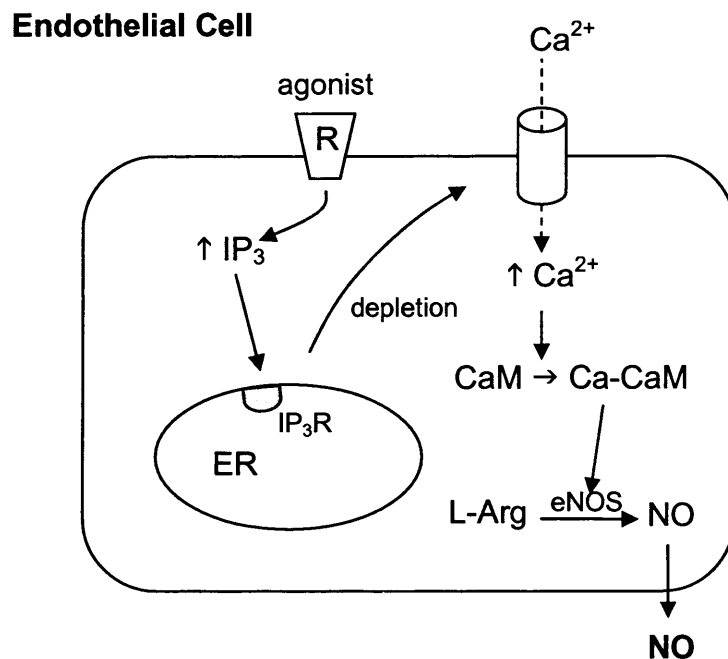
**Fig. 1.2** Schematic representation of the basic steps of agonist-evoked generation of nitric oxide (NO) in the endothelium of arteries. The production of NO is a calcium-dependent process that relies on the activation of the endothelial subtype of the enzyme nitric oxide synthase (eNOS). eNOS catalyses the conversion of L-arginine (L-Arg) into NO and L-citrulline. R: receptor, ER: endoplasmic reticulum, IP$_3$: inositol (1,4,5)-trisphosphate, CaM: calcium calmodulin.

It has been suggested that an NO-induced hyperpolarizing effect might take place in some vessels. Indeed, in guinea-pig uterine and coronary arteries, and in rat mesenteric resistant arteries, stimulation of endogenous NO or application of NO-donors led to a variable decrease in membrane potential from –60mV to approximately –80mV (Tare *et al.*, 1990; Parkington *et al.*, 1993; Garland & McPherson, 1992). The stimulation of smooth muscle hyperpolarizations by NO was primarily attributed to the direct cGMP-dependent or the indirect cGMP-independent activation of BK$_{Ca}$ channels (Archer *et al.*, 1994; Bolotina *et al.*, 1994; Peng *et al.*, 1996; Li *et al.*, 1998; Mistry & Garland, 1998; Plane *et al.*, 2001; Yu *et al.*, 2002). Nevertheless, in carotid and femoral arteries of the rabbit, NO failed to evoke hyperpolarizations in resting tissues, but instead repolarized them if they had been previously depolarised by agonists, such as noradrenaline (Cohen *et al.*, 1997; Plane *et al.*, 1995). In other vessels, such as porcine coronary and rat

hepatic arteries, NO did not affect the membrane potential at all, therefore excluding the possibility of being a 'universal' inducer of hyperpolarizations (Ito *et al.*, 1980; Zygmunt *et al.*, 1998).

### 1.1.2 Prostanoids

Prostacyclin ($PGI_2$) is a principal metabolite of arachidonic acid synthesized by cyclo-oxygenase (COX) in the endothelium of arteries and veins (Moncada *et al.*, 1976; 1977; Moncada & Vane, 1978). Other endothelium-derived prostaglandins such as $PGE_2$ can also show vasodilatory properties albeit to a lesser extent. Endogenous $PGI_2$ selectively activates IP receptors (a rhodopsin type G-protein coupled cell surface receptor; Ruan *et al.*, 2005) in smooth muscle cells and dilates vessels through the intracellular accumulation of cAMP. In some arteries, $PGI_2$ has also been shown to hyperpolarize the media by a mechanism involving the protein kinase A (PKA)-dependent activation of $K_{Ca}$ channels (Schubert *et al.*, 1997). However, other reports, failed to demonstrate smooth muscle hyperpolarizations to $PGI_2$ or its analogue iloprost (Parkington *et al.*, 1993), therefore suggesting that $PGI_2$ is not always a hyperpolarizing factor. In addition to this finding, there is adequate evidence, which supports that transient hyperpolarizing responses are still detectable in many blood vessels preincubated with COX inhibitors, such as indomethacin (Hutcheson *et al.*, 1994; Dong *et al.*, 2000; Bychkov *et al.*, 2002; Ungvari *et al.*, 2002; Hinton & Langton, 2003; Weston *et al.*, 2005). Thence, it was speculated that smooth muscle hyperpolarizations were stimulated by another factor, which was generated in the endothelium and could diffuse freely to the media.

### 1.1.3 Endothelium-Derived Hyperpolarizing Factor

The term Endothelium-Derived Hyperpolarizing Factor (EDHF) was used to describe the hypothetical factor, which modulates smooth muscle cell potential, followed by a decrease in vascular contractility (De Mey *et al.*, 1982; Bolton *et al.*, 1984; Beny & Brunet, 1988; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Griffith, 2004). EDHF-type responses have been demonstrated in variety of vessels including guinea-pig carotid and basilar arteries, rabbit mesenteric and iliofemoral arteries, porcine coronary arteries and rat hepatic arteries (Corriu *et al.*, 1996; Petersson *et al.*, 1997; Fujimoto *et al.*, 1999; Murphy & Brayden, 1994; Chaytor *et al.*, 2000; 2001; 2002; 2003; Ge *et al.*,

2000; Zygmunt *et al.*, 1997). EDHF-type relaxations have been defined as a distinct dilatation mechanism on the basis that they are insensitive to eNOS and/or COX inhibitors. Several lines of evidence implied that EDHF is the main vasorelaxant mechanism in small size arteries where NO bioavailability is limited (Shimokawa *et al.*, 1996; Busse *et al.*, 2002; Inokuchi *et al.*, 2003). An inverse relation between NO and EDHF has been demonstrated in rabbit carotid and mesenteric arteries and the mesenteric arterial bed of the rat (Bauersachs *et al.*, 1996; McCulloch *et al.*, 1997). For instance, in rat mesenteric arteries the magnitude of the NO-independent component was significantly suppressed in the presence of 8-Br-cGMP, a cell permeable analogue of cGMP, which indicated that in these vessels basal release of NO might inversely modulate EDHF-type responses (McCulloch *et al.*, 1997). The inverse relation between NO release and the EDHF component has also been demonstrated with bioassays using the NO-donor C87-3786 (Bauersachs *et al.*, 1996).

## 1.2 Putative transferable EDHF candidates

In previous years, several studies have attributed the EDHF-type responses to factors such as potassium cations ($K^+$), epoxyeicosatrienoic acids (EETs), C-natriuretic peptide (CNP), hydrogen peroxide ($H_2O_2$), adenosine, carbon monoxide and endocannabinoids (Edwards *et al.*, 1998; Fleming *et al.*, 2001; Honing *et al.*, 2001; Matoba *et al.*, 2000; Prior *et al.*, 1999; Kemp & Cocks, 1999; Wang *et al.*, 1997; Nishikawa *et al.*, 2004; Randall *et al.*, 1997). Indeed, all these investigations provided evidence to support the proposal that each of the aforementioned agents might be an EDHF. Nevertheless, a number of conflicting reports in the literature suggested that it is more likely for them to be 'EDHFs' in certain species and tissues, rather than the 'universal' factor. For instance, in rat mesenteric and hepatic arteries, $K^+$ was considered to be an EDHF on the basis that extracellular accumulation of the ion (Fig. 1.3) leads to smooth muscle hyperpolarizations whereas other studies contradicted this hypothesis (Edwards *et al.*, 1998; Andersson *et al.*, 2000; Doughty *et al.*, 2000; 2001; Lacy *et al.*, 2000).

In recent years, a much simpler theory suggested that the EDHF phenomenon might involve the electrotonic spread of hyperpolarizations from the endothelium to smooth muscle cells via myoendothelial gap junctions (Chaytor *et al.*, 2003; Griffith *et al.*, 2005). There is growing evidence showing that agonist stimulated endothelium-derived

hyperpolarizations may be conducted electrotonically to subjacent smooth muscle cells with the resulting hyperpolarization causing reductions in $[Ca^{2+}]_i$ and vasorelaxation.
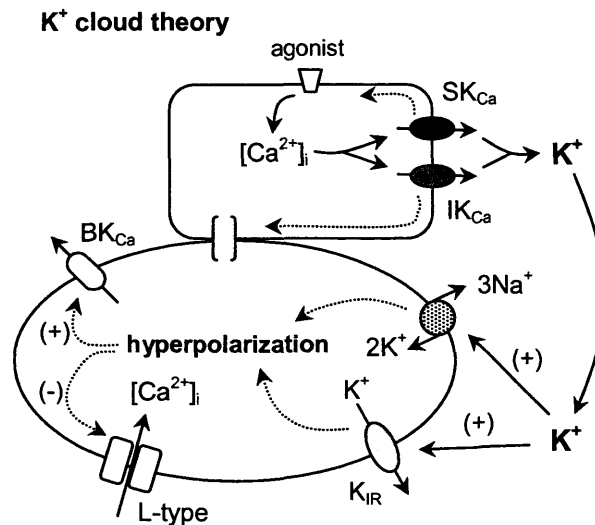


**Fig. 1.3** The potassium cloud hypothesis. According to this theory, increases in intracellular $Ca^{2+}$ in the endothelium lead to efflux of $K^+$ through $K_{Ca}$ channels, which accumulates in the intercellular space forming a 'cloud' of ions. This 'cloud' subsequently activates inward rectifying potassium channels $(K_{IR})$ and $Na^+/K^+$-ATPases in smooth muscle causing hyperpolarization.

### 1.2.1 Gap junctional communication

Gap junctions are formed at points of cell-cell contact and they consist of two connexons or hemichannels, each formed from six connexin subunits (Griffith, 2004; de Wit, 2004; Takano *et al.*, 2005)(Fig. 1.4). Three main connexin protein subtypes are known to exist in the vascular wall, namely Cx37, 40 and 43, although in some vessels Cx45 may also be present (Ujie *et al.*, 2003; Lang *et al.*, 2007; Ceroni *et al.*, 2007). It should be noted that in the same connexon different connexin subtypes often co-localize forming heterotypic gap junctions, while in some tissues homotypic gap junctions, containing only one connexin subtype may be present (Griffith, 2004). These differences in structure have been extensively investigated electrophysiologically by patch-clamp techniques, which revealed more complex conductances in gap junctions with more than one connexin subtype (Wang *et al.*, 2001; Yamazaki & Kitamura,

2003). Furthermore, individual gap junctions aggregate in plaques that consist of focal clusters of many hundreds of units forming a characteristic pentalaminar structure (Griffith, 2004). These gap junction plaques form heterocellular contacts between endothelial and smooth muscle cells or homocellular contacts between adjacent endothelial or smooth muscle cells (Sandow & Hill, 2000; Bukauskas *et al.*, 2000; de Wit, 2004).

To investigate the role of gap junctions in cell-cell coupling, a number of experimental methods have been employed, such as fluorescent dye transfer and electrophysiological techniques. The use of fluorescent probes such as biocytin and calcein AM, which is cleaved intracellularly thereby facilitating its diffusion, was employed to give insight into the passage of molecules from the endothelium to the smooth muscle (Little *et al.*, 1995). However, this method failed to demonstrate dye coupling between adjacent cells, and therefore conventional electrophysiology was used as an alternative approach. Indeed, failure to demonstrate dye coupling, did not exclude the possible presence of electrical continuity between adjacent cells (Little *et al.*, 1995). In segments of guinea-pig submucosal arterioles, electrophysiological studies confirmed that ACh-evoked endothelial hyperpolarizations, propagate to the smooth muscle through an electrotonic mechanism, and conversely, hyperpolarizations originating in the smooth muscle could also be detected in the endothelium (Coleman *et al.*, 2001; Yamamoto *et al.*, 2001). Similar results were demonstrated in porcine coronary arteries, in rat aortas and rabbit iliac arteries (von der Weid & Bény, 1993; Marchenko & Sage, 1994; Griffith *et al.*, 2002).

More conclusive data about the role of gap junctions in cell-cell coupling were obtained by means of pharmacological agents, and more specifically the connexin-mimetic peptides. The connexin-mimetic peptides are short synthetic peptides that are homologous to the conserved Gap 26 and 27 domains of gap junctions and correspond to amino acid sequences in the extracellular loops of Cxs 37, 40 and 43. These peptides have been developed by minor variations in the amino acid sequences of the first and second extracellular loops of Cxs 37,40 and 43 (Griffith, 2004; Evans *et al.*, 2006). In experiments carried out on vascular tissues, the peptides [37,40]Gap26, [37,43]Gap27, [40]Gap27 and [43]Gap26 have been extensively used either on their own or more commonly in combination so as to determine the role of gap junctions in EDHF-type responses

(Chaytor *et al.*, 1997; 1998; 1999; 2000; 2001; 2002; 2003; 2005; Griffith *et al.*, 2002, 2004; Berman *et al.*, 2002; Dora *et al.*, 1999). Notably, these peptides modulate the gating of the channels with no concomitant changes in the structure of the plaques (Martin *et al.*, 2005). This property is also in agreement with findings showing that the effects of gap peptides on EDHF-type responses are reversible, and that the responses are restored following consecutive washing of the tissues of interest (Chaytor *et al.*, 2001).

## 1.2.2 Receptor-dependent and receptor-independent stimulation of EDHF

EDHF-type relaxations are evoked by agonists, which cause a global increase in endothelial $Ca^{2+}$ levels linked to depletion of the endoplasmic reticulum $Ca^{2+}$ store (ER)(Sedova *et al.*, 2000; Nilius & Droogmans, 2001)(Fig. 1.5). In most cell types, depletion of ER signals the activation of capacitative calcium entry (CCE), occurring through store-operated calcium channels (SOCs) in the plasmalemma (Fleming *et al.*, 1996; Barritt, 1999). Activation of these channels leads to increases in $[Ca^{2+}]_i$, which provides the physiological stimulus for the EDHF phenomenon and also NO release (see section 1.1.1). It is established that agonists that evoke EDHF-type relaxations promote the receptor-dependent stimulation of CCE. ACh, BK and substance P elevate endothelial $Ca^{2+}$ levels in a receptor-dependent fashion. These agonists induce depletion of the ER through the activation of phospholipase C (PLC), followed by the formation of $IP_3$, which binds to $IP_3$ receptor $Ca^{2+}$ channels ($IP_3R$) on the store (Fleming *et al.*, 1996; Barritt, 1999). By contrast, agents such as cyclopiazonic acid and thapsigargin promote direct ER depletion by inhibiting $Ca^{2+}$ uptake by SERCA (Pasyk *et al.*, 1995; Fukao *et al.*, 1995; 1997; Davis & Sharma, 1997), thereby stimulating store-operated $Ca^{2+}$ entry. Similarly, agents such as calcium ionophore A23187 can also be employed to evoke receptor-independent activation of EDHF-type responses (Plane *et al.*, 1997; Zygmunt *et al.*, 1997; Hutcheson *et al.*, 1999; Kagota *et al.*, 2001; Chaytor *et al.*, 2003). Although the exact mechanism of action of A23187 is not entirely understood, it has been postulated that depletion of ER and changes in the function of mitochondrial electron transport chain due to changes in mitochondrial $Ca^{2+}$ loading, might underpin endothelial $Ca^{2+}$ influx (Pfeiffer *et al.*, 1974; Reed & Lardy, 1972; Kauffman *et al.*, 1980; Gill *et al.*, 1986).
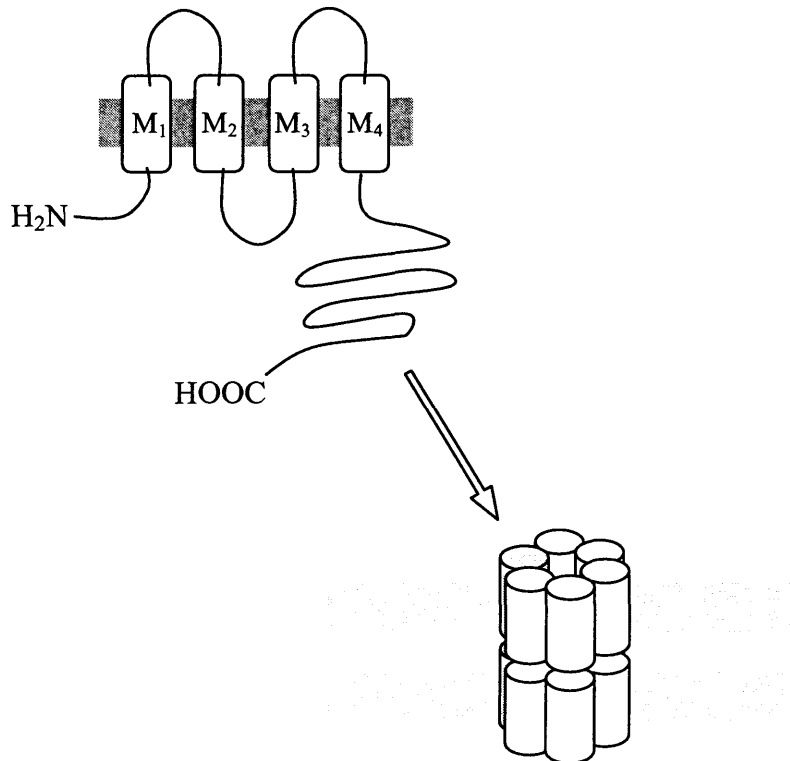
**Fig. 1.4** Representation showing the structure of one connexin protein, and how six connexins form a connexon. Docking of connexons from apposing cells results in the formation of the aqueous pore of the gap junction.

## 1.2.3 Membrane potential: Endothelial hyperpolarizations in the EDHF phenomenon

A *membrane potential* arises when there is a difference in the electrical charge on two sides of a membrane, due to a slight excess of positive ions over negative ones on one side and a slight deficit on the other. Such differences in charge can be the result of both active electrogenic pumping and diffusion of ions. Potassium, sodium, calcium and chloride are the ionic species that establish the membrane potential. The equilibrium condition, in which there is no net flow of ions across the plasma membrane, is known as the *resting membrane potential* of the cell. This equilibration is mainly performed by $K^+$ ions, which are pumped into the cells by the $Na^+/K^+$-ATPase (an electrogenic pump) in exchange for $Na^+$ ions. Efflux of $K^+$ ions is performed by potassium channels which

are located in the plasma membrane of the cell. As $K^+$ begins to move out, an unbalanced negative charge is left behind which creates a membrane potential that tends to oppose the further efflux of the ion. Efflux of $K^+$ ceases when the electrical driving force on $K^+$ balances the effect of its concentration gradient. However, the resting membrane potential describes an ideal condition since flow of ions always takes place even in unstimulated cells. Depending on the ions that flow through the plasma membrane, the membrane potential can either increase or decrease.

*Depolarization* is the term that is used to describe the decreases in the absolute value of a cell's membrane potential. Depolarization is often caused by influx of cations e.g. influx of $Ca^{2+}$ through calcium channels. Indeed, vasoconstrictors such as phenylephrine, cause increases in vascular tension by facilitating influx of $Ca^{2+}$ in smooth muscle cells through voltage-dependent L-type $Ca^{2+}$ channels. The inward movement of the ion is accompanied by a change of the membrane potential towards the reversal potential for $Ca^{2+}$ ions, and will thus depolarize the cell. By contrast, increasing the permeability to $K^+$ will have the opposite effect.

*Hyperpolarization* is the term that is used to describe the increases in the absolute value of a cell's membrane potential. It has become evident that agonists that evoke EDHF-type relaxations cause a shift in the membrane potential towards the reversal potential for $K^+$ ions (~-80mV; hyperpolarization)(Mehrke & Daut, 1990; Marchenko & Sage, 1993). This endothelial hyperpolarizing response is determined by the efflux of $K^+$, which is a consequence of the increases in $[Ca^{2+}]_i$ in this cell. Indeed, it has been demonstrated that suppression of the extracellular $[Ca^{2+}]$ with EGTA, or inhibition of CCE with agents such as 2-aminoethoxydiphenyl borate (2-APB), attenuates endothelial hyperpolarization (Chen & Suzuki, 1990; Iwasaki *et al.*, 2001). Accordingly, there is accumulating evidence, which shows that the opening of endothelial $Ca^{2+}$-dependent $K^+$ channels ($K_{Ca}$) contributes to this phenomenon on the basis that EDHF-type relaxations can be abolished by application of $K_{Ca}$ inhibitors (Doughty *et al.*, 1999; Quignard *et al.*, 1999; Dora *et al.*, 2001; Walker *et al.*, 2001; Crane *et al.*, 2003; Petersson *et al.*, 1997; Plane *et al.*, 1997; Edwards *et al.*, 1998; Ungvari *et al.*, 2002; Brakemeier *et al.*, 2003)(Fig. 1.5).

### 1.2.4 Relation between endothelial hyperpolarization and smooth muscle relaxation

As previously discussed, a smooth muscle hyperpolarizing response occurs in parallel to that in the endothelium. Based on the fact that both hyperpolarizations have the same magnitude, it was speculated that the EDHF phenomenon might be associated with the electrotonic coupling between the two vascular layers (Fig. 1.5). In addition, the number of myoendothelial gap junctions increases with the diminution of the size of the artery, which parallels the role of EDHF as a vasorelaxant mechanism in small size vessels (Shimokawa *et al.*, 1996; Hill *et al.*, 2002). Indeed, inhibition of myoendothelial gap junctions with connexin-mimetic peptides possessing homology with the conserved domains of these proteins, attenuated the propagation of hyperpolarizations to smooth muscle (Chaytor *et al.*, 1997; 1998; 2001; 2005; Griffith *et al.*, 2002). The connexin-mimetic peptides also uncouple vascular smooth muscle cells reflecting an ability to inhibit the spread of endothelial hyperpolarizations through successive layers of the media (Chaytor *et al.*, 1997, 2005; Edwards *et al.*, 2000). In support of this concept, suppression of gap junctional communication attenuates EDHF-type relaxations in several blood vessels, such as the rat hepatic and rabbit iliac arteries (Chaytor *et al.*, 2001; 2003).

Nevertheless, it has been reported that, in some cases, smooth muscle relaxations may be independent of endothelial hyperpolarization. First, Plane *et al.* (1995), suggested that A23187-evoked NO-independent smooth muscle relaxations are triggered by a diffusible factor that is generated in endothelial cells. In support to this notion, studies in rabbit superior mesenteric arteries, showed that A23187-dependent responses are attributable to an agent that diffuses to smooth muscle via the extracellular space, whereas similar responses by ACh were attributable to an agent that crosses to the muscle through myoendothelial gap junctions (Hutcheson *et al.*, 1999). Chaytor *et al.* (2003) subsequently demonstrated that in rabbit iliac arteries this discrepancy was due to the ability of A23187 to generate $H_2O_2$ in the endothelium, which in these vessels may be regarded as a relaxing factor, but not as a hyperpolarizing factor. Notably, in these vessels, inhibition of gap junctions with connexin-mimetic peptides failed to inhibit the concomitant relaxations to A23187, therefore effectively dissociating the hyperpolarizing from the relaxant mechanism (Chaytor *et al.*, 2003).
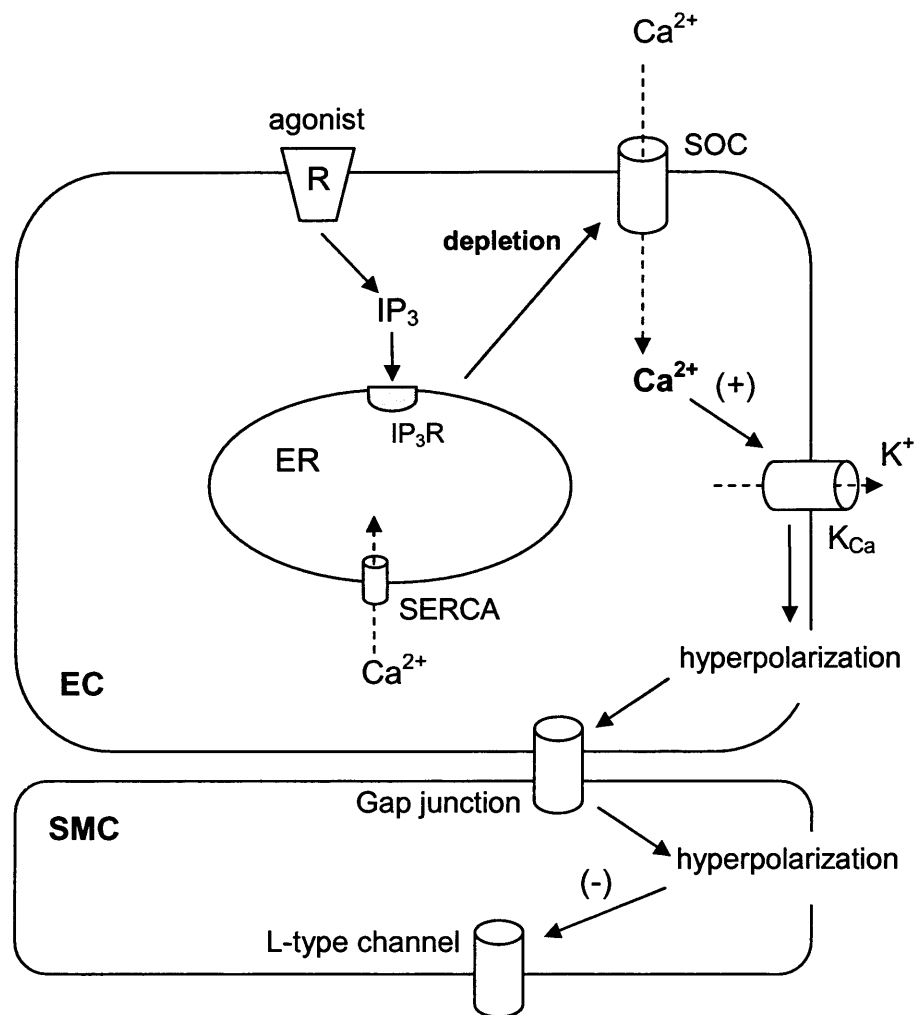
**Fig. 1.5** Endothelial interactions that participate in the EDHF phenomenon. The image shows that depletion of the store upon stimulation with an agonist leads to an increase in $Ca^{2+}$ influx through store-operated channels (SOCs) in the endothelium. This increase opens endothelial $K_{Ca}$ channels and causes efflux of $K^+$ which ultimately leads to endothelial hyperpolarization. This hyperpolarization propagates to adjacent smooth muscle cells via myoendothelial gap junctions, and subsequently to other smooth muscle cells.

In addition to the ability of some agonists to stimulate smooth muscle relaxation via the propagation of endothelium-dependent hyperpolarizations, there are reports that suggest that both hyperpolarization and depolarization may also be conducted electrotonically from smooth muscle to the endothelium (Yamamoto *et al.*, 1999; Allen *et al.*, 2002). Although this 'reverse' mechanism is not considered to be a hallmark of the EDHF phenomenon, it comprises a complementary pathway that depends on myoendothelial communication and may influence vascular contractility through secondary effects on endothelial function. Indeed, experiments carried out in hamster arteries with the $Ca^{2+}$

fluorescent probe Fluo-3, demonstrated that changes in $[Ca^{2+}]_i$ were similar in smooth muscle and endothelial cells following stimulation with a vasoconstrictor such as PE (Dora *et al.,* 1997). It was thereby suggested that the diffusional transfer of some low molecular mass agent from the smooth muscle to the endothelium, such as $IP_3$ or $Ca^{2+}$, might be responsible for the similar time courses observed in both layers (Dora *et al.,* 1997). Indeed, investigations carried out on a vascular cell coculture system that forms myoendothelial gap junctions, demonstrated that stimulation of smooth muscle cells is accompanied by an $IP_3$- and $Ca^{2+}$-mediated response in the endothelium, whereas secondary responses in smooth muscle following endothelial stimulation required diffusion of $Ca^{2+}$ only (Isakson *et al.,* 2007).

## 1.2.5 Calcium-Activated Potassium Channels – general properties

The calcium-activated potassium channel family consists of six or seven transmembrane-helix domains and one pore domain (Fig. 1.7). There are two main subfamilies of $K_{Ca}$ channels categorized according to their single channel conductance. The first one consists of the small and intermediate conductance channels ($SK_{Ca}$: 2-25pS and $IK_{Ca}$: 25-100pS), while the second one consists of the large conductance channels ($BK_{Ca}$: 100-300pS). In the vascular wall, $K_{Ca}$ channels are located in the endothelium and the smooth muscle, and their localization underpins their variable physiological role, such as endothelial hyperpolarization, $Ca^{2+}$ entry and smooth muscle excitability (Ledoux *et al.,* 2006).

$SK_{Ca}$ channels were the first members to be identified on the basis of their pharmacology. These channels are sensitive to apamin (a toxin extracted from the venom of the European bee *Apis mellifera*) and the plant alkaloid *d*-tubocurarine (Ishii *et al.,* 1997; Shah & Haylett, 2000). Three highly homologous subtypes of $SK_{Ca}$ have been cloned, and classified as $K_{Ca}2.1$ (SK1), $K_{Ca}2.2$ (SK2) and $K_{Ca}2.3$ (SK3) (Köhler *et al.,* 1996; Wei *et al.,* 2005). The cloned $SK_{Ca}$ pore results from the coassembly of four subunits (also termed α-subunits) which share a conserved transmembrane region (80-90% identical), but vary in their amino and carboxy terminals (Köhler *et al.,* 1996). Nevertheless, all three variants are sensitive to changes in $[Ca^{2+}]_i$, and their function is independent of the membrane potential of the cell. It is noteworthy that $SK_{Ca}$ channels do not contain a $Ca^{2+}$-binding domain, but respond to changes in $[Ca^{2+}]_i$ indirectly by

interacting with calcium calmodulin (Xia *et al.*, 1998). In the vascular wall, $SK_{Ca}$ are expressed in endothelial cells (Marchenko & Sage, 1996; Edwards *et al.*, 1998; Burnham *et al.*, 2002; Eichler *et al.*, 2003; Taylor *et al.*, 2003), while SK3 has also been identified in smooth muscle of human colonic arterioles (Chen *et al.*, 2004).

Similarly, $IK_{Ca}$ channels are voltage-independent and sensitive to changes in intracellular $[Ca^{2+}]$. The α subunits of the channel are 50% homologous to those of $SK_{Ca}$, and therefore $IK_{Ca}$ are considered to be an $SK_{Ca}$ channel subtype ($K_{Ca}3.1$, SK4 or IK1). Like $SK_{Ca}$, $IK_{Ca}$ do not bind to $Ca^{2+}$ directly, but $Ca^{2+}$ sensing depends on the binding of the carboxy terminal region with calmodulin (Fanger *et al.*, 1999). Despite these common traits, the pharmacological properties of $IK_{Ca}$ differ considerably from those of $SK_{Ca}$. $IK_{Ca}$ are inhibited by clotrimazole (a cytochrome P450 mono-oxygenase inhibitor) and its more selective analogues 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) and (2-(-chlorophenyl)-2,2-diphenylacetonitrile (TRAM-39) (these analogues lack P450 mono-oxygenase inhibitory properties), while the $SK_{Ca}$ inhibitors apamin and tubocurarine have no effect on this channel (Wulff *et al.*, 2000; Eichler *et al.*, 2003; Chandy *et al.*, 2004; Weston *et al.*, 2005; Burnham *et al.*, 2006a). $IK_{Ca}$ are also inhibited by charybdotoxin (ChTX), a peptide found in the venom of the scorpion *Leiurus quinquestriatus* (Ishii *et al.*, 1997; Joiner *et al.*, 1997). $IK_{Ca}$ are constitutively expressed in vascular endothelium (Edwards *et al.*, 1998; Eichler *et al.*, 2003; Weston *et al.*, 2005).

$BK_{Ca}$ channels (also known as $K_{Ca}1.1$, Maxi-K or Slo1) are both $Ca^{2+}$ and voltage-regulated. In contrast to $SK_{Ca}$ and $IK_{Ca}$, $BK_{Ca}$ sensitivity to $Ca^{2+}$ relies on the direct binding of the cation to a region of negatively charged aspartate residues in the C-terminus of the α-subunit of the channel, which is known as the "$Ca^{2+}$ bowl" (Bao *et al.*, 2004). Additionally, $BK_{Ca}$ consists of two subunits, namely α and β (Tanaka *et al.*, 1997). The α subunits are the pore-forming portion of the channel, while the β subunits modulate their $Ca^{2+}$ sensitivity (Toro *et al.*, 1998)(Fig. 1.6). So far, up to eight β-subunit proteins have been identified, each providing different modulatory effects on the pharmacology and gating of the channel (Knaus *et al.*, 1994; Xia *et al.*, 2000). Although activation of the channel is facilitated by increases in $[Ca^{2+}]_i$ above the threshold of 100 nM, it has been inferred that it can also be activated directly by depolarizing voltages in the absence of $Ca^{2+}$ (Kaczorowski *et al.*, 1996). $BK_{Ca}$ are selectively inhibited by

iberiotoxin (IbTX), a toxin derived from the scorpion *Buthus tamulus*, that seems to interact selectively with the β subunits of the channel (Brenner *et al.*, 2000; Löhn *et al.*, 2001). ChTX can also be used to inhibit these channels, in addition to its effects on $IK_{Ca}$. $BK_{Ca}$ are mainly located in vascular smooth muscle cells (Ledoux *et al.*, 2005; Wei *et al.*, 2005), although some reports suggest that the channel is also located in the endothelium of rat gracilis muscle arterioles and the rabbit ductus arteriosus (Ungvari *et al.*, 2002; Thebaud *et al.*, 2002).
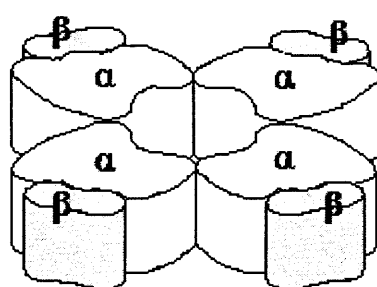


**Fig. 1.6** Representation of the spatial distribution of the α and β subunits that form the $BK_{Ca}$ channel pore. The channel consists of four α-subunits, which are the main structural components, and four accessory β-subunits that modulate the $Ca^{2+}$-sensitivity of the former.

## 1.2.6 Identification of $K_{Ca}$ channels involved in the EDHF phenomenon

The identity of the $K_{Ca}$ channels involved in EDHF-type relaxations is controversial (Griffith, 2004; Feletou & Vanhoutte, 2006). It has been established that differences in expression of $K_{Ca}$ channels among species and vascular beds, reflect: 1] the differential involvement of the three subtypes in endothelium-dependent hyperpolarizations (Busse *et al.*, 2002; Griffith, 2004), and 2] the different role of these channels under resting or depolarizing conditions (Crane *et al.*, 2003). The involvement of $K_{Ca}$ channels in the EDHF phenomenon has been demonstrated by mechanical and electrophysiological studies using a combination of pharmacological probes.

In rat and rabbit mesenteric arteries, EDHF-type responses were virtually abolished by apamin, suggesting that in these vessels $SK_{Ca}$ channels might be the prevailing $K_{Ca}$ subtype (Chen & Cheung, 1997; Murphy & Brayden, 1995). Similar effects were also

19

observed in bovine and porcine coronary arteries, and bovine oviductal arteries (Hecker *et al.*, 1994; García-Pascual *et al.*, 1995). Nevertheless, apamin failed to modulate EDHF-type responses in other blood vessels, such as rabbit carotid arteries (Lischke *et al.*, 1995), suggesting that other $K_{Ca}$ channels might be involved. Indeed, in rabbit carotid arteries, in guinea pig basilar and canine coronary arteries, EDHF-type relaxations were completely or partially inhibited by the $BK_{Ca}/IK_{Ca}$ inhibitor ChTX (Lischke *et al.*, 1995; Petersson *et al.*, 1996; Nakashima *et al.*, 1997).

Due to the inevitable selectivity problems that arise with this toxin, definitive identification of $BK_{Ca}$ and $IK_{Ca}$ was subsequently made with more selective agents, such as IbTX and TRAM-34. Although IbTX cannot substitute for ChTX in most cases, it can attenuate EDHF-type relaxations in some vessels, including first order rat mesenteric arteries and bovine coronary arteries, therefore illustrating the participation of $BK_{Ca}$ (Hilgers *et al.*, 2006; Weston *et al.*, 2005). Similarly, in rat carotid arteries, TRAM-34 significantly attenuated ACh-evoked hyperpolarizations, providing evidence for the involvement of $IK_{Ca}$ (Eichler *et al.*, 2003). It has been established that in many EDHF-type responses each toxin on its own has a minor or no effect, while their combination abolishes the residual effects. The effects of such combinations on EDHF-type relaxations will be discussed in more detail in Chapter 2.

### 1.2.7 Other potassium channels in the EDHF phenomenon

Although it is now widely accepted that $K_{Ca}$ is the main potassium channel family involved in the EDHF phenomenon, there are also reports suggesting that in some species and vessels inward-rectifying, voltage-dependent and ATP-dependent potassium channels might be involved (Zygmunt & Hogestatt, 1996; Edwards *et al.*, 1998; Doughty *et al.*, 2000; Corriu *et al.*, 1996)(Fig. 1.7).

*Inward-rectifying $K^+$ channels ($K_{IR}$)* – The involvement of $K_{IR}$ channels in EDHF-type responses has been described in the context of the 'potassium cloud' theory (Edwards *et al.*, 1998; Edwards & Weston, 2004; Jiang *et al.*, 2005). According to this model, $K^+$ efflux from the endothelium generates local elevations in extracellular $K^+$ surrounding the smooth muscle cells, thus stimulating the activation of $K_{IR}$ channels in smooth muscle, and subsequently allowing the outward movement of $K^+$ with resultant
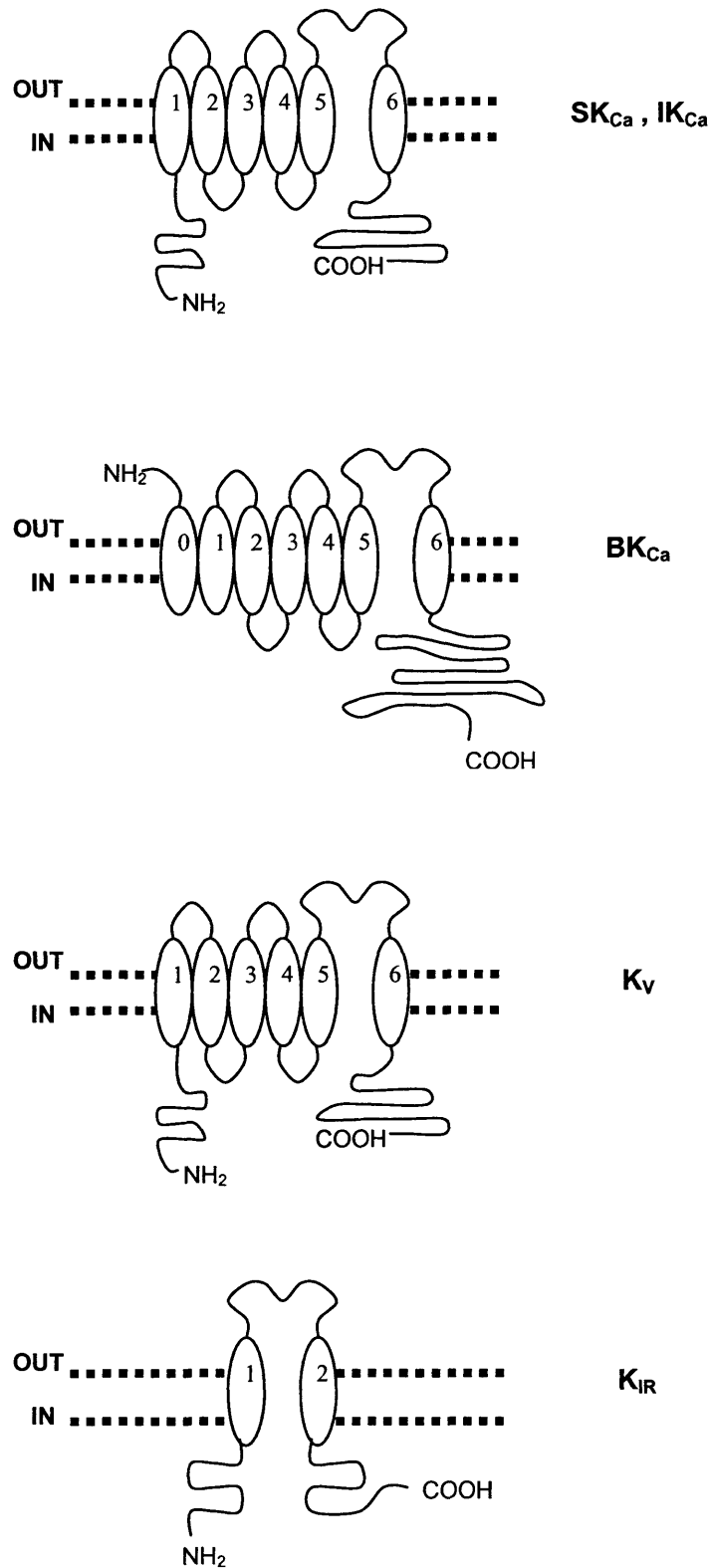
**Fig. 1.7** The potassium channel family. The four panels (above) show the structure of the subunits of $SK_{Ca}$, $IK_{Ca}$, $BK_{Ca}$, $K_V$ and $K_{IR}$. For $BK_{Ca}$ only the α-subunit is shown. The structure of the $BK_{Ca}$ β-subunit is similar to that of $K_{IR}$.

hyperpolarization (Edwards *et al.*, 1998; Jiang *et al.*, 2005). Although in some reports inhibition of endothelial $K_{IR}$ channels with $Ba^{2+}$, an inhibitor of these channels, led to a significant leftward shift in both the hyperpolarizing and relaxant responses (Goto *et al.*, 2004), many studies have failed to demonstrate a role for $K_{IR}$ in the EDHF phenomenon. For instance, $Ba^{2+}$ did not affect EDHF-type responses in both porcine coronary arteries and guinea-pig arterioles (Beny & Schaad, 2000; Quignard *et al.*, 1999; Coleman *et al.*, 2001). However, there are reports suggesting that the involvement of $K_{IR}$ in the EDHF phenomenon depends on the level of depolarization of the tissue. Indeed, in rat mesenteric arteries, KCl-induced hyperpolarizations were significantly inhibited by PE, therefore emphasizing that differences in experimental conditions might underpin the differential role of these channels in the hyperpolarizing mechanism (Richards *et al.*, 2001).

*Voltage-dependent $K^+$ channels (Kv)* - In rabbit femoral, rat hepatic and guinea-pig basilar arteries, 4-aminopyridine (4-AP), an inhibitor of Kv channels, significantly attenuated EDHF-type relaxations, suggesting that Kv channels play a pivotal role in endothelial hyperpolarizations in these vessels (Nilius & Droogmans, 2001; Zygmunt & Hogestatt, 1996; Kwon *et al.*, 1999; Zygmunt *et al.*, 1997). However, it should be noted that the interpretation of these data could be complicated by the fact that 4-AP is also known to inhibit $SK_{Ca}$ (SK3) channels in a concentration-dependent manner (Grunnet *et al.*, 2001). For instance, 4-AP inhibits SK3 channels at 100 µM, while at higher concentrations it inhibits both $K_{IR}$ and SK3 (Grunnet *et al.*, 2001; Hinton & Langton, 2003). By contrast, EDHF-type responses in rabbit mesenteric, rat cerebral and human resistance arteries were virtually unaffected by the inhibitor (Murphy & Brayden, 1995; Petersson *et al.*, 1997; Ohlmann *et al.*, 1997). In some studies, the combination of 4-AP with inhibitors of $K_{Ca}$, such as apamin, has been more effective than each inhibitor on its own (Hinton & Langton, 2003). Although the double combination fails to abolish ACh-evoked EDHF-type relaxations, it suggests that a synergy between Kv and $SK_{Ca}$ channels might partially contribute to endothelial hyperpolarizations in these arteries (Hinton & Langton, 2003).

*ATP-dependent $K^+$ channels ($K_{ATP}$)* – Inhibition of EDHF-type responses by the sulphonylurea glibenclamide, a selective inhibitor of $K_{ATP}$ channels, has been demonstrated only in few vessels, including rabbit mesenteric and piglet pial arteries

(Murphy & Brayden, 1995; Lacza *et al.*, 2002). In rabbit iliac arteries, glibenclamide had no effect on ACh- and A23187-evoked EDHF-type relaxations and hyperpolarizations, therefore excluding the proposed role of $K_{ATP}$ in mesenteric arteries from the same species (Chaytor *et al.*, 2002). Similarly, glibenclamide failed to inhibit EDHF-type relaxations in bovine and porcine coronary arteries, and guinea-pig carotid artery (Hecker *et al.*, 1994; Corriu *et al.*, 1996).

## 1.3 Reactive oxygen species (ROS)

ROS such as superoxide ($O_2{}^{\cdot -}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($\cdot OH$) are by-products of oxygen metabolism and play a major role in determining the cellular redox status. Although the production of ROS was initially linked to various pathological conditions, it has now been established that the basal release of ROS also takes place under physiological conditions. Both endothelial and smooth muscle cells generate ROS either spontaneously or in response to receptor-dependent and – independent stimuli (Heinzel *et al.*, 1992; Zafari *et al.*, 1998). From a physiological standpoint, $H_2O_2$ is an important determinant of vascular tone, and possesses both vasodilator and vasoconstrictor properties, depending on the tissue and the experimental conditions. $H_2O_2$ induces relaxation in porcine and human coronary arteries, in rabbit and rat mesenteric arteries, in rabbit iliofemoral arteries and guinea-pig aorta (Fujimoto *et al.*, 2001; 2003; Chaytor *et al.*, 2003; Gao *et al.*, 2003; Hattori *et al.*, 2003; Matoba *et al.*, 2003; Miura *et al.*, 2003; Sato *et al.*, 2003). In some blood vessels, $H_2O_2$ has been considered to be an EDHF, on the basis that in these arteries NO/prostanoid-independent relaxations and hyperpolarizations are catalase sensitive (Matoba *et al.*, 2000). In addition, application of exogenous $H_2O_2$ mimics EDHF-type responses through a mechanism that involves the activation of $K_{Ca}$ channels in the smooth muscle (Hayabuchi *et al.*, 1998). By contrast, in pathological conditions such as hypertension, diabetes and hypercholesterolemia, the release of $H_2O_2$ and other ROS is augmented, leading to endothelial dysfunction, increased contractility, inflammation and apoptosis.

### 1.3.1 Production of $H_2O_2$

ROS are formed as intermediates in the redox process that leads to the production of $H_2O$ from molecular oxygen. The initial step for the production of ROS is the reduction

of molecular oxygen to $O_2^{\cdot-}$ (Fig. 1.8). $O_2^{\cdot-}$ is a negatively charged radical, which is highly unstable in aqueous solution, and depending on the conditions, it can act as an oxidizing or as a reducing agent. As an oxidizing agent $O_2^{\cdot-}$ is reduced to $H_2O_2$, while as a reducing agent it donates electrons to NO, generating peroxynitrite (ONOO⁻). $O_2^{\cdot-}$ has a half-life of a few seconds, and it is rapidly dismutated to $H_2O_2$. Although the dismutation reaction can occur spontaneously between two $O_2^{\cdot-}$ molecules, it can be significantly accelerated in the presence of the enzyme superoxide dismutase (SOD).

NADPH oxidase
Mitochondria
Xanthine oxidase
Endothelial NOS
Cyclooxygenase
Lipoxygenase
$CYP_{450}$ enzymes

$$O_2^{\cdot-} \xrightarrow[2H^+]{SOD} H_2O_2$$

GSSG
$2GSH \quad GSHP_x \longrightarrow 2H_2O$

catalase $\longrightarrow H_2O + \frac{1}{2} O_2$

$Fe^{2+}$

$\cdot OH$
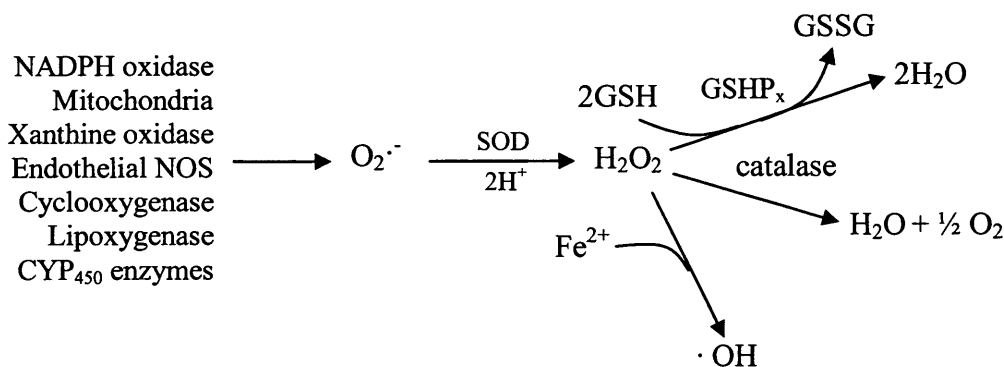
**Fig. 1.8** Schematic representation summarizing the steps that lead to the generation and degradation of $H_2O_2$ in the cell.

## 1.3.1.1 Superoxide dismutases

There are three major SODs: the Cu,ZnSOD (SOD1), the MnSOD (SOD2) and the extracellular SOD (EC-SOD; SOD3). SOD1 is mainly found in the cytosolic and lysosomal regions of the cell, although some reports have suggested that the enzyme might also be located in the intermembrane space of mitochondria (Okado-Matsumoto & Fridovich, 2001). SOD2 is the main mitochondrial isoform and is located in the mitochondrial matrix. By contrast, SOD3 localizes to the extracellular space by binding to heparan sulphate proteoglycans (Fattman *et al.*, 2003). Due to this extracellular localization, the enzyme plays a central role in scavenging $O_2^{\cdot-}$ especially that generated from membrane-bound NADPH oxidase and inflammatory cells (Oury *et al.*, 1992; Laude *et al.*, 2005). In the vasculature, SOD3 is mainly expressed by smooth muscle cells and macrophages, and is distributed at high concentrations in the interstitium of the arterial wall (Stralin *et al.*, 1995). Because of their distinct subcellular localization,

the catalytic action of the three SOD isoenzymes therefore takes place in distinct compartments since $O_2^-$ crosses cell membranes very poorly (Mendez *et al.,* 2005).

Among the three isoforms, SOD1 is the most abundant (accounting for 80% of the total SOD) and seems to be the most essential for the regulation of redox homeostasis in cells, SOD2 is much less preponderant (accounting for only the 2% of the total SOD), while the SOD3 content varies among mammalian species (Stralin *et al.,* 1995). Studies with SOD2 knockout mice have demonstrated that animals with the homozygous phenotype SOD2$^{-/-}$ die soon after birth, while those with the heterozygous SOD2$^{+/-}$ can survive despite the development of oxidative stress, which is attributed to mitochondrial dyfunction (Andresen *et al.,* 2004). Similarly, lucigenin-enhanced chemiluminescence experiments, which are used for the detection of $O_2^-$, demonstrated that in aortas and carotid arteries from SOD1-deficient mice the complete absence of the dismutase (SOD1$^{-/-}$ phenotype) is associated with $O_2^-$ levels that are 2-fold higher in corresponding vessels from wild type animals (Didion *et al.,* 2002). By contrast, the absence of SOD3 was found to have no effect on the lifespan of mice (Sentman *et al.,* 2006). The reason for these differences remains to be elucidated.

## 1.3.2 Degradation of $H_2O_2$

In conjunction to the enzymatic dismutation of $O_2^-$ radicals into $H_2O_2$, the cells have also evolved homeostatic mechanisms for the limitation of $H_2O_2$ levels in the vasculature. It has been established that the elimination of $H_2O_2$ is critical, as it forms a major protective mechanism that protects the cells against oxidative stress (Warner *et al.,* 2004). So far, three main mechanisms that reduce intracellular $H_2O_2$ levels have been identified. In the presence of (i) catalase or (ii) glutathione peroxidase, $H_2O_2$ is dismutated into $H_2O$ and molecular oxygen, whereas in the presence of (iii) transition metals, such as iron, $H_2O_2$ generates highly reactive hydroxyl radicals through the Haber-Weiss reaction (Fig. 1.8).

## 1.3.2.1 Degradation by catalase

Catalases are haem-containing enzymes that are responsible for the degradation of $H_2O_2$, thereby maintaining a stable redox status in the cells and preventing the damage of cellular components such as proteins (Ellis & Triggle, 2003). The catalase reaction is

a two-step process that depends on the oxidation/reduction of haem. In the first reaction, one $H_2O_2$ molecule oxidizes haem to an oxyferryl species, and subsequently the generation of a porphyrin cation radical known as Compound I (Cpd I)(2). In the second reaction, an $H_2O_2$ molecule reduces Cpd I to the resting state enzyme with a concomitant release of water and molecular oxygen (3).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (1)$$
$$Enz(Por - Fe^{III}) + H_2O_2 \rightarrow Cpd\ I(Por^+ \bullet - Fe^{IV} = O) + H_2O \qquad (2)$$
$$Cpd\ I(Por^+ \bullet - Fe^{IV} = O) + H_2O_2 \rightarrow Enz(Por - Fe^{III}) + H_2O + O_2 \qquad (3)$$
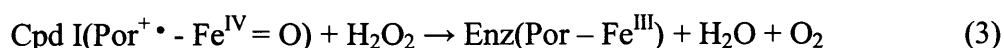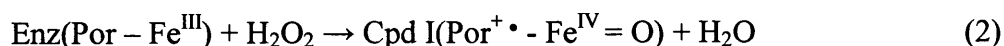
The catalase activity of animal and plant tissues is largely located in subcellular organelles, known as peroxisomes (Chance *et al.,* 1979). However, in some tissues such as in guinea-pig liver and rat heart, non-peroxisomal catalase is also located in mitochondria (Bulita *et al.,* 1996; Radi *et al.,* 1991). Other organelles such as the ER contain little, if any, catalase, while a small amount of the enzyme has been identified in the cytosol (Subramani, 1992).

Catalase extracts and synthetic catalase have been widely used as experimental tools to determine the role of drug-induced $H_2O_2$-dependent responses in the vasculature. In rabbit iliac arteries, for instance, application of bovine liver catalase has been used to investigate the putative role of endogenous $H_2O_2$ in EDHF-type responses induced by calcium ionophore A23187 and ACh (Chaytor *et al.,* 2003; Iesaki *et al.,* 1994; Mian & Martin, 1997). In these investigations the potential role of catalase in preventing $H_2O_2$-evoked responses was further elucidated by the application of 3-amino-1,2,4-triazol (ATZ), an inhibitor of the active site of the enzyme. The inhibitory action of ATZ is exerted on compound I, which means that it can only inhibit the enzyme in the presence of $H_2O_2$, and acts by modifying a histidine ligand to the haem (Margoliash & Novogrodsky, 1958). ATZ significantly impaired the catalase activity in rat aortas and restored the ability of $H_2O_2$ to depress the ACh-induced responses (Mian & Martin, 1997).

### 1.3.2.2 Degradation by glutathione peroxidase

An alternative mechanism of enzymatic $H_2O_2$ degradation is one that involves the oxidation of glutathione by the enzyme glutathione peroxidase (GSHpx)(Fig. 1.8). Although GSHpx and catalase serve the same purpose, it has been established that the ability of GSHpx to cope with $H_2O_2$ is 7-fold greater than that of catalase (Marklund *et al.*, 1982). Also, in tissues such as skeletal muscle, spermatozoa and certain regions of the brain it has been demonstrated that the amount of GSHpx present is significantly higher than that of catalase (Chance *et al.*, 1979). Nevertheless, it is now known that *in vivo* both enzymes co-operate in the removal of $H_2O_2$. In fact, studies carried out on mammalian red blood cells have shown that GSHpx regulates the normal low rate of $H_2O_2$ production, while as the concentration of $H_2O_2$ increases catalase becomes more important (Gaetani *et al.*, 1994).

At least four isoforms of GSHpx have been identified, with the GSHpx-1 being the most prevalent. The importance of the antioxidant properties of GSHpx-1 has been demonstrated in human T47D transfectants overexpressing the enzyme. In this mammary adrenocarcinoma cell line, overexpression of GSHpx-1 decreases $H_2O_2$ levels and increases resistance to cell damage (KretzRemy *et al.*, 1996). The crucial role of GSHpx in the determination of the redox status of the cells has also been demonstrated by studies carried out on GSHpx-1$^{-/-}$ knockout animals. These animals were shown to be highly susceptible to various forms of oxidative injury, and were predisposed to endothelial dysfunction, especially when fed with a hyperhomocysteinemic diet (Arthur, 2000; Dayal *et al.*, 2002).

The substrate for GSHpx is the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH), which depending on the redox status of the cells can be found either as a dimer or a monomer. The dimerization requires the oxidation of the cysteine sulfhydryl groups of two GSH molecules, followed by the subsequent formation of a disulphide bridge to form glutathione disulphide (GSSG). In this reaction, GSH serves as an electron donor for the reduction of $H_2O_2$ to $H_2O$ (Dringen *et al.*, 2005).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$
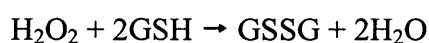
## 1.3.2.3 The Haber-Weiss / Fenton reaction - Metal induced oxidative stress

The iron-dependent decomposition of $H_2O_2$ is known as the Haber-Weiss reaction and generates $\cdot OH$, which can react at diffusion-limited rates with proteins, lipids and other biomolecules (Sawyer *et al.*, 1993; Lloyd *et al.*, 1997). It has been demonstrated that, although cells have evolved mechanisms to protect themselves from damage by $H_2O_2$ overproduction (see section 1.3.2.2), they are particularly vulnerable to iron-mediated oxidation of proline, histidine, arginine, lysine and in particular cysteine residues (Stadtman, 1990; Saurin *et al.*, 2004). Several lines of evidence have confirmed that the production of $\cdot OH$ requires the reduction / oxidation of iron, which has been previously released from iron-binding proteins, such as haemoglobin and cytochromes (Lloyd *et al.*, 1997; Valko *et al.*, 2004).

The two main reactions of iron-dependent $H_2O_2$ decomposition are:

$$Fe^{III} + O_2^{\cdot -} \leftrightarrow Fe^{II} + O_2 \tag{1}$$

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + \cdot OH + OH^- \text{ (Fenton reaction)} \tag{2}$$
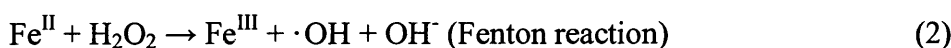
The overall reaction is called Haber-Weiss reaction:

$$O_2^{\cdot -} + H_2O_2 \leftrightarrow O_2 + \cdot OH + OH^- \tag{3}$$

*In situ*, generation of highly reactive $\cdot OH$ is associated with the iron-mediated oxidative damage that is implicated in cardiovascular disease, including cerebral ischaemia and reperfusion (Liu *et al.*, 2004; Valko *et al.*, 2005). Studies with the iron chelators deferoxamine and deferiprone have been used to probe these reactions in both vascular and brain tissues (Nelson *et al.*, 1992; Chaytor *et al.*, 2003; Shi *et al.*, 2007). For instance, in an experimental model of cerebral ischaemia in cats, treatment with deferoxamine has been associated with both reduced lipid peroxidation and improved post-ischaemic vasoreactivity (Nelson *et al.*, 1992).

## 1.3.3 Sources of $H_2O_2$

As described in a previous section, $O_2^{\cdot -}$ serves as a precursor for other ROS, such as $H_2O_2$, and modulates several signalling processes in the cellular compartments where it is released. Therefore, the sources of $H_2O_2$ are mainly the intracellular sources of $O_2^{\cdot -}$,

which include NADPH oxidases, mitochondria, eNOS, xanthine oxidase, cytochrome $P_{450}$ monoxygenase enzymes, COX and lipoxygenase.

### 1.3.3.1 NADPH oxidase

NADPH oxidase is widely considered to be the primary source of $O_2$ in the vascular wall, and it is known to be active in the endothelial, smooth muscle and adventitial layers (Touyz *et al.*, 2002; Rey & Pagano, 2002; Sorescu *et al.*, 2002; Muzaffar *et al.*, 2003; Yamawaki *et al.*, 2003).

The best characterized NADPH oxidase is found in phagocytes such as neutrophils and macrophages. The phagocytic NADPH oxidase consists of five major components, namely p47phox, p67phox, p40phox, p22phox and gp91phox. Additional components include the small size G-proteins Rac and Rap1A. In resting cells, these subunits form two complexes, one cytosolic and one bound to the plasma membrane (Fig. 1.9). The cytosolic complex comprises of p47phox, p67phox and p40phox, while p22phox and gp91phox form the integral membrane complex known as cytochrome $b_{558}$. The catalytic subunit of NADPH oxidase is gp91phox, and is mainly bound to FAD and two haem molecules. Conversely, the role of p40phox is unclear, although there is accumulating evidence which suggests that this subunit might be a negative regulator of the enzyme (Lopes *et al.*, 2004).

Two main events are required for the activation of NADPH oxidase. The first one is the exchange of GTP for GDP by Rac, and the second one is the phosphorylation of p47phox by protein kinase C (PKC) (Heyworth *et al.*, 1993; Xu *et al.*, 1994; Ungvari *et al.*, 2003; Bey *et al.*, 2004). Notably, the phosphorylation of p47phox triggers a conformational change, which leads to the release of p40phox and the subsequent association of the cytosolic complex with $b_{558}$ (Fig. 1.9). Following this interaction, the resulting functional enzyme reduces NADPH in the cytoplasm with a concomitant release of two electrons. These two electron pass from the cytoplasmic side to the extracellular space, through FAD and the two haem groups, and they are finally accepted by molecular oxygen.
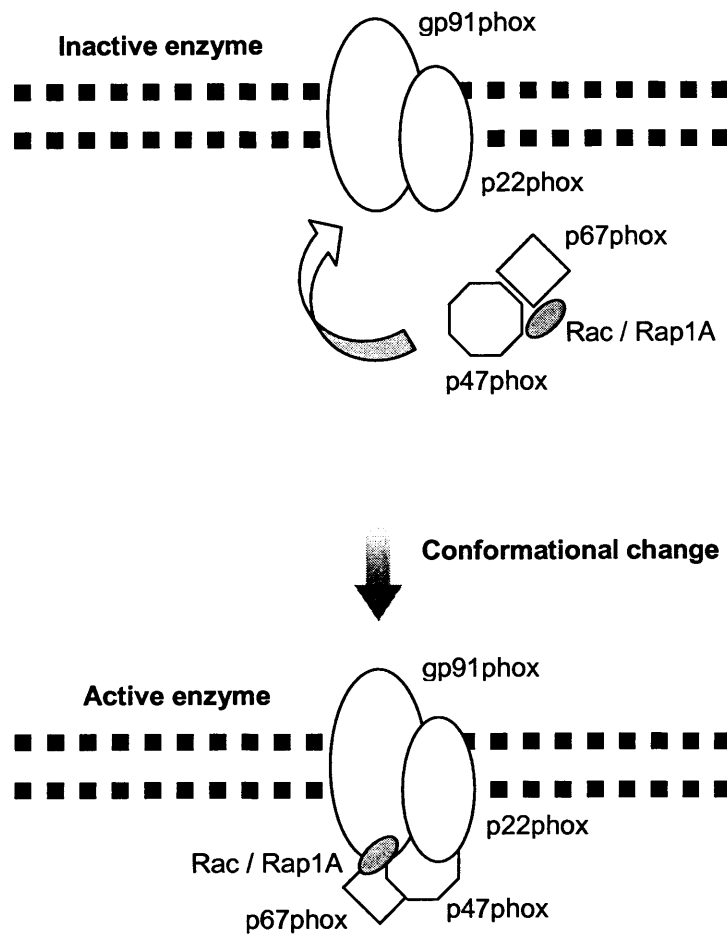
**Fig. 1.9** Regulation of NADPH oxidase by protein interactions. The figure shows the enzymatic components prior to cell activation and the active enzyme following the assembly of the regulatory cytosolic subunits with those that reside on the cell membrane.

Similarly to the phagocytic oxidase, the vascular homologues transfer electrons from the reduced substrate to molecular oxygen, producing $O_2^{\cdot-}$. Nevertheless there are still major differences between the vascular and the phagocytic NADPH oxidases. For instance, vascular NADPH oxidases are constitutively active and produce $O_2^{\cdot-}$ in a slow and sustained fashion, while the phagocytic one is inducible by cytokines and pathogens (Babior *et al.*, 2002; Li & Shah, 2002). Furthermore, in vascular tissues a family of gp91phox-like proteins called non-phagocytic NADPH oxidase (Nox) proteins has been identified (Suh *et al.*, 1999; Cheng *et al.*, 2001). According to their structure these proteins are classified as Nox1, Nox2 (also known as gp91phox), Nox3, Nox4 and Nox5 (Suh *et al.*, 1999). In the vasculature, Nox1, Nox4, Nox5 and Nox2 are located in

the endothelium of the vessels, while smooth muscle contains all homologues except for Nox2 (Banfi *et al.,* 2001; Lambeth *et al.,* 2002; Sorescu *et al.,* 2002). Notably, proteins homologous to p47phox and p67phox have also been identified. These proteins are known as 'NOX organizer 1' and 'NOX activator 1', and it has been suggested that they regulate NADPH oxidase function through the modulation of Nox1 activity (Takeya *et al.,* 2003).

The vascular NADPH oxidases are activated by a variety of hormones and factors, such as platelet-derived growth factor (PDGF), tumor necrosis factor (TNFα), oxidized LDL and angiotensin II (Griendling *et al.,* 2000). Endogenous NO derived from the vascular endothelium has also been proposed to affect the function of this enzyme. Indeed, in human cultured endothelial cells it has been demonstrated that NO has anti-inflammatory properties, and it can suppress $O_2^-$ production by NADPH oxidase (Selemidis *et al.,* 2007). Further investigations by the same group showed that in human microvascular endothelial cells (HMEC-1) addition of NO donors, namely DETA-NONOate, can significantly reduce $O_2^-$ production by ~50%, an effect which was attributed to the S-nitrosylation of the p47phox subunit (Selemidis *et al.,* 2007). Similar effects have also been demonstrated by other studies with other NO donors and related S-nitrosothiols, which suggested that these agents might interfere with the assembly of the functional enzyme (Park, 1996; Fujii *et al.,* 1997). However, in this thesis all experiments were carried out in the presence of the NOS inhibitor L-NAME and therefore the aforementioned interaction is irrelevant in this context.

## 1.3.3.2 Mitochondrial electron transport chain

Production of ROS by mitochondria takes place predominantly in the electron transport chain (ETC) in the inner mitochondrial membrane. ETC comprises of more than 80 proteins, grouped into five redox centres (complexes), which may leak electrons to molecular oxygen during respiration. The monovalent reduction of oxygen is favoured by the fact that most ETC steps involve single-electron reactions.

## 1.3.3.2.1 The mechanism of electron transport and ATP production

In general terms, reducing equivalents are transferred from NADH and FADH2, during the metabolism of carbohydrates and fatty acids, to molecular oxygen with a

31

concomitant extrusion of protons across the mitochondrial membrane, which is known as 'respiration'. The transport of protons generates a proton motive force – an electrochemical gradient – that is dissipated when ATP levels are low. It has been established that this gradient has two components: 1] a pH-related component, which is the result of the pH difference ($\Delta pH$) across the inner membrane of the organelle, and 2] a difference in membrane potential ($\Delta\Psi m$), due to charge separation (Lee *et al.*, 2001; Hunte *et al.*, 2003). The rate of ATP synthesis is determined by these components, as well as the cellular concentration of ADP, which is approximately 10-fold lower than that of ATP. The process of ATP synthesis, known as oxidative phosphorylation, takes place in the inner mitochondrial membrane at the five complexes of ETC. Each complex exerts a different degree of control over respiration, and thereby the generation of ATP.
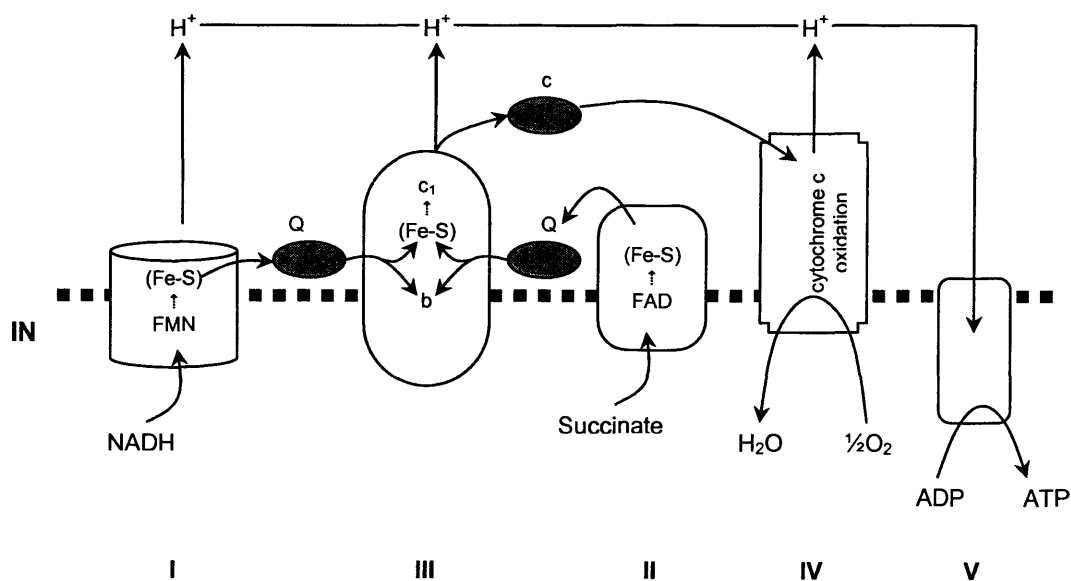


**Fig. 1.10** Schematic representation of the mitochondrial electron transport chain (ETC), showing the five complexes (I, II, III, IV and V), and how consumption of the substrates NADH and succinate leads to the development of the proton motive force needed for the production of ATP from ADP in complex V. Q: ubiquinone, Fe-S: iron-sulphur centre, $c_1$: cytochrome $c_1$, c: cytochrome c, b: cytochrome b, FAD: flavin adenine dinucleotide, FMN: flavin mononucleotide. OUT: outer mitochondrial membrane. IN: inner mitochondrial membrane.

NADH:ubiquinone oxidoreductase is the first and largest enzyme in ETC (Complex I)(Fig. 1.10), and its main function is the catalysis of electron transport from NADH to ubiquinone (Q). This translocation is coupled to the efflux of protons from the mitochondrial matrix to the intermembrane space.

The flavoprotein succinate:ubiquinone oxidoreductase (Complex II) oxidizes succinate to fumarate, transferring electrons to ubiquinone (Fig. 1.10). The second complex is the only part of the respiratory chain that forms a link with the citric acid cycle. In terms of its structure, complex II consists of a hydrophilic domain located in the matrix and a hydrophobic one bound on the mitochondrial membrane. The hydrophilic domain comprises of a flavoprotein folded in four domains, bound to an iron-sulfur subunit. The flavoprotein contains a flavin adenine dinucleotide (FAD) domain, which is the primary electron acceptor in this complex. The iron-sulfur subunit has a C- and an N- terminus. The N-terminal domain contains the [2Fe-2S] centre, whereas the C-terminal domain contains the [3Fe-4S] and the [4Fe-4S] centres. During respiration, electrons flow through the [3Fe-4S] centre to ubiquinone, forming ubisemiquinone, before becoming fully reduced.

Complex III, also known as ubiquinol:cytochrome c reductase, is the component of ETC that is responsible for the transfer of electrons from ubiquinone to cytochrome c (Fig. 1.10). Like all the reactions in ETC, this one is coupled to the pumping of protons from the matrix to mitochondrial inner membrane, thereby contributing to the proton gradient required for the ATP synthesis. Complex III is a multi-subunit entity, which consists of two cytochromes, b and c, a low ($b_L$ or b-566) and high ($b_H$ or b-560) potential b-type haem, and the Rieske protein, which is bound to the [2Fe-2S] centre. The mechanism of electron transport by complex III is known as the Q cycle (Fig. 1.11), and relies on the transfer of electrons between two ubiquinone sites, Qo and Qi, which face the intermembrane space and the matrix, respectively (Fig. 1.11). During a complete cycle, two molecules of ubiquinol are oxidized to ubiquinones, and one molecule of ubiquinol is regenerated from the re-reduction of one of the two ubiquinones (Trumpower, 1990). In the first step, ubiquinol is oxidized at the Qo site, forming an ubisemiquinone anion. This electron transfer from ubiquinol is bifurcated (Fig. 1.11). The first electron is transferred to cytochrome $c_1$ and then cytochrome c,
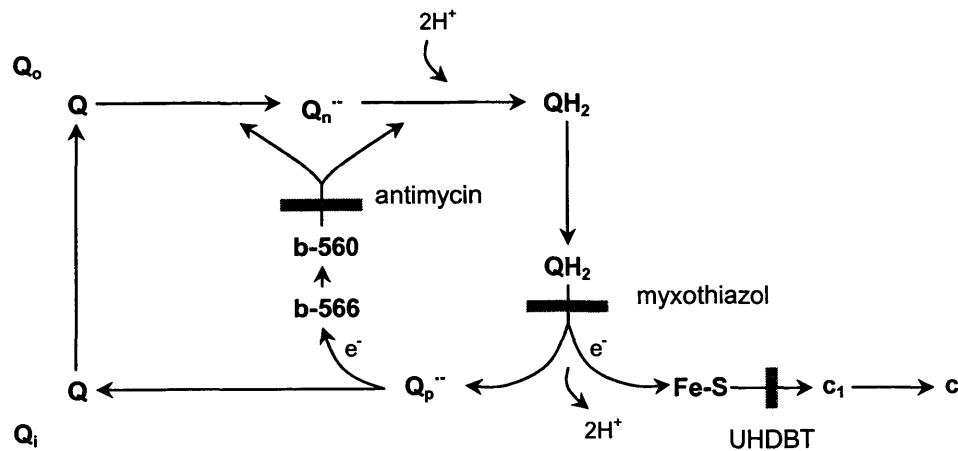
**Fig. 1.11** The protonmotive Q cycle. The diagram shows the branched cyclic pathway of electron transfer from ubiquinol ($QH_2$) to cytochrome c through the four redox centres of the cytochrome b and c1 complex. In the first step $QH_2$ is oxidized and one electron is transferred to the iron-sulfur protein (Fe-S) with the concomitant generation of low potential ubisemiquinone ($Q_p^-$). $Q_p^-$ subsequently reduces the b-566 heam group with the concomitant production of ubiqionone (Q). In the second step the electron that was transferred to Fe-S is transferred to cytochrome $c_1$ and then cytochrome c, while simultaneously one electron is transferred from b-566 to b-560 heam. In the third step b-560 heam reduces Q to the relatively stable ubisemiquinone anion ($Q_n^-$). When b-560 is re-reduced by repeat of the described cycle, it reduces $Q_n^-$ to $QH_2$. The diagram also shows the sites of action of three agents, which inhibit the Q cycle; antimycin, myxothiazol and 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT)(adopted from Trumpower, 1990). $Q_o$: intermembrane space ubiquinone site, $Q_i$: matrix ubiquinone site.

through the Fe-S proteins, whereas the second one propagates from haem $b_L$ to haem $b_H$. At this point, $b_H$ reduces ubiquinone to ubisemiquinone anion. Then, a second molecule of ubiquinol is oxidised by the Fe-S proteins by the same pathway, and electrons are transferred to the haem molecules. Haem $b_H$ finally reduces the previously formed ubisemiquinone anion to ubiquinol, thereby completing the cycle. Subsequently, cytochrome c carries the electrons from complex III to cytochrome c oxidase, also known as complex IV. This enzyme couples the reduction of molecular oxygen to water, to the pumping of protons from the mitochondrial matrix to the intermembrane space, contributing to the proton motive force generated across the membrane. These protons, in conjunction to those originating from complexes I and III are used by F1-F0-ATP synthase (complex V) to drive ATP synthesis (Fig. 1.10).

**1.3.3.2.2 Topological sites of $O_2$· release in mitochondria**

It has been established that mitochondria are one of the major sources of ROS in various experimental models. There is accumulating evidence that supports that complexes I and III of the ETC are important sites of $O_2$· generation (Lenaz *et al.*, 1998; Tompkins *et al.*, 2006; Grivenikova & Vinogradov, 2006; Guzy *et al.*, 2006). Indeed, several respiratory components, such as flavoproteins, Fe-S clusters and ubisemiquinone are capable of promoting the monovalent reduction of oxygen. Nevertheless, the relative contribution of each component varies among organs, and also depends on the respiration state, that is if the mitochondria are actively respiring or if the respiratory chain is highly reduced (Barja, 1999). It has been demonstrated that the rate of $O_2$· production depends on the law of mass action, therefore increasing when the concentration of oxygen increases, or the degree of reduction of the autoxidizable electron carriers of the ETC (Barja, 1999). In complex I, the primary source of $O_2$· seems to be one of the iron-sulfur clusters, whereas in complex III, the radical is formed during the autoxidation of ubisemiquinone in the mitochondrial membrane (Herrero *et al.*, 2000; Genova *et al.*, 2001; Lambert & Brand, 2004).

Studies carried out with the complex I inhibitor rotenone and the complex III inhibitor myxothiazol, demonstrated that occupation of the Q-binding site triggers $O_2$· production by promoting the reaction with molecular oxygen (Lambert & Brand, 2004). Similarly, in vivo experiments showed that $O_2$· production by mitochondria is particularly prominent in situations of metabolic perturbations, hypoxia-reoxygenation and ischaemia-reperfusion (Du *et al.*, 2001; Pearlstein *et al.*, 2002). Nevertheless, there is a large number of reports that suggest that interruption of electron transport by the aforementioned agents, and other ETC inhibitors does not always increase ROS production. For instance, in isolated rat pulmonary arteries, rotenone attenuates production of ROS, whereas in renal arteries from the same species, it has the opposite effect (Michelakis *et al.*, 2002). Other reports in human coronary resistance arteries and hypoxic pulmonary myocytes also confirm the fact that ETC inhibitors can either inhibit or stimulate ROS production (Liu *et al.*, 2003; Waypa *et al.*, 2002).

### 1.3.3.3 Endothelial Nitric Oxide Synthase (eNOS)

eNOS is a complex homodimeric oxidoreductase that requires the cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), NAD(P)H and *6R*-tetrahydrobiopterin ($BH_4$). The production of NO from L-arginine involves two independent steps (Gao *et al.*, 2007). The first one is associated with the NADPH-dependent oxidation of the substrate into $N^G$-hydroxy-arginine, while the second one involves the haem-dependent reduction of molecular oxygen. One essential feature of eNOS is that the activity of the enzyme requires the formation of an active dimer, and it is thought that NO is synthesized when electrons are transferred from the reductase domain (a C-terminal-bound NAD(P)H) of one subunit to the oxidase domain (an N-terminal haem centre) of the other (Nishino *et al.*, 2007). It has been suggested that $BH_4$ is perhaps the most essential cofactor in NO generation, as it plays a key role for the structural stability of the dimer (Landmesser *et al.*, 2003).

Moreover, there is increasing evidence that suggests that $BH_4$ availability determines the balance between NO and $O_2^-$ production by eNOS (Bendall *et al.*, 2005). Decreases in $BH_4$ levels, due to catabolism or oxidative degradation, are associated with eNOS uncoupling and concomitant increases of NOS-dependent $O_2^-$ production (Landmesser *et al.*, 2003). Taken that $BH_4$ is a reducing agent, it has been suggested that the cofactor is highly susceptible to oxidant stress, which ultimately leads to eNOS malfunction (Milstien & Katusic, 1999).

The generation of $O_2^-$ from eNOS has been demonstrated in stroke-prone spontaneously hypertensive rats (SPSHR), in animal models of angiotensin II-induced hypertension and in patients with diabetes mellitus (Kerr *et al.*, 1999; Mollnau *et al.*, 2002; Heitzer *et al.*, 2000). It has also been observed that eNOS function is impaired in ischemic hearts (Dumitrescu et al., 2007). Indeed, in the ischaemic and reperfused heart there is a significant increase in $O_2^-$ levels in the endothelium of coronary vessels, which was increased 10-fold 1 in a 30 min time-point following the induction of ischaemia in hearts from Sprague-Dawley rats. This increase was significantly inhibited by the eNOS inhibitor L-NAME, therefore confirming that the uncoupled enzyme is the major source of $O_2^-$ release in these vessels (Dumitrescu *et al.*, 2007).

## 1.3.3.4 Xanthine oxidase

Xanthine oxidase (XO) is one of the two inter-convertible forms of the molybdenum hydroxylase flavoprotein xanthine oxidoreductase (XOR), the other form being xanthine dehydrogenase (XD). The distribution of XOR in the vascular wall has been a controversial topic mainly due to the inconsistent results obtained by different methods of enzymatic detection. It has been demonstrated that XOR is present in the cytoplasm of the cells and on cell membranes bound to glycosaminoglycans, such as heparin (Rouquette *et al.*, 1998; Radi *et al.*, 1997). In addition, immunohistochemical studies have located XOR in bovine capillary endothelial cells and human endothelial cells (Jarasch *et al.*, 1981; Bruder *et al.*, 1984). However, other reports suggested that the enzyme might be located in peroxisomes, lysosomes and the rough endoplasmic reticulum (Fredericks & Vreeling-Sindelarova, 2002).

XO and XD catalyse the oxidation of hypoxanthine to xanthine and xanthine to uric acid respectively, with a concomitant reduction of $NAD^+$ or molecular oxygen (Pritsos, 2000; Meneshian & Bulkley, 2002; Nishimoto *et al.*, 2005). It has been demonstrated that the $NAD^+$-dependent XD is more preponderant than the oxidase isoform in mammalian tissues (Stripe & Della Corte, 1969). Nevertheless, XD can be reversibly converted into XO through the thiol oxidation of sulfhydryl residues, or irreversibly by proteolytic cleavage of the active site loop Gly-423-Lys-433 (Nishino & Nishino, 1997; Nishino *et al.*, 2005; Kuwabara *et al.*, 2003).

Whereas XD requires $NAD^+$ as an electron acceptor, XO requires the reduction of molecular oxygen. It has been proposed that the utilization of molecular oxygen by XO potentially leads to the formation of $O_2^{\cdot-}$, which has been involved in endothelial dysfunction, as demonstrated in experimental models of ischaemia-reperfusion, hypercholesterolemia, and double transgenic rats harbouring the human renin and angiotensin genes (Angelos *et al.*, 2006; White *et al.*, 1996; Mervaala *et al.*, 2001). Additionally, XO can function in a molybdenum-independent manner, which generates $O_2^{\cdot-}$ at the expense of NADH (Zhang *et al.*, 1998). However, it has also been demonstrated that the XD-dependent generation of $O_2^{\cdot-}$ was increased 4-fold in the presence of $O_2$ and NADH than that generated when hypoxanthine was the substrate (Zhang *et al.*, 1998). This effect is particularly significant for the interpretation of the effects of XOR inhibition, since $O_2^{\cdot-}$ generated by this mechanism is not blocked by

37

oxypurinol, but by the flavoprotein inhibitor diphenyliodonium (DPI)(Zhang *et al.*, 1998).

### 1.3.3.5 Cytochrome P450 enzymes

As described in section 1.2, cytochrome P450 enzymes (CYP) are potential modulators of vascular function, through the catalytic generation of vasoactive metabolites. Although CYP activity has been linked to the development of EDHF-type responses, there is accumulating evidence to suggest that these enzymes also modulate vascular contractility through the generation of ROS.

CYP enzymes are haem-containing terminal oxidases that catalyse the mixed-function oxidase reaction ie. the oxidation of highly lipid-soluble substrates including drugs and chemicals. The catalytic cycle of cytochrome P450 is a six step process that depends on the reduction/oxidation of iron, as well as the binding of molecular oxygen to the CYP-substrate complex. It has been suggested that, during this redox cascade, the generation of ROS takes place when electrons escape from the P450 cycle, and subsequently bind to free oxygen molecules. These electrons are mainly derived from the consumption of NADPH by mono-oxygenases, and they are transferred to the substrate-bound ferric ($Fe^{3+}$) CYP by the flavoprotein NADPH-CYP reductase. Moreover, it has been demonstrated that different CYP enzymes generate varying amounts of free radicals (Puntarulo & Cererbaum, 1998). Although, it is still unclear why these differences might take place, there is consensus on the fact that it might reflect putative differences in electron leakage from each enzymatic complex.

### 1.3.3.6 Cyclooxygenases and Lipoxygenases

Cyclooxygenase (COX) is a rate-limiting enzyme in prostaglandin biosynthesis, a two-step enzymatic process in which ROS can potentially be generated. COX catalyze both the reaction that converts arachidonate to prostaglandin $G_2$ ($PGG_2$) and a peroxidase reaction in which $PGG_2$ is converted into prostaglandin $H_2$ ($PGH_2$)(Smith *et al.*, 2000). Once arachidonic acid is available, the constitutive enzyme COX-1 uses molecular oxygen to generate the endoperoxides $PGG_2$ and $PGH_2$. The cyclooxygenase reaction initiates with the abstraction of a hydrogen atom from arachidonate to yield an

arachidonyl radical, while oxygen is sequentially added to the molecule to yield $PGG_2$. One requirement for this reaction to take place is the presence of a hydroperoxide, either an alkyl peroxide or peroxynitrite derived from the condensation of nitric oxide with superoxide, to oxidize the haem group at the active site of the peroxidase (Smith et al., 2000; Liu et al., 2007). The oxidized haem group then oxidizes the COX active site (Landino *et al.*, 1997; Smith *et al.*, 2000). Efficient peroxide removal or prevention of peroxide formation by antioxidants reduces the rate of prostaglandin synthesis, at least until sufficient $PGG_2$ is formed to activate COX-1 to its full potential. It should be noted that the peroxidase activity of the enzyme can function independent of the cyclo-oxygenase, while the cyclooxygenase reaction is peroxide-dependent (Smith & Lands, 1972a).

The role of COX in the generation of $O_2^{\cdot-}$ has been demonstrated by gas chromatography and mass spectrometry, carried out in microsomes from ram seminal vesicles (Kukreja *et al.*, 1986). It has been suggested that $O_2^{\cdot-}$ is not produced directly by the enzyme, but it is a product of a side-chain reaction, which takes place only when suitable co-substrates, such as NADH and NADPH are available. Notably, $O_2^{\cdot-}$ generation depended on the peroxidase activity of the enzyme, since the radical was generated when $PGG_2$ was used as substrate, but not with $PGH_2$ (Kukreja *et al.*, 1986). $O_2^{\cdot-}$ is generated when molecular oxygen interacts with $\cdot NAD$ and $\cdot NADP$ radicals that are respectively derived from NADH and NADPH with the following reactions:

with NADH co-substrate:
peroxidase + ROOH $\rightarrow$ compound I +ROH
compound I + NADH $\rightarrow$ compound II + $\cdot NAD$
compound II + NADH $\rightarrow$ peroxidase + $\cdot NAD$

with NADPH co-substrate:
peroxidase + ROOH $\rightarrow$ compound I +ROH
compound I + NADPH $\rightarrow$ compound II + $\cdot NADP$
compound II + NADPH $\rightarrow$ peroxidase + $\cdot NADP$

reaction with oxygen:
$\cdot NAD + O_2 \rightarrow NAD^+ + O_2^{\cdot-}$
$\cdot NADP + O_2 \rightarrow NADP^+ + O_2^{\cdot-}$
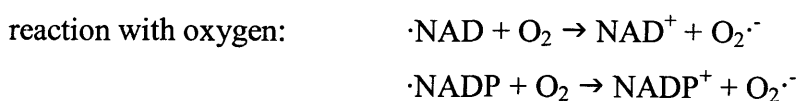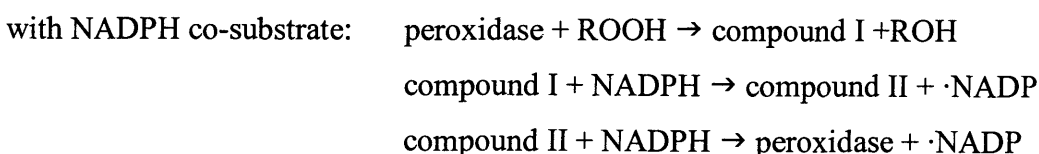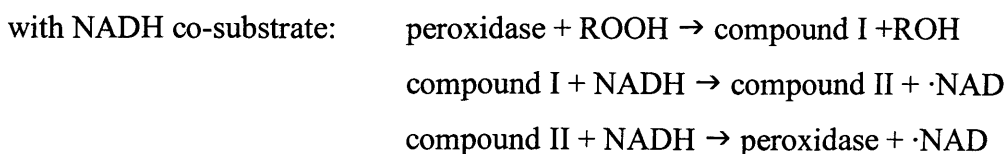
where ROOH is the hydroperoxide substrate, ROH is the hydroxide product, compound I and compound II are intermediate products (enzyme-centered radicals). These aforementioned reactions can be generalized to explain the generation of $O_2^{\cdot-}$ from both COX and lipoxygenase.

Lipoxygenases (LOX) are iron-containing dioxygenases that catalyse the direct reaction of polyunsaturated fatty acids (PUFAs) with oxygen to hydroperoxides and other metabolites using a non-haem iron active site (Smith & Lands, 1972b). LOXs are also involved in the biosynthesis of lipid hormones such as leukotrienes, lipoxins and hydroxy fatty acids. It has been reported that certain LOX subtypes generate $O_2^{\cdot-}$ during their action on linoleic acid or arachidonate in the presence of NADH and NADPH. The mechanism that has been proposed to generate ROS is similar to that of COX and requires the generation of enzyme-centered radical intermediates (Kukreja *et al.*, 1986). Nevertheless, the time course of $O_2^{\cdot-}$ production by this enzyme declines much more rapidly than COX, and it reaches zero in less than ten minutes (Kukreja *et al.*, 1986).

### 1.3.4 Vascular effects of $H_2O_2$

Endogenously produced $H_2O_2$ has been demonstrated to directly relax vascular smooth muscle and to contribute to endothelium-dependent responses, through the modulation of potassium channel function, alterations in calcium homeostasis, and by activating pathways that subsequently mediate relaxation (Barlow *et al.*, 2000; Fujimoto *et al.*, 2003; Miura *et al.*, 2003).

*Potassium channels* – The ability of $H_2O_2$ to modulate the opening of potassium channels has been attributed to the oxidative modification of suphydryl groups located in cysteine residues (Ruppersberg *et al.*, 1991). The $H_2O_2$-evoked effects on potassium channels vary among tissues and species, and depend on the potassium channel subtype involved. However, there are reports, which indicate that the oxidative modification of thiol groups might not have the same outcome on the same channel in all the tissues studied (Cai & Sauve, 1997; Tang *et al.*, 2001). For instance, $H_2O_2$ has been shown to decrease the open probability of $BK_{Ca}$ channels in synthetic lipid bilayers (Soto *et al.*, 2002), whereas in isolated porcine coronary myocytes it had the opposite effect (Barlow & White, 1998). Similarly, in rat cerebral arteries, exogenous $H_2O_2$ or endogenous $H_2O_2$

evoked by bradykinin increased arteriolar diameter in a concentration-dependent manner (Sobey *et al.*, 1997). Dilator responses to $H_2O_2$ were inhibited by tetraethylammonium (TEA), a non-selective $K_{Ca}$ blocker, and IbTX (Sobey *et al.*, 1997). Studies in intact canine cerebral and mouse mesenteric arteries and human umbilical vein endothelial cells (HUVECs) have also supported these findings (Iida *et al.*, 2000; Matoba *et al.*, 2000; Bychkov *et al.*, 1999). By contrast, investigations carried out on endothelial cells from bovine aorta demonstrated that the activity of $IK_{Ca}$ channels is reduced, possibly due to the oxidation of sulfhydryl groups that regulate channel gating by $H_2O_2$ (Cai & Sauvé, 1997). However, no similar studies appear to have been performed for $SK_{Ca}$ channels in the vasculature.

In addition to $K_{Ca}$, the opening of $K_{ATP}$ channels is subject to oxidative modification, as demonstrated by studies carried out on feline cerebral vessels and rat mesenteric arteries (Wei *et al.*, 1996; Nakazaki *et al.*, 1995). In these studies, $H_2O_2$-induced relaxations were significantly attenuated by glibenclamide, but not TEA. An appraisal of the available literature, suggests that the effects of $H_2O_2$ on $K_{ATP}$ channels can be direct or indirect. Notably, in ventricular myocytes from guinea-pig hearts, $H_2O_2$ can directly modulate the opening of these channels by interacting with the ATP-binding sulfonylurea subunit, and subsequently reducing its affinity for ATP (Ichinary *et al.*, 1996). Other reports in neuronal tissue, however, have suggested an indirect inhibitory effect of $H_2O_2$ on $K_{ATP}$ opening, mainly resulting from a reduction of oxidative phosphorylation, and consequently intracellular ATP levels (Hyslop *et al.*, 1988; Teepker *et al.*, 2007). Finally, a small number of reports have suggested that $H_2O_2$ might also interact with $K_V$ and $K_{IR}$ channels (Bychkov *et al.*, 1999; Muller & Bittner, 2002). However, the available evidence on vascular tissue is limited.

*Calcium homeostasis* – The role of $H_2O_2$ in the modulation of $Ca^{2+}$ mobilization has been previously demonstrated in various species and disease models, which are associated with ROS release. In an experimental model of ischaemia-reperfusion, accumulating ROS inactivate SERCA of smooth muscle cells in porcine coronary arteries (Grover & Samson, 1997). Further studies on the same vessels showed that $H_2O_2$ has a direct effect on SERCA on smooth muscle cells, with no similar effects taking place in the endothelium. This discrepancy was attributed to the presence of different SERCA variants in the two vascular layers, with the endothelial SERCA3

variant being more resistant to $H_2O_2$ than the smooth muscle SERCA2b variant (Grover *et al.*, 1997). It has been suggested that the mechanism through which $H_2O_2$ inhibits SERCA is probably through the oxidation of sulfhydryl groups or by direct attack on the ATP binding site (Grover *et al.*, 2003; Scherer & Deamer, 1986).

Moreover, it has been accepted that $H_2O_2$ impairs vascular contractility through a direct effect on L-type $Ca^{2+}$ channels (Gill *et al.*, 1995; Tabet *et al.*, 2004). The differential activation of L-type $Ca^{2+}$ channels has been demonstrated in the WKY rat and was attributed to $H_2O_2$, on the basis that it time-dependently increased $[Ca^{2+}]_i$, primarily via extracellular $Ca^{2+}$ influx (Tabet *et al.*, 2004). However, in these investigations, neither verapamil or diltiazem, two L-type channel inhibitors, completely blocked the $H_2O_2$-mediated $[Ca^{2+}]_i$ effects in the smooth muscle, which suggested that mechanisms independent of the activation of these channels might also be involved. Enhanced activation of L-type channels by $H_2O_2$ has also been observed in spontaneously hypertensive rats (SHR) (Tabet *et al.*, 2004). Notably, in cells from these species, the magnitude of the inhibition by verapamil and diltiazem was greater relative to that of the normotensive WKY, which suggested that $H_2O_2$-induced effects on L-type channels are enhanced in hypertension (Tabet *et al.*, 2004). Indeed, this finding was consistent with an increase in expression of L-type channels in SHR, as well as a significant increase in the whole-cell and single-channel currents relative to those of WKY (Ohya *et al.*, 1993; 1998).

Several studies have suggested that alterations in $Ca^{2+}$ sensitivity and the ability of smooth muscle cells to contract to agonists might be associated with the ability of $H_2O_2$ to increase tissue concentrations of cGMP and phosphorylation of myosin light chain (MLC) (Lorenz *et al.*, 1999; Fujimoto *et al.*, 2001; 2003). Indeed, it has been shown that $H_2O_2$ indirectly reduces noradrenaline- and ACh-evoked increases in MLC phosphorylation in canine airway smooth muscle and guinea-pig aortae (Lorenz *et al.*, 1999; Fujimoto *et al.*, 2003). However, there are contradictory reports suggesting that in arteries depolarized with $K^+$, $H_2O_2$ produces a transient increase in intracellular $Ca^{2+}$ concentration without a concomitant activation of the contractile machinery (Krippeit-Drews *et al.*, 1995; Iesaki *et al.*, 1996). One possible explanation for this effect is that the relaxation induced by $H_2O_2$ might be associated with a cGMP-mediated inhibition of $Ca^{2+}$ sensitivity following agonist stimulation (Fujimoto *et al.*, 2003). However, in

this study, Fujimoto *et al.* (2003), demonstrated that the $H_2O_2$-induced relaxations were only partially blocked by guanylate cyclase inhibitors, such as 1H-[1,2,4]oxadiazolo[4,3,α]quinoxalin-1-one (ODQ). Hence, it is more likely that other mechanisms might participate in the $H_2O_2$-dependent effects on $Ca^{2+}$ homeostasis. Indeed, it has been demonstrated that $H_2O_2$ induces relaxation by forming interprotein disulfide bonds between two cysteine residues on adjacent chains in the PKG homodimer complex, independently of cGMP. Such effect seems to occur specifically at cysteine residues since it was not evident in A10 cells, overexpressed with the redox-insensitive Cys42Ser mutant (Burgoyne *et al.*, 2007). $H_2O_2$ has also been reported to directly inhibit actomyosin ATPase, thereby reducing $Ca^{2+}$ sensitivity directly and producing relaxation (Perkins *et al.*, 1997; 2003).

### 1.3.4.1 $H_2O_2$ as a vasoconstrictor

Taken that in some vessels $H_2O_2$ increases $[Ca^{2+}]_i$, it had been suggested that it might also act as a vasoconstrictor depending on the tissue and the conditions involved (Katušić *et al.*, 1993; Yang *et al.*, 1998; 1999). Although most of the studies have failed to demonstrate a direct relation between $H_2O_2$-evoked contractions and $[Ca^{2+}]_i$ increases, it has been accepted that the stimulation of enzymes such as phospholipase A2 ($PLA_2$), COX, phospholipase C (PLC), and tyrosine kinases, might underpin these responses in quiescent vessels (Chakraborti *et al.*, 1989; Rodriguez-Martinez *et al.*, 1998; Yang *et al.*, 1998; Jin & Rhoades, 1997). For instance, it has been suggested that in endothelial cells U73122, a specific PLC inhibitor, abolished $H_2O_2$-induced $Ca^{2+}$ mobilization, and that $H_2O_2$ caused hydrolysis of inositol phospholipids (Shasby *et al.*, 1988; Volk *et al.*, 1997). Also, these results together with the fact that PKC inhibition with staurosporin attenuates $H_2O_2$-induced contractions in rat aorta support further the fact that a PLC-PKC signalling cascade might be involved (Yang *et al.*, 1998).

Furthermore, exogenous $H_2O_2$ has been reported to cause transient contractions in vessels such as the rat aorta and mesenteric arteries (Malmsjo *et al.*, 2000; Gao & Lee, 2001; Yang *et al.*, 2003). It is likely that these responses might be associated with pathways involving arachidonate metabolism, since inhibitors of COX and thromboxane synthase attenuated contractions (Gao & Lee, 2001; Yang *et al.*, 2003). Other studies, however, attributed the $H_2O_2$-induced contractions to products of lipid

peroxidation, such as isoprostanes, which have been suggested to activate a tyrosine kinase pathway following the stimulation of thromboxane receptors on smooth muscle (Hoffman *et al.*, 1997; Janssen, 2002). However, the role of these metabolites in $H_2O_2$-induced vasoconstriction needs to be investigated further, as some reports in the available literature have proposed isoprostanes as a stimulus of the EDHF phenomenon (Janssen, 2002).

Finally, recent studies have suggested that in rat mesenteric arteries, the aforementioned contractile and relaxant properties of $H_2O_2$ might be concentration-dependent (Gao *et al.*, 2003). Indeed, incubation of PE preconstricted arteries with micromolar concentrations of $H_2O_2$ led to an additional increase in tone, while addition of concentrations in the milimolar range relaxed the muscle by ~95% (Gao *et al.*, 2003). Notably, the transient contractile response was significantly depressed by inhibitors of $PLA_2$ and COX, therefore being in agreement with previous studies by Gao & Lee (2001), which inferred that this pathway contributes to $H_2O_2$-induced contraction (Gao *et al.*, 2003). Biphasic responses to $H_2O_2$ have also demonstrated in porcine cerebral arteries and in rat gracilis skeletal muscle arterioles (Leffler *et al.*, 1990; Cseko *et al.*, 2004).

## 1.3.4.2 $H_2O_2$ as an EDHF

It has been suggested that $H_2O_2$ might be an EDHF, on the basis that in certain blood vessels ACh-evoked EDHF-type relaxations are catalase sensitive. Indeed, a critical appraisal of the available literature, suggests that $H_2O_2$ has EDHF-like effects in porcine pial, rat, mouse and human mesenteric arteries, and in canine, porcine and human coronary arteries (Lacza *et al.*, 2002; Kimura *et al.*, 2002; Liu *et al.*, 2006; Matoba *et al.*, 2000; 2002; 2003; Miura *et al.*, 2003; Yada *et al.*, 2003). The first available evidence which showed that $H_2O_2$ might be an EDHF was provided by studies carried out in eNOS-knockout and C57BL/6 mice (Matoba *et al.*, 2000). In small mesenteric arteries from these animals, mechanical and electrophysiological investigations showed that catalase inhibited both ACh-evoked relaxations and hyperpolarizations, the effects of which were abolished in the presence of ATZ. Also, the gap junction inhibitor 18β-GA had no significant effect on EDHF-type relaxations, which suggested that gap junctional communication does not play a role in these responses (Yamamoto *et al.*,

1998; Matoba *et al.*, 2000). The role of $H_2O_2$ as a putative EDHF was also proposed by *in vivo* studies carried out on canine coronary arteries, which showed that ACh-induced vasodilations could be significantly attenuated by catalase, an effect which was more profound in arterioles than small size arteries (Yada *et al.*, 2003). It was also suggested that the differences in catalase sensitivity might concur with the notion that EDHF-type responses play a more important role as the size of the vessels decreases (Yada *et al.*, 2003).

Further investigations carried out on homozygous $SOD1^{-/-}$ and heterozygous $SOD1^{+/-}$ mice suggested that the endothelial SOD1 might probably be the main source of $H_2O_2$-associated EDHF, at least in mice, as in this model endothelium derived $H_2O_2$ is mainly generated in the membrane (Morikawa *et al.*, 2003). Indeed, in $SOD1^{-/-}$ animals, EDHF-type relaxations were significantly depressed relative to the control in both mesenteric arteries and coronary microvessels (Morikawa *et al.*, 2003). Investigations with heparin, an inhibitor of SOD3, excluded the involvement of this enzyme in $H_2O_2$-associated EDHF, while the role of SOD2 was ruled out because this enzyme is located in mitochondria, an orgenelle that is not the source of $H_2O_2$ in these animals (McIntyre *et al.*, 1999; Morikawa *et al.*, 2003).

Other studies carried out on arterial preparations showed that $H_2O_2$ can potentially produce endothelium-dependent hyperpolarizations and relaxations through the differential activation of potassium channels and $Na^+/K^+$-ATPase (Pomposiello *et al.*, 1999; Barlow *et al.*, 2000; Kimura *et al.*, 2002; Gao *et al.*, 2003; Miura *et al.*, 2003). For instance, in eNOS knockout mice, the combination of ChTX and apamin significantly inhibits $H_2O_2$/EDHF-type relaxations, whereas tetrabutyl-ammonium (TBA), a non-selective $K_{Ca}$ inhibitor, abolishes similar responses in wild-type animals (Matoba *et al.*, 2000). In porcine pial arteries, catalase-sensitive BK-evoked responses were significantly reduced by glibenclamide, suggesting that, in these vessels, the opening of $K_{ATP}$ prevails in NO/prostanoid-independent relaxations (Lacza *et al.*, 2002).

By contrast, in the rabbit, A23187-evoked $H_2O_2$ triggers relaxations that are independent of the concomitant hyperpolarizing effects (Chaytor *et al.*, 2003). As previously discussed, although catalase attenuates vasorelaxation in these vessels in a concentration-dependent manner, it fails to inhibit smooth muscle hyperpolarizations,

and in essence $H_2O_2$ is more likely to be a relaxing factor, rather than an EDHF. In conjunction to A23187, ACh-evoked relaxations comprise of a catalase-sensitive component, but it is smaller in magnitude than that of A23187 (Chaytor *et al.*, 2003). Similarly, in canine coronary arteries, generation of $H_2O_2$ by A23187 was significantly attenuated by the eNOS inhibitor L-NAME, therefore suggesting that in these vessels eNOS-generated $H_2O_2$ might compensate for the loss of NO (Cosentino & Katušić, 1995).

Interestingly, electrochemical measurements in the rat demonstrated that application of exogenous $H_2O_2$ to aortas fails to mimic the effects of authentic $H_2O_2$ produced following stimulation of the endothelium with A23187 (Cosentino *et al.*, 1998). In piglet coronary microvessels, electron spin resonance revealed that BK-evoked EDHF-type responses are accompanied by a nanomolar production of $H_2O_2$ (Matoba *et al.*, 2003). Nevertheless, mechanical studies in rabbit iliac arteries showed that exogenous $H_2O_2$ fails to show any significant effects at concentrations lower than 100 μM (Chaytor *et al.*, 2003). One possible explanation for this discrepancy is that authentic $H_2O_2$ is generated in close proximity to its site of action (Miura *et al.*, 2003), and therefore its exposure to enzymatic degradation might be limited. The autocrine effects of $H_2O_2$ and its contribution to the EDHF phenomenon need to be elucidated further.

### 1.3.4.3 Other ROS and myogenic response

In addition to $H_2O_2$, ROS such as $O_2^{\cdot-}$ and $\cdot OH$ are also involved in the regulation of the vascular tone. $O_2^{\cdot-}$ is the primary product of oxygen metabolism, but it is highly unstable and it is directly converted into $H_2O_2$ by SODs (see section 1.3.1). Nevertheless, a small amount of $O_2^{\cdot-}$ is still able to escape dismutation and have a significant effect on the myogenic response (Table 1.1). $O_2^{\cdot-}$ is known to interact with NO to generate $ONOO^-$, which can potentially interact with regulatory systems that are of biological significance (Liu *et al.*, 1998). One potent effect of $ONOO^-$ seems to be thiol modification, which affects the function of signalling systems that possess regulatory function or when produced at high concentrations it forms NO donors through the modification of alcohols and sugars to nitrated species, which release NO in the presence of thiols (Moro *et al.*, 1995; Wolin *et al.*, 1998)(Table 1.1). However, in the presence of eNOS inhibitors, such as L-NAME, it is unlikely that $ONOO^-$ is

| Species | Site of Reaction | Signaling Action |
|---|---|---|
| $O_2^{\cdot-}$ | NO | Inactivation of NO prevents sGC stimulation; $ONOO^-$ generation |
| | Fe-S complexes | Inhibition of aconitase and mitochondrial respiration by releasing Fe, which forms $\cdot OH$ |
| | Catecholamines | Inactivation of adrenaline or noradrenaline inhibitors interactions with adrenergic receptors |
| $H_2O_2$ | Catalase | Stimulates sGC |
| | GSH peroxidase | GSSG formation |
| | COX | Activation at low levels of peroxide |
| | $PGI_2$ synthase | Inactivation at high levels of ROS and RNS |
| | $IP_3R$ | Activation of receptor |
| | RyR | Activation of receptor |
| $\cdot OH$ | Thiols | Oxidation |
| | Lipids | Generation of vasoactive isoprostanes and lipid oxidation products |

**Table 1.1** Summary of signalling actions of $O_2^{\cdot-}$, $H_2O_2$ and $\cdot OH$ on the vascular wall of arteries (modified from Wolin, 2000).

produced, and therefore interactions of $O_2^{\cdot-}$ with other vascular components should be taken into account (Table 1.1). Furthermore, $H_2O_2$ can react with iron to produce the highly toxic $\cdot OH$ radical, which promotes oxidative stress-associated tissue injury (see section 1.3.1). However, previous investigations carried out in rabbit iliac arteries with SOD and the cell-permeant iron chelator deferiprone failed to affect relaxations evoked by X/XO (Chaytor *et al.,* 2003), thereby suggesting that neither $O_2^{\cdot-}$ nor $\cdot OH$ generated intracellularly from $H_2O_2$ via Fenton reaction contributes to the EDHF-type responses in this species.

## 1.4 General aims of thesis

The aim of this thesis has been to provide a clearer understanding of the EDHF phenomenon and the putative role of oxidative stress in the dilatory responses induced

by agents which are known to trigger hyperpolarizations in the endothelium of rabbit iliac arteries. More specifically, investigations were carried out to assess the importance of different isoforms of the $K_{Ca}$ subfamily in EDHF-type relaxations evoked by both ACh and calcium ionophore A23187, and to demonstrate the localization of these channels in the endothelium and smooth muscle of rabbit iliac arteries. In a separate series of experiments, the role of $H_2O_2$ in A23187-evoked EDHF-type relaxations was investigated in conjunction with its putative effects on smooth muscle $K_{Ca}$ channels. Finally, mechanical investigations were performed to determine the putative sources of $H_2O_2$ in the endothelium of these vessels upon stimulation with A23187 and ACh.

*Chapter Two*


*The role of endothelial calcium-dependent
potassium channels in EDHF-type
responses in the rabbit*

## 2.1 Introduction

### 2.1.1 Activation of $K_{Ca}$ channels in EDHF-type responses

Agonists such as ACh and calcium ionophore A23187, evoke EDHF-type relaxations through a NO/prostanoid-independent pathway that involves the activation of endothelial $K_{Ca}$ channels (Chaytor *et al.*, 2002; Weston *et al.*, 2005; Gluais *et al.*, 2005; Sandow *et al.*, 2006; Sainsbury *et al.*, 2007). It has been established that sustained opening of these channels depends on the elevation of intracellular $Ca^{2+}$ via the mechanism of capacitative $Ca^{2+}$ entry, which is secondary to the depletion of the endoplasmic reticulum $Ca^{2+}$ store (ER)(Sedova *et al.*, 2000; Nilius & Droogmans, 2001). Increases in $[Ca^{2+}]_i$ lead to activation of the channels, which allow the efflux of $K^+$ into the extracellular space. This outward current is accompanied by a concomitant change in the membrane potential, which moves towards the equilibrium potential for $K^+$ at $\sim$-90mV, thereby causing membrane hyperpolarization. In the rabbit iliac artery, the endothelial hyperpolarization then propagates to the adjacent smooth muscle cells via myoendothelial gap junctions and ultimately leads to relaxation of the vessel (Chaytor *et al.*, 2005).

### 2.1.2 The role of different $K_{Ca}$ isoforms in EDHF-type responses

$K_{Ca}$ channels are separated into three different classes, namely the small conductance $K_{Ca}$ ($K_{Ca}2.1$, $SK_{Ca}$), the intermediate conductance $K_{Ca}$ ($K_{Ca}3.1$, $IK_{Ca}$) and the large conductance $K_{Ca}$ ($K_{Ca}1.1$, $BK_{Ca}$)(see section 1.2.5). Because of the differences in the biophysical properties of the three $K_{Ca}$ subtypes, each channel can be distinguished by the application of selective pharmacological agents. For instance, apamin is a potent inhibitor of both native and cloned $SK_{Ca}$ channels, while ChTX and IbTX has been extensively used to inhibit $BK_{Ca}$ (see section 1.2.5). Interestingly, $IK_{Ca}$ comprises a distinct category of channels, which has been characterized on the basis that it is structurally related to the $SK_{Ca}$ subfamily, but still has a distinct pharmacological profile. Indeed, the $SK_{Ca}$ subfamily of channels comprises of three main members, namely SK1, SK2 and SK3, which are all sensitive to apamin, and a forth apamin-insensitive member, namely SK4 which is now known to be the $IK_{Ca}$ channel (Köhler *et al.*, 1996). Selective inhibition of $IK_{Ca}$ has been achieved recently with two analogues of clotrimazole, TRAM-34 and TRAM-39, which are devoid of $P_{450}$ mono-oxygenase

inhibitory properties (Wulff *et al.*, 2000), while less selective inhibitors, such as ChTX have also been widely used.

In most blood vessels both $SK_{Ca}$ and $IK_{Ca}$ are expressed in the endothelium (Sakai, 1990; Marchenko & Sage, 1996; Doughty *et al.*, 1999; Köhler *et al.*, 2000; Walker *et al.*, 2001). Indeed, investigations in the guinea-pig and the rat, demonstrated that the combination of apamin and TRAM-34 significantly attenuates ACh-evoked EDHF-type responses in most endothelium-intact vessels (Edwards *et al.*, 1998; Gluais *et al.*, 2005). One possible explanation for this combined effect was that $SK_{Ca}$ and $IK_{Ca}$ form a heteromultimer, which requires the presence of two toxins in order to be inhibited, as demonstrated by radioligand-binding studies conducted with homogenates of rat brain cortex (Zygmunt *et al.*, 1997). However, immunohistochemical investigations carried out on rat mesenteric arteries, suggested that differences in the spatial distribution of these channels in the endothelium are more likely to underpin the inhibitory effects of apamin and TRAM-34 when administered (Sandow *et al.*, 2006). Indeed, these studies showed that $SK_{Ca}$ channels are localized in proximity to gap junctions between adjacent endothelial cells, while $IK_{Ca}$ are localized in proximity to myoendothelial gap junctions. This difference in localization could potentially explain why the incubation of arteries with TRAM-34 has a different physiological effect on EDHF-type responses than apamin, and why the co-administration of the two agents is necessary to abolish the responses (Corriu *et al.*, 1996; Chataigneau *et al.*, 1998). Differences in the role of these channels have also been demonstrated with studies carried out on both quiescent and depolarized rat mesenteric arteries, which demonstrated that in resting vessels ACh-evoked EDHF-type responses are mainly triggered by endothelial $SK_{Ca}$ channels, while in depolarized vessels the co-activation of $SK_{Ca}$ and $IK_{Ca}$ is required (Crane *et al.*, 2003).

Similarly, in most arteries apamin and ChTX are individually each only partially effective, while their co-administration is necessary to attenuate EDHF-type responses completely (Edwards *et al.*, 1998; Doughty *et al.*, 1999; Corriu *et al.*, 1996; Plane & Garland, 1996). The dual combination of apamin and ChTX abolishes EDHF-type responses in guinea pig carotid and basilar arteries, in rat mesenteric and hepatic arteries, and rabbit mesenteric and femoral arteries (Corriu *et al.*, 1996; Plane & Garland, 1996; Petersson *et al.*, 1997; Chen & Cheung, 1997; Fujimoto *et al.*, 1999;

Kwon *et al.*, 1999). In fact, it has been established that, each inhibitor on its own has a minor or no effect on the EDHF phenomenon, while their combination abolishes the residual effects. In most species studied so far, co-administration of apamin and ChTX attenuated both relaxations and hyperpolarizations evoked by ACh, A23187 and BK, suggesting that $K_{Ca}$ channels have a complementary role in the EDHF phenomenon (Chataigneau *et al.*, 1998; Fujimoto *et al.*, 1999; Ge *et al.*, 2000; Hinton & Langton, 2003; Eichler *et al.*, 2003; Gluais *et al.*, 2005; Weston *et al.*, 2005). Although according to those findings it would be reasonable to attribute the endothelial hyperpolarizing component to the activation of all three $K_{Ca}$ subtypes, investigations with the more selective $BK_{Ca}$ inhibitor IbTX failed to mimic the effects of ChTX in porcine coronary endothelial cells, and thereby disputed the fact that $BK_{Ca}$ might be functionally active in the endothelium (Bychkov *et al.*, 2002). Furthermore, some electrophysiological studies carried out on non-depolarized rabbit mesenteric arteries suggested that there is a possibility that a functional $BK_{Ca}$ channel might not be present on the endothelium of these vessels, on the basis that addition of IbTX had no effect on ACh-evoked hyperpolarizations, which opposed the findings of electrophysiological studies carried out on porcine renal arteries that identified a channel on the endothelium (Murphy & Brayden, 1995; Brakemeier *et al.*, 2003). However, it should be noted that in these investigations lack of inhibition of endothelial hyperpolarizations by IbTX does not exclude the fact that these responses might be triggered by more than one $K_{Ca}$ subtype e.g. $BK_{Ca}$ and $IK_{Ca}$, and therefore the combination of more than one inhibitor might be required to unmask the contribution of different channels to EDHF-type responses.

Further evidence about the effects of IbTX in the rabbit was obtained in renal arteries, in which application of the toxin almost completely inhibited EDHF-type relaxations (Kagota *et al.*, 1999). Notably, addition of apamin on its own had no effect on ACh-induced responses, while the effects of apamin plus IbTX did not differ from those obtained in the presence of IbTX only (Kagota *et al.*, 1999). It was therefore suggested that inhibition of $BK_{Ca}$ channels might be sufficient to attenuate the EDHF phenomenon in these arteries (Kwon *et al.*, 1999; Kagota *et al.*, 1999). By contrast, dual administration of apamin and IbTX is in most blood vessels ineffective or partially effective, thus reflecting the differences in putative interactions between the three channel subtypes (Zygmunt *et al.*, 1997; Chataigneau *et al.*, 1998; Yamanaka *et al.*, 1998; Fujimoto *et al.*, 1999). It should be noted that a functional $BK_{Ca}$ channel is also

expressed in cultured endothelial cells from rabbit aorta, in intact porcine renal and rat gracilis muscle arterioles (Rusko *et al.*, 1992; Baron *et al.*, 1996; Ungvari *et al.*, 2002). In intact rat gracilis muscle arterioles, the presence of $BK_{Ca}$ in the endothelium has been demonstrated by immunohistochemical techniques, and could potentially support the role of these channels in the signal transduction of EDHF-type dilatation, which was also demonstrated as a reduction in arteriolar diameter following the intraluminal administration of ChTX in the same vessels (Ungvari *et al.*, 2002). Similarly, in the rabbit aorta, whole cell voltage-clamp recordings revealed the presence of spontaneous transient outward currents (STOCs) in the endothelium, which represent the simultaneous activation of several $K_{Ca}$ channels and are inhibited by ChTX and TEA (Rusko *et al.*, 1992). Nevertheless, in endothelial cells from rat aorta and porcine coronary arteries, $BK_{Ca}$ channels are poorly expressed and no single IbTX-sensitive currents can be detected (Marchenko *et al.*, 1996; Bychkov *et al.*, 2002). This can be attributed possibly to the absence in these cells of regulatory $BK_{Ca}$ β subunits that enhance $Ca^{2+}$ sensitivity or in changes in the distribution of these channels during culture maintenance and passage (Bao & Cox, 2005; Frieden *et al.*, 1999). Indeed, substance P and BK cause hyperpolarization in the native endothelium of porcine coronary arteries by opening $SK_{Ca}$ and $IK_{Ca}$ channels only, while in cultured cells from the same arteries an IbTX-sensitive $BK_{Ca}$ is also known to be activated by BK (Frieden *et al.*, 1999). $BK_{Ca}$ channels are also expressed in vascular myocytes in which their activation may function as a negative feedback mechanism that limits increases in $[Ca^{2+}]_i$ in response to various stimuli (Weston *et al.*, 2005; Burnham *et al.*, 2006; Dong *et al.*, 1997).

## 2.1.3 Studies with $K_{Ca}$ channel activators

More conclusive data about the localization of $K_{Ca}$ channels have been obtained with the derivatives of the muscle relaxant chlorzoxazone 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619) and 1-ethyl-2-benzimidazolinone (1-EBIO), both of which can induce responses that are independent of ER depletion (Xia *et al.*, 1998; Fanger *et al.*, 1999; Cao *et al.*, 2001; Marrelli *et al.*, 2003). Despite their structural similarities, each compound shows a distinct level of selectivity for different $K_{Ca}$ isoforms. For instance, NS1619 triggers relaxations by opening $BK_{Ca}$ channels, as demonstrated by studies in which both ChTX and IbTX significantly attenuated the responses induced by this agent (Holland *et al.*,

1996; Edwards *et al.*, 2001). Notably, it is now known that these properties depend on a direct intracellular interaction of NS1619 with $BK_{Ca}$ (Holland *et al.*, 1996), and an indirect action through $Ca^{2+}$ release from the stores (Yamamura *et al.*, 2001). By contrast, 1-EBIO is the most commonly used positive modulator of $SK_{Ca}$ channels, including $SK_4$ ($IK_{Ca}$)(Pedarzani et al., 2001; Hougaard *et al.*, 2007). Although 1-EBIO is a relatively weak and non-specific activator, it has nevertheless been a useful experimental tool for the localization of the $SK_{Ca}$ isoforms in vascular and non-vascular tissues. Notably, 1-EBIO causes a leftward shift in the $Ca^{2+}$-activation curves for $SK_{Ca}/IK_{Ca}$ and reduces the rate of deactivation of the channels upon intracellular $Ca^{2+}$ removal in human embryonic kidney cells (HEK293) and rat pulmonary cortical neurones (Pedersen *et al.*, 1996; Pedarzani *et al.*, 2001). Application of 1-EBIO to the endothelium of intact arteries evokes EDHF-type responses without increasing the intracellular concentration of $Ca^{2+}$, an advantageous property for studies which require a stable $[Ca^{2+}]_i$ (Marrelli *et al.*, 2003).

## 2.1.4 Aims of study

The involvement of $K_{Ca}$ channels in ACh- and A23187-evoked EDHF-type responses has been previously demonstrated in the rabbit iliac artery (Chaytor *et al.*, 2002). However, the evidence provided by the study of Chaytor et al. (2002) was not sufficient to demonstrate which $K_{Ca}$ subtypes contribute to the EDHF phenomenon in this vessel. For this reason the main focus of the current chapter was to provide greater insights into the role of $K_{Ca}$ channels in EDHF-type responses in rabbit iliac arteries. More specifically the aims were:

- to identify the $K_{Ca}$ channels that participate in the development of relaxations following the stimulation of endothelium intact rings with ACh and A23187.

- to identify the $K_{Ca}$ subtypes which are located on the endothelium and smooth muscle of rabbit iliac arteries.

## 2.2 Methods

### 2.2.1 Isolated ring preparations

Male New Zealand White rabbits (2-2.5 kg) were killed with sodium pentobarbitone (120 mg/kg; i.v.; according to Home Office and Cardiff University guidelines). Iliac arteries were dissected out, cleaned of the adherent adipose and connective tissue, and cut into segments 2 mm long. The segments were mounted on a four channel multi-myograph (610M Danish Myo Technology (DMT), Denmark) containing oxygenated (95% $O_2$, 5% $CO_2$) Holmans buffer (composition: 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 10 mM sucrose) at 37 °C. The rings were equilibrated for 30 min at a resting tension of approximately 2 mN, during which the tissues were washed with fresh Holman's buffer and the tension readjusted following stress relaxation to ensure that the experiments were conducted from the same basal tension. Data was acquired with the Myodaq 2.01 program (DMT) stored on a PC and analysed with Myodata 2.02 (DMT) Depending on the protocol employed, endothelium-denuded arteries were prepared by gentle abrasion of the intimal layer with a roughened probe. Prior to any investigation, all arteries were treated with the NOS inhibitor $N^G$-nitro-L-arginine methyl ester (L-NAME; 300 μM) and the COX inhibitor indomethacin (Indo; 10 μM) for 40 min. To verify the presence or absence of endothelium, all vessels were constricted with phenylephrine (PE; 1 μM), and the endothelium function was assessed with ACh. If ACh failed to induce relaxation, the vessel was considered to have been successfully denuded of its endothelium.

### 2.2.2 Protocols

Following the initial standarization and equilibration protocol, endothelium-intact arteries were incubated with IbTX (100 nM; Holland *et al.,* 1996), the $IK_{Ca}$ inhibitor 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; 10 μM; Gluais *et al.,* 2005) or the $SK_{Ca}$ inhibitor apamin (1 μM) for 30 min. Apamin and TRAM-34 were added either individually or in combination. In some vessels, the triple combination of apamin, TRAM-34 and IbTX was used to assess the contribution of all three $K_{Ca}$ subtypes to EDHF-type responses. Following incubation with these inhibitors, cumulative concentration-response curves were constructed for either ACh or the calcium ionophore A23187, and compared with control responses from vessels that had not been

treated with the $K_{Ca}$ channel inhibitors. Because some of the $K_{Ca}$ inhibitors used in the current study have been previously shown to affect the basal tone of endothelium-intact rat arteries (Dora *et al.*, 2000), the tension of endothelium-intact segments was measured before and after stimulation with PE (1 μM) so as to investigate their effects in the rabbit iliac artery. Endothelium-denuded vessels were also incubated with IbTX (100 nM) or the combination of apamin (1 μM), TRAM-34 (10 μM) plus IbTX (100 nM) and then constricted with PE (1 μM).

In a separate group of experiments, cumulative concentration-response curves were constructed for the $BK_{Ca}$ channel opener 1-EBIO over the concentration range 1 μM to 100 μM. This range was determined by investigations carried out on iliac artery segments and it was limited to 100 μM maximum due to the solubility of both agents, as previously described in the available literature (Holland *et al.*, 1996; Köhler *et al.*, 2005). Experiments with NS1619 were performed on arterial segments in the presence or absence of IbTX (100 nM), whereas investigations with 1-EBIO were carried out in the presence or absence of apamin (1 μM) plus TRAM-34 (10 μM). Endothelium-denuded rings were used to elucidate the direct effects of the two agents on smooth muscle.

### 2.2.3 Immunohistochemistry and confocal microscopy

Iliac arteries were cryopreserved in OCT compound and frozen by liquid $N_2$. Cryosections 10μm thick were mounted onto polylysine-coated slides, air-dried, and stored at –20 °C. Prior to immunostaining the sections were fixed in –20 °C methanol for 10min and rehydrated in phosphate buffered saline (PBS; 120 mM NaCl, 2.7 mM $Na_2PO_4 \cdot 2H_2O$, pH 7.4) for 10min.The sections were permeabilized in PBS containing 0.1% $vv^{-1}$ Triton X-100 for 30min, and then blocked with PBS containing 1% $wv^{-1}$ bovine serum albumin (BSA) for 30min at room temperature. Sections were labelled with the following primary antibodies: for $BK_{Ca}$, an affinity purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of $MaxiK_\alpha$ of human origin (200 μg/ml; code: sc-14747) was used; for $IK_{Ca}$, an affinity purified goat polyclonal antibody raised against a peptide mapping within an internal region of IK1 of human origin (200 μg/ml; code: sc-27081) was used; and for $SK_{Ca}$, an affinity purified goat polyclonal antibody raised against a peptide mapping within an internal region of

SK3 of human origin (200 µg/ml; code: sc-16027) was used. Primary antibodies were incubated for 2 hrs at 37 °C, followed by washes in PBS for 30 min. The secondary antibody of Cy5-conjugated donkey anti-goat IgG as incubated for 45 min at 37 °C, followed by consecutive washes (2 hrs) to remove excess antibody. Sections were mounted on Fluorsave and imaged using a Leica TCS-SP2 RS confocal laser scanning microscope. The autofluorescence of the internal elastic lamina (IEL) was used as a marker to separate the endothelial and smooth muscle layers. Images of IEL were overlaid with the images obtained for the protein of interest using Adobe Photoshop 7.0. The resultant image showed the distribution of the protein on the endothelium and smooth muscle relative to IEL. The presence of the intimal layer was demonstrated with an endothelium specific FITC-conjugated anti-von Willebrand factor (anti-vWF) antibody. Separate iliac artery transverse sections were incubated with the secondary Cy5-conjugated antibody only and were used as the control. All images were acquired at a magnification of x40 using Leica Confocal Software. Cy5 fluorescence was visualized with excitation wavelength of 635 nm and peak emission at 670 nm. FITC fluorescence was visualized with excitation wavelength of 488 nm and peak emission at 520 nm.

## 2.2.4 Data analysis

Responses to added drugs were plotted into sigmoidal concentration-response curves and expressed as percentages of relaxation. The curves were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. For all experiments $pEC_{50}$ values (negative logarithm of the molar concentration of an agonist that produces 50% of the maximum possible response for that agonist) were calculated, with the exception of NS1619 and 1-EBIO for both of which the $pIC_{50}$ values (negative logarithm of the molar concentration of these agents that reduces the response to a fixed concentration of PE to 50% of its original value) were given instead. $pEC_{50}$ values were obtained from concentration response curves by fitting the data to the following logistic equation: $Y = A + (B - A) / (1+10^{\wedge} ((LogEC_{50} - X)))$ where Y is the response, X is the logarithm of concentration, A is the response observed with zero drug, and B is the response observed with maximal concentration of drug. $pIC_{50}$ values were obtained by fitting the data to the following logistic equation: $Y = 100 - (A + (B - A) / (1+10^{\wedge} ((LogIC_{50} - X))))$ where Y is the response, X is the logarithm of

concentration, A is the response observed with zero drug, and B is the response observed with maximal concentration of drug. $R_{max}$ (maximal % relaxation) was calculated for all vasorelaxant responses except for those of NS1619 and 1-EBIO, where the % relaxation produced by the highest concentration of NS1619 and 1-EBIO employed (100 µM) was calculated instead. Tensions before and after the addition of PE were measured and compared by two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. All data were expressed as mean ± SEM. $P < 0.05$ was considered statistically significant. n denotes the number of animals studied. Data analysis was carried out with GraphPad Prism 4.0 (San Diego, USA).

## 2.2.5 Drugs and reagents

All drugs were supplied by Sigma Aldrich (Gillingham, UK), except for 1-EBIO which was purchased from Tocris Cookson (Avonmouth, UK). All drugs were dissolved in water, apart from indomethacin which was dissolved in 5% NaHCO₃ and A23187, TRAM-34 and NS1619 which were dissolved in dimethylsulfoxide (DMSO). ACh and PE stock solutions were freshly prepared on a daily basis prior to any investigations. For immunohistochemistry, the OCT compound was purchased from Agar Scientific (Stansted, UK), the polylysine-coated slides from Surgipath Europe (Bretton, UK), Triton-X 100 and BSA from Sigma Aldrich (Gillingham, UK), and the Fluorsave reagent from Calbiochem (Nottingham, UK). All primary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The Cy5-conjugated donkey anti-goat antibody (code: 705-175-003) was from Jackson Immunoresearch Laboratories (Suffolk, UK). The FITC-conjugated anti-vWF antibody was purchased from Serotec Laboratories (Kindlington, UK).

## 2.3 Results

### 2.3.1 Effects of IbTX on ACh- and A23187-induced relaxations

EDHF-type relaxations to ACh were maximal at a concentration of ~3 μM and equivalent to 69.3 ± 10.1% of PE preconstriction, with a $pEC_{50}$ value of 6.8 ± 0.1 (Fig. 2.1, 2.2; Table 2.1). Preincubation with IbTX (100 nM) inhibited the relaxation evoked by ACh to 35.1 ± 4.7% with a $pEC_{50}$ value of 7.2 ± 0.2 (Fig. 2.1, 2.2; Table 2.1). EDHF-type relaxations to calcium ionophore A23187 were maximal at a concentration of ~3 μM and equivalent to 83.2 ± 12.6% of PE preconstriction with a $pEC_{50}$ value of 6.4 ± 0.1 (Fig. 2.3, 2.4; Table 2.1). Preincubation with IbTX (100 nM) inhibited the relaxation evoked A23187 to 56.9 ± 7.8% with a $pEC_{50}$ of 6.3 ± 0.1 (Fig. 2.3, 2.4; Table 2.1).

### 2.3.2 Effects of TRAM-34 and apamin on ACh- and A23187-induced relaxations – combination with IbTX

EDHF-type relaxations to ACh were maximal at a concentration of ~3 μM and equivalent to 74.4 ± 11.1% of PE preconstriction with a $pEC_{50}$ value of 6.9 ± 0.1 (Fig. 2.5, 2.7; Table 2.1). Preincubation with apamin (1 μM) had no effect, whereas TRAM-34 (10 μM) significantly reduced the relaxation evoked by ACh (Fig. 2.5, 2.7; Table 2.1), whereas (Fig. 2.5, 2.7; Table 2.1). Preincubation with the combination of apamin (1 μM) plus TRAM-34 (10 μM) inhibited the relaxation evoked by ACh even further (Fig. 2.6, 2.7; Table 2.1), while the triple combination of apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM) abolished the responses (Fig. 2.6, 2.7). EDHF-type relaxations to A23187 were maximal at a concentration of ~3 μM and equivalent to 90.7 ± 13.9 % of PE preconstruction with a $pEC_{50}$ value of 6.6 ± 0.1 (Fig. 2.8, 2.9; Table 2.1). Preincubation with apamin (1 μM) had no significant effect on relaxations evoked by A23187, while TRAM-34 (10 μM) caused a small, but significant rightward shift in $pEC_{50}$ value (Fig. 2.8, 2.9; Table 2.1). Preincubation with apamin (1 μM) plus TRAM-34 (10 μM), inhibited the relaxation evoked by A23187 to 33.7 ± 4.7% and a $pEC_{50}$ value of 6.2 ± 0.2 (Fig. 2.9, 2.10; Table 2.1). Preincubation of iliac arteries with apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM) abolished the responses (Fig. 2.9, 2.10; Table 2.1).

## 2.3.3 Tension changes to $K_{Ca}$ inhibitors in quiescent iliac arteries

In quiescent endothelium-intact vessels a small increase in tension was observed in the presence of L-NAME (300 μM) and indomethacin (10 μM), the magnitude of this increment being on average 3.1 ± 0.6 mN. Addition of PE led to a further increase in tension to 32.7 ± 2.0 mN (P<0.05; Fig. 2.11; Table 2.2). Preincubation with apamin (1 μM) and TRAM-34 (10 μM) had no significant effect on the basal tone of quiescent vessels or the maximal contractions induced by PE (P>0.05; Fig. 2.11; Table 2.2).

In a separate series of experiments in endothelium intact vessels addition of L-NAME (300 μM) and indomethacin (10 μM) led to a small increase in basal tone of 3.1 ± 0.5 mN (not shown). Basal tone was further increased by preincubation with IbTX (100 nM) although there was considerable variability in the contractile response evoked by this toxin in individual preparations (Fig. 2.12, 2.13; Table 2.2). In the absence of endothelium, the contractions to PE in the presence or absence of IbTX (100 nM) were 24.2 ± 1.3 mN and 28.9 ± 4.8 mN, respectively, and were not statistically different (Table 2.2). By contrast no significant difference was observed between IbTX-treated and IbTX-untreated vessels following the addition of PE in both endothelium-intact and endothelium-denuded vessels (Fig. 2.12, 2.13, 2.14, 2.15; Table 2.2).

Incubation of iliac arteries with the triple combination of $K_{Ca}$ inhibitors led to an increase in basal tone, that was not significantly different from that of arteries pre-treated with IbTX only. Preincubation with apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM) led to a significant increase of the basal tension to 18.5 ± 2.8 mN and a PE-evoked contraction equal to 30.9 ± 3.1 mN (Fig. 2.12, 2.13; Table 2.2). Similarly, in endothelium-denuded vessels preincubation with the three inhibitors led to a significant increase in tension before and after addition of the constrictor (Fig. 2.14, 2.15; Table 2.2). The effects of the three inhibitors on the tension of endothelium-intact iliac arteries were statistically similar to those of endothelium-denuded preparations.

## 2.3.4 NS1619-evoked EDHF-type responses

Cumulative addition of NS1619 to rabbit iliac arteries resulted in a decrease in tone equivalent to 98.6 ± 15.2% of PE preconstriction with a $pIC_{50}$ value of 5.1 ± 0.1 when 100 μM of the agent was added (Fig. 2.16, 2.17; Table 2.3). Removal of the endothelium led to an increase in $pIC_{50}$ to 4.4 ± 0.1 without significantly affecting the

relaxation obtained at an NS1619 concentration of 100 µM. Similarly, preincubation with IbTX (100 nM) led to a significant increase in $pIC_{50}$ value to 4.4 ± 0.3, without affecting the relaxation obtained at an NS1619 concentration of 100 µM (Fig. 2.16, 2.17; Table 2.3).

### 2.3.5 1-EBIO-evoked EDHF-type responses

Cumulative addition of 1-EBIO to rabbit iliac arteries resulted in a decrease in tone equivalent to 90.5 ± 13.2% of PE preconstriction and with a $pIC_{50}$ value of 4.6 ± 0.2 (Fig. 2.18, 2.19; Table 2.3). Removal of the endothelium led to an increase in $pIC_{50}$ value to 4.3 ± 0.6 without significantly affecting the relaxation obtained at an 1-EBIO concentration of 100 µM. Similarly, preincubation with apamin (1 µM) plus TRAM-34 (10 µM) led to an increase in $pIC_{50}$ value to 3.5 ± 1.6 with no significant effect on the relaxation obtained at an 1-EBIO concentration of 100 µM (Fig. 2.18, 2.19; Table 2.3).

### 2.3.6 Immunohistochemical localization of $K_{Ca}$ channels

Immunostaining revealed heterogeneity in the distribution of $K_{Ca}$ channels in the endothelium and smooth muscle of rabbit iliac arteries. The absence of Cy5 stain in the control samples excludes the presence of non-selective binding (Fig. 2.20), while immunolabelling directed against the endothelium-specific cell marker von Willebrand factor (vWF) illustrated the presence of the intimal layer (Fig. 2.21). Incubation of transverse sections with an antibody directed against the $BK_{Ca}$ α-subunit showed that this channel is highly expressed in both the endothelial and smooth muscle layers (Fig. 2.22). By contrast, $IK_{Ca}$ was mainly detectable in the endothelium of the vessels, and only weakly expressed in the smooth muscle (Fig. 2.23). Finally, investigations carried out for $SK_{Ca}$ revealed distinct punctate staining in the endothelium of rabbit iliac arteries, while an increase in the fluorescence of the smooth muscle relative to the control was also detected (Fig. 2.24).
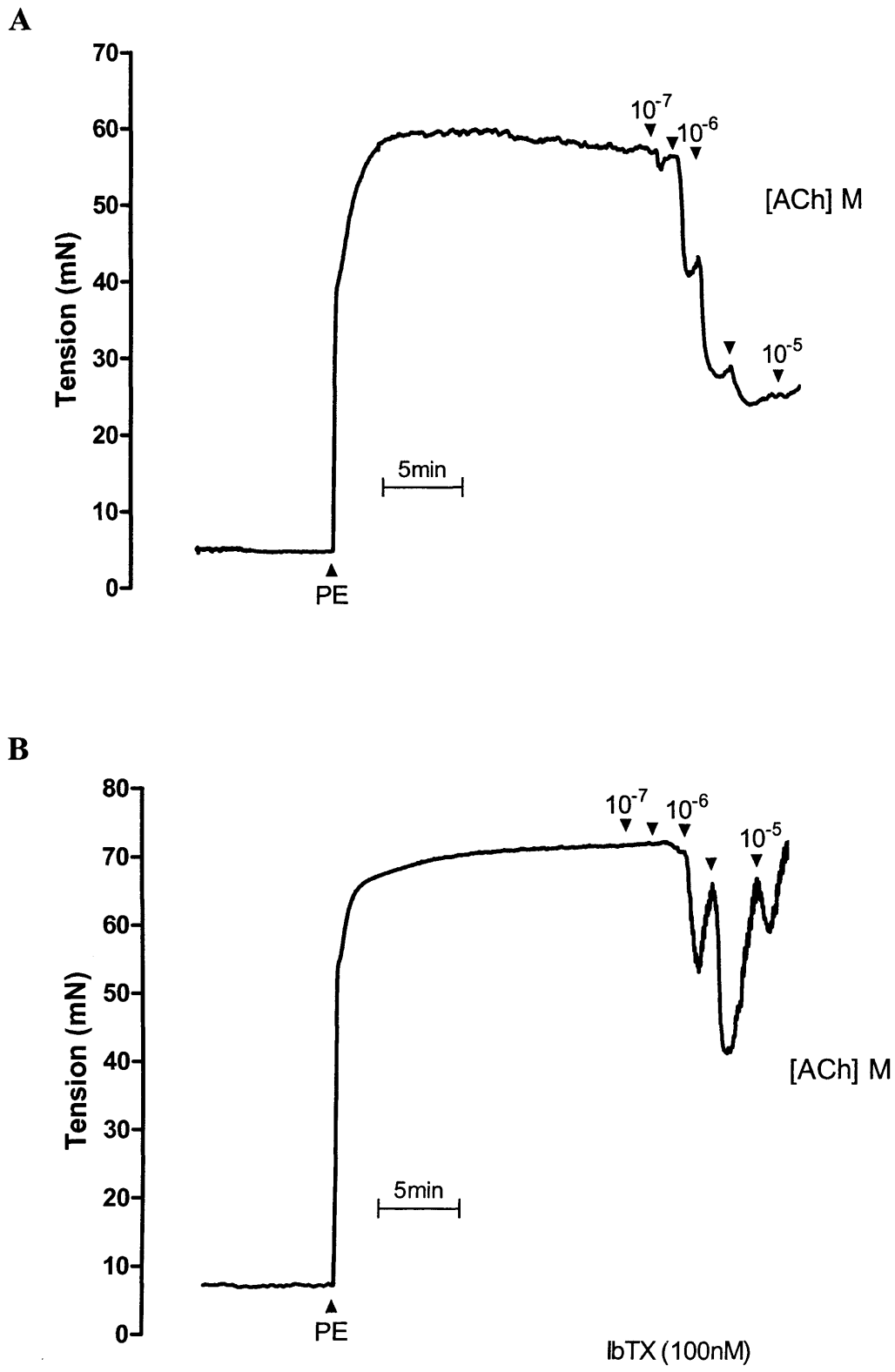
**Fig. 2.1** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to ACh and associated changes in the presence of (B) IbTX (100 nM). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).
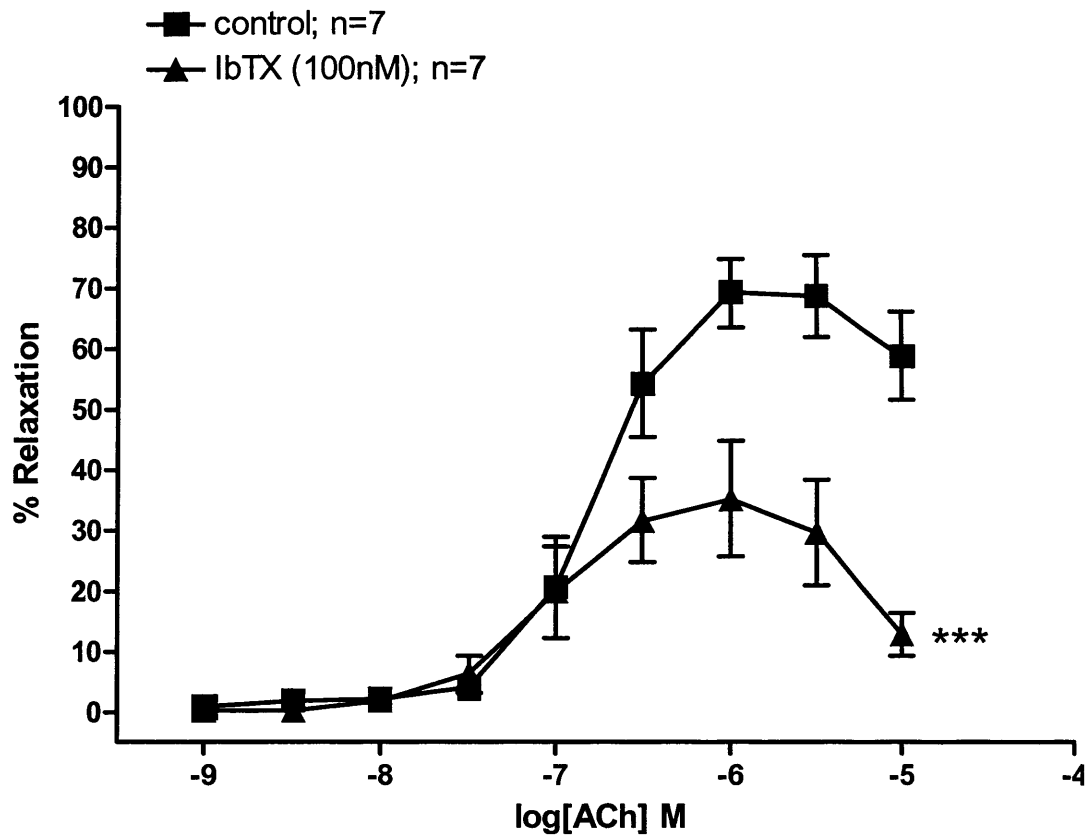
**Fig. 2.2** Concentration-response curves for ACh-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of IbTX (100 nM). Experiments were carried out with IbTX to assess the involvement of $BK_{Ca}$ channels in relaxations evoked by ACh. It is demonstrated that the toxin significantly inhibits the responses. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. ***, $P<0.001$ for whole curves compared with the control.

**Fig. 2.3** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) IbTX (100 nM). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).

**Fig. 2.4** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of IbTX (100 nM). Experiments were carried out with IbTX to assess the involvement of $BK_{Ca}$ channels in relaxations evoked by A23187. It is demonstrated that the toxin significantly inhibits the responses. Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM). Data are expressed as mean ± SEM. *, $P<0.05$ for whole curves compared with the control.
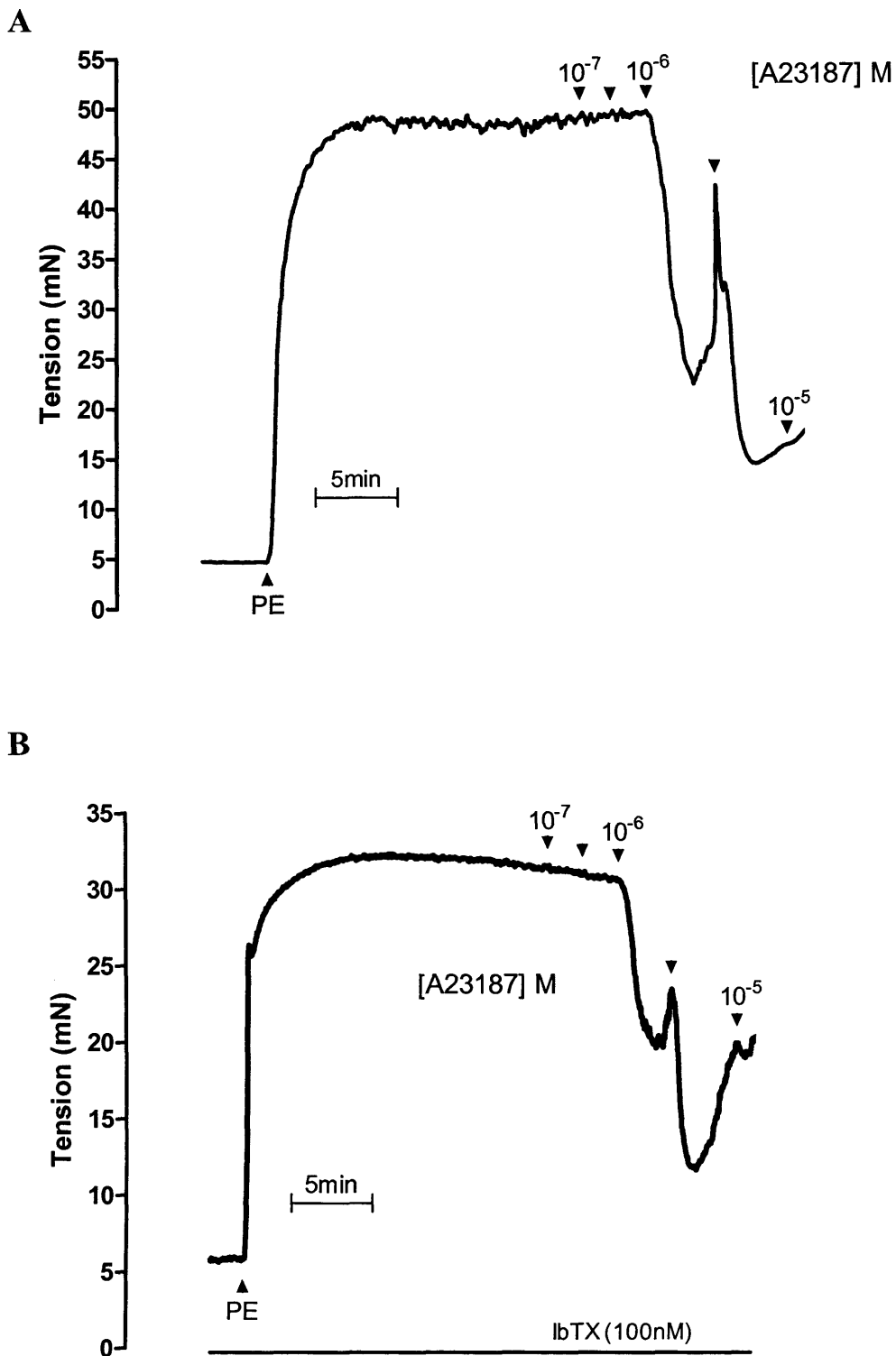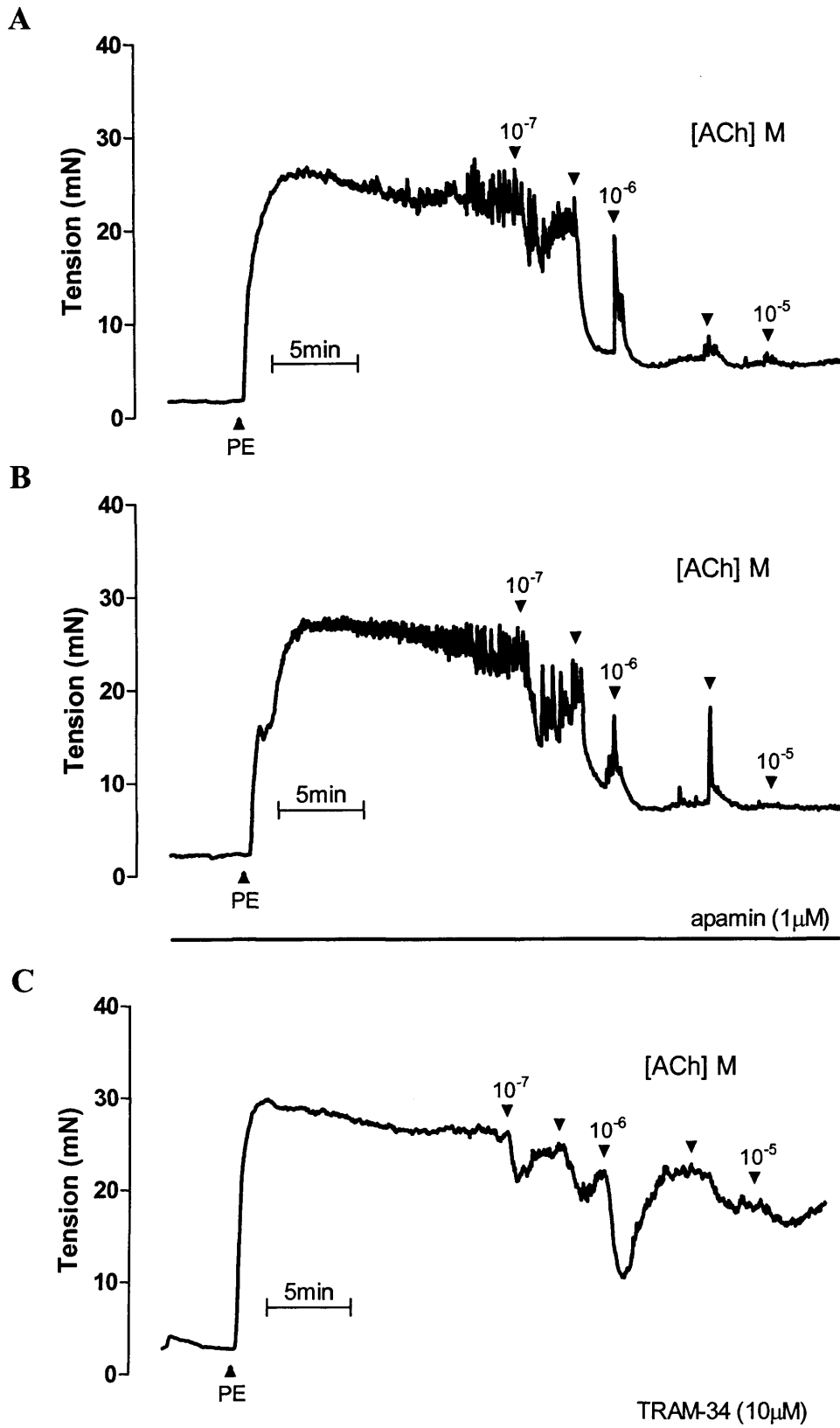
**Fig. 2.5** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to ACh and associated changes in the presence of (B) apamin (1 μM) and (C) TRAM-34 (10 μM). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).

**D**

40

Tension (mN)

30

20

10

0

$10^{-7}$  $10^{-6}$  $10^{-5}$

[ACh] M

5min

PE

apamin (1μM) + TRAM-34 (10μM)

**E**

40

Tension (mN)

30

20

10

0

[ACh] M

$10^{-7}$  $10^{-6}$  $10^{-5}$

PE

5min

apamin (1μM) +TRAM-34 (10μM) +IbTX (100nM)

**Fig. 2.6** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing relaxations to ACh in the presence of (D) the double combination of apamin (1 μM) plus TRAM-34 (10 μM) and (E) the triple combination of apamin (1 μM) plus TRAM-34 (10μM) plus IbTX (100 nM). The elevation in basal tone observed in the lower panel is attributable to IbTX (see below). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).

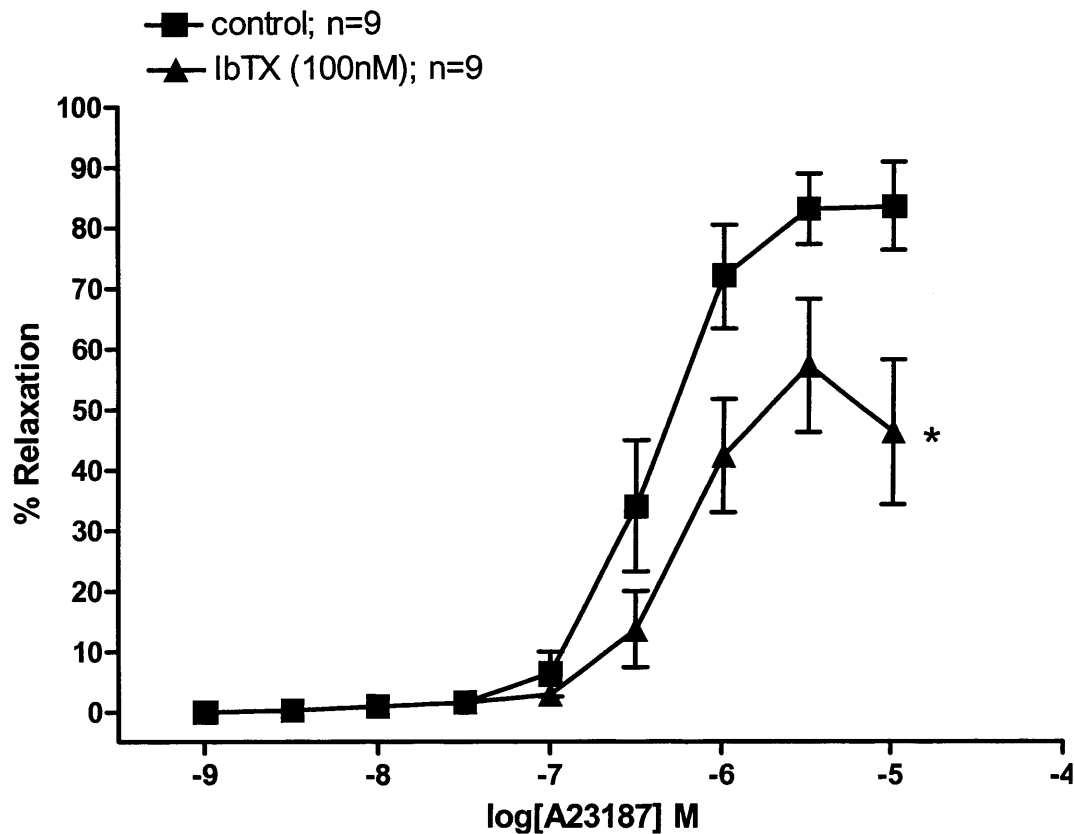**Fig. 2.7** Concentration-response curves for ACh-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of apamin (1 μM), TRAM-34 (10 μM) and IbTX (100 nM). Experiments were carried out with each inhibitor on its own or in combination to assess the involvement of SK$_{Ca}$ and IK$_{Ca}$ channels. Although apamin did not affect ACh-evoked EDHF-type relaxations, the combination of apamin plus TRAM-34 caused significant inhibition. The triple combination of apamin plus TRAM-34 plus IbTX demonstrated that the residual relaxation was BK$_{Ca}$-dependent. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. *, $P<0.05$ and ***, $P<0.001$ for whole curves compared with the control.
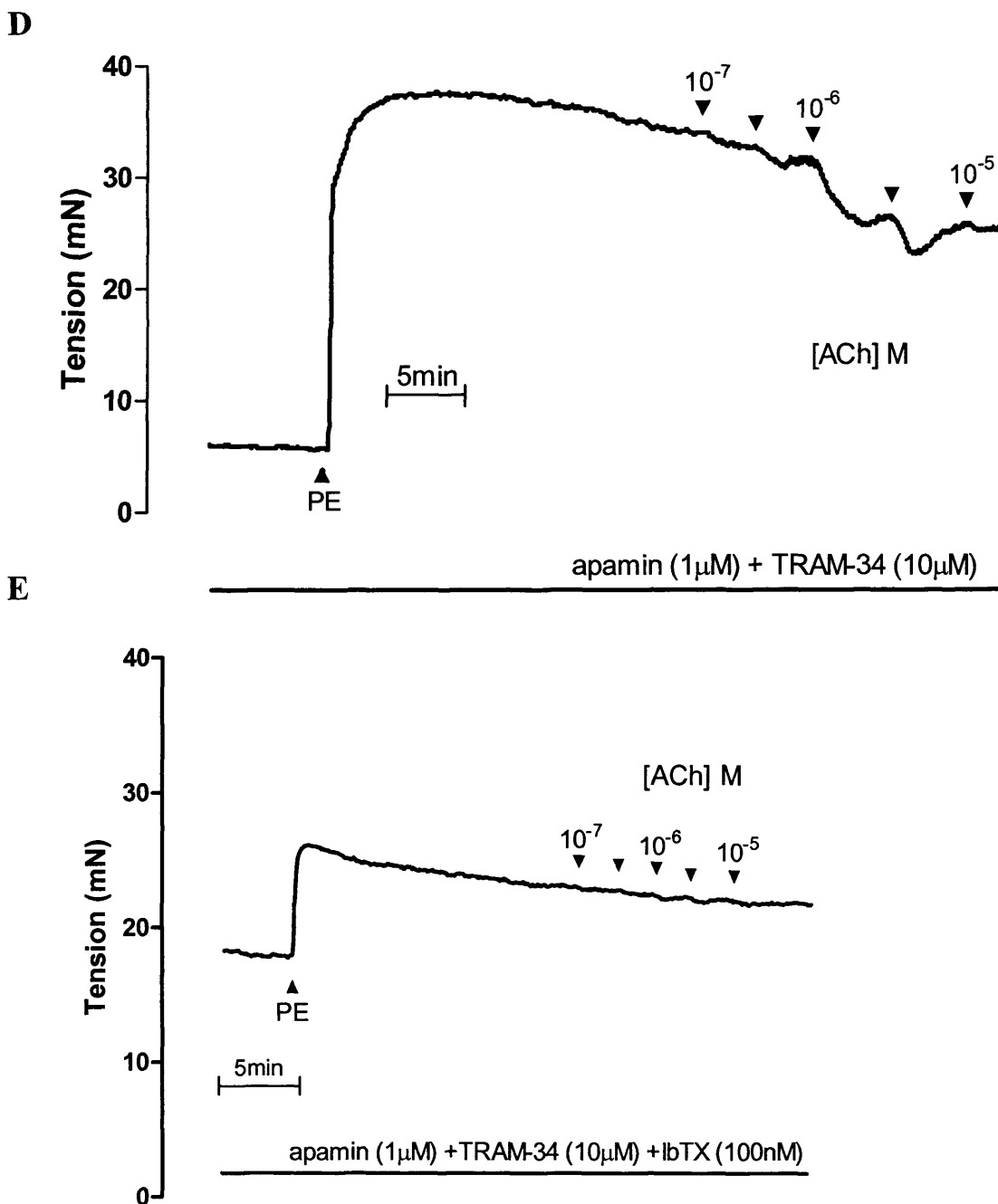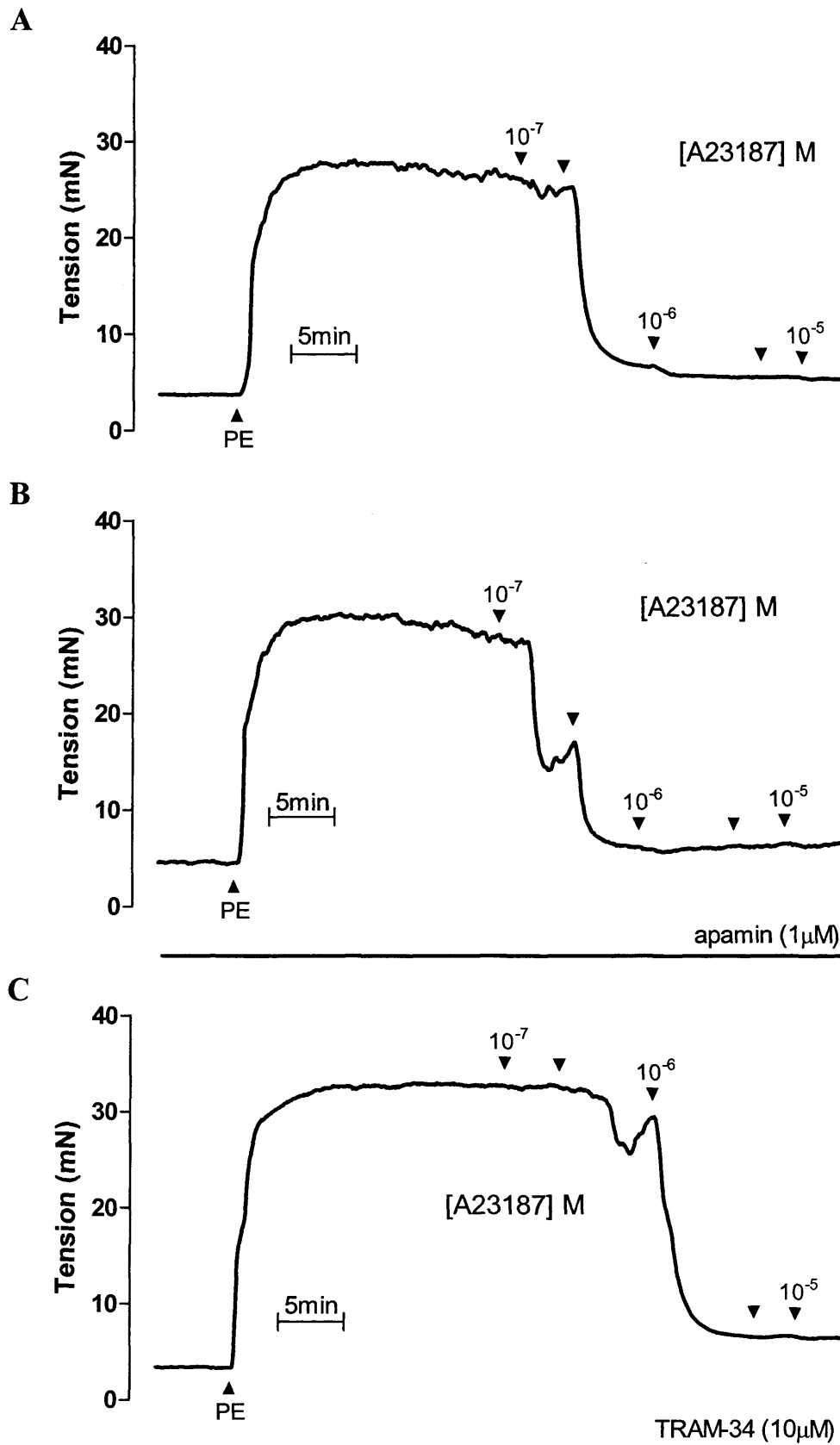
**Fig. 2.8** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) apamin (1 µM) and (C) TRAM-34 (10 µM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
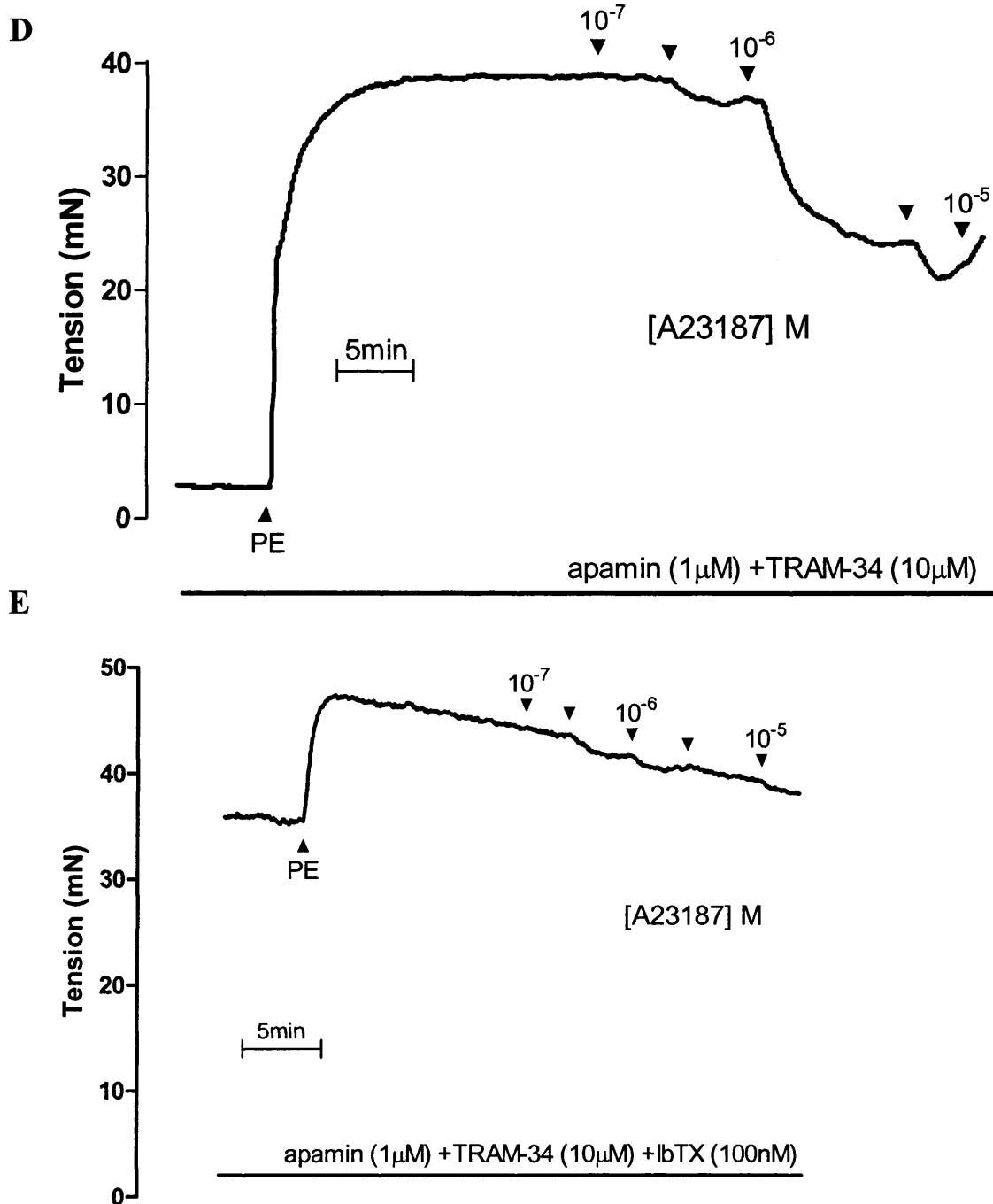
**Fig. 2.9** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing relaxations to A23187 in the presence of (D) the double combination of apamin (1 μM) plus TRAM-34 (10 μM) and (E) the triple combination of apamin (1 μM) plus TRAM-34 (10μM) plus IbTX (100 nM). The elevation in basal tone observed in the lower panel is attributable to IbTX (see below). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).
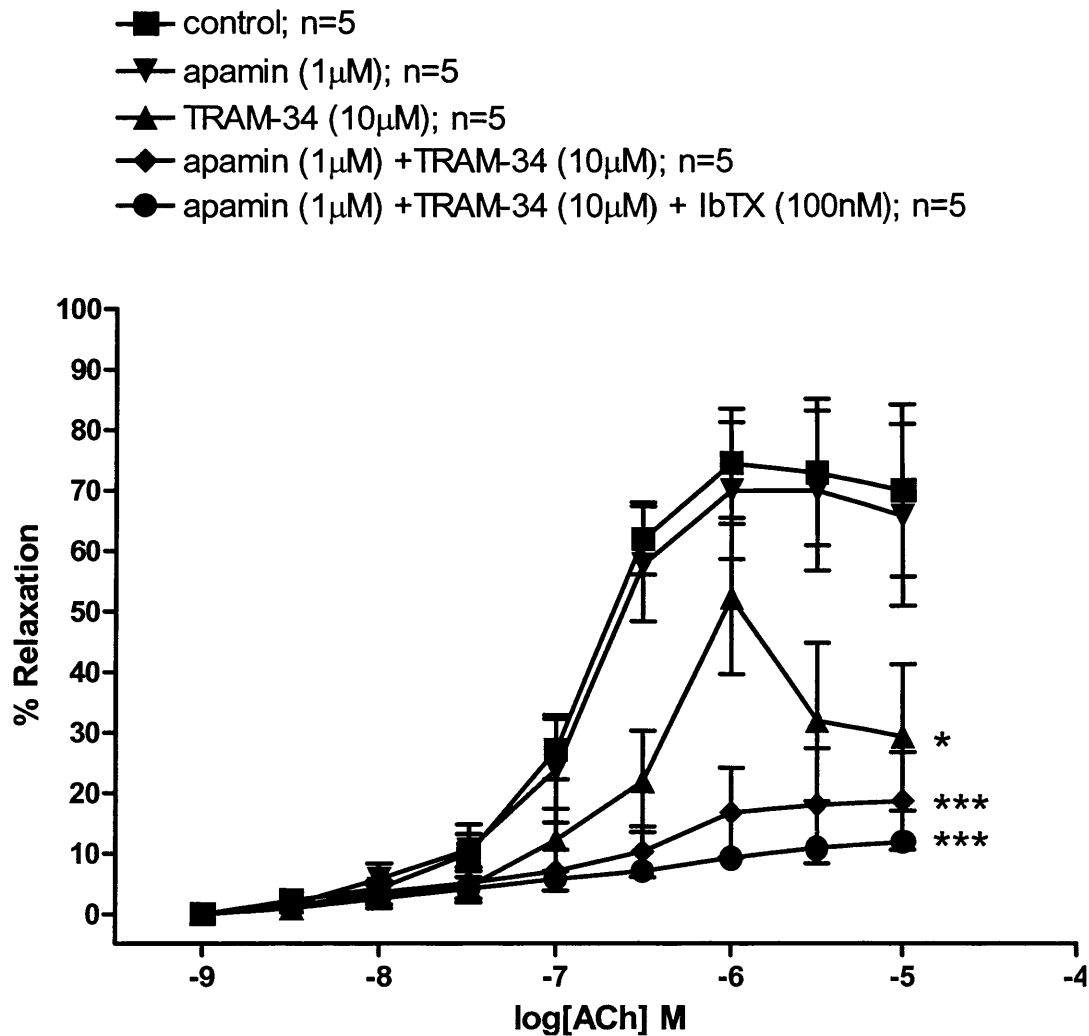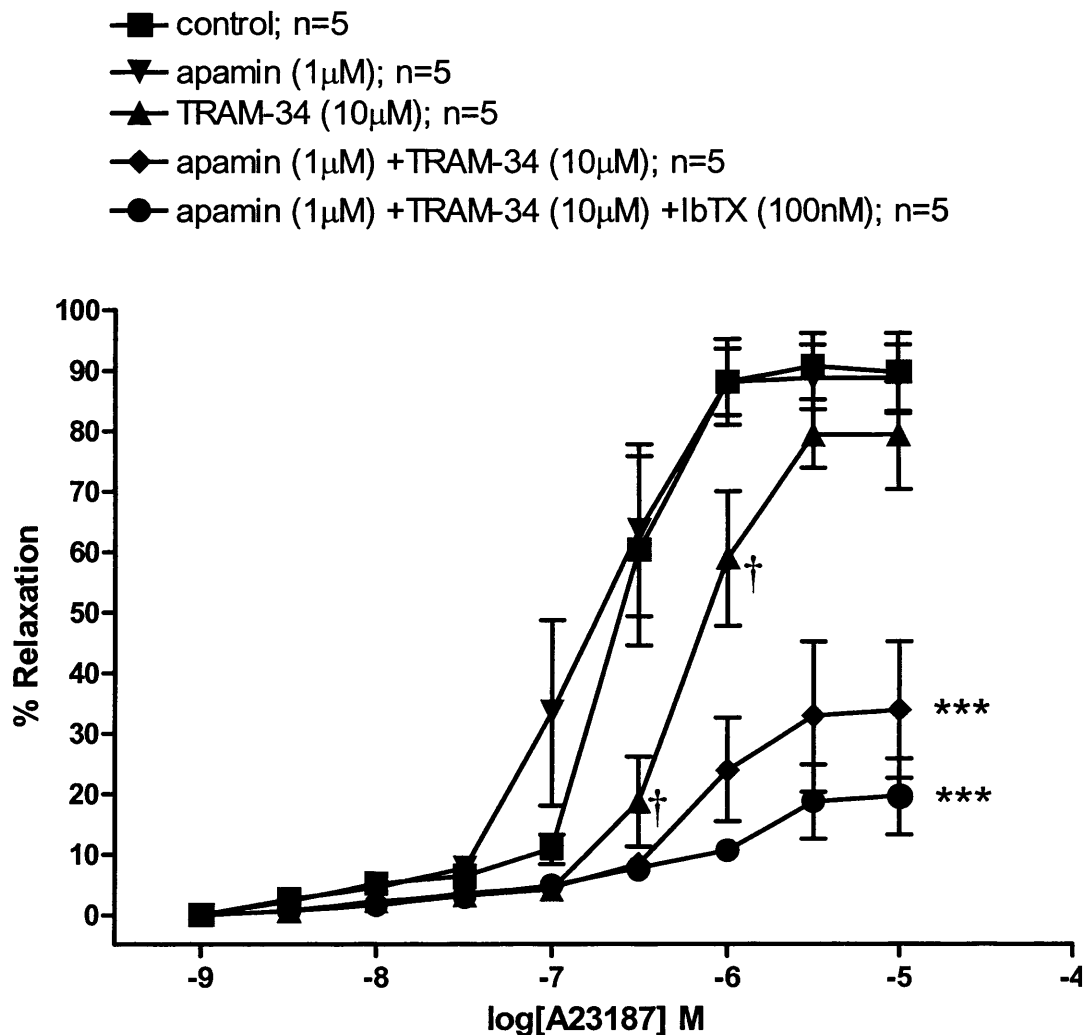
**Fig. 2.10** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of apamin (1 μM), TRAM-34 (10 μM) and IbTX (100 nM). Experiments were carried out with each inhibitor on its own or in combination to assess the involvement of $SK_{Ca}$ and $IK_{Ca}$ channels. TRAM-34 on its own caused a small but significant rightward shift of the curve relative to the control. Although apamin did not affect A23187-evoked EDHF-type relaxations, the combination of apamin plus TRAM-34 caused significant inhibition. The triple combination of apamin plus TRAM-34 plus IbTX demonstrated that the residual relaxation was $BK_{Ca}$-dependent. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. †, $P<0.05$ for specific A23187 concentration compared with the control. ***, $P<0.001$ for whole curves compared with the control.

| Intervention | $R_{max}$ % | $pEC_{50}$ | n |
|---|---|---|---|
| | | | |
| ACh control | 69.2±10.1 | 6.8±0.1 | 7 |
| IbTX (100 nM) | 35.1±4.7*** | 7.2±0.2 | 7 |
| | | | |
| A23187 control | 83.2±12.6 | 6.4±0.1 | 9 |
| IbTX (100 nM) | 56.9±7.8*** | 6.3±0.1 | 9 |
| | | | |
| ACh control | 74.4±11.1 | 6.9±0.1 | 5 |
| apamin (1 μM) | 69.9±10.4 | 6.8±0.1 | 5 |
| TRAM-34 (10 μM) | 52.1±5.9 | 6.7±0.2* | 5 |
| apamin (1μM) + TRAM-34 (10 μM) | 18.6±2.4*** | n/a | 5 |
| apamin (1 μM) + TRAM-34 (10 μM) + IbTX (100 nM) | n/a | n/a | 5 |
| | | | |
| A23187 control | 90.7±13.9 | 6.6±0.1 | 5 |
| apamin (1 μM) | 88.7±13.4 | 6.8±0.1 | 5 |
| TRAM-34 (10 μM) | 79.4±13.4 | 6.2±0.1* | 5 |
| apamin (1 μM) + TRAM-34 (10 μM) | 33.7±4.7*** | 6.2±0.2* | 5 |
| apamin (1 μM) + TRAM-34 (10 μM) + IbTX (100 nM) | n/a | n/a | 5 |
| | | | |

**Table 2.1** Summary of effects of $K_{Ca}$ channel inhibitors on EDHF-type relaxations evoked by ACh and the calcium ionophore A23187 in the rabbit iliac artery. Potency (negative log $EC_{50}$) and maximal percentage relaxation ($R_{max}$) are expressed as a function of the constrictor response to PE and given as mean ± SEM. *$P<0.05$; ***$P<0.001$ compared with the corresponding intra-group control. n denotes the number of animals studied. n/a denotes non-applicable data.
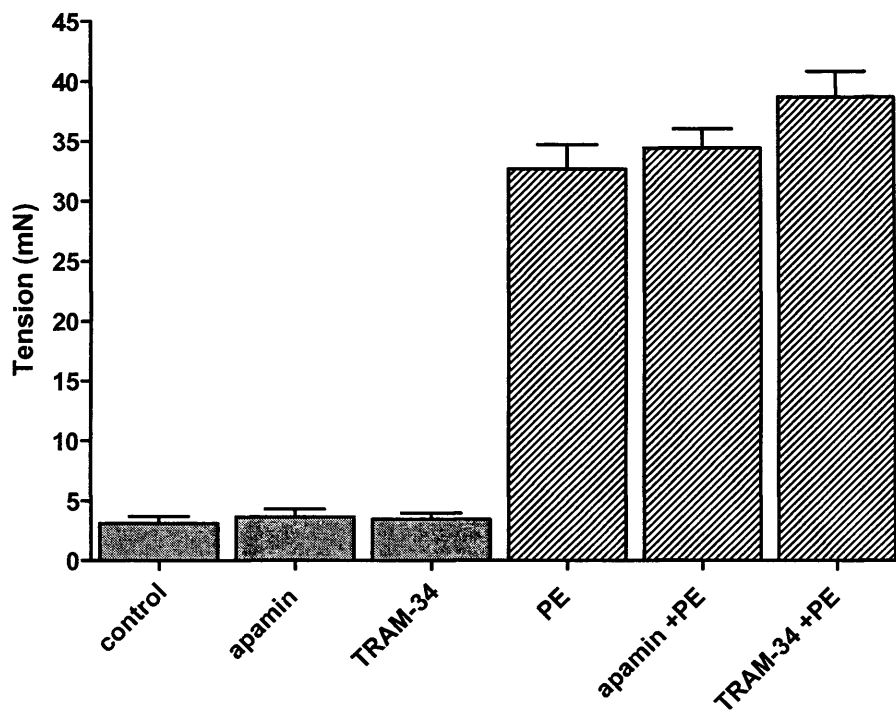
**Fig. 2.11** Bar graphs summarizing the changes in the tone of endothelium-intact rabbit iliac arteries following the addition of apamin ($1\mu$ M) and TRAM-34 ($10$ $\mu$M). Data are presented for both quiescent and PE preconstricted arteries. Apamin and TRAM-34 did not affect the tensions of any vessels. Tensions were obtained in the presence of L-NAME ($300$ $\mu$M) and Indo ($10$ $\mu$M).
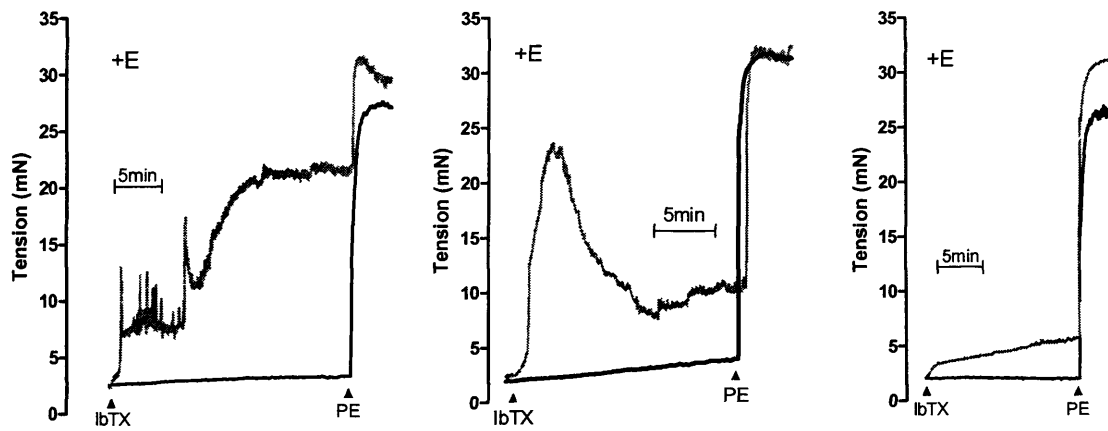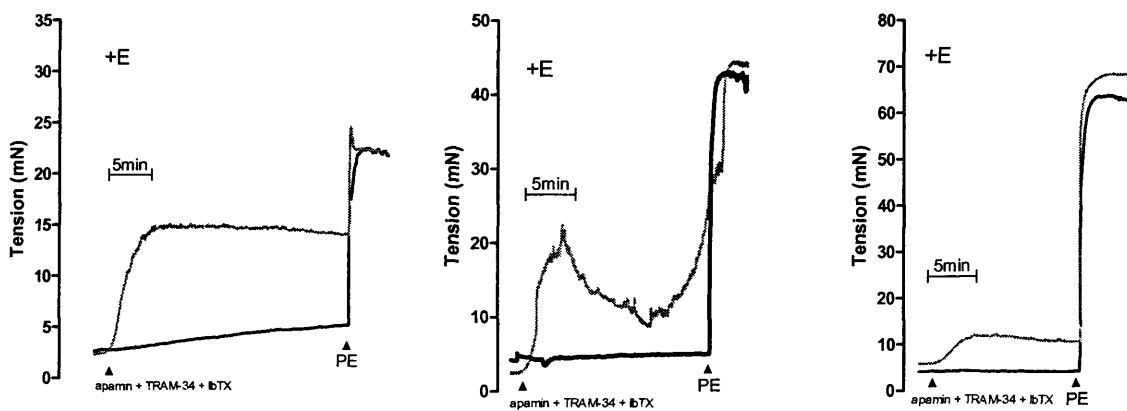
**Fig. 2.12** Representative traces showing the variable changes in tension of endothelium-intact rabbit iliac arteries following the addition of (A) IbTX (100 nM) and (B) the triple combination of apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM). L-NAME (300 μM) and Indo (10 μM) were present in all experiments. +E denotes the presence of an intact endothelium.
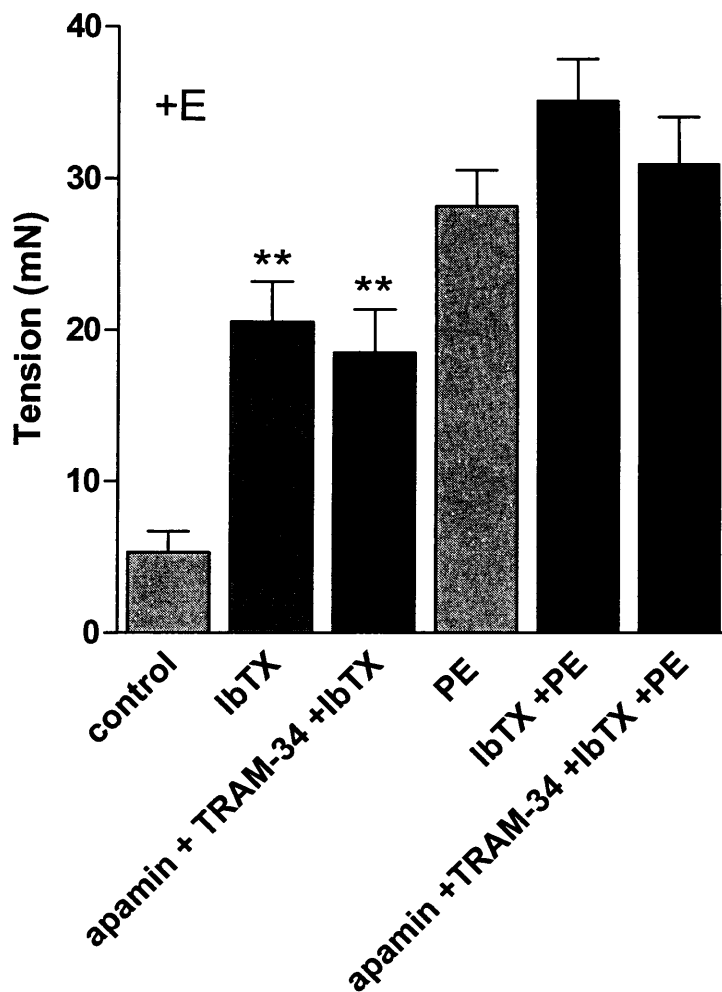
**Fig. 2.13** Bar graphs summarizing the changes in the tone of endothelium-intact rabbit iliac arteries following addition of IbTX (100 nM) and the triple combination of apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM). Data are presented for both quiescent and PE-preconstricted vessels. No statistically significant changes were observed between rings treated with IbTX alone or the triple combination. Tensions were obtained in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. **$P<0.01$ compared with the control.

**A**



**B**
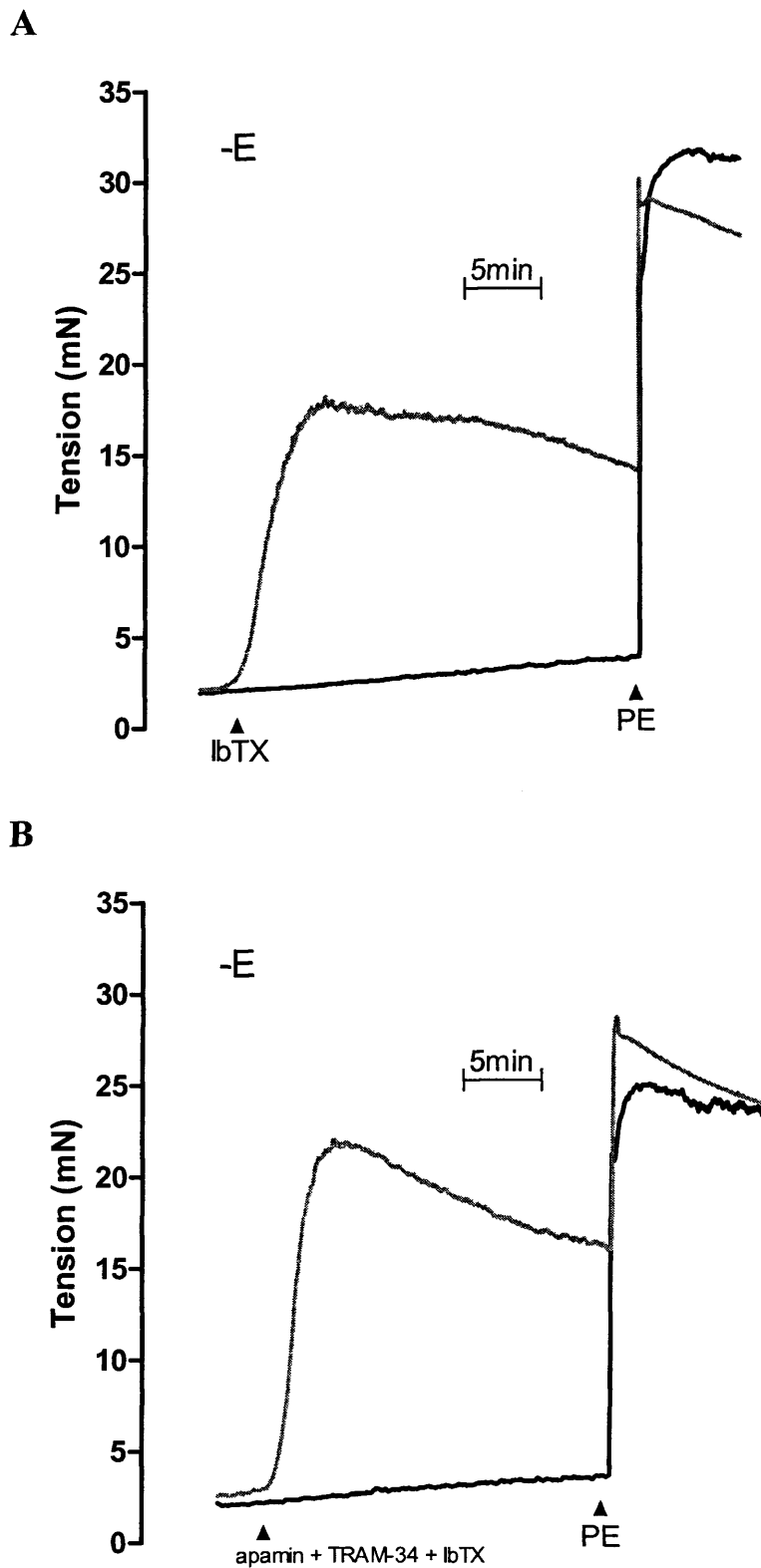


**Fig. 2.14** Representative traces showing changes in tension of endothelium-denuded rabbit iliac arteries following the addition of (A) IbTX (100 nM) and (B) the triple combination of apamin (1 µM) plus TRAM-34 (10 µM) plus IbTX (100 nM). L-NAME (300 µM) and Indo (10 µM) were present in all experiments. –E denotes the absence of endothelium.

76
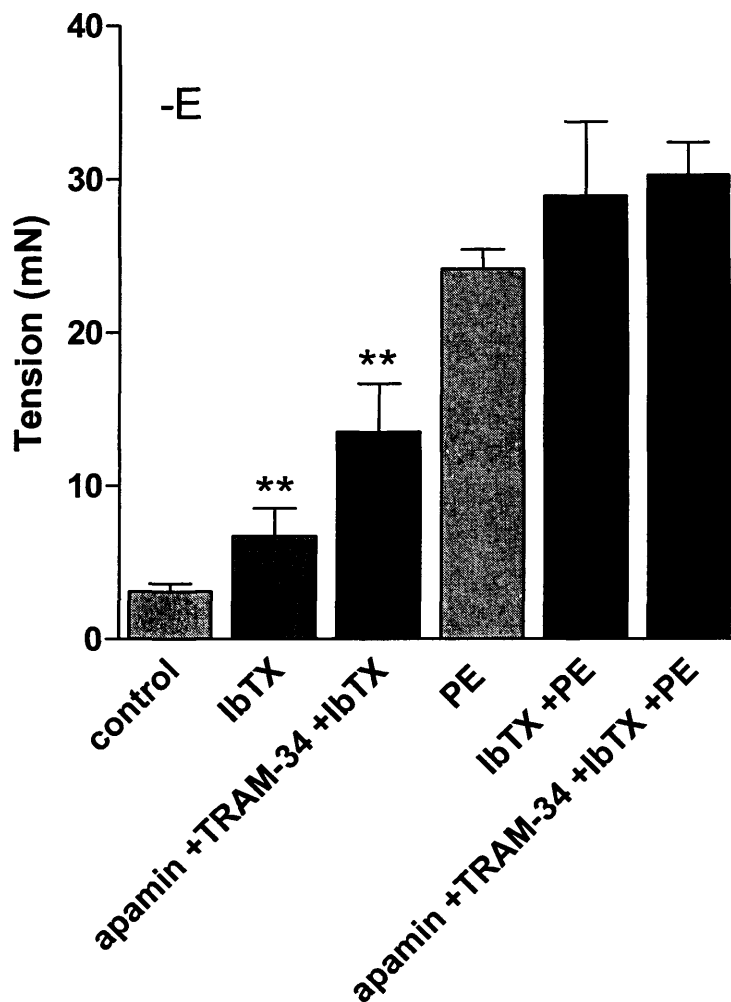
**Fig. 2.15** Bar graps summarizing the changes in the tone of endothelium-denuded rabbit iliac arteries following addition of IbTX (100 nM) and the triple combination of apamin (1 µM) plus TRAM-34 (10 µM) plus IbTX (100 nM). A significant increase in the resting tension was observed directly after the addition of the drugs, although PE-induced contraction was not significantly affected by IbTX or the triple inhibitor combination. Tensions were obtained in the presence of L-NAME (300 µM) and Indo (10 µM). Data are expressed as mean ± SEM. **$P<0.01$ compared with the control.

| Intervention | basal tension | PE tension | n |
|---|---|---|---|
| control | 3.1±0.6 | 32.7±2.0 | 9 |
| apamin (1 μM) | 3.6±0.7 | 34.4±1.6 | 9 |
| TRAM-34 (10 μM) | 3.5±0.5 | 38.7±2.1 | 9 |
| | | | |
| *endothelium intact* | | | |
| | | | |
| control | 5.3±1.4 | 28.2±2.4 | 11 |
| IbTX (100 nM) | 20.5±2.7** | 35.1±2.8 | 5 |
| apamin (1 μM) + TRAM-34 (10 μM) + IbTX (100 nM) | 18.5±2.8** | 30.9±3.1 | 6 |
| | | | |
| *endothelium denuded* | | | |
| | | | |
| control | 3.1±0.5 | 24.2±1.3 | 10 |
| IbTX (100 nM) | 6.7±1.8** | 28.9±4.8 | 4 |
| apamin (1 μM) + TRAM-34 (10 μM) + IbTX (100 nM) | 13.5±3.1** | 30.3±2.1 | 6 |
| | | | |

**Table 2.2** Summary of effects of $K_{Ca}$ inhibitors on the basal tension and the tension of rabbit iliac arteries following stimulation with PE. Tensions are given as means ± SEM. **$P<0.01$ compared with the corresponding intra-group control. n denotes the number of animals studied.
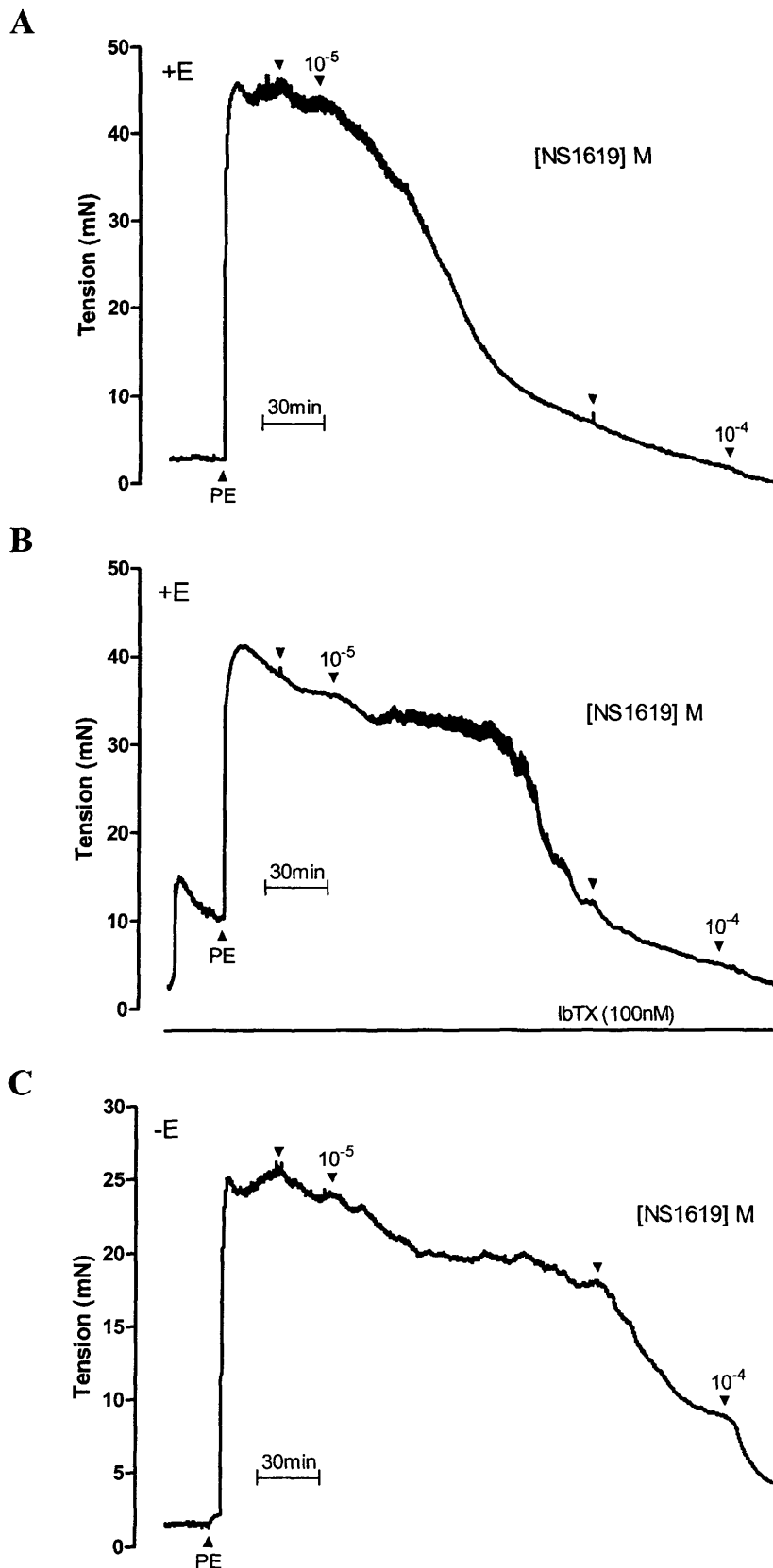
**Fig. 2.16** Representative traces from ring preparations of rabbit iliac arteries showing (A) relaxations to NS1619 and associated changes in the presence of (B) IbTX (100 nM) and (C) relaxations to NS1619 in endothelium-denuded rings. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). ±E denotes the presence or absence of an intact endothelium.
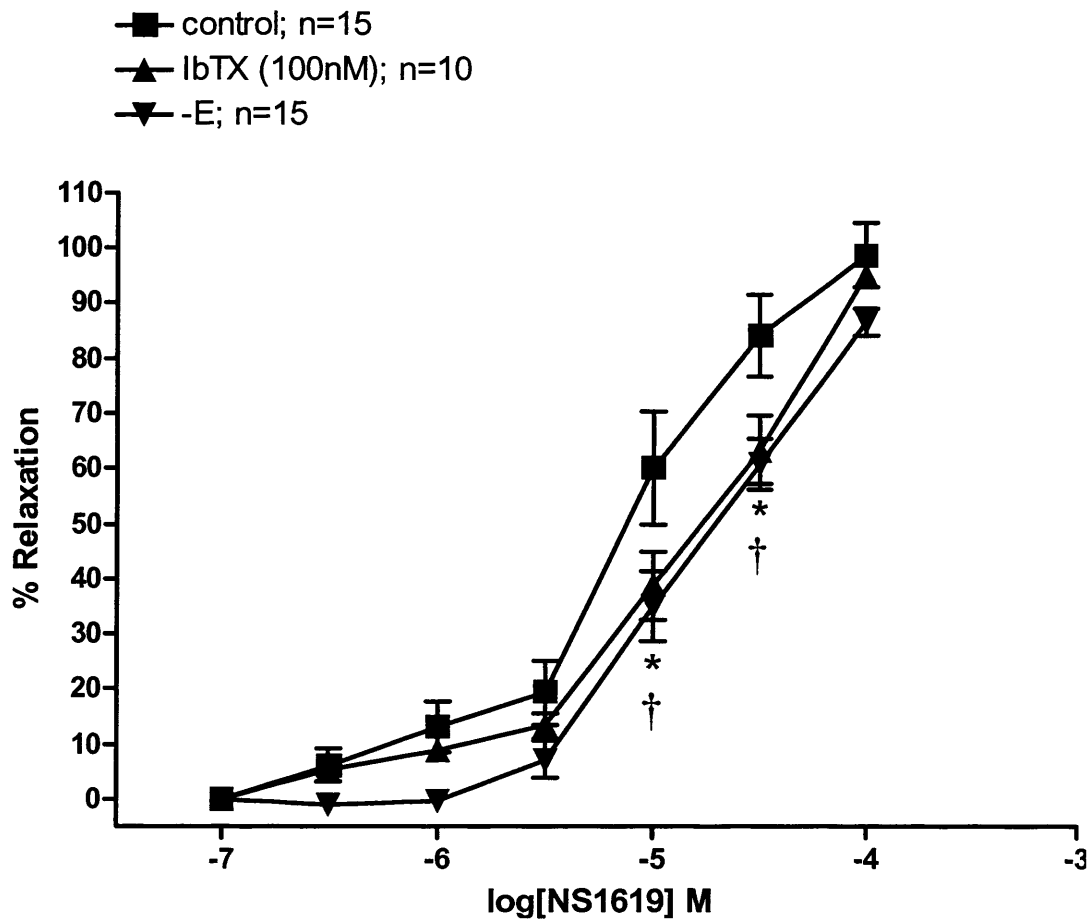
Fig. 2.17 Concentration-response curves for NS1619-evoked relaxations of rabbit iliac arteries and associated changes in the presence of IbTX (100 nM). Experiments were carried out with NS1619 to confirm the presence of $BK_{Ca}$ channels in the endothelium of these vessels. The endothelium of some arteries was removed to dissociate direct effects of NS1619 on $BK_{Ca}$ channels in this layer from the channels in the smooth muscle. IbTX and ablation of the endothelium caused a small but significant rightward shift of the curve relative to the control. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. †, $P < 0.05$ for specific NS1619 concentration in the presence of IbTX compared with the control. *, $P < 0.05$ for specific NS1619 concentration in endothelium-denuded vessels compared with the control.
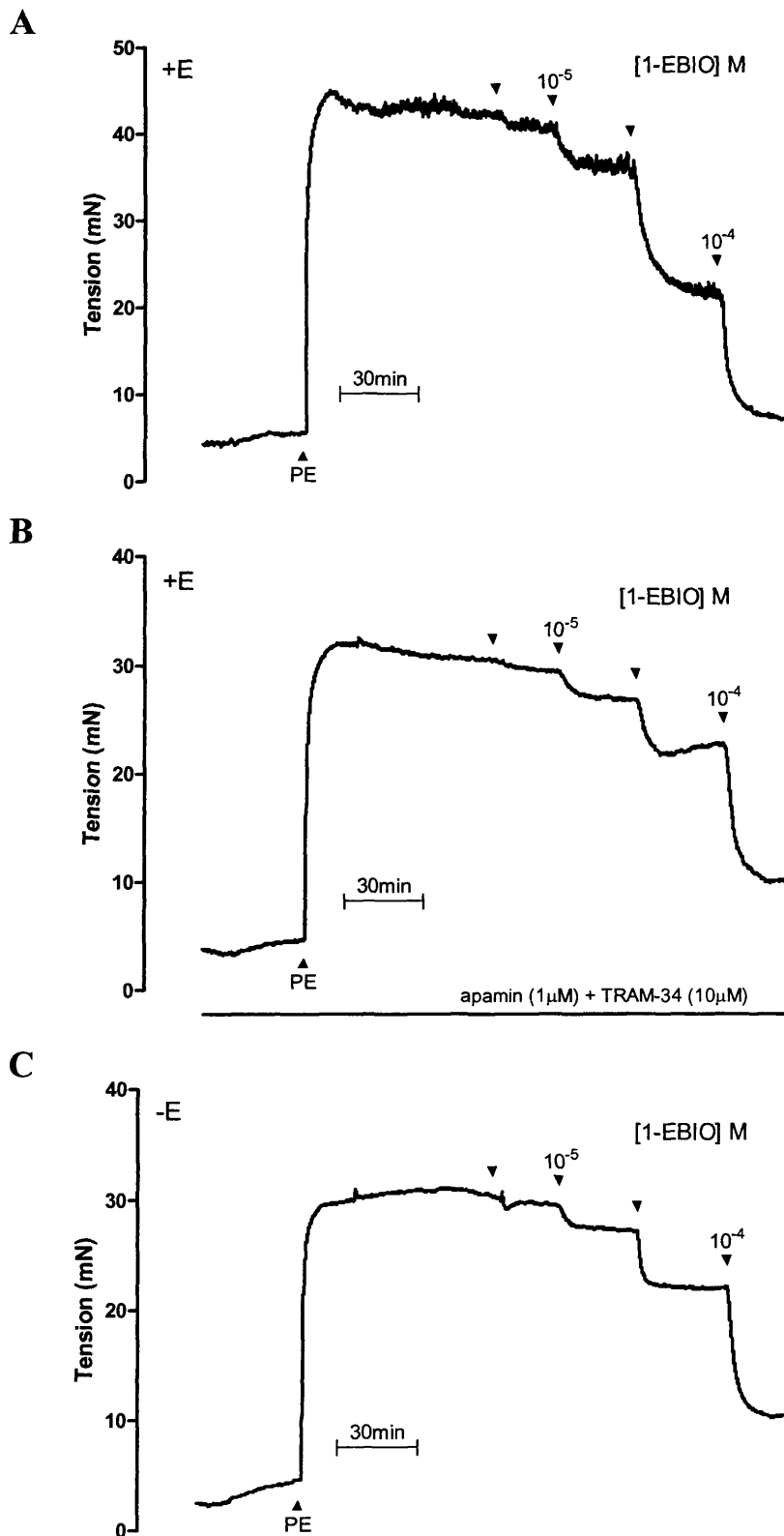
**Fig. 2.18** Representative traces from ring preparations of rabbit iliac arteries showing (A) relaxations to 1-EBIO and associated changes in the presence of (B) apamin (1 μM) plus TRAM-34 (10 μM) and (C) relaxations to 1-EBIO in endothelium-denuded rings. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). ±E denotes the presence or absence of an intact endothelium.
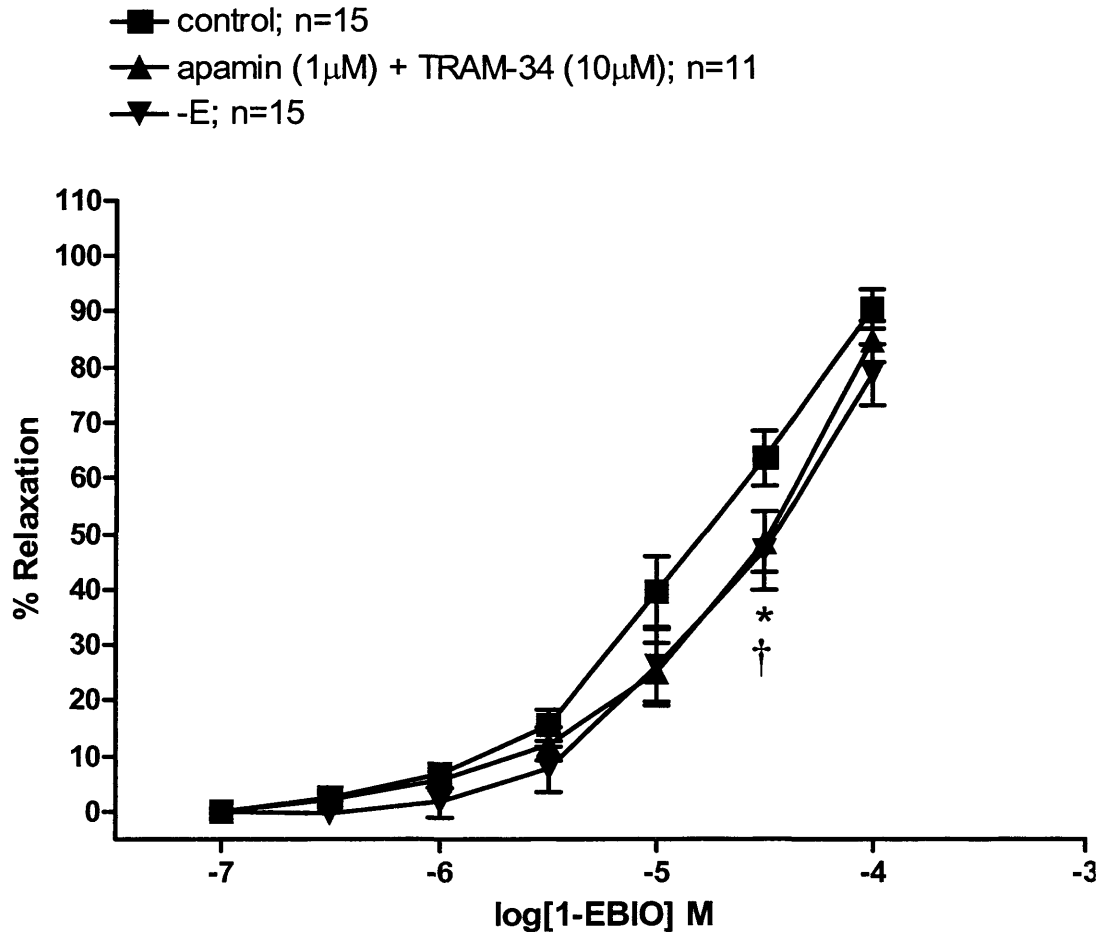
**Fig. 2.19** Concentration-response curves for 1-EBIO-evoked relaxations of rabbit iliac arteries and associated changes in the presence of apamin (1 μM) plus TRAM-34 (10 μM). Experiments were carried out with 1-EBIO to confirm the presence of $SK_{Ca}/IK_{Ca}$ channels in the endothelium of these vessels. The endothelium of some arteries was removed to dissociate direct effects of 1-EBIO on $SK_{Ca}/IK_{Ca}$ channels in this layer from any effects of this agent in the smooth muscle. The combination of apamin plus TRAM-34 or ablation of the endothelium caused a small but significant rightward shift of the curve relative to the control. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. †, $P<0.05$ for specific 1-EBIO concentration in the presence of apamin plus TRAM-34 compared with the control. *, $P<0.05$ for specific 1-EBIO concentration in endothelium-denuded vessels compared with the control.

| Intervention | % relaxation at 100μM | pIC$_{50}$ | n |
|---|---|---|---|
| | | | |
| NS1619 control | 98.6±15.2 | 5.1±0.1 | 15 |
| IbTX (100 nM) | 95.2±13.5 | 4.4±0.3* | 10 |
| denuded | 86.6±13.3 | 4.7±0.1* | 15 |
| | | | |
| 1-EBIO control | 90.5±13.2 | 4.6±0.2 | 15 |
| apamin (1 μM) + TRAM-34 (10 μM) | 84.7±11.8 | 3.5±1.6* | 11 |
| denuded | 78.7±11.4 | 4.3±0.6* | 15 |
| | | | |

**Table 2.3** Summary of effects of $K_{Ca}$ inhibitors and endothelial ablation on relaxations induced by NS1619 and 1-EBIO. Potency (negative log IC$_{50}$) and relaxations at 100 μM of agent are expressed as a function of the constrictor response to PE and given as mean ± SEM. *$P<0.05$ compared with the corresponding intra-group control. n denotes the number of animals studied.
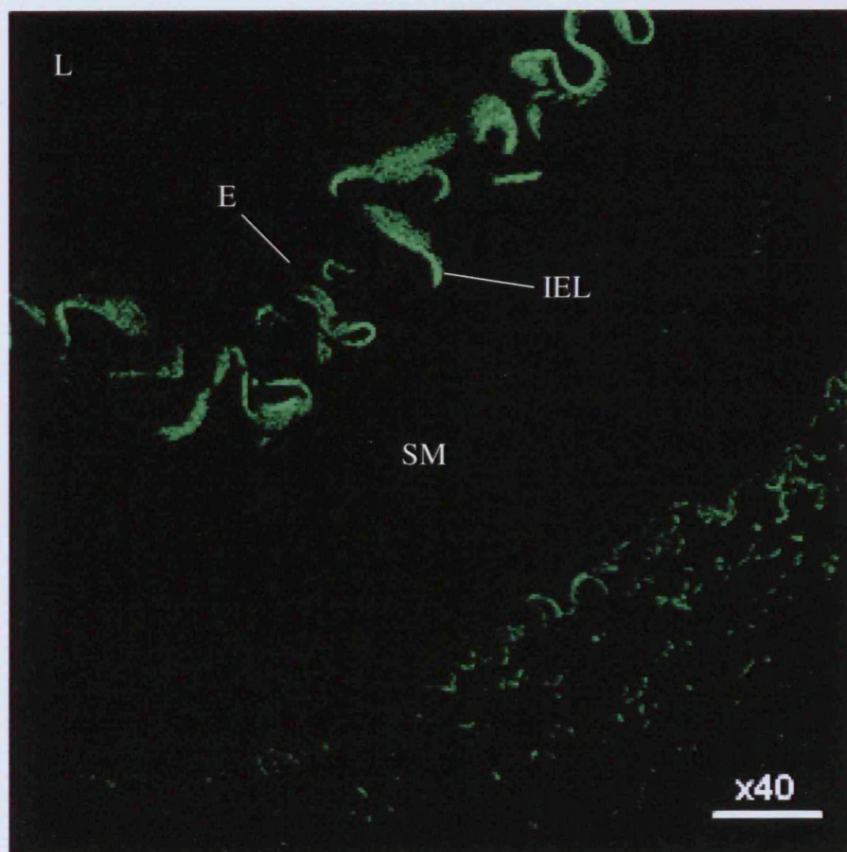
**Fig. 2.20** Transverse section of rabbit iliac arteries stained with a secondary Cy5-conjugated donkey anti-goat IgG in the absence of primary antibodies. The image shows lack of staining of the section excluding the possibility of non-selective binding of the secondary Cy5 with the tissue. The autofluorescence of the internal elastic lamina was used for the distinction of the endothelium from the rest of the vessel. Cy5 fluorescence was visualized with an excitation wavelength of 635 nm and peak emission at 670 nm, while the autofluorescence of the internal elastic lamina was visualized using the FITC filter at an excitation wavelength of 488 nm and peak emission at 520 nm. The two images were obtained at a magnification of x40 and they were superimposed to demonstrate lack of staining in both the endothelium and the smooth muscle. E = endothelium, SM = smooth muscle, IEL = internal elastic lamina, L = lumen
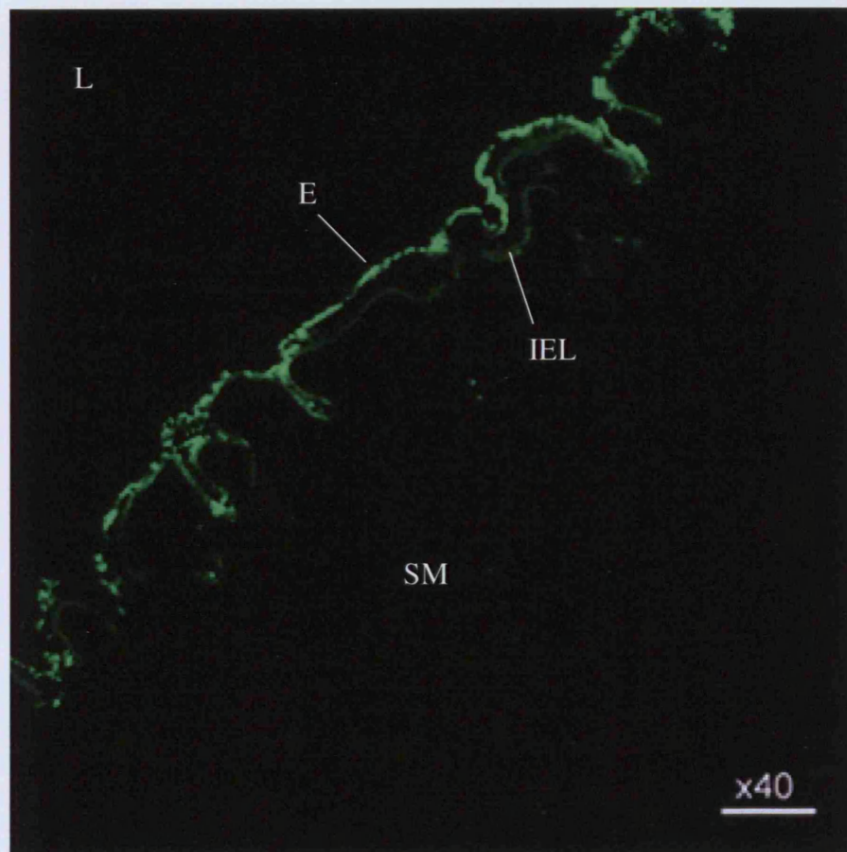
**Fig. 2.21** Transverse section of rabbit iliac artery stained for von Willebrand factor with an FITC-conjugated anti-vWF antibody. Immunohistochemical detection of the von Willebrand fcactor indicated the presence of intact endothelium on these vessels. The image was obtained at a magnification of x40 and was visualized using the FITC filter at an excitation wavelength of 488 nm and peak emission at 520 nm. Although visualization of the internal elastic lamina was not required in this instance, autofluorescence was obtained at the same wavelength. E = endothelium, SM = smooth muscle, IEL = internal elastic lamina, L = lumen
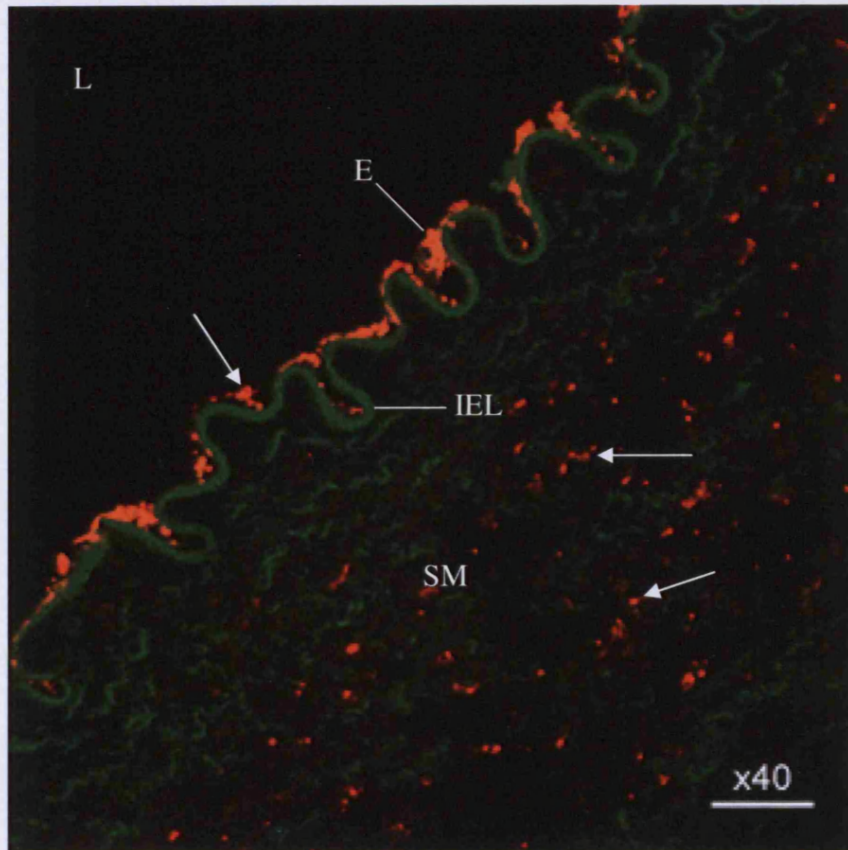
**Fig. 2.22** Transverse section of the rabbit iliac artery stained with a primary goat polyclonal antibody to $BK_{Ca}$ and a secondary Cy5-conjugated donkey anti-goat IgG. Punctate fluorescence in the endothelium and the smooth muscle (arrows) indicates the presence of $BK_{Ca}$ channels in these layers. The autofluorescence of the internal elastic lamina was used for the distinction of the endothelium from the rest of the vessel. Cy5 fluorescence was visualized with an excitation wavelength of 635 nm and peak emission at 670 nm, while the autofluorescence of the internal elastic lamina was visualized using the FITC filter at an excitation wavelength of 488 nm and peak emission at 520 nm. The two images were obtained at a magnification of x40 and they were superimposed to demonstrate $BK_{Ca}$ localization relative to the internal elastic lamina. E = endothelium, SM = smooth muscle, IEL = internal elastic lamina, L = lumen
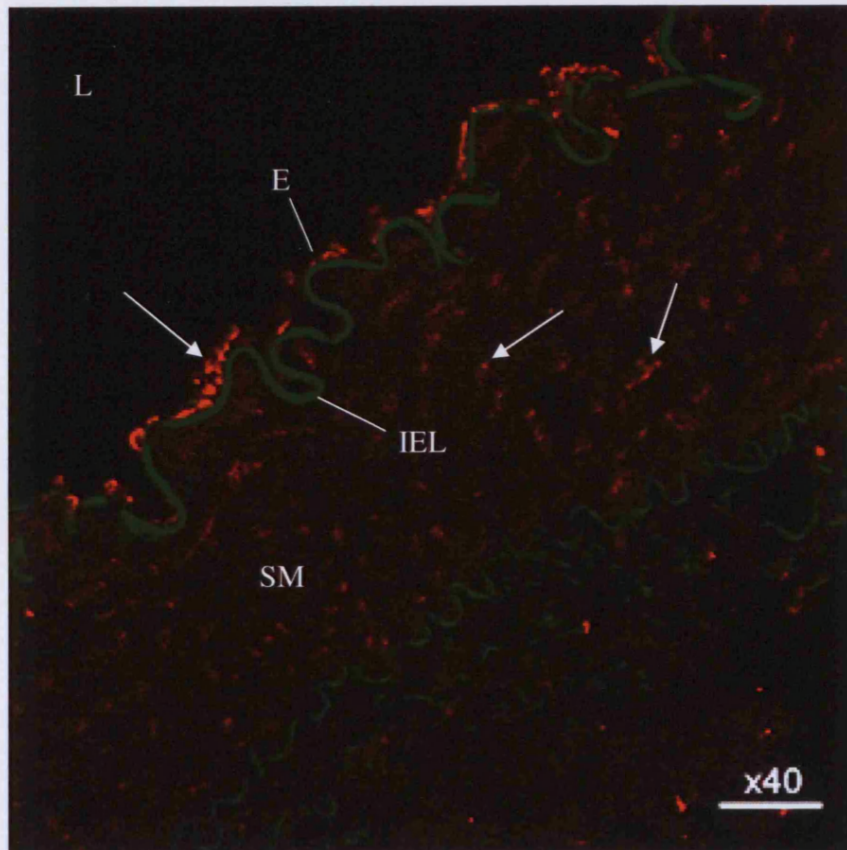
**Fig 2.23** Transverse section of the rabbit iliac artery stained with a primary goat polyclonal antibody to $IK_{Ca}$ and a secondary Cy5-conjugated donkey anti-goat IgG. Punctate fluorescence in the endothelium and the smooth muscle (arrows) indicates the presence of $IK_{Ca}$ channels in these layers. The autofluorescence of the internal elastic lamina was used for the distinction of the endothelium from the rest of the vessel. Cy5 fluorescence was visualized with an excitation wavelength of 635 nm and peak emission at 670 nm, while the autofluorescence of the internal elastic lamina was visualized using the FITC filter at an excitation wavelength of 488 nm and peak emission at 520 nm. The two images were obtained at a magnification of x40 and they were superimposed to demonstrate $IK_{Ca}$ localization relative to the internal elastic lamina. E = endothelium, SM = smooth muscle, IEL = internal elastic lamina, L = lumen
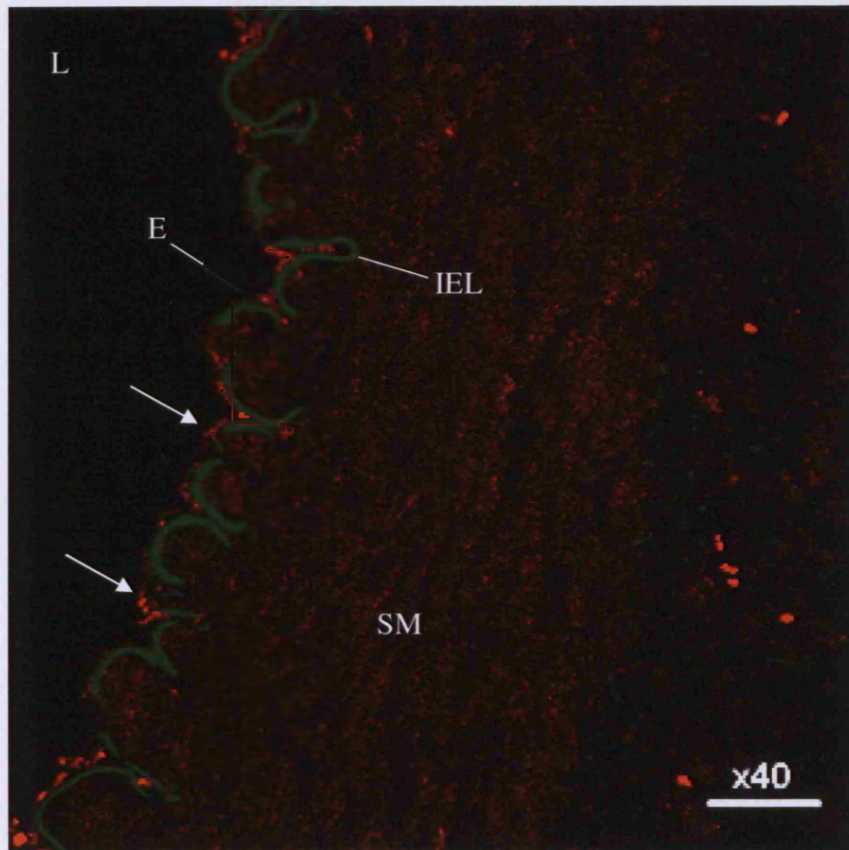
**Fig 2.24** Transverse section of the rabbit iliac artery stained with a primary goat polyclonal antibody to $SK_{Ca}$ and a secondary Cy5-conjugated donkey anti-goat IgG. Punctate fluorescence in the endothelium (arrows) indicates the presence of $SK_{Ca}$ channels in this layer. An increase in fluorescence is also detected in the smooth muscle of this section, thereby indicating that $SK_{Ca}$ is also located in the media of rabbit iliac arteries. The autofluorescence of the internal elastic lamina was used for the distinction of the endothelium from the rest of the vessel. Cy5 fluorescence was visualized with an excitation wavelength of 635 nm and peak emission at 670 nm, while the autofluorescence of the internal elastic lamina was visualized using the FITC filter at an excitation wavelength of 488 nm and peak emission at 520 nm. The two images were obtained at a magnification of x40 and they were superimposed to demonstrate $SK_{Ca}$ localization relative to the internal elastic lamina. E = endothelium, SM = smooth muscle, IEL = internal elastic lamina, L = lumen

## 2.4 Discussion

### 2.4.1 ACh and A23187 promote activation of all $K_{Ca}$ subtypes

The present study has highlighted the role of $K_{Ca}$ channels in EDHF-type relaxations induced by ACh and A23187 in rabbit iliac arteries. Although the involvement of $K_{Ca}$ channels in this species has been previously demonstrated by Chaytor *et al.* (2002), the relative contribution of each channel to the development of NO/prostanoid-independent relaxations was not defined. Therefore, it was necessary to identify which $K_{Ca}$ channels participate in the induction of EDHF-type responses by each agent, and more importantly the degree to which each subtype contributes to each response. Accordingly, the current study focused on identifying the $K_{Ca}$ channel subtypes present in the endothelium of these vessels. It was demonstrated that $SK_{Ca}$, $IK_{Ca}$ and $BK_{Ca}$ channels may contribute to EDHF-type responses evoked by ACh and A23187, but that the relative contribution of each channel depends on the mode of stimulation. Supporting immunohistochemical data demonstrated that in rabbit iliac arteries all $K_{Ca}$ channels are expressed in both the endothelium and the smooth muscle.

### 2.4.2 $K_{Ca}$ channels in EDHF-type responses

In intact rings pre-incubated with L-NAME and indomethacin, ACh and A23187 generated maximal relaxations respectively equivalent to ~70% and ~80% of PE-induced tone at a concentration of ~3 µM. The selective $BK_{Ca}$ inhibitor IbTX attenuated these responses, thus confirming that a functional $BK_{Ca}$ channel is present in rabbit iliac arteries. Although IbTX does not affect EDHF-type responses in all artery types and species, there is substantial evidence that this toxin is devoid of non-specific effects (Galvez et al., 1990; Waldron & Cole, 1999; Gao & Garcia, 2003). Indeed, the major advantage of IbTX is therefore that it does not inhibit $K_V$ or $IK_{Ca}$ channels, two effects which have been reported for ChTX, which is another widely used, but consequently less specific $BK_{Ca}$ inhibitor (Giangiacomo *et al.,* 1992; Kaczorowski *et al.,* 1996).

Apart from its effects on EDHF-type relaxations in rabbit iliac arteries, IbTX significantly attenuates ACh-evoked EDHF-type hyperpolarizations in rabbit renal arteries, whereas in branches of rabbit superior mesenteric artery such responses were unaffected, thereby suggesting the existence of differences in expression of $BK_{Ca}$ across vessels (Kagota et al., 1999; Kwon et al., 2001; Brayden & Murphy, 1995; Fujimoto et al., 1999). Indeed, the toxin elicits only a partial inhibitory effect in rabbit iliac arteries,

thus suggesting that $BK_{Ca}$ is not the sole channel mediating hyperpolarization in this species. Inhibitory effects of IbTX against EDHF-type responses have also been reported in the guinea-pig cerebral, mesenteric and spiral modiolar arteries (Dong *et al.*, 1997; Jiang *et al.*, 2007). Nevertheless, other studies carried out in human gastroepiploic arteries and guinea-pig carotid artery have excluded the involvement of $BK_{Ca}$ channels on the basis that in these tissues ACh-evoked EDHF-type responses were unaffected by IbTX (Urakami-Harasawa *et al.*, 1997; Chataigneau *et al.*, 1998; Fujimoto *et al.*, 1999; Murphy & Brayden, 1995). Also, investigations in guinea pig basilar and rabbit iliac arteries with ChTX demonstrated that this toxin did not attenuate A23187-induced responses, thereby emphasizing that $BK_{Ca}$ might not be involved in the EDHF phenomenon in different vessels from the same species (Petersson *et al.*, 1997).

Notably, in reports that demonstrate that $BK_{Ca}$ channels participates in the EDHF phenomenon, activation of the channel coincides with the activation of other $K_{Ca}$ subtypes. For instance, in isolated rat gracilis muscle arterioles, ChTX blocks EDHF-type responses more effectively when co-administered with apamin, rather than on its own (Ungvari *et al.*, 2002). Similar effects have also been shown in guinea pig carotid, submucosal, basilar and coronary arteries, porcine coronary and renal arteries, human pial and renal arteries, and rabbit mesenteric arteries (Gonzales-Nunez *et al.*, 2001; Petersson *et al.*, 1997; Yamanaka *et al.*, 1998; Ge *et al.*, 2000; Büssemaker *et al.*, 2002, 2003; Fujimoto *et al.*, 1999).

It has been suggested that in EDHF-type responses, $SK_{Ca}$ and $IK_{Ca}$ channels might be activated simultaneously (Bychkov *et al.*, 2002; Crane *et al.*, 2003; Gluais *et al.*, 2004). To confirm this hypothesis, functional experiments were carried out with apamin and TRAM-34 in the rabbit iliac artery. It was demonstrated that apamin alone had no effect on ACh- and A23187-induced relaxations, thus indicating that the sole activation of apamin-sensitive $SK_{Ca}$ channels might not be sufficient to trigger a response in these vessels. Conversely, TRAM-34 suppressed EDHF-type relaxations by significantly increasing the $pEC_{50}$ of both ACh and A23187, thereby reflecting a pivotal role for $IK_{Ca}$. To elucidate further the implication of both channels on EDHF-type responses, investigations were carried out with the double combination of apamin plus TRAM-34. It was demonstrated that in rabbit iliac arteries, the two inhibitors combined are more effective than either apamin or TRAM-34 on their own, by decreasing vascular tone by

~60%. Indeed, in most studies carried out so far, addition of each inhibitor separately has little or no effect on the EDHF phenomenon, while their co-administration suppresses the response. Although the absence of apamin-induced inhibition in rabbit iliac arteries is consistent with studies in hepatic arteries from female Sprague-Dawley rats (Zygmunt *et al.*, 1997), it contrasts with other studies demonstrating an apamin-sensitive component, therefore suggesting that $SK_{Ca}$ expression is different among species (Murphy & Brayden, 1995; Chen & Cheung, 1997; Hinton & Langton, 2003; Crane *et al.*, 2003). On the other hand, the identification of $IK_{Ca}$ concurs with the fact that this channel is partially responsible for the development of the EDHF phenomenon in some species, such as the rat, and it is known to occur in conjunction with $SK_{Ca}$ opening (Crane *et al.*, 2003; Gluais *et al.*, 2004). Although it is not well understood how apamin potentiates the inhibitory effect of TRAM-34, insight was given by recent studies carried out on rat superior mesenteric arteries (Crane *et al.*, 2003; Sandow *et al.*, 2006). According to these investigations $SK_{Ca}$ and $IK_{Ca}$ are activated simultaneously but separately which was speculated to occur due to the spatial separation of the two channels. Indeed, immunohistochemical observations showed that $IK_{Ca}$ is present at the IEL close to myoendothelial gap junctions, while $SK_{Ca}$ is localized at adjacent endothelial gap junction plaques (Sandow *et al.*, 2006). However, whether this proposed spatial separation reflects the putative differences in the contribution of each channel in the development of endothelial hyperpolarizations or the modulation of cell-cell coupling requires further investigation.

In rabbit iliac arteries, the residual relaxations obtained in the presence of apamin and TRAM-34 were abolished when iberiotoxin (100nM) was co-administered in the organ chamber, thereby suggesting that the effects of $SK_{Ca}$, $IK_{Ca}$ and $BK_{Ca}$ might be synergistic. Complementary effects of these three $K_{Ca}$ inhibitors have also been demonstrated in guinea pig carotid and pig anterior descending coronary arteries (Quignard *et al.*, 2000; Gluais *et al.*, 2005; Bychkov *et al.*, 2002; Weston *et al.*, 2005). Nevertheless, it has been suggested that differences in cell type underpin the different role of these channels in signalling processes. For instance, endothelial $SK_{Ca}$ and $IK_{Ca}$ channels have been thought to be the main channels responsible for endothelial hyperpolarizations, whereas $BK_{Ca}$ channels have been thought to mediate smooth muscle hyperpolarization evoked by freely diffusible EDHFs (Köhler *et al.*, 2000; Bychkov *et al.*, 2002; Sollini *et al.*, 2002; Ding *et al.*, 2003; Eichler *et al.*, 2003;

McNeish *et al.,* 2006; Plane *et al.,* 1996; Zhao *et al.,* 1997). For this reason immunostaining was carried out in a separate section of this study, in order to identify the location of the three $K_{Ca}$ channels in the rabbit iliac artery.

## 2.4.3 Identification of endothelial $K_{Ca}$ subtypes

Experiments were carried out so as to determine the localization of $BK_{Ca}$ and $SK_{Ca}/IK_{Ca}$ channels on the endothelium and/or smooth muscle of rabbit iliac arteries. It has been demonstrated that in many blood vessels, $BK_{Ca}$ channels are mainly expressed in the vascular smooth muscle (Mistry & Garland, 1998; Barlow *et al.,* 2000; Weston *et al.,* 2005; Wu *et al.,* 2005; Zhu *et al.,* 2006). Nevertheless, some reports involving immunohistochemical and patch-clamp studies suggested that $BK_{Ca}$ might also be located on the intimal layer (Rusko *et al.,* 1992; Yousif *et al.,* 2002; Ungvari *et al.,* 2002; Papassotiriou *et al.,* 2000; Brakemeier *et al.,* 2003). To elucidate the role of endothelial $BK_{Ca}$ channels on EDHF-type relaxations in the rabbit, functional experiments were carried out with the $BK_{Ca}$ channel opener NS1619. In intact rings, NS1619 generated relaxations equal to ~85% at a concentration of 100 µM with a threshold of 1-3 µM. Denudation of the endothelium caused a small but significant rightward shift of the response, thus indicating that in these vessels NS1619 has both an endothelial and smooth muscle site of action. Similar results to those carried out with endothelium-denuded vessels were also obtained with IbTX, which suggested that one of the components of the NS1619-associated response is $BK_{Ca}$-dependent. However, the available evidence is not sufficient to demonstrate that ablation of the endothelium consequently removes the IbTX-sensitive component. In fact, the findings of this study concur with those of investigations carried out in rat basilar arteries, which demonstrated that apart from the $BK_{Ca}$–dependent component NS1619-induced relaxations comprise of a residual response, which was attributed to the concurrent closure of voltage operated L-type $Ca^{2+}$ channels (Holland *et al.,* 1996). Hence, it is still unclear whether NS1619 activates $BK_{Ca}$ channels in the endothelium or in the smooth muscle.

To elucidate the role of endothelial $SK_{Ca}$ and $IK_{Ca}$ channels on EDHF-type relaxations in the rabbit, mechanical investigations were carried out with the benzimidazolone 1-EBIO. It was demonstrated that 1-EBIO generates relaxations equivalent to ~80% of the

PE preconstriction. Denudation of the endothelium caused a small but significant increase in the $pIC_{50}$ value of the agonist, thus confirming that both $SK_{Ca}$ and $IK_{Ca}$ might be located on the endothelium. Similar results were also observed in vessels preincubated with apamin and TRAM-34, therefore confirming that both channels are present in the endothelium of iliac arteries. However, 1-EBIO does have other actions apart from activating $SK_{Ca}$ and $IK_{Ca}$. Although it is now established that it triggers EDHF-type hypepolarizations in a variety of tissues, including rat mesenteric and middle cerebral arteries, there is evidence that this agent also stimulates smooth muscle relaxation (Walker *et al.*, 2001; Crane *et al.*, 2002; Marelli *et al.*, 2003). Indeed, it has been previously demonstrated that the mechanism of action of 1-EBIO is strongly dependent on intracellular $Ca^{2+}$ and its interaction with calmodulin (Pedarzani *et al.*, 2001). For instance, in human embryonic kidney cells (HEK 293) stably expressing hIK channels, patch clamp studies showed that in the presence of 1-EBIO, the $Ca^{2+}$– activation curve was shifted to the left, and maximal currents were attained at 100 nM (Pedersen *et al.*, 2002). This observation suggests that changes in the underlying $[Ca^{2+}]$ not only affect the functionality of $SK_{Ca}$ and $IK_{Ca}$, but also other cellular components that depend on $Ca^{2+}$, such as the ER and mitochondria.

More conclusive data about the localization of $K_{Ca}$ channels were obtained by immunohistochemical investigations carried out on transverse sections of rabbit iliac arteries. It was demonstrated that $BK_{Ca}$ is expressed in both endothelial and smooth muscle layers, which was consistent with the hypothesis that the channel might trigger endothelium-derived hyperpolarizations and the development of tonic contractions in the presence of IbTX. These findings were also in agreement with the involvement of a $BK_{Ca}$-dependent component in NS1619-evoked EDHF-type relaxations, and concurred with the fact that in rabbit iliac arteries IbTX inhibits both ACh- and A23187-induced EDHF-type responses. Endothelial localization of $BK_{Ca}$ has also been demonstrated immunohistochemically in other blood vessels such as the rabbit ductus arteriosus and the rat gracilis muscle arterioles (Thebaud *et al.*, 2002; Ungvari *et al.*, 2002). By contrast, $IK_{Ca}$ expression was restricted in the endothelium and to a lesser extent the smooth muscle of rabbit iliac arteries, a finding which was consistent with data from studies carried out in porcine coronary arteries and in rat mesenteric and middle cerebral arteries (Marrelli *et al.*, 2003; Bychkov *et al.*, 2002; Walker *et al.*, 2001). It should be noted that although $IK_{Ca}$ seems to be one of the two main $K_{Ca}$ channels that induce

endothelial hyperpolarizations in the rat, the other being $SK_{Ca}$, in the rabbit the contribution of this channel in EDHF-type relaxations seems to occur in conjunction with endothelial $BK_{Ca}$ (Edwards *et al.,* 1998; McNeish *et al.,* 2006).

Finally, immunohistochemical studies carried out on rabbit iliac arteries demonstrated the presence of $SK_{Ca}$ in both the endothelium and the smooth muscle of this vessel. Although there are three different $SK_{Ca}$ subtypes, namely SK1, SK2 and SK3, in the current study emphasis was given only to SK3, as this is the subtype known to be more preponderant on endothelial cells and therefore is more relevant to the development of EDHF-type hyperpolarizations (Burnham *et al.,* 2002; Bychkov *et al.,* 2002; Ceroni *et al.,* 2007; Chaytor *et al.,* 2003). The presence of SK3 channels in the endothelium concurs with the aforementioned mechanical investigations (section 2.4.2), which illustrated that addition of apamin potentiates the inhibitory effects of TRAM-34 against both ACh- and A23187-evoked EDHF-type relaxations. The presence of SK3 in the endothelium has also been demonstrated with molecular techniques in $1^{st}$, $2^{nd}$, $3^{rd}$ and $4^{th}$ order mesenteric arteries from rats and eNOS-knockout mice, in which the expression of the channel increases as the size of the vessels decreases (Doughty *et al.,* 1999; Shandow *et al.,* 2006; Ceroni *et al.,* 2007). Notably, SK3 channels were detected in the smooth muscle of rabbit iliac arteries, a finding that was surprising knowing that this $SK_{Ca}$ subtype is mainly located in the intimal layer. Such finding is also contradictory to the mechanical data presented in this thesis, which demonstrate that in rabbit iliac arteries $SK_{Ca}$ has no tonic role in apamin pretreated vessels. Therefore, based on the current evidence is difficult to understand how smooth muscle $SK_{Ca}$ channels might contribute to ACh- and A23187-evoked EDHF-type relaxations.

## 2.4.4 Effects of $K_{Ca}$ inhibitors on smooth muscle

It was demonstrated that addition of IbTX to the organ chamber, has a significant effect on the tension of resting arteries, thus implying that the toxin might interact with $BK_{Ca}$ located on smooth muscle. Indeed, the tonic contraction was observed in all experiments with IbTX, with the average magnitude of the tension being the same in both endothelium-intact and endothelium-denuded vessels. This suggested that in these vessels the endothelium might not play a significant role in the determination of the IbTX-induced tonic contraction, and therefore these effects are only restricted in smooth

muscle. It was also demonstrated that addition of apamin or TRAM-34 on their own failed to increase the basal tone of endothelium intact and endothelium-denuded vessels. These results are consistent with studies carried out in rat mesenteric arteries, where both $SK_{Ca}$ and $IK_{Ca}$ are localized in the endothelium (Edwards *et al.*, 1998; Crane *et al.*, 2003). Notably, it has been demonstrated that in resting rat mesenteric arteries, hyperpolarizations can be explained solely by the activation of $SK_{Ca}$ channels, while during PE-induced depolarization and contraction, the activation of $IK_{Ca}$ is also necessary (Crane *et al.*, 2003). However, in the current study the available evidence suggests that $SK_{Ca}$ and $IK_{Ca}$ do not play a significant role in smooth muscle depolarization and contraction, but they might both be necessary for the development of EDHF-type relaxations as demonstrated in section 2.4.2. This finding was also supported by the absence of any significant changes in the magnitude of tonic contractions in the presence of the triple combination of apamin plus TRAM-34 plus IbTX in both endothelium-intact and endothelium-denuded vessels. If smooth muscle $K_{Ca}$ channels, other than $BK_{Ca}$, contributed to the development of the tonic contraction, then a further increase in the magnitude of the tonic contractions would have been observed in the presence of apamin and TRAM-34. Hence, it is reasonable to speculate that the only $K_{Ca}$ subtype that might be responsible for the tonic contractions observed in rabbit iliac arteries is $BK_{Ca}$.

$BK_{Ca}$ channels are expressed in almost all vascular smooth muscle cells, and their physiological role could more appropriately be seen as a feedback inhibitor of contraction or a physiological break against increases in intravascular pressure. Indeed, in the resting state, the activation of clusters of smooth muscle $BK_{Ca}$ channels leads to the development of spontaneous transient outward currents (STOCs) in response to localized $Ca^{2+}$ microelevations, known as $Ca^{2+}$ sparks (Nelson *et al.*, 1995; Bychkov *et al.*, 1997). Due to $BK_{Ca}$ activation, $Ca^{2+}$ sparks may therefore lead to a paradoxical decrease in the overall intracellular $Ca^{2+}$ concentration, which could be responsible for a decrease in vascular tone (Jaggar *et al.*, 1998). However, some studies have suggested that even at membrane potentials close to the resting state in isolated arteries (-40mV to -50mV), $BK_{Ca}$ channels may be tonically active (Quignard et al., 2000). Hence, there is a possibility that the inhibition of $BK_{Ca}$ with IbTX might lead to a significant increase in $[Ca^{2+}]_i$ and subsequently contraction.

Furthermore, other studies have suggested that in the muscle activation of these channels might depend on both $Ca^{2+}$ sparks and $Ca^{2+}$ entry through L-type $Ca^{2+}$ channels (Herrera & Nelson, 2002). Although the exact mechanism underlying the association between $BK_{Ca}$ and L-type channels is yet unkown, it is possible that inhibition of smooth muscle $BK_{Ca}$ could have a major effect on the opening of L-type channels through the development of a depolarizing response. Indeed, the physiological functions of $BK_{Ca}$ arise from their allosteric activation by two factors: 1] membrane depolarisation and 2] changes in $[Ca^{2+}]_i$ (Cui *et al.*, 1997). A connection between $BK_{Ca}$ and L-type channels has been previously demonstrated in the rat, in which the sustained phase of the myogenic response was sensitive to both IbTX and D600, an L-type channel inhibitor (Chlopicki *et al.*, 2001). Other studies carried out on femoral arteries from both SHR and WKY rats have also demonstrated that in the resting state increases in $Ca^{2+}$ efflux are coupled to the appearance of myogenic tone and changes in $K^+$ fluxes through $BK_{Ca}$ channels (Asano *et al.*, 1995).

Nevertheless, the identification of $BK_{Ca}$ channels in the smooth muscle of rabbit iliac arteries and the development of variable increases in tension during incubation with IbTX raised the possibility that the effects of the toxin on EDHF-type relaxations might be indirect. Speculatively, the IbTX-induced constriction of the vessel could inhibit relaxations to ACh and A23187 through the propagation of depolarizations from the smooth muscle to the endothelium. Furthermore, it was demonstrated that removal of the endothelium of rabbit iliac arteries did not affect the tonic contractions evoked by IbTX, thereby suggesting that the augmentation of the tone occurs due to inhibition of smooth muscle $BK_{Ca}$ channels only. However, it was observed that the magnitude of the inhibition of EDHF-type relaxations by IbTX was the same in all experiments, while the magnitude of the tonic contractions was variable. Although it cannot be excluded that inhibition of smooth muscle $BK_{Ca}$ channels might have an indirect effect on EDHF-type relaxations, such discrepancy indicates that IbTX is more likely to inhibit these responses directly. Therefore, the role of an indirect effect of IbTX on EDHF-type relaxations cannot be fully supported by the data of this study, and in this regard it needs to be investigated further.

The immunohistochemical detection of $IK_{Ca}$ in the smooth muscle of rabbit iliac arteries indicated that this channel could also be implicated in the development of vascular tone.

A functional smooth muscle $IK_{Ca}$ is known to be essential to negatively modulate the rhythmical activity of vessels, that is the changes in contractility which are associated with underlying oscillations in $Ca^{2+}$ and rhythmical changes in membrane potential (Hill *et al.,* 2001; Peng *et al.,* 2001; Haddock & Hill, 2002). However, in rabbit iliac arteries $IK_{Ca}$ has no tonic role in TRAM-34 pretreated vessels as previously demonstrated, and therefore it is difficult to establish the role of this channel in the smooth muscle. Notably, studies carried out on mature and immature cultured smooth muscle cells from aortae of WKY rats demonstrated that the expression of $IK_{Ca}$ channels is more profound at early stages of cell development while $BK_{Ca}$ is the main $K_{Ca}$ subtype in mature cells (Neylon *et al.,* 1999). Although the current study cannot provide any insights on these developmental changes, it does concur with the fact that both $BK_{Ca}$ and $IK_{Ca}$ channels can be found on the smooth muscle and that the distribution of the former is significantly higher than the latter.

Following the constriction of iliac arteries with PE, the maximum tension of IbTX pre-treated vessels did not differ significantly from that of the control. This indicated that PE stimulation might be sufficient to mask the tonic contraction induced by $BK_{Ca}$ closure. Similar results were observed for both endothelium-intact and endothelium-denuded vessels, which once again emphasized the fact that the IbTX-evoked tonic contractions are independent of the endothelium. However, a functional $BK_{Ca}$ channel is present in the endothelium of rabbit iliac arteries, and therefore pharmacological blockade of the channel would not be sufficient to distinguish its contribution from that of the corresponding channels in the muscle. Furthermore, it was demonstrated that IbTX significantly inhibits both ACh- and A23187-evoked EDHF-type relaxations of the rabbit iliac artery, suggesting that endothelial hyperpolarizations might be associated with a functional $BK_{Ca}$ channel in this layer. It is therefore possible that smooth muscle depolarization might have a counteractive effect on the development of hyperpolarizations in the endothelium. In fact, there are reports which suggest that smooth muscle depolarization induced by PE might be conducted to adjacent endothelial cells via myoendothelial gap junctions (Little *et al.,* 1995; Dora *et al.,* 1997, 2000; Griffith *et al.,* 2004). However, the evidence provided in this study is not sufficient to demonstrate how the localization of $BK_{Ca}$ channels or smooth muscle depolarization might affect the development of EDHF-type responses, and therefore no direct conclusion can be drawn.

## 2.4.5 Concluding remarks

In conclusion, the present Chapter has confirmed that $K_{Ca}$ channels participate in the induction of EDHF-type responses by ACh and calcium ionophore A23187 in the rabbit iliac artery. It was demonstrated that the relative contribution of each channel subtype varies depending on the stimulus, and that $BK_{Ca}$ channels in the endothelium might play a significant role in both ACh- and A23187-evoked responses. It was also demonstrated that simultaneous inhibition of $BK_{Ca}$, $IK_{Ca}$ and $SK_{Ca}$ channels attenuates ACh- and A23187-evoked EDHF-type relaxations. Immunohistochemical studies showed that all $K_{Ca}$ subtypes are present on both the endothelium and the smooth muscle of rabbit iliac arteries. More conclusive data about the localization and the putative role of $BK_{Ca}$ and $SK_{Ca}/IK_{Ca}$ were obtained in NS1619- and 1-EBIO-treated vessels.

*Chapter Three*


*The mitochondria and endothelial $H_2O_2$ generation in ACh- and A23187-evoked EDHF-type relaxations*

## 3.1 Introduction

Endothelial cells and smooth muscle cells may both generate large amounts of reactive oxygen species depending on the conditions and the physiological stimulus involved. It is now known that $H_2O_2$ produced in endothelial cells in response to agents such as ionophore A23187, and to a lesser extent ACh, has vasoactive properties that are associated with effects on cellular components such as actin-myosin and $K_{Ca}$ channels (Ellis & Triggle, 2000; Chaytor *et al.*, 2003). $H_2O_2$ induces relaxations in porcine, canine and human coronary arteries, and rabbit, rat and human mesenteric arteries (Matoba *et al.*, 2003; Pomposiello *et al.*, 1999; Rubanyi & Vanhoutte, 1986; Beny & von der Weid, 1991; Consentino & Katusic, 1995; Barlow & White, 1998; Hayabuchi *et al.*, 1998; Miura *et al.*, 2003; Sato *et al.*, 2003; Thengchaisri & Kuo, 2003), whereas in other vessels such as the bovine coronary artery and the canine cerebral artery it causes contraction (Oeckler *et al.*, 2003; Katusic *et al.*, 1993).

In human submucosal microvessels application of exogenous $H_2O_2$ triggered two different effects depending on the presence or absence of the endothelium (Hatoum *et al.*, 2005). $H_2O_2$ induced a dose-dependent relaxation in endothelium intact sections, while application of $H_2O_2$ on endothelium-denuded arterioles caused a significant increase in tension (Hatoum *et al.*, 2005). The ability of $H_2O_2$ to cause either relaxation or contraction on the same vessel has also been demonstrated in rat skeletal muscle arterioles, with $H_2O_2$ causing vasoconstriction at low concentrations and vasodilatation at concentrations higher than 60 μM (Cseko *et al.*, 2004). Notably, in the rat removal of the endothelium did not change the pattern of the $H_2O_2$-induced response, as observed in human submucosal microvessels, but it significantly reduced the magnitude of both contractions and relaxations (Hatoum *et al.*, 2005; Cseko *et al.*, 2004). It has been suggested that in porcine coronary arteries $H_2O_2$ reduces vascular tone through two different mechanisms; one endothelium-dependent and one endothelium-independent (Thengchaisri & Kuo, 2003). In these vessels, endothelial denudation in combination with indomethacin led to the conclusion that the COX-PGE2 pathway accounts for the endothelium-dependent component, while the endothelium independent was associated with the activation of $K_{Ca}$ channels in the smooth muscle (Thengchaisri & Kuo, 2003). Such differences in the effects of $H_2O_2$ on the endothelium and the smooth muscle raised the possibility that apart from its properties as an EDRF, $H_2O_2$ could be a factor that triggers endothelial and subsequently smooth muscle hyperpolarizations. Indeed,

some reports have suggested that $H_2O_2$ might be an EDHF on the basis that in the rat, catalase significantly inhibited the underlying hyperpolarizations (Matoba *et al.,* 2000). However, studies carried out in rabbit iliac arteries demonstrated that upon stimulation with A23187, an $H_2O_2$-dependent component of relaxation was concomitant with but distinct from an associated hyperpolarizing response (Chaytor *et al.,* 2003). This finding suggests that either A23187 stimulates simultaneously two separate pathways, or the release of $H_2O_2$ is a consequence of the endothelial hyperpolarization.

### 3.1.1 Effects of $H_2O_2$ on $K_{Ca}$ channels

Numerous investigations have been carried out to elucidate the possible effects of $H_2O_2$ on $K_{Ca}$ channels in the endothelium and smooth muscle. Although it has been suggested that $H_2O_2$ affects the opening of $K_{Ca}$ by targeting cysteine residues near the $Ca^{2+}$ bowl of the $\alpha$ subunit (Tang *et al.,* 2004), there is consensus on the fact that this mechanism affects $K_{Ca}$ channels differently among vessels and species, and is dependent on the stimulus employed, the localization of the channels and the $K_{Ca}$ subtype (Wei *et al.,* 1996; Sobey *et al.,* 1997; Hattori *et al.,* 2003). For instance, in isolated porcine coronary arteries patch clamp techniques have demonstrated that exposure to $H_2O_2$ opens smooth muscle $BK_{Ca}$ channels via the $PLA_2$ – arachidonate cascade (Barlow *et al.,* 2000), while in the renal artery endothelium from the same species application of $H_2O_2$ inactivates these channels (Brakemeier *et al.,* 2003). Activation of $BK_{Ca}$ channels by $H_2O_2$ has also been demonstrated in the smooth muscle of rat cerebral arteries, in small mesenteric arteries from eNOS-knockout mice, and the smooth muscle of porcine coronary arteries (Sobey *et al.,* 1997; Matoba *et al.,* 2000; Thengchasri & Kuo, 2003). In human coronary microvessels, it has been reported that smooth muscle $K_{Ca}$ channels sensitive to ChTX and apamin are activated by $H_2O_2$ (Miura *et al.,* 2003).

However, studies carried out so far have not explored the possibility of coupling between $K_{Ca}$ activation and the release of $H_2O_2$ from the endothelium. Indeed, in Chapter 2, it was demonstrated that the combined application of apamin, TRAM-34 and IbTX, significantly attenuates EDHF-type relaxations to A23187. Hence, considering that A23187-evoked relaxations exhibit a major catalase-sensitive component, it is possible that the release of this species is a consequence of endothelial $K_{Ca}$ activation (Chaytor *et al.,* 2003). It is also evident that in order to understand the mechanisms that underpin A23187-evoked EDHF-type relaxations, it is necessary to identify the sources

of $H_2O_2$ in these tissues. Because all studies questioning the role of the EDHF mechanism in the regulation of vascular tone were carried out in the presence of NOS and prostanoid inhibitors in the present thesis, it was considered necessary to focus on three sources of ROS only, namely, NADPH oxidase, xanthine oxidase and the mitochondrial electron transport chain (ETC).

## 3.1.2 Putative sources of ROS in the vasculature

### 3.1.2.1 The NADPH oxidase system

The NADPH oxidase system is an assembly of membrane-associated heterodimer of gp91phox and p22phox (also known as cytochrome $b_{558}$) and the cytosolic components p67phox, p47phox and p40phox. In the novel Nox terminology, gp91phox is called Nox2, and can be regarded as a redox transmembrane chain that connects the cytosolic electron donor NADPH with oxygen, which plays the role of the electron acceptor on the outer membrane (see section 1.3.3.1). Although Nox2 was initially identified in macrophages, it may also be located in non-phagocytic cells such as endothelial cells and cardiac and skeletal myocytes (Zhang *et al.*, 2006; Wang *et al.*, 2007). In non-phagocytic cells production of $H_2O_2$ occurs by dismutation of $O_2^{\cdot-}$, which occurs when electrons move from the reduced substrate to molecular oxygen. It should be noted that the movement of electrons takes place only once the cytosolic components have translocated to the plasmalemma. The plant phenol 4-hydroxy-3-methoxyacetophenone (apocynin) has been extensively used for the elucidation of the interactions between the various subunits of the oxidase, and their role in ROS production. In fact, it has been established that its inhibitory properties are associated with the blockade of the translocation of the cytosolic components and their interaction with Nox2 (Stolk *et al.*, 1994). Apocynin has been previously used to demonstrate the role of NADPH oxidase in ROS production in a number of vascular tissues, including rabbit carotid arteries, porcine pulmonary arteries and rat cerebral arteries (Chen *et al.*, 2007; Cai *et al.*, 2003; Paravicini *et al.*, 2002; Miller *et al.*, 2005).

### 3.1.2.2 Xanthine oxidase and ROS production

The xanthine oxidoreductase (XOR) system is also relevant as a putative source of $H_2O_2$ in the vasculature (White *et al.*, 1994; Kelley *et al.*, 2006). One of the primary roles of this system is the conversion of hypoxanthine to xanthine and xanthine to uric acid.

XOR exists in two interconvertible forms, the xanthine dehydrogenase (XDH), which reduces nicotinamide adenine dinucleotide, and xanthine oxidase (XO), which oxidizes molecular oxygen to $O_2 \cdot^-$ (Berry & Hare, 2004). The enzyme is a 270 kDa protein which incorporates two FAD molecules, two molybdenum atoms forming a molybdopterin unit which is the active site of the enzyme, and eight iron atoms which participate in electron transport reactions (Fridovich & Handler, 1958). Allopurinol was the first inhibitor of this enzyme to be developed, and was found to reduce uric acid levels in both serum and urine (Springer *et al.*, 1996; Perez-Ruiz & Liote, 2007). Allopurinol reacts with XOR at the molybdopterin unit to yield alloxanthine (or oxypurinol), which binds to XOR via direct coordination to molybdenum atoms, thereby inhibiting the interaction of the enzyme with its substrate (Berry & Hare, 2004; Truglio *et al.*, 2002; also see Chapter 1). Oxypurinol inhibits XO in a non-competitive manner and together with its long persistence in tissues, is responsible for much of the pharmacological activity of allopurinol.

### 3.1.2.3 The mitochondrial electron transport chain (ETC)

ROS are produced in mitochondria, and more specifically by the ETC, the site of respiration (see section 1.3.3.2). Although molecular oxygen is reduced to water by the ETC, a small fraction is incompletely reduced to $O_2 \cdot^-$, which is subsequently dismutated into $H_2O_2$ by mitochondrial SODs. Generation of $O_2 \cdot^-$ takes place in complexes I and III, but it still remains unclear which complex is the primary source. Indeed, it has become evident that each of these sites might play a distinct role depending on different stimuli. For instance, complex I preferentially produces $O_2 \cdot^-$ in response to shear stress in human coronary resistance arteries, while in human umbilical vein endothelial cells complex III preferentially produces the anion in response to hypoxia-reoxygenation (Liu *et al.*, 2003; Therade-Matharan *et al.*, 2005; see section 1.3.3.2). Accordingly, the use of mitochondrial antagonists and uncouplers has been instrumental in evaluating the contribution of mitochondria to ROS generation. Two widely used inhibitors of ETC are rotenone, a blocker of complex I, and myxothiazol, a blocker of complex III. These agents have been demonstrated to either enhance or diminish $H_2O_2$ production consistent with functional differences between the two ETC sites (Grivennikova & Vinogradov, 2006; Guidarelli *et al.*, 2007). Notably, in investigations carried out on HEK293, U87 and HeLa cells rotenone significantly increased the production of $H_2O_2$ which ultimately led to irreversible cell damage (Chen *et al.*, 2007), while other studies

have shown that application of rotenone in human coronary resistance arterioles and rat coronary arteries inhibits the production of this species (Liu *et al.,* 2003; Saitoh *et al.,* 2006). Such differences implied that in contrast to myxothiazol, the mitochondrial effects of rotenone are not always inhibitory and might be strongly influenced by the experimental conditions employed.

### 3.1.3 Aims of study

The aims of the current study were two-fold. Firstly, a series of mechanical investigations was carried out to elucidate the putative connection between endothelial $K_{Ca}$ activation and subsequent $H_2O_2$ release. These investigations included experiments designed to show how $H_2O_2$ produced in the endothelium might affect the $K_{Ca}$ channels located in smooth muscle. Given the potential role of the different ROS sources in A23187-evoked $H_2O_2$ release in the endothelium, the second objective of the current study was to identify the extent to which NADPH oxidase, xanthine oxidase or mitochondria might contribute to ROS production in rabbit iliac arteries.

## 3.2 Methods

### 3.2.1 Isolated ring preparations

Iliac artery rings were isolated and prepared for mechanical investigations as described in section 2.2.1

### 3.2.2 Protocols

Cumulative concentration-response curves were constructed for the calcium ionophore A23187 following the constriction of the vessels with 1 μM PE. To confirm that the previously described inhibitory effects of catalase are due to the interaction of the enzyme with $H_2O_2$, some rings were incubated with catalase (2000 $U.ml^{-1}$) or the combination of catalase (2000 $U.ml^{-1}$) plus the catalase inhibitor ATZ (50 mM) for 30 min. In conjunction, some catalase-treated arteries were stimulated with ACh. To provide further evidence about the role of $H_2O_2$ release on A23187-treated iliac arteries, endothelium-intact rings were also incubated with manganese porphyrin (MnTMPyP; 500 μM), an SOD/catalase-mimetic in a separate series of investigations.

To investigate the relationship between $H_2O_2$ production and $K_{Ca}$ activation in A23187-treated vessels, iliac arteries were incubated with IbTX (100 nM) or apamin (1 μM) plus TRAM-34 (10 μM) in the presence or absence of catalase (2000 $U.ml^{-1}$). Control concentration response curves were derived from responses to A23187 with rings that had not been incubated with the $K_{Ca}$ inhibitors. Endothelium-denuded rings were also used to test the effects of $H_2O_2$ on $K_{Ca}$ channels on smooth muscle, and therefore elucidate the putative role of these channels in A23187-generated $H_2O_2$-induced relaxations. The vessels were incubated with IbTX (100 nM) or apamin (1 μM) plus TRAM-34 (10 μM) for 30 min, and then cumulative concentration response curves were constructed for exogenous $H_2O_2$.

To identify putative sources of endogenous $H_2O_2$ production in the endothelium of rabbit iliac arteries, the effects of A23187 were investigated under control conditions and in the presence of apocynin (1 mM; Chan *et al.*, 2007), oxypurinol (100 μM; McNally *et al.*, 2003), rotenone (300 nM) and myxothiazol (300 nM). Following a 40 min incubation period with these agents, constriction was induced by phenylephrine (PE; 1 μM), and cumulative concentration response curves constructed for A23187. In further experiments, arteries were incubated with rotenone (300 nM) and myxothiazol

(300 nM), and cumulative concentration response curves to ACh constructed to investigate whether these inhibitors also affected ACh-evoked relaxations.

### 3.2.3 Data analysis

Responses to added drugs were plotted into sigmoidal concentration response curves and expressed as percentages of relaxation. The curves were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. $pEC_{50}$ values were obtained from concentration response curves by fitting the data to the following logistic equation: $Y = A + (B - A) / (1 + 10^{\wedge} ((LogEC_{50} - X)))$ where Y is the response, X is the logarithm of concentration, A is the response observed with zero drug, and B is the response observed with maximal concentration of drug. $pEC_{50}$ values are expressed as mean $\pm$ SEM. $R_{max}$ (maximal % relaxation) was calculated for all vasorelaxant responses. When $R_{max}$ was not reached, the % relaxation obtained with 10 $\mu M$ of agonist was calculated instead. To assess the effects of apocynin, oxypurinol, rotenone and myxothiazol on vascular tone, tensions before and after the addition of PE were measured and compared by two-way analysis of variance (ANOVA) following by the Bonferroni multiple comparison test. In experiments with IbTX data were analysed relative to the basal tension in the presence of L-NAME and indomethacin. All results were expressed as mean $\pm$ SEM. $P < 0.05$ was considered statistically significant. n represents the number of animals. Analysis was carried out with GraphPad Prism 4.0 (San Diego, USA).

### 3.2.4 Drugs and reagents

All drugs were supplied by Sigma Aldrich (Gillingham, UK). Similarly to the procedures described in Chapter 2, indomethacin was dissolved in 5% $NaHCO_3$. Stock solutions for A23187 and TRAM-34, rotenone and myxothiazole were prepared in DMSO; thereafter serial dilutions were carried out in water. All other drugs were water soluble. ACh and PE stock solutions were made daily prior to any investigations.

## 3.3 Results

### 3.3.1 Effects of catalase and MnTMPyP on EDHF-type relaxations

EDHF-type relaxations to A23187 were maximal at a concentration of ~3 μM and equivalent to 73.9 ± 11.8% of PE preconstriction with a $pEC_{50}$ value of 7.0 ± 0.1 (Fig. 3.1, 3.2; Table 3.1). Preincubation with catalase (2000 $U.ml^{-1}$) inhibited the relaxation evoked by A23187 and caused a significant rightward shift of the threshold of the relaxation from a concentration of ~0.1 μM to ~1 μM (Fig. 3.1, 3.2; Table 3.1). Preincubation with catalase (2000 $U.ml^{-1}$) plus ATZ (50 mM) partially restored the relaxation to A23187 towards its control value (Fig. 3.1, 3.2; Table 3.1). EDHF-type relaxations to ACh were maximal at a concentration of ~3 μM and equivalent to 65.8 ± 1.6% PE preconstriction with a $pEC_{50}$ value of 6.5 ± 0.1 (Fig. 3.3, 3.4; Table 3.1). Preincubation with catalase (2000 $U.ml^{-1}$) inhibited the relaxation evoked by ACh to 41.4 ± 7.6% with a $pEC_{50}$ value of 6.9 ± 0.3 (Fig. 3.3, 3.4; Table 3.1). In a separate series of investigations, EDHF-type relaxations to A23187 were maximal a concentration of ~3 μM and equivalent to 57.9 ± 9.5% of PE preconstriction with a $pEC_{50}$ of 6.9 ± 0.1 (Fig. 3.5, 3.6; Table 3.1). Preincubation with MnTMPyP (500 μM) led to a significant rightward shift of the curve relative to the control relaxation (Fig. 3.5, 3.6; Table 3.1).

### 3.3.2 Effects of $K_{Ca}$ inhibitors and catalase on A23187-induced relaxations

EDHF-type relaxations to A23187 were maximal at a concentration of ~3 μM and equivalent to 73.7 ± 11.3% of PE preconstriction with a $pEC_{50}$ value of 6.4 ± 0.8 (Fig. 3.7, 3.8; Table 3.2). Preincubation with IbTX (100 nM) inhibited the response by approximately 23% (Fig. 3.7, 3.8; Table 3.2), while the combination of IbTX (100 nM) plus catalase (2000 $U.ml^{-1}$) inhibited relaxations to A23187 even further leaving a residual response equal to ~14% (Fig. 3.7, 3.8; Table 3.2). Similar investigations were carried out with apamin (1 μM) plus TRAM-34 (10 μM). In these experiments, EDHF-type relaxations to A23187 were maximal at a concentration of ~3 μM and equivalent to 79.6 ± 11.5% of PE preconstriction with a $pEC_{50}$ value of 6.4 ± 0.1 (Fig. 3.9, 3.10; Table 3.2). Preincubation with apamin (1 μM) plus TRAM-34 (10 μM) inhibited the relaxation evoked by A23187 to 40.9 ± 5.7% with a $pEC_{50}$ value of 6.2 ± 0.1 (Fig. 3.9, 3.10; Table 3.2). Preincubation with apamin (1 μM) plus TRAM-34 (10 μM) plus catalase (2000 $U.ml^{-1}$) further inhibited the relaxation evoked by A23187 and caused a significant rightward shift of the curve, but it failed to abolish it.

### 3.3.3 Effects of $K_{Ca}$ inhibitors on relaxations induced by exogenous $H_2O_2$

In endothelium-denuded arteries, relaxations to $H_2O_2$ were maximal at ~ 300 μM and equivalent to 70.2 ± 13.1% of PE preconstriction with a pEC$_{50}$ value of 4.0 ± 6.6 (Fig. 3.11, 3.12; Table 3.2). Preincubation with IbTX (100 nM) on its own did not affect either the relaxations evoked by $H_2O_2$ or the corresponding pEC$_{50}$ value (Fig. 3.11, 3.12; Table 3.2). In a separate series of experiments in endothelium-denuded vessels, relaxations to $H_2O_2$ were maximal at ~ 300 μM and equivalent to 78.1 ± 12.7% of PE preconstriction with a pEC$_{50}$ value of 4.5 ± 0.3 (Fig. 3.13, 3.14; Table 3.2). Preincubation with apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM) had no effect on $H_2O_2$-evoked relaxations (Fig. 3.13, 3.14; Table 3.2).

### 3.3.4 Effects of apocynin and oxypurinol on A23187-induced relaxations

EDHF-type relaxations to A23187 were maximal at ~1 μM and equivalent to 80.5 ± 13.3% of PE preconstriction with a pEC$_{50}$ value of 6.6 ± 0.1 (Fig. 3.15, 3.16; Table 3.3). Preincubation with apocynin (1 mM) did not significantly affect either the relaxations evoke by A23187 or the corresponding pEC$_{50}$ values. In a separate series of experiments, EDHF-type relaxations to A23187 were maximal at ~1 μM and equivalent to 73.1 ± 11.3% of PE preconstriction with a pEC$_{50}$ value of 6.9 ± 0.1 (Fig. 3.17, 3.18; Table 3.3). Preincubation with oxypurinol (100 μM) did not affect the relaxations.

### 3.3.5 Effects of ETC inhibitors on A23187- and ACh-evoked relaxations

In a separate series of experiments, the ETC complex I inhibitor rotenone (300 nM) significantly inhibited the maximum relaxation to A23187 from 80.7 ± 12.9% to 1.1 ± 3.7% (Fig. 3.19, 3.20; Table 3.3), and those induced by ACh from 67.6 ± 9.4% to 20.3 ± 3.7% (Fig. 3.21, 3.22; Table 3.3). Similarly, the ETC complex III inhibitor myxothiazole (300 nM) significantly inhibited EDHF-type responses to both A23187 and ACh, and reduced the maximal relaxations to 20.7 ± 2.6% and 15.9 ± 2.2%, respectively (Fig. 3.23, 3.24, 3.25, 3.26; Table 3.3).

### 3.3.6 Effects of inhibitors on the tension on PE-preconstricted arteries

Under control conditions, addition of PE (1 μM) led to a significant increase in tension, which was equal to ~38 mN (Table 3.4). Application of rotenone (300 nM) significantly decreased the magnitude of the control tension to 9.7 ± 0.7 mN from 45.3 ± 2.3mN, but other inhibitors did not significantly affect PE-evoked contraction (Table 3.4).
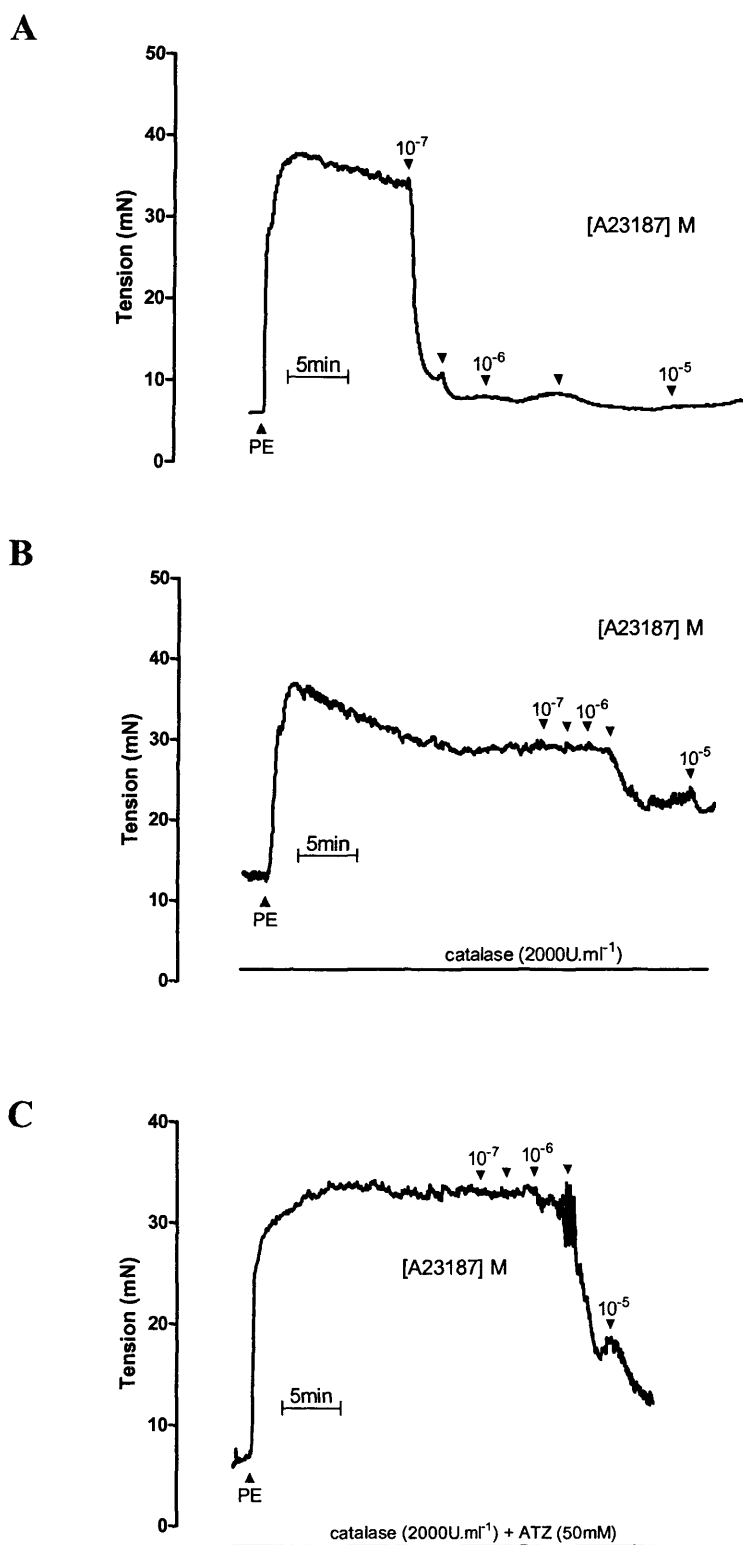
**Fig. 3.1** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) catalase (2000 $U.ml^{-1}$) and (C) catalase (2000 $U.ml^{-1}$) plus ATZ (50 mM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
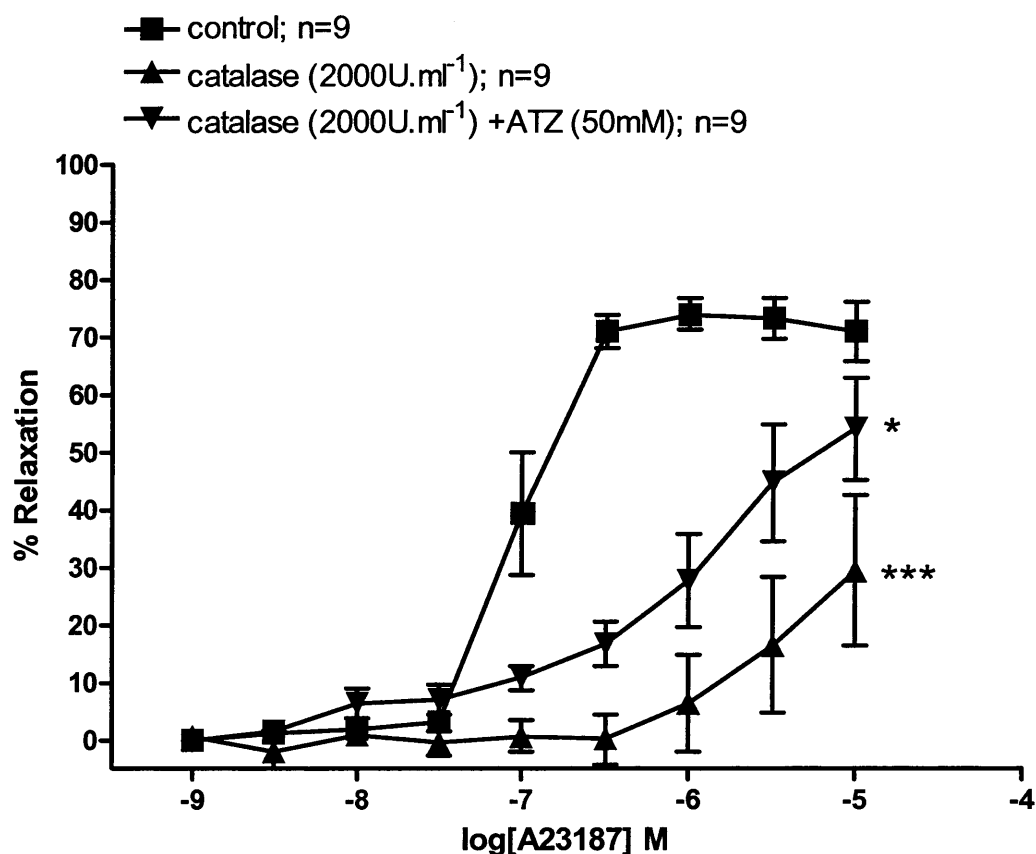
Fig. 3.2 Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of catalase (2000 U.ml$^{-1}$) and the dual combination of catalase (2000 U.ml$^{-1}$) plus ATZ (50 mM). Experiments were carried out with catalase to assess the role of endogenous $H_2O_2$ in relaxations evoked by A23187. Addition of ATZ, an inhibitor of the active site of catalase, was used to demonstrate the selectivity of the enzyme for $H_2O_2$. Catalase significantly inhibited the relaxation evoked by A23187 and caused a significant rightward shift of the threshold of the relaxation. ATZ partially restored the response to A23187 relative to the control. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. *, $P<0.05$; ***, $P<0.001$ for maximal concentration compared with the control.

**Fig. 3.3** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to ACh and associated changes in the presence of (B) catalase (2000 U.ml$^{-1}$). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).

**Fig. 3.4** Concentration-response curves for ACh-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of catalase (2000 $U.ml^{-1}$). Experiments were carried out with catalase to assess the role of endogenous $H_2O_2$ in relaxations evoked by ACh. Catalase inhibited the relaxation evoked by this agonist to $41.4 \pm 7.6\%$. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. **, $P<0.01$ for maximal concentration compared with the control.

**Fig. 3.5** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) MnTMPyP (500 µM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
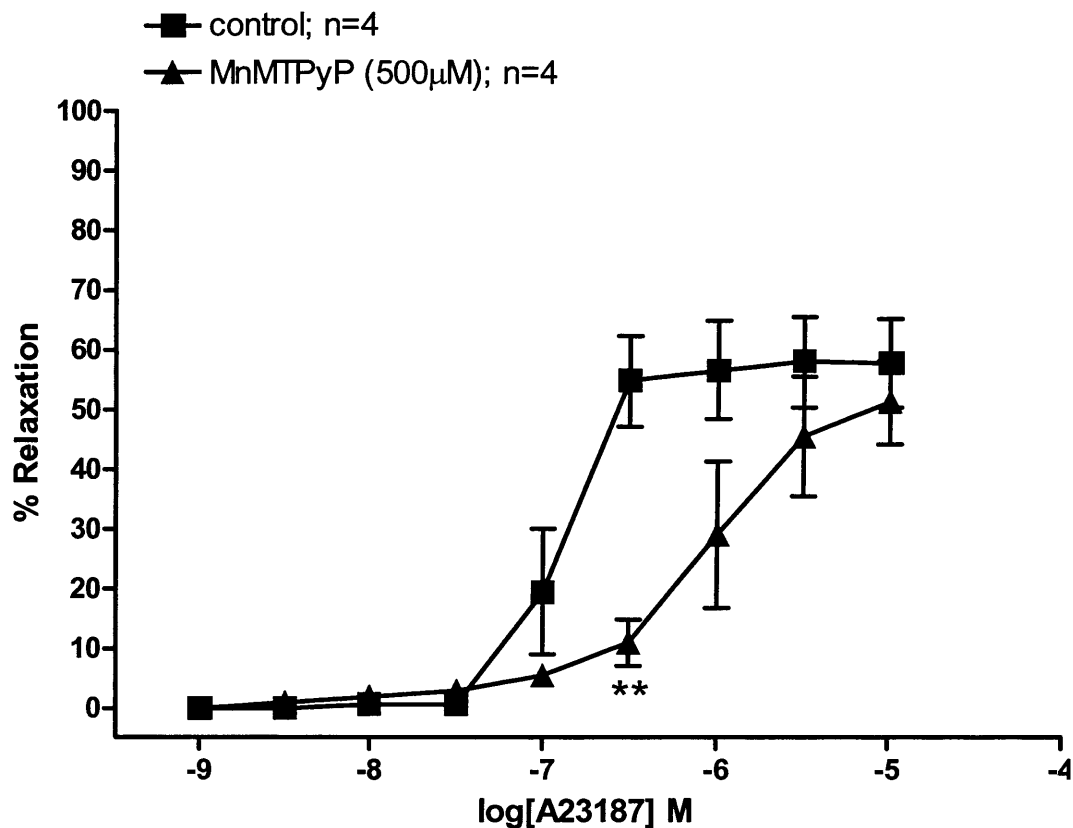
**Fig. 3.6** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of the SOD/catalase-mimetic agent manganese porphyrin (MnMTPyP; 500 μM). Experiments were carried out with MnMTPyP to assess the involvement of an $H_2O_2$-dependent component in relaxations induced by A23187. Preincubation with MnTMPyP led to a significant rightward shift of the curve relative to the control, with no statistically significant effect on the response obtained with 10 μM of A23187. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. **, $P<0.01$ for specific A23187 concentration compared with the control.

| Intervention | % relaxation at 10μM | pEC$_{50}$ | n |
|---|---|---|---|
| | | | |
| A23187 control | 73.9±11.8 | 7.0±0.1 | 9 |
| catalase (2000 U.ml$^{-1}$) | 29.4±3.5*** | n/a | 9 |
| catalase (2000 U.ml$^{-1}$) + ATZ (50 mM) | 54.0±6.5* | n/a | 9 |
| | | | |
| ACh control | 65.8±1.6 | 6.5±0.1 | 4 |
| catalase (2000 U.ml$^{-1}$) | 41.4±7.6** | 6.9±0.3 | 4 |
| | | | |
| A23187 control | 57.9±9.5 | 6.9±0.1 | 4 |
| MnTMPyP (500 μM) | 51.2±6.7 | 6.1±0.2* | 4 |
| | | | |

**Table 3.1** Summary of effects of catalase and MnTMPyP on A23187-evoked EDHF-type responses in rabbit iliac arteries. Potency (negative log EC$_{50}$) and % relaxation at 10 μM of agonist are expressed as a function of the constrictor response to PE and given as means ± SEM. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared with the corresponding intra-group control. n denotes the number of animals studied. n/a denotes non-applicable data.
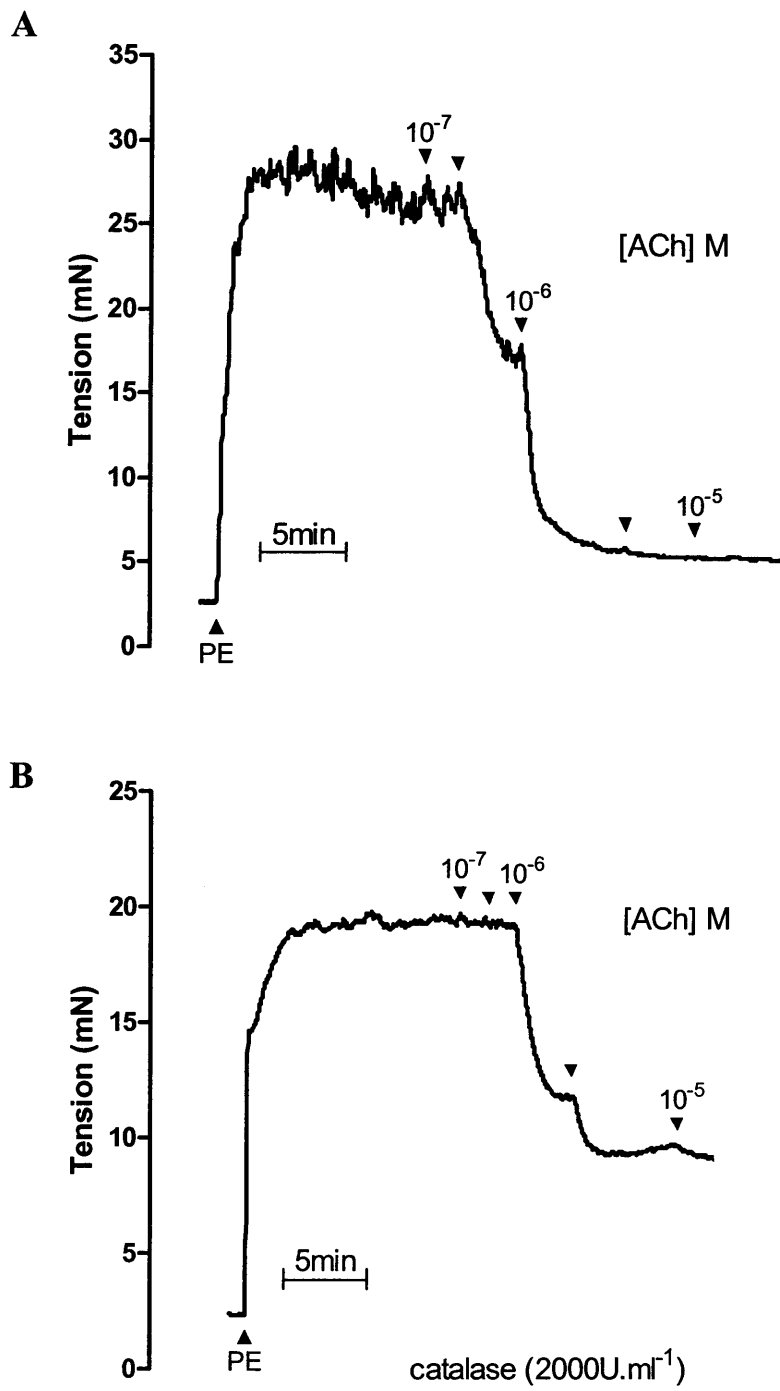
**Fig. 3.7** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) IbTX (100nM) and (C) IbTX (100 nM) plus catalase (2000 U.ml$^{-1}$) In the presence of IbTX (100nM), the resting tension of some vessels was significantly increased as illustrated in the lower panel. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).
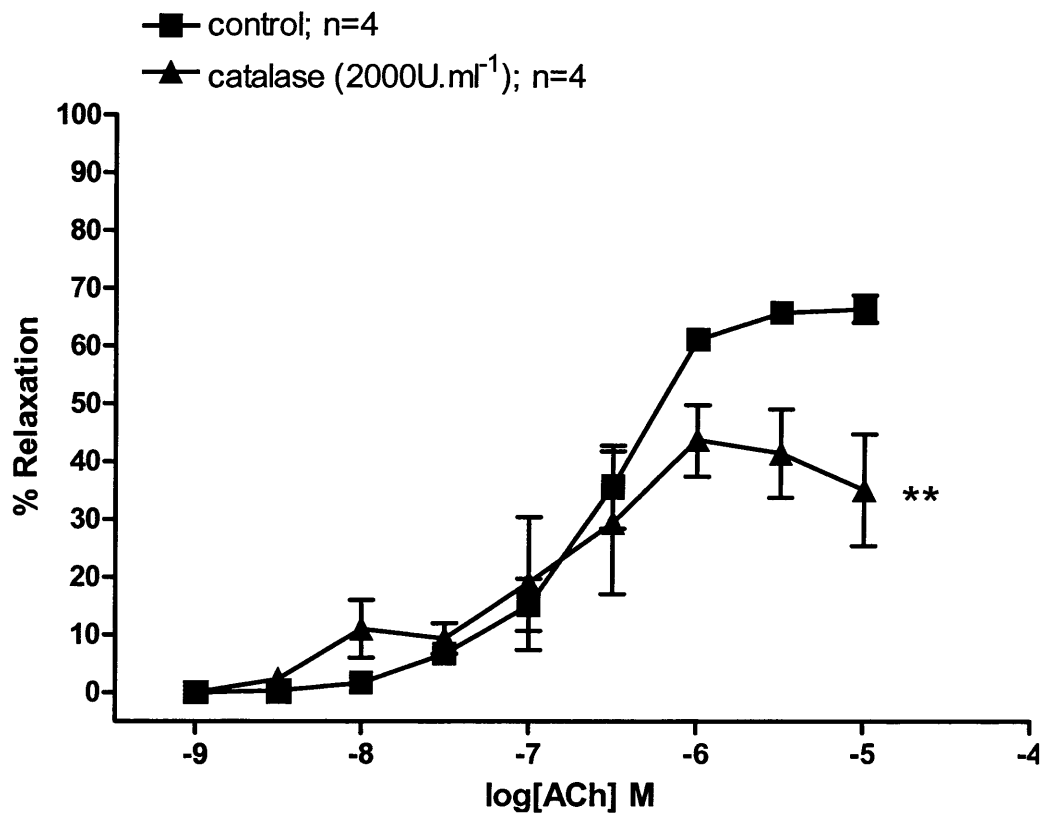
**Fig. 3.8** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of IbTX (100 nM) and the combination of IbTX (100 nM) plus catalase (2000 U.ml$^{-1}$). Experiments were carried out with IbTX and catalase to assess whether the residual relaxation in IbTX-pretreated vessels is $H_2O_2$-dependent. IbTX significantly inhibited the relaxation evoked by A23187, while the double combination of IbTX plus catalase abolished the response. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. *, $P<0.05$; ***, $P<0.001$ for whole curves compared with the control.
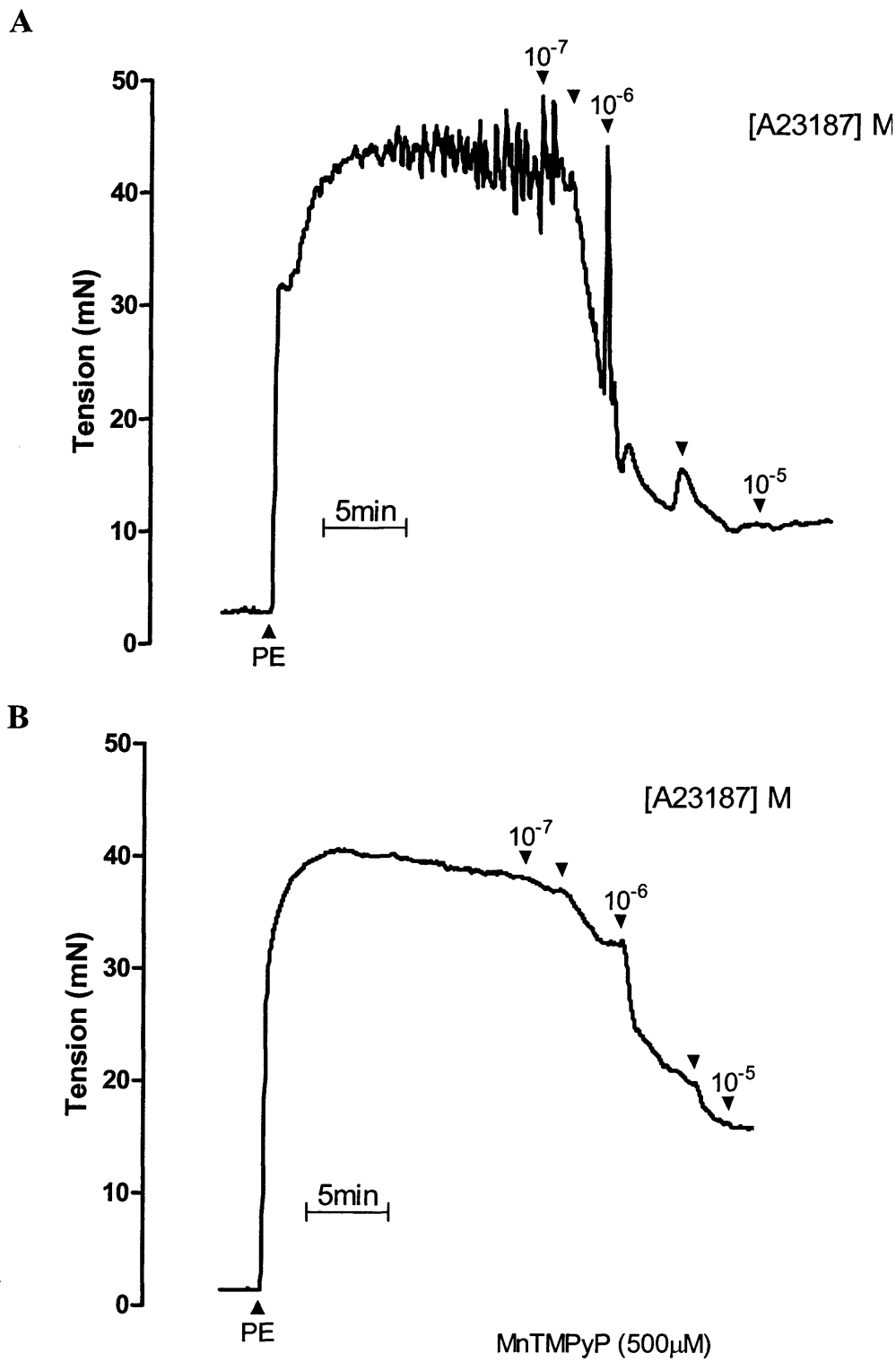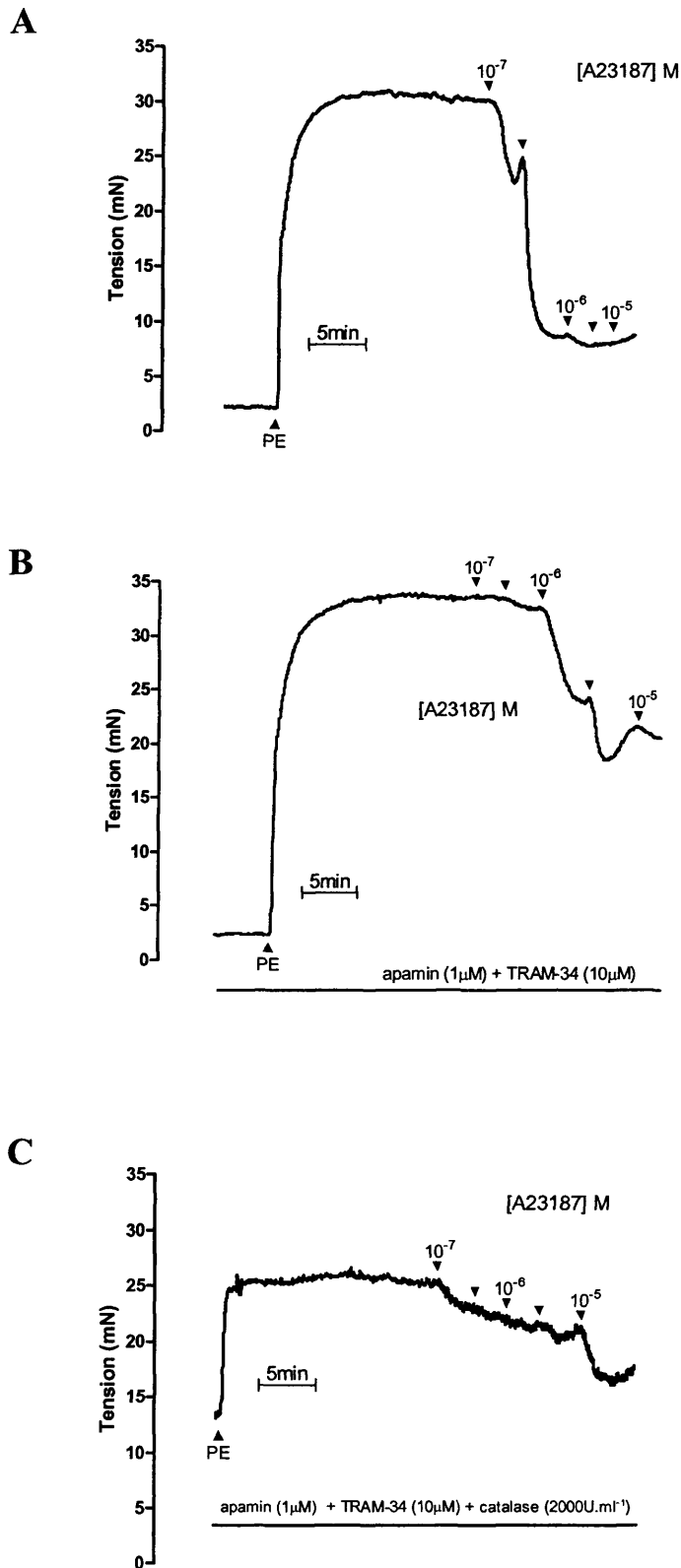
**Fig. 3.9** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) apamin (1 μM) plus TRAM-34 (10 μM) and (C) apamin (1 μM) plus TRAM-34 (10 μM) plus catalase (2000 U.ml⁻¹). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).

**Fig. 3.10** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of apamin (1 μM) plus TRAM-34 (10 μM) and the combination of apamin (1 μM) plus TRAM-34 (10 μM) plus catalase (2000 U.ml$^{-1}$). Experiments were carried out with apamin, TRAM-34 and catalase to assess whether the residual relaxation in vessels pretreated with apamin plus TRAM-34 is $H_2O_2$-dependent. The double combination of apamin (1 μM) plus TRAM-34 (10 μM) significantly depressed the relaxation evoked by A23187, with the residual response being inhibited further by catalase. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. ***, $P<0.001$ for whole curves compared with the control.
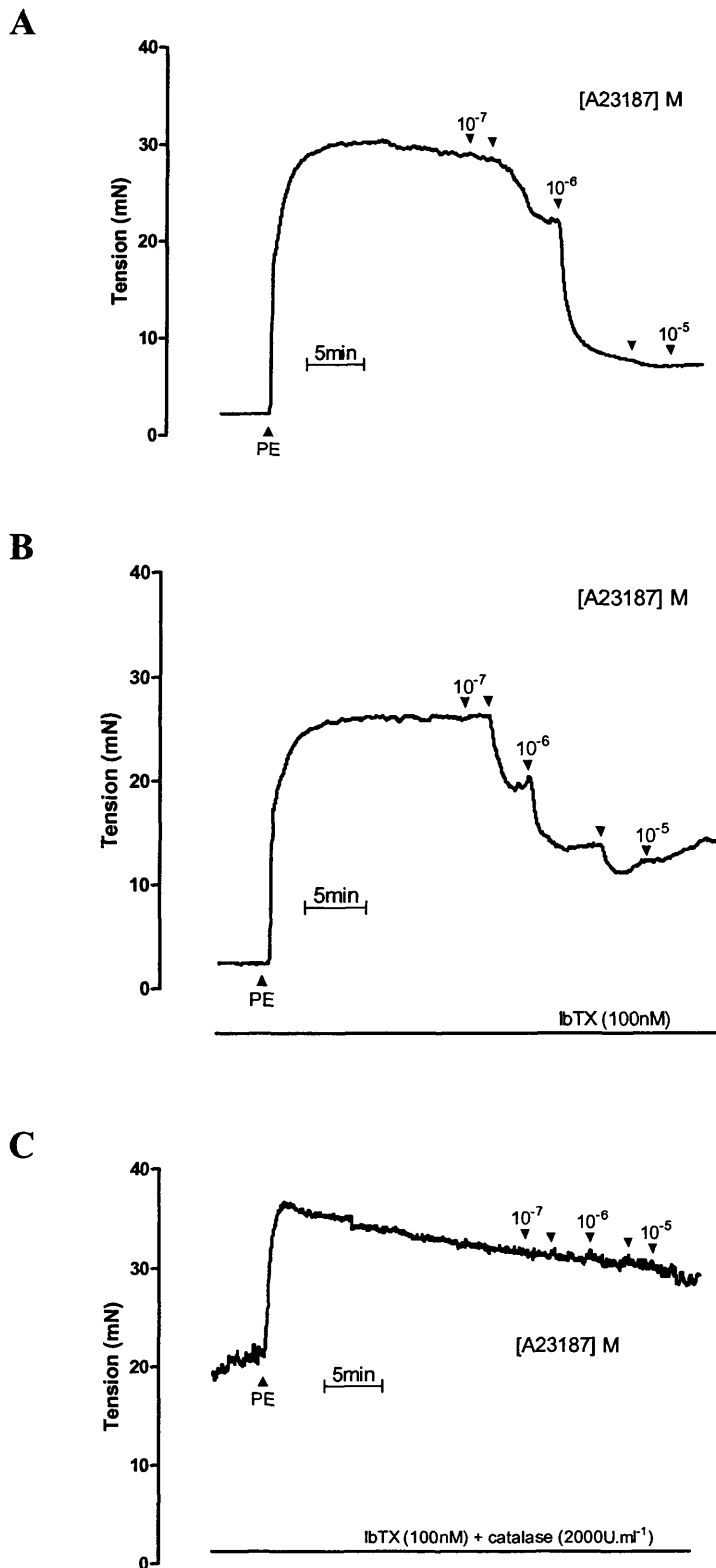
**A**



**B**



**Fig. 3.11** Representative traces from endothelium-denuded ring preparations of rabbit iliac arteries showing (A) relaxations to $H_2O_2$ and associated changes in the presence of (B) IbTX (100 nM). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).

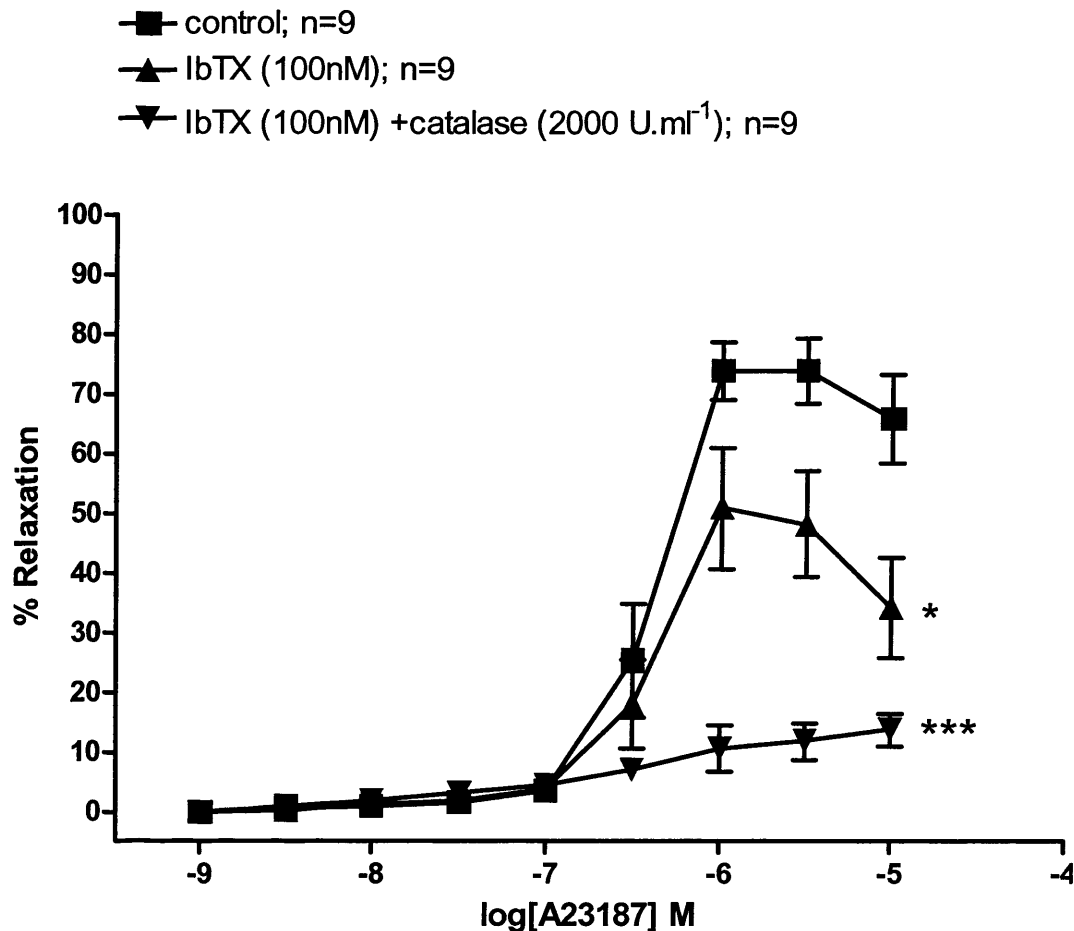**Fig. 3.12** Concentration-response curves for authentic $H_2O_2$-induced relaxations in endothelium-denuded rabbit iliac arteries and associated changes in the presence of IbTX (100 nM). Experiments were carried out to assess the effects of authentic $H_2O_2$ on $BK_{Ca}$ channels in the smooth muscle of these vessels. It is demonstrated that IbTX has no effect on $H_2O_2$-induced relaxations. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM.

**Fig. 3.13** Representative traces from endothelium-denuded ring preparations of rabbit iliac arteries showing (A) relaxations to $H_2O_2$ and associated changes in the presence of (B) the triple combination of apamin (1 μM) plus TRAM-34 (10 μM) plus IbTX (100 nM). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).
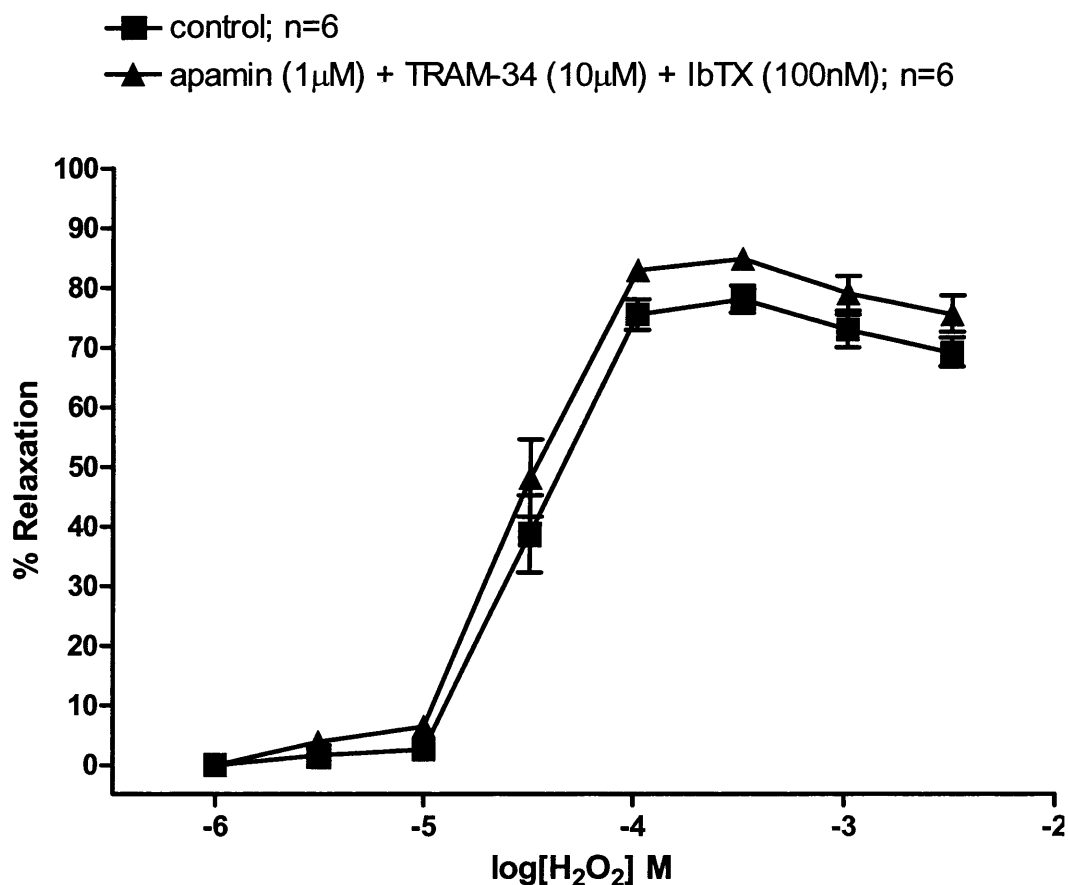
**Fig. 3.14** Concentration-response curves for authentic $H_2O_2$-induced relaxations in endothelium-denuded rabbit iliac arteries and associated changes in the presence of the triple combination of apamin (1 µM) plus TRAM-34 (10 µM) plus IbTX (100 nM). Experiments were carried out to assess the effects of authentic $H_2O_2$ on all $K_{Ca}$ channels in the smooth muscle of these vessels. It is demonstrated that the combination of the three inhibitors has no effect on $H_2O_2$-induced relaxations. Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM). Data are expressed as mean ± SEM.

| Intervention | %$R_{max}$ | $pEC_{50}$ | n |
|---|---|---|---|
| A23187 control | 73.7±11.3 | 6.4±0.8 | 9 |
| IbTX (100 nM) | 50.8±7.1** | 6.5±0.0 | 9 |
| IbTX (100 nM) + catalase (2000 U.ml$^{-1}$) | n/a | n/a | 9 |
| | | | |
| A23187 control | 79.6±11.5 | 6.4±0.1 | 11 |
| apamin (1 μM) + TRAM-34 (10 μM) | 40.9±5.7*** | 6.2±0.1 | 11 |
| apamin (1 μM) + TRAM-34 (10 μM) + catalase (2000 U.ml$^{-1}$) | n/a | n/a | 11 |
| | | | |
| $H_2O_2$ | 70.2±13.1 | 4.0±6.6 | 5 |
| IbTX (100 nM) | 80.5±15.3 | 3.9±5.6 | 5 |
| | | | |
| $H_2O_2$ | 78.1±12.7 | 4.5±0.3 | 6 |
| apamin (1 μM) + TRAM-34 (10 μM) + IbTX (100nM) | 84.8±13.5 | 4.5±0.03 | 6 |
| | | | |

**Table 3.2** Summary of effects of $K_{Ca}$ inhibitors plus catalase on A23187-evoked EDHF-type responses, and $K_{Ca}$ inhibitors on their own on relaxations evoked by authentic $H_2O_2$. Potency (negative log $EC_{50}$) and maximal percentage relaxation ($R_{max}$) are expressed as a function of the constrictor response to PE and given as means ± SEM. **$P<0.01$; ***$P<0.001$ compared with the corresponding intra-group control. n denotes the number of animals studied. n/a denotes non-applicable data.

**Fig. 3.15** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) apocynin (1 mM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
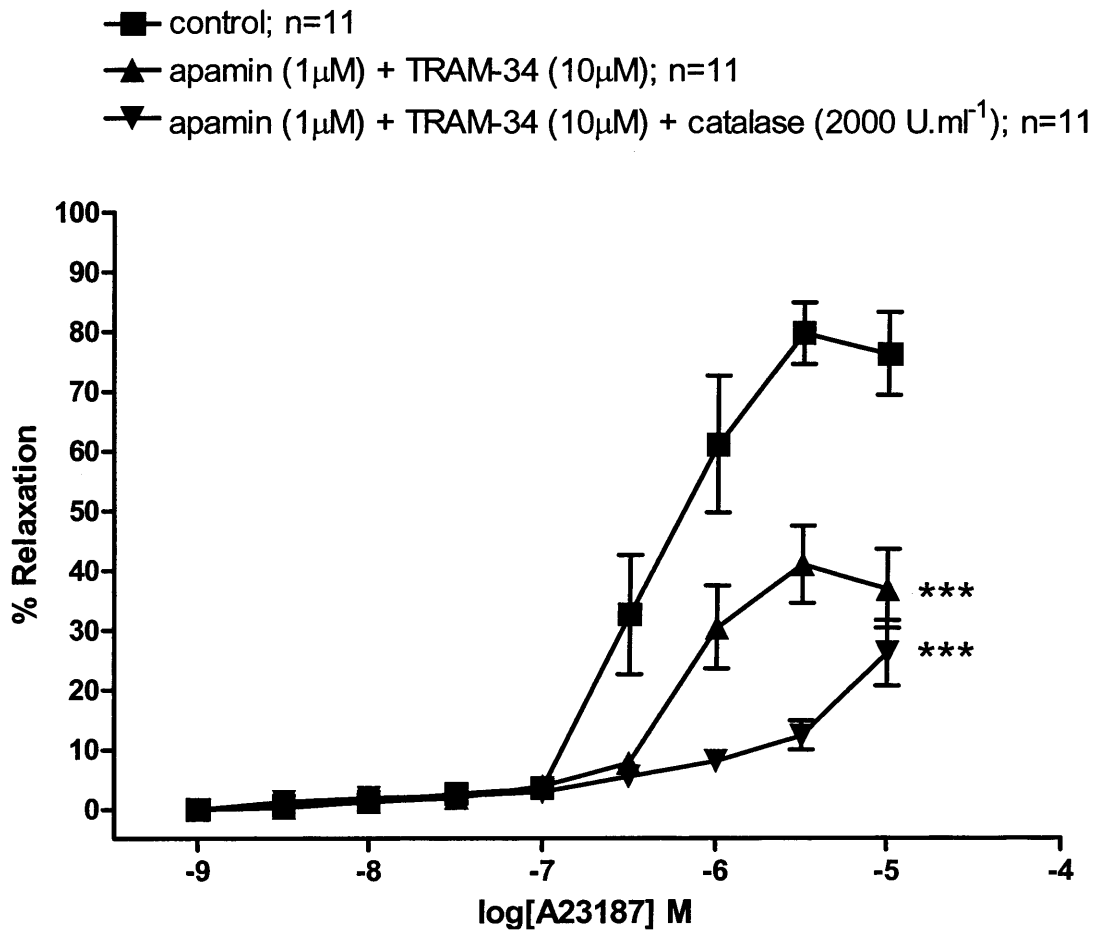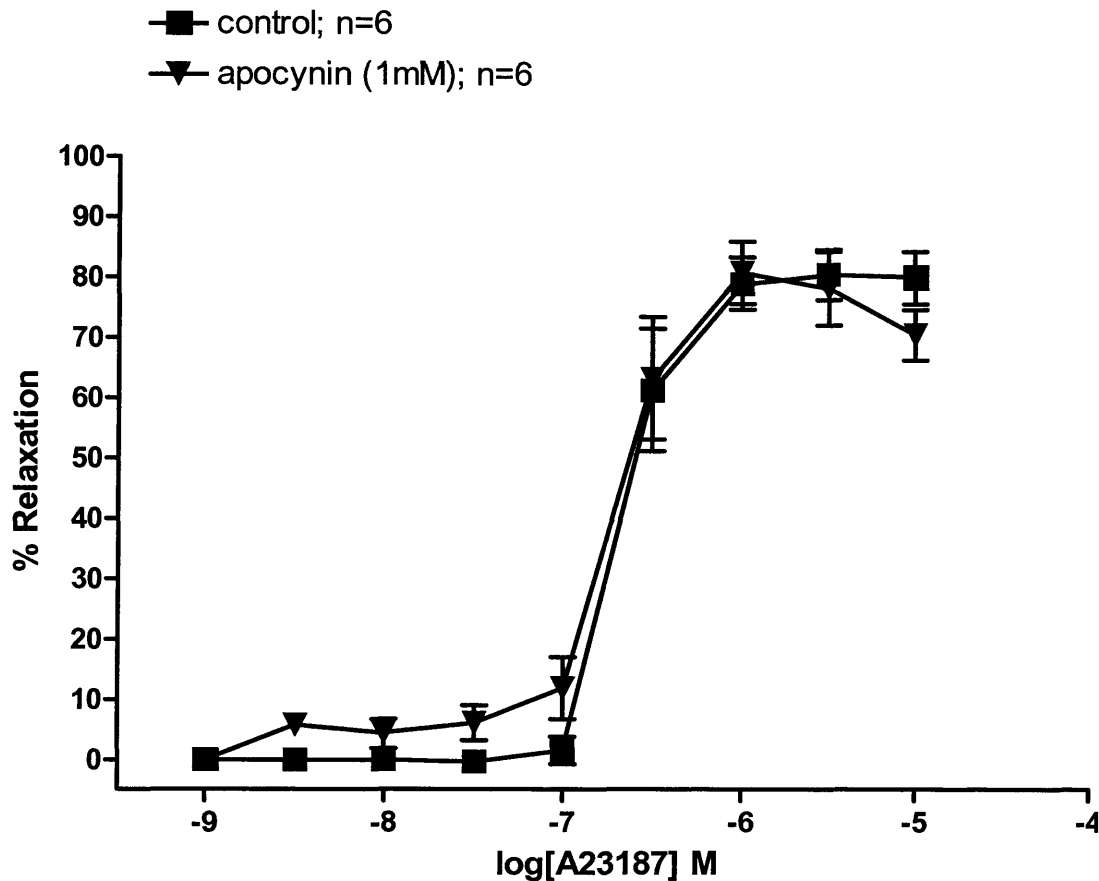
**Fig. 3.16** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of apocynin (1 mM). Experiments were carried out with apocynin to assess the role of NADPH oxidase as a source of $H_2O_2$ in responses evoked by A23187. It is demonstrated that relaxations to A23187 are unaffected by apocynin. Experiments were carried out in the presence of L-NAME (300 $\mu$M) and Indo (10 $\mu$M). Data are expressed as mean $\pm$ SEM.
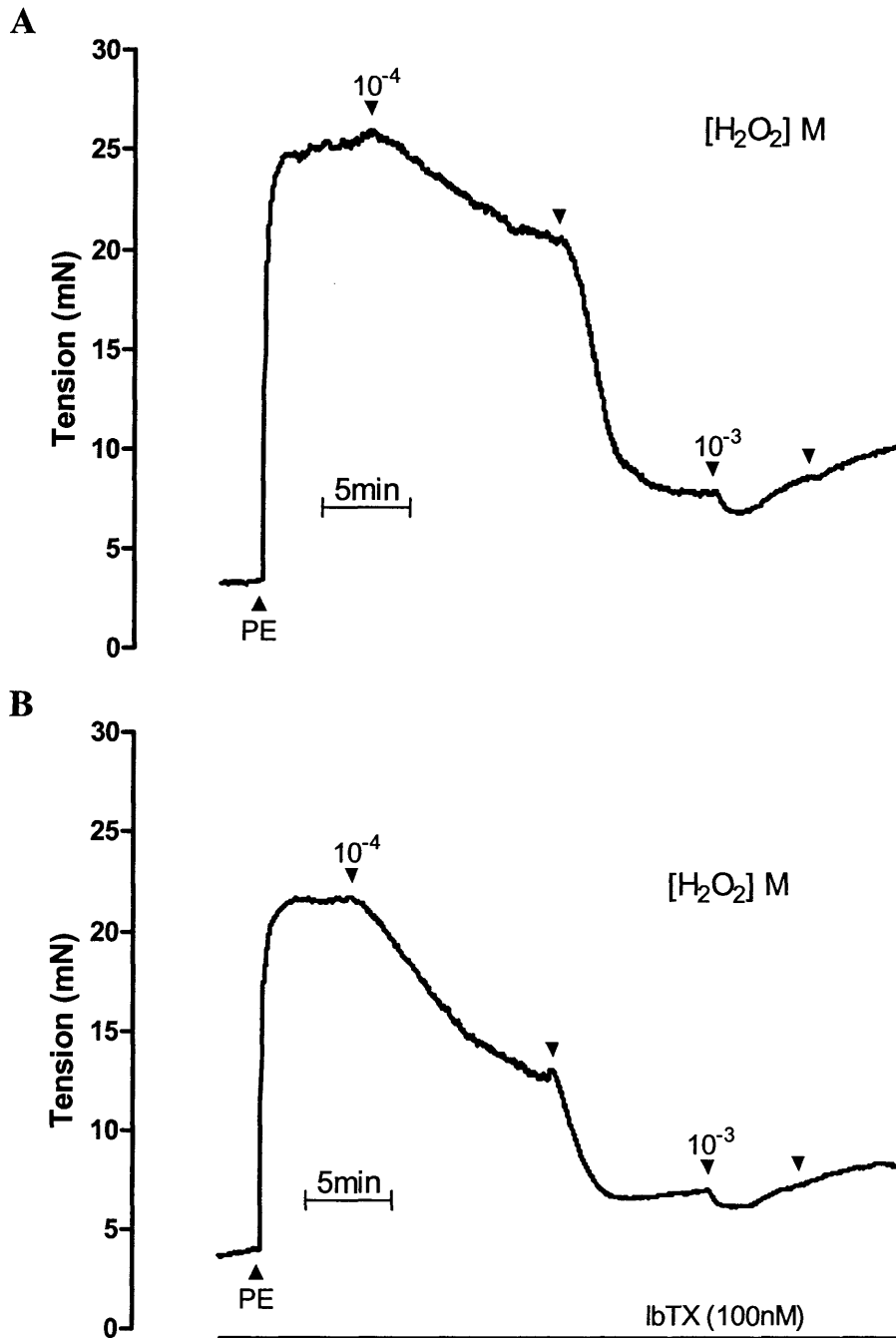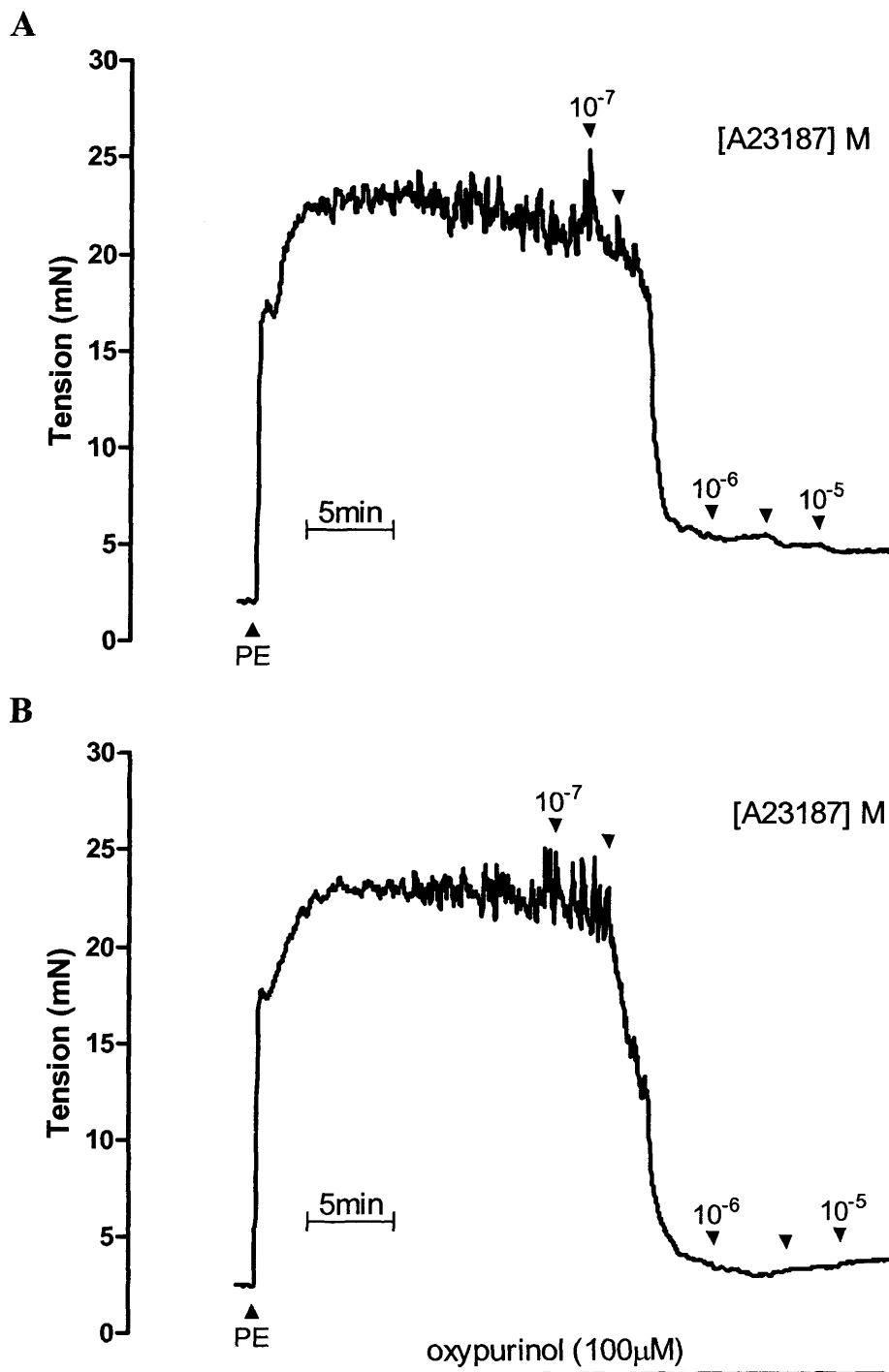
**Fig. 3.17** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) oxypurinol (100 $\mu$M). Experiments were carried out in the presence of L-NAME (300 $\mu$M) and Indo (10 $\mu$M).
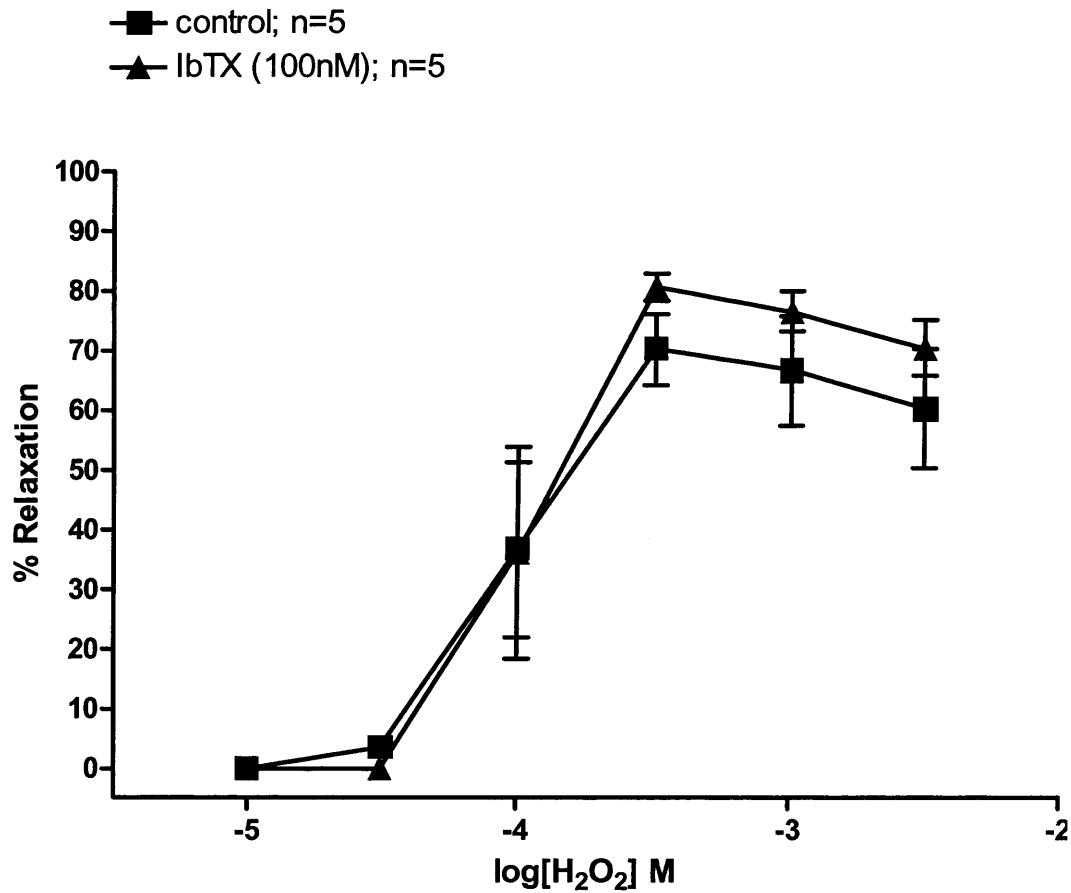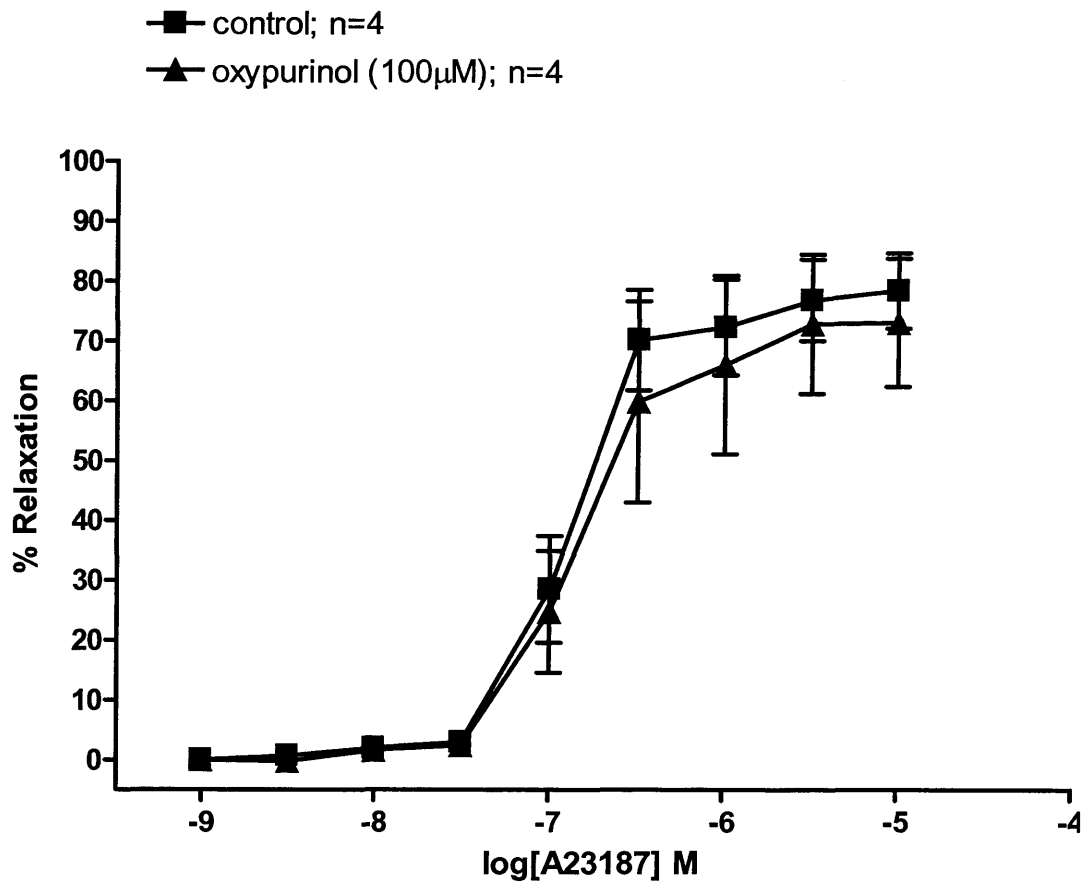
**Fig. 3.18** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of oxypurinol (100 μM). Experiments were carried out with oxypurinol to assess the role of xanthine oxidase as a source of $H_2O_2$ in responses evoked by A23187. It is demonstrated that relaxations to A23187 are unaffected by oxypurinol. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM.

**Fig. 3.19** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) rotenone (300 nM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
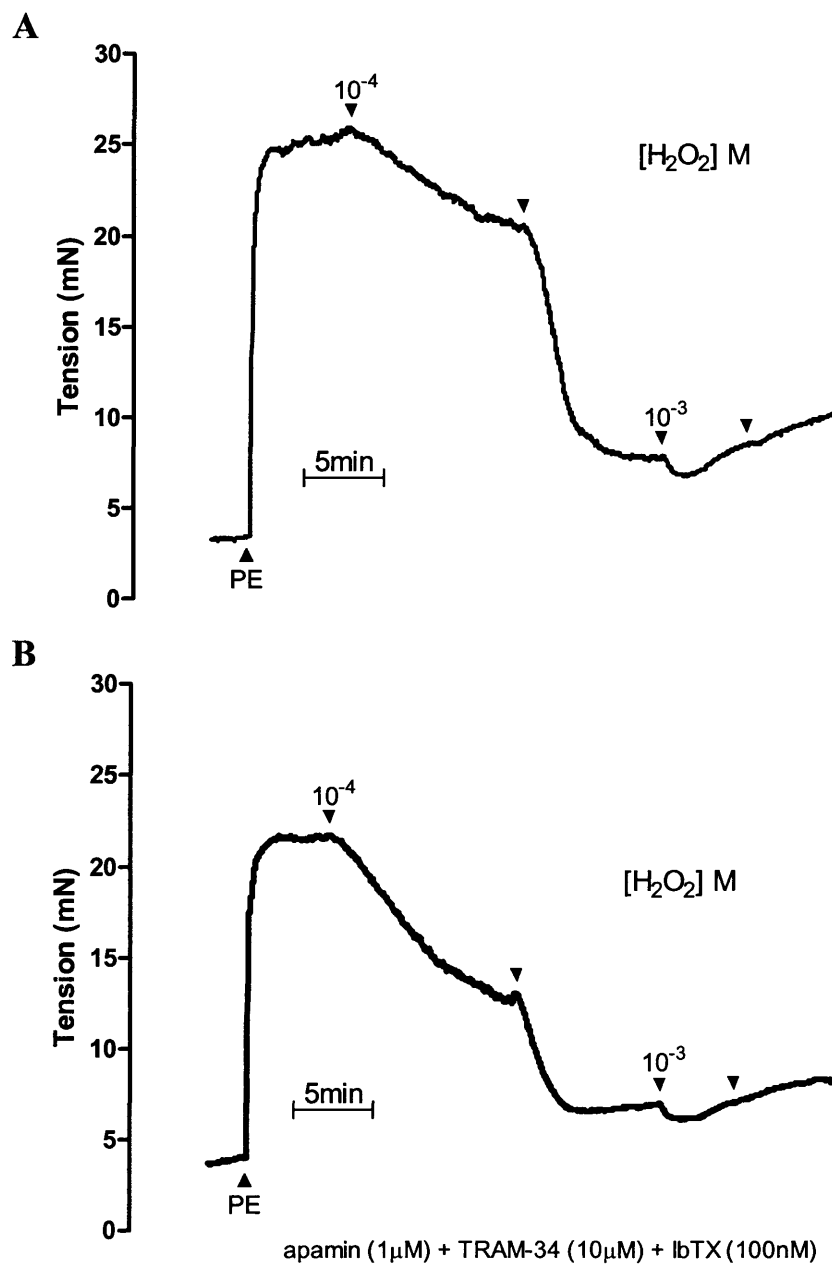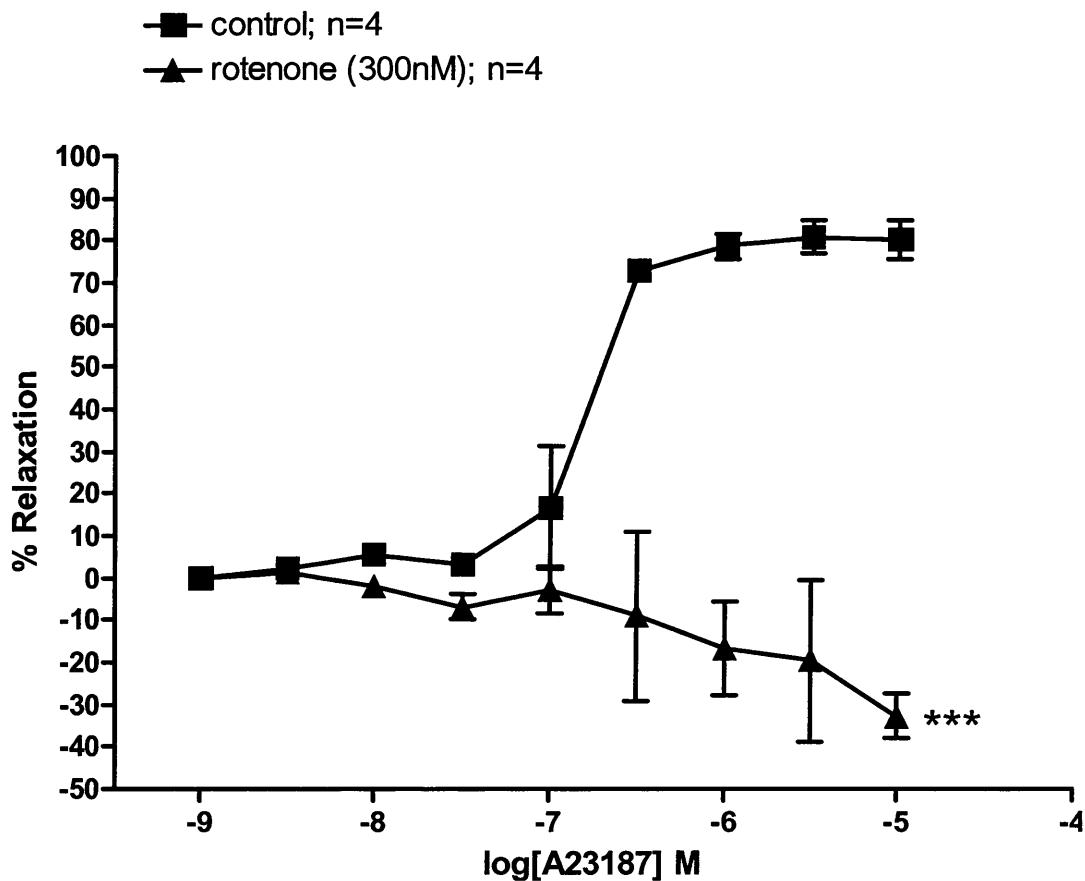
**Fig. 3.20** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of rotenone (300 nM). Experiments were carried out with rotenone to assess the role of complex I of the mitochondrial ETC as a source of $H_2O_2$ in responses evoked by A23187. It is demonstrated that rotenone abolishes the relaxations evoked by A23187. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. ***, $P<0.001$ for whole curve compared with the control.

**Fig. 3.21** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to ACh and associated changes in the presence of (B) rotenone (300 nM). Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM).
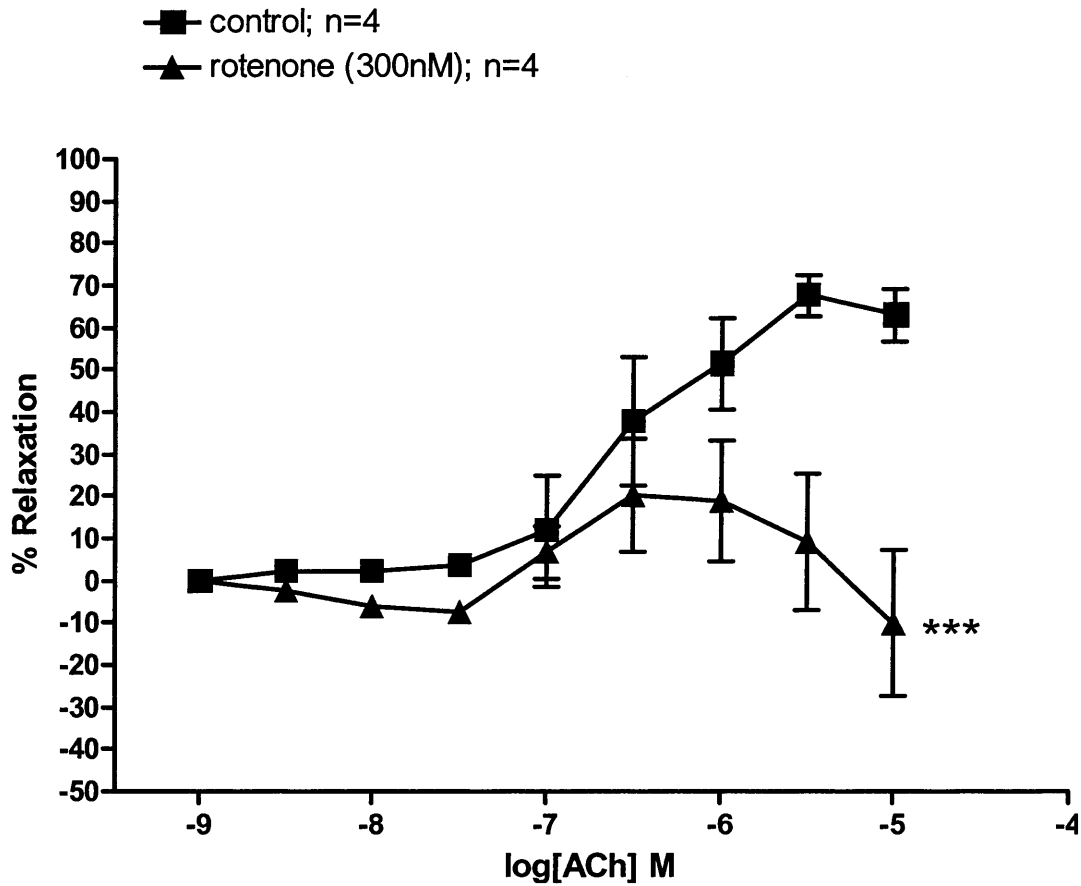
**Fig. 3.22** Concentration-response curves for ACh-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of rotenone (300 nM). Experiments were carried out with rotenone to assess the role of complex I of the mitochondrial ETC as a source of $H_2O_2$ in responses evoked by ACh. It is demonstrated that rotenone abolishes the relaxations evoked by ACh. Experiments were carried out in the presence of L-NAME (300 μM) and Indo (10 μM). Data are expressed as mean ± SEM. ***, $P<0.001$ for whole curve compared with the control.
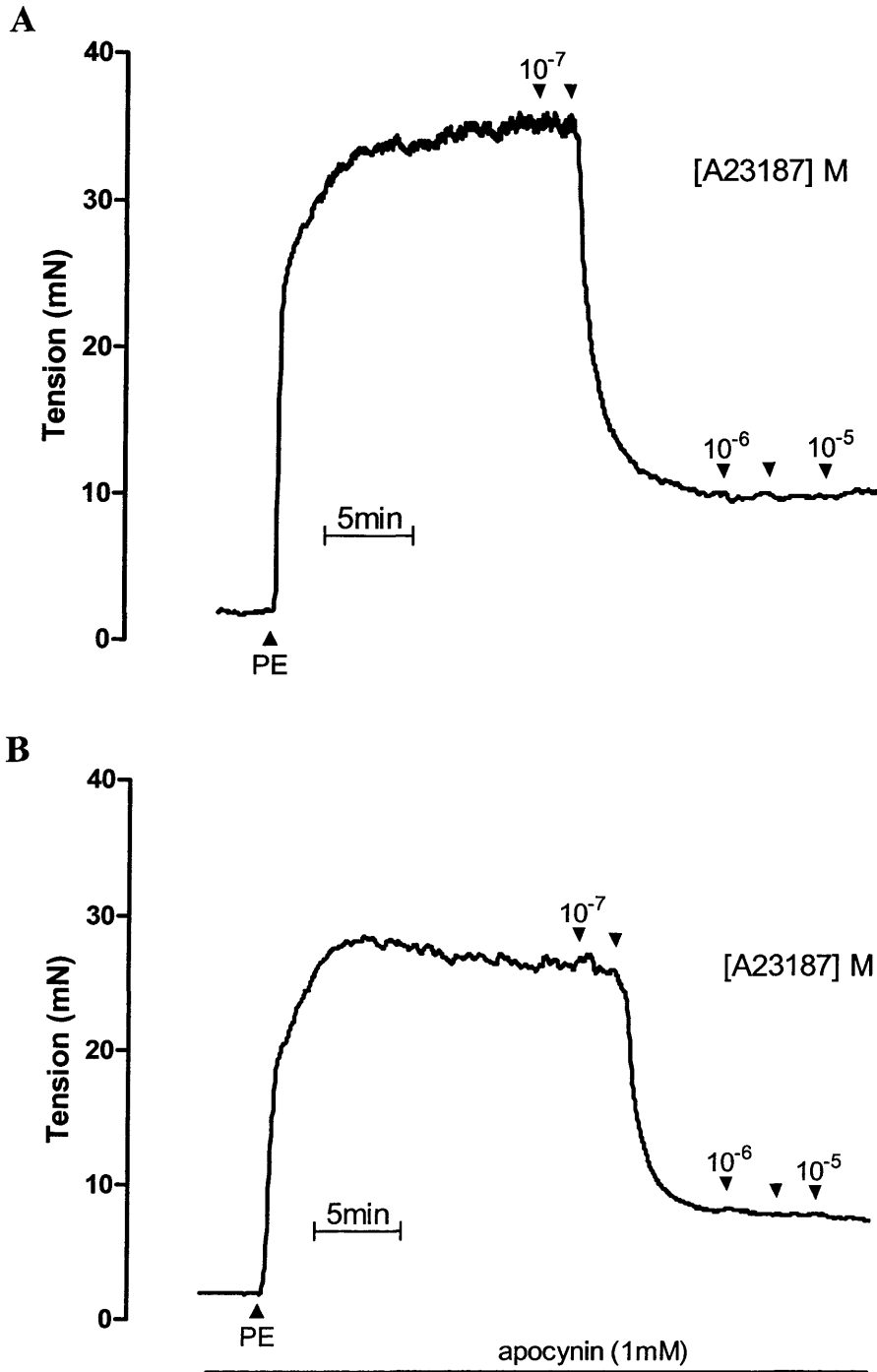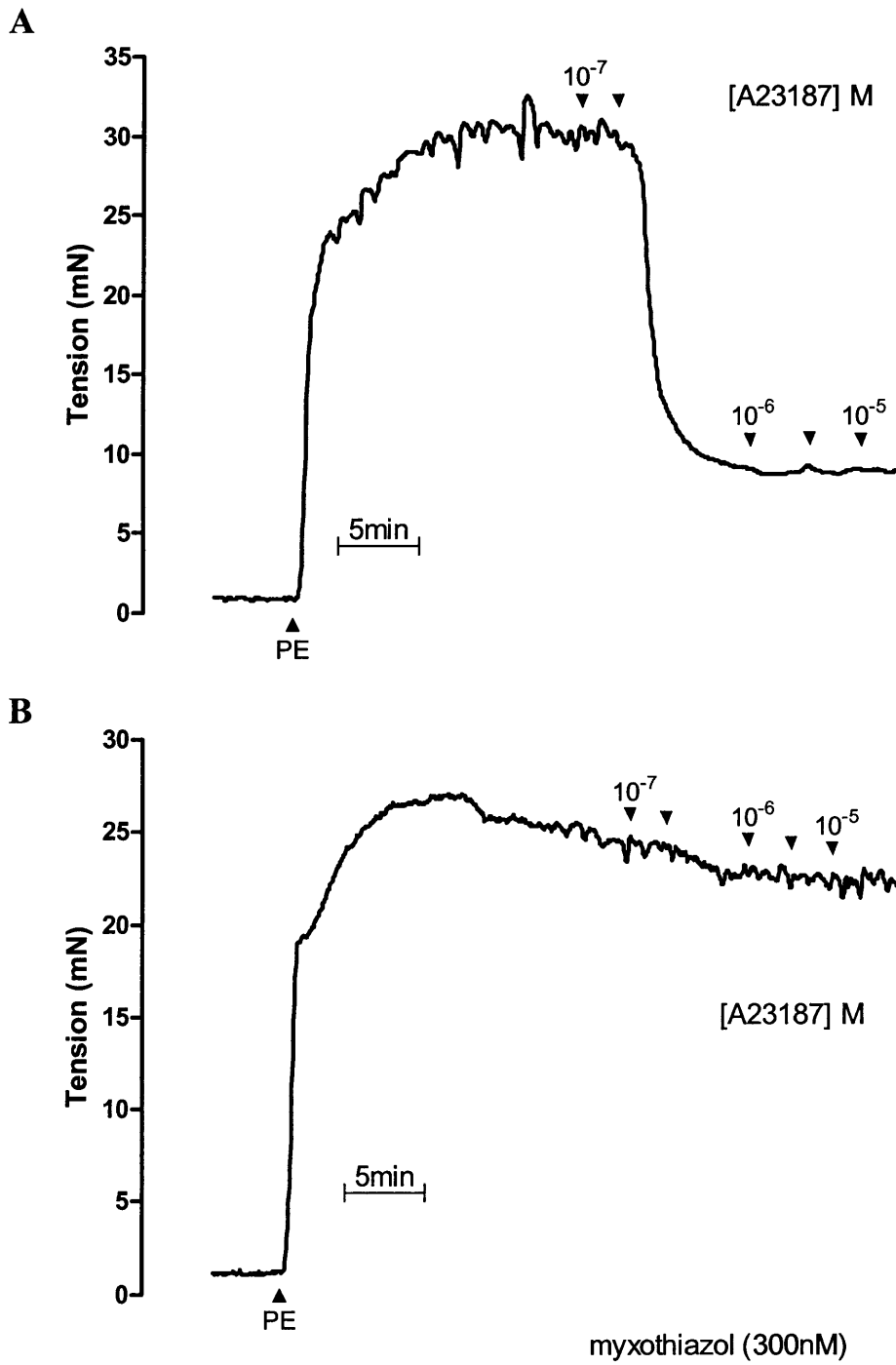
**Fig. 3.23** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) myxothiazol (300 nM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
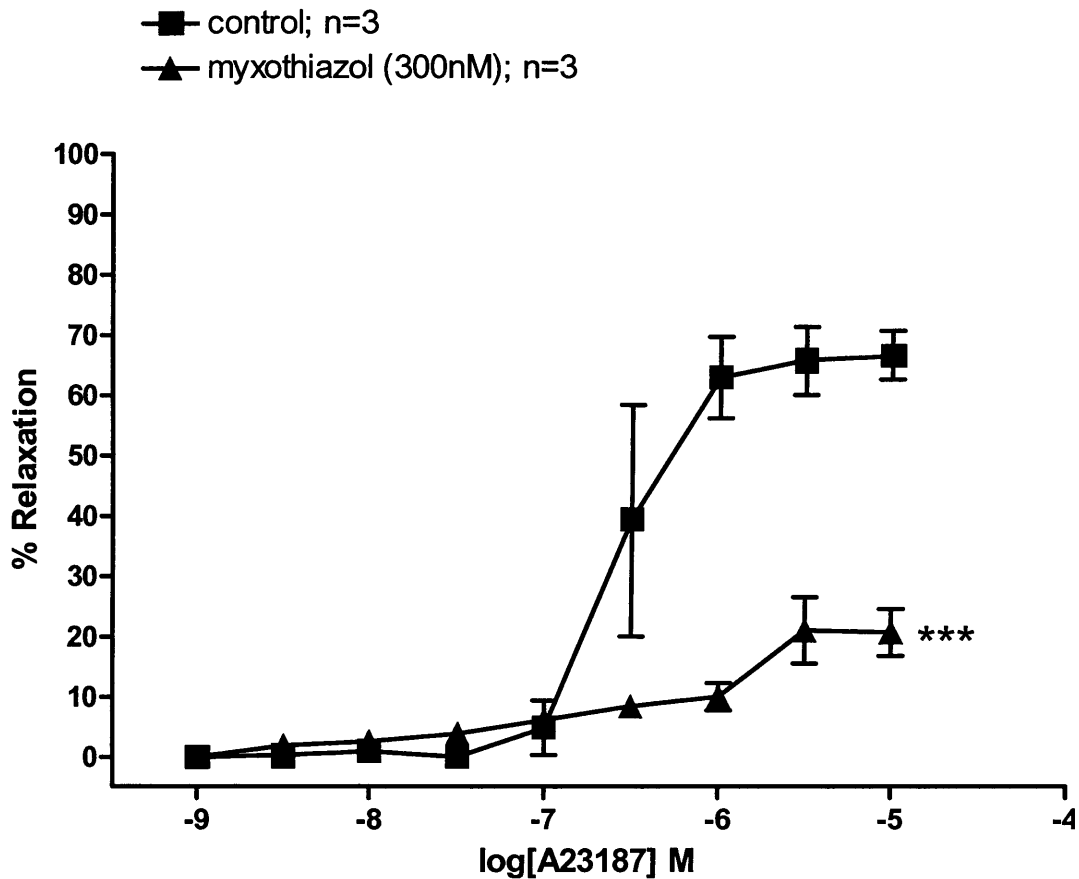
**Fig. 3.24** Concentration-response curves for A23187-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of myxothiazol (300 nM). Experiments were carried out with myxothiazol to assess the role of complex III of the mitochondrial ETC as a source of $H_2O_2$ in responses evoked by A23187. Myxothiazol significantly inhibited the responses evoked by A23187 with a residual relaxation equal to 20.7 ± 2.6%. Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM). Data are expressed as mean ± SEM. ***, $P<0.001$ for whole curve compared with the control.
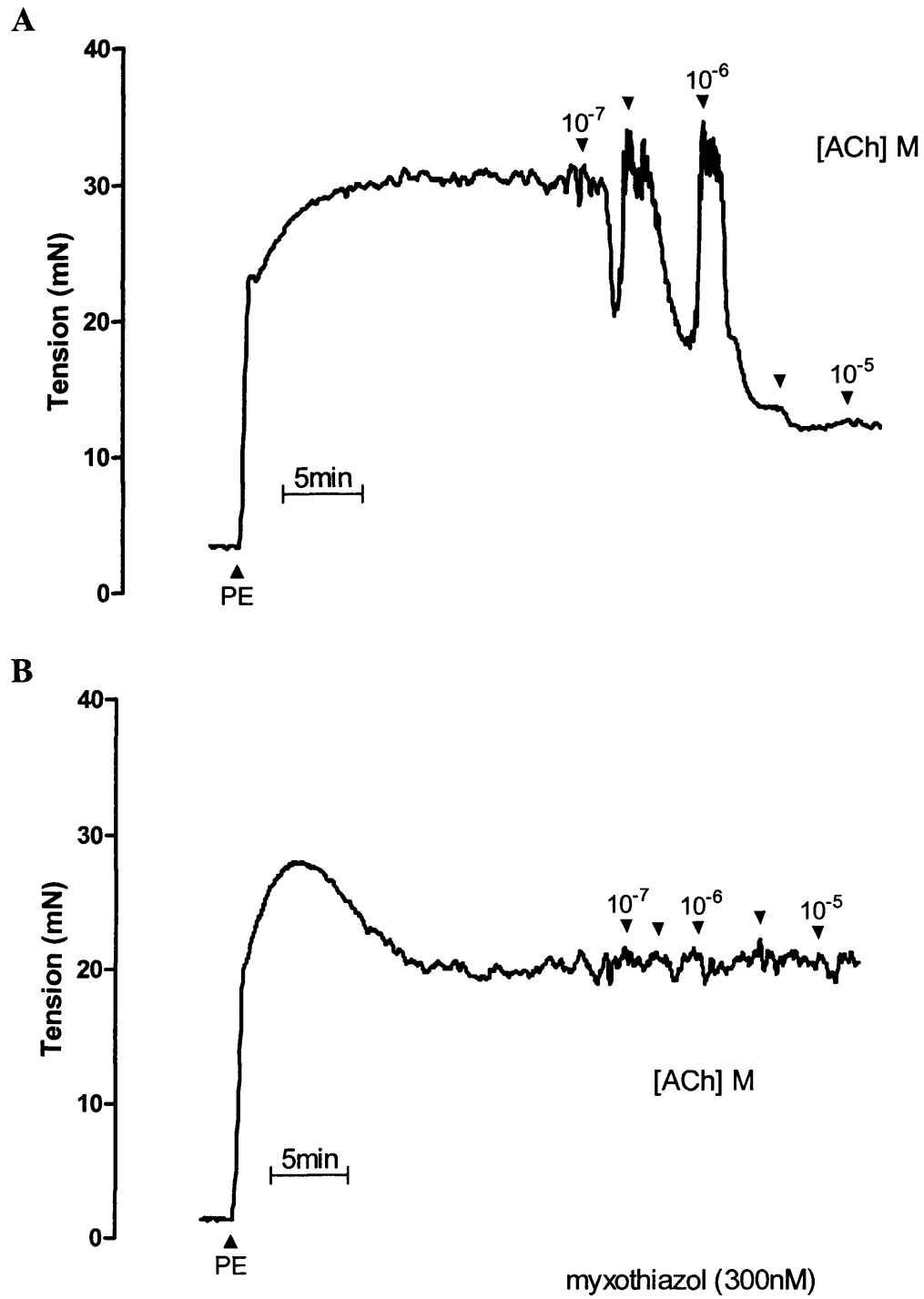
**Fig. 3.25** Representative traces from ring preparations of rabbit iliac arteries with intact endothelium showing (A) relaxations to A23187 and associated changes in the presence of (B) myxothiazol (300 nM). Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM).
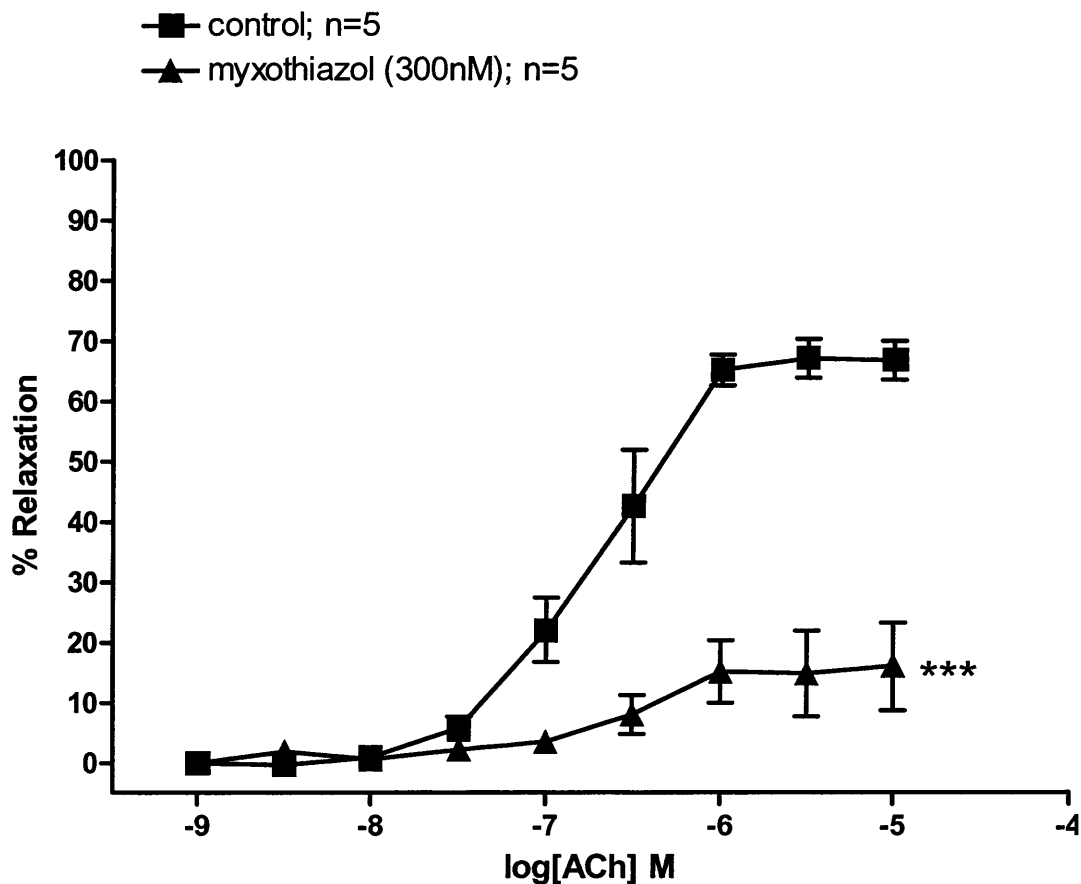
**Fig. 3.26** Concentration-response curves for ACh-evoked EDHF-type relaxations of rabbit iliac arteries and associated changes in the presence of myxothiazol (300 nM). Experiments were carried out with rotenone to assess the role of complex I of the mitochondrial ETC as a source of $H_2O_2$ in responses evoked by ACh. Myxothiazol significantly inhibited the responses evoked by ACh with a residual relaxation equal to $15.9 \pm 2.2\%$. Experiments were carried out in the presence of L-NAME (300 µM) and Indo (10 µM). Data are expressed as mean $\pm$ SEM. ***, $P<0.001$ for whole curve compared with the control.

| Intervention | % relaxation at 10μM | pEC$_{50}$ | n |
|---|---|---|---|
| A23187 | 83.5±3.3 | 6.6±0.1 | 6 |
| A23187 + apocynin (1 mM) | 68.83±3.8 | 6.9±0.1 | 6 |
|  |  |  |  |
| A23187 | 78.4±6.3 | 6.9±0.1 | 4 |
| A23187 + oxypurinol (100 μM) | 73.1±10.7 | 6.9±0.1 | 4 |
|  |  |  |  |
| A23187 | 80.2±4.5 | 6.8±0.1 | 4 |
| A23187 + rotenone (300 nM) | n/a | n/a | 4 |
|  |  |  |  |
| ACh | 62.8±6.3 | 6.5±0.2 | 4 |
| ACh + rotenone (300 nM) | 3.1±15.7*** | n/a | 4 |
|  |  |  |  |
| A23187 | 66.5±4.1 | 6.6±0.1 | 3 |
| A23187 + myxothiazol (300 nM) | 16.6±0.1*** | n/a | 3 |
|  |  |  |  |
| ACh | 66.7±3.1 | 6.7±0.1 | 5 |
| ACh + myxothiazol (300 nM) | 15.9±7.2*** | n/a | 5 |
|  |  |  |  |

**Table 3.3** Summary of effects of apocynin, oxypurinol, rotenone and myxothiazol on EDHF-type relaxations in rabbit iliac arteries. Potency (negative log EC$_{50}$) and % relaxation at 10 μM of agonist are expressed as a function of the constrictor response to PE and given as means ± SEM. ***$P<0.001$ compared with the corresponding intra-group control. n denotes the number of animals studied. n/a denotes non-applicable data.

| Intervention | PE contraction (mN) | n |
|---|---|---|
| control | 41.9±3.9 | 6 |
| apocynin (1mM) | 37.3±6.1 | 6 |
| | | |
| control | 26.1±1.8 | 4 |
| oxypurinol (100μM) | 24.8±1.2 | 4 |
| | | |
| control | 45.3±2.3 | 16 |
| myxothiazol (300nM) | 43.2±3.3 | 8 |
| rotenone (300nM) | 9.7±0.7*** | 8 |
| | | |

**Table 3.4** Summary of effects of apocynin, oxypurinol, rotenone and myxothiazol on contractions induced by PE in rabbit iliac arteries. All data are given as means ± SEM. ***$P$<0.001 compared with the corresponding intra-group control. n denotes the number of animals studied.

## 3.4 Discussion

### 3.4.1 The $K_{Ca}$-dependent $H_2O_2$ release from mitochondria might account for the A23187 and ACh-evoked EDHF-type relaxations in rabbit iliac arteries.

In the second part of this study, a series of mechanical investigations were carried out so as to elucidate the role of reactive oxygen species on A23187- and ACh-evoked EDHF-type relaxations. It has been previously demonstrated that the mechanism of action of A23187 differs from that of ACh in that the relaxant response 1) does not require functional endothelial gap junctions and 2) depends on a 'factor' which diffuses from the endothelium to smooth muscle (Plane *et al.*, 1995; Hutcheson *et al.*, 1999). Further investigations performed on rabbit iliac arteries, including 'sandwich' preparations and *en face* imaging with the $H_2O_2$-sensitive probe dihydrochlorofluorescein (DCF), suggested that this diffusible factor is $H_2O_2$ (Chaytor *et al.*, 2003). It was also demonstrated that $H_2O_2$ is the only ROS responsible for A23187-evoked EDHF-type relaxations, as neither SOD inhibition or deferiprone affected the responses (Chaytor *et al.*, 2003; see section 1.3.2.3). Notably, an $H_2O_2$-dependent component was observed in both A23187- and ACh-evoked responses, but it is only in the former that this component is distinct from the concomitant hyperpolarizing response. In ACh-evoked EDHF-type relaxations inhibition of gap junctional communication with connexin-mimetic peptides abolished the entire response (Chaytor *et al.*, 2003).

Because EDHF-type responses depend on the opening of endothelial $K_{Ca}$ channels and subsequently the development of endothelial hyperpolarizations (see Chapter 1), the idea of a functional relationship between $K_{Ca}$ opening and the release of $H_2O_2$ from endothelial cells requires further elucidation. Although in rabbit iliac arteries the propagation of hyperpolarizations from the endothelium to smooth muscle does not seem to be the dominant relaxation mechanism as previously suggested by Chaytor *et al.* (2003), it is possible that the underlying hyperpolarization and changes in intracellular $Ca^{2+}$ levels might be a prerequisite for $H_2O_2$ release. Based on this hypothesis, the investigations undertaken in this study focused primarily on the acquisition of evidence regarding $K_{Ca}$-$H_2O_2$ coupling, and secondarily, on the identification of the endothelial source of $H_2O_2$. The results obtained suggest that changes in $[Ca^{2+}]_i$ might stimulate independently but simultaneously $K_{Ca}$ channel opening and $H_2O_2$ release from the endothelium of rabbit iliac arteries. Further

investigations supported the hypothesis that the endothelial source of $H_2O_2$ might be the ETC of mitochondria.

### 3.4.2 Both A23187- and ACh-evoked EDHF-type relaxations are $H_2O_2$-dependent

It was demonstrated that catalase significantly impairs relaxations to calcium ionophore A23187, with a residual response equal to ~30% of the PE-preconstriction. Similar investigations carried out with ACh revealed a significantly smaller catalase-sensitive component which accounted for 40% of the maximal response. These findings, which concur with those of studies carried out by Chaytor *et al.* (2003), suggested that the endothelial release of $H_2O_2$ is more significant in A23187-evoked EDHF-type responses than the corresponding ACh-induced relaxation. The generation of $O_2 \cdot^-$ by A23187 and its subsequent dismutation to $H_2O_2$ have also been demonstrated in other experimental models, such as the rat aorta, rat thymocytes and bovine aortic endothelial cells (BAECs) (Cosentino *et al.*, 1998; Azmi *et al.*, 1994; McNally *et al.*, 2005). For instance, in Wistar (WKY) and spontaneously hypertensive rat (SHRs) aortae, chemiluminescence methods showed a significant increase in the corresponding $H_2O_2$ levels upon stimulation with A23187, an effect that was further potentiated after incubation with SOD (Consentino *et al.*, 1998). Nevertheless, in rabbit iliac arteries catalase failed to abolish the responses to both agents, thereby indicating that a residual $H_2O_2$-independent response is also involved. Indeed, it has been previously demonstrated that incubation of rabbit iliac arteries with catalase plus inhibitors of myoendothelial gap junctions abolishes both A23187- and ACh-evoked EDHF-type relaxations (Chaytor *et al.*, 2003). These findings confirmed that the residual $H_2O_2$-independent component occurs due to the propagation of hyperpolarizations from the endothelium to smooth muscle, but the magnitude of the hyperpolarization-dependent component is greater in ACh-evoked responses that the A23187 equivalent.

In rabbit iliac arteries, production of $H_2O_2$ by A23187 has also been suggested by studies using the $H_2O_2$-sensitive probe DCF (Chaytor *et al.*, 2003). In these investigations, acute application of A23187 caused a significant increase in endothelial fluorescence, which confirmed that a peroxide is released in this layer. Indeed, there is accumulating evidence which suggests that DCF is not selective for $H_2O_2$, but it could potentially interact with any peroxide that is present (Hempel *et al.*, 1999). It is therefore reasonable to say that the use of DCF cannot give conclusive data about $H_2O_2$

generation in rabbit iliac arteries. However, the current study and the study of Chaytor *et al.* (2003) both demonstrated that catalase significantly inhibits responses to A23187, thereby indicating that the peroxide detected by DCF might be $H_2O_2$. Notably, the form of catalase used for the purpose of the current study does not penetrate cell membranes and therefore the enzyme can only degrade $H_2O_2$ that has diffused into the extracellular space. However, in small mesenteric arteries of eNOS-knockout mice application of catalase abolishes DCF fluorescence which suggested that the enzyme does affect endogenous $H_2O_2$ production (Matoba *et al.*, 2000).

To ensure that the inhibitory effects of catalase are entirely associated with $H_2O_2$ scavenging, experiments were carried out with ATZ, an inhibitor of the active site of the enzyme (Margoliash & Novogrodsky, 1958). It was demonstrated that preincubation of iliac arteries with ATZ partially restores relaxations to A23187 in the presence of catalase, therefore suggesting that an interaction between the enzyme and $H_2O_2$ is likely to take place. These findings are also consistent with studies carried out in isolated rat aortae, which aimed to emphasize the role of endogenous catalase as a protective cellular component and as the site of conversion of nitrovasodilators into NO (Mian & Martin, 1997; Waldman & Murad, 1987). In these vessels, application of ATZ potentiated the inhibitory effects of exogenous $H_2O_2$ on relaxations induced by glyceryl nitrate (Mian & Martin, 1997), while inhibition of the enzyme blocked the relaxations induced by sodium azide and hydroxylamine (Waldman & Murad, 1987). Nevertheless, the inability of ATZ to completely restore relaxations to A23187 in rabbit iliac arteries indicated that either the concentration of ATZ might have been submaximal or catalase might have triggered effects other than $H_2O_2$ scavenging. If indeed catalase has other effects in rabbit iliac arteries, future investigations should aim to identify them and explain how these effects might alter A23187-evoked EDHF-type responses.

More conclusive data about the release of $H_2O_2$ in A23187-evoked relaxations were obtained with the superoxide-quenching agent manganese prophyrin (MnTMPyP). The scavenging effects of MnTMPyP have been previously demonstrated in rat cerebral and mesenteric arteries, in rabbit aorta and the retractor penis muscle (Xi *et al.*, 2005; MacKenzie & Wadsworth, 2003; MacKenzie & Martin, 1998; Mok *et al.*, 1998). In rabbit iliac arteries it was demonstrated that MnTMPyP causes a significant rightward shift of the A23187 curve with a 10-fold increase in the $pEC_{50}$ value, but with no

apparent effect on the maximal relaxation. This change provided further support to the aforementioned findings with catalase, and concurred with the fact that $H_2O_2$ plays a major role in A23187-induced EDHF-type relaxations. However, there are reports in the literature that demonstrate that MnTMPyP also possesses SOD-like properties (Faulkner *et al.,* 1994). Indeed, assays carried out on samples of *Escherichia coli* mutants lacking SOD, showed that MnTMPyP can substitute for the enzyme and catalyse the dismutation of $O_2^{\cdot-}$ to $H_2O_2$ (Faulkner *et al.,* 1994). Paradoxically, in PE-preconstricted rat aortic rings, cumulative concentration response curves to MnTMPyP unravelled a double concentration-dependent effect. More specifically, concentrations between 10 nM and 30 μM MnTMPyP augmented the aortic tone due to destruction of basal NO, while at higher concentrations it caused an endothelium-independent vasodilatation through a direct smooth muscle action (MacKenzie *et al.,* 1999). By contrast, in carotid arteries from spontaneously hypertensive stroke prone rats (SHRSP) MnTMPyP triggered a contractile response that was significantly potentiated by the removal of the endothelium and inhibited in the presence of the L-type channel inhibitor verapamil (Sekiguchi *et al.,* 2003). In the current study, no apparent changes were detected in the basal tensions or the tension of PE-preconstricted rabbit iliac arteries following incubation with this agent. Such finding excluded the possibility that MnTMPyP might have a direct effect on the smooth muscle of the vessels, and that $O_2^{\cdot-}$ radicals do not directly contribute to A23187-evoked EDHF-type relaxations. Hence, it is reasonable to speculate that the effects of MnTMPyP in A23187-induced relaxations might be a result of its catalase-like properties only.

### 3.4.3 Relation between $K_{Ca}$ activation and $H_2O_2$ release

Experiments were carried out with the $K_{Ca}$ inhibitors apamin, TRAM-34 and IbTX in combination with catalase. Similarly to Chapter 2 it was demonstrated that inhibition of $K_{Ca}$ causes a significant suppression of A23187-induced EDHF-type relaxations, with a residual response being evident in all investigations. Interestingly addition of catalase almost abolished the remaining relaxation when combined with apamin and TRAM-34 or singly with IbTX. Such findings indicate that the release of $H_2O_2$ in the endothelium of iliac arteries might occur simultaneously with the activation of $K_{Ca}$ channels. Indeed, previous investigations have demonstrated that $H_2O_2$ released following the application of a stimulus, can either increase or decrease the opening of $BK_{Ca}$ and $IK_{Ca}$ channels

according to the tissue and the experimental conditions employed (Barlow *et al.*, 2000; Gao *et al.*, 2003; Brakemeier *et al.*, 2003). However, in the current study the available evidence is not sufficient to prove such an interaction and therefore it is not possible to propose that $H_2O_2$ release affects endothelial $K_{Ca}$ channels. By contrast, it is more likely that activation of endothelial $K_{Ca}$ channels might be a prerequisite for the release of $H_2O_2$, since the residual relaxation could also be inhibited if other inhibitors of these channels are added. For instance in Chapter 2, it was demonstrated that addition of the triple combination of apamin plus TRAM-34 plus IbTX abolishes A23187-evoked EDHF-type relaxations, which suggests that the residual catalase-sensitive component is also $K_{Ca}$-dependent. Nevertheless, it could be possible that changes in intracellular $Ca^{2+}$ levels upon stimulation with A23187 might initiate two different pathways, one which involves the activation of endothelial $K_{Ca}$ channels and one which involves the $Ca^{2+}$-dependent release of $H_2O_2$ leading ultimately to relaxation.

Indeed, it has been suggested that apart from the effects that changes in $[Ca^{2+}]_i$ have in the opening of $K_{Ca}$ channels, the release of $H_2O_2$ in the endothelium is also $Ca^{2+}$-dependent (Guidarelli *et al.*, 2007). Hence, it would be reasonable to speculate that A23187-induced depletion of the store causes a significant increase in $[Ca^{2+}]_i$, which both stimulates $K_{Ca}$ opening and triggers the independent release of $H_2O_2$. Interestingly, in cultured endothelial cells from human umbilical veins (HUVECs), fluorescent measurements and patch-clamp techniques demonstrated that inhibition of $K_{Ca}$ channels with apamin, TRAM-34 and ChTX significantly reduces the elevations in cytosolic free $Ca^{2+}$ with a modest $Ca^{2+}$ transient still being evident (Sheng & Braun, 2007). It was thereby suggested that this transient might reflect the combination of $Ca^{2+}$ released from the stores and the residual entry of external $Ca^{2+}$ (Sheng & Braun, 2007). In accordance to these findings, it is possible that in the current study, inhibition of $K_{Ca}$ channels might reduce intracellular $Ca^{2+}$ levels, which could potentially affect the release of $H_2O_2$. Besides, in the presence of apamin plus TRAM-34 or IbTX on their own, a residual catalase-sensitive relaxation was still evident, which was consistent with the presence of a remaining $Ca^{2+}$ transient as proposed by Sheng & Braun (2007). However, it should be noted that in intact arterial preparations, agonist-evoked elevations in endothelial cytosolic $Ca^{2+}$ are reported to be unaltered in the presence of $K_{Ca}$ inhibitors (Ghisdal & Morel, 2001; McSherry *et al.*, 2005; Stankevicius *et al.*, 2006). Hence, the available

evidence is not sufficient to exclude the involvement of another putative mechanism that couples $K_{Ca}$ channel opening to $H_2O_2$ release.

To investigate whether $H_2O_2$ release in the endothelium has an effect on the opening of smooth muscle $K_{Ca}$ channels, endothelium-denuded vessels were treated with exogenous $H_2O_2$ in the presence and absence of $K_{Ca}$ inhibitors. In a previous study, it was demonstrated that the combination of apamin plus ChTX does not affect relaxations evoked by X/XO or authentic $H_2O_2$ in these vessels (Chaytor *et al.*, 2003). However, differences in the pharmacological profile of ChTX and the more selective $BK_{Ca}$ inhibitor IbTX made the latter a more specific pharmacological probe. For this reason experiments were carried out with IbTX on its own or the triple combination of apamin plus TRAM-34 plus IbTX so as to elucidate the putative involvement of all $K_{Ca}$ subtypes. It was demonstrated that incubation of rabbit iliac arteries with IbTX or the triple combination has no significant effect on any of the responses induced by $H_2O_2$. Interestingly, these findings differ from those of other studies which showed that inhibition of $K_{Ca}$ channels either potentiates or inhibits the effects of $H_2O_2$ depending on the tissues and the experimental conditions employed. Also, patch-clamp investigations in porcine coronary smooth muscle cells and human embryonic kidney cells demonstrated a significant change in $K^+$ currents respectively upon stimulation with $H_2O_2$ (Barlow & White, 1998; Hayabuchi *et al.*, 1998; Barlow *et al.*, 2000; Tang *et al.*, 2004). By excluding the products of arachidonate metabolism due to the inevitable use of COX inhibitors in EDHF-related studies, in these tissues the endothelium-independent effects of $H_2O_2$ on arterial myocytes could be attributed to three main factors: 1) a direct interaction of $H_2O_2$ with $K_{Ca}$ channels, 2) a direct interaction of $H_2O_2$ with PKG and 3) an indirect effect through the action of cGMP (Sato *et al.*, 2003; Iesaki *et al.*, 1999; Burgoyne et al., 2007; Fujimoto *et al.*, 2001, 2003). However, the current investigations demonstrated the absence of any effects of $K_{Ca}$ inhibitors on relaxations evoked by authentic $H_2O_2$ thereby indicating that smooth muscle $K_{Ca}$ channels are unaffected by the peroxide in rabbit iliac arteries. Notably, such effects cannot be directly compared to those of endogenous $H_2O_2$ produced by A23187, neither do they indicate that endogenous $H_2O_2$ has no effect on smooth muscle $K_{Ca}$ channels. In fact, it has been established that authentic $H_2O_2$ induces significant direct smooth muscle relaxation at 'supraphysiological' concentrations between 100 µM – 1 mM, while the concentration of endogenous $H_2O_2$ produced by A23187 does not exceed 100 nM

(Matoba *et al.*, 2000; Gao *et al.*, 2003; Chaytor *et al.*, 2003; Cosentino *et al.*, 1998). Although it is not clear why such differences exist, it is possible that the higher biological activity of endogenous $H_2O_2$ might be due to its enzymatic generation in close proximity to its site of action. In accordance to these findings and based on the data of the current study, it could be speculated that since $H_2O_2$ has no effect on $K_{Ca}$ channels at a concentration range of 100 µM – 1mM, it is highly unlikely that it will have any effects on these channels at much lower concentrations. However, the effects of A23187 on the vessels are not entirely $H_2O_2$-dependent, which means that other parameters need to be taken into account when comparing the two responses.

### 3.4.4 Potential sources of $H_2O_2$ in the endothelium of rabbit iliac arteries

### 3.4.4.1 Role of NADPH oxidase and xanthine oxidase in A23187-evoked relaxations

Previous investigations have indicated that NADPH oxidase is a major source of $H_2O_2$ in the vascular system (Griendling *et al.*, 1994; Mohazzab *et al.*, 1994), and it is now known that it can either play a physiological role in vasodilatory responses or contribute to endothelial dysfunction depending on the underlying conditions (Cai *et al.*, 2000). To investigate the role of NADPH oxidase in A23187-evoked $H_2O_2$ release, rabbit iliac arteries were incubated with the naturally occurring methoxy-substituted catechol apocynin. Apocynin has been shown to be a powerful antioxidant and anti-inflammatory agent and is capable of blocking the activity of NADPH oxidase by interfering with the assembly of the cytosolic components with the membrane subunits (Stolk *et al.*, 1994). In rabbit iliac arteries it was demonstrated that apocynin has no significant effect on A23187-induced EDHF-type relaxations, which suggested that NADPH oxidase is not the main source of $O_2^{\cdot-}$ in the endothelium of these vessels.

Because NADPH oxidase was excluded as a possible source of $H_2O_2$ in rabbit iliac arteries, further investigations were carried out to identify other putative sources of this species. Experiments were therefore performed to investigate the role of xanthine oxidase as a putative source of $H_2O_2$ (Zhang *et al.*, 1998). Assessment of XO activity was carried out with the non-competitive inhibitor oxypurinol, a XO-inhibitory metabolite of allopurinol. It was demonstrated that incubation of rabbit iliac arteries with the inhibitor had no significant effect on A23187-evoked EDHF-type relaxations,

thereby excluding the involvement of XO in responses induced by this agent in this vessel. Due to the known specificity of oxypurinol, no further investigations were deemed necessary for this enzyme. Hence, the remaining part of this study focused on the putative role of complexes I and III of the mitochondrial ETC.

### 3.4.4.2 Mitochondrial electron transport chain and $H_2O_2$ release

In the final part of this study experiments were carried out to elucidate the role of the mitochondrial ETC on A23187- and ACh-evoked EDHF-type relaxations in rabbit iliac arteries. Production of ROS in mitochondria is associated with the leakage of electrons from membrane-associated enzyme complexes, with ubisemiquinone serving as an electron donor and molecular oxygen as the acceptor, thereby generating $O_2^{\cdot-}$ (see Chapter 1; Trumpower, 1981; Chakraborti *et al.*, 1999; Lenaz *et al.*, 2002). There are two main ETC sites where ROS are produced, namely complex I and complex III (Lenaz *et al.*, 1998, 2002). Since, mitochondrial-derived $H_2O_2$ originates from $O_2^{\cdot-}$ that is formed at these two complexes, mechanical investigations were carried out to determine the relative contribution of each complex to the total release of ROS upon stimulation with A23187 and ACh. In accordance to the available literature, rotenone was used for the inhibition of complex I and myxothiazol for the inhibition of complex III (see Chapter 1). The properties of both inhibitors have been extensively studied in both tissue preparations and isolated mitochondria, and they were chosen for the current study because of their known selectivity.

It was demonstrated that rotenone and myxothiazol significantly inhibit both A23187- and ACh-evoked EDHF-type relaxations, which suggested that the first and third complexes of ETC might play a major role in $H_2O_2$ production. It was also demonstrated that although rotenone abolishes both A23187- and ACh-evoked EDHF-type responses, a residual relaxation was still observed in the presence of myxothiazol. As described in the general introduction, complex I is the primary site of electron transport in ETC, and complex III accepts electrons from both complex I and complex II. Therefore, if both complexes produced $O_2^{\cdot-}$, rotenone and myxothiazol would have had a partial effect in these relaxations. Indeed, inhibition of complex I with rotenone only prevents electron transport from this complex to complex III, but transport of electrons from complex II to complex III remains intact, and therefore complex III is

still able to generate $O_2\cdot^-$. Accordingly, inhibition of complex III with myxothiazol has no effect on $O_2\cdot^-$ production from complex I. However, based on the evidence provided in this study, it is difficult to understand whether the residual relaxation observed in the presence of myxothiazol is a rotenone-sensitive component or a catalase-insensitive hyperpolarization-dependent component as previously suggested by Chaytor *et al.* (2003). Furthermore, it should be noted that the effects of rotenone and myxothiazol on A23187- and ACh-evoked EDHF-type relaxations are not proportional to the effects of catalase on these responses (see section 3.4.2). Such discrepancy suggests that both inhibitors might not only attenuate the $H_2O_2$-dependent component of A23187- and ACh-evoked EDHF-type relaxations, but they might also have other effects.

Indeed, the current study revealed that the most significant difference between the two agents was their effect on the PE-induced contraction. Paradoxically, it was demonstrated that addition of rotenone irreversibly suppresses the contraction to PE by approximately 25mN relative to the control, while equimolar amounts of myxothiazol had no effect at all. Although inhibition of complex I has a significant effect on ATP synthesis, inhibition of complex III with myxothiazol would inhibit ETC in its entirety, and therefore myxothiazol would attenuate the contraction to phenylephrine. This difference indicates that the effects of rotenone on the smooth muscle might not be entirely the result of suppression of ATP synthesis, but also due to the known toxicity of the agent (Pei *et al.,* 2003). Consequently, inhibition of PE-induced contraction by rotenone is very likely to have a direct impact on the magnitude of relaxations to both A23187 and ACh. Hence, more investigations with tone-matched controls are required to discriminate between the effects of rotenone on $O_2\cdot^-$ production, the concomitant ATP synthesis and its toxic effects.

Finally, it is worth mentioning that previous investigations have suggested that it is possible for ER and mitochondria to be directly attached with each other and communicate through a link between the IP$_3$ receptor of the former and the PTP of the latter (Verrier *et al.,* 2004). It was subsequently inferred that such a connection between the two organelles would make it possible for $Ca^{2+}$ released from ER to be rapidly taken up into mitochondria. Indeed, in guinea-pig single colonic myocytes mitochondria effectively reduce the $Ca^{2+}$ concentration in a restricted space near the store, as it would be high enough to inhibit further release (McCarron *et al.,* 1999). $Ca^{2+}$ uptake has been

involved in $H_2O_2$ release in some experimental models, which contrary to the notion that $H_2O_2$ diffuses to the cytosol through the mitochondrial membrane, showed that its release is controlled by the $Ca^{2+}$-dependent opening of PTP (Verrier *et al.*, 2004). It has also been suggested that inhibition of $Ca^{2+}$ uptake by mitochondria may decrease store-operated $Ca^{2+}$ entry or $Ca^{2+}$ pump activity by localized ATP depletion or alteration of the ATP/ADP ratio (Innocenti *et al.*, 1996; Landolfi *et al.*, 1998). The physiological connection between the two organelles has also been demonstrated by studies which showed that mitochondrial inhibition reduces the amount of $Ca^{2+}$ released by $IP_3$ (Gurney *et al.*, 2000). Hence, the findings of the current study may partially reflect this connection, and therefore the inhibitory effects of rotenone or myxothiazol might be due to the inhibition of $Ca^{2+}$ uptake by mitochondria.

### 3.4.5 Concluding remarks

In conclusion, investigations were carried out so as to elucidate the role of $H_2O_2$ on A23187- and ACh-evoked EDHF-type relaxations. It was demonstrated that $H_2O_2$ plays a major role in both responses, although the $H_2O_2$-dependent component was more profound on A23187-induced relaxations rather than the ACh equivalent. It was also proposed that although A23187-evoked hyperpolarizations and relaxations are distinct, $H_2O_2$ release from the endothelium of iliac arteries and the opening of endothelial $K_{Ca}$ channels might be equally dependent of increases in intracellular $Ca^{2+}$ levels upon stimulation with the agent. This finding was supported by investigations carried out with the combination of catalase and the $K_{Ca}$ blockers apamin, TRAM-34 and IbTX. Additional studies on endothelium-denuded vessels excluded the involvement of smooth muscle $K_{Ca}$ channels. Finally, experiments were carried out so as to determine the putative sources of $H_2O_2$ and it was proposed that the mitochondrial ETC might possibly be the source of this species.

*Chapter Four*

# General Discussion

## 4.1 Overview

It is widely recognized that in vascular beds from many species, an NO/prostanoid-independent mechanism accounts for a residual endothelium-dependent relaxation, which is known as the 'EDHF phenomenon'. There is growing evidence that the 'EDHF phenomenon' is an electrotonic mechanism that is more preponderant in small size vessels and it is also believed that its manipulation could be used for the treatment of disorders in which there is a significant impairment of other vasodilatory mechanisms (Csanyi *et al.*, 2006). Nevertheless, it does appear that in some cardiovascular diseases, such as diabetes and hyperhomocysteinemia, the changes that underpin their main pathological manifestation are accompanied by the excessive generation of ROS, which outstrip the endogenous defence mechanisms and contribute further to the pathogenesis of these disorders (De Vriese *et al.*, 2000; 2004). Indeed, EDHF-type responses are exposed to the same oxidative stress that inactivates NO-evoked responses, and thereby its ability to compensate for loss of NO might be potentially suppressed. The effects of ROS, such as $H_2O_2$, on electrotonically mediated endothelium-dependent vascular relaxations have been previously attributed to the ability of intracellular redox mechanisms to modulate the function of gap junctions by regulating the phosphorylation status of connexins, which can be normalized by addition of antioxidants such as 5-methyltetrahydrofolate and tetrahydrobiopterin (Griffith *et al.*, 2005). Furthermore, it has been suggested that endogenous production of $H_2O_2$, might compensate for the loss of NO- and EDHF-dependent responses in disease (Triggle *et al.*, 2003). Nevertheless, the available evidence is still limited and there is only one study carried out in tetrahydrobiopterin-deficient mice showing that $H_2O_2$ plays such a role (Consentino *et al.*, 2001). Although endothelium-derived $H_2O_2$ is more likely to be an EDRF (Gluais *et al.*, 2005; Griffith, 2004), there are reports which have suggested that under certain experimental conditions, such as those described by Matoba et al. (2000), it may have EDHF-type properties. In the rabbit iliac artery $H_2O_2$ is not an EDHF (Chaytor *et al.*, 2003), but this finding did not exclude its importance as a modulator of vascular tone. For this reason the aims of the current project were two-fold.

The first aim was to elucidate further the mechanism of EDHF-type relaxations in rabbit iliac arteries by identifying the $K_{Ca}$ channels that participate in responses induced by ACh and calcium ionophore A23187. The effects of ACh and A23187 on the

endoplasmic reticulum differ in that the former requires an interaction with cholinergic receptors on plasmalemma and an increase in $IP_3$ levels, while the latter has been proposed to interact directly with the stores. Such a difference suggested that it is possible for these agents to trigger the EDHF phenomenon at a different extent, which meant that the contribution of each $K_{Ca}$ channel could potentially vary depending on the stimulus employed. The protocol for this part of the thesis was adopted on the basis that previous studies have shown that blockade of $K_{Ca}$ channels with inhibitors, such as apamin, TRAM-34 and IbTX attenuates EDHF-type relaxations. The use of these inhibitors either on their own or in combination aimed to determine the relative contribution of each channel individually to the phenomenon and to assess the overall contribution of $K_{Ca}$ channels opening to ACh- and A23187-evoked responses.

The second aim of this thesis was to analyse the role of $H_2O_2$ in EDHF-type relaxations induced by ACh and A23187. It has been previously demonstrated that both agents stimulate the release of $H_2O_2$, although the amount of this species generated by A23187 is significantly greater than that generated by ACh (Chaytor *et al.,* 2003). Also, the release of $H_2O_2$ in the endothelium following stimulation with both agents is sufficient to mask the functional consequences of an associated hyperpolarization, a response which in A23187-evoked responses was still evident in the presence of inhibitors of myoendothelial gap junctions (Chaytor *et al.,* 2003). However, the fact that the endothelium-dependent hyperpolarizations and the concomitant endothelium-dependent relaxations were distinct did not necessarily exclude the possibility that the two mechanisms could be potentially linked together. For this reason investigations were carried out so as to identify the connection between the activation of $K_{Ca}$ and the release of $H_2O_2$. To obtain a more conclusive idea about the mechanism of ACh- and A23187-evoked $H_2O_2$ release experiments were also performed so as to identify the sources of this species.

The results of the investigations carried out in this thesis demonstrated that:

- In rabbit iliac arteries $BK_{Ca}$ channels are localized in both the endothelium and the smooth muscle of the vessels (Fig. 4.1). These findings were supported by data obtained by 1] mechanical investigations carried out with the selective $BK_{Ca}$ channel inhibitor IbTX and the $BK_{Ca}$ channel opener NS1619. 2]

Immunohistochemical investigations carried out with an antibody selective for the α-subunit of the channel confirmed these findings.

- $SK_{Ca}$ and $IK_{Ca}$ channels were also identified on the endothelium of rabbit iliac arteries (Fig. 4.1). It was demonstrated that inhibition of these channels with apamin and TRAM-34 has a significant effect on EDHF-type relaxations induced by both ACh and A23187. These findings were supported further by investigations with the $SK_{Ca}$ / $IK_{Ca}$ channel opener 1-EBIO and immunohistochemistry.

- ACh- and A23187-evoked EDHF-type relaxations are sensitive to catalase, thereby suggesting that both responses consist of a significant $H_2O_2$-dependent component (Fig. 4.1). The role of $H_2O_2$ in A23187-evoked EDHF-type relaxations was confirmed with MnTMPyP. It was also revealed that a residual catalase-insensitive component plays a major role in both responses, although the magnitude of this component was significantly greater in ACh-evoked EDHF-type relaxations.

- Investigations were carried out to determine the role of $K_{Ca}$ activation in A23187-evoked $H_2O_2$-dependent relaxation. Inhibition of the responses with IbTX plus catalase or apamin plus TRAM-34 plus catalase indicated that it is possible for the activation of the three $K_{Ca}$ channels to be a prerequisite for the endothelial generation of $H_2O_2$. The involvement of smooth muscle $K_{Ca}$ channel was excluded by investigations carried out with exogenous $H_2O_2$ on endothelium-denuded iliac arteries.

- According to mechanical investigations the mitochondrial ETC is likely to be the main source of reactive oxygen species in both ACh- and A23187-treated arteries (Fig. 4.1). Experiments were carried out with inhibitors of the three main sources of ROS, i.e. apocynin for NADPH oxidase, oxypurinol for xanthine oxidase and rotenone and myxothiazol for mitochondria. Because the vessels were treated with L-NAME and indomethacin, it was not necessary to investigate other putative sources, namely eNOS and COX.

In summary, the findings of this study lead to the conclusion that the contribution of endothelial $K_{Ca}$ channels, including $BK_{Ca}$, is equally important in responses induced by both ACh and A23187. The presence of a functional $BK_{Ca}$ channel in the endothelium of iliac arteries is perhaps one of the major findings of this thesis, since it emphasizes the fact that some physiological characteristics of the rabbit are different from those of other species, such as the rat which mainly possesses $SK_{Ca}$ and $IK_{Ca}$ in the intimal layer of arteries. By focusing further on the mechanisms of EDHF-type relaxation when ACh or A23187 are used as stimuli, it can also be proposed that the role of $K_{Ca}$ opening is similar in ACh- and A23187-evoked relaxations, on the basis that complete inhibition of these channels abolishes both responses. In accordance to these findings separate investigations aimed to determine the role of $K_{Ca}$ activation on $H_2O_2$ release from its endothelial source, which in rabbit iliac arteries seems to be the mitochondrial ETC. It should be noted that the available evidence is not sufficient to draw a definite conclusion, but it is possible that changes in $[Ca^{2+}]_i$ might underpin the distinct activation of $K_{Ca}$ and $H_2O_2$ release.
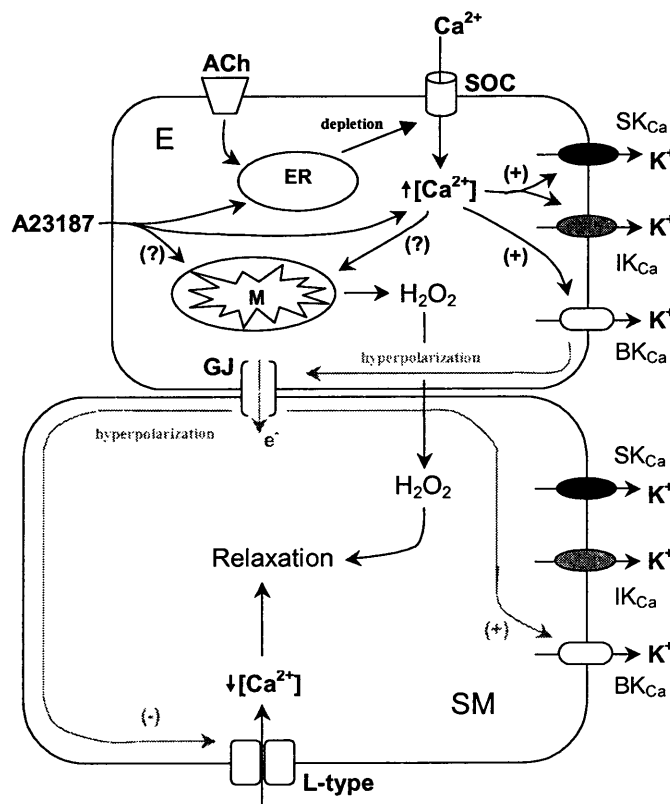


**Fig. 4.1** Schematic summary of the major findings of the current study. The image demonstrates the presence of $K_{Ca}$ channels in the endothelium and the smooth muscle of rabbit iliac arteries, and the pathways that might lead to generation of endothelial $H_2O_2$ from mitochondria. E: endothelial cell, SM: smooth muscle cell, GJ: gap junction, ER: endoplasmic reticulum, M: mitochondrion, SOC: store-operated $Ca^{2+}$ channel, L-type: L-type $Ca^{2+}$ channel.

## 4.2 EDHF in disease

In order to appreciate the importance of the observations of this study it is necessary to understand how the 'EDHF phenomenon' is affected by various disease states and how pharmacological manipulation of this pathway could be used for the treatment of these diseases. Indeed, there is a substantial amount of evidence to suggest that the 'EDHF phenomenon' can play the role of a compensatory mechanism when the production of NO or prostacyclin is significantly attenuated (Kilpatrick & Cocks, 1994; Corriu *et al.*, 1998; McCulloch *et al.*, 1997), as previously demonstrated in some experimental models of atherosclerosis, hypertension and heart failure (Ayajiki *et al.*, 2000; Fujii *et al.*, 1992; Malmsjo *et al.*, 1999).

However, EDHF-type responses are not always enhanced when NO production is reduced. For instance, in spontaneously hypertensive rats (SHR) it has been demonstrated that the role of these responses is limited, which appeared to be age-dependent and was also associated with concomitant changes in the expression of Cx37 and Cx40 (Rummery & Hill, 2004; Büssemaker *et al.*, 2003). Similarly, in isolated gastroepiploic arteries from atherosclerotic patients, endothelium-dependent hyperpolarizations are inhibited (Urakami-Harasawa *et al.*, 1997), while the 'EDHF phenomenon' is also attenuated in insulin-dependent and non-insulin-dependent diabetes, as demonstrated by various experimental models of the disease in mice and rats (Table 4.1).

Therefore, the use of drugs that enhance EDHF-type responses could be a putative solution for these disorders. Currently, there are agents such as angiotensin converting enzyme inhibitors (ACEI), diuretics and anti-diabetic drugs, which are known to potentiate electrotonically mediated endothelium-dependent vascular relaxations (Mombouli *et al.*, 1995; Onaka *et al.*, 1998)). For instance, in SHR chronic treatment with hydrochlorothiazide, in combination with hydralazine, normalizes the blood pressure by potentiating the EDHF-type responses (Onaka *et al.*, 1998). However, in many cases the concomitant development of oxidative stress forms an additional obstacle in the treatment of these disorders. For this reason it is crucial to understand how ROS release might affect the 'EDHF phenomenon'.

| Models | EDHF | NO | References |
|--------|------|-----|------------|
| Streptozocin | ↓ | ↓ | Kamata *et al.*, 2000; De Vriese *et al.*, 2000 |
| Zucker rat | ↓ | -- | Burnham *et al.*, 2006; Wu *et al.*, 1996 |
| OLETF | ↓ | -- | Kagota *et al.*, 2000; Matsumoto *et al.*, 2006 |
| Db/db$^{-/-}$ mouse | -- | ↓ | Pannirselvam *et al.*, 2002 |
| | | | |

**Table 4.1** A summary of the changes in EDHF-type and NO-mediated responses in five different models of diabetes. ↓ = decreased contribution, -- = no change.

## 4.3 EDHF-type responses and ROS

Under physiological conditions, ROS are known to affect vascular contractility through a direct interaction on channels and signalling molecules which contribute to the regulation of vascular tone (see section 1.3.4). The relaxation response caused by endothelium-generated $H_2O_2$ may have both endothelium-dependent and endothelium-independent components. The endothelium-dependent relaxation is mainly mediated through the direct activation of $IP_3R$ in the endothelium and an increase in $[Ca^{2+}]_i$, which ultimately leads to: 1) NO synthesis and 2) the development of EDHF-type hyperpolarizations (Zheng & Shen, 2005; Thengchaisri & Kuo, 2003). The endothelium-independent component is associated with sGC activation and cGMP formation (Theresa *et al.*, 1991), and in some vessels the direct effect of $H_2O_2$ on potassium channels, such as $K_{Ca}$, $K_v$ and $K_{ATP}$ (Gao *et al.*, 2003; Wei *et al.*, 1996).

In the presence of eNOS inhibitors, such as L-NAME, the increase in $[Ca^{2+}]_i$ in the endothelium triggers an EDHF-type hyperpolarization followed by relaxation, which also forms the basis for the development of distinct hyperpolarizing and relaxant responses upon stimulation with ACh and A23187. It has already been established that inhibition of gap junctional communication has a different effect on responses induced by both agents. However, these effects did not seem to affect the $H_2O_2$-dependent component, and therefore, what remained unclear was the putative link between the hyperpolarizing response as a result of $K_{Ca}$ activation and the generation of the endothelial $H_2O_2$. By demonstrating the link between $K_{Ca}$ activation and $H_2O_2$ release it would be possible to understand:

- whether increases in $H_2O_2$ production could be a consequence of the augmentation of the 'EDHF phenomenon' in certain diseases, or

- whether changes in intracellular $H_2O_2$ levels due to these diseases could have an effect on the development of the 'EDHF phenomenon', and subsequently vascular tone.

*In vivo* the release of ROS is a complex process that involves the convergence of several redox-sensitive signalling pathways and the balance between pro-oxidant and anti-oxidant mechanisms (Griendling *et al.*, 2000). In some disorders, it has been suggested that $K_{Ca}$ opening and the concomitant hyperpolarization could potentially lead to an increase in ROS production. Indeed, in an experimental model of monocrotaline-induced pulmonary hypertension upregulation of $K_{Ca}$ expression led to the augmentation of tonic EDHF activity, which in a separate study was shown to be accompanied by the production of oxygen radicals (Morio *et al.*, 2007; Chen *et al.*, 2001). However, at the moment there is a limited amount of evidence which shows how potassium channel opening could potentially lead to the production of ROS. In fact, the majority of investigations have focused on the effects of ROS on the opening of these channels as one of the consequences of excessive release of this species in diseases, such as coronary atherosclerosis (Gutterman *et al.*, 2005). ROS affect $K_{Ca}$ opening either directly by targeting the cysteine residues of the channel or indirectly through changes in intracellular $Ca^{2+}$ levels (Tang *et al.*, 2004; Lin *et al.*, 2007). Indeed, in rat intralobar pulmonary arterial smooth muscle cells $H_2O_2$ has been suggested to mobilize intracellular $Ca^{2+}$ through multiple pathways, including the $IP_3$ and ryanodine receptor-gated $Ca^{2+}$ stores, which could potentially affect $K_{Ca}$ opening (Tang *et al.*, 2004; Lin *et al.*, 2007). However, it remains unclear whether this coupling is a direct one since 1] the relaxant and hyperpolarizing responses are distinct (Chaytor *et al.*, 2003), 2] mitochondria on their own participate in intracellular $Ca^{2+}$ homeostasis via several $Ca^{2+}$ uptake and release pathways (Benardi, 1999) and 3] inhibition of endothelial $K_{Ca}$ channels decreases $[Ca^{2+}]_i$ (Sheng & Braun, 2007).

Increases in cytosolic $[Ca^{2+}]$ in the endothelium are known to induce $Ca^{2+}$ entry across the mitochondrial inner membrane and result in an elevation in the mitochondrial matrix $Ca^{2+}$ concentration ($[Ca^{2+}]_m$). According to the available literature, the effects of

increased $[Ca^{2+}]_m$ on mitochondrial ROS production are complex, but there is consensus that release of $H_2O_2$ from these organelles depends on $Ca^{2+}$ influx through the mitochondrial $Ca^{2+}$ uniporter (Guidarelli *et al.,* 2006; 2007). Paradoxically, in ACh-evoked EDHF-type relaxations, the underlying changes in the endothelial $[Ca^{2+}]_i$ may only weakly stimulate $H_2O_2$ release, which suggested that in contrast to A23187, ACh might cause smaller transient changes in $[Ca^{2+}]_i$. However, it should be noted that the evidence provided by this study is not sufficient to demonstrate how the two agents might affect $[Ca^{2+}]_i$. Besides, inhibition of $K_{Ca}$ channels with the same toxins had the same effect on EDHF-type relaxations evoked by both agents, therefore suggesting that the mechanism that leads to $H_2O_2$ generation might involve more complex changes than simply the spread of $Ca^{2+}$ signals.

## 4.4 Limitations of study

There are several limitations of the current study that should be considered:

➡ *IbTX-dependent tonic contractions might affect the development of EDHF-type responses indirectly.*

It was demonstrated that IbTX causes a significant increase in tension of rabbit iliac arteries. As mentioned in chapter 2 this increase could affect the magnitude of EDHF-type relaxations, and therefore the results of the mechanical investigations might not reflect the direct inhibition of the EDHF phenomenon *per se.* It was also demonstrated that the magnitude of the tonic contractions was variable, while the magnitude of the relaxations in the presence of IbTX was consistent in all experiments. Although such discrepancy indicates that the effect of IbTX on the EDHF phenomenon might be a consequence of inhibition of endothelial $BK_{Ca}$ channels, the evidence provided in this thesis cannot exclude the possibility that inhibition of $BK_{Ca}$ channels in the smooth muscle might still affect the development of the endothelium-dependent response.

➡ *Catalase might have other effects that are independent of $H_2O_2$ scavenging.*

The second part of this thesis aimed to identify the role of $H_2O_2$ in ACh- and A23187-evoked EDHF type relaxations. It was demonstrated that both catalase and MnTMPyP

significantly attenuate both responses, thereby suggesting that one of the components of these relaxations is $H_2O_2$-dependent. However, ATZ, an inhibitor of the active site of catalase did not fully restore the relaxations to A23187. It is therefore possible that the residual ATZ-insensitive component might be due to secondary effects of the enzyme, e.g. catalase might interact with $K_{Ca}$ channels directly. Hence, future studies should aim to clarify the effects of catalase, and elucidate further the role of $H_2O_2$ on both ACh- and A23187-evoked EDHF-type relaxations.

➡ *The inhibitors of mitochondrial ETC, rotenone and myxothiazol, might affect ATP synthesis instead of $H_2O_2$ production. Toxicity of rotenone should also be taken into account.*

It was demonstrated that both rotenone and myxothiazol inhibit responses to ACh and A23187. This finding indicated that it is possible that complexes I and III of the mitochondrial ETC might generate $O_2^{\cdot-}$, and subsequently $H_2O_2$ through dismutation. Notably, a small but significant relaxation was still evident in the presence of myxothiazol, which could potentially be a rotenone-sensitive component or a hyperpolarization-dependent component. It was also demonstrated that rotenone significantly attenuates the contractions to phenylephrine, an effect which was disproportionate to that obtained with equimolar amounts of myxothiazol (see section 3.4.4.2). Therefore, the available evidence is not sufficient to demonstrate whether the effects of rotenone and myxothiazol are indeed due to direct inhibition of $O_2^{\cdot-}$ release by ETC or due to other effects such as a reduction in ATP levels and toxicity.

➡ *Changes in endothelial $[Ca^{2+}]_i$ might have a distinct but concomitant effect on $K_{Ca}$ opening and $H_2O_2$ release.*

Investigations were carried out to demonstrate whether $H_2O_2$ release is coupled to $K_{Ca}$ opening in the endothelium. Although the $H_2O_2$-dependent component was abolished in the presence of the triple combination of apamin, TRAM-34 and IbTX, the evidence provided in this study is not sufficient to demonstrate that inhibition of $K_{Ca}$ channels directly affects the release of $H_2O_2$ from the endothelium. Furthermore, it has been speculated that changes in $[Ca^{2+}]_i$ might have a concomitant but distinct effect on $K_{Ca}$ activation and the release of $H_2O_2$ from mitochondria. This notion is mainly supported

by two studies which demonstrated that: 1) in HUVECs inhibition of $K_{Ca}$ channels with TRAM-34 and ChTX significantly reduces the elevation in cytosolic free calcium (Sheng & Braun, 2007), and 2) in intact U937 cells the release of $H_2O_2$ from mitochondria is $Ca^{2+}$-dependent (Guidarelli *et al.*, 2006; 2007). It is therefore reasonable to consider that inhibition of $K_{Ca}$ channels in rabbit iliac arteries, could potentially affect $[Ca^{2+}]_i$ and subsequently the release of $H_2O_2$ from mitochondria. Future investigations should demonstrate the changes in $[Ca^{2+}]_i$ in rabbit iliac arteries.

## 4.5 Future directions

In the light of the aforementioned limitations, several experiments could be carried out to provide greater insights into the role $K_{Ca}$ channels and $H_2O_2$ in the EDHF phenomenon. First of all, the involvement of endothelial $BK_{Ca}$ channels in EDHF-type responses needs to be clarified further. One approach that could be used is to investigate the effects of IbTX on the development of endothelial hyperpolarizations. Microelectrode studies in endothelium-intact and endothelium-denuded iliac artery strips could demonstrate how inhibition of $BK_{Ca}$ channels might affect the development of endothelial hyperpolarizations or how removal of the endothelium would affect the membrane potential of the smooth muscle cells in the presence or absence of IbTX. Furthermore, studies carried out with IbTX in the presence of connexin-mimetic peptides (see section 1.2.1) could aim to demonstrate how changes in endothelial membrane potential might affect the membrane potential of the subjacent smooth muscle cells. Inhibition of gap junctions with connexin-mimetic peptides would prevent the propagation of depolarizations from the smooth muscle to the endothelium and therefore the development of endothelial hyperpolarizations would be unaffected by any changes in the membrane potential of the smooth muscle.

Secondly, studies should clarify further the role of $H_2O_2$ in both ACh- and A23187-evoked EDHF-type responses. For instance, mechanical investigations could be carried out to demonstrate whether higher concentrations of ATZ would completely restore the relaxation to A23187 in the presence of catalase. These experiments would exclude the possibility that the concentration of ATZ used in the current study might have been submaximal. Also, patch clamp investigations could be carried out in the presence of catalase and inhibitors of $K_{Ca}$ channels to demonstrate any direct effects of the enzyme

on the opening of $K_{Ca}$ in the rabbit iliac artery. If catalase directly affected these channels, changes in $K^+$ current would be detected and reversed in the presence of the $K_{Ca}$ inhibitors. However, if catalase had no effect, then the activity of the enzyme could be attributed entirely to the scavenging of endothelial $H_2O_2$.

Thirdly, a series of separate experiments could aim to elucidate the role of mitochondria as a source of $H_2O_2$ by demonstrating the effects of rotenone and myxothiazol in A23187-evoked responses in greater detail. For instance, mechanical investigations could be carried out with: 1) tone-matched controls in the presence or absence of rotenone and myxothiazol, and 2) the combination of connexin-mimetic peptides plus myxothiazol or the double combination of rotenone plus myxothiazol. These experiments would demonstrate whether the residual A23187-evoked relaxation in the presence of myxothiazol is a complex I-dependent component. In support of these investigations, confocal microscopy and flow cytometry with the novel fluoroprobe MitoSox Red could be carried out. MitoSox Red was introduced for the selective detection of $O_2^{\cdot-}$ in mitochondria (Mukhopadhyay *et al.*, 2007), and therefore it could be used to clarify the role of mitochondria in $H_2O_2$ release in rabbit iliac arteries. These investigations should be repeated with ACh so as to confirm the role of mitochondria in these responses.

Finally, imaging of cytosolic and mitochondrial $[Ca^{2+}]$ using fluo-4 and rhod-2 fluorescence could be used to determine how these concentrations change in the presence of A23187 and ACh. For instance, it has been shown that exposure of HepG2 cells to A23187 raises both concentrations simultaneously (Abramov & Duchen, 2003). Such changes need to be demonstrated in the rabbit in the presence or absence of $K_{Ca}$ inhibitors and connexin-mimetic peptides for both A23187- and ACh-evoked EDHF-type responses. Inhibition of gap junctions would ensure that: 1) no calcium diffuses from the intimal layer to the smooth muscle and vice versa, and 2) no electrotonic signalling occurs between the two layers. Therefore, any changes in $[Ca^{2+}]_i$ induced by A23187 and ACh in the presence of apamin, TRAM-34 and IbTX would be restricted to the endothelium only. Additionally, studies could be performed in rabbit iliac arteries to demonstrate that release of $H_2O_2$ from mitochondria is indeed $Ca^{2+}$-dependent. To achieve this, two separate mechanical investigations could be carried out to demonstrate how inhibition of the mitochondrial $Ca^{2+}$ uniporter and the permeability transition pore

(PTP) might affect A23187- and ACh-evoked EDHF-type relaxations, and specifically the $H_2O_2$-dependent component of these responses. Agents such as CGP37157 (an inhibitor of $Ca^{2+}$ uniporter) and CsA (an inhbitor of PTP) could be used in combination with $K_{Ca}$ inhibitors so as to demonstrate how changes in mitochondrial $Ca^{2+}$ loading might affect the function of $K_{Ca}$ channels. Imaging of cytosolic and mitochondrial $[Ca^{2+}]$ using fluo-4 and rhod-2 fluorescence could also be used to determine how these concentrations change in the presence of A23187, ACh, and the aforementioned inhibitors.

## 4.6 Concluding remarks

In conclusion, this thesis emphasized the role of $K_{Ca}$ activation on EDHF-type responses induced by both ACh and A23187. Furthermore, this study elucidated the putative role of $K_{Ca}$ opening on $H_2O_2$ release in the endothelium of rabbit iliac arteries and its role on the hyperpolarization-dependent component of EDHF-type relaxations upon stimulation with both agents. Although the findings of these investigations are not sufficient to totally support the fact that the $H_2O_2$-dependent relaxations might be a consequence of a hyperpolarizing response in the endothelium, they do provide further insights on the events that might take place. In accordance to the evidence collected in the previous chapters, the changes in $[Ca^{2+}]_i$ and their distinct effects on $K_{Ca}$ and $H_2O_2$ release by mitochondria might be a necessary step to be investigated next.

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163

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174

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181

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