CHARACTERISATION OF $P2X_7$ RECEPTORS IN HUMAN OSTEOBLASTS AND MODULATION BY OESTROGEN

A thesis submitted with the conditions governing
candidates for the degree of
PHILOSOPHIAE DOCTOR
in the **University of Cardiff**

Presented by

Sayed Mahmood Alqallaf *BPharm, MPharm*

Division of Pharmacology
Welsh School of Pharmacy
Cardiff University, U.K.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Acknowledgments

Studying towards the PhD degree was my dream since I was in high school, and I would not have been able to perform this big achievement without the help and support of countless people since my high school years. First of all, I would like to thank my great supervisors, Dr. Emma Kidd and Dr. Bronwen Evans, for their ideas, tremendous support and major influence on this thesis. I would like to thank them for their great assistance in reading and correcting this thesis and the papers we are going to publish, and for helping me to attend several interesting national and international conferences. I learned a lot of skills during this time and I am sure that this knowledge will help in my future. I wish to thank the Ministry of Health in Bahrain for their financial support during my PhD and Dr. Shawqi Ameen, the Dean of the College of Health Sciences in Bahrain, for his great support towards giving me the chance to achieve my dream of studying for a PhD.

I would like to thank all the people in the Pharmacology and Child Health labs especially Martina Fehler, Borzo Gharibi, Carole Elford and Lynne Murphy whom it was a pleasure for me to work with over the years. Additionally, I would like to thank the graduate students, postdocs and staff in the Pharmacology department which helped make my time in the PhD program more fun and interesting. I wish to thank my office mates Dr. Rhian Thomas and Dr. Amy Herbert, for their insights and comments, Dr. Jack Ham for donating human pancreas cell (BON-1) cDNA, and Veronica Thiongo for her help during her final year MPharm undergraduate project.

Finally, I thank my parents for nurturing in me the love of science and learning. Lastly but certainly not least, I would like to thank my wonderful wife, Ramla and children, Afaf, Sayed Hadi, Batool and my newborn baby Jenan Alzahra for their love, unlimited support, and patience with my long absences away from home.

Abstract

Purinergic $P2X_7$ receptors are ligand-gated ion channels characterized by the formation of a membrane pore upon continuous activation. This receptor has been shown to be expressed and functional in osteoclasts. $P2X_7$ receptor knockout mice studies suggested that this receptor has a role in bone formation. The aim of this project was to characterise the expression and function of $P2X_7$ receptors in human osteoblasts and to investigate the modulation of $P2X_7$ receptor expression and function by oestrogen and glucocorticoids. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting were used to study $P2X_7$ receptor expression at the mRNA and protein levels, respectively. $P2X_7$ receptor pharmacology was studied using pore formation and YO-PRO 1 influx in the presence of different agonists and antagonists. The effects of $P2X_7$ receptor activation on physiological aspects of osteoblast function, namely alkaline phosphatase (ALP) production, interleukin-6 (IL-6) and IL-1β release and mineralisation, were also studied.

The general findings are:

- Human osteoblasts (cell lines and primary cells) express functional $P2X_7$ receptors with higher expression seen in primary cells.
- $P2X_7$ receptor protein is expressed in all the osteoblast-like cell line samples used (lysate, nuclei, and membranes).
- The functional form of the $P2X_7$ receptor protein appears to correspond to the 67 kDa band and not the 85 kDa band as suggested by the Western blotting results.

- The pharmacology of $P2X_7$ receptors in human osteoblasts is atypical as evinced by the low affinity of the agonist, benzoyl-benzoyl-adenosine triphosphate (DBzATP) and the weak action of the antagonists, KN62 and brilliant blue-G (BBG).
- 17β-oestradiol and dexamethasone modulate $P2X_7$ receptor function probably by a non-classical, non-genomic mechanism.
- ALP production and IL-6 release are increased by $P2X_7$ receptor activation.
- $P2X_7$ receptor activation appears to decrease mineralisation in primary human osteoblasts.
- In addition to $P2X_7$ receptors, human osteoblasts express $P2X_4$ and $P2X_5$ receptors.

These findings suggest that $P2X_7$ receptors play an important role in osteoblast bone formation and in osteoblast-osteoclast signalling. Targeting $P2X_7$ receptors is a novel approach for the treatment of conditions such as osteoporosis and rheumatoid arthritis. However, further research is needed to develop selective agonists and antagonists.

List of abbreviations

ABC: ATP-binding cassette

ALP: Alkaline phosphatase

AMV: Avian myeloblastosis virus

ANOVA: Analysis of variance

ADP: Adenosine diphosphate

AEBSF: 4-(2-Aminoethyl)-bezenesulfonylfluoride.HCl

ALL: Acute Lymphoblastic leukaemia

AML: Acute Myelogenous Leukemia

AMP: Adenosine monophosphate

APS: Ammonium persulfate

ARRF: Activation–resorption–reversal–formation

ATP: Adenosine triphosphate

ATPγS: adenosine-5'-O-(3-thiotriphosphate)

BBG: Coomassie brilliant blue G

BMD: Bone mineral density

Bp: Base pairs

cAMP: Cyclic adenosine triphosphate

CCR: Chemokine receptor

cDNA: Complementary deoxyribonucleic acid

CLL: Chronic Lymphocytic Leukemia

CML: Chromic Myelogenous Leukemia

COX-1, 2: Cyclo-oxygenase-1, 2

Ct: Threshold cycle

D: Dalton

DBzATP: 2'- and 3'-O-(4benzoylbenzoyl) adenosine 5′-triphosphate triethylammonium salt

DMEM: Dulbecco's modified eagle's medium

dNTP: deoxynucleotides triphosphate

DVT: Deep vein thrombosis

DXA: Dual-energy X-ray absorptiometry

EBV: Epstein-Barr virus

EC_{50}: Effective concentration 50 %

ECACC: European Collection of Cell Cultures

EDTA: Ethylene diamine tetra acetic acid

EGTA: 1,2-di(2-1 aminoethoxy)ehane-N,N,N',N'-tetra-acetic acid disodium salt

ELISA: Enzyme linked immunosorbent assay

ER: Oestrogen receptor

ERT: Oestrogen Replacement Therapy

FCS: Foetal calf serum

FPT: Fracture prevention trial

GABA: γ-aminobutyric acid

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GI: Gastro-intestinal

GPCR: G protein-coupled receptor

GR: Glucocorticoid receptor

HEK293: Human embryonic kidney 293 cells

HEK7: HEK293 cells transfected with the $P2X_7$ receptor

HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid

HMA: 5- (N,N – Hexamethylene) – Amiloride

HOB: Human primary osteoblasts

HRP: Horseradish peroxidase

HRT: Hormone replacement therapy

icIL-1ra: Intracellular interleukin-1 receptor antagonist type-1

IgG: Immunoglobulin G

IL: Interleukin

IP3: Inositol triphosphate

KN62: 4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo- 3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

M-CSF: Macrophage-colony stimulating factor

MDS: Myelo-Dysplastic Syndrome

αβMeATP: αβMethylene adenosine triphosphate

βγmeATP: βγMethylene adenosine triphosphate

2Me-S-ATP: 2-methylthio- adenosine 5′-triphosphate tetrasodium salt

NANC: Non-adrenergic, non-cholinergic

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NDP: Nucleotide di-phosphate

NF279: 8,8′-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)) bis (1,3,5-naphthalenetrisulfonic acid)

NMDG: N-methyl-D-glucamine

NMP: Nucleotide mono-phosphate

NP-40: Tergitol

NSAID: Non-steroidal anti-inflammatory drug

NTP: Nucleotide tri-phosphate

oATP: Adenosine 5′-triphosphate periodate oxidized sodium salt

OD: Optical density

OPG: Osteoprotegrin

PCR: Polymerase chain reaction

PBS: Phosphate buffered saline

PBST: Phosphate-buffered saline with Tween

PGH2, E2: Prostaglandin H2, E2

PLC: Phospholipase C

PMSF: Phenylmethylsulfonyl fluoride

P5P: pyridoxal-5'-phosphate

PPADS: Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate

PTH: Parathyroid hormone

PTHrP: Parathyroid hormone-related peptide

QPCR: Quantitative PCR

RANK: Receptor activator of nuclear factor Kappa-B

RANKL: Receptor activator of nuclear factor Kappa-B ligand

RA: Rheumatoid arthritis

RFU: relative fluorescence unit

RNA: Ribonucleic acid

RT: Reverse transcriptase

SARM: Selective androgen receptor modulators

SDS: Sodium dodecyl sulphate

SEM: Standard error of mean

SERM: Selective oestrogen receptor modulator

SLE: Systemic lupus erythematosus

SOTI: Spinal Osteoporosis Therapeutic Intervention

TBE: Tris base, boric acid, EDTA

TBST: Tris-buffered saline with Tween-20

TEMED: N,N,N',N'-Tetramethylethylenediamine

TM: Transmembrane domain

TNF-α: Tumour necrosis factor-α

TROPOS: treatment of peripheral osteoporosis

TUNEL: Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling

UDP: Uridine diphosphate

UTP: Uridine triphosphate

YO-PRO 1: Quinolinium 4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio) propyl]- diiodide

Chapter 1: General Introduction

1.1 Adenosine triphosphate

Adenosine triphosphate (ATP) is synthesized in the mitochondria from adenosine diphosphate (ADP) and inorganic phosphate under the influence of ATP synthase (Schon et al., 2001). ATP synthase consists of two segments, F_0 and F_1 (Pedersen et al., 2000; Schon et al., 2001; Senior et al., 2002), where the former consists of at least 7 subunits and the latter consists of 6 subunits (Schon et al., 2001). The F_0 segment is found in the mitochondrial inner membrane and is responsible for proton transport from the intermembrane spaces to the mitochondrial matrix (Schon et al., 2001; Senior et al., 2002). The F_1 segment is found in the matrix and utilizes the protons transported by the F_0 segment to convert ADP to ATP (Schon et al., 2001). The exact detail of ATP synthesis is still not clear, but it is believed to be complicated and involve conformational changes and/or rotations of different subunits of ATP synthase (Pedersen et al., 2000; Schon et al., 2001; Senior et al., 2002). It has been suggested that the pathway of ATP synthesis and hydrolysis is common (Pedersen et al., 2000; Schon et al., 2001; Senior et al., 2002; Weber and Senior, 2003) and that the F_1 segment is responsible for the hydrolysis (Pedersen et al., 2000; Weber and Senior, 2003).

The relationship between the different purinergic receptors arises from the relationship between their natural ligands such as ATP, which is metabolized by ectoenzymes to ADP, then to adenosine monophosphate (AMP), and finally to adenosine which is taken up by the cell to resynthesize ATP (Lazarowski et al., 2003; Schwiebert and Zsembery, 2003; Bours et al., 2006) as shown in Figure 1.1.

Due to the role of ATP as the intracellular energy source, it is believed to be available in every cell (Bours et al., 2006). The intracellular ATP

concentration is approximately 3-10 mM, but the extracellular concentration is much lower (Schwiebert and Zsembery, 2003; Bours et al., 2006). Normal ATP concentrations in the plasma have been reported to be 400-700 nM (Bours et al., 2006). Large amounts of ATP are released non-lytically (Pellegatti et al., 2005; Ferrari et al., 2006) possibly by ATP-binding cassette (ABC) transporters (Schwiebert and Zsembery, 2003; Gallagher, 2004). Another pathway of ATP release is believed to be by cell lysis following cell injury (Gartland et al., 2003a; Ferrari et al., 2006) which causes its release in very high concentrations (1-5 mM) resulting in cell apoptosis (Gartland et al., 2003a). In osteoblasts intracellular ATP concentration has been reported to be approximately 3-5 mM (Gallagher and Buckley, 2002).

Extracellular ATP has been implicated in many functions including immunity and inflammation (Bours et al., 2006), as a neurotransmitter (Cunha and Ribeiro, 2000; Khakh and North, 2006; Burnstock, 2006b) and as a co-neurotransmitter with many other neurotransmitters (Cunha and Ribeiro, 2000; Burnstock, 2006b). The modulatory action of ATP on inflammatory and immune processes is believed to involve many immune cells such as neutrophils, monocytes and macrophages and non-immune cells (Bours et al., 2006). The ATP metabolite, adenosine, is believed to have similar effects to ATP on inflammatory and immune processes (Bours et al., 2006). In the heart, ATP was found to have a positive inotropic effect and induced various types of arrhythmias when applied rapidly to cells (Vassort, 2001). In epithelial cells, ATP has been shown to have many physiological effects. An example of this is in the lung, where ATP was found to increase the release of chloride in cystic fibrosis patients, which made purinergic receptors a potential approach for cystic fibrosis therapy (Schwiebert and Zsembery, 2003). In the ear, ATP was found to regulate hearing sensitivity and development and was

implicated in the treatment of Meniere's disease and tinnitus (Burnstock, 2006b).

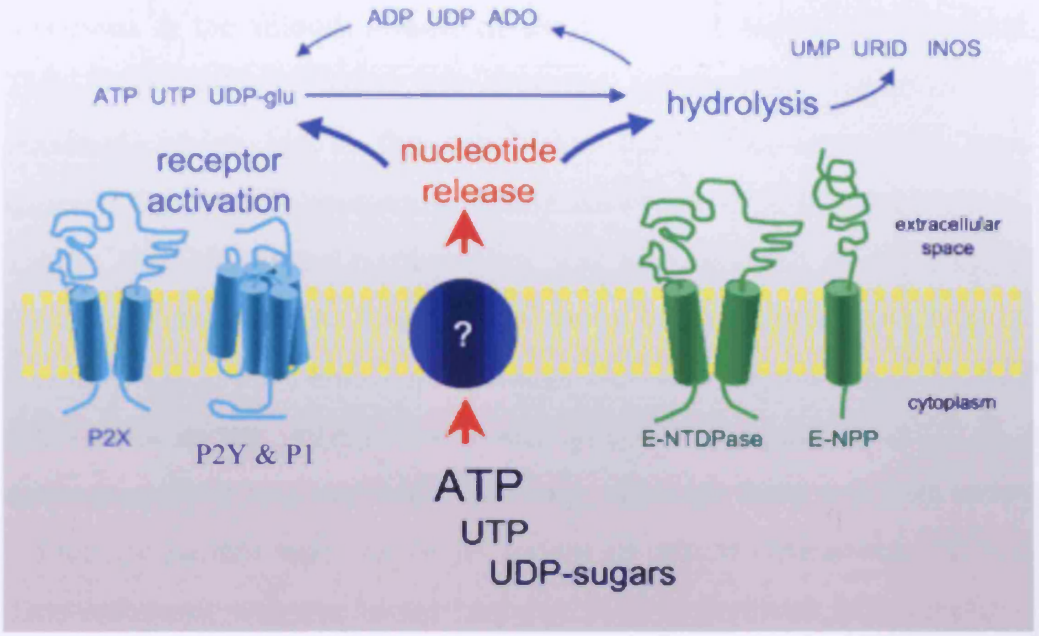


Figure 1.1: Cellular release and fate of nucleotides

A diagram showing the cellular release of nucleotides, their metabolism, and their receptors. Nucleotides are believed to be released via lytical or non-lytical mechanism. ATP, UTP, and UDP-sugar stimulate P2Y receptors and ATP stimulates P2X receptors. E-NTPDases degrade nucleotides tri-phosphate (NTPs) to nucleotides di-phosphate (NDPs), and then to nucleotides mono-phosphate (NMPs). Adenosine, which is produced from ATP, stimulates adenosine receptors. ADO, adenosine; E-NTPDases Ecto-Nucleotidase 5'-Triphosphate Diphosphohydrolase; E-NPP, Ecto-phosphodiesterase / Nucleotide Pyrophosphate; UDP-glu, UDP-glucose; URID, uridine; INOS, inosine. Adapted from Lazarowski, et al (2003).

1.2 History of purinergic receptors

The physiological actions of ATP were discovered as early as 1929, when ATP and adenosine were found to have an effect on the heart and coronary blood vessels (Drury and Szent-Györgyi, 1929). The idea of ATP as a neurotransmitter started when acetylcholine and noradrenaline responses in the smooth muscle of the guinea pig taenia were blocked. This block did not cause the expected contractions, but relaxation occurred, which led to the conclusion that a non-adrenergic, non-cholinergic (NANC) neurotransmitter must be involved (Burnstock et al., 1963). This NANC neurotransmitter was also reported in cat stomach (Martinson and Muren, 1963). After many experiments in a variety of animal organs, the NANC neurotransmitter was suggested to be ATP (Burnstock et al., 1970). For many years acceptance of ATP as a neurotransmitter was met with resistance, although there was very strong evidence that this was one of its modes of action (Burnstock, 2006b). This resistance was due to the fact that ATP is involved in intracellular biochemical processes, and was thought unlikely to have an extracellular role (Burnstock, 2007). In addition to acting as a neurotransmitter, ATP was suggested to act as a co-neurotransmitter in sympathetic and parasympathetic nerves (Burnstock, 1976; Sneddon and Burnstock, 1984; Cunha and Ribeiro, 2000; Burnstock, 2006b). The proportion of ATP and noradrenaline or acetylcholine was found to be tissue- and species-dependent (Burnstock, 2006b). ATP was reported to act as a co-neurotransmitter with many other neurotransmitters including, 5-hydroxytryptamine, glutamate, dopamine, and γ-aminobutyric acid (GABA) (Cunha and Ribeiro, 2000; Burnstock, 2006b).

In 1978 the first description of purinergic receptors and their subdivision into two main classes, P1 and P2 activated by adenosine and ATP,

respectively was published (Burnstock, 1978) and then the two P2 subtypes, X and Y were distinguished (Burnstock and Kennedy, 1985). However, distinction of the different purinergic receptors was originally based on their pharmacological properties (Burnstock, 2006b). An important stage in the history of purinergic receptors was the cloning of P2X receptors which occurred in the mid 1990s (Gever et al., 2006). The first P2X receptor complementary deoxyribonucleic acid (cDNA) cloned was in 1994 (Valera et al., 1994), the $P2X_4$ receptor cDNA in 1996 (Wang et al., 1996), and the $P2X_7$ receptor cDNA later in 1996 (Suprenant et al., 1996). Cloning of some of the P2Y members occurred in the 1990s (Webb et al., 1993; Tokuyama et al., 1995) and others in the early 2000s (Zambon et al., 2000; Zambon et al., 2001). Figure 1.2 shows the most important stages in the discovery of P2X receptors.

The $P2X_7$ receptor was formerly known as P2Z since it was thought to belong to a different family than the P2X (Suprenant et al., 1996). This was based mainly on the characteristic pore formation (discussed later in Chapter 4) observed first with this receptor and later with other P2X receptors namely $P2X_2$ and $P2X_4$ (Di Virgilio, 1995).

Nowadays, research is concentrating on the production of more potent and selective purinergic agonists and antagonists for effective and safe use in humans. For example P2X receptor antagonists are in different stages of research in a variety of areas (Stokes et al., 2006; Donnelly-Roberts and Jarvis, 2007; King, 2007) such as analgesics (Donnelly-Roberts and Jarvis, 2007; King, 2007).

The A1 and A2 adenosine (originally called P1) receptors were first described in 1979 based on their pharmacology (Van Calker et al., 1979). Adenosine receptors were cloned in the late 1980s and early 1990s

(Ralevic and Burnstock, 1998). For example, the A_{2A} adenosine receptor cDNA was cloned in 1989 (Libert et al., 1989) and the A_1 adenosine receptor cDNA in 1991 (Mahan et al., 1991).

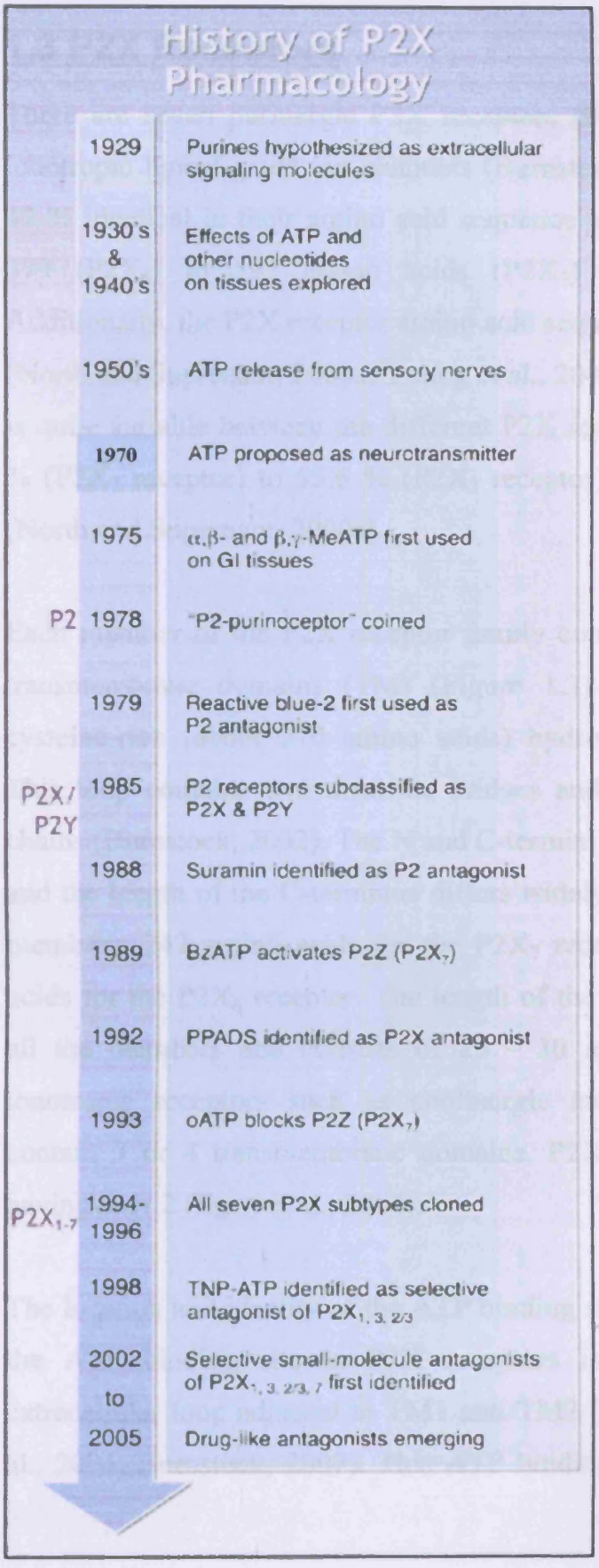


Figure 1.2: A summary of the history of P2X receptor pharmacology.
A diagram showing a timeline of the discovery of P2 receptors and some of the compounds acting on them. Adapted from Gever, et al (2006).

1.3 P2X Receptors

There are seven purinergic P2X receptors ($P2X_1$ – $P2X_7$) and all are ionotropic ligand-gated ion channels (Burnstock, 2007). They are 26 to 47 % identical in their amino acid sequence and their sizes range from 379 ($P2X_6$) to 595 amino acids ($P2X_7$) (Khakh et al., 2001b). Additionally, the P2X receptor amino acid sequence is species-dependent (North and Suprenant, 2000a; Young et al., 2007). The species homology is quite variable between the different P2X receptors ranging from 79.7 % ($P2X_7$ receptor) to 95.6 % ($P2X_3$ receptor) between human and rat (North and Suprenant, 2000a).

Each member of the P2X receptor family consists of two hydrophobic transmembrane domains (TM) (Figure 1.3) attached together by a cysteine-rich (about 270 amino acids) hydrophilic extracellular loop. This loop contains two disulfide bridges and three N-linked glycosyl chains (Burnstock, 2002). The N and C-termini are located intracellularly and the length of the C-terminus differs widely between the P2X family members; 242 amino acids for the $P2X_7$ receptor, but only 28 amino acids for the $P2X_6$ receptor. The length of the N-terminus is similar for all the members and consists of 28 – 30 amino acids. While other ionotropic receptors such as cholinergic and serotonergic receptors contain 3 or 4 transmembrane domains, P2X receptors are unique in having only 2 (Egan et al., 2004).

The location and identity of the ATP binding site is debatable. However, the ATP binding site in P2X receptors is believed to be in the extracellular loop adjacent to TM1 and TM2 (Burnstock, 2004; Egan et al., 2004; Burnstock, 2007). This ATP binding site could be a positive

charge loci in the extracellular loop attracting the negative charge of the phosphate group of the agonists (Egan et al., 2004).

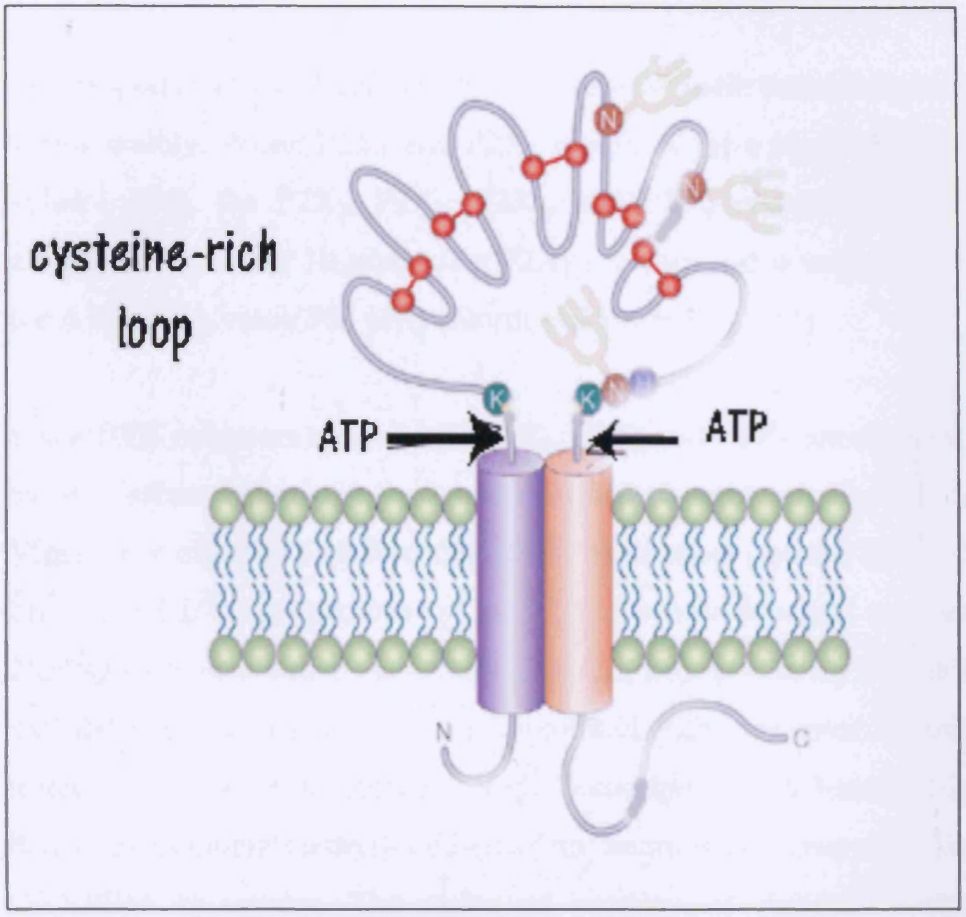


Figure 1.3: General Topology of the P2X receptors

Illustration describing the general topology of P2X receptors showing the N- and C-termini, two transmembrane domains attached together by a cysteine-rich (270 amino acids) hydrophilic extracellular loop containing disulfide bridges and three N-linked glycosyl chains. Adapted from Khakh, et al (2001a) and Burnstock (2002). The ligand binding site is believed to be in the extracellular loop adjacent to TM1 and TM2 (Burnstock, 2004; Egan et al., 2004; Burnstock, 2007).

Although all the P2X receptors show non-selective permeability to small cations such as sodium and potassium, some of them have considerable permeability to calcium. This feature is more pronounced if the receptor

is activated repeatedly or for a prolonged time as is the case with the $P2X_7$ receptor as it becomes permeable to large molecules as big as 900 Dalton (D) on repetitive activation (North, 2002).

The response of the P2X family members to their natural ligand ATP varies widely. While $P2X_1$ and $P2X_3$ receptors have high affinity (EC_{50} value:1 μM), the $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$ receptors have lower affinity (EC_{50} value 10 μM). The $P2X_7$ receptor has a very low affinity for ATP (EC_{50} value 300 μM) (North and Barnard, 1997).

Some P2X receptors such as the $P2X_2$, $P2X_4$, and $P2X_7$ are characterized by the effect of monovalent and/or divalent ions on their function. Virginio et al (1997) studied the effect of divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+}) and protons on rat $P2X_7$ receptor function activated by 2',3'-O-(4-benzoyl-benzoyl)-ATP (DBzATP). In this study it was found that divalent cations and protons inhibited $P2X_7$ receptor function as tested by whole-cell currents and Quinolinium 4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio) propyl]- diiodide (YO-PRO 1) uptake. The order of potency of divalent cations in inhibiting $P2X_7$ receptor function was reported to be: $Cu^{2+} > Cd^{2+} = Zn^{2+} > Ni^{2+} >> Mg^{2+} = Co^{2+} > Mn^{2+} > Ca^{2+} > Ba^{2+} >> Sr^{2+}$(Virginio et al., 1997). Michel et al (1999) have shown that the response of the $P2X_7$ receptor to the agonists ATP and DBzATP was decreased by monovalent and divalent cations (Mg^{2+}, Ca^{2+}, Cu^{2+}, and Na^{+}), monovalent anions (Cl^{-}), and by changes in pH (Michel et al., 1999). Xiong et al (1999) found that Cu^{2+} and Zn^{2+} increased ATP-induced currents mediated by $P2X_2$ and $P2X_4$ receptors (Xiong et al., 1999).

One possible explanation for the inhibitory effect of divalent cations on P2X receptor activation by agonists such as ATP and DBzATP may be

that these compounds bind the divalent cations leading to decreased or abolished activity of the agonists (Michel et al., 1999). However, this may not be correct as Xiong et al (1999) reported that ATP-induced currents mediated by $P2X_2$ and $P2X_4$ receptors increased in the presence of Cu^{2+} and Zn^{2+}. Another suggested explanation is that these cations have a direct modulatory effect on P2X receptors (Virginio et al., 1997).

Many P2X receptor heteromultimers have been recognised. Examples are $P2X_{1/2}$, $P2X_{1/4}$, $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$, and $P2X_{4/6}$, and it has been suggested that these cause the difference in the observed P2X pharmacology between transfected and native cells (Burnstock, 2007). One of the extensively studied P2X heteromultimers is the $P2X_{2/3}$ (Ueno et al., 1998; Koshimizu et al., 2002) and it has been found to have some of the properties of the $P2X_2$ receptor and other properties of the $P2X_3$ receptor (North, 2002). For example, it was found that the $P2X_{2/3}$ receptor is activated by αβmeATP, a property contributed by the $P2X_3$ subunit, and potentiated by low pH, a property contributed by the $P2X_2$ subunit (North and Suprenant, 2000a). The $P2X_{2/3}$ receptor was reported to be expressed by some neurons and sympathetic ganglion cells (North, 2002; Burnstock, 2007). The $P2X_6$ receptor exists only in a heteromultimeric form (Burnstock, 2004; Egan et al., 2004; Jacobson et al., 2004; Burnstock, 2007). $P2X_7$ receptors were believed not to form heteromultimers (Burnstock, 2004; Egan et al., 2004; Jacobson et al., 2004; Burnstock, 2007) but a recent study reported that $P2X_7$ and $P2X_4$ receptors might form heteromultimers together (Guo et al., 2007).

P2X receptors are widely distributed and are expressed by many body tissues such as neurons, muscles, bones, and epithelia (North, 2002). For example, neurons were found to express $P2X_2$, $P2X_3$ and $P2X_4$ receptors (North, 2002; Burnstock, 2007), while smooth muscles were found to

express $P2X_1$ and $P2X_2$ receptors (Burnstock, 2007). Heart was found to express $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_5$ receptors, while $P2X_7$ receptor was implicated in airway epithelium and salivary glands (North, 2002). The distribution and physiological function of purinergic receptor in bone cells will be discussed later in this introduction (Section 1.7).

1.4 $P2X_7$ Receptor

The $P2X_7$ receptor is the largest member of this family (595 amino acids) and has the longest C-terminus (242 amino acids) (Khakh, 2001a). It is 39.2 to 49.8 % identical in its amino acid sequence with other P2X receptors (North, 2002). Differences in the $P2X_7$ receptor amino acid sequence were observed between different species, where for example human and rat receptors have a 79.7 % identical amino acid sequence (North and Suprenant, 2000a). This was suggested as an explanation for the differences in response to agonists and antagonists between different species (Young et al., 2007).

An important characteristic of the $P2X_7$ receptor is the formation of a non-selective pore in the cell membrane permeable to molecules as large as 900 D after prolonged activation. The characteristics of this $P2X_7$ receptor pore formation and its physiological effects are discussed in detail in Chapters 4 and 5. The $P2X_7$ receptor has been described as a death receptor, because cell death by either necrosis or apoptosis resulting from its stimulation has been reported in many studies (Di Virgilio et al., 1998; Sluyter and Wiley, 2002; Wang et al., 2004a). This cell death was found to have the characteristics of both necrosis and apoptosis, therefore it is sometimes called "aponecrosis" (Elliott et al., 2005).

The P2X$_7$ receptor has been found to play an important role in immune and inflammatory responses (Di Virgilio et al., 2001; Bours et al., 2006). An important effect of P2X$_7$ receptors on inflammatory process is on cytokine release (Di Virgilio et al., 2001; Bulanova et al., 2005; Bours et al., 2006; Hughes et al., 2007). The P2X$_7$ receptor was described as the cytokine release pore (Ferrari et al., 1997), because its stimulation induced the release of many cytokines such as interleukin (IL)-1β, IL-4, IL-6, IL-13, and tumour necrosis factor-α (TNF-α) (Ferrari et al., 1997; Bulanova et al., 2005; Di Virgilio, 2007; Hughes et al., 2007; Qu et al., 2007).

1.4.1 P2X$_7$ receptor agonists

Although ATP is the natural ligand of the P2X receptors, and is the most potent of the nucleotide tri-phosphates (NTPs), the response of the P2X$_7$ receptor to it is very weak (EC_{50}= 300 μM) (King and Townsend-Nicholson, 2003). In fact DBzATP was reported to be the most potent P2X$_7$ agonist (EC_{50}= 3 μM) (North and Suprenant, 2000a). In addition to P2X$_7$ receptors, other P2X receptors are also activated by DBzATP namely P2X$_1$, P2X$_2$, and P2X$_5$, with EC_{50} values of: 3, 30, and >300 μM, respectively. As mentioned earlier the presence of extracellular divalent cations affects the activity of the agonists, which makes comparisons of agonist potencies between studies difficult (North and Suprenant, 2000b).

ADP, adenosine 5'-*O*-(3-thiotriphosphate) (ATPγS), and 2-methylthio-adenosine 5'-triphosphate tetrasodium salt (2Me-S-ATP) when tested were found to be less effective compared to ATP, while adenosine, αβmeATP, βγMethylene adenosine triphosphate (βγmeATP), and uridine

triphosphate (UTP) were found to be ineffective at 300-1000 μM (North and Suprenant, 2000b).

1.4.2 $P2X_7$ receptor antagonists

One of the most important difficulties in studying the $P2X_7$ receptor is the lack of selective antagonists. Many antagonists have been studied, but none were found to be selective (North and Suprenant, 2000a). Divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} were found to have an inhibitory effect on $P2X_7$ receptor agonists and hence were regarded as blockers (North, 2002).

The suramin analogue, 8,8'-(carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)) bis (1,3,5-naphthalenetrisulfonic acid) (NF279) was reported to be a highly effective antagonist against the human $P2X_1$ receptor, but has moderate potency at human $P2X_7$ receptors with IC_{50} values of 0.05 μM and 2.8 μM, respectively (Klapperstuck et al., 2000).

Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) and 4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo- 3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester (KN62) were found to have partially reversible antagonist activity, but the effect of adenosine 5'-triphosphate periodate oxidized sodium salt (oATP) was irreversible. IC_{50} values were found to be 30 – 100 nM for KN62, and 50 μM for PPADS (the PPADS IC_{50} value was much lower against other P2X receptors) (Humphreys et al., 1998; Michel et al., 2000; North and Suprenant, 2000b). Humphreys et al (1998) found that the rat $P2X_7$ receptor was not blocked by KN62, while it fully blocked the action of ATP on human receptors, but only partially

that of DBzATP (Humphreys et al., 1998). KN62 was found to be 500-fold more selective for human than rat receptors (Hibell et al., 2001b).

In addition to their findings of the substantial species selectivity of the $P2X_7$ receptor antagonists, Hibell et al (2001) also documented the effect of the experimental conditions on the antagonist's potency, as they studied agonist concentration, temperature, and buffer composition. This study found that the activity of oATP, KN62 and Coomassie Brilliant Blue G (BBG) on the human $P2X_7$ receptor is reduced at 22°C. Additionally PPADS, pyridoxal-5'-phosphate (P5P), oATP and suramin were found to have 3- to 10-fold lower activity in sodium chloride (NaCl) than in sucrose buffer (Hibell et al., 2001b).

The effect of several antagonists has been compared using whole cell patch clamp, and the following rank order of antagonist potency on the human P2X receptor was found: KN62 > Calmidazolium > PPADS = Cu^{2+} > Suramin = 5- (N,N – Hexamethylene) – Amiloride (HMA) (Chessell et al., 1998).

BBG was regarded as a selective antagonist as it succeeded in inhibiting the current, YO-PRO 1 uptake, and membrane blebbing resulting from $P2X_7$ receptor stimulation at nanomolar concentration (IC_{50} values: 10 and 200 nM for rat and human receptors, respectively). $P2X_1$, $P2X_2$, $P2X_3$, $P2X_{2/3}$, $P2X_4$, and $P2X_{1/5}$ receptors were tested and BBG was found to be either ineffective or much higher concentrations in the micromolar range were needed to block activity (Jiang et al., 2000).

Many other new antagonists have been studied utilizing $P2X_7$ receptor pore formation, and claimed to be potent such as the 4,5-diarylamines,

where one compound has an IC_{50} value of 0.01 μM, but their selectivity needs to be established (Alcaraz et al., 2003; Merriman et al., 2005).

1.4.3 Tissue distribution of $P2X_7$ receptors

$P2X_7$ receptors were initially only found in haemopoietic cells but now they have been identified in other cells as well (Gartland et al., 2001). The $P2X_7$ receptor is often found internally in cells, but tends to become externalized in pathological conditions like cancer (Burnstock, 2004).

In human blood the $P2X_7$ receptor was reported to be expressed by the following cells: macrophages, dendritic cells, B-lymphocytes, T-lymphocytes, polymorphonuclear cells, and erythrocytes (Di Virgilio et al., 2001; Volonte et al., 2006). In addition to macrophages, expression of $P2X_7$ receptors in microglia, brain, spinal cord, lung and spleen has been reported by some other studies (Suprenant et al., 1996; Chiozzi et al., 1997). Collo et al 1997 studied the distribution of $P2X_7$ receptor in hemopoietic cells using Northern blotting, in situ hybridization and immunohistochemistry. This study showed expression of the $P2X_7$ receptor in cells derived from myeloid progenitors (granulocytes and monocytes). B cells and a subset of T cells were also found to be positive for the receptor ribonucleic acid (RNA) (Collo et al., 1997). Murine mast cells were found to express the $P2X_7$ receptor (Bulanova et al., 2005).

To examine the role of the $P2X_7$ receptor in pathological blood cells, Zhang et al., (2004) studied its expression in human leukaemic cells. It was found that the relative receptor expression levels were significantly higher in acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), and myelo-dysplastic syndrome (MDS) groups compared to the normal group.

Interestingly, after one induction therapy regimen, the remission rate in the high $P2X_7$ expression group was lower than those in negative or low expression groups suggesting its involvement in disease pathogenesis (Zhang et al., 2004). In chronic lymphocytic leukemia (CLL) a single nucleotide polymorphism in the $P2X_7$ receptor gene causes replacement of glutamic acid with alanine at amino acid position 496, and results in loss of receptor function. It was reported that this polymorphic mutation could be inherited and could contribute to the disease pathogenesis, and its clinical outcome (Thunberg et al., 2002; Wiley et al., 2002).

In non-haemopoietic cells, expression of $P2X_7$ receptors in epithelial tissues (namely airway epithelium, salivary gland, exocrine pancreas, anterior pituitary gland, and skin) was shown either at the mRNA level, or implied from the pharmacological evidence (North, 2002). Based on pharmacological properties, the $P2X_7$ receptor was found to be expressed in the toad stomach (North, 2002). In addition, the receptor is expressed in human intestinal epithelial carcinoma cells (Coutinho-Silva et al., 2005), and human epidermal and monocyte-derived Langerhans cells (Georgiou et al., 2005).

In addition to hemopoietic cells, Collo et al. (1997) studied the expression of the $P2X_7$ receptor in the rat nervous sytem, and found that it is expressed in the brain by microglia and ependymal cells. Expression of $P2X_7$ receptors in rat neurons was also documented (Deuchars et al., 2001). Kobayashi et al (2005) reported expression of $P2X_7$ mRNA by glial cells in the rat lumbar dorsal root ganglion (Kobayashi et al., 2005).

Additionally, functional receptor was found to be expressed by human melanoma cells and receptor activation caused their apoptosis (White et al., 2005).

Human fibroblasts from diabetic patients were found to have enhanced $P2X_7$ receptor responses (Solini et al., 2004). Enhanced receptor responses were also found in diabetic retinal microvasculature, and suggested to be the mechanism of diabetic retinopathy (Sugiyama et al., 2004).

Expression of $P2X_7$ receptor on the nucleus and then in the cytoplasm of prostate tissue was found to suggest prostate cancer in its early stage. The $P2X_7$ receptor labelling was proposed as a method to detect prostate cancer and to monitor progression of the disease and its treatment (Slater et al., 2005). Similarly, the $P2X_7$ receptor was suggested as a good marker for human thyroid cancer (Solini et al., 2008).

The distribution and physiological function of $P2X_7$ receptor in bone cells will be discussed later in this introduction (Section 1.7).

1.5 Other purinergic receptors

1.5.1 Adenosine Receptors

The adenosine or P_1 receptor family consists of four members (A_1, A_{2A}, A_{2B}, and A_3). They are members of the rhodopsin – like G protein-coupled receptor (GPCR) family (King and Townsend-Nicholson, 2003; Burnstock, 2007). Their sizes range between 318 and 412 amino acids (Ralevic and Burnstock, 1998; King and Townsend-Nicholson, 2003). They are 39-61% identical in amino acid sequence and share 11-18 % identity with P2Y receptors (King and Townsend-Nicholson, 2003; Burnstock, 2007). Adenosine receptors are coupled mainly to activation or inhibition of adenylate cyclase (Burnstock, 2007). Adenosine receptors are widely distributed, and have been proven to modulate many

of the body's systems, such as the cardiovascular, immune, skeletal and central nervous systems (King and Townsend-Nicholson, 2003; Evans et al., 2006; Burnstock, 2006b).

1.5.2 P2Y Receptors

These are GPCR composed of seven transmembrane domains. As with all GPCRs the ligand binding site is in the N-terminus, and available extracellularly, while the C-terminus is available intracellularly (King and Townsend-Nicholson, 2003; Burnstock, 2006a; Burnstock, 2007). They are 15-65 % identical in their amino acid sequence and their sizes range between 328 and 538 amino acids (von Kugelgen and Wetter, 2000; King and Townsend-Nicholson, 2003). It is believed that several amino acids in transmembrane domains 3, 5, 6, and 7 are involved in ligand binding (von Kugelgen and Wetter, 2000).

This family consists of eight members, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, which are all present in human tissues (Sak and Webb, 2002; Hoebertz et al., 2003; Burnstock, 2007). $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are coupled to phospholipase C (PLC) resulting in the formation of inositol triphosphate (IP3). $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors are negatively coupled to adenylate cyclase. $P2Y_{11}$ receptor is coupled to both PLC and adenylate cyclase leading to increases in cyclic adenosine triphosphate (cAMP) (Volonte et al., 2006).

They are widely distributed in the human body including the heart ($P2Y_1$, $P2Y_2$, $P2Y_6$), skeletal muscles ($P2Y_1$, $P2Y_2$), lung ($P2Y_2$, $P2Y_4$, $P2Y_6$), and spleen ($P2Y_2$, $P2Y_6$, $P2Y_{11}$) (von Kugelgen and Wetter, 2000).

ATP may act either as an agonist or antagonist at the human $P2Y_1$ receptor. Conversely, it is a pure agonist at the $P2Y_1$ receptor in other species (Sak and Webb, 2002). Some members are activated by nucleotide diphosphates (such as ADP for $P2Y_1$ and uridine diphosphate (UDP) for $P2Y_6$), while others by nucleoside triphosphates (such as ATP and UTP for $P2Y_2$ and UTP for $P2Y_4$) (von Kugelgen and Wetter, 2000; King and Townsend-Nicholson, 2003).

1.6 Bone biology

1.6.1 Bone Composition

Bone composition varies according to anatomical site, age, and dietary and health status (Bostrom et al., 2000; Sommerfeldt and Rubin, 2001; Baron, 2003). Generally bone is composed of 60-70 % mineral or inorganic phase, 5-8 % water, and the rest is organic matrix. Collagen type I is the main constituent of the organic matrix and accounts for about 90 %, while the remainder is non-collagenous protein. Examples of non-collagenous proteins found in bone are osteonectin, osteopontin, osteocalcin, and bone sialoprotein. Some of these non-collagenous proteins may be produced by bone cells, and others may be produced in other organs and absorbed by the bone matrix. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) makes up the inorganic phase and may contain some impurities such as carbonate, magnesium, and fluoride. These impurities can substitute for the calcium, phosphate, or hydroxyl groups of the hydroxyapatite leading to a major change in some of its physical properties and thus affecting its normal function (Bostrom et al., 2000; Sommerfeldt and Rubin, 2001; Baron, 2003). The mineral content of bone provides its mechanical rigidity and load bearing strength, while the

collagen provides bone elasticity and flexibility (Robey and Boskey, 2006).

Bone can be divided into two types, cortical bone (also called compact bone (Seeley et al., 2008a)) representing approximately 80 % of the adult skeleton, and cancellous bone (also called trabecular bone (AMA, 2004; Slomianka, 2006)) representing approximately 20 % of the adult skeleton (Figure 1.4) (Dempster et al., 2006). However, the ratio of cancellous: cortical bone is widely variable between different bones (Felsenberg and Boonen, 2005; Dempster et al., 2006). It is estimated that this ratio is approximately 75:25 in vertebrae, but 50:50 in the femoral head (Dempster et al., 2006). The first difference between these two types of bones is that cortical bone is dense, solid, and has more bone matrix and less space while cancellous bone is spongy and has more space and less matrix with a honey comb-like network (Dempster et al., 2006; Zaidi, 2007; Seeley et al., 2008b). The second difference is in the function of the two types of bone. Cancellous bone provides bone scaffolding without extra weight and is considered to be more metabolically active (Dempster et al., 2006; Seeley et al., 2008a), while cortical bone provides support and is involved in mechanical loading (Felsenberg and Boonen, 2005; Dempster et al., 2006; Zaidi, 2007; Seeley et al., 2008a). However, their functions are believed to be species- and situation-dependent (Dempster et al., 2006).

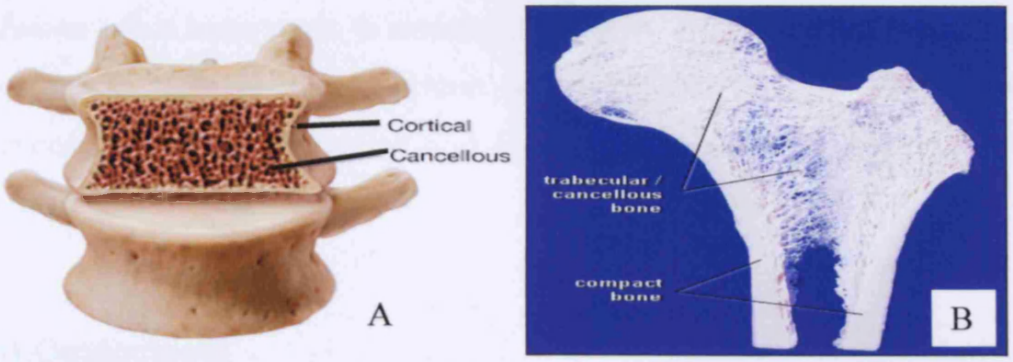


Figure 1.4: Bone architecture

A diagram showing the two types of bones; cortical (compact), and cancellous (trabecular). A adapted from AMA (2004) and B adapted from Slomianka (2006).

1.6.2 Bone Cells

Bone has three major types of bone cells; osteoclasts, osteoblasts, and osteocytes (Arnett and colleagues, 2004) (Figure 1.5).

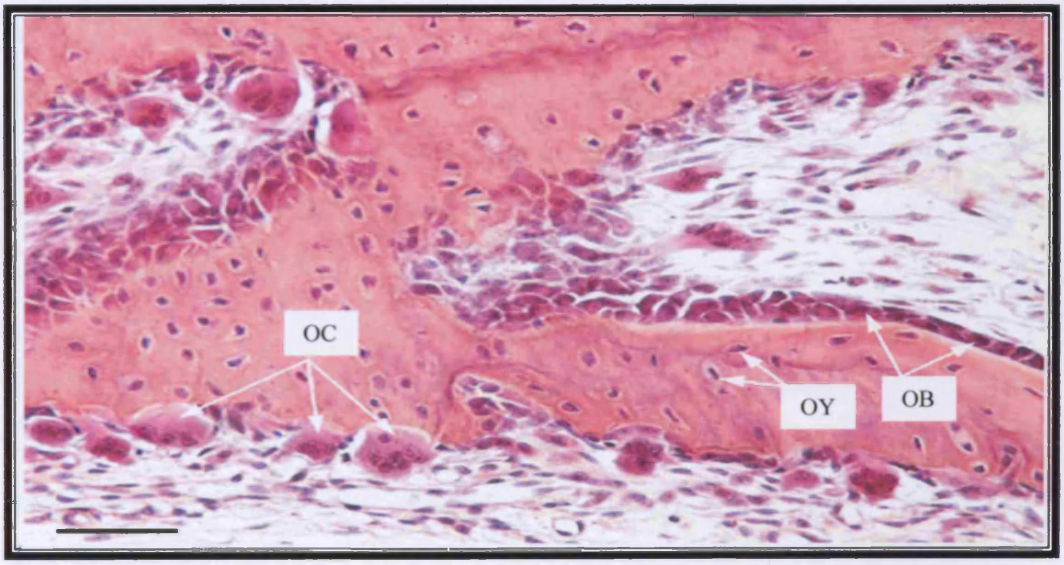


Figure 1.5: Bone cells

A diagram showing the three major bone cells; osteoclasts (OC), osteoblasts (OB), and osteocytes (OY). Adapted from Arnett and colleagues (2004).

Human adult bone mass is maintained locally by the balance between osteoclasts and osteoblasts which are responsible for the two major processes in bone, resorption and formation, respectively (Harada and Rodan, 2003; Zaidi, 2007).

A) Osteoclasts

Osteoclasts are the bone cells responsible for resorption (bone destruction). Osteoclasts are multinucleated (4 – 20 nuclei) (Figure 1.5) and originate from a monocyte / macrophage hemopoietic lineage (Teitelbaum, 2000; Baron, 2003; Boyle et al., 2003; Seeley et al., 2008b). For osteoclastogenesis, osteoblasts or stromal cells secrete macrophage colony stimulating factor (M-CSF) and receptor activator for nuclear factor kappa-B ligand (RANKL) (Teitelbaum, 2000; Baron, 2003; Boyle et al., 2003; Teitelbaum, 2007). M-CSF and RANKL act on M-CSF receptor and receptor activator for nuclear factor kappa-B (RANK), respectively (Zaidi, 2007). Additionally, contact between osteoclast precursors and osteoblasts or stromal cells is needed for osteoclastogenesis (Teitelbaum, 2000; Baron, 2003; Boyle et al., 2003; Teitelbaum, 2007).

The osteoclast starts its function of resorption by adhering to the bone surface and forming a sealed extracellular area. Then, across its ruffled membrane, the osteoclast secretes protons to acidify the environment, and lysosomal enzymes such as cathepsin K, and carbonic anhydrase II to dissolve the minerals and degrade the collagenous matrix (Teitelbaum, 2000; Baron, 2003; Boyle et al., 2003; Zaidi, 2007).

The action of many anti-osteoporosis agents such as oestrogens and bisphosphonates arises from their action on osteoclast apoptosis (Teitelbaum, 2000; Zaidi, 2007).

B) Osteoblasts

Osteoblasts are the bone cells responsible for bone formation which is "the synthesis and deposition of the bone extracellular matrix" (Ducy et al., 2000).

Osteoblasts are mononucleated cells derived from local mesenchymal stem cells under the influence of local growth factors such as fibroblast growth factors, bone morphogenetic proteins and Wnt proteins (Baron, 2003; Spangler, 2008). The precursor stem cells proliferate into preosteoblasts and then into mature osteoblasts (Baron, 2003). Osteoblasts never work individually (Baron, 2003). Their plasma membrane is rich in alkaline phosphatase (ALP) which is used as an index of bone formation (Baron, 2003; Cohen, 2006). Osteoblasts have been found to express receptors for parathyroid hormone(Manen et al., 1998), prostaglandins(Nemoto et al., 1995), oestrogen receptors (Penolazzi et al., 2004; Miki et al., 2007), and vitamin D_3 (van Leeuwen et al., 1992).

Bone formation by osteoblasts is involved in bone growth, mineralisation and remodelling (Ducy et al., 2000). A differentiated osteoblast starts bone formation by secreting bone matrix proteins (type I collagen and other non-collagenous proteins) followed by mineral (hydroxyapatite) deposition on the network of type I collagen (Mackie, 2003). ALP contributes to the process of osteoblast mineralisation (Mackie, 2003).

After finishing bone formation osteoblasts differentiate into osteocytes or bone lining cells or undergo apoptosis (Baron, 2003; Jilka, 2007).

C) Osteocytes

Osteocytes are cells which were originally osteoblasts and became trapped in the bone matrix (Baron, 2003; Jilka, 2007; Seeley et al., 2008b). On osteoclastic bone resorption, osteocytes are phagocytosed and digested along with other bone components (Baron, 2003; Jilka, 2007).

1.6.3 Bone modelling and remodelling

Bone modelling is a formation process that is usually not preceded by resorption, and is involved in bone growth. Bone remodelling is a continuous physiological process throughout life in which resorption by osteoclasts is followed by bone formation by osteoblasts, and is intended for the replacement of old bone with new bone, in addition to its role in mineral metabolism (Ducy et al., 2000; Sommerfeldt and Rubin, 2001; Baron, 2003; Seeley et al., 2008b). Although mechanical load has a minor effect on bone modulation, it was found that strenuous sports such as tennis make the player's bones stronger. Conversely, immobilization can increase bone loss significantly (Harada and Rodan, 2003). The period needed for one full remodelling cycle is 3-6 months, and this cycle sequence is called activation–resorption–reversal–formation (ARRF) as shown in Figure 1.6. During activation, RANKL, produced by osteoblasts and stromal cells, simulates the preosteoclasts to fuse and proliferate into mature osteoclasts which migrate to the remodelling site. After activation the osteoclasts start to resorb bone in two stages; a rapid stage achieved by multinucleated osteoclasts which takes about eight

days, followed by a slow stage achieved by mononuclear cells which takes about thirty four days. The reversal phase is an intermediate stage between resorption and formation where macrophage-like, uncharacterized mononuclear cells are observed. In this phase stopping resorption and cementing together of old and new bone occurs. Lastly, formation occurs where the osteoblasts secrete new collagen matrix and then mineralise it (Sommerfeldt and Rubin, 2001; Baron, 2003; Prentice, 2007).

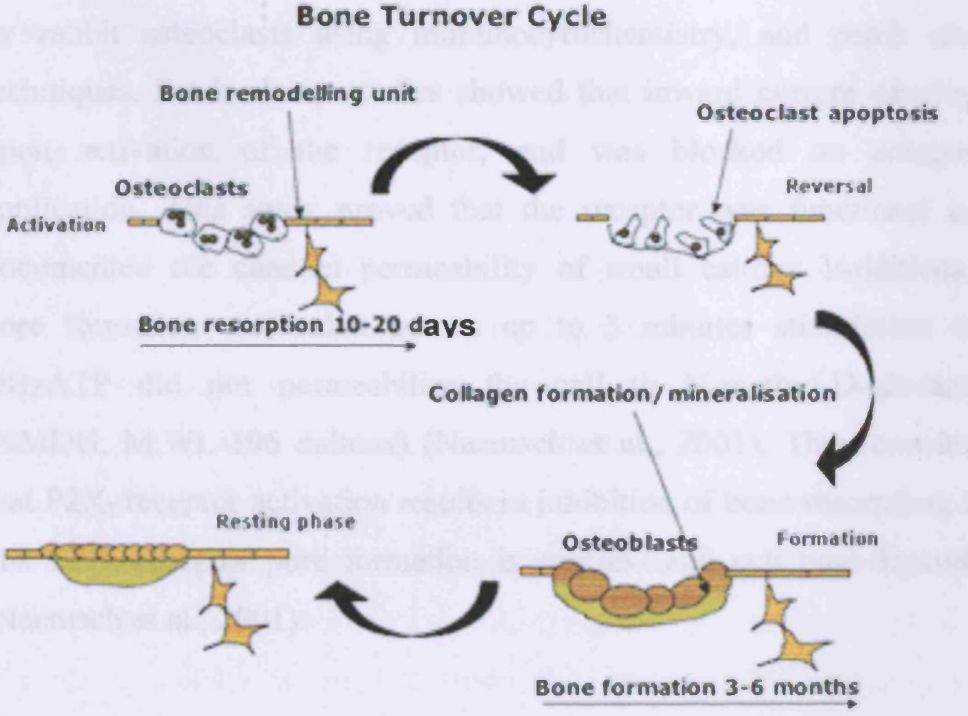


Figure 1.6: Bone remodelling

A diagram showing activation–resorption–reversal–formation (ARRF) in the bone remodelling cycle sequence. Adapted from Prentice (2007).

Many hormones are involved in the remodelling process. Some of these are: oestrogen, testosterone, thyroid, parathyroid and growth hormones which are anabolics, but thyroid and parathyroid hormones in excess act as pro-resorptive agents (Zaidi, 2007).

1.7 Purinergic receptors in bone

1.7.1 Osteoclasts

Many studies have confirmed the expression of $P2X_7$ receptors by osteoclasts, and clearly established their function. The first, carried out by Hoebertz et al (2000), reported the presence of $P2X_7$ receptors in the nuclei of cultured rat osteoclasts using immunocytochemical studies (Hoebertz et al., 2000).

Naemsch et al (2001) demonstrated the expression of the $P2X_7$ receptor by rabbit osteoclasts using immunocytochemistry, and patch clamp techniques. Patch clamp studies showed that inward current developed upon activation of the receptor, and was blocked on antagonist application. This study proved that the receptor was functional as it documented the channel permeability of small cations. Additionally, pore formation was ruled out as up to 3 minutes stimulation with DBzATP did not permeabilize the cell to N-methyl-D-glucamine (NMDG, M.Wt. 196 daltons) (Naemsch et al., 2001). They concluded that $P2X_7$ receptor activation results in inhibition of bone resorption, and that $P2X_7$ receptor pore formation is species- and cell type-dependent (Naemsch et al., 2001).

The involvement of $P2X_7$ receptors in the process of calcium signalling between osteoblasts and osteoclasts was studied by Jorgensen et al (2002). This group confirmed that human osteoclasts express $P2X_7$ receptors using reverse transcriptase-polymerase chain reaction (RT-PCR), and proved that the receptor is functional by utilizing Ca^{2+} uptake and pore formation properties. To achieve the objective of the study, osteoblast $P2X_7$ receptors were blocked using oATP which led to diminished calcium signal propagation to osteoclasts. This demonstrates

the presence of osteoblast-osteoclast calcium signalling and the involvement of the $P2X_7$ receptor in this process (Jorgensen et al., 2002).

Gartland et al (2003b) studied the effect of blocking the $P2X_7$ receptor on the formation of human multinucleated osteoclasts by utilizing the receptor pore formation characteristic, in addition to RT-PCR, immunohistochemistry, and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL). This study showed that human osteoclasts express the $P2X_7$ receptor and its activation mediates pore formation. Additionally, blockade of the receptor prevented the formation of multinucleated osteoclasts from human peripheral blood, increased osteoclast apoptosis and eventually inhibited osteoclastic resorption (Gartland et al., 2003b).

In a study by Ke et al (2003) to find the importance of $P2X_7$ receptors in bone resorption, it was revealed that deletion of the receptor in mice caused increased resorption proving the significance of the receptor in osteoclast function. An interesting finding in this study is that multinucleated osteoclasts can be produced even in the absence of $P2X_7$ receptors, although electrophysiological studies showed that the $P2X_7$ receptor is expressed only in the wild-type, but not the knock-out mice (Ke et al., 2003). In another study the formation of multinucleated osteoclasts from mononuclear precursor cells was not impaired in $P2X_7$ receptor knockout mice (Gartland et al., 2003c).

Gartland et al (2003b), and Jorgensen et al (2002) showed pore formation by osteoclasts, while Naemsch et al (2001) ruled it out. This may be due to insufficient time for agonist stimulation as Gartland et al (2001) stated that 5 minutes is usually needed for pore formation in haemopoietic cells. The above mentioned studies established the vital

role of the $P2X_7$ receptor in osteoclast formation and function. Additionally, $P2X_2$ and $P2X_4$ receptors have been identified in osteoclasts, and it has been reported that $P2X_2$ receptor stimulation increases osteoclast activity (Hoebertz et al., 2003). Expression of $P2X_1$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors has also been reported in osteoclasts and ATP was found to play a role in osteoclast activation by increasing expression of RANKL (Buckley et al., 2002).

$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors have also been identified in human osteoclasts, and it has been claimed that $P2Y_1$ receptor activation causes stimulation of osteoclast formation and function (Buckley et al., 2002; Hoebertz et al., 2003).

1.7.2 Osteoblasts

Expression of $P2X_7$ receptors in osteoblasts has been reported in very few studies. The first, carried out by Nakamura et al (2000), showed the expression of $P2X_7$ receptors mRNA by human osteoblast-like MG63 cells (Nakamura et al., 2000).

Gartland et al (2001) studied the expression of $P2X_7$ receptors by two human osteosarcoma cell lines (SaOS2 and Te85) and primary human bone-derived cells. The first finding in this study was the expression of $P2X_7$ receptors by osteosarcoma cells and primary human cells using RT-PCR. The immunocytochemical technique showed high expression of the receptor in the SaOS2 cells, but no expression was observed in the Te85 cells, and varying degrees were found in the primary human cells. $P2X_7$ receptor pore formation upon activation was studied using lactate dehydrogenase (LDH) release and ethidium bromide uptake. Both were reported in SaOS2 cells, but not in Te85 cells. Pore formation in SaOS2

cells was confirmed by blocking the receptor using the antagonist PPADS. Changes in the morphology of SaOS2 and human primary cells were also noticed upon activation which eventually led to apoptosis, and again this effect was blocked by the antagonist PPADS. One of the interesting findings in this study is that pore formation in osteoblasts needed 60 minutes incubation with the agonists, while only 5 minutes is usually needed for haemopoietic cells, which led the authors to assume an atypical pharmacology of the receptor. This study concluded that the $P2X_7$ receptor is functional in osteoblast cells, and its expression is dependent on the differentiation of the osteoblast population (Gartland et al., 2001).

The importance of $P2X_7$ receptors in bone formation was established in the study by Ke et al (2003). They compared $P2X_7$ receptor expression and function in knockout and wild-type mouse osteoblasts using RT-PCR and pore formation studies. It was found that it was only expressed and functional in wild-type, but not knockout mice. The authors concluded that "Loss of $P2X_7$ receptors increased osteoclastic bone resorption, while decreasing osteoblastic bone formation. Thus, $P2X_7$ receptor agonists may be useful as a combined antiresorptive and anabolic therapy in skeletal disorders such as osteoporosis" (Ke et al., 2003). Additionally, $P2X_7$ receptor expression has been reported in other studies (Li et al., 2005; Orriss et al., 2006; Panupinthu et al., 2007). Furthermore, $P2X_5$ and $P2X_6$ receptors have been identified in osteoblasts (Nakamura et al., 2000; Hoebertz et al., 2003) where $P2X_5$ receptors were reported to be involved in osteoblast proliferation and differentiation (Hoebertz et al., 2003). $P2X_2$ (Hoebertz et al., 2003) and $P2X_4$ receptors (Nakamura et al., 2000) have also been identified in osteoblasts.

$P2Y_1$ (Maier et al., 1997; Hoebertz et al., 2000), $P2Y_2$ (Maier et al., 1997; Hoebertz et al., 2000; Orriss et al., 2007), $P2Y_4$ (Maier et al., 1997), and $P2Y_6$ (Maier et al., 1997) receptors have been identified in osteoblasts. $P2Y_2$ receptor was found to be involved in communication between osteoblasts and in inhibiting bone formation (Maier et al., 1997; Hoebertz et al., 2000).

Nothing is known about the expression of adenosine receptors by osteoclasts. However, adenosine was reported to stimulate osteoblast proliferation (Shimegi, 1996) . Additionally, human preosteoblastic cells were found to express all four adenosine receptors and adenosine stimulated IL-6 release from these cells and inhibited osteoprotegerin (OPG) expression (Evans et al., 2006). In another study, expression of A_1, A_{2a}, and A_{2b} but not A_3 was reported in the MG63 cell line (Russell et al., 2007). In this study adenosine was found to inhibit IL-6 release, therefore it was suggested that adenosine receptors have a role in inflammation and osteoclastogenesis (Russell et al., 2007). The difference in the findings of Evans et al (2006) and Russell et al (2007) is believed to be the cells used, preosteoblastic cells used by the first group and mature osteoblasts by the second.

1.7.3 Osteocytes

Nothing is known about the expression of purinergic receptors by osteocytes.

1.8 Bone diseases

Due to the discovery of their importance in bone biology (Ke et al., 2003) and cytokine release (Ferrari et al., 1997; Bulanova et al., 2005; Di

Virgilio, 2007; Hughes et al., 2007; Qu et al., 2007), P2X$_7$ receptors have been implicated in bone diseases such as osteoporosis (Ke et al., 2003) and rheumatoid arthritis (Ferrari et al., 2006).

1.8.1 Osteoporosis

Osteoporosis is a metabolic bone disease characterized by increased skeletal fragility, thus exposing the patients to fractures arising from trivial activities (Mackie, 2003; Marie, 2006). Figure 1.7 shows the change that occurs in cancellous bone in osteoporosis (Langton, 2000). Advanced age, and the menopause are known to be the key risk factors for osteoporosis (Manolagas, 2000; Mundy, 2001; Ebeling, 2004; Gonzales-Macias et al., 2005; Reginster and Burlet, 2006), in addition to female sex, low Ca^{2+} diet, Cushing's syndrome and corticosteroid therapy (Manolagas, 2000; Mundy, 2001; Ebeling, 2004; Gonzales-Macias et al., 2005; Reginster and Burlet, 2006).

Bone mineral density (BMD) is an approach to measure bone mass and to diagnose osteoporosis. The most accurate BMD test is called dual-energy X-ray absorptiometry (DXA), which compares the patient's BMD to an established standard and gives a score. The score is either a T-score (BMD compared to that of a healthy 30 year-old adult), or a Z-score (BMD compared to a person of matching age) (NIAMS, 2006).

Pathophysiology

Fractures in osteoporotic patients are most likely to occur in the hip, wrist and vertebrae (Mundy, 2001; Ortolani and Vai, 2006) as a result of a decrease in BMD (AMA, 2004). The increased bone fragility in osteoporosis is due to two types of changes in bone. The first type of

change is the decrease in bone mass occurring with advanced age, while the other type is specific, occurring in the cancellous bone microarchitecture of the vertebral bodies and the neck of the femur (Mundy, 2001). The advanced age bone mass decrease is thought to be related to genetic factors (Mundy, 2001; Sambrook and Cooper, 2006). The main gene shown to be involved is the vitamin D receptor gene (Mundy, 2001; Sambrook and Cooper, 2006), but other genes have also been suggested including those for IL-6 (Mundy, 2001), the oestrogen receptor and type-I collagen (Mundy, 2001; Sambrook and Cooper, 2006). Bone loss in females of advanced age is rapid because of the abrupt withdrawal of sex hormones after the menopause, but in males it is slow because of the gradual decrease in sex hormones.

At the cellular level, a balance between osteoblast and osteoclast activity exists in normal adults, but in osteoporosis this balance is negative, as osteoclast activity is increased (Mackie, 2003; Meunier et al., 2004; Marie, 2006; Sambrook and Cooper, 2006; Teitelbaum, 2007). It has been shown that the rates of osteoblast and osteoclast production and apoptosis are crucial in physiological bone regeneration (Manolagas, 2000).

Sex hormone deficiency (oestrogen and androgens) causes increased osteoclastogenesis and osteoblastogenesis, but the life span of osteoblasts is decreased in contrast to that of osteoclasts which is increased. This is translated into increased bone resorption with decreased bone formation. The effect of sex hormone loss on bone cells was reported to be due to the upregulation of cytokines such as IL-6, IL-1 and TNF-α which are responsible for osteoblastogenesis and osteoclastogenesis. Additionally, sex hormone deficiency causes a decrease in osteocyte life span which

could cause further weakness of bone (Manolagas, 2000; Fini et al., 2004).

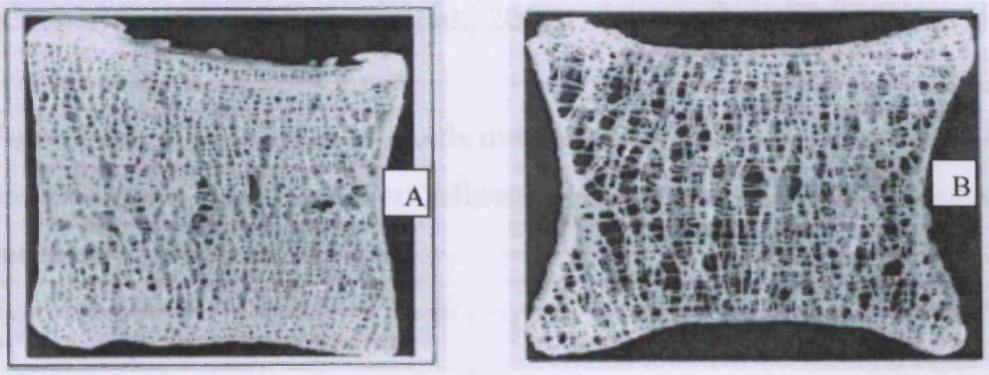


Figure 1.7: Change in cancellous Bone in osteoporosis.
A diagram showing normal (A) and osteoporotic (B) cancellous bone. Adapted from Langton (2003).

In advanced age, bone formation after each remodelling cycle is decreased. This is reflected by a decrease in bone wall thickness, which is used as a measure of bone formation. The reasons for this were reported to be a decrease in osteoblastogenesis and osteoclastogenesis, and the life span of osteocytes (Manolagas, 2000).

Secondary osteoporosis is a potential complication of excess glucocorticoids in the body, which may arise from their chronic use or from Cushing's syndrome (Boling, 2004; Gregório et al., 2006; Kanis et al., 2007). The mechanism for bone loss due to excess glucocorticoid is a decrease in osteoblastogenesis and osteoclastogenesis, an increase in the life span of osteoclasts, and a decrease in the life span of osteoblasts and osteocytes. In addition, bone resorption resulting from hyperparathyroidism increases. Hyperparathyroidism in this case results because glucocorticoids increase calcium renal excretion and decrease intestinal absorption leading to an increase in parathyroid hormone

(PTH) release (Manolagas, 2000; Boling, 2004; Gregório et al., 2006; Kanis et al., 2007). Another reason for the increased bone resorption induced by glucocorticoids is an increase in the production of RANKL (Gregório et al., 2006; Kanis et al., 2007).

Aging, disuse and glucocorticoids may affect the remodelling process by slowing down bone formation following resorption resulting in low bone turnover (Zaidi, 2007).

Current pharmacological treatment

Calcitonin was the first therapy used for the treatment of osteoporosis. It acts through an antiresorptive mechanism by inhibiting the action of osteoclasts. It has a rapid action by stopping osteoclasts producing a ruffled border, and a long-term action in reducing the number of osteoclasts. It has been shown to reduce the risk of vertebral fracture by up to 36 % (Zaidi et al., 2002; Mulder et al., 2006). Calcitonin has largely been superseded with newer drugs because of their potency and long-acting effect, but it may be considered if the patient can't tolerate other anti-osteoporotic drugs (Zaidi et al., 2002; Cohen and Adachi, 2004; Gregório et al., 2006; Mulder et al., 2006).

Bisphosphonates such as etidronate, and zoledronate are widely used nowadays for the management of osteoporosis because of their potent effect on reducing bone turnover, increasing bone mass, and decreasing fracture risk (Boling, 2004; Gregório et al., 2006; Mulder et al., 2006). The bisphosphonates have a potent antiresorptive action arising from inhibition of osteoclast activity, and induction of osteoclast apoptosis (Licata, 2005; Mulder et al., 2006). Additionally, bisphosphonates decrease the development of osteoclast progenitors (Manolagas, 2000).

Based on their intracellular action on osteoclasts, bisphosphonates are divided into two classes. Class 1 are those which have no nitrogen atom in their chemical structure such as etidronate and acts by inhibiting ATP-dependent intracellular enzymes causing induction of osteoclast apoptosis. Class 2 bisphosphonates are the nitrogen-containing compounds such as zoledronate and act by inhibiting osteoclast activity, and inducing their apoptosis (Licata, 2005; Mulder et al., 2006; Zaidi, 2007). Class 2 bisphosphonates are much more potent than class 1 compounds, for example zoledronate has an antiresorptive relative potency at least 10,000-fold higher compared to etidronate (Licata, 2005). Oral bisphosphonates are associated with poor patient compliance due to their high incidence of gastro-intestinal side effects and complicated dosing requirement (Epstein and Zaidi, 2005; Black et al., 2007; Cramer et al., 2007; Kanis et al., 2007). Hence, interest is directed currently towards the use of long acting and / or parenteral dosage forms such as a once-yearly infusion of zoledronic acid (Mulder et al., 2006; Black et al., 2007).

Recombinant human PTH (teriparatide) is the first anti-osteoporotic drug which acts by stimulation of bone formation (Quattrocchi and Kourlas, 2004; Deal, 2005; McClung et al., 2005; Marie, 2006; Chen et al., 2007). Teriparatide is composed of the first 34 amino acids of native human PTH (Quattrocchi and Kourlas, 2004; Deal, 2005; McClung et al., 2005; Marie, 2006; Chen et al., 2007). A constant high dose of teriparatide induces resorption (Quattrocchi and Kourlas, 2004; Mulder et al., 2006), but an intermittent low dose induces bone formation (Quattrocchi and Kourlas, 2004; Mulder et al., 2006; Jilka, 2007). It acts by stimulating osteoblast production, and inhibiting osteoblast apoptosis (Quattrocchi and Kourlas, 2004; Jilka, 2007). It was reported that bone formation markers increase after about 1 month of treatment with teriparatide,

while bone resorption markers increase after about 6 months of treatment indicating an early stimulation of bone formation and delayed bone resorption (Deal, 2005; Jilka, 2007). As a reflection of the increase in osteoblast life span, osteocytes in the newly formed bone in mice receiving PTH were found to be more numerous, and closely spaced (Manolagas, 2000). In a clinical trial, teriparatide reduced the fracture rate by 50 % compared to placebo (Davidson, 2003). The fracture prevention trial (FPT) study showed that teriparatide significantly increased BMD (Chen et al., 2007) and significantly reduced the risk of vertebral and non-vertebral fractures by 65 % and 53 %, respectively, in osteoporotic postmenopausal women (Mulder et al., 2006; Chen et al., 2007). Additionally, back pain was reported less frequently in patients using teriparatide compared to alendronate (McClung et al., 2005). The use of a combination of bisphosphonates and teriparatide would be more potent than using a single agent alone, since each one acts by a different mechanism (Quattrocchi and Kourlas, 2004; Deal, 2005; Mulder et al., 2006). However, a study found that bone density responses to teriparatide were impaired in patients pretreated with alendronate (Deal, 2005; Mulder et al., 2006), which led to a recommendation of leaving a 6 month drug-free period between alendronate and teriparatide (Deal, 2005). This effect has not been reported with any other bisphosphonate (Deal, 2005).

Hormone replacement therapy / oestrogen replacement therapy (HRT / ERT) is an approach which compensates for the reduction in oestrogen in menopausal women, and acts by inhibiting bone resorption (Mackie, 2003; Zaidi, 2007). Recently a question has been raised about the rationale of the indefinite use of HRT, especially after the publication of studies which showed that the risks from its long-term use outweigh the benefits. These risks include breast cancer, coronary heart disease, and

deep vein thrombosis (DVT) (Cohen and Adachi, 2004; Mulder et al., 2006).

SERMs (Selective Oestrogen Receptor Modulators) such as raloxifene have a similar action to oestrogen and are used for the prevention of breast cancer (Reid, 2002). Studies showed that raloxifene increased spinal BMD by 1-3 % and reduced vertebral fractures by 30 %. The incidence of DVT with raloxifene is similar to that caused by HRT (Reid, 2002; Mulder et al., 2006).

Fluoride acts by increasing osteoblast proliferation (Davidson, 2003). Studies showed that it increased BMD by 8 %, but its continuous use in high doses leads to osteomalacia (soft bones), which may explain its failure in decreasing the fracture rate (Reid, 2002). Studies recommend its use as an adjunct therapy with antiresorptive agents in patients with severe bone loss (Cohen and Adachi, 2004).

Vitamin D and calcium supplements are not recommended as a sole therapy for osteoporosis since the results of the studies done are variable. Instead, the combination may be used as an adjunct therapy with other anti-osteoporotic agents (Cohen and Adachi, 2004; Sambrook and Cooper, 2006; Rizzoli et al., 2008). Additionally, Vitamin D and calcium supplements are recommended for patients using corticosteroids to prevent bone loss (Gregório et al., 2006).

One of the most recent treatments for osteoporosis is strontium ranelate. Its efficacy and safety were proved by many animal and human studies (Fogelman and Blake, 2005; Ammann, 2006; Marie, 2006; Ortolani and Vai, 2006; Bonnelye et al., 2008). It acts by stimulating bone formation and inhibiting resorption (Meunier et al., 2004; Fogelman and Blake,

2005; Ortolani and Vai, 2006; Bonnelye et al., 2008), which makes it the first agent to have the opposite actions of being antiresorptive and bone-forming (Marie, 2006). At the cellular level strontium ranelate was found to decrease the differentiation and activity of osteoclasts and increase their apoptosis and increase preosteoblastic cell replication and collagen synthesis with no effect on bone mineralisation (Marie, 2006). Strontium ranelate was found to produce both early and long-term reductions in the risk of vertebral fractures (Meunier et al., 2004). The treatment of peripheral osteoporosis (TROPOS) and spinal osteoporosis therapeutic intervention (SOTI) studies showed that strontium ranelate reduced significantly the risk of vertebral and non-vertebral fractures in postmenopausal women, and that it was well tolerated (Reginster et al., 2005; Adami, 2006; Ortolani and Vai, 2006).

Future treatment

Novel PTH derivatives including oral dosage forms are in phase II clinical trials (Rosen, 2004).

Another potential treatment approach is through the RANK/RANKL/OPG system, which is known to play a major role in osteoclastic bone resorption (Theoleyre et al., 2004; Mazziotti et al., 2006; McClung et al., 2006; Schwarz and Ritchlin, 2007; Teitelbaum, 2007). OPG inhibits osteoclast formation by inhibiting RANKL which is involved in osteoclast differentiation and activity (Davidson, 2003; Theoleyre et al., 2004; Teitelbaum, 2007; Zaidi, 2007). Denosumab is a human monoclonal antibody that inhibits binding of RANKL to RANK and hence inhibits osteoclast differentiation, activation and survival (McClung et al., 2006; Schwarz and Ritchlin, 2007). Denosumab was found to decrease bone resorption markers (McClung et al., 2006) and is

in Phase 2 clinical trials for the treatment of osteoporosis (Lewiecki et al., 2007). In a clinical study involving postmenopausal women, denosumab administered subcutaneously every 3 or 6 months was found to increase BMD and decrease bone resorption (McClung et al., 2006) and this effect was sustained for up to 2 years (Cramer et al., 2007). Many other studies have proved the efficacy and safety of denosumab in osteoporosis (Schwarz and Ritchlin, 2007).

Another anabolic approach could involve targeting sclerostin which is produced by osteocytes and inhibits bone formation (Mazziotti et al., 2006; Mulder et al., 2006; Jilka, 2007). Part of the action of PTH on bone formation is believed to be via reduction of sclerostin expression in osteocytes (Jilka, 2007; Leupin et al., 2007). A monoclonal antibody against sclerostin is under development (Zaidi, 2007).

Purinergic receptors, particularly the $P2X_7$ receptor, represent a promising new field in the treatment of osteoporosis especially with the discovery of their expression by osteoclasts, and role in apoptosis of these cells (Hoebertz et al., 2003). Additionally, $P2X_7$ receptor knockout mice were found to have low bone formation and high bone resorption (Ke et al., 2003), therefore a $P2X_7$ receptor agonist could be a good treatment.

Table 1.1 shows some of the other potential agents or targets for the treatment of osteoporosis and their mechanism of action.

Agent or target	*Mechanism of action*
Calcium sensing receptor antagonist	Increase endogenous PTH (Mulder et al., 2006)
Integrin antagonist	Inhibit osteoclast interaction with extracellular matrix (Mulder et al., 2006)
Cathepsin-K inhibitor	Inhibit bone resorption (Mulder et al., 2006; Sambrook and Cooper, 2006; Stoch and Wagner, 2007)
Statins	Stimulation of bone formation (Soubrier and Roux, 2006; Zaidi, 2007)
Leptin	Stimulation of bone formation (Mackie, 2003; Zaidi, 2007)
Cannabinoid receptors	Stimulation of bone formation and inhibition of bone resorption (Pacher et al., 2006; Bab and Zimmer, 2008)
Thiazide diuretics	Renal calcium reabsorption and reduction of osteoclasts activity (O'Neill et al., 2004)
Full length (84 amino acid) human PTH	stimulation of bone formation (Deal, 2005; Mulder et al., 2006)

Table 1.1: Some of the potential agents or targets for the treatment of osteoporosis and their mechanism of action.

1.8.2 Rheumatoid Arthritis

"Rheumatoid arthritis (RA) is an inflammatory joint disease characterized by inflammation of synovial tissues, often leading to destruction of joint cartilage and bone" (Walsh et al., 2005). It is the commonest auto-immune and chronic systemic disease affecting a patient's morbidity and mortality (Sacre et al., 2005). It has higher prevalence in females compared to males (Lee and Weinblatt, 2001; Brooks, 2006) and is more common between the age of 40 and 70 years (Lee and Weinblatt, 2001). The disease is progressive, unless treatment is started early and aggressively (Kuritzky and Weaver, 2003; Blom and van Riel, 2007). Approximately 50 % of patients become too disabled to work within 10 years of disease onset (Kuritzky and Weaver, 2003;

Brooks, 2006). The influence of genetic factors has been implied by various studies performed (Lee and Weinblatt, 2001), and it is thought that genes are responsible for about 50 % of the risk of developing RA (Edwards and Cooper, 2006). Additionally smoking (Brooks, 2006; Edwards and Cooper, 2006) and some infectious diseases such as tuberculosis (Edwards and Cooper, 2006) were found to play a role in developing RA.

Pathophysiology

The initial signs and symptoms of RA are a symmetrical polyarthritis targeting many joints such as the metacarpaphalangeal and proximal interphalangeal joints of the hands and joints of the feet, wrists, etc (Walsh et al., 2005; Brooks, 2006; Combe, 2007). The major target of inflammation in RA is the joint synovium, which is infiltrated by inflammatory cells, causing expansion of the synovial tissues due to their proliferation and accompanied by neovascularisation forming what is known as a "pannus" (Walsh et al., 2005). The inflammatory mediators released by these inflammatory cells in the synovium contribute to the bone and joint destruction (Walsh et al., 2005; Knedla et al., 2007). The major inflammatory cells involved in RA pathogenesis include monocytes, B-cells, and T-cells (Chaiamnuay and Bridges, 2005; Fournier, 2005; Loetscher, 2005; Knedla et al., 2007).

Cytokines such as IL-1 and 6, RANKL and TNF-α play a major role in the RA inflammatory process. They can either act as inflammatory mediators contributing to tissue damage, or have direct or indirect effects on osteoclast differentiation, activity or survival (Andreakos et al., 2002; Boyce et al., 2005; Clark et al., 2005; Walsh et al., 2005). TNF-α was

found to be directly related to the release of many of the proinflammatory cytokines such as IL-1 and 6 (Andreakos et al., 2002).

Chemokines are chemotactic proteins found to be involved in the RA inflammatory process by stimulating migration of the inflammatory cells to the synovium (Knedla et al., 2007; Smolen et al., 2007; Tayal and Kalra, 2008). Additionally, the release of chemokines was found to be stimulated by some cytokines such as IL-4 and 13 (Knedla et al., 2007).

Prostaglandins (PG) are potent inflammatory mediators formed from arachidonic acid by cyclo-oxygenase type-1 and 2 (COX-1 and 2) to form Prostaglandin H2 (PGH2). Prostaglandin E2 (PGE2) is formed from PGH2 by PGE synthase. PGE2 is produced by some inflammatory cells such as monocytes, and was found to be elevated in RA synovial fluid (Walsh et al., 2005).

Effects on osteoblast differentiation, function and survival of many of the inflammatory mediators mentioned above have been shown in in vitro studies (Walsh et al., 2005). Osteoporosis in RA patients at sites distant from the inflamed joints was also reported. Early studies indicated that this was due to decreased bone formation, but more recent studies reported this to be mainly due to increased bone resorption (Walsh et al., 2005).

Current Treatment

Due to their analgesic action, NSAIDs (non-steroidal anti-inflammatory drugs) such as ibuprofen and diclofenac were and still are the initial medications used in RA treatment (Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Combe, 2007). They exert their action by

inhibition of COX responsible for the production of PGs from arachidonic acid (Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Vuolteenaho et al., 2008). The action of NSAIDs is only symptomatic, alleviating pain and swelling, but without any effect on the disease process and progression. Based on this, the current recommendation is that they should not be used alone (Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Majithia and Geraci, 2007). NSAIDs carry the risk of gastro-intestinal (GI) adverse effects due to inhibition of the COX-1 isoenzyme responsible for production of the prostaglandins which provide GI mucosal protection (Scheiman, 2005).

COX-2 inhibitors (or the Coxibs) such as celecoxib and rofecoxib are NSAIDs that selectively inhibit the COX-2 isoenzyme responsible for the production of the inflammatory PGs, with a minor action on COX-1. They have similar efficacy to the non-selective agents, but with a significantly lower incidence of GI adverse effects (Kuritzky and Weaver, 2003; Scheiman, 2005; Bannwarth, 2006; Combe, 2007). Recently, rofecoxib has been withdrawn from the market due to increased risk of cardiovascular events (Scheiman, 2005; Bannwarth, 2006). Based on this, restrictions were placed on the use of all the COX-2 inhibitors, although it was stated that the risk of cardiovascular side effects appears to differ across this class and they should be considered only as a second or third line agents (Becker, 2005).

Corticosteroids in low doses (<10 mg/day prednisone or equivalent) play a major role in alleviating RA symptoms because of their potent anti-inflammatory actions (Geletka and St Clair, 2003; Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Combe, 2007). They are claimed to have a degree of disease-modifying action, by slowing the joint damage

(Geletka and St Clair, 2003; Kuritzky and Weaver, 2003; Srikanth and Deighton, 2006; Combe, 2007), but this damage may increase following treatment withdrawal (Kuritzky and Weaver, 2003). Corticosteroid dosages and duration of treatment should be kept to the minimum to minimize the risk of adverse effects which include osteoporosis, hypertension, and hyperglycemia (Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Combe, 2007). Rheumatic patients on corticosteroids are at higher risk of osteoporosis due to the disease process and the additional complications of the drug (Kuritzky and Weaver, 2003). Therefore it is recommended that these patients receive vitamin D and calcium supplement (Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Majithia and Geraci, 2007).

Disease-modifying anti-rheumatic drugs (DMARDs) have been proven to reduce joint damage, and delay disease progression (Kuritzky and Weaver, 2003; Blom and van Riel, 2007). Studies support their early use for all RA patients (Geletka and St Clair, 2003; Kuritzky and Weaver, 2003; Rindfleisch and Muller, 2005; Uhlig and Kvien, 2005; Combe, 2007). They include gold agents, anti-malarial agents, and methotrexate. Anti-malarial agents are the least effective and least toxic, but gold agents have moderate efficacy and the highest toxicity of this group. Methotrexate is considered the standard to which other DMARDs are compared due to its good therapeutic index (Kuritzky and Weaver, 2003; Majithia and Geraci, 2007). Studies have proved that the use of combination therapy of DMARDs is more beneficial than using a single agent (Geletka and St Clair, 2003; Kuritzky and Weaver, 2003; Feldmann et al., 2005; Rindfleisch and Muller, 2005; Uhlig and Kvien, 2005; Blom and van Riel, 2007).

Biological agents are the most recent and biggest development in the management of RA (Brown, 2005; Majithia and Geraci, 2007). Anti-TNF-α monoclonal antibodies such as adalimumab and infliximab are examples of these agents (Sacre et al., 2005; Haraoui and Keystone, 2006; Srikanth and Deighton, 2006; Blom and van Riel, 2007; Williams et al., 2007). TNF-α was found to be overexpressed in RA, and blockade of its actions caused a reduction in the release of the proinflammatory cytokines (Feldmann et al., 2005). These agents are indicated for severe RA not controlled by the conventional DMARDs (Kuritzky and Weaver, 2003; Feldmann et al., 2005). They have proved to be highly effective (Kuritzky and Weaver, 2003; Blom and van Riel, 2007), but a minority of patients do not respond, therefore methotrexate is added, which improves efficacy significantly (Feldmann et al., 2005; Blom and van Riel, 2007). These agents carry a risk of infection (Feldmann et al., 2005; Rindfleisch and Muller, 2005; Park and Pillinger, 2007), and malignancy (Feldmann et al., 2005; Park and Pillinger, 2007).

Other biological agents are, anakinra which is an IL-1 receptor antagonist (Rindfleisch and Muller, 2005; Smolen et al., 2007; Williams et al., 2007; Tayal and Kalra, 2008) and rituximab which is a monoclonal antibody initiated recently for the treatment of RA acting by inducing B-cell apoptosis (Brown, 2005; Rindfleisch and Muller, 2005; Sacre et al., 2005; Blom and van Riel, 2007; Majithia and Geraci, 2007). Rituximab was used previously for the treatment of non-Hodgkin's lymphoma (Smolen et al., 2007). Abatacept is another new biological agent acting by inhibiting T-cell activation (Blom and van Riel, 2007; Majithia and Geraci, 2007; Smolen et al., 2007).

Future Treatment

Many new agents are in different stages of study, most of which target different cytokines such as IL-8, IL-12, and IL-15 (Sacre et al., 2005; Smolen et al., 2007; Williams et al., 2007; Tayal and Kalra, 2008). Tocilizumab is a monoclonal antibody against the IL-6 receptor (Abramson and Yazici, 2006; Park and Pillinger, 2007; Smolen et al., 2007), which is in Phase III clinical trials for the treatment of RA (Park and Pillinger, 2007; Smolen et al., 2007; Tayal and Kalra, 2008).

Other treatment approaches are through targeting chemokine receptors (CCR) such as CCR1 (Loetscher, 2005; Knedla et al., 2007; Smolen et al., 2007) T-cell depletion which plays a major role in RA pathogenesis (Brown, 2005), and P38 mitogen-activated protein kinases (MAPK) (Knedla et al., 2007).

Denosumab, which is undergoing clinical trials for the treatment of osteoporosis, is also under consideration to slow joint destruction resulting from RA (Smolen et al., 2007). Many other therapeutic approaches for the treatment of RA are in different phases of research, and some of them (e.g. cathepsin K) are also being considered for the treatment of osteoporosis (Smolen et al., 2007).

A novel approach in the treatment of RA is targeting the Epstein-Barr virus (EBV) which was found in the synovial tissue of RA patients. The remission of patients with active RA when treated with anti-herpes medication represents further support for the involvement of EBV in RA (Sawada et al., 2007).

Targeting the P2X$_7$ receptor represents a promising area in RA management especially with the discovery of its expression by

osteoclasts (Hoebertz et al., 2003) and that its stimulation causes the release of cytokines, namely IL-4, IL-6, IL-13, and TNF-α, from mast cells (Bulanova et al., 2005) and IL-1β from macrophages (Ferrari et al., 1997; Labasi et al., 2002).

Expression of functional $P2X_7$ receptors by human osteoclasts has been shown by many studies, but its expression by osteoblasts was only reported in very few studies. Additionally, the exact role of the $P2X_7$ receptor on bone formation has not been identified, although low bone formation was observed in $P2X_7$ receptor knockout mice. Since the $P2X_7$ receptor is implicated in bone cell function and novel potent therapies for osteoporosis are needed, it is important to answer the question "could the $P2X_7$ receptor be the next drug target?" To answer this question, it is necessary first to know more about $P2X_7$ receptors in human osteoblasts. Hence my project was aimed at the characterisation of the expression and function of $P2X_7$ receptors in human osteoblasts and the investigation of the modulation of receptor expression and function by oestrogen and glucocorticoids.

1.9 General aims

- To study the expression of $P2X_7$ receptors by osteoblast-like osteosarcoma cell lines (MG63 and SaOS2).
- To investigate the pharmacology of $P2X_7$ receptors in human osteoblast-like cells using P2X receptor agonists and antagonists.
- To study the effects of $P2X_7$ receptor activation and inhibition on aspects of osteoblast function.
- To study the effects of glucocorticoids and oestrogen, known to affect bone formation, on $P2X_7$ receptor function.
- To characterise $P2X_7$ receptor expression and function in primary human osteoblasts (HOB).

Chapter 2: Materials and Methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) or Fisher Scientific UK Ltd (Leicester, UK), unless otherwise stated.

2.2 Cell culture

MG63 and SaOS2 human osteosarcoma cells were obtained from European Collection of Cell Cultures (ECACC). The cells were grown in 25 cm^2 flasks (Nunc, Fisher Scientific, Loughborough, UK) at approximately passages 14 - 34, or on glass coverslips coated with 3-aminopropyl triethoxy silane for immunocytochemistry, in Dulbecco's modified Eagle's medium (DMEM; Cambrex, Berks, UK) supplemented with 5 % foetal calf serum (FCS; Invitrogen, Paisley, UK), 2 mM glutamine, 4500 mg/l glucose, 50 U/ml penicillin G sodium and 50 µg/ml streptomycin sulfate. This medium will be referred to as DMEM throughout this thesis. To routinely passage the cells in 25 cm^2 flasks, 350 µl trypsin - ethylenediaminetetraacetic acid disodium salt (EDTA) (0.025 % trypsin, Lorne Laboratories, Reading, UK, 0.025 % EDTA) was added, and incubated at 37°C for 5 minutes, and then the cells were split in a ratio of 1:5. The cells were maintained at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2, and the medium changed every three or four days.

Human embryonic kidney cells (HEK293) stably transfected with the $P2X_7$ receptor (Hibell et al., 2000) were used as a positive control and referred to as HEK7 in this thesis.

2.3 Preparation of cell lysate

Medium was removed from the flask where cells had been grown to confluence. 1 ml of lysis buffer (Tris base 50 mM, 1,2-di(2-1 aminoethoxy)ehane-N,N,N',N'-tetra-acetic acid disodium salt [EGTA] 5 mM, sodium chloride 150 mM, Triton 1 %, sodium vanadate 0.4 mM, sodium fluoride 50 mM, phenylmethylsulfonyl fluoride [PMSF] 1 mM, phenylarsine oxide 20 μM, sodium molybdate 10 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml) was added, and the flask incubated on ice for 15 minutes. Cells were removed from the bottom of the flask using a scraper, centrifuged at 15,306g (centrifuge: Sanyo Harrier 18/80) at 4°C for 15 minutes, and the supernatant was kept in aliquots at -20°C until used.

2.4 Isolation of nuclei

The medium was removed from the flask where cells had been grown to confluence, and 1 ml trypsin-EDTA was added, and incubated at 37°C for 10 minutes. 1 ml of growing medium was added and the cells were resuspended. The cells were centrifuged at 500 g at 4°C for 5 minutes. The pellet was gently loosened by vortexing and resuspended in 4 ml NP-40 lysis buffer (10 mM Tris base, 10 mM sodium chloride, 5 mM magnesium chloride, 0.5 % tergitol (NP-40), pH 7.4) and incubated on ice for 5 minutes. The lysed cells were centrifuged at 500 g at 4°C for 5 minutes, and the pellet resuspended in 4 ml lysis buffer, vortexed well, and centrifuged as above. The pellet containing the nuclei was resuspended in 150 μl 2:1 50 mM Tris pH 7.4: 3x Laemmli sample buffer (9.4 mM Tris base, 20.8 mM sodium doedcyl sulphate [SDS], 30 % glycerol, 15 % β-mercaptoethanol, 3 % bromophenol blue [1mg/ml], (Laemmli, 1970)) by gentle vortexing and stored at -20°C until needed.

2.5 Cell membrane preparation

The media was removed from cells grown to confluence. The cells were collected with a scraper in Dulbecco's phosphate-buffered saline (PBS), and centrifuged at 1,000g at 4°C for 5 minutes. The pellet was resuspended in 20 ml ice-cold buffer containing: Tris base 50 mM, sodium chloride 150 mM, EDTA 1 mM, 4-(2-Aminoethyl)-bezenesulfonylfluoride.HCl (AEBSF) 1mM, bacitracin 0.1mg/ml. The pellet was homogenised with a Polytron homogenizer (3x10 seconds) and centrifuged at 500g at 4°C for 10 minutes to pellet nuclei. The supernatant was kept on ice, and the pellet resuspended in 10 ml buffer with a Polytron homogeniser as above and centrifuged again as above. The supernatant was combined with the earlier one and centrifuged at 48,000g at 4°C for 15 minute. The final pellet was resuspended in 15 ml of the above buffer by vortexing and centrifuged as above. The pellet was resuspended in 1 ml buffer without peptidase inhibitors (bacitracin & AEBSF), using a syringe and a fine gauge needle. Membrane aliquots were stored at -80 °C until needed.

2.6 Protein assay

Serial dilutions of a standard bovine serum albumin 2 mg/ml (Pierce, Perbio Science UK Ltd. Cramlington, Northumberland, UK) were prepared. 25 µl of each standard dilution and 5 µl of the samples were used to determine the protein concentration using BCA™ assay reagents A and B (Pierce) according to the manufacturer's instructions. The samples were incubated at 37°C for 30 minutes, and then allowed to cool at room temperature for 5 minutes. The optical densities (OD) of the standards and the samples were measured at 540 nm using an MRX microplate reader-MRXTC Revelation (Dynex Technologies Limited,

Worthing, West Sussex, UK). A standard curve was produced from which the protein concentration of the samples was calculated using the Prism 4.0 programme (Graphpad software, San Diego, USA). The BCA™ protein assay method is based on a reduction reaction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. The Cu^{1+} ion formed is detected colorimetrically after its chelation by bicinchoninic acid forming a water soluble complex. This complex shows linear absorbance with increasing protein concentrations (manufacturer's instructions).

2.7 Primary Antibodies

Two anti-$P2X_7$ receptor primary antibodies were used in this thesis. One was a rabbit polyclonal anti-$P2X_7$ receptor antibody (Caltag-Medsystems Ltd., Buckingham, Bucks, UK) with antigeneic sequence (KIRKEFPKTQGQYSGFKYPY) corresponding to residues 576 – 595 of the rat $P2X_7$ receptor. This antibody recognises the human receptor and is referred to as the $rP2X_7$ antibody. A monoclonal anti-human $P2X_7$ receptor antibody (L4, (Buell et al., 1998)) and referred to as the L4 antibody in this thesis was also used. Other primary antibodies used were rabbit polyclonal C-terminal anti-$P2X_2$ and anti-$P2X_4$ receptor antibodies (Caltag-Medsystems Ltd., Buckingham, Bucks, UK), referred to as $rP2X_2$, and $rP2X_4$ antibodies, respectively. Primary antibodies for housekeeping gene protein (β-actin (anti-β-actin antibody, Sigma-Aldrich) and glyceraldehyde-3-phosphate dehydrogenase [GAPDH] (anti-GAPDH antibody, Ambion, Huntingdon, UK) were used.

2.8 Western Blotting

Samples were boiled at 95°C for 5 minutes, and then loaded onto 10 % polyacrylamide gels (acrylamide 10 %, Tris.HCL 375 mM, SDS 0.1 %, ammonium persulfate [APS] 0.005 %, N,N,N',N'-Tetramethylethylenediamine [TEMED] 0.05 %). A Precision Plus protein standards marker (Bio-Rad Laboratories Ltd. Bio-Rad House, Hertfordshire, UK) was loaded onto the gel to help in determining the size of the separated proteins. Membrane samples of HEK7 cells were used as a positive control (Michel et al., 1999). Tank buffer (Tris base 2.5 mM, glycine 19 mM, SDS 0.005 %, pH 8.3) was used to run the gel. Then the gels were blotted on to 0.2 μm nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK) using a semi-dry blotter (Pharmacia Biotech, St. Albans, UK) at 0.8 mÅ/cm^2 for one hour, in blotting buffer (Tris base 42.9 mM, glycine 38.9 mM, SDS 0.038 %, methanol 20 %).

After blotting, the membranes were washed in Tris-buffered saline with Tween-20 (TBST, Tris base 2 mM, sodium chloride 15 mM, Tween-20 0.1 %, pH 7.5), and then blocked at room temperature for one hour in 5 % weight/volume fat-free dried milk (Blotto; Marvel, Premier International Ltd., Spalding, UK) in TBST. The membranes were then incubated in the primary antibody (at a concentration of 1: 1000 in 1 % w/v Blotto) at 4°C overnight. For readsorption of the primary antibody with the peptide used to raise it, the peptide was incubated at 4°C overnight with the antibody in 1 % w/v Blotto before use. Control antibody was incubated without peptide.

After incubation with the primary antibody, the membranes were washed twice for 5 minutes in TBST, and once for 15 minutes, then incubated for

60 minutes at room temperature in the secondary antibody anti-rabbit immunoglobulin G (IgG) (Vector, Burlingame, California) conjugated to horseradish peroxidase (HRP) at 1:20,000 in 1 % w/v Blotto. The membranes were washed as above in TBST, the bands visualized using Enhanced Chemiluminiscent detection (Super Signal®, West Dura, Pierce), and the membranes exposed to chemiluminiscent X-ray film (Amersham Biosciences). Films were scanned using a Lexmark X1180 scanner.

2.9 Immunocytochemistry

Cells were grown on 13 mm coverslips treated with 2 % 3-aminopropyltriethoxy silane in acetone. The media was removed from the coverslips, and the cells rinsed three times with 0.1 M PBS (0.145 M sodium chloride, 0.0964 M sodium hydrogen orthophosphate, 0.0215 M sodium dihydrogen orthophosphate, pH 7.4). The cells were fixed for 15 minutes with 2 % formaldehyde in 0.1 M PBS, and then washed three times (5 minutes apart) with 0.1 M PBS and stored in this buffer until use in a sealed plate.

On the day of use, the cells were washed again as above. The cells were blocked for 30 minutes with 400 µl PBS containing 3 % serum from the animal used to raise the secondary antibody (horse for anti-mouse secondary or goat for anti-rabbit secondary), 1 % bovine serum albumin, and 0.1 % Triton X-100® to permeabilise the cells if needed. The cells were then incubated in 400 µl blocking solution containing the primary antibody (1:600 for the L4 antibody (personal communication, E.J. Kidd), and 1:1000 for the $rP2X_7$ antibody) for 24-72 hours at 4°C. The blocking solution alone was used as a control. To confirm the labelling seen when using the polyclonal $rP2X_7$ receptor antibody, it was

preincubated for 48 hours in PBS containing 3 % goat serum with 0.8 μg of the peptide used to raise it.

After incubation, the cells were washed three times (5 minutes apart) with 0.1 M PBS, and incubated in 400 μl blocking solution containing the relevant secondary antibody conjugated to Cy3 at 1:800 (anti-rabbit: Millipore, Watford, UK and Anti-Mouse: Amersham Biosciences) for 1 hour at room temperature in the dark. The cells were washed again as above, then the coverslips dipped in distilled water to remove buffer salts and left to dry in the dark. Dry coverslips were mounted on glass slides, rinsed with 100 % ethanol using an anti-fade agent (Fluorescent mounting medium, DAKO UK Ltd, Cambridge House, Cambridgeshire, UK) and stored in the dark in the fridge until examination under the fluorescent microscope (Leica DMRA2, Wetzlar, Germany). Images were recorded using a digital camera (Leica DC 500, Wetzlar, Germany) and the Leica FW 4000 software (Wetzlar, Germany). Images were processed using Adobe Photoshop 6.0 (Adobe, USA).

2.10 RT–PCR

RNA was extracted from the cells using the MIDAS RNA extraction kit (Biogene, Cambridge, UK) according to the manufacturer's instructions, and then the RNA samples were treated with a DNase kit (Applied Biosystems, Lingley House, Warrington, UK). The RNA content in the samples was measured at 260 nm and 280 nm using a Genesys 10 UV spectrophotometer (Fisher Scientific UK Ltd). 1 OD unit measured at 260 nm corresponds to 40 μg of RNA/ml. The ratio of A_{260}/A_{280} of pure RNA acid is 2.0, and a ratio of 1.8-2.0 corresponds to 90-100% pure RNA acid (manufacturer's instructions). The A_{260}/A_{280} ratios obtained for the samples were in this range.

RT reactions were carried out to prepare cDNA from the RNA in a 40 µl RT reaction ($MgCl_2$ 25 mM, reverse transcription 10x buffer 2 µl, deoxynucleotides triphosphate (dNTP) mixture 10 mM, Recombinant RNasin® Ribonuclease inhibitor 0.5 µl, high concentration avian myeloblastosis virus (AMV) RT 15 U, oligo $(dt)_{15}$ Primer 0.5 µg, RNA 1 µg, nuclease free water to a final volume of 40 µl). The RT kit was from Promega (Promega UK, Southampton, UK). Another 20 µl RT reaction was done with the concentrations as above, but without AMV. RT reactions were incubated in a Perkin Elmer 480 Thermal Cycler (Global Medical Instruments, Minnesota, USA) at 42°C for 1 hour, then at 99°C for 5 minutes to inactivate the AMV enzyme. cDNA samples were stored at -20°C until needed.

A PCR master-mix for each of the samples, and 1 blank, was prepared: 10x buffer 2.5 µl (Promega UK), PCR nucleotide mix 0.5 µl (Promega UK), sense primer 10 pmol, anti-sense primer 10 pmol, $MgCl_2$ 1.5 µl (Promega UK), Taq DNA Polymerase 0.2 µl (Promega UK), sterile water to 25 µl. 1 µl of the cDNA for each sample was added to 24 µl of the master mix and 1 µl of distilled water added to the blank. The PCR reactions were run in a Perkin Elmer 480 Thermal Cycler, and the conditions were: 94°C for 3 minutes, 60°C for 1 minute, 72°C for 1 minute, and then 35 cycles at: 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute. The conditions were based on previous PCR experience in our laboratory and were the same for all the receptors. Details of all the primers pairs used are presented in Chapter 3.

1x TBE Buffer (Tris base 90 mM, boric acid 90 mM, EDTA 2.5 mM) was used to prepare 1.5 % agarose gels and 1.27 µM ethidium bromide was added. Ethidium bromide fluoresces on binding to the DNA and thus

allows visualization of the bands. The marker ladder (0.3 µg/lane, New England Biolabs, Herts, UK), and 18 µl samples were loaded into the gel, and run at 100 V for about 50-60 minutes. The gel was observed under a transilluminator (Fotodyne Incorporated, New Berlin, USA), and a photograph taken using a Polaroid MP-4 land camera. To confirm the identity of the bands obtained, they were excised from the gels, incubated in 50 µl sterile water, kept in the fridge for 48 hours, and the cDNA eluted into the water sequenced using the Big Dye® Terminator V 3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK). The sequencing was performed by the Central Biotechnology Services available on site at Cardiff University.

2.11 Quantitative real-time RT- PCR

Standard curves for the human P2X$_7$ and β-actin (house-keeping gene) primer pairs and cDNA prepared from HEK7 cells were prepared to confirm that the expression of these two genes can be compared against each other, reflected by the standard curve efficiency (has to be 100±10%). "100 % reaction efficiency indicates occurrence of a doubling of product for every reaction cycle. Reaction efficiencies < 80% indicate inhibition of amplification, poor primer or probe binding or loss of linearity at high concentrations in the standard curve. Reaction efficiency > 115 % can indicate amplification of more than one product, template-independent probe degradation or loss of linearity at low concentrations" (Stratagene, personal communication). For these standard curves, serial dilutions of cDNA from HEK7 cells were prepared in triplicate. PCR reactions (25 µl) containing: 12.5 µl 2x Brilliant® SYBR Green quantitative PCR (QPCR) Mastermix (Stratagene, Texas, USA), 10 pmol sense primer, 10 pmol antisense primer, 9.5 µl sterile water, and 1 µl cDNA were prepared for each

standard sample. The reactions were run in an MX 3000P-Multiplex QPCR System (Stratagene, Texas, USA), and the conditions were: 94°C for 3 minutes, 60°C for 1 minute, 72°C for 1 minute, and then 40 cycles at: 94°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute. The master mix contained SYBR® Green which is a dye freely available in the reaction solution which fluoresces on binding to the double-stranded DNA produced during the PCR reaction. This fluorescence is monitored on a real-time basis as the PCR product is generated over the PCR cycles. However, fluorescence may result from non-specific product such as primer-dimers. To check for primer-dimers, melting curves were checked to make sure that there was only one peak at a high temperature with each pair of primers used. In addition, samples were also run on agarose gels and gave the expected bands. The fluorescence signal produced is monitored in real-time and can be displayed as amplification plots, which shows the change in fluorescence during the PCR cycles, and can be used to quantitate the threshold cycle (Ct), which is defined as "the cycle at which fluorescence is determined to be statistically significant above background" (manufacturer's instructions).

2.12 YO-PRO 1 assay

YO-PRO 1 is a dye that fluoresces on binding to nucleic acids. The YO-PRO 1 uptake method used has been described previously by Michel et al. (1999) to study P2X$_7$ receptor activation.

Cells were grown in 25 cm^2 flasks until confluent, then the medium was removed and cells were collected using trypsin-EDTA. The cells were resuspended in 1 ml of growing medium and centrifuged at 200g at room temperature for 5 minutes. The cell pellet was then resuspended in 10 ml Dulbecco's PBS, the cells were counted with a haemocytometer and

centrifuged again at 200g for 5 min at room temperature. The cell pellet was resuspended in 10 ml ice-cold assay buffer pH 7.4 (KCl 5 mM, $CaCl_2$ 0.5 mM, glucose 10 mM, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid (HEPES) 10 mM, N-methy-D-glucamine 10 mM, and sucrose 280 mM) and centrifuged at 200 g for 5 min at room temperature and resuspended again in 37°C assay buffer. Cells were added to Greiner 96-well black plates (Greiner Bio-One Ltd., Stonehouse, Gloucestershire, UK) (100,000 cells/well for MG63 and SaOS2 cells) containing agonist and 1 μM YO-PRO 1 in assay buffer, and then the plate was incubated at 37°C. YO-PRO 1 fluorescence was monitored in a 96-well plate reader (Fluostar Optima, BMG Labtechnologies Ltd., Aylesbury, UK) using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data were expressed as % of maximum uptake for each agonist.

To determine the best incubation time for YO-PRO 1 uptake, MG63 and SaOS2 cells were incubated with ATP $8x10^{-4}$ M for different periods (5-30 minutes) and then the uptake was monitored. Another experiment was carried out to find the best assay buffer for YO-PRO 1 uptake in MG63 and SaOS2 cells. Five different buffers were studied, and these are shown in Table 2.1.

Buffer	Ingredient
Buffer-A	KCl 5 mM, $CaCl_2$ 0.5 mM, glucose 10 mM, HEPES 10 mM, NMDG 10 mM, and sucrose 280 mM
Buffer-B	KCl 5 mM, $CaCl_2$ 0.1 mM, glucose 10 mM, HEPES 10 mM, NMDG 10 mM, and sucrose 280 mM
Buffer-C	KCl 5 mM, EDTA 0.1 mM, glucose 10 mM, HEPES 10 mM, NMDG 10 mM, and sucrose 280 mM
Buffer-D	Dulbecco's PBS
Buffer-E	KCl 125 mM, EDTA 1 mM, glucose 5 mM, and HEPES 20 mM, pH 7.4 (Gartland et al., 2001)

Table 2.1: Buffers tested to find the best assay buffer for YO-PRO 1 uptake in MG63 and SaOS2 cells.

All other experiments (outlined below) with YO-PRO 1 were carried out for 5 minutes and in buffer-A.

Different P2X receptor antagonists were studied to investigate whether they could block pore formation induced by ATP and DBzATP. For this purpose cells were pre-incubated with different concentrations of the antagonists for one hour in the presence of 1 μM YO-PRO 1 in assay buffer-A at 37°C, then ATP or DBzATP was added and the assay performed as above in assay buffer-A and an incubation time of 5 minutes. The antagonist pre-incubation period was based on an earlier experiment to determine the best incubation time for the antagonists to inhibit YO-PRO 1 uptake (details are given in Chapter 4). Data were expressed as % of maximum uptake of each agonist and EC_{50} values of the agonists were calculated using GraphPad Prism 4.0 programme (Graphpad software, San Diego, USA).

Experiments were also performed to investigate receptor desensitization and/or the involvement of other pore forming purinergic receptors (namely $P2X_2$ and $P2X_4$). In this experiment flasks of MG63 or SaOS2 cells were incubated with or without ATP $3x10^{-3}$ M at 37°C for 20 minutes. Following this, the YO-PRO 1 uptake assay was carried out using assay buffer-A and an incubation time of 5 minutes.

To study the effect of cell density and cell culture period on $P2X_7$ receptor-induced pore formation, experiments were carried out as described in Chapter 4.

2.13 ALP assay

MG63 or SaOS2 cells were grown in 25 cm^2 flasks until confluent, then the growing medium was removed and cells were collected using trypsin-EDTA. The cells were resuspended in 1 ml of medium and centrifuged at 522 g at room temperature for 3 minutes. The cell pellet was then resuspended in 7 ml medium and the cells counted as before. The cells were added to 96 well plates at 1,500 cells per well and incubated overnight. For each experiment 2 plates were set up, and for control wells and each concentration of ATP, twelve replicates per plate were set up. Next day (day 0) the medium was removed and ATP-containing medium was added. In the control wells the ATP was replaced with an equal volume of water (ATP vehicle). The cells were treated again with ATP or water on days 2 and 5.

ALP assays were performed on days 2, 5, and 7. The medium was removed from wells in one plate of each pair and cells were washed with 100 µl Dulbecco's PBS per well, and then treated with 50 µl freshly

mixed equal volumes (1:1) of alkaline buffer solution (2-amino-2-methyl-1-propanol 1.5 M, pH 10.3) and phosphatase substrate (4 mg/ml p-nitrophenylphosphate, disodium). In the case of the SaOS2 cells (based on previous experiments by B. Evans, personal communication) a ratio of alkaline buffer solution to phosphatase substrate of 9:1 was used. The treated cells were incubated at 37°C and the absorbance was then read at 20, 30, 40, and 50 minutes in a Packard Spectracount plate reader (Coningen, Netherlands) at 405 nm. The lowest sensitivity of the assay is 0.4 Sigma unit/ml (approximately 0.06 OD). A blank well (i.e. no cells) was included in each assay.

The second plate from each pair was used to assess cell number on each assay day using a Cell Titer 96® Aqueous One Solution Cell Proliferation assay (Promega, Southampton, UK). According to the manufacturer's instructions, 20 µl of the reagent was added to each well, the plate incubated at 37°C for one hour and the absorbance read at 490 nm in the above plate reader. Appropriate blank wells were always included. The results were expressed as OD mean ± the standard error of the mean (SEM).

Following an ALP assay and cell number assay on any set of 2 plates, the ALP data was corrected for cell number by correcting the ALP data to the equivalent of an OD of 10 in the cell number assay.

In addition to investigating the effect of ATP on ALP activity, experiments were performed to determine the effect of DBzATP on ALP. The cells were treated with DBzATP 24 hours after plating, and ALP activity and cell number were measured after 2 days of treatment. The results were expressed as ALP activity corrected for cell number as described above.

2.14 IL-6 and IL-1β ELISA

Human IL-6 and IL-1β enzyme linked immunosorbent assay (ELISA) Ready-SET-Go sets (eBioscience, Insight Biotechnology Limited, Wembley, UK) were used to measure IL-6 and IL-1β concentrations in cell culture medium. The manufacturer's instructions were followe, and the assay sensitivities for IL-6 and IL-1β were 2 pg/ml and 4 pg/ml, respectively.

A NUNC Maxisorp 96 well ELISA plate was coated with 100 µl/well of capture antibody in coating buffer, and the plate was sealed and incubated overnight at 4°C. Next day, the wells were washed 5 times with >250 µl/well of Dulbecco's PBS with 0.05 % Tween (PBST), allowing about 1 minute for soaking during each wash step, then the plate was blotted on absorbent paper to remove any residual buffer. In the case of the IL-6 ELISA, the number of washes in this step was 3. Next, wells were blocked with 200 µl/well of assay diluent, and incubated at room temperature for 1 hour, then washed as before 5 times. In the next step 100 µl/well of each standard and sample was added, and the plate sealed.

After incubation at room temperature for 2 hours, wells were washed 5 times as before. Then, 100 µl/well of detection antibody was added, the plate sealed and incubated at room temperature for 1 hour, followed by 5 washes as before. Next, 100 µl/well of Avidin-HRP was added, the plate sealed and incubated at room temperature for 30 minutes, followed by 7 washes with 1-2 minutes soaking during each wash step. Then 100 µl/well of Substrate Solution (Tetramethylbenzidine) was added to each well, and the plate incubated at room temperature for 15 minutes. In the last step, 50 µl of Stop Solution (2N sulphuric acid) was added to each

well, and the plate read at 450 and 570 nm. The 570 nm values represent the background and were subtracted from those at 450 nm. A standard curve was produced from which the concentration of the samples was calculated using the Prism 4.0 programme (Graphpad software, San Diego, USA). Protein concentrations were measured for each well using the BCA™ method described previously and the data were expressed as pg/mg protein.

For collecting cells for protein assay, medium was removed and cells washed with Dulbecco's PBS and fixed with 100 % methanol for 1 minute, and then the methanol was removed and cells allowed to dry. Next, cells were solubilised with 0.5 M sodium hydroxide (NaOH) for 30 minutes, and the solubilised cells transferred to 1.5 ml tubes and stored at -20°C. The method used for protein estimation is described in this Chapter (section 2.6).

2.15 Mineralisation

SaOS2 cells were seeded into 6 well plates at 500,000 cells per well and incubated overnight. For each experiment 2 plates were set up, one for the mineralisation assay and another one for protein assay. The next day, the DMEM was changed to one containing ascorbic acid (50 μg/ml). On the next day, DMEM was changed to one containing ascorbic acid (50 μg/ml) and β-glycerophosphate (8 mM), and different treatments. This treatment was repeated twice weekly until day 15, when cells were stained with freshly prepared 1 % alizarin red S in water as follows.

On the day of staining, the medium was removed and cells were washed with approximately 1.5 ml Dulbecco's PBS per well. Then, the PBS was removed and the cells fixed in 1 ml per well with formaldehyde in saline

(5 ml formaldehyde 40 % solution + 45 ml Dulbecco's PBS) for 15 minutes. Next, the formaldehyde in saline was removed and the cells washed in 1.5 ml per well with distilled water. The distilled water was then removed and the cells were stained with 1 ml per well of alizarin red S for 5 minutes. Next, the stain was removed and the cells were washed 5 times with approximately 2 ml per well of 50 % ethanol leaving the last wash on the cells for 15 minutes, and then the ethanol was removed and the cells left to air dry. The plates were then scanned using an Epson Perfection 4990 photo flatbed scanner, and the alizarin red S stain OD of the scans was analyzed using Image-pro plus version 6.1 (Media Cybernetics, UK).

2.16 Statistical analysis

Results were analysed using one-way analysis of variance (ANOVA). Multiple comparisons were then made using Newman-Keuls post-hoc tests in Prism 4.0 programme (Graphpad software, San Diego, USA). If two groups of samples were analysed, Student's t-Test was used. P-values less than 0.05 were considered to be significant. All results are expressed as the mean ± SEM.

Chapter 3: Expression of the $P2X_7$ receptor by human osteoblasts

3.1 Introduction

3.1.1 Tissue distribution of $P2X_7$ Receptors

The $P2X_7$ receptor (initially known as P2Z) is widely distributed in the body. $P2X_7$ receptors were initially found in haemopoietic cells only but now they have been identified in other cells as well. In human blood the $P2X_7$ receptor has been reported to be expressed by the following cells: macrophages, dendritic cells, B-lymphocytes, T-lymphocytes, polymorphonuclear cells, and erythrocytes (Di Virgilio et al., 2001; Volonte et al., 2006). $P2X_7$ receptor were also found in cells derived from myeloid progenitors (granulocytes and monocytes), B cells and a subset of T cells (Collo et al., 1997) and murine mast cells (Bulanova et al., 2005). In pathological blood cells, expression of the $P2X_7$ receptor has been reported in human leukaemic cells (Thunberg et al., 2002; Wiley et al., 2002; Zhang et al., 2004).

In non-haemopoietic cells, expression of $P2X_7$ receptors in epithelial tissues (namely airway epithelium, salivary gland, exocrine pancreas, anterior pituitary gland, and skin) has been demonstrated (North, 2002). Additionally, $P2X_7$ receptor has been found in toad stomach (North, 2002), human intestinal epithelial carcinoma cells (Coutinho-Silva et al., 2005), and human epidermal and monocyte-derived Langerhans cells (Georgiou et al., 2005). In the nervous system, $P2X_7$ receptors were reported in the rat brain in microglia and ependymal cells (Collo et al., 1997), rat neurons (Deuchars et al., 2001) and glial cells in the rat lumbar dorsal root ganglion (Kobayashi et al., 2005).

$P2X_7$ receptor expression has also been shown in many other cells such as human melanoma cells (White et al., 2005), fibroblasts from diabetic

patients' (Solini et al., 2004; Sugiyama et al., 2004) and prostate cancer (Slater et al., 2005).

A more detailed discussion of the tissue distribution of P2X$_7$ receptors is presented in Chapter 1.

3.1.2 P2X$_7$ receptors in bone

The expression and function of P2X$_7$ receptors in osteoclasts is well established in the literature (Hoebertz et al., 2000; Naemsch et al., 2001; Jorgensen et al., 2002; Ke et al., 2003; Gartland et al., 2003b). in contrast, at the beginning of my Ph.D., expression of P2X$_7$ receptor in osteoblasts had been reported in few studies only (Nakamura et al., 2000; Gartland et al., 2001; Ke et al., 2003). More details of the distribution of P2X$_7$ receptors in bone is presented in Chapter 1.

Since P2X$_7$ receptors appear to be involved in bone formation as revealed from the work with P2X$_7$ receptor knockout mice (Ke et al., 2003), and so little was known about their expression in osteoblasts, the work in this chapter aimed to characterise these receptors more fully in two human osteoblast-like cell lines, MG63 and SaOS2, which are at different stages of development of the osteoblast phenotype (Hughes and Aubin, 1998). The MG63 cells are early osteoblasts, whilst the SaOS2 cells are at a much later stage of osteoblast differentiation.

3.2 Aims

To study the expression of $P2X_7$ receptors by osteoblast-like osteosarcoma cell lines (MG63 and SaOS2) and to show the subcellular localization of the receptor in these cells. More specifically:

A. To investigate receptor expression at the mRNA level using RT-PCR. Additionally, QPCR was used to study the effect of cell density on receptor expression.

B. To investigate receptor expression at the protein level using Western blotting. Additionally, this technique was used to determine the subcellular localisation of the receptor using samples of cell membranes, lysate, and nuclei. Furthermore, the effect of receptor deglycosylation was studied.

C. To investigate the effect of cell density on receptor expression at the protein level using Western blotting.

D. To confirm receptor expression at the protein level using immunocytochemistry, and to show the subcellular localization of the receptor in these cells.

E. To study the expression of other purinergic pore-forming receptors, $P2X_2$, $P2X_4$, and $P2X_5$.

3.3 Experimental protocols

The protocols used to study the expression of P2X$_7$ receptors in osteoblasts were described in detail in Chapter 2.

3.3.1 RT-PCR

Specific primers for the human P2X$_2$, P2X$_4$, P2X$_5$, and P2X$_7$ receptors and β-actin and their product sizes are shown in table 3.1. All primers were obtained from Invitrogen (Paisley, UK) and were checked against Genbank for specificity.

Gene	*Primers (5' – 3')*	*Product Size (bp)*
P2X$_2$ Receptor	Sense-CCCAAATTCCACTTCTCCAA	204
	Antisense-GTCCAGGTCACAGTCCCAGT	
P2X$_4$ Receptor	Sense- ACCGTGCTGTGTGACATCAT	190
	Antisense- TGAGTGCTTGTGGAGTGGAG	
P2X$_5$ Receptor	Sense - AGCTGGAAACGGAGTGAAGA	212
	Antisense- CCTTGACGTCCATCACATTG	
P2X$_7$ Receptor	Sense -GTTCCTCTGACCGAGGTT	100
	Antisense-CAGGTCTTCTGGTTCCCT	
β-actin	Sense - CCCAGCCATGTACGTTGCTA	126
	Antisense- GGGCATACCCCTCGTAGATG	

Table 3.1: Gene primers for the human P2X$_2$, P2X$_4$, P2X$_5$, and P2X$_7$ receptors and β-actin used in this project and their product sizes. bp=base pairs.

Human pancreas cell (BON-1) cDNA, used as a positive control for P2X$_2$ receptors was obtained from Dr. J. Ham, School of Medicine, Cardiff University.

3.3.2 Western Blotting

To study the expression of P2X$_7$ receptors at the protein level, Western blotting was carried out using the method and antibodies described in Chapter 2. Membranes of HEK7 cells were used as a positive control.

P2X$_7$ receptor deglycosylation

Deglycosylation of the P2X$_7$ receptor in MG63 and SaOS2 cell membranes was studied using a Glycoprotein Deglycosylation kit (Merck chemicals Ltd., Nottingham, UK). According to the manufacturer's instructions, samples were centrifuged at 15,306 g at 4°C for 15 minutes, and the pellet resuspended in 30 µl deionized water. The sample was divided into two tubes (15 µl each), 5 µl of 5x reaction buffer (250 mM sodium phosphate buffer, pH 7.0) and 1.25 µl denaturation solution (2 % SDS, 1 M β-mercaptoethanol) were added to each tube and mixed gently. Both tubes were heated at 100°C for 5 minutes, and cooled to room temperature. 1.25 µl Triton X-100® solution was added to each tube and mixed gently. 0.5 µl each of: N-glycosidase F, α2-3,6,8,9-neuroaminidase, and Endo- α-N-acetylgalactosaminidase was added to the deglycosylated tube, and 1.5 µl distilled water added to the non-deglycosylated tube. Both tubes were incubated for 3 hours at 37°C, half the volume of 3x sample buffer added, and they were then stored at -20°C until use. The samples were separated on 10 % polyacrylamide gels (as described in Chapter 2).

3.3.3 Immunocytochemistry

Immunocytochemistry was used to confirm the expression of P2X$_7$ receptors in the osteoblast-like cells (MG63 and SaOS2) at the protein level, and to show the subcellular localization of the receptor in these

cells. The monoclonal L4 antibody and the polyclonal $rP2X_7$ receptor were used for this purpose. Details of the methodology can be found in Chapter 2.

3.3.4 Effect of cell density on $P2X_7$ receptor expression

a) mRNA level

To investigate the effect of cell density on $P2X_7$ receptor expression at the mRNA level, different cell densities ($2x10^5$, $4x10^5$, $1x10^6$, and $3x10^6$ cells per flask) of both cell lines (MG63 and SaOS2) were prepared, maintained in culture medium for 24 hours, and then the RNA extracted. The RNA preparations were treated with DNAse before undertaking RT and QPCR for the $P2X_7$ receptor and β-actin genes. The QPCR system software produced a relative quantity chart comparing the receptor expression of the samples (i.e. comparing expression of the $P2X_7$ receptor and β-actin genes). A detailed discussion of the QPCR is presented in Chapter 2.

b) Protein level

To study the effect of cell density on $P2X_7$ receptor protein expression, cells (MG63 and SaOS2) were seeded at different densities ($2x10^5$, $4x10^5$, $1x10^6$, and $3x10^6$ cells per 25 cm^2 flask) in medium, incubated for 24 hours, lysed and Western blotting performed. The densities of the bands were compared to a housekeeping gene protein (β-actin or GAPDH).

Additionally, Mg63 and SaOS2 cells at densities of $4x10^5$, and $1x10^6$ cells per 25 cm^2 flask were maintained in culture for one and seven days,

lysed, and Western blotting performed. The densities of the bands were compared to a housekeeping gene protein (β-actin or GAPDH).

3.4 Results

3.4.1 Expression at the mRNA level

RT-PCR for $P2X_4$ receptors (Figure 3.1B), $P2X_5$ receptors (Figure 3.1C), and $P2X_7$ receptors (Figure 3.1D), in MG63 and SAOS2 cell mRNA samples gave bands of the expected size (200 bp for the $P2X_4$, and $P2X_5$ and 100 bp for the $P2X_7$ receptor). Control samples (water or no AMV in the RT reaction) did not show any bands. To confirm these results sequencing was carried on the bands excised from the gel and the expected sequence of nucleotides was obtained in the DNA fragments (Figures 3.3, 3.4, and 3.5). No bands were seen using the $P2X_2$ receptor primers in either cell line (Figure 3.1A). Human pancreatic cell line mRNA (BON-1 cells) was used as a positive control to demonstrate that the primers did amplify the expected product (200bp, Figure 3.1A) which was sequenced to confirm the expected sequence of nucleotides (Figure 3.2).

3.4.2 Expression at the protein level

a) Western Blotting:

Membranes of HEK7 cells were used as positive controls. Using the $rP2X_7$ receptor antibody, bands were seen in the positive control, SaOS2 and MG63 cell membranes, lysate, and nuclei at 89.1±0.7 kDa (n=10) and 67.9±0.8 kDa (n=10) (figure 3.6). The 89 kDa band was absent in some of the membrane samples. A representative example with bands at 91.7 and 73 kDa (SaOS2) and 106.6 and 71.4 kDa (MG63) is shown in

Figure 3.6C. Bands larger than 100 kDa were also seen in the SaOS2 and MG63 lysate and nuclei (data not shown). A band at 55 kDa was also seen in some of the samples. Following pre-incubation of the $rP2X_7$ receptor antibody with the peptide used to raise it, all of the bands in the positive control, SaOS2 and MG63 membranes, lysate and nuclei were greatly reduced or disappeared.

Additionally, Western blotting was performed to investigate the expression of other pore-forming P2X receptors ($P2X_2$ and $P2X_4$). Rat brain cell membranes were used as a positive control. Figure 3.7 shows bands in the positive control, and MG63 and SaOS2 cell lysate samples at 59 kDa (61.3±0.6 kDa; n=5) for the $P2X_4$ receptor. Following pre-incubation of the $rP2X_4$ receptor antibody with the peptide used to raise it, this band in the positive control, and MG63 and SaOS2 cell lysates disappeared. Using the anti-$P2X_2$ receptor antibody, bands at 56.5±1.0 kDa (n=7) and 65.7±1.0 kDa (n=7) were seen in all samples but, following pre-incubation of the $rP2X_2$ receptor antibody with the peptide used to raise it, only the positive control band disappeared (figure 3.8).

Deglycosylation of MG63 and SaOS2 cell membranes reduced the apparent molecular weight of the $P2X_7$ receptor from 68 kDa±1.7 to 60kDa ±2 (n=3) and from 70 kDa±1.9 to 61 kDa±2 (n=3) in MG63 and SaOS2 cells, respectively (figure 3.9).

b) Immunocytochemistry

Localization of the $P2X_7$ receptor with the L4 antibody

The L4 monoclonal antibody binds to an external epitope on the $P2X_7$ receptor (Buell et al., 1998), and therefore it is not necessary to permeabilize the cell to see staining. To observe the effect of

permeabilizing the cell and allowing the antibody to enter inside the cell, a surfactant, Triton X-100® was also used. The blocking solution in which the antibody was diluted was used either in the presence or absence of Triton X-100®. Figures 3.10 and 3.11 show MG63 and SaOS2 cells incubated with the L4 antibody in the presence and absence of Triton X-100®. In the presence of Triton X-100®, the labelling was seen throughout the cell, but in its absence, only the cell membrane was labelled. C and D in Figures 3.10 and 3.11 show MG63 and SaOS2 control cells in which the blocking solution was used alone (without addition of antibody) plus or minus Triton X-100®. As can be seen, only very faint labelling of the cells was present compared to the cells incubated with the antibody.

Localization of the P2X$_7$ receptor with the rP2X$_7$ receptor antibody

Since the epitope to which the rP2X$_7$ receptor antibody binds is located intracellularly on the C-termini of the receptor, Triton X-100® was always added to the blocking solution used to dilute the antibody. In Figure 3.12 A and C MG63 and SaOS2 cells were incubated with the rP2X$_7$ antibody, and showed much more intense labelling of the nuclei compared to the cytoplasm. No labelling was seen in the control cells incubated with the blocking solution in the absence of the antibody (data not shown).

To confirm that the labelling seen with the polyclonal rP2X$_7$ receptor antibody was specific, it was incubated for 48 hours with the peptide used to raise it, and thus the antibody would no longer be available to recognise and bind to the receptor. Following pre-adsorption the labelling disappeared or was much weaker (Figure 3.12 B and D).

3.4.3 Effect of cell density on P2X$_7$ receptor expression

a) Quantitative real-time RT-PCR

Standard curves for the P2X$_7$ receptor and β–actin cDNA were generated by preparing serial dilutions of the cDNAs. These are shown in Figure 3.13 (A and B). The standard curves for β-actin and P2X$_7$ receptor cDNAs gave efficiencies of 105.7%, and 95.6% respectively. To confirm that the fluorescence resulted from the P2X$_7$ receptor cDNA and not from non-specific product such as primer-dimers, the final PCR reaction samples were run on an agarose gel and only gave bands of the expected size (100 bp) (data not shown). In addition melting curves showed one peak only (data not shown).

To determine the influence of cell density on receptor mRNA expression, QPCR was carried out on RNA prepared from cells at different densities ($2x10^5$, $4x10^5$, $1x10^6$, and $3x10^6$ cells per flask). The relative quantity of the receptor expression based on the mRNA level for all the cell densities of both cell lines is shown in Figure 3.14. In this figure the software built in the system compared P2X$_7$ receptor expression to that of β-actin in SaOS2 and MG63 cells. In each cell line the ratio of P2X$_7$ receptor: β-actin expression in $2x10^5$ cells per flask was set at 1.0. P2X$_7$ receptor expression in the $3x10^6$ SaOS2 cells per flask was found to be significantly higher than for $2x10^5$ cells per flask. Expression of β-actin did not change with cell density (data not shown).

b) Western blotting

To find the effect of cell density and days in culture on the P2X$_7$ receptor protein expression, cell lysate samples of MG63 and SaOS2 cells at $4x10^5$ and $1x10^6$ cells per 25 cm2 flask and cultured for 1 or 7 days were separated on 10 % gels. Membranes of HEK7 cells were used as positive

control. In all experiments the $P2X_7$ receptor band was seen at 69 kDa ± 1.35 (n=2), as was a band for β-actin at 42.7 kDa±0.42 (n=2) (Figure 3.15). An 88kDa±0.9 (n=2) band was seen in the MG63 $4x10^5$ sample cultured for 1 day, and $4x10^5$ and $1x10^6$ samples cultured for 7 days. Other bands (75, 70, 52, and 36 kDa) were also seen in some samples. The band intensity was similar for all samples of the $P2X_7$ receptor (69 kDa band) while the 85 kDa band was stronger in the higher cell density and longer growth time. However, the β-actin band intensity varied with cell number.

In other experiments, to find the effect of cell density on the $P2X_7$ receptor expression, cell lysate samples of MG63 and SaOS2 cells at $2x10^5$, $4x10^5$, $1x10^6$ and $3x10^6$ cells per 25 cm^2 flask grown for one day were separated on 10% gels. Membranes of HEK7 cells were used as positive controls. The $P2X_7$ receptor band was seen at 60.1 kDa±3.6 for MG63 and 61.7 kDa±3.1 for SaOS2 and a band for β-actin at 39.8 kDa±1.3 for MG63 and 40.4 kDa±1 for SaOS2. In addition, a band at 88.1 kDa±1 was seen in all the samples except the positive control, and another 92.8 kDa band was seen in the MG63 $4x10^5$, $1x10^6$, $3x10^6$, and SaOS2 $1x10^6$ and $3x10^6$ samples (Figure 3.16). Other smaller band in some samples was also seen at 23 kDa (data not shown). The intensity of the $P2X_7$ receptor at 60 kDa band was not altered by cell density, but the intensity of the 88 kDa and β-actin bands increased as the cell density increased.

Since the intensity of the β-actin band was shown to increase with increasing cell density, an experiment was performed to examine another housekeeping gene protein, GAPDH. In both experiments the positive control gave a band at 72-72.5 kDa ($P2X_7$ receptor protein) and 34.1-35.3 kDa (GAPDH protein), but other samples gave strong bands at 61-

65.2 kDa and 35.3-35.6 kDa (Figure 3.17). A band of 86.2-105.8 kDa size was seen in the $1x10^6$ and $3x10^6$ samples. Other smaller band in some samples was also seen at 25 kDa (data not shown). The intensity of the $P2X_7$ receptor did not alter with increasing cell density but the intensity of the 86 kDa and GAPDH bands increased as the cell density increased.

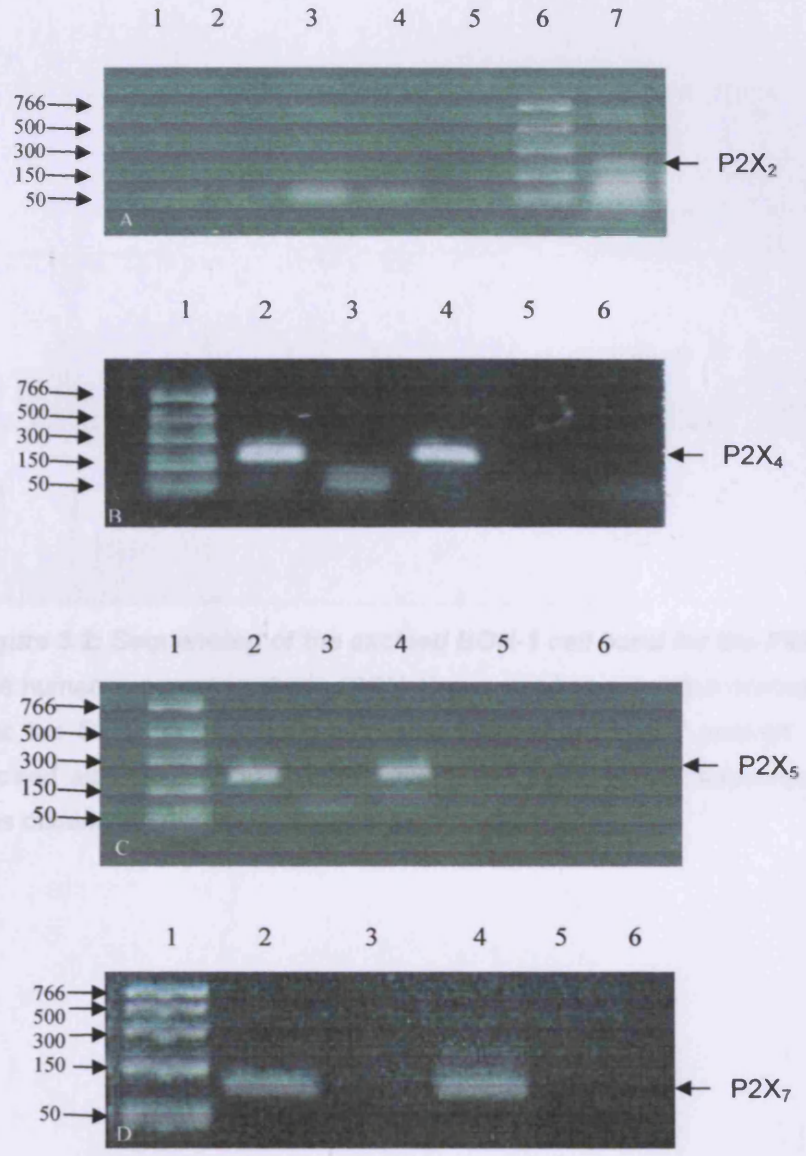


Figure 3.1: ***Representative example of RT-PCR of $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_7$ receptor mRNA in MG63 and SaOS2 samples.***

RT-PCR was performed on RNA extracted from MG63 and SaOS2 cells using primers for the human $P2X_2$ (A), $P2X_4$ (B), $P2X_5$ (C) and $P2X_7$ (D) receptors. In A the marker ladder is shown in lane 6, and human pancreas cell cDNA used as a positive control showed a band of 200 bp in lane 7, while no band was seen in all other samples. In B, C, and D the marker ladder is shown in lane 1, and in lanes 2 and 4 200 bp (or 100 bp) bands were seen for MG63 and SaOS2 samples, respectively, corresponding to $P2X_4$ and $P2X_5$ (or $P2X_7$) receptors; lanes 3, 5, and 6, no bands were seen when MG63 (lane 3) and SaOS2 (lane 5) samples were incubated without the RT enzyme or when sterile water was used in the PCR reaction instead of the RT reaction (lane 6). [n=2-5]

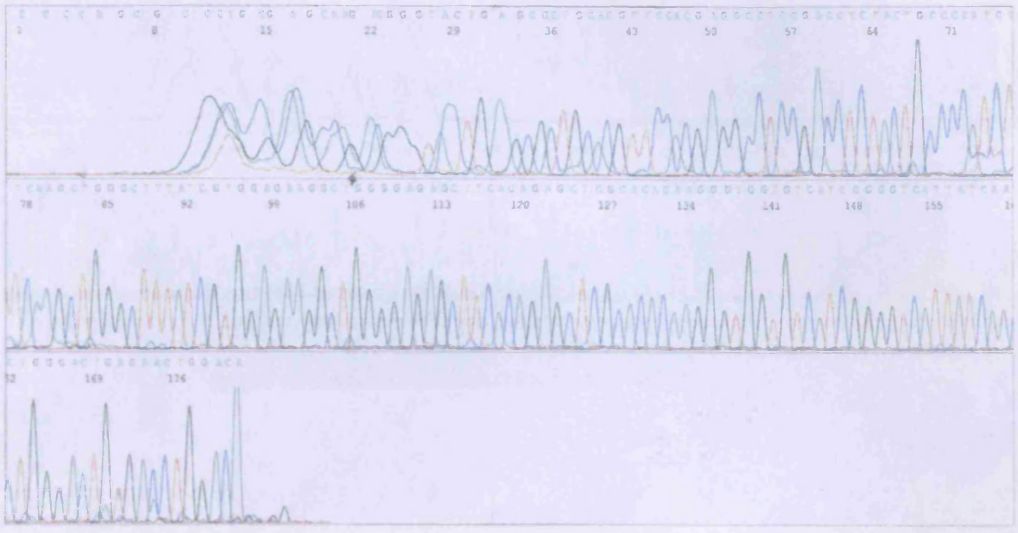


Figure 3.2: Sequencing of the excised BON-1 cell band for the $P2X_2$ receptor.
The human pancreatic cell line (BON-1) was used as a positive control to demonstrate that the $P2X_2$ receptor primers did amplify the expected product. The band was excised and sequencing was performed and the expected sequence of nucleotides was obtained in the DNA fragment. [n=1]

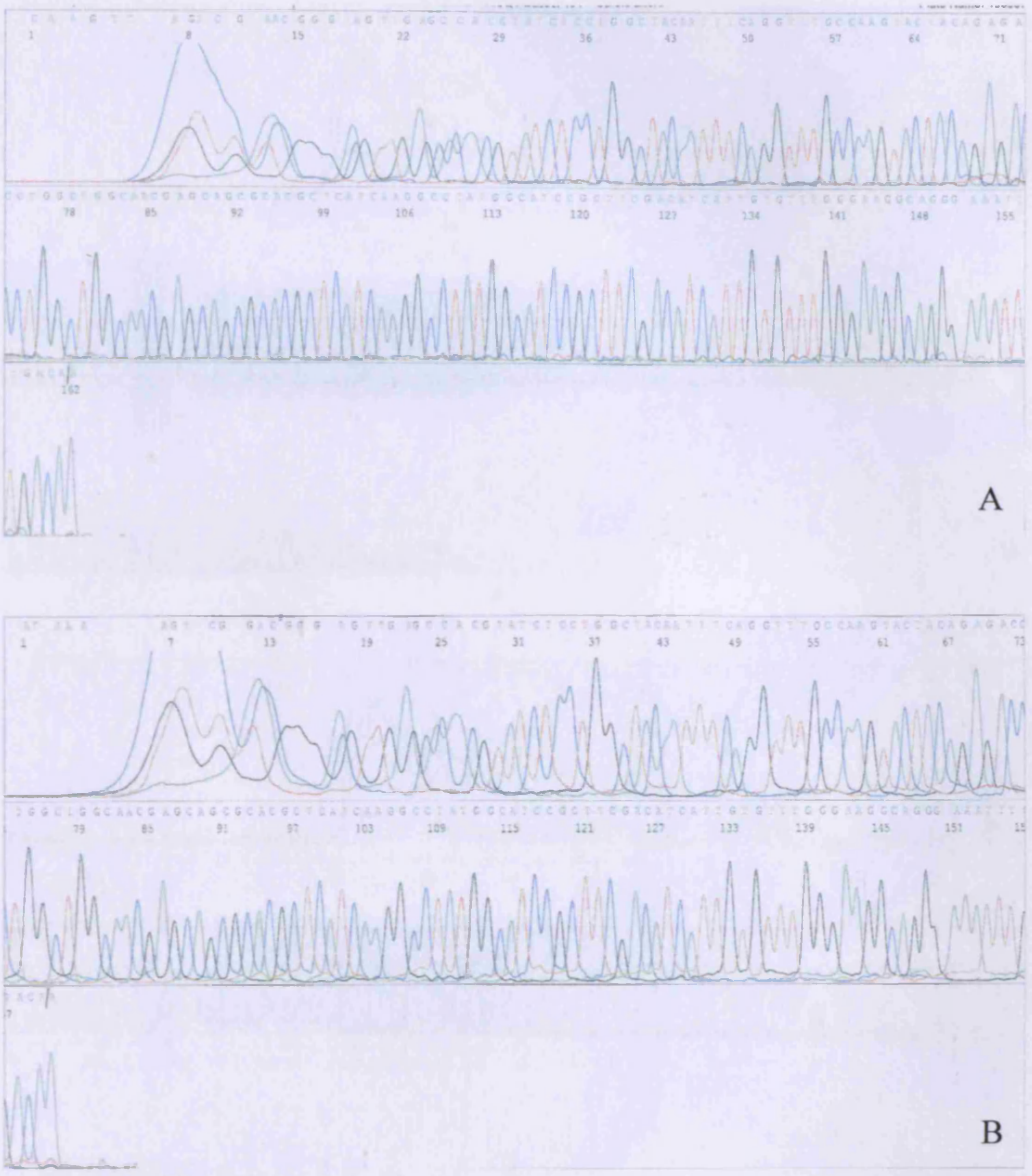


Figure 3.3: Sequencing of the excised SaOS2 and MG63 bands for the $P2X_4$ receptor.

Sequencing of the excised SaOS2 (A) and MG63 (B) sample bands for the $P2X_4$ receptor was performed and the expected sequence of nucleotides was obtained in the DNA fragments. [n=1]

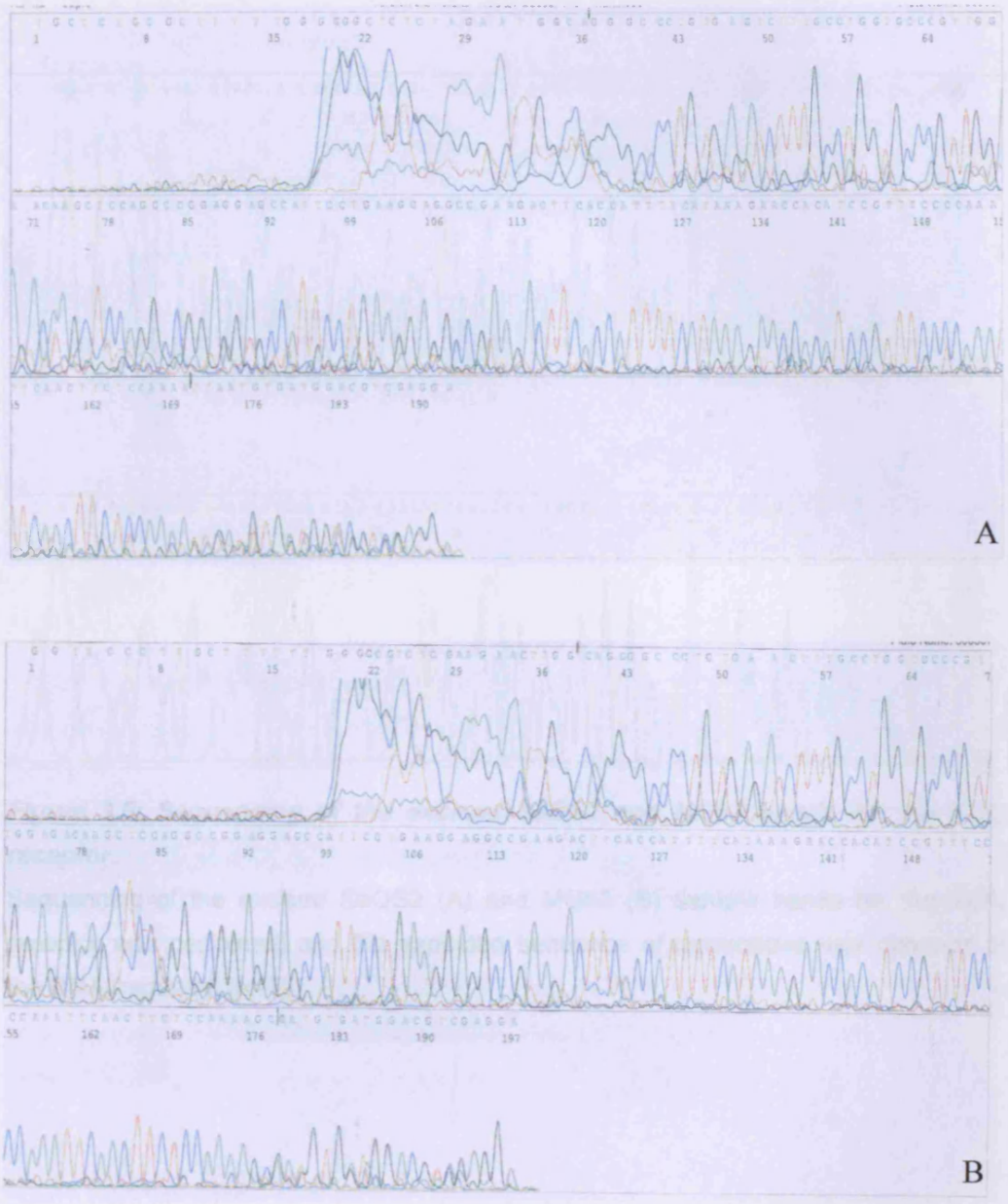


Figure 3.4: Sequencing of the excised SaOS2 and MG63 bands for the $P2X_5$ receptor.

Sequencing of the excised SaOS2 (A) and MG63 (B) sample bands for the $P2X_5$ receptor was performed and the expected sequence of nucleotides was obtained in the DNA fragment. [n=1]

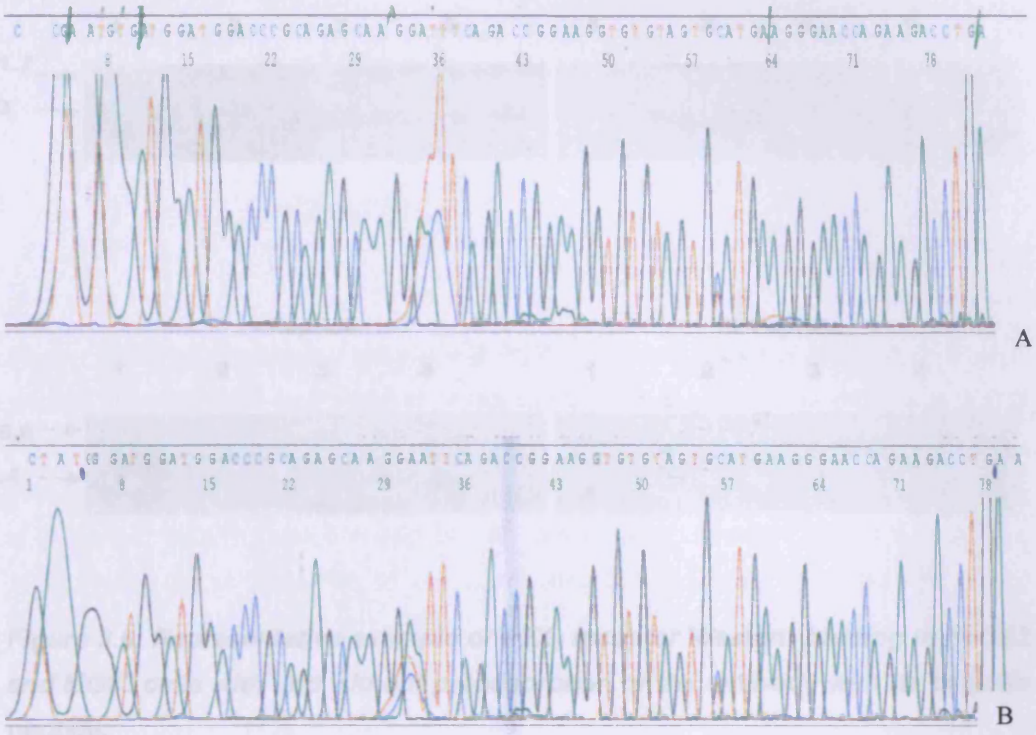


Figure 3.5: Sequencing of the excised SaOS2 and MG63 bands for the P2X$_7$ receptor.

Sequencing of the excised SaOS2 (A) and MG63 (B) sample bands for the P2X$_7$ receptor was performed and the expected sequence of nucleotides was obtained in the DNA fragment. [n=1]

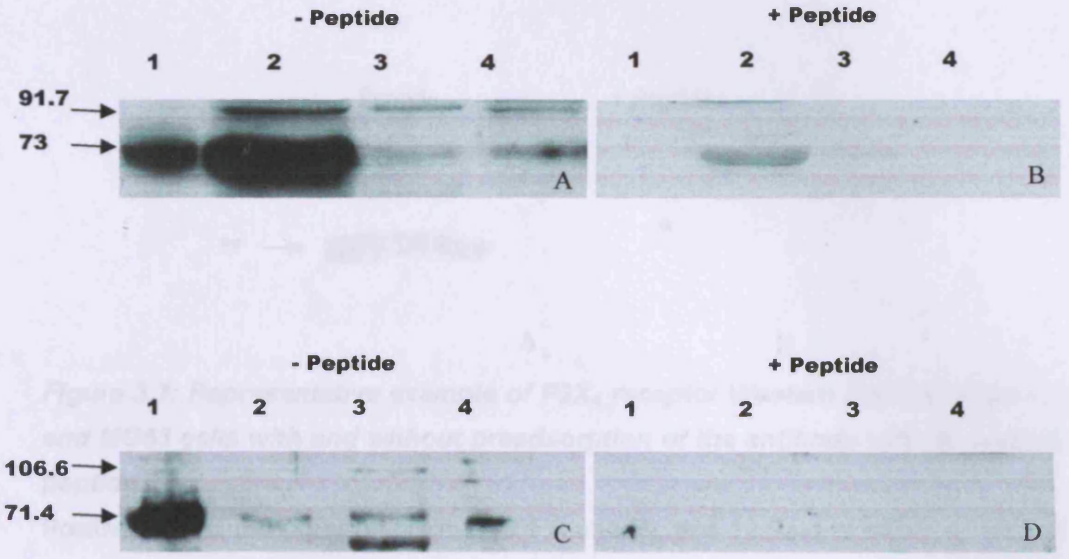


Figure 3.6: Representative example of P2X$_7$ receptor Western blotting in SaOS2 and MG63 cells with and without preadsorption of the antibody with its cognate peptide.

Positive control (10 µg of HEK7 cell membrane in lane 1), and 25 µg of SaOS2 (A and B) or MG63 (C and D) cell membranes (lane 2), lysate (lane 3), and nuclei (lane 4) were separated on 10 % gels and probed after prior incubation of the rP2X$_7$ receptor antibody (1:1000) with (B & D) or without (A & C) the peptide (1.2 µg) used to raise it. Bands were seen at at 73 kDa and 91.7 kDa in SaOS2 cells (A) and at 71.4 kDa in MG63 cells (C). All these bands were greatly reduced or disappeared after preincubation with the peptide (B & D). [n=10]

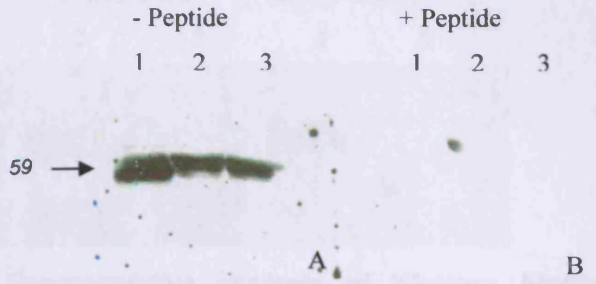


Figure 3.7: Representative example of $P2X_4$ receptor Western blotting in SaOS2 and MG63 cells with and without preadsorption of the antibody with its cognate peptide.

Positive control (60 µg of rat brain lane 1 A and B), and 12.5 µg of MG63 (lanes 2 A and B) and SaOS2 (lane 3 A and B) cell lysate were separated on 10 % gels and probed after prior incubation of the $rP2X_4$ receptor antibody (1:1000) with (B) or without (A) the peptide (3.2 µg) used to raise it. A band was seen in all the samples at 59 kDa (A), which disappeared after preincubation with the cognate peptide (B). [n=5]

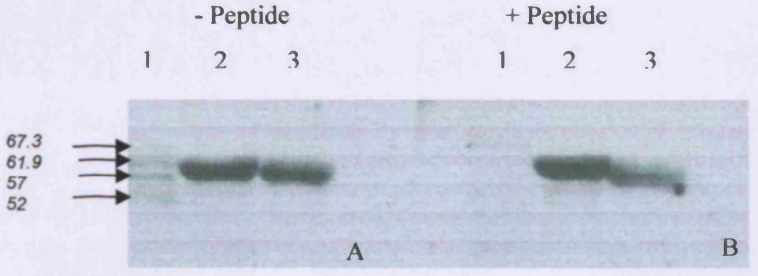


Figure 3.8: Representative example of $P2X_2$ receptor Western blotting in SaOS2 and MG63 cells with and without preadsorption of the antibody with its cognate peptide.

Positive control, 60 µg of rat brain (lane 1 A and B), 12.5 µg of MG63 (lane 2 A and B), and SaOS2 (lane 3 A and B) cell lysate were separated on 10 % gels and probed after prior incubation of the $rP2X_2$ receptor antibody (1:1000) with (B) or without (A) 4 µg of the peptide used to raise it. The positive control gave two bands at 67.3, 61.9, 57 and 52 kDa, MG63 and SaOS2 cell lysate samples gave bands at 61.9 and 52 kDa, but only the positive control bands (lane 1 in B) disappeared following preincubation with the cognate peptide. [n=7]

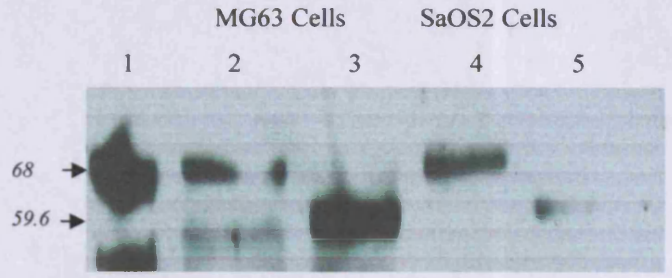


Figure 3.9: Representative example of Western blotting of the effect of deglycosylation on MG63 and SaOS2 cell membranes.

Positive control (5 µg of HEK7 cell membranes in lane 1), and 100 µg of non-deglycosylated MG63 (lane 2) and SaOS2 (4) cell membranes, deglycosylated MG63 (lane 3) and SaOS2 (lane 5) cell membranes. The nitrocellulose membrane was probed with the rP2X$_7$ antibody (1:2000). The positive control gave a band at 66.6 kDa, non-deglycosylated MG63 cell membranes at 68 kDa, deglycosylated MG63 cell membranes at 59.6 kDa, non-deglycosylated SaOS2 cell membranes at 69.4 kDa, and deglycosylated SaOS2 cell membranes at 50.7 kDa. [n=3]

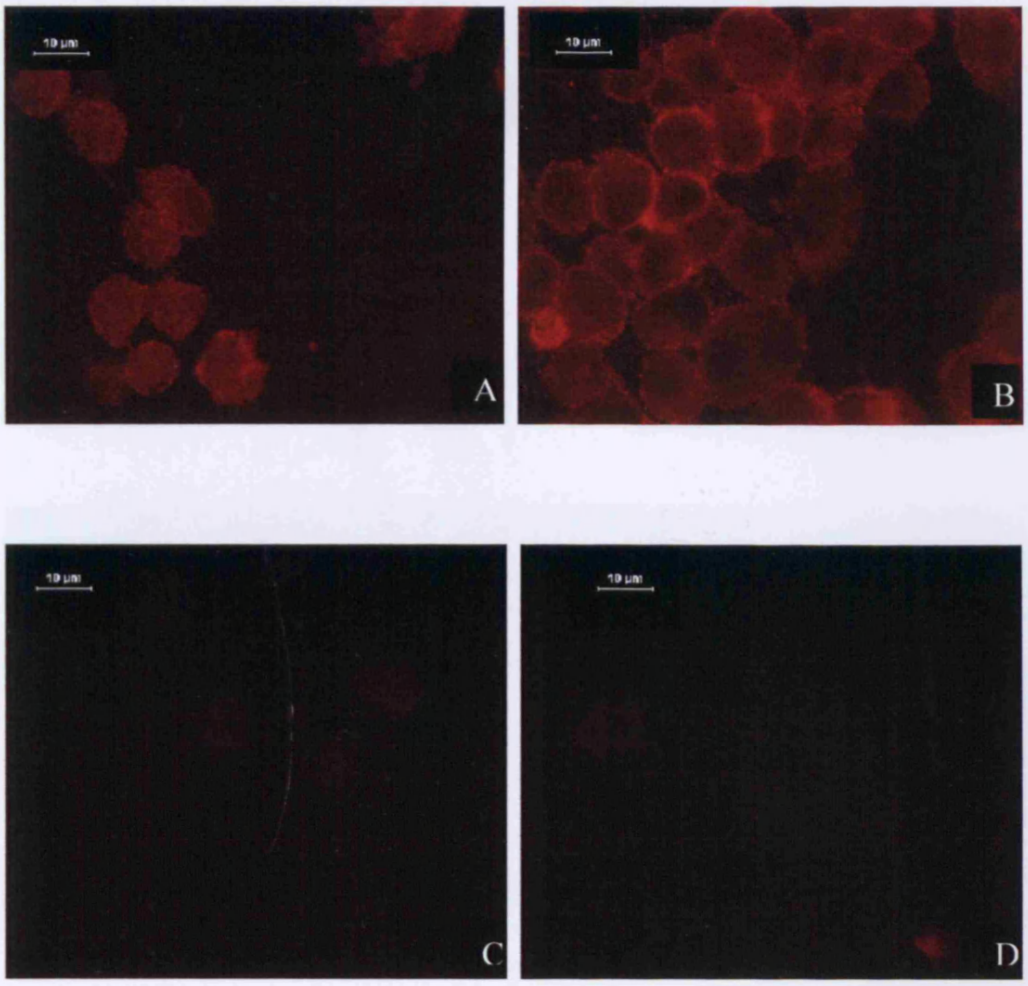


Figure 3.10: Representative example of immunocytochemistry in MG63 cells using the L4 antibody.

MG63 cells were incubated in the presence (A & B) and absence (C & D) of the L4 antibody (1:600) and in the presence (A & C) and absence (B & D) of Triton X-100® and detected with an anti-mouse secondary antibody conjugated to CY3 (1:800). In A labelling was seen throughout the cells, but in B the staining was located at the cell membrane. C and D are control images and show very little labelling of the cells compared to (A) and (B). Scale bar=10 µm. [n=3]

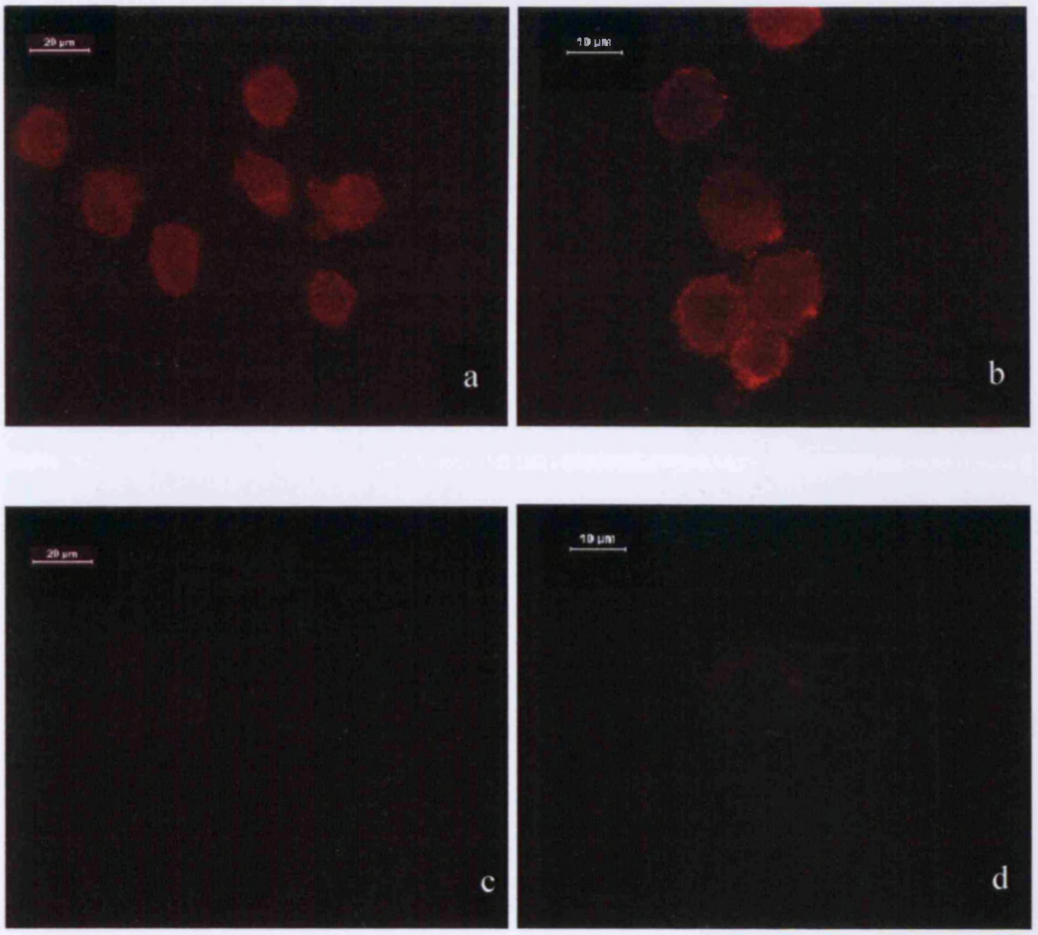


Figure 3.11: Representative example of immunocytochemistry in SaOS2 cells using the L4 antibody.

SaOS2 cells were incubated in the presence (A & B) and absence (C & D) of the L4 antibody (1:600) and in the presence (A & C) and absence (B & D) of Triton X-100® and detected with an anti-mouse secondary antibody conjugated to CY3 (1:800). In A labelling was seen throughout the cells, but in B the staining was located at the cell membranes. C and D are control images and show very little labelling of the cells compared to (A) and (B). Scale bar=10 µm in (A & C) and 20 µm in (B & D). [n=3]

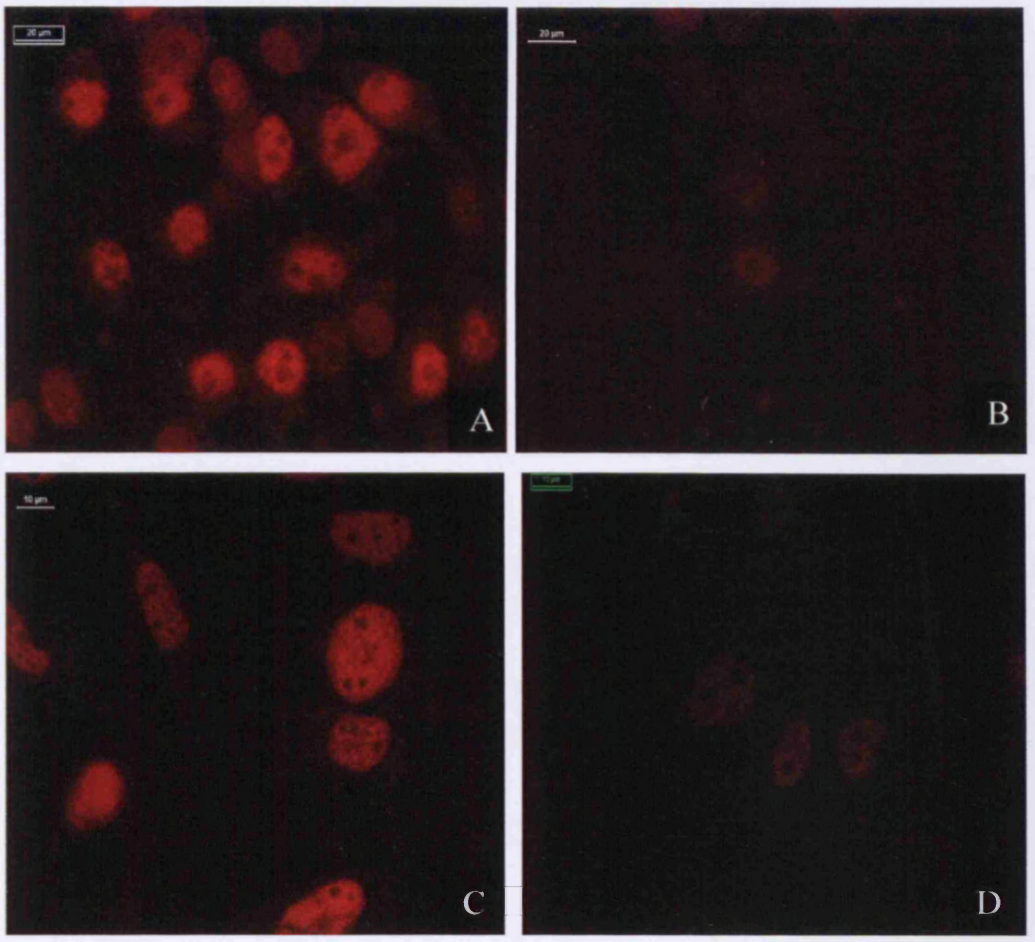


Figure 3.12: Representative example of immunocytochemistry in MG63 and SaOS2 cells using the rP2X$_7$ receptor antibody in the presence and absence of peptide.

MG63 (A and B) and SaOS2 cells (C and D) were incubated with the rP2X$_7$ receptor antibody (1:1000) after prior incubation with (B and D) or without (A and C) the peptide used to raise it (0.8 µg) and detected with an anti-rabbit secondary antibody conjugated to Cy3 (1:800). A and C showed intense staining in the nuclei compared to the cytoplasm, while in B and D the staining disappeared or was much weaker. Scale bar=10 µm in (A & B) and 20 µm in (C & D) [n=3].

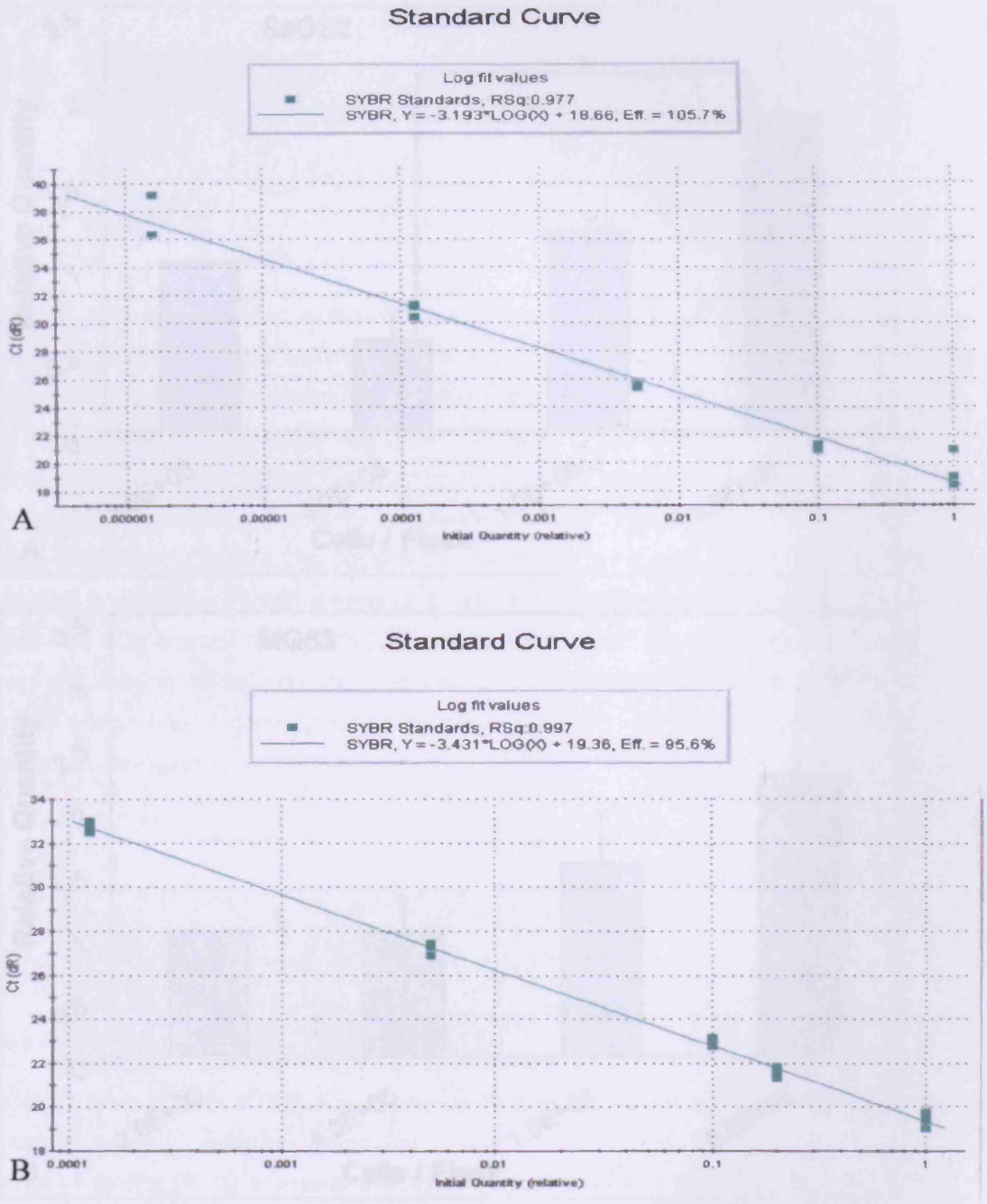


Figure 3.13: Standard curve for β-actin and P2X$_7$ receptor cDNAs.

An efficiency of 105.7% for β-actin (A) and 95.6% for P2X$_7$ (B) cDNAs was achieved. Ct: threshold cycle; "the cycle at which fluorescence is determined to be statistically above background"(Manual). [n=1]

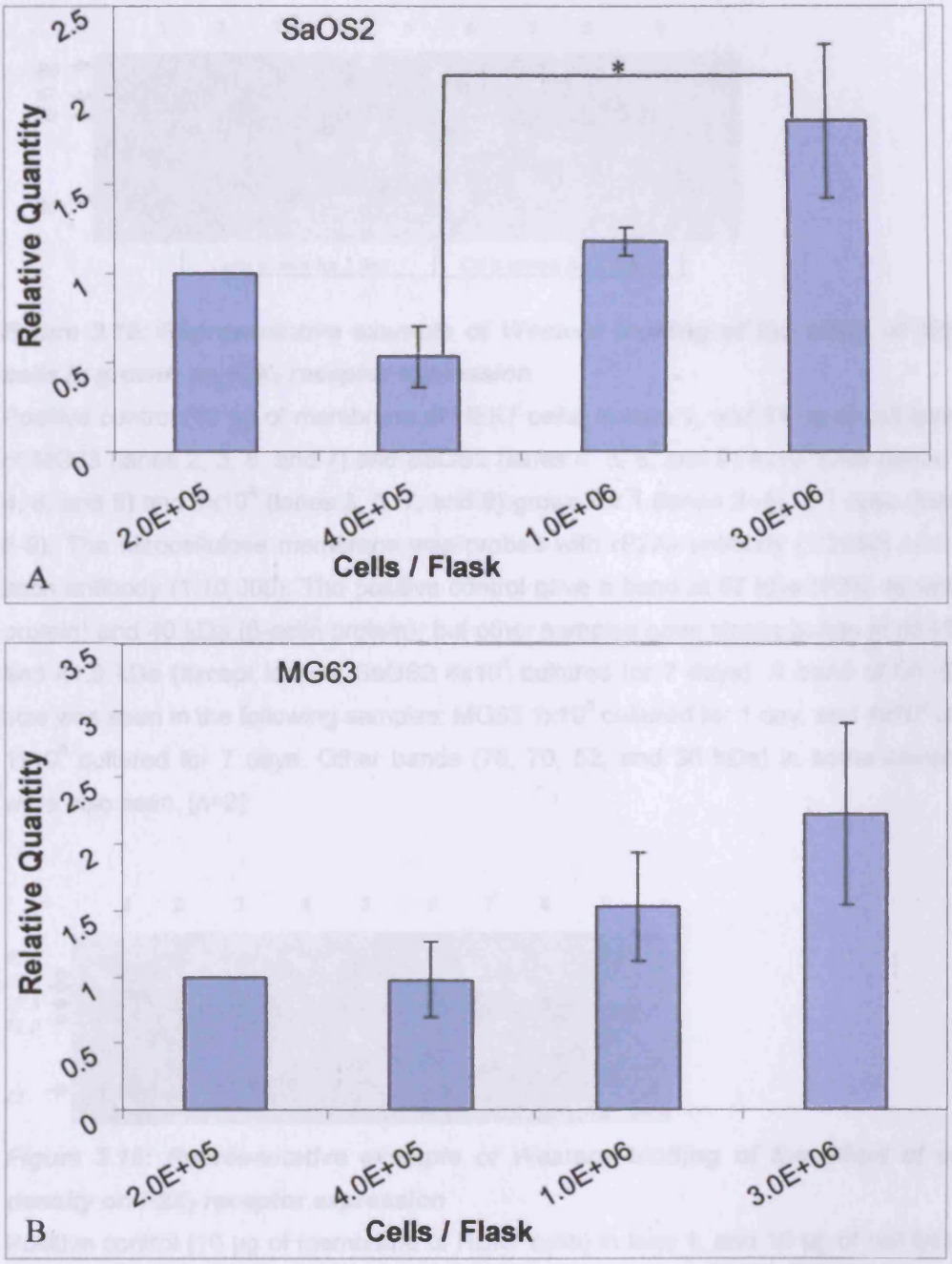


Figure 3.14: Relative expression of the P2X7 receptor mRNA for both cell lines. $2x10^5$, $4x10^5$, $1x10^6$, and $3x10^6$ SaOS2 (A) or MG63 (B) cells per flask were grown overnight, and the next day the RNA was extracted, and RT reactions followed by qPCR were performed. The system assumes the $2x10^5$ cells/flask = 1. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *$P<0.05$ vs. $4x10^5$ cells per flask. [n=3]

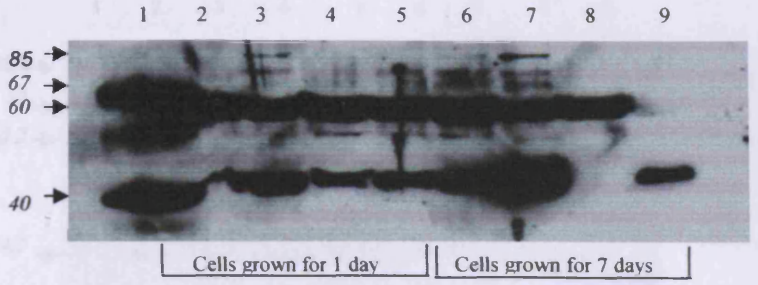


Figure 3.15: Representative example of Western blotting of the effect of days cells in growth on $P2X_7$ receptor expression

Positive control (10 µg of membrane of HEK7 cells) in lane 1, and 11 µg of cell lysate of MG63 (lanes 2, 3, 6, and 7) and SaOS2 (lanes 4, 5, 8, and 9) $4x10^5$ cells (lanes 2, 4, 6, and 8) and $1x10^6$ (lanes 3, 5, 7, and 9) grown for 1 (lanes 2 -5) or 7 days (lanes 6-9). The nitrocellulose membrane was probed with $rP2X_7$ antibody (1:2000) and β-actin antibody (1:10,000). The positive control gave a band at 67 kDa ($P2X_7$ receptor protein) and 40 kDa (β-actin protein), but other samples gave strong bands at 60 kDa and 41.9 kDa (except lane 8; SaOS2 $4x10^5$ cultured for 7 days). A band of 85 kDa size was seen in the following samples: MG63 $1x10^6$ cultured for 1 day, and $4x10^5$ and $1x10^6$ cultured for 7 days. Other bands (75, 70, 52, and 36 kDa) in some samples were also seen. [n=2]

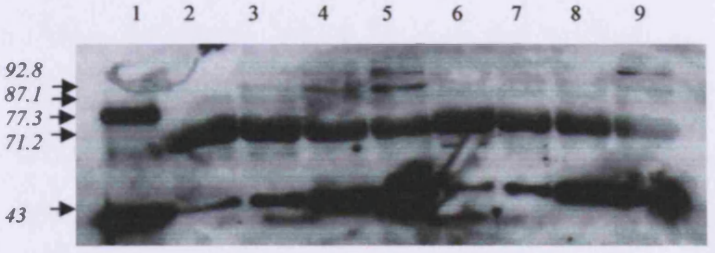


Figure 3.16: Representative example of Western blotting of the effect of cell density on $P2X_7$ receptor expression

Positive control (10 µg of membrane of HEK7 cells) in lane 1, and 10 µg of cell lysate of MG63 (lanes 2, 3, 4, and 5) and SaOS2 (lanes 6, 7, 8, and 9) $2x10^5$ cells (Lanes 2 and 6) $4x10^5$ cells (lanes 3 and 7), $1x10^6$ cells (lanes 4 and 8) and $3x10^6$ cells (lanes 5 and 9). The nitrocellulose membrane was probed with $rP2X_7$ antibody (1:2000) and β-actin antibody (1:10,000). The positive control gave a band at 77.3 kDa ($P2X_7$ receptor protein) and 43 kDa (β-actin protein), but other samples gave strong bands at 71.2 kDa and 43 kDa. A band of 87.1 kDa was seen in all the samples except the positive control, and another 92.8 kDa band was seen in the following samples: MG63 $4x10^5$, $1x10^6$, $3x10^6$, SaOS2 $1x10^6$, and $3x10^6$. [n=5]

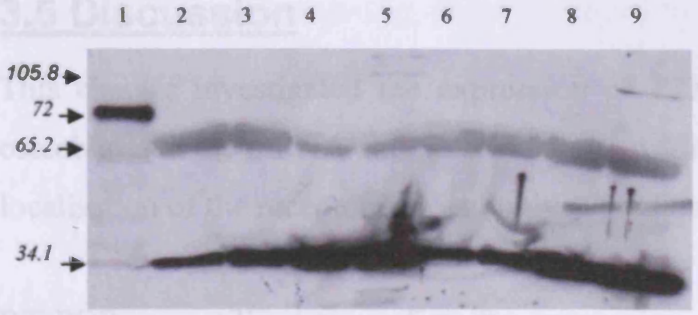


Figure 3.17: Representative example of Western blotting of the effect of cell density on $P2X_7$ receptor expression

Positive control (10 µg of membrane of HEK7 cells) in lane 1, and 10 µg of cell lysate of MG63 (lanes 2, 3, 4, and 5) and SaOS2 (lanes 6, 7, 8, and 9), $2x10^5$ cells (Lanes 2 and 6), $4x10^5$ cells (lanes 3 and 7), $1x10^6$ cells (lanes 4 and 8) and $3x10^6$ cells (lanes 5 and 9). The nitrocellulose membrane was probed with $rP2X_7$ antibody (1:2000) and GAPDH antibody (1:4000). The positive control gave a band at 72 kDa ($P2X_7$ receptor protein) and 34.1 kDa (GAPDH protein), but other samples gave strong bands at 65.2 kDa and 35.6 kDa. A band of 105.8 kDa size was seen in the $1x10^6$ and $3x10^6$ samples. [n=2]

3.5 Discussion

This chapter investigated the expression of $P2X_7$ receptors by human osteoblasts at the mRNA and protein levels. Additionally, the subcellular localisation of the receptor and its deglycosylation was studied.

RT-PCR was utilized to confirm the expression of the $P2X_7$ receptor at the mRNA level in both cell lines. This was confirmed by sequencing the bands excised from the gel and the expected sequence of nucleotides was attained in the cDNA fragments.

Since $P2X_2$ and $P2X_4$ receptors can both form pores (Virginio et al., 1999; Gever et al., 2006), the expression of these receptors was investigated in both cell lines. Neither $P2X_2$ receptor mRNA nor protein were detected in MG63 or SaOS2 cells suggesting that the receptor is not expressed or present in very low amounts in these cells. Positive controls for the PCR primers and antibody demonstrated that the $P2X_2$ receptor mRNA or protein could be detected by both methods. However, $P2X_4$ receptor mRNA and protein were identified in both cell lines. Therefore, MG63 and SaOS2 cells express $P2X_4$ and $P2X_7$ receptors. Another study reported pore formation with $P2X_5$ receptors (Bo et al., 2003). Hence, its expression was also examined using RT-PCR, and the presence of its mRNA was identified. Previous studies reported expression of $P2X_4$ receptor (Nakamura et al., 2000) and $P2X_5$ receptor (Hoebertz et al., 2000; Nakamura et al., 2000; Orriss et al., 2006). Therefore, MG63 and SaOS2 cells express three potential pore-forming purinergic receptors, $P2X_4$, $P2X_5$ and $P2X_7$.

Initial results demonstrated that the labelling seen with antibodies in Western blotting and immunocytochemsitry was specific for the $P2X_7$

receptor. This was proved by prior overnight incubation of the primary antibody ($rP2X_7$ receptor antibody) with the peptide used to raise it. This incubation allows the antibody to recognise the immobilized peptide and bind to it. The peptide and the antibody are then removed and the antibody is no longer available to recognise and bind to the receptor. This preincubation caused the relevant labelling to disappear, which suggests that it is caused by the anti-$P2X_7$ receptor antibody.

It was found that the receptor is expressed in all the samples used (lysate, nuclei, and membrane), but apparently at different levels which suggests that the level of expression of the receptor is different between the cell parts. The Western blotting results revealed in this study are consistent with those of Wang et al. (2005), as the band sizes are similar. They carried out their experiments on human cervical epithelial cells and reported a band of 85 kDa which they claimed to be the mature and functional form of the receptor, and another band of 65 kDa, believed to be a deglycosylated form, which was proved by deglycosylating the receptor. A previous study described only a band of approximately 70 kDa in human primary fibroblast lysate (Solini et al., 1999). Wang et al. (2005) described another band of 18 kDa which was believed to be a degradation product of the receptor. My experiments showed similar bands of 67.9±5.4 kDa, and 89.1±3.4 kDa. The 18 kDa band couldn't be seen in my study possibly due to longer gel running time, but I observed a band around 24 kDa, and other slightly larger bands (up to 55 kDa) in some experiments especially in the nuclei, which could be receptor deglycosylated forms. In the majority of my experiments, the 85 kDa receptor form, which was described by Feng et al (2005) and Wang et al (2005) in human cervical epithelial cells to be the mature and functional form of the receptor was either faint or absent in the membrane samples and the positive control (membranes of HEK7 cells). This may be

because the membrane is expressing the receptor at a lower level compared to the cytoplasm and the nuclei. However, this is unlikely because, if this is the functional form of the receptor, it would be expected to be strongly expressed at the membrane where the natural ligand (ATP) will activate it. Feng et al (2005) studied transfected HEK293 and CasKi (human cervical epithelial cells) cell membrane expression of the $P2X_7$ receptor and reported the three bands that were seen by Wang et al. (2005) and suggested also that the 85 kDa receptor was the functional form of the receptor. A possible reason for the difference between the results of this group and our results is that the method used to extract the cell membranes in our laboratory is highly efficient with no other cellular components available in the sample. To prove this a lysate sample of the HEK7 cells was run and it gave a band of 91.7 kDa, which may correspond to the 85 kDa band reported by Feng et al. (2005). An alternative explanation is that the 85 kDa is not the functional form of the receptor in our cells. To determine if the receptor in osteoblasts follows the same pattern as the epithelial cells, receptor deglycosylation was done.

$P2X_7$ receptor deglycosylation of membrane samples showed that the size of the $P2X_7$ receptor band decreased by approximately 9 kDa in the deglycosylated samples in both cell lines. North (2002) proposed that the glycosylated form of the P2X receptor is functional. My results suggest that the 67 kDa band possibly represents the functional form of the receptor in these cells (as it is the glycosylated form) and not the 85 kDa band claimed in epithelial cells (Wang et al., 2004a; Wang et al., 2004b; Wang et al., 2005; Feng et al., 2005b). An explanation of the difference of the functional receptor size is that it might differ from one cell type to another, and that it is approximately 67 kDa in osteoblasts. Another explanation is that there is more than one fully glycosylated form of the

receptor, but they are not all found in the membrane fraction. I have seen previously that the 85 kDa form of the receptor was either absent in membrane samples or only gave a faint band. The above groups used cell lysate samples and not membranes for their deglycosylation experiments. Efforts to deglycosylate $P2X_7$ receptors in my lysate samples were not successful probably due to the expression level of the receptor.

From the immunocytochemistry results I can conclude that the receptor is strongly expressed in intact cells of both the MG63 and SaOS2 cell lines. To investigate the receptor expression using this technique, immunolabelling using two different antibodies was carried out. The first antibody was the L4 monoclonal antibody, which binds to an external epitope of the receptor (Buell et al., 1998), therefore Triton X-100® is not needed for it to work. The other antibody was the $P2X_7$ receptor polyclonal antibody, which binds to an epitope located intracellularly on the C-terminus of the receptor (Manufacturer's Instructions), therefore Triton X-100® was needed to permeabilize the cells. The cell labelling with the L4 antibody was compared in the presence and absence of Triton X-100® which permeabilizes the cell allowing the antibody to enter. The purpose of this was to determine intracellular L4 antibody labelling, and to compare this labelling with non-permeabilized cells. On permeabilizing the cells with Triton X-100®, the staining was seen throughout the cells and found to be weak. In contrast, in the absence of Triton X-100®, the labelling was slightly stronger (same antibody concentration and exposure), and located at the cell membrane. For labelling with the polyclonal $P2X_7$ receptor antibody, Triton X-100® was used for all the experiments. In both cell lines I observed that the labelling was stronger in the nuclei compared to the membrane and the cytoplasm. Therefore the results show that the receptor is expressed strongly in the nuclei compared to the cytoplasm. This difference in

staining between the two antibodies raises a question as to whether the polyclonal antibody is labelling only the $P2X_7$ receptor or other proteins in addition. To help answer this question, the antibody was incubated for 48 hours with the peptide used to raise it. This preincubation resulted in little or no immunolabelling, thus confirming it was due to the antibody. The strong nuclear labelling may be seen because the receptor is strongly expressed in the nuclei, especially given that Western blotting of some of the nuclei samples showed stronger bands compared to lysate and membrane samples (around 70 kDa, 85 kDa and other smaller bands) that disappeared on incubation of the antibody with the peptide used to raise it. In support of this, a previous study found strong nuclear expression of the $P2X_7$ receptor in guinea pig smooth muscle cells, (Menzies et al., 2003). An explanation might be that the $P2X_7$ receptors have a role in calcium transport into the nuclear envelope, especially as the intracellular ATP concentration is sufficient to activate the receptor. Previous results from my laboratory have identified labelling on THP-1 monocyte nuclei (Kidd, E. J., unpublished data). The difference in labelling between the L4 antibody and the $P2X_7$ receptor antibody may be due to the contributions of the tertiary or quaternary structures of the receptor to the external epitope to which the L4 antibody binds.

QPCR showed that there is a tendency for an increase in receptor expression with increasing cell density in both cell lines. The significant difference was seen only in $3x10^6$ SaOS2 cells/flask compared to $4x10^5$ cells/flask. A possible reason for this effect may be that an increase in cell density represents a stress for the cells, and stress has been shown to induce nucleotide release via a non-lytic mechanism (Gartland et al., 2001), causing upregulation of the receptor. The most important nucleotide here is the $P2X_7$ receptor natural ligand (ATP) itself, especially as UTP proved to be ineffective on this receptor (Gartland et

al., 2001). Feng et al (2005) found that ATP affected the expression of the three main receptor bands (85, 65, and 18 kDa) in Western blotting experiments and stimulated expression of the functional 85 kD form of the receptor. Another possible reason for my finding is inter-cellular communication, which has been reported to increase with an increase in the density of some cells (Long et al., 2002). In my case, ATP may be involved resulting in receptor mRNA upregulation. Wang et al. (2005) studied human cervical epithelia cells and found that the band intensity in Western blotting differed according to the number of days the cells were grown for (studied on days 2 and 6). They found that the receptor expression and function was greater on day 6 compared to day 2. The difference between day 6 and day 2 cell growth would be the cell density. To my knowledge this is the first evidence demonstrating an increase in receptor mRNA expression with an increase in cell density.

To determine if there was any effect of cell density on receptor expression at the protein level, Western blotting was performed using a range of cell densities. The results show that cell density does not affect expression of the receptor protein, as all the samples had approximately the same intensity of the 67 kDa band. The only difference seen involved the 85 kDa band which appeared only in the higher cell densities ($1x10^6$ and $3x10^6$ cells/flask) and the longer growth period of the cells (7 days) especially in the MG63 cell line. This 85 kDa form of the receptor was claimed by Wang et al (2005) to be the functional form, and found to give a stronger band on day 6 of incubation compared to day 2. Again the differences between the results could be due to the availability of more than one functional form of the receptor, 67 and 85 kDa. Hence, it appears that the influence of cell density on receptor expression is seen at the mRNA level only, possibly because this mRNA is not translated into protein. To try and quantify $P2X_7$ receptor expression seen in Western

blotting, two house-keeping proteins were examined (β-actin and GAPDH) based on previous studies in other cell lines (Park et al., 2002; Wang et al., 2004a; Wang et al., 2004b). An interesting and unexpected finding in my experiments was that the house-keeping proteins used (β-actin and GAPDH) gave bands of increasing intensity with the increase in cell density, although they are generally thought to be expressed to the same levels in different cell samples. This may be due to their modulation by stress caused by cell density, which might be a phenomenon of these cell lines only. A previous study found that GAPDH expression was altered by mechanical stimulation (Saunders et al., 2006). Since the band intensity of the house-keeping proteins used increased with cell density, comparison with the data for P2X$_7$ receptor expression was not possible, and hence no quantification was done.

3.6 Conclusion

The expression of the $P2X_7$ receptor in human osteoblast-like cell lines (MG63 and SaOS2 osteosarcoma cell lines) was demonstrated at the mRNA and protein levels. It was observed that the relative receptor expression at mRNA level is significantly higher at the highest SaOS2 cell density. Efforts to prove this concept at the protein level failed due to the effect of cell density on the housekeeping protein used. With immunocytochemistry, the immunolabelling of the $P2X_7$ receptor antibody was found to be stronger on the nuclei compared to the L4 antibody. In addition, immunolabelling of the L4 antibody was spread throughout the cell when the cells were permeabilized. In Western blotting, receptor deglycosylation in cell membranes was performed and the results suggested that the fully functional form of the receptor is that giving a band at approximately 67 kDa. However, the intensity of the band of the 85 kDa form of the receptor (claimed to be the functional form by some studies) appeared to be increased by increasing cell density. Additionally, expression of other pore-forming purinergic receptors was investigated in both cell lines. Only expression of $P2X_4$ and $P2X_5$ receptors mRNA and protein was demonstrated. No evidence was obtained for $P2X_2$ receptor expression. Since the expression of $P2X_7$ receptor was shown, its functionality needed to be investigated.

Chapter 4: Effect of $P2X_7$ receptor agonists and antagonists on YO-PRO 1 uptake

4.1 Introduction

One characteristic of the $P2X_7$ receptor is the formation of a non-selective pore in the cell membrane permeable to molecules as large as 900 D (North, 2002; Liang and Schwiebert, 2005; Gever et al., 2006; Donnelly-Roberts and Jarvis, 2007; King, 2007). However, not all $P2X_7$ receptor-expressing cells form a pore (Donnelly-Roberts and Jarvis, 2007).

Steinberg et al (1987) documented that the mouse macrophage plasma membrane was permeable to compounds as large as 831 daltons upon activation by ATP. This effect was found to be inhibited in the presence of divalent cations (Mg^{2+} and Ca^{2+}) (Steinberg et al., 1987). At that time the $P2X_7$ receptor was still not identified, but the effect of the divalent cations on cell permeabilization allows us to conclude that it is due to this receptor.

This was also documented by Chessell et al (1997) who showed that prolonged activation of $P2X_7$ receptors using ATP led to the uptake of ethidium bromide (M.Wt.: 314 D) (Chessell et al., 1997). Other studies have documented the formation of this pore using larger molecules such as YO-PRO 1 (M.Wt.: 629 D for the di-iodide salt, 375 D for free base) (Hibell et al., 2000).

Dyes reported in the literature for the pharmacological study of $P2X_7$ receptor-induced pore formation are either ethidium bromide (dimensions 1.4x1.1x 0.52 nm) or YO-PRO 1 (dimensions 1.9x1.0x0.55 nm) (North, 2002). The molecular weight of YO-PRO 1 is higher than that of ethidium bromide (see above). It has been reported that uptake of ethidium bromide is faster than YO-PRO 1 (North, 2002). However, a

study on osteoblasts found that an hour is needed for the uptake of ethidium bromide (Gartland et al., 2001). This delay in uptake is unusual and led the authors to speculate that there was an atypical pharmacology of $P2X_7$ receptors in human osteoblasts.

Although pore formation has been observed in cells expressing $P2X_2$, $P2X_4$, and $P2X_7$ receptors, only in the case of $P2X_7$ receptors does this lead to cell lysis (Virginio et al., 1999; Gever et al., 2006). Another study reported pore formation with the $P2X_5$ receptor (Bo et al., 2003). Since the pore formed by all of these receptors is permeable to large cations, NMDG, ethidium bromide, and YO-PRO 1, the size of the pore the receptors are forming is probably similar.

It is believed that pore formation is induced mainly by the long C-terminus of the $P2X_7$ receptor. This was concluded when the receptor was truncated to 418 amino acids, which resulted in a significant decrease in intracellular YO-PRO 1 uptake (Suprenant et al., 1996). However, $P2X_2$, $P2X_4$, and $P2X_5$ receptors have shorter C-termini, but still form pores so this can not be the only factor involved.

Gu et al (2001) reported that $P2X_7$ receptor is expressed by human leukocytes, but it lost its function (pore formation) upon mutation of adenine to cytosine at position 1513 of the cDNA, causing the replacement of alanine with glutamic acid at amino acid position 496 (Gu et al., 2001).

North (2002) suggested two mechanisms for the progressive permeability of the $P2X_7$ receptor to cations and larger molecules on activation (Figure 4.1). The first mechanism is the dilation hypothesis, where several units of the receptor are involved to form the pore. This

pore forms upon binding of ATP and dilates progressively by undergoing conformational change. In the beginning the pore is permeable to small cations, but later it becomes permeable to larger compounds (~900 daltons) (North, 2002). It is believed that the subunits are arranged where the first transmembrane domain of one subunit is located in close proximity to the second (Jiang et al., 2003). The second mechanism is the extra protein hypothesis, where a special channel protein is involved. Binding of ATP causes permeability of small cations. After activation, the receptor interacts with this special protein causing opening of the channel protein which is permeable to larger compounds (North, 2002). Based on recent studies, the extra protein hypothesis looks more likely (Donnelly-Roberts and Jarvis, 2007).

The pore has been reported to be up to approximately 4 nm in size, and it may be due to a homomultimer consisting of 2 or 3 molecules of the receptor (Ziganshin et al., 2002; Burnstock, 2004).

A second messenger was reported to be needed for $P2X_7$ receptor pore formation (Faria et al., 2005; Liang and Schwiebert, 2005). This was suggested to be Ca^{2+} and MAPK in the 2BH4 mouse thymic epithelial cell line (Faria et al., 2005). Another study reported that MAPK and caspase signalling pathways are required for $P2X_7$ receptor pore formation in the THP-1 human monocytic cell line (Donnelly-Roberts et al., 2004). The difference in the messenger might be due to differences in cell type.

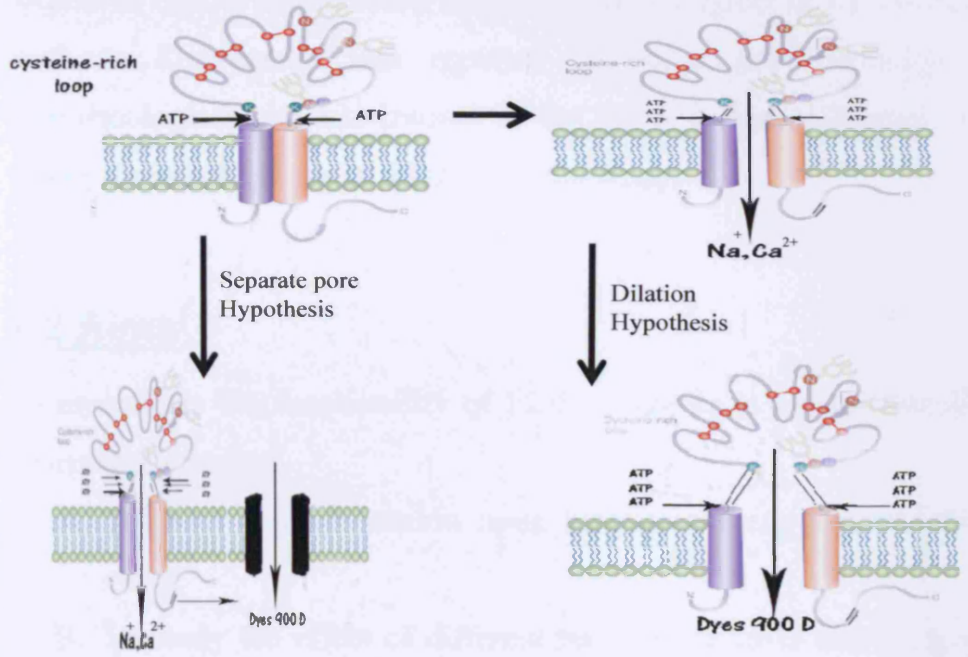


Figure 4.1: P2X$_7$ receptor pore formation

A diagram showing two suggested mechanisms for P2X$_7$ receptor pore formation. This pore forms upon binding of ATP and dilates progressively by undergoing a conformational change. Brief activation of the receptor forms a channel permeable to small cations such as sodium and calcium. Prolonged activation of receptors results in formation of a pore permeable to larger compounds (~900 daltons). Two suggested hypotheses for the pore formation are shown here, the separate pore and the dilation hypothesis. Adapted from North (2002); Liang et al (2005).

The other characteristic of the P2X$_7$ receptor is its very low affinity for ATP (EC_{50} value 300 μM) compared to other P2X family members (North and Barnard, 1997; Gever et al., 2006; Donnelly-Roberts and Jarvis, 2007). Additionally, DBzATP is reported to be the most potent agonist, though it is not selective (North, 2002; Gever et al., 2006).

Since osteoblast-like cell lines have been shown to express P2X$_7$ and other pore forming P2X receptors, the next step was to characterise them pharmacologically. For pharmacological study of these receptors, pore

formation can be tested either by electrophysiological or by dye-uptake methods. Dye-uptake was reported to be a good technique for pharmacological characterization of the $P2X_7$ receptor (Michel et al., 1999), therefore I chose to utilize it in my study.

4.2 Aims

To investigate the functionality of $P2X_7$ receptors in human osteoblasts pharmacologically:

A. To study pore formation upon activation using the YO-PRO 1 assay.
B. To study the effect of different receptor agonists and antagonists on pore formation.
C. To study the involvement of other $P2X_7$ pore-forming receptors in YO-PRO 1 uptake.
D. To determine the effect of cell density and cell culture period on pore formation and $P2X_7$ receptor expression.

4.3 Experimental protocols

To study the function of the $P2X_7$ receptor, the pore formation characteristic was utilized. For this purpose, the YO-PRO 1 uptake method was used.

To determine the best incubation time for YO-PRO 1 uptake, MG63 and SaOS2 cells were incubated with ATP $8x10^{-4}$ M for different periods (5-30 minutes) and then the uptake was monitored. This experiment was performed in assay buffer-A pH 7.4 (KCl 5 mM, $CaCl_2$ 0.5 mM, glucose 10 mM, HEPES 10 mM, NMDG 10 mM, and sucrose 280 mM). For details see Chapter 2.

To find the best assay buffer for YO-PRO 1 uptake in MG63 and SaOS2 cells, five different buffers were studied as described in Chapter 2.

4.3.1 Effect of $P2X_7$ receptor agonists on YO-PRO 1 uptake

The effects of different P2X agonists were tested using the YO-PRO 1 uptake method. The agonists used were: ATP, DBzATP, ATPγS, and 2Me-S-ATP.

Experiments were also performed to investigate receptor desensitization and/or the involvement of other pore-forming purinergic receptors (namely $P2X_2$, $P2X_4$ and $P2X_5$) as described in Chapter 2.

4.3.2 Effect of $P2X_7$ receptor antagonists on YO-PRO 1 uptake induced by agonists

Since there is no agreement in the literature on the pre-incubation period with antagonists, an experiment was performed to determine the best incubation time for the antagonists to inhibit YO-PRO 1 uptake. MG63 and SaOS2 cells were incubated in Greiner 96-well black plates with ATP $8x10^{-4}$ M in the presence of different antagonists for different periods (30-60 minutes) and then the uptake was monitored. The antagonists used were: BBG, KN62, oATP, and PPADS. The antagonist concentrations used were adapted from the literature (Michel et al., 2000).

4.3.3 Effect of cell density on YO-PRO 1 uptake induced by agonists

To study the effect of cell density and cell culture period on $P2X_7$ receptor-induced pore formation, MG63 and SaOS2 cells were seeded at different densities ($1x10^6$, $2x10^6$, and $3x10^6$ cells per flask) in medium, incubated for 1 or 3 days, and YO-PRO 1 uptake was determined. Another experiment was performed to find the effect of time cells were in culture on receptor function, where $1x10^6$ cells per 25 cm^2 flask were grown for 1, 3, or 6 days, then the YO-PRO 1 uptake was determined. In each case cells were treated with ATP ($5x10^{-4}$ M or $1x10^{-4}$ M) or DBzATP ($5x10^{-4}$ M or $1x10^{-4}$ M) for 5 minutes prior to the YO-PRO 1 assay.

4.3.4 Effect of ATP on $P2X_7$ receptor expression

Since it is possible that ATP modulates the expression of the $P2X_7$ receptor, an experiment was performed to find the effect of incubating

cells with ATP on receptor expression using Western blotting (described in Chapter 2). In this experiment SaOS2 cells were incubated with medium containing ATP 3×10^{-3} M or water (ATP vehicle) at 37°C for 30 minutes, then trypsinised and lysed. Lysate samples were separated on 10 % gels (as described in Chapter 2). The films were scanned using a Lexmark scanner, and the bands produced quantified by densitometry using NIH Image software by comparing their OD to those for the house-keeping protein, β-actin. This experiment was performed in conjunction with Veronica Thiongo (Final year MPharm undergaduate project student).

4.4 Results

To find the best conditions for the YO-PRO 1 uptake method in MG63 and SaOS2 cells, different incubation times with ATP (5-30 minutes) and different buffers were studied (see Methods section). Figure 4.2 shows the effect of incubation time with ATP on YO-PRO 1 uptake in MG63 and SaOS2 cells. In the MG63 cell line the 5 minute incubation time gave the same or 3.6 - 19 % higher uptake compared to other incubation times. In the case of the SaOS2 cells, the uptake was 6.6 - 41.2 % higher at 5 minutes compared to other incubation times. In SaOS2 cells the uptake was found to be significantly higher after 5 minute compared to 20 and 30 minutes incubation, while no significant difference was seen in case of MG63 cells. Therefore 5 minutes incubation was used for further studies in both cell lines. Figure 4.3 shows the effect of different assay buffers on YO-PRO 1 uptake in MG63 and SaOS2 cells. It was found that the assay buffer-A gave the best YO-PRO 1 uptake in both cell lines and was therefore used in all experiments. No uptake was seen with buffer-C, buffer-D or buffer-E (for details of buffers see Chapter 2).

Additionally, preliminary results showed that no uptake was seen with buffer-E when the cells were incubated for 60 minutes (data not shown).

4.4.1 Effect of $P2X_7$ agonists on YO-PRO 1 uptake

All the agonists used, ATP, DBzATP, ATPγS, and 2Me-S-ATP were found to induce YO-PRO 1 uptake in SaOS2 cells and their concentration-effect curves (Figure 4.4) gave EC_{50} values of 0.43 mM±0.02, 0.36 mM±0.04, 0.6 mM±0.05 and 0.42 mM±0.04, respectively. In MG63 cells the agonist concentration-effect curves (Figure 4.5) gave EC_{50} values of 0.36 mM±0.03 and 0.5 mM±0.04 for ATP and DBzATP, respectively. YO-PRO 1 uptake induced by ATP and 2Me-S-ATP (in the case of the SaOS2 cells) at concentrations higher than $1x10^{-3}$ M was less than that induced by $1x10^{-3}$ M.

Table 4.1 compares the EC_{50} values of the above mentioned agonists obtained in this project with those reported in the literature for human $P2X_2$, $P2X_4$, and $P2X_7$ receptors.

To compare the results obtained in this project with those reported by Gartland et al (2001), Figure 4.6 shows the YO-PRO 1 uptake obtained using the same concentrations, 0.5 and 1 mM ATP and DBzATP, in MG63 and SaOS2 cells. There were small variations between the same concentrations of ATP and DBzATP in both cell lines, but none showed significant difference.

In the receptor desensitization experiment in MG63 and SaOS2 cells, the concentration-effect curve for ATP was identical in the ATP-treated cells compared to the control cells (Figure 4.7). The ATP EC_{50} values of the treated and control MG63 cells were 0.43 mM±0.07 and 0.44 mM±0.02,

respectively, while in SaOS2 cells the EC_{50} value was 0.58 mM±0.05 in both treated and control cells.

4.4.2 Effect of $P2X_7$ antagonists on YO-PRO 1 uptake induced by agonists

One hour was found to be the best incubation time for the antagonists to inhibit YO-PRO 1 uptake induced by ATP (data not shown), therefore this incubation period was used for further studies in both cell lines.

Figures 4.8, 4.9, 4.10, and 4.11 show the concentration-effect curves for ATP in the presence of different concentrations of BBG, KN62, PPADS, and oATP, respectively in MG63 cells. Figures 4.12, 4.13, 4.14, and 4.15 show the concentration-effect curves for ATP in the presence of different concentrations of the above mentioned antagonists in SaOS2 cells. Figures 4.16 and 4.17 show the concentration-effect curves for DBzATP in the presence of different concentrations of BBG and KN62 in SaOS2 cells.

In MG63 cells BBG at a concentration of 5 μM significantly reduced the maximum YO-PRO 1 uptake induced by ATP by approximately 20 %, but at 0.2 mM it significantly increased the uptake induced by ATP 3x 10^{-5} M and 1x 10^{-4} M (Figure 4.8). KN62 reduced the maximum YO-PRO 1 uptake induced by ATP in a concentration-related manner but this was not significant (Figure 4.9). PPADS at concentrations of $3x10^{-7}$ M and $3x10^{-8}$ M significantly decreased the maximum uptake by approximately 17 and 11 %, respectively (Figure 4.10). oATP was found to have little or no effect on YO-PRO 1 uptake (Figure 4.11).

In SaOS2 cells BBG at a concentration of 5 μM reduced the maximum YO-PRO 1 uptake induced by ATP by approximately 33 % but this was not significant (Figure 4.12). KN62 at concentration of 10 μM significantly reduced the maximum YO-PRO 1 uptake induced by ATP by approximately 11 % (Figure 4.13). PPADS and oATP potentiated YO-PRO 1 uptake at the lower concentrations of ATP, but decreased it at the higher concentrations but these changes were not significant (Figures 4.14 and 4.15). In the case of DBzATP, BBG and KN62 were found to have some effect on blocking YO-PRO 1 uptake, but this was not significant (Figure 4.16 and 4.17). EC_{50} values for ATP and DBzATP were not significantly altered by any of the antagonists used. The EC_{50} values obtained in the presence of the highest antagonist concentration used are shown in Table 4.2.

4.4.3 Effect of cell density on YO-PRO 1 uptake induced by agonists

YO-PRO 1 uptake was determined in MG63 and SaOS2 cells grown at different cell densities (Figures 4.18 and 4.19) or following one cell density ($1x10^6$ per 25 cm^2 flask) maintained for different numbers of days in culture (Figures 4.20 and 4.21). This experiment was performed to identify the effect of the cell density and days cells in culture on the receptor function.

In MG63 cells, it was found that the cell density and days cells in culture inversely affected YO-PRO 1 uptake induced by both ATP and DBzATP (Figures 4.18 and 4.20). The effect of the number of days MG63 cells were kept in culture was found to be significant with ATP and DBzATP $1x10^{-4}$ M. In SaOS2 cells, there was a tendency to increase YO-PRO 1 uptake with the increase in cell density, but a decrease in uptake with

increasing days cells in culture for both ATP and DBzATP. None of these changes were significant (Figures 4.19 and 4.21).

4.4.4 Effect of ATP on $P2X_7$ receptor expression assessed by Western blotting

Western blotting was carried out with cells pre-treated with ATP $3x10^{-3}$ M for 30 minutes in order to determine whether the band pattern obtained would be different to that described in Chapter 3. Figure 4.22A shows a representative example of the effect of ATP on $P2X_7$ receptor protein expression. Bands were seen for the $P2X_7$ receptor at 60 and 67 kDa, while the β-actin band was seen at 39 kDa. In order to quantify $P2X_7$ receptor expression, it was compared to labelling for β-actin using densitometric analysis as seen in Figure 4.22B. ATP did not have a significant effect on $P2X_7$ receptor expression. Quantification of the 85 kDa band could not be performed because this band was not seen in all the samples used (data not shown).

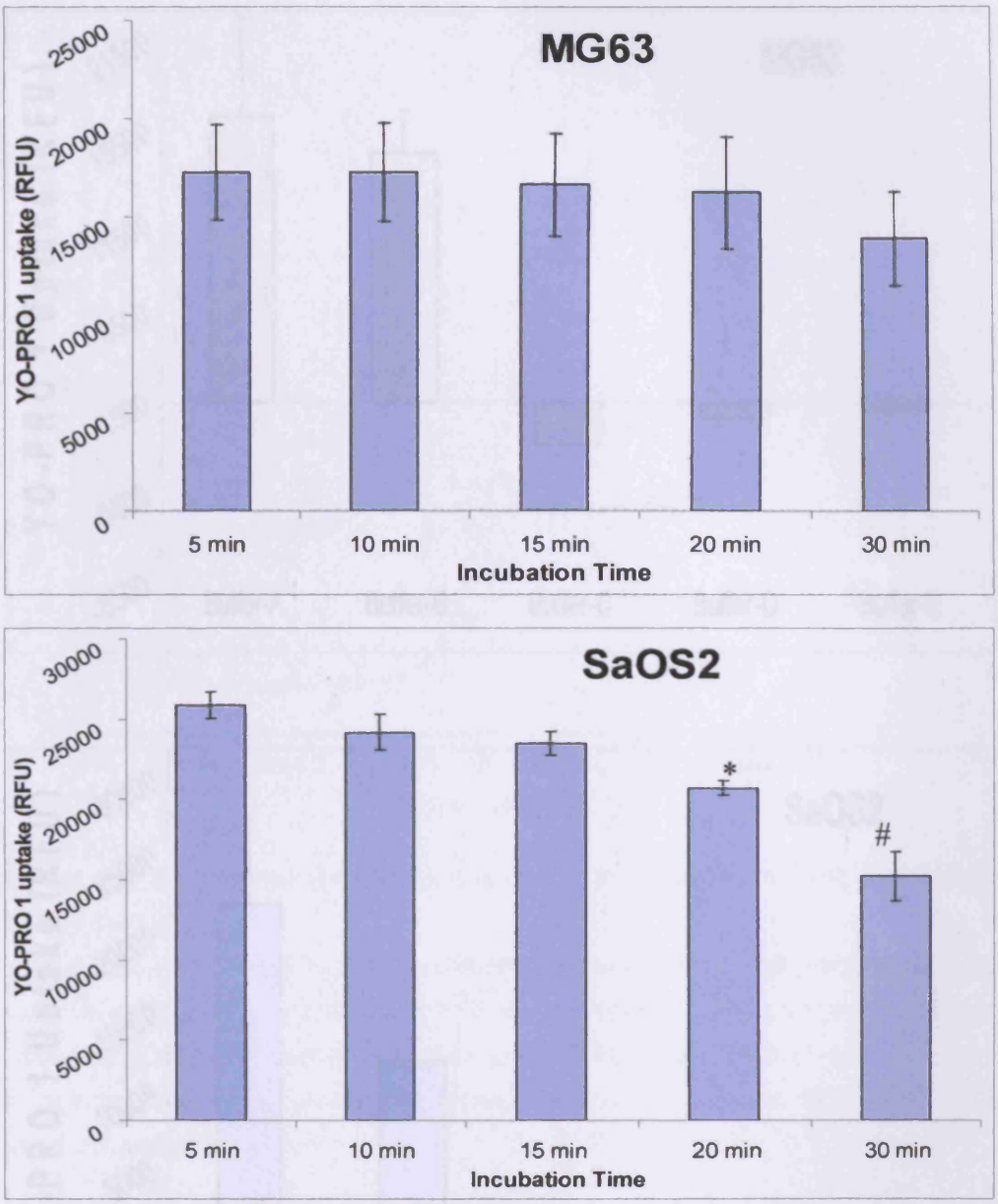


Figure 4.2: Effect of incubation time with ATP on YO-PRO 1 uptake in MG63 and SaOS2 cells.

MG63 and SaOS2 cells (approximately 100,000 cells/well) were incubated with ATP $8x10^{-4}$ M and 1 µm YO-PRO 1 in the assay buffer at 37°C for 5-30 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. In MG63 cells the YO-PRO 1 uptake at 5 minutes incubation time was the same or higher than other incubation times, but in SaOS2 cells it was higher at 5 minutes compared to all other incubation times. [n=4]. RFU: relative fluorescence unit. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 and # P<0.001 vs. 5 minutes incubation.

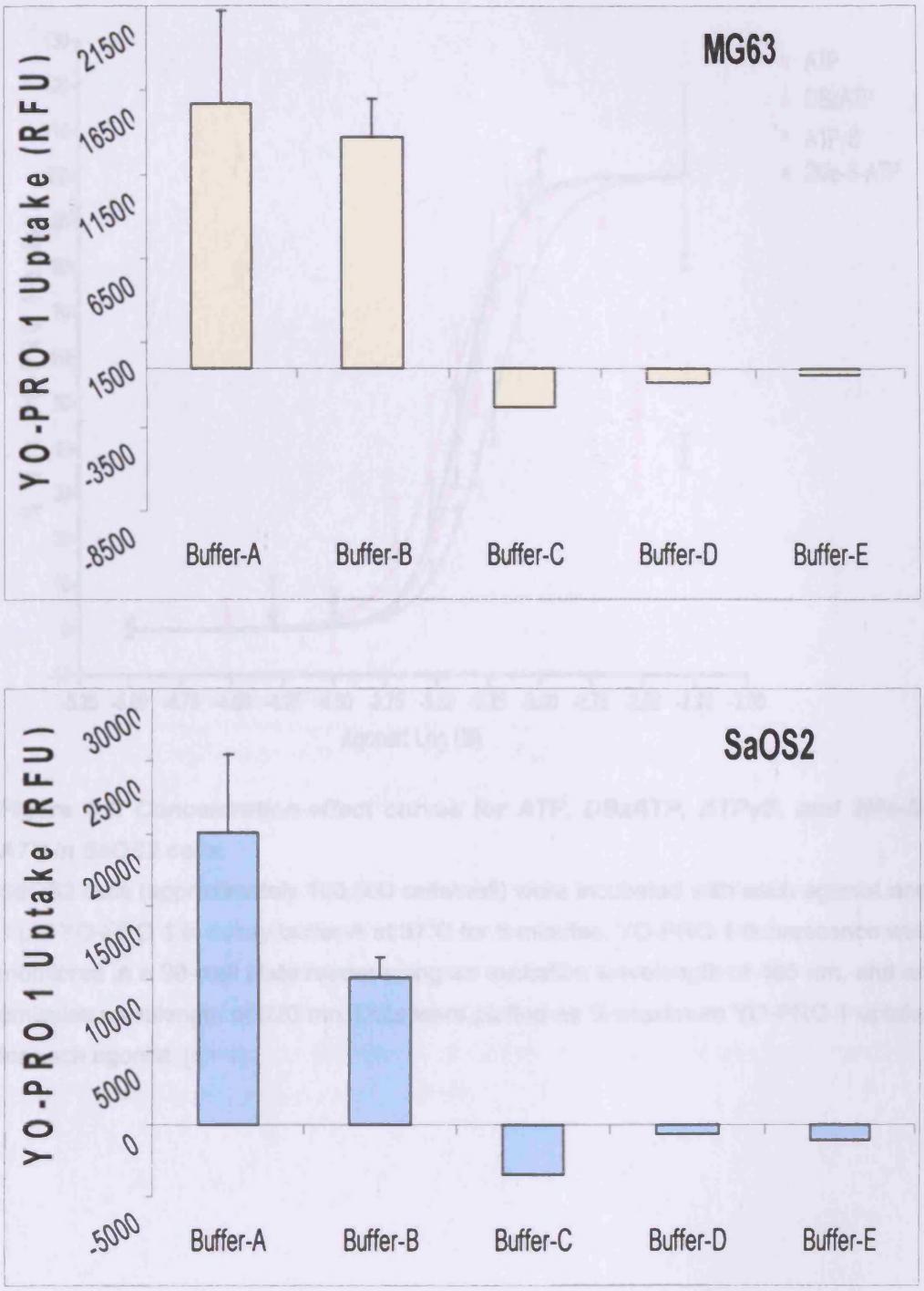


Figure 4.3: Effect of assay buffer on YO-PRO 1 uptake in MG63 and SaOS2 cells. MG63 and SaOS2 cells (approximately 100,000 cells/well) were incubated with ATP $8x10^{-4}$ M and 1 μM YO-PRO 1 in different assay buffers (described in Chapter 2) at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. In both cell lines the YO-PRO 1 uptake was greatest in the 0.5 mM calcium chloride-containing buffer. [n=2]. RFU: relative fluorescence unit.

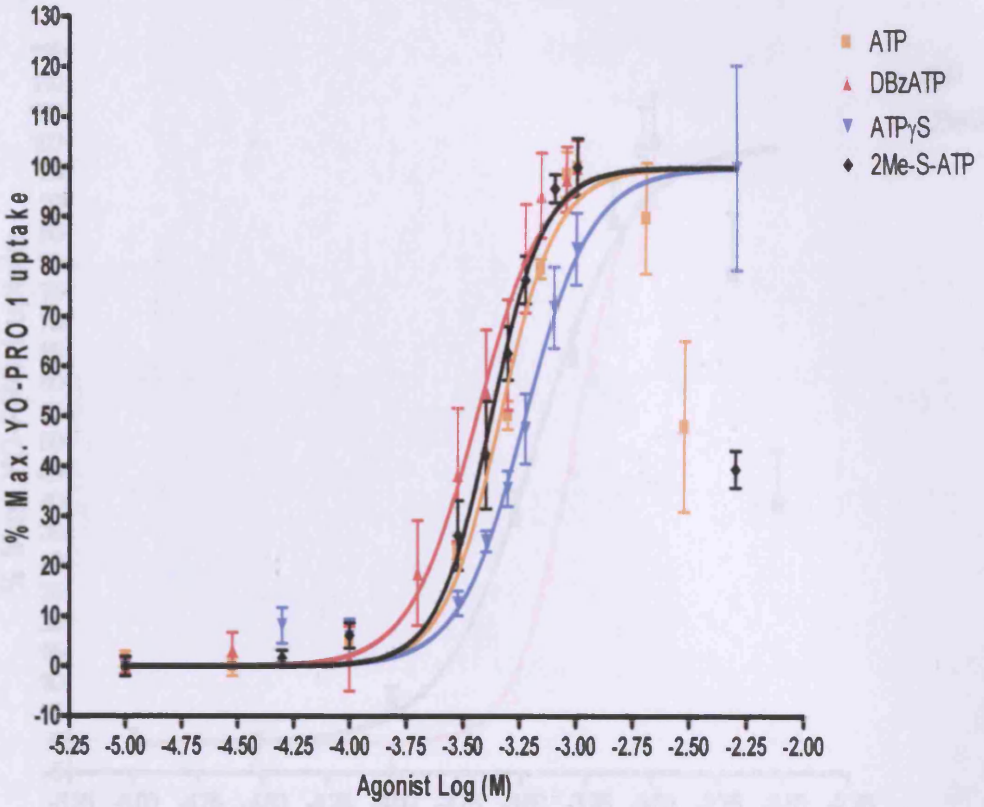


Figure 4.4: Concentration-effect curves for ATP, DBzATP, ATPγS, and 2Me-S-ATP in SaOS2 cells.

SaOS2 cells (approximately 100,000 cells/well) were incubated with each agonist and 1 μM YO-PRO 1 in assay buffer-A at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data were plotted as % maximum YO-PRO 1 uptake for each agonist. [n= 4].

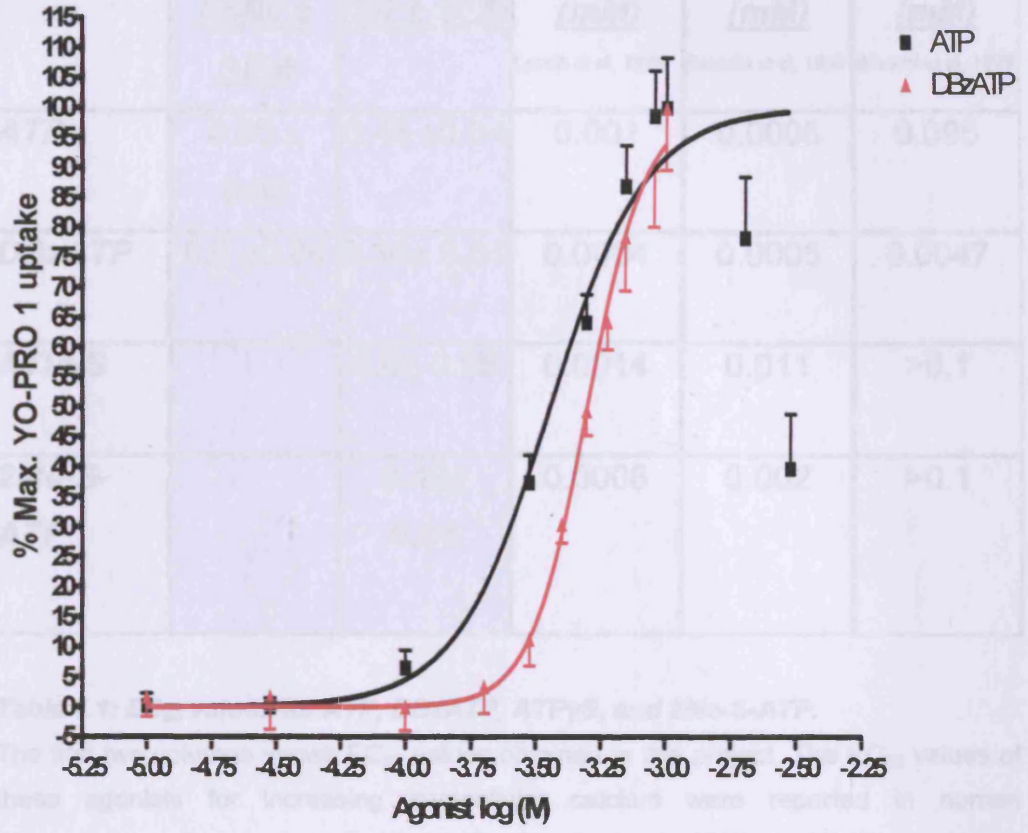


Figure 4.5: Concentration-effect curves of ATP and DBzATP in MG63 cells.

MG63 cells (approximately 100,000 cells/well) were incubated with each agonist and 1 μM YO-PRO 1 in 0.5 mM assay buffer-A at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data were plotted as % maximum YO-PRO 1 uptake for each agonist [n=6-15].

Agonist	MG63 [mM] ± SEM	SaOS2 [M] ± SEM	hP2X$_2$ (mM) Lynch et al, 1999	hP2X$_4$ (mM) Bianchi et al, 1999	hP2X$_7$ (mM) Bianchi et al, 1999
ATP	0.36 ± 0.03	0.43 ±0.04	0.001	0.0005	0.095
DBzATP	0.5 ±0.04	0.36± 0.04	0.0004	0.0005	0.0047
ATPγS		0.6± 0.05	0.0014	0.011	>0.1
2Me-S-ATP		0.42± 0.04	0.0008	0.002	>0.1

Table 4.1: EC_{50} values for ATP, DBzATP, ATPγS, and 2Me-S-ATP.

The first two columns shows EC_{50} values obtained in this project. The EC_{50} values of these agonists for increasing intracellular calcium were reported in human astrocytoma cells transfected with hP2X$_2$ (Lynch et al., 1999) or hP2X$_4$ or hP2X$_7$ (Bianchi et al., 1999) receptors.

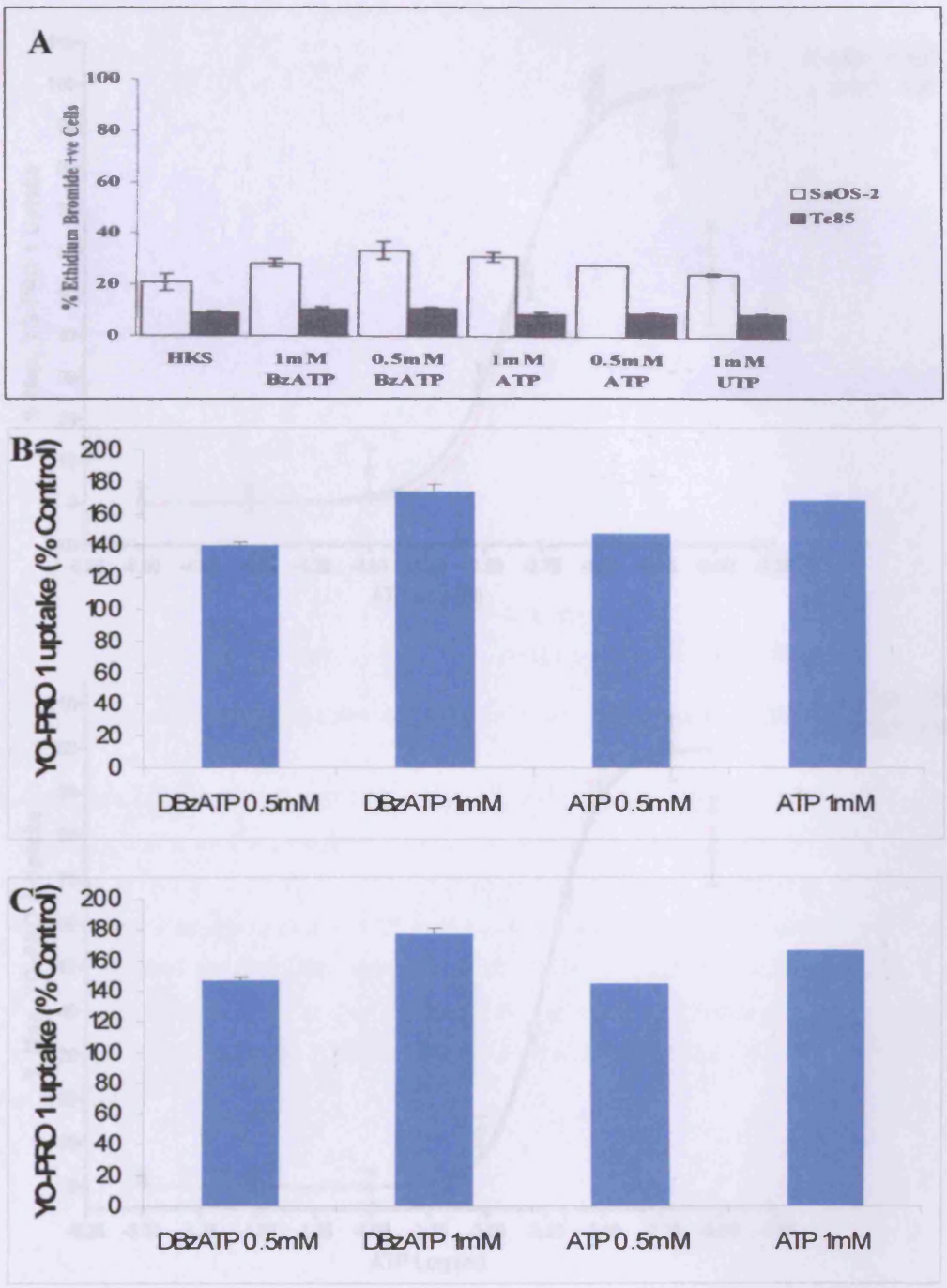


Figure 4.6: Comparison of the data obtained by Gartland et al (2001) with the results of this study.

A shows the results obtained by Gartland et al (2001) using ethidium bromide and an hour incubation with 0.5 and 1mM ATP and DBzATP. YO-PRO 1 uptake in MG63 (B) or SaOS2 (C) cells induced by 5 minutes incubation with 0.5 and 1mM ATP and DBzATP. Data are expressed as % of control. Statistical analyses were performed using Student's t-Test between each concentration of ATP and DBzATP. [n=6 - 45]. A adapted from Gartland et al (2001).

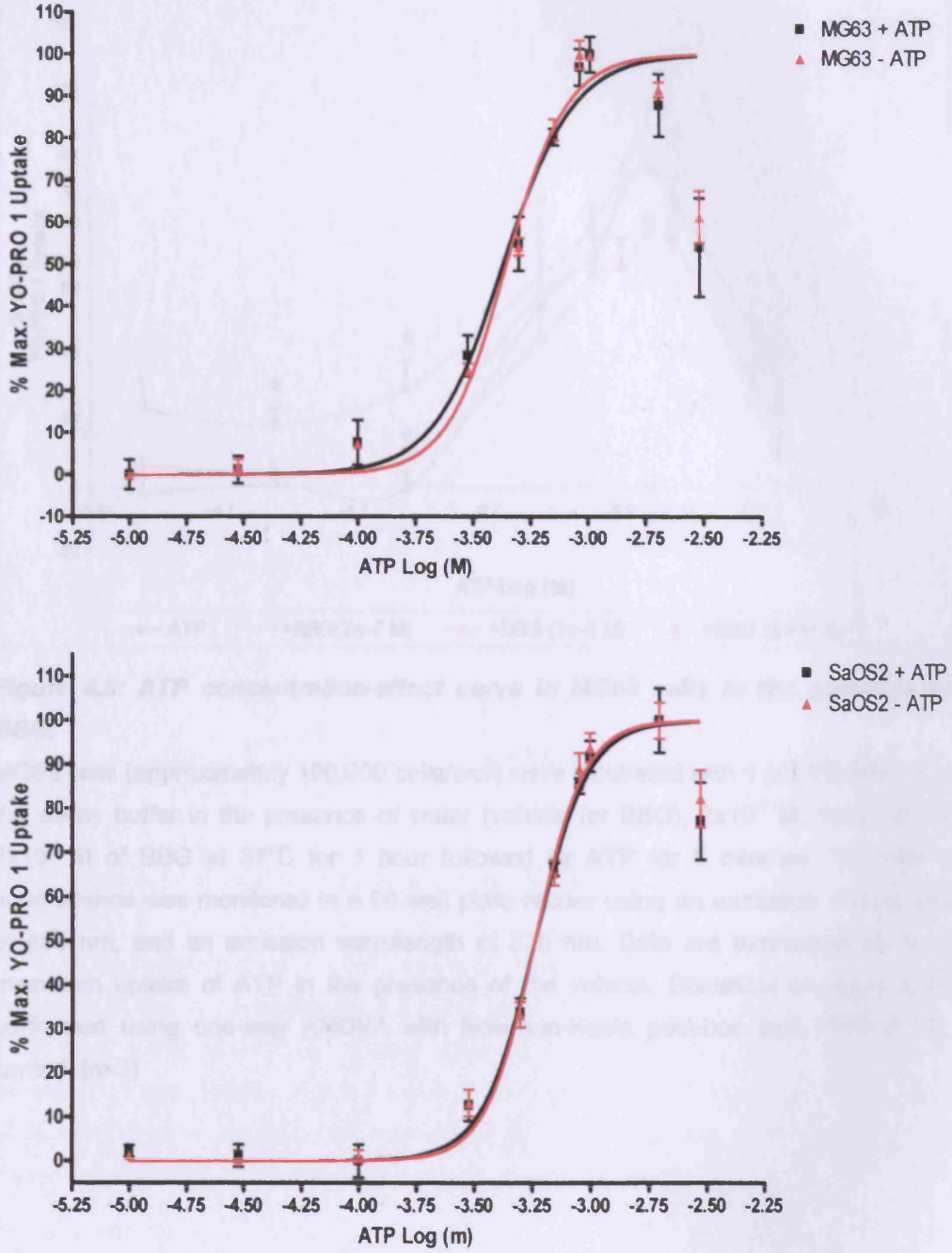


Figure 4.7: P2X$_7$ receptor desensitization in MG63 and SaOS2 cells.

SaOS2 and MG63 cells (approximately 100,000 cells/well) were incubated with ATP $3x10^{-3}$ M or Dulbecco's PBS for 20 minutes at 37°C and were then incubated with ATP and 1 μM YO-PRO 1 at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. [n=3]

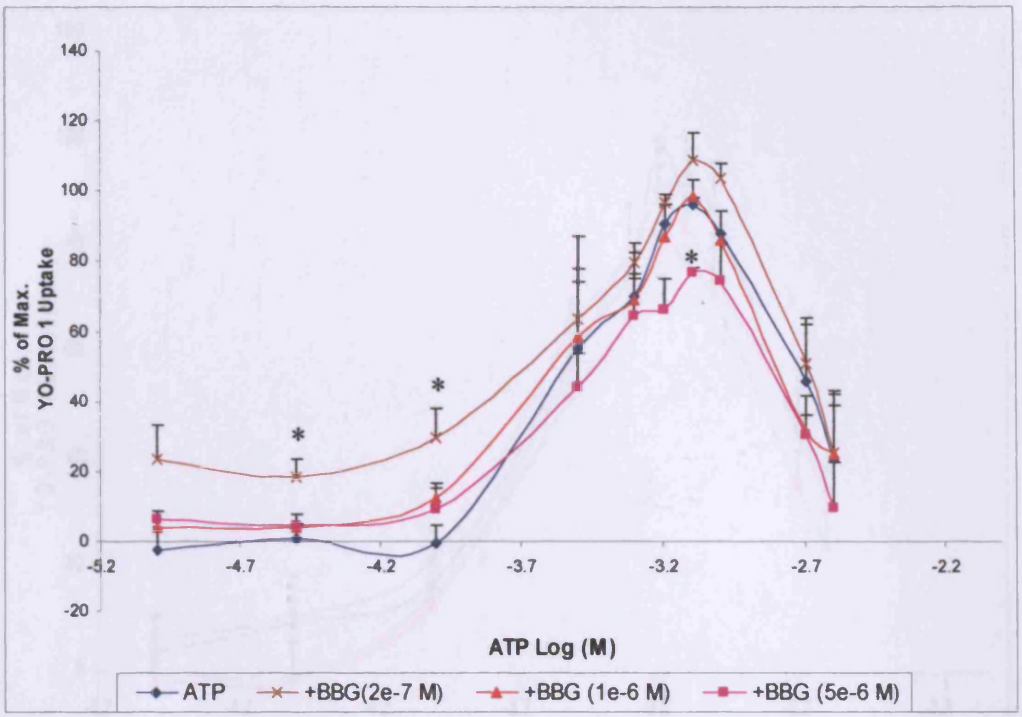


Figure 4.8: ATP concentration-effect curve in MG63 cells in the presence of BBG.

MG63 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of water (vehicle for BBG), $2x10^{-7}$ M, $1x10^{-6}$ M, or $5x10^{-6}$ M of BBG at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. [n=3]

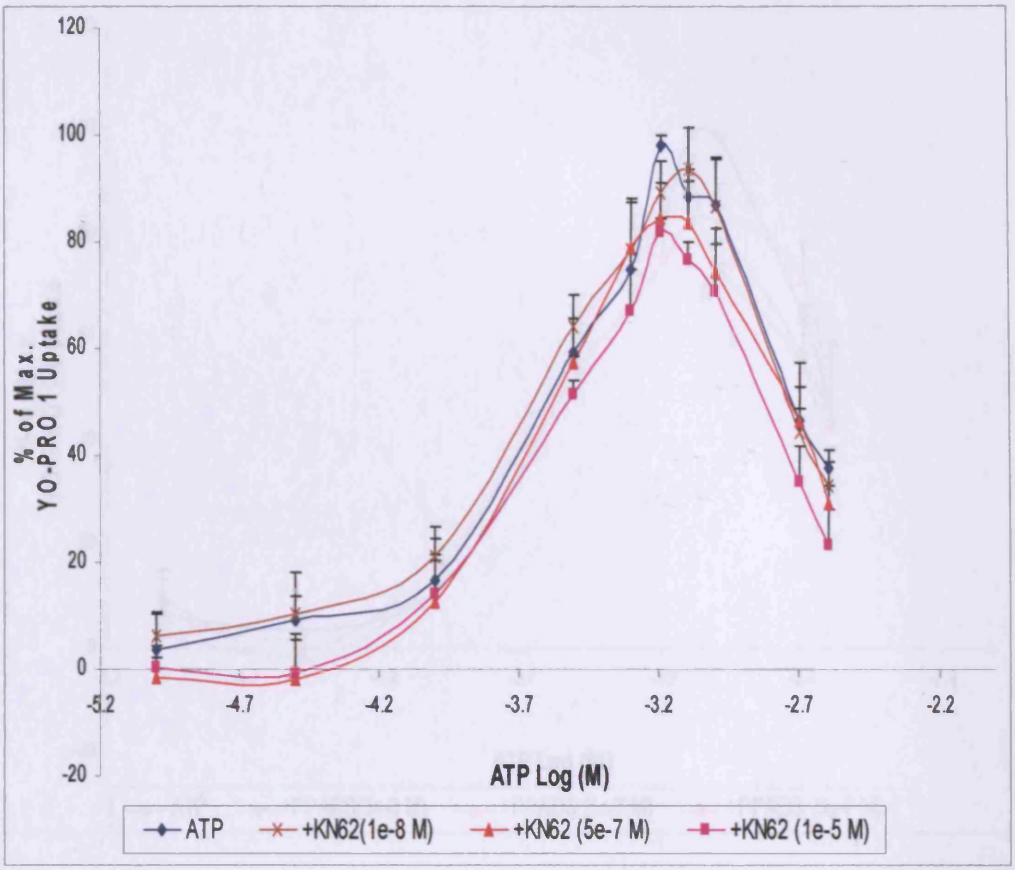


Figure 4.9: ATP concentration-effect curve in MG63 cells in the presence of KN62.

MG63 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of DMSO (vehicle for KN62), $1x10^{-8}$M, $5x10^{-7}$ M, or $1x10^{-5}$M of KN62 at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. [n=3]

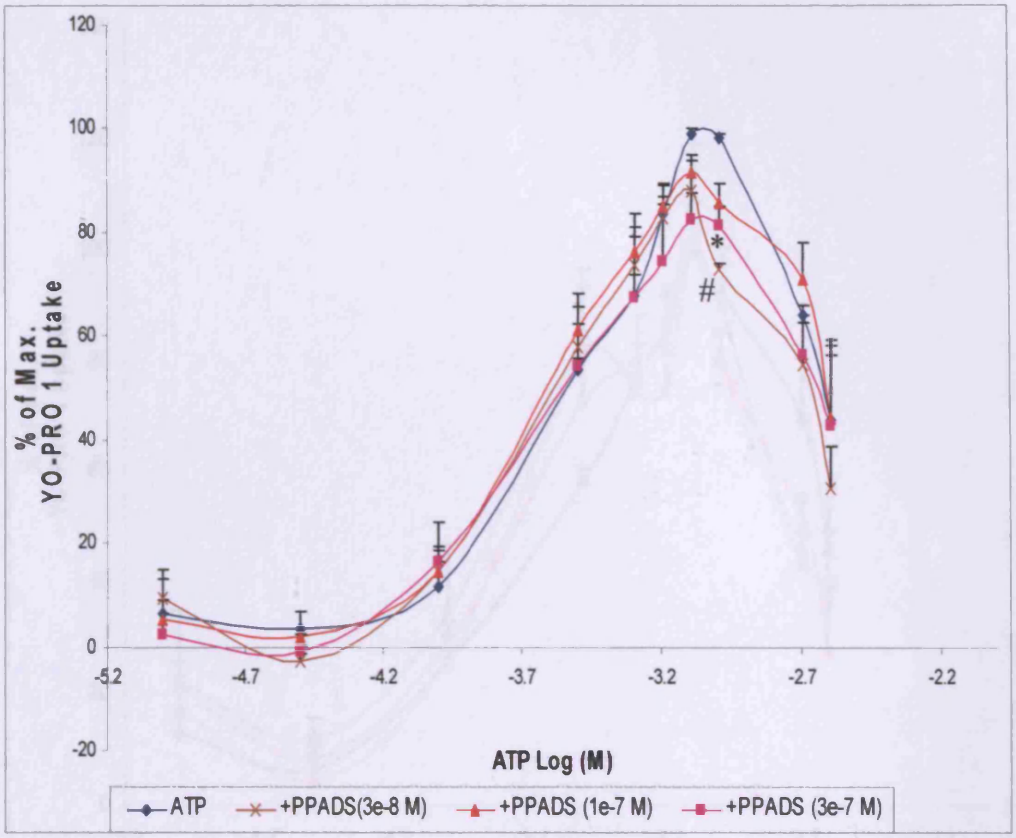


Figure 4.10: ATP concentration-effect curve in MG63 cells in the presence of PPADS.

MG63 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of water (vehicle for PPADS), $3x10^{-8}$M, $1x10^{-7}$ M, or $3x10^{-7}$M of PPADS at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 and # P<0.001 vs. control. [n=3]

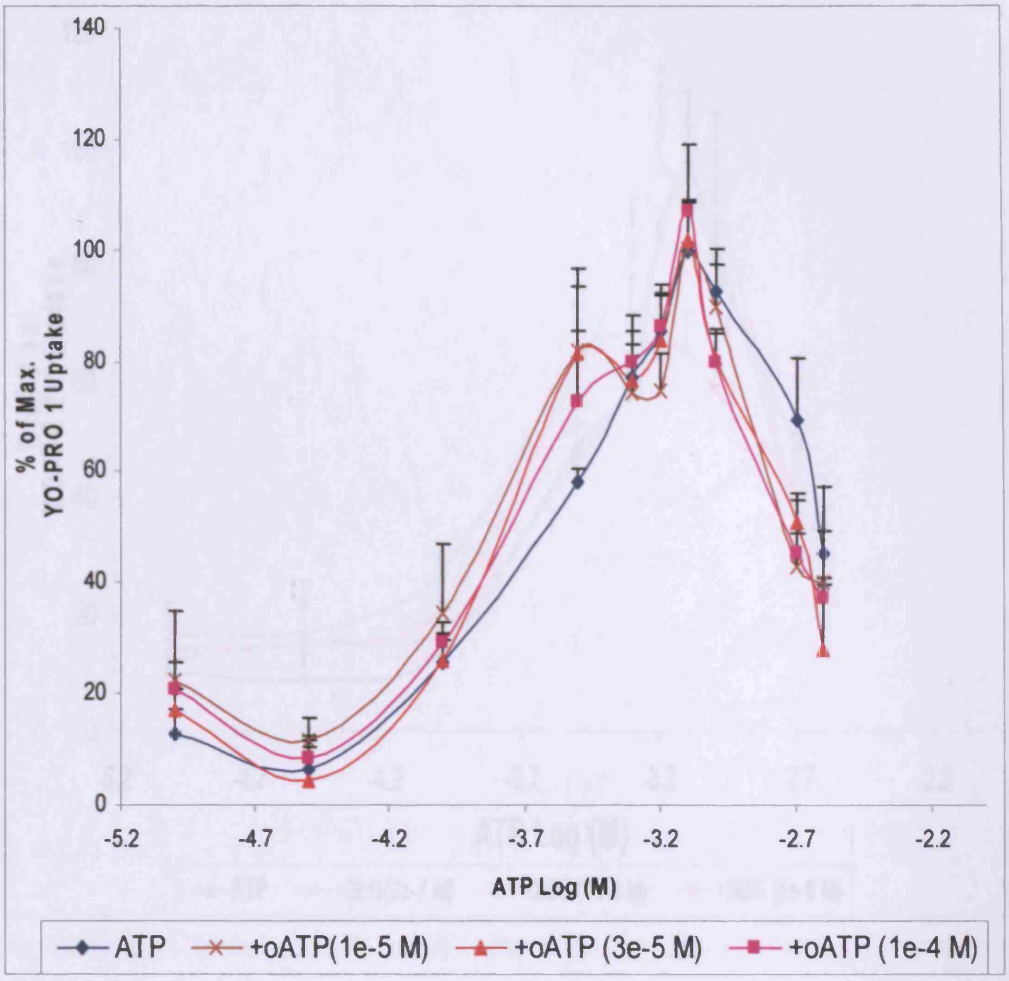


Figure 4.11: ATP concentration-effect curve in MG63 cells in the presence of oATP.

MG63 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of water (vehicle for oATP), $1x10^{-5}$M, $3x10^{-5}$ M, or $1x10^{-4}$M of oATP at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. [n=3]

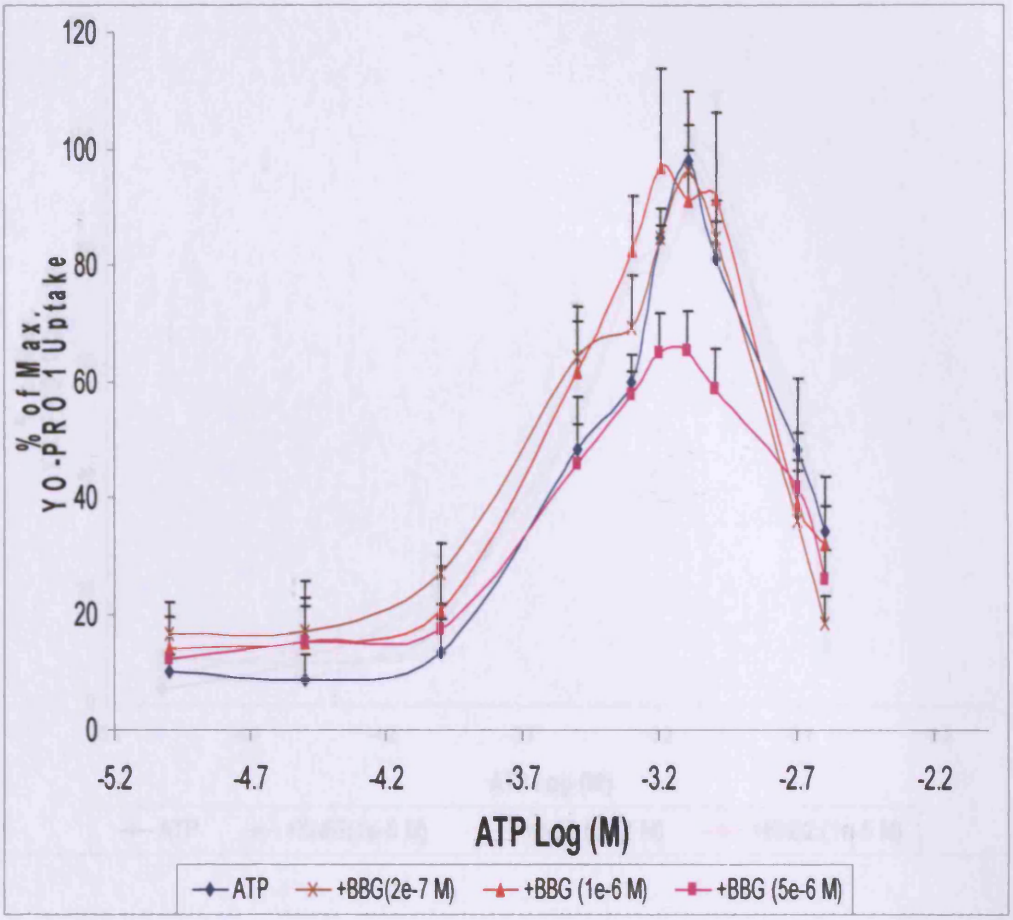


Figure 4.12: ATP concentration-effect curve in SaOS2 cells in the presence of BBG.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 µM YO-PRO 1 in the assay buffer in the presence of water (vehicle for BBG), $2x10^{-7}$M, $1x10^{-6}$M, or $5x10^{-6}$M of BBG at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. [n=4]

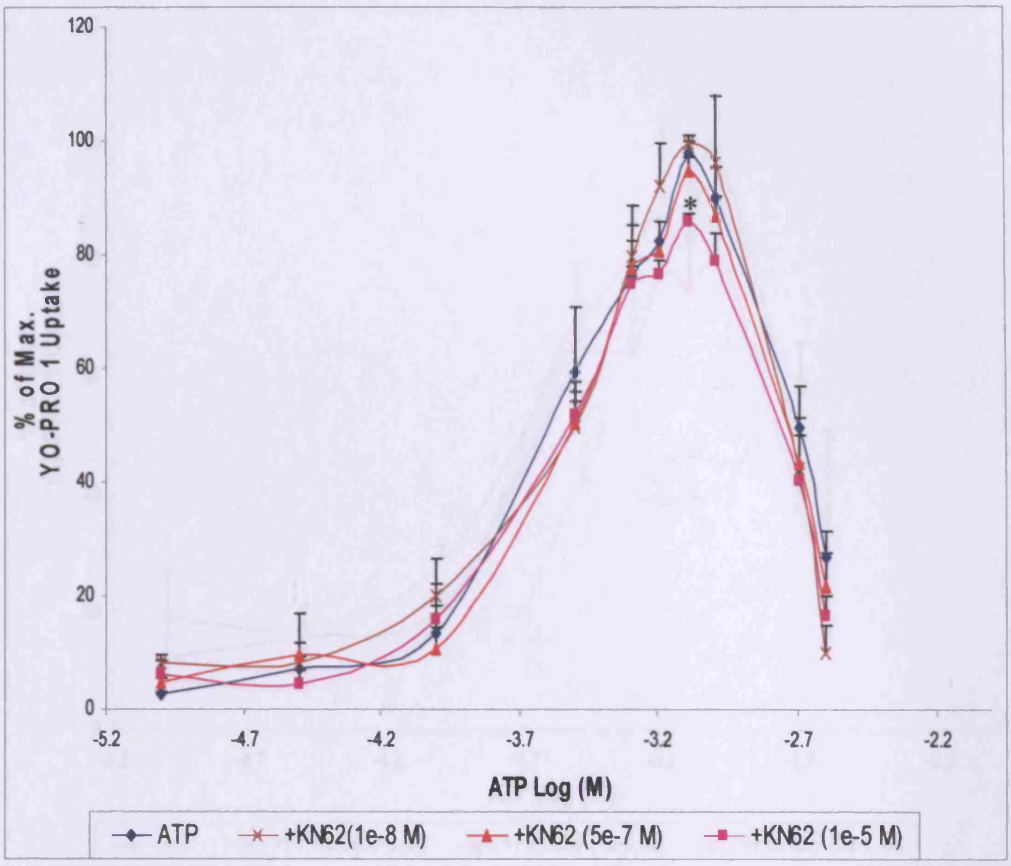


Figure 4.13: ATP concentration-effect curve in SaOS2 cells in the presence of KN62.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of DMSO (vehicle for KN62), $1x10^{-8}$M, $5x10^{-7}$M, or $1x10^{-5}$M of KN62 at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. [n=3]

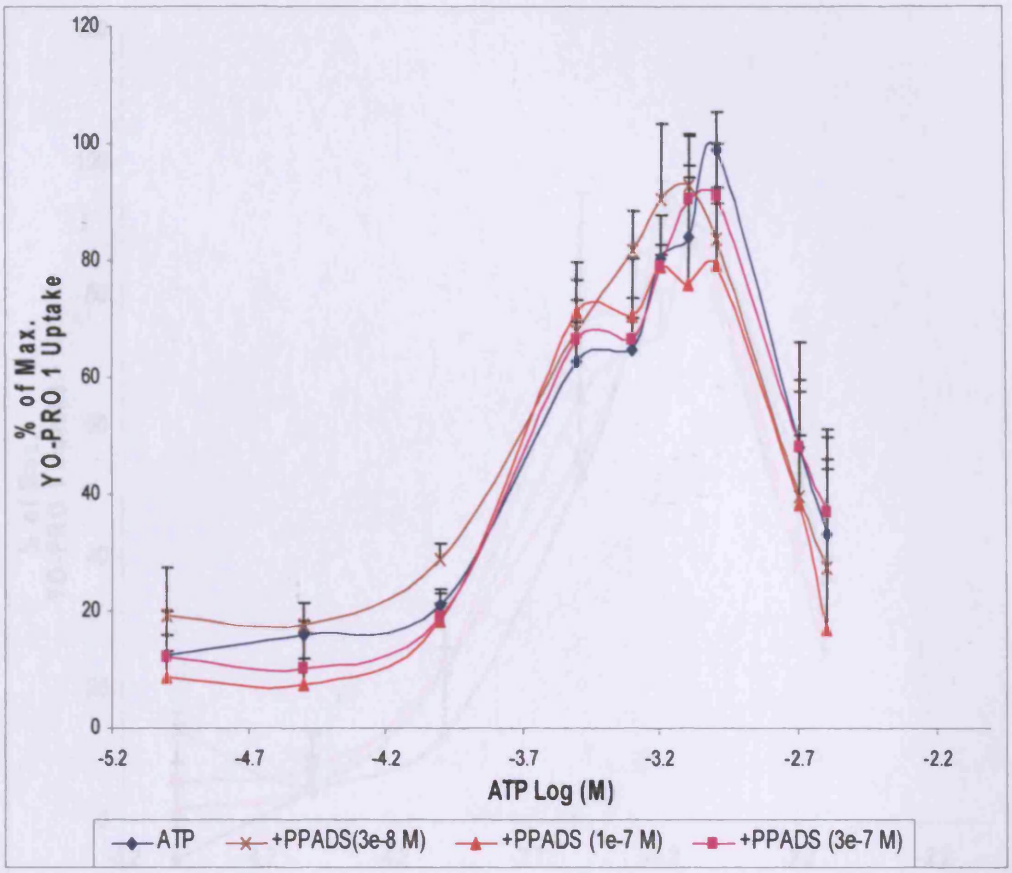


Figure 4.14: ATP concentration-effect curve in SaOS2 cells in the presence of PPADS.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 µM YO-PRO 1 in the assay buffer in the presence of water (vehicle for PPADS), $3x10^{-8}$M, $1x10^{-7}$M, or $3x10^{-7}$M of PPADS at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. [n=3]

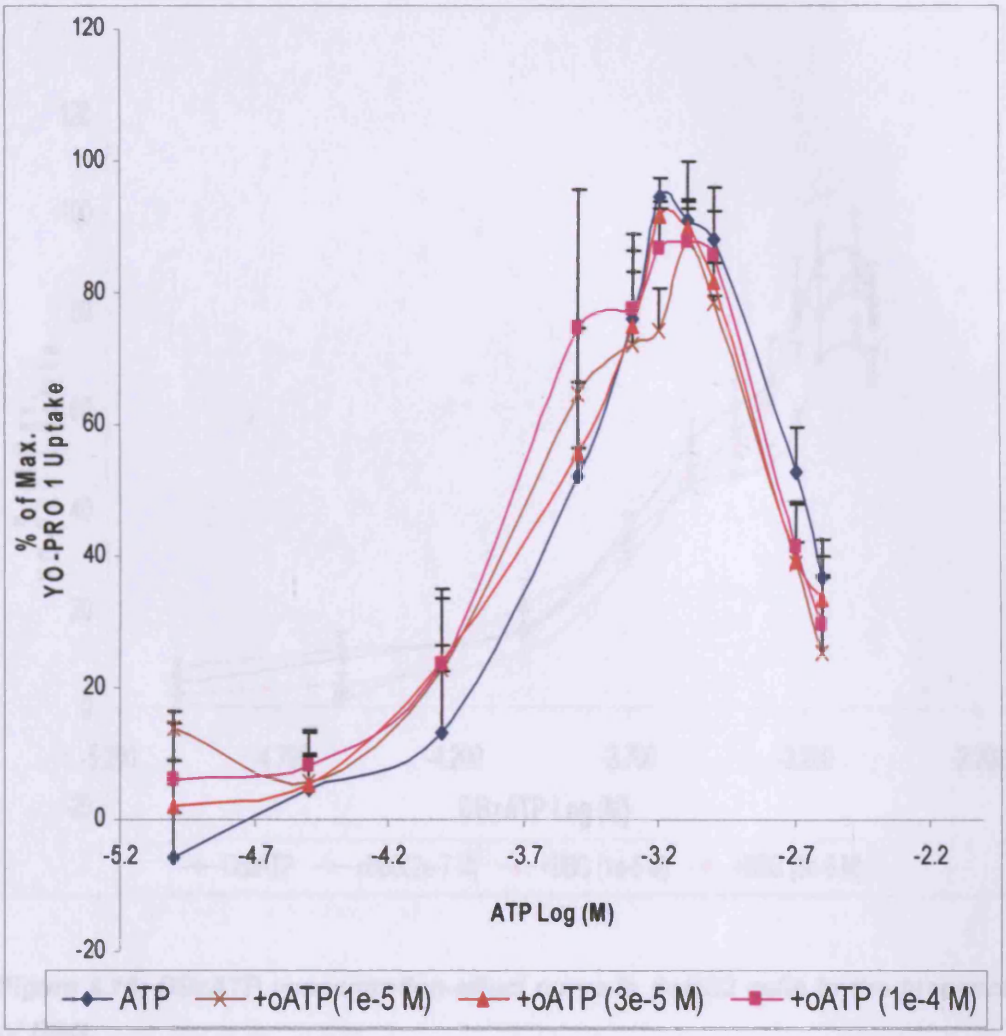


Figure 4.15: ATP concentration-effect curve in SaOS2 cells in the presence of oATP.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of water (vehicle for oATP), $1x10^{-5}$M, $3x10^{-5}$M, or $1x10^{-4}$M of oATP at 37°C for 1 hour followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the presence of the vehicle. [n=3]

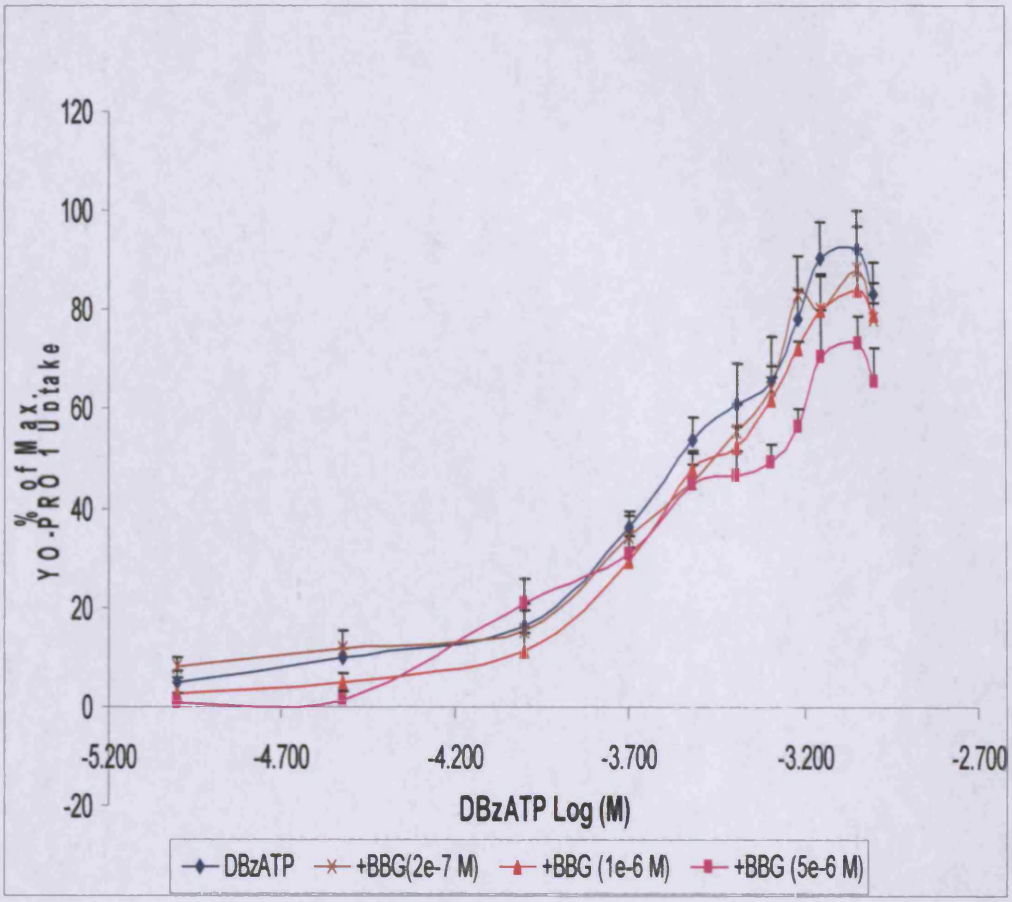


Figure 4.16: DBzATP concentration-effect curve in SaOS2 cells in the presence of BBG.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of water (vehicle for BBG), $2x10^{-7}$M, $1x10^{-6}$M, or $5x10^{-6}$M of BBG at 37°C for 1 hour followed by DBzATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of DBzATP in the presence of the vehicle. [n=4]

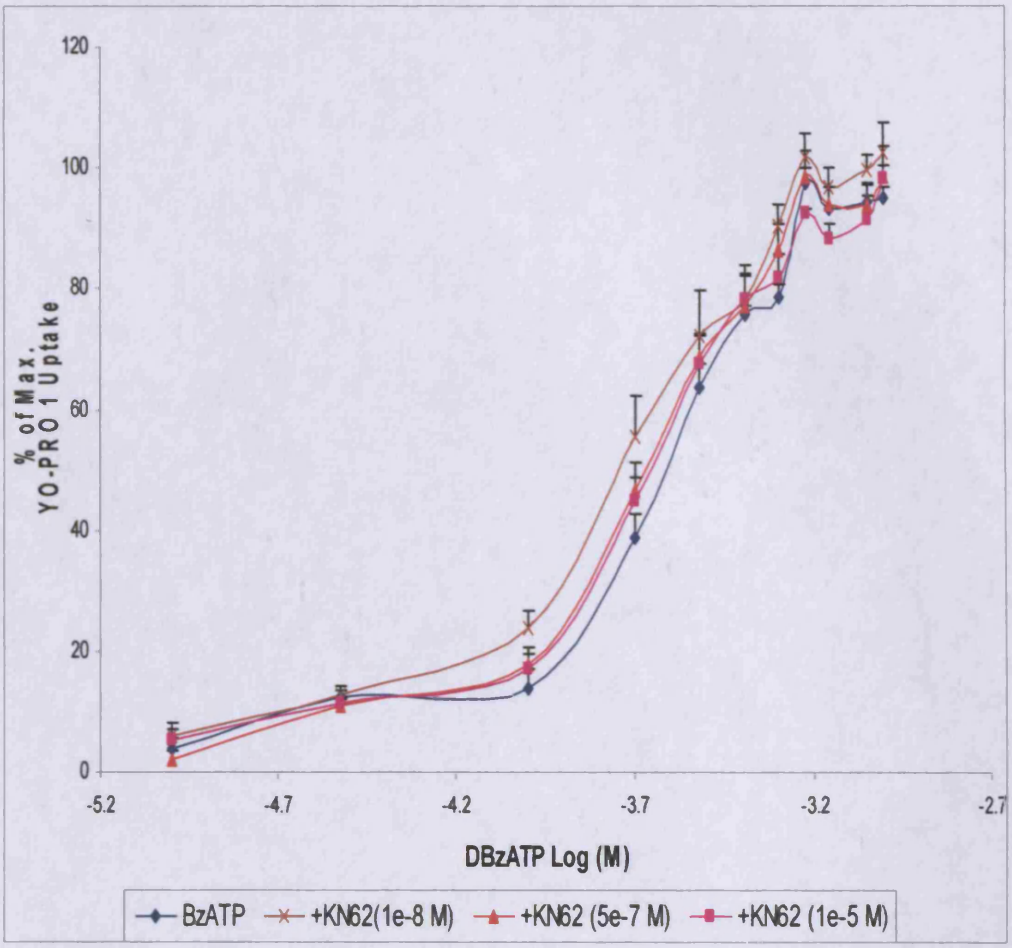


Figure 4.17: DBzATP concentration-effect curve in SaOS2 cells in the presence of KN62.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of DMSO (vehicle for KN62), $1x10^{-8}$M, $5x10^{-7}$M, or $1x10^{-5}$M of KN62 at 37°C for 1 hour followed by DBzATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of DBzATP in the presence of the vehicle. [n=4]

	MG63 (mM)	*SaOS2 (mM)*
ATP	0.28	0.29
ATP + BBG 5x10^{-6}M	0.28	0.29
ATP	0.17	0.19
ATP + KN62 1x10^{-5}M	0.13	0.16
ATP	0.18	0.29
ATP + oATP 1x10^{-4}M	0.12	0.11
ATP	0.26	0.17
ATP + PPADS 3x10^{-7}M	0.16	0.15
DBzATP	ND	0.34
DBzATP + BBG 5x10^{-6}M	ND	0.44
DBzATP	ND	0.26
DBzATP + KN62 1x10^{-5}M	ND	0.23

Table 4.2: EC_{50} values for ATP and DBzATP in the presence or absence of different antagonists.

MG63 or SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of water or DMSO (vehicle), or BBG, KN62, oATP, or PPADS at 37°C for 1 hour followed by ATP or DBzATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. EC_{50} values were calculated using Prism. The EC_{50} values obtained with the highest antagonist concentration used are shown here. No significant differences were seen with any of the concentrations used. ND= not done. *n=3-4*

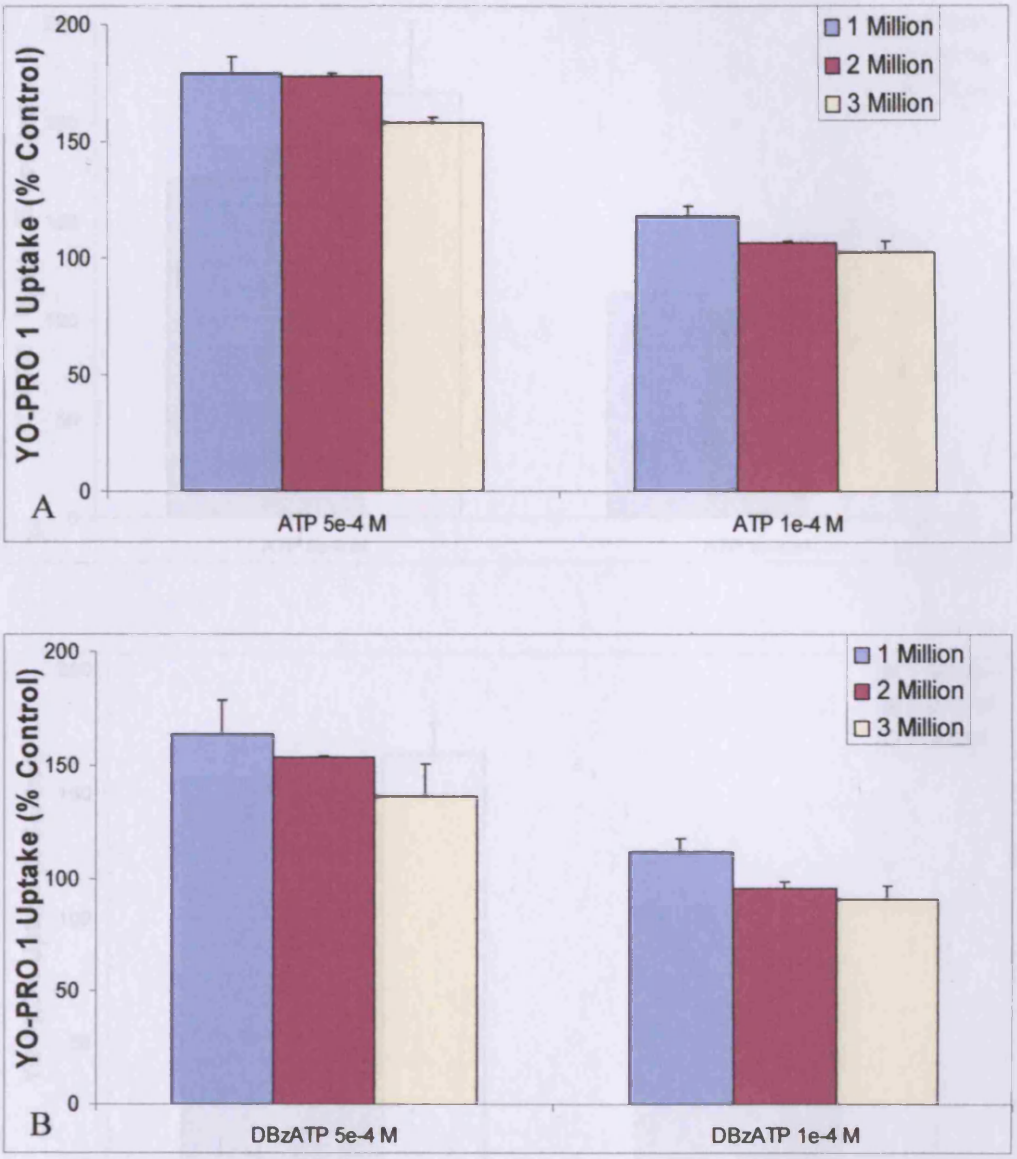


Figure 4.18: Effect of cell density on YO-PRO 1 uptake in MG63 cells.

Cells at different cell densities ($1x10^6$, $2x10^6$, and $3x10^6$ cells/25 cm^3 flask) were grown overnight and were then incubated (approximately 100,000 cells/well) with ATP (A) or DBzATP (B) and 1 μM YO-PRO 1 at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of control in the presence of vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test, but no significant differences were seen. [n=3].

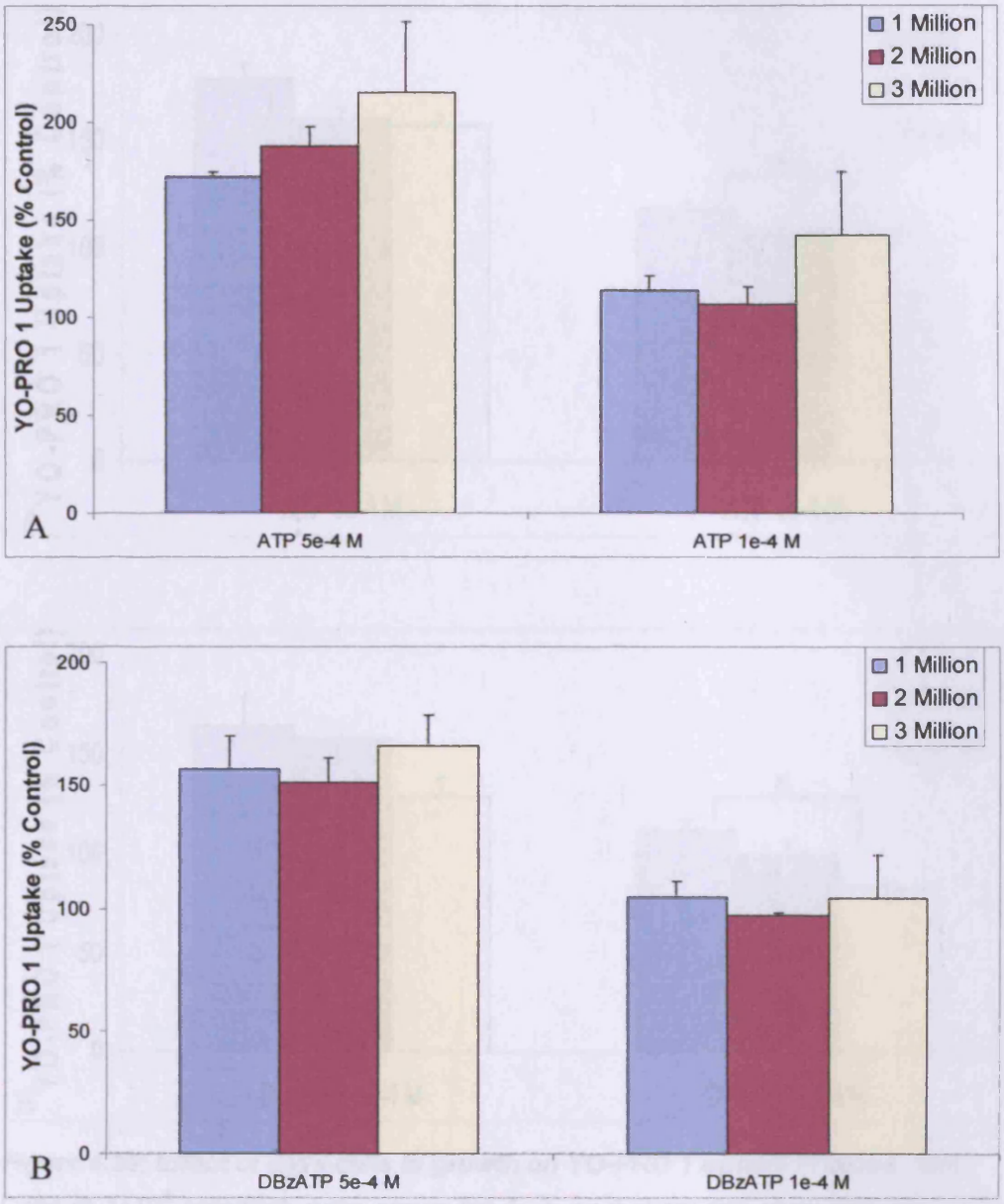


Figure 4.19: Effect of cell density on YO-PRO 1 uptake in SaOS2 cells.

Cells at different cell densities ($1x10^6$, $2x10^6$, and $3x10^6$ cells/ cm^3 flask) were grown overnight and were then incubated (approximately 100,000 cells/well) with ATP (A) or DBzATP (B) and 1 μM YO-PRO 1 at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of control in the presence of vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test, but no significant differences were seen. [n=3].

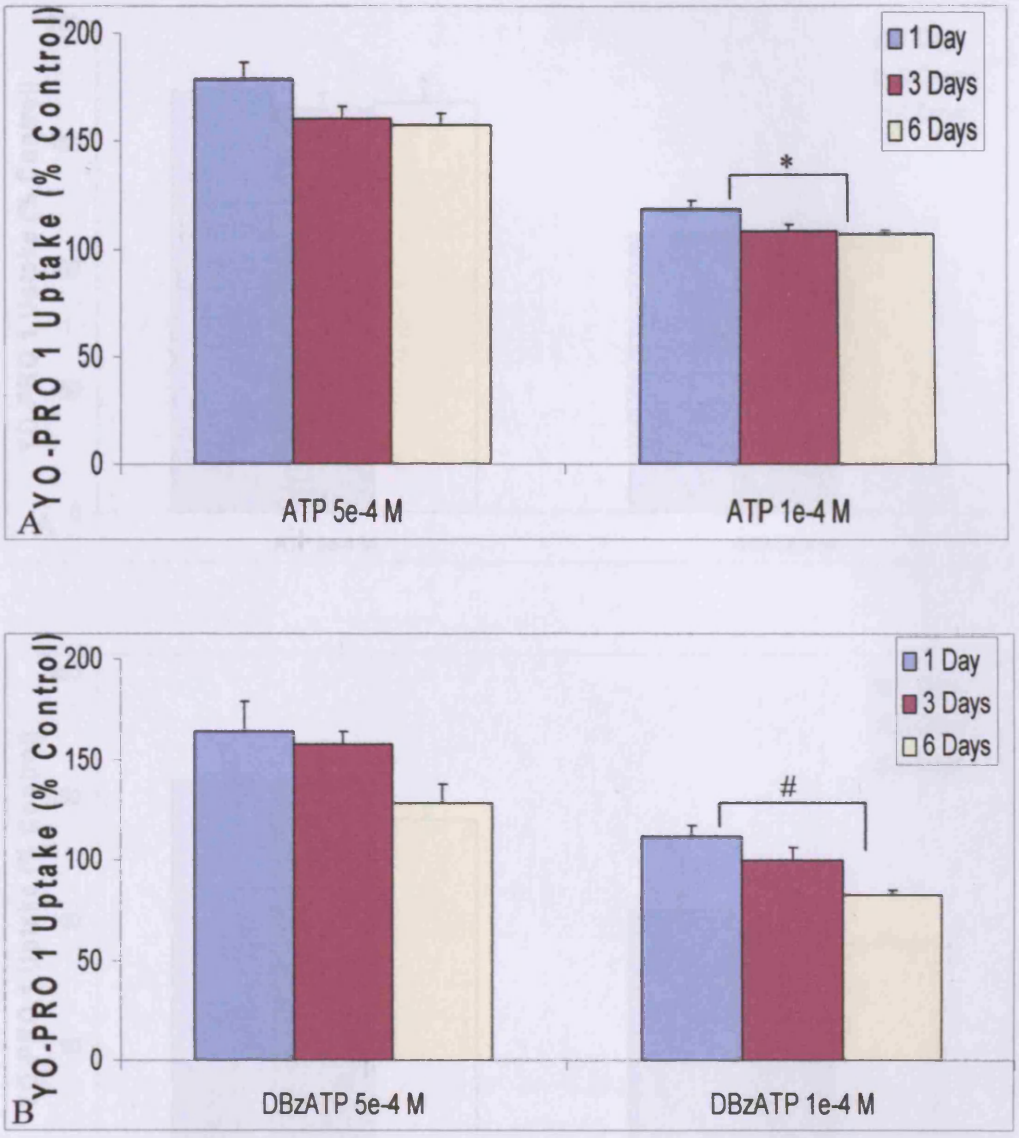


Figure 4.20: Effect of days cells in growth on YO-PRO 1 uptake in MG63 cells.
Cells at 1x10^6 cells/flask were grown for 1, 3, or 6 days and were then incubated (approximately 100,000 cells/well) with ATP (A) or DBzATP (B) and 1 μM YO-PRO 1 at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of control in the presence vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. * P<0.05, #<0.01 [n=3-4].

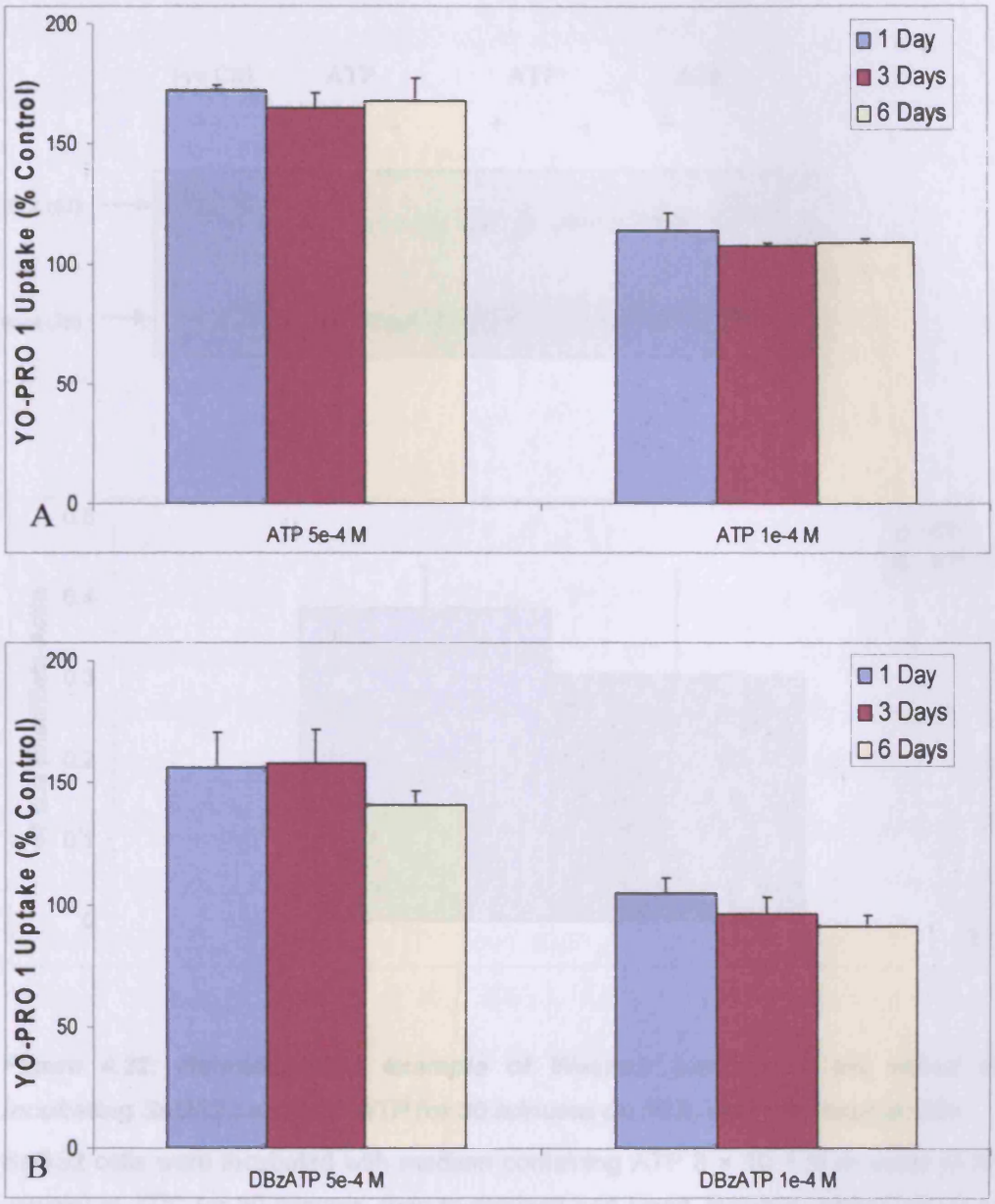


Figure 4.21: Effect of days cells in growth on YO-PRO 1 uptake in SaOS2 cells. Cells at 1x10^{6}cells/flask) were grown for 1, 3, or 6 days and were then incubated (approximately 100,000 cells/well) with ATP and DBzATP and 1 μM YO-PRO 1 at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of control in the presence vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test, but no significant differences were seen. [n=3-4].

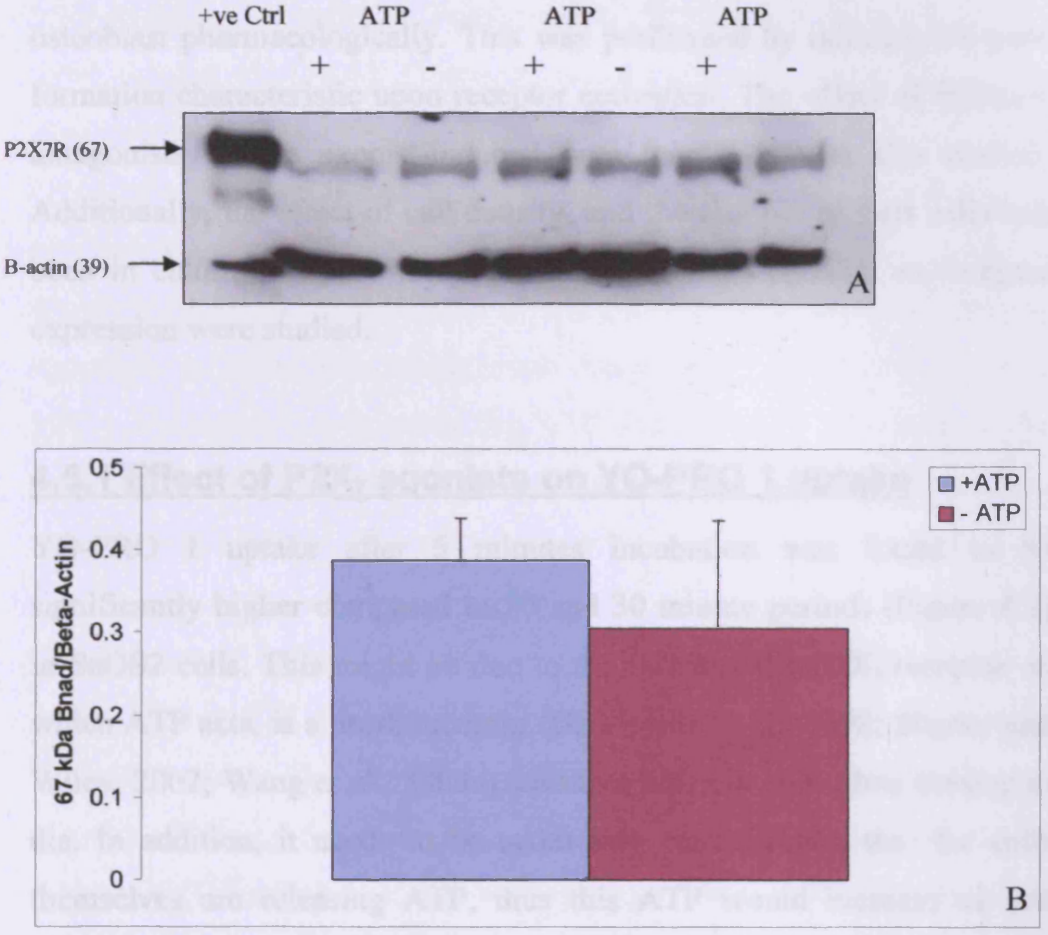


Figure 4.22: Representative example of Western blotting of the effect of incubating SaOS2 cells with ATP for 30 minutes on P2X$_7$ receptor expression.

SaOS2 cells were incubated with medium containing ATP 3 × 10^{-3} M or water (ATP vehicle) at 37°C for 30 minutes, then trypsinized and lysed. Positive control (10 µg of HEK7 cell membrane in lane 1), and 25 µg of SaOS2 cell lysates from 3 different passages were separated on 10 % gels. A shows P2X$_7$ receptor labelling. Bands were seen at 60 and 67 kDa, while the β-actin band was seen at 39 kDa. B shows P2X$_7$ receptor: β-actin ratio quantified using densitometric analysis. Statistical analyses were performed using Student's t-Test, but no difference was seen [n=3]

4.5 Discussion

This chapter investigated the functionality of $P2X_7$ receptors in human osteoblast pharmacologically. This was performed by utilizing the pore formation characteristic upon receptor activation. The effect of different antagonists on the agonist-induced pore formation was also studied. Additionally, the effect of cell density, and the number of days cells had been in culture, on pore formation and the effect of ATP on receptor expression were studied.

4.5.1 Effect of $P2X_7$ agonists on YO-PRO 1 uptake

YO-PRO 1 uptake after 5 minutes incubation was found to be significantly higher compared to 20 and 30 minute periods (Figure 4.2) in SaOS2 cells. This might be due to the fact that the $P2X_7$ receptor on which ATP acts, is a death receptor (Di Virgilio et al., 1998; Sluyter and Wiley, 2002; Wang et al., 2004a), and that the cells were thus starting to die. In addition, it needs to be taken into consideration that the cells themselves are releasing ATP, thus this ATP would increase as cell number increase (Buckley et al., 2003). Another explanation, might be that the ATP is being broken down as it has been reported that the ATP added to SaOS2 cells was degraded completely within 120 minutes (Buckley et al., 2003).

Different agonists were used, to compare their potency on the $P2X_7$ receptor. Additionally, this comparison was useful to confirm that the $P2X_7$ receptor is mainly responsible for the pore formation upon activation. The results demonstrated that all the agonists used were weak (EC_{50} values in the millimolar level), although DBzATP was slightly more potent than the others. These results are consistent with those obtained by Gartland et al 2001 where ATP and DBzATP showed

similar effects in opening the pore in SaOS2 cells (Figure 4.5). DBzATP was not more potent than ATP, unlike results from experiments with cells containing transfected receptors (Chessell et al., 1998; Michel et al., 1999; Lee et al., 2006). However, other studies in native cells have demonstrated DBzATP to be equipotent to ATP (Hickman et al., 1994; Gartland et al., 2001) or to be inactive in opening the pore when used at concentrations up to 100 μM (Cario-Toumaniantz et al., 1998). Another study found that ATP and DBzATP had comparable potency in inducing apoptosis in cells with endogenous receptors (Coutinho-Silva et al., 1999). Therefore, it is possible that ATP and DBzATP are equipotent in cells expressing endogenous $P2X_7$ receptors and/or that the high receptor number in transfected cells affects potency.

Comparison of agonist potencies for $P2X_2$, $P2X_4$, and $P2X_7$ receptors suggested that $P2X_7$ receptors are most likely to be involved in my pore formation assay. This agrees with the results from the RT-PCR and Western blotting for the $P2X_2$ receptor (described in Chapter 3). Although $P2X_4$ receptors are expressed in these cells (described in Chapter 3), the agonist potencies results suggest that they do not contribute significantly to pore formation. Also, the lack of receptor desensitization supports the involvement of the $P2X_7$ receptor, rather than $P2X_2$ or $P2X_4$ receptors as the latter desensitize upon long exposure (seconds in electophysiological studies) to agonists (North and Barnard, 1997; North, 2002; Fountain and North, 2006; Sperlagh et al., 2006). $P2X_5$ receptor has been shown to have little desensitization (North, 2002). Direct comparison of the agonist potencies for $P2X_5$ to that for $P2X_4$ and $P2X_7$ receptors could not be done as the studies performed on $P2X_5$ receptors utilized electrophysiological assays (Collo et al., 1996; Le et al., 1999). However, the study that reported pore formation with $P2X_5$ receptor showed that YO-PRO 1 uptake occurred with 10 and 100 μM

ATP and DBzATP, respectively (Bo et al., 2003). Additionally, they reported that the YO-PRO 1 uptake occurred after 60 seconds of agonist application and that divalent cations had no effect on the uptake (Bo et al., 2003).

Interestingly, ATP and 2Me-S-ATP at concentrations higher than 1 mM were found to reduce YO-PRO 1 uptake, an effect observed on inducing ethidium uptake by ATP in HEK7 cells (Humphreys et al., 1998; Lee et al., 2006). This may be due to the agonists binding to the calcium available in the buffer (Dweck et al., 2005; Saris and Carafoli, 2005) thus reducing the effective concentration. Another explanation may be that these agents are exposed to more breakdown at their higher concentrations. It was reported in the literature that ATP and 2Me-S-ATP potency was decreased by 100-1000 fold by breakdown (Kennedy and Leff, 1995). Another, more likely explanation, given the low concentration of calcium used in the current study, is that concentrations higher than $1x10^{-3}$ M cause dephosphorylation of the $P2X_7$ receptor on tyrosine-343. A similar effect was seen with repeated application of DBzATP (100 μM) on rat $P2X_7$ receptors, where a decline in current recorded was observed (Kim et al., 2001). This dephosphorylation might cause either a change in channel conformation, or disrupt the protein-protein interactions needed for pore formation. Receptor desensitization can be ruled out as an explanation as it was not shown in the experiments performed on the MG63 and SaOS2 cells and is not reported by other studies.

In osteoblasts, Gartland et al 2001 found that one hour incubation with agonists was needed for pore formation. In contrast, in my study, a 5 minute period was found to give maximal pore formation. This discrepancy might be due to methodological differences in the buffer and

dye used (ethidium bromide used by Gartland et al. (2001)). Different times were found to be needed by others for the uptake of ethidium bromide and YO-PRO 1 through $P2X_7$ receptor-induced pores (Dixon, J, Personal communication) which might be due to the dimensions of these dyes (See chapter 4). Based on the hour incubation period needed for pore formation, Gartland et al (2001) suggested that the $P2X_7$ receptors have atypical pharmacology in osteoblasts. Atypical pharmacology is also seen in my study, but is based on the low potency of DBzATP compared to ATP as pore formation was seen within 5 minutes.

4.5.2 Effect of $P2X_7$ antagonists on YO-PRO 1 uptake induced by agonists

Different P2X antagonists (BBG, KN62, PPADS, and oATP) were studied to investigate their effect on blocking the pore formation induced by the agonists (ATP and DBzATP) and reflected by the YO-PRO 1 uptake.

An experiment was performed to determine the best pre-incubation period for the antagonists to inhibit YO-PRO 1 uptake and one hour was found to be the best pre-incubation time (data not shown). Pre-incubation period of P2X receptor antagonists was found to vary from one study to another. A pre-incubation period of 30 minutes with PPADS, oATP and KN62 was found to be enough to study the antagonist effect of these compunds on DBzATP-induced YO-PRO 1 uptake in HEK7 cells (Michel et al., 2000). Additionally, this study reported that 60 minutes pre-incubation did not increase these compounds antagonist effect (Michel et al., 2000). Another study used 20 minutes pre-incubation period for KN62 in DBzATP-induced YO-PRO 1 uptake assay (White et al., 2005). A pre-incubation period of one hour with PPADS was used to

block DBzATP-induced blebbing in human osteoblasts (Gartland et al., 2001).

Efforts to generate concentration-effect curves of all of the above mentioned antagonists with one concentration of agonist failed, which may be due to the atypical pharmacology of the receptor in these cells. Therefore, concentration-effect curves of the agonists in the presence of different concentrations of the antagonists were studied. The concentration-effect curves showed that the main action of the antagonists, BBG, KN62 and PPADS, was to reduce the maximum YO-PRO 1 uptake induced by ATP and DBzATP. There was no shift in the EC_{50} values. oATP had no effect on YO-PRO 1 uptake in either cell lines.

The antagonists were found to behave in a similar non-competitive manner in human osteoblasts compared to data reported in the literature in other cell-lines (Michel et al., 2000). BBG was reported to be more potent at $P2X_7$ receptors compared to other receptors (IC_{50} values in the nanomolar range (Hibell et al., 2000; Jiang et al., 2000; Khakh et al., 2001b; Sperlagh et al., 2006)), and the effect found here is probably on the $P2X_7$ receptor. KN62 is reported in the literature to be the most potent $P2X_7$ receptor antagonist with an IC_{50} value in the nanomolar range (Sperlagh et al., 2006). However, that was not found here. The reason for this difference is not clear, but may be related to the atypical pharmacology of the receptor in the human osteoblasts. Another explanation for these results may be the involvement of the $P2X_4$ and/or $P2X_5$ receptor in pore formation, but this is unlikely due to affinities of the agonists and the reported higher potency of PPADS on $P2X_5$ compared to $P2X_7$ receptors (North and Suprenant, 2000a; Gever et al., 2006), but it was found to be weakly active in this study. Alternatively,

the $P2X_7$ receptor may form a heterodimer with $P2X_4$ receptor, a possibility that very recently has been reported in other cells (Guo et al., 2007). A heterodimer would probably have different affinities for agonists and antagonists compared to homomeric receptors.

4.5.3 Effect of cell density on YO-PRO 1 uptake induced by agonists

The effect of cell density, and the days cells had been maintained in culture, on YO-PRO 1 uptake were studied in relation to $P2X_7$ receptor protein expression. The purpose of these experiments was to find if cell density and the days cells had been kept in culture affected the receptor pore formation, and if the functional form of the receptor in osteoblasts is the 85 kDa band reported in the literature. It needs to be taken into consideration that RT-PCR illustrated that receptor mRNA expression was significantly higher at the highest SaOS2 cell density used (see Chapter 3).

Although most of the results obtained were not significant, a trend was observed in the modulation of the receptor function by cell density and the days of cells in culture as reflected by YO-PRO 1 uptake. It was observed here that there is an inverse relationship between cell density and days of culture and the YO-PRO 1 uptake in MG63 cells, but this relationship was less clear in SaOS2 cells. The effect of ATP and DBzATP on YO-PRO 1 uptake supports earlier data suggesting that the 85 kDa form of the $P2X_7$ receptor might not be the functional form (see Chapter 3). This is based on the observation that the intensity of this band increased as the cell density and the days in culture increased, while the YO-PRO 1 uptake was observed to decrease with increasing cell density and days of cells in culture, especially in the MG63 cells. The

reason for the effect of cell density and days of cells in culture on the receptor function is not known, but other pore-forming purinergic receptors ($P2X_4$ and $P2X_5$) may be involved, or the $P2X_7/P2X_4$ heteromer may exist (Guo et al., 2007).

4.6 Conclusion

Both the MG63 and SaOS2 cells were found to form a pore upon activation with ATP and DBzATP using the YO-PRO 1 uptake method. My results suggest that the $P2X_7$ receptor is likely to be mainly responsible for YO-PRO 1 uptake in these two osteoblast cell lines, although the $P2X_4$ and $P2X_5$ receptors might also be involved, although unlikely. This is based on the affinities of the four agonists used to induce pore formation and the lack of receptor desensitisation. The unexpectedly low affinity of DBzATP, reported to be more potent than ATP in the literature, and the weak action of the antagonists confirms earlier studies showing the atypical pharmacology of the $P2X_7$ receptor in osteoblast cells. Additionally, the observed effects of cell density and days of culture on YO-PRO 1 uptake supports earlier findings with Western blotting that the 85 kDa form is not the functional form of the $P2X_7$ receptor in human osteoblasts.

Chapter 5: Effect of $P2X_7$ activation on osteoblast function

5.1 Introduction

5.1.1 Bone metabolism markers

Bone metabolism is an active process that includes bone modelling and remodelling, and in the normal situation results in a balance between bone resorption and formation (Christenson, 1997) (see detailed section in Chapter 1). Biochemical markers are used for monitoring bone metabolism and diagnosing diseases. Some of these markers are related to osteoblastic bone formation such as ALP and others are related to osteoclastic bone resorption such as hydroxyproline (Bikle, 1997; Christenson, 1997; Weisman and Matkovic, 2005; Hannon and Eastell, 2006).

ALP

ALP is the most commonly used osteoblastic bone formation marker (Bikle, 1997). It is a plasma membrane enzyme released extracellularly. Although its exact function is not clear, it is thought to be involved in the shift of some substances (such as inorganic phosphate and calcium) from the intracellular to the extracellular region (Christenson, 1997). Four widely distributed isoenzymes exist. One specific isoenzyme is associated with liver, bone, placental and intestinal tissues (Whyte, 1994; Christenson, 1997; Balcerzak et al., 2003).

Bone ALP is found in the plasma membrane of osteoblasts, and is released in large amounts during bone formation, therefore it is a good marker of bone formation (Bikle, 1997; Christenson, 1997). Its importance in bone formation was proven by its deficiency in hypophosphatasia which causes bone diseases such as rickets (Whyte, 1994; Bikle, 1997; Wennberg et al., 2000). Interestingly, it was found

that osteoblasts from ALP-knockout mice can differentiate normally, but their initiation of mineralisation in vitro was impaired (Wennberg et al., 2000). In bone, ALP has been suggested to be involved in the transport of inorganic phosphate extracellularly and its increase locally, cellular uptake of calcium, and destruction of inhibitors of hydroxyapatite crystal growth (Whyte, 1994).

In addition to its role in the diagnosis and monitoring of metabolic bone diseases such as osteoporosis, bone ALP can be used in the diagnosis of bone metastases (Bikle, 1997; Hannon and Eastell, 2006).

ALP contribution from other tissues is problematic in the accurate measurement of bone-ALP, but sensitive immunoassay kits that can identify the bone ALP isoenzyme have been developed (Bikle, 1997; Christenson, 1997; Hannon and Eastell, 2006). However, this is not a problem in experiments such as mine on osteoblasts in cell culture.

5.1.2 Cytokine release and bone

Many cytokines are involved in bone metabolism (Lowik, 1992; Roodman, 1993) and the pathogenesis of some of bone diseases such as osteoporosis (Lowik, 1992; Roodman, 1993; Manolagas, 2000; Fini et al., 2004) and RA (Lowik, 1992; Roodman, 1993; Andreakos et al., 2002; Boyce et al., 2005; Clark et al., 2005; Walsh et al., 2005). Two of the cytokines which have been studied extensively and demonstrated to play a major role in bone metabolism and diseases are IL-6 and IL-1 (α and β). IL-6 is produced by osteoblasts and acts as a signalling pathway for the stimulation of osteoclastic bone resorption (Canalis et al., 1991; Lowik, 1992; Manolagas, 2000; Fini et al., 2004). The action of IL-6 on resorption is believed to be via stimulation of osteoclast differentiation

and activity (Lowik, 1992; Roodman, 1993; Connell and McInnes, 2006). High levels of IL-6 have been detected in the serum and synovial fluid of RA patients (Abramson and Yazici, 2006; Connell and McInnes, 2006). Additionally, many cytokines including IL-6 are released by osteoclasts and stimulate the growth of multiple myeloma cells (Hideshima et al., 2005). IL-1 is also involved in bone resorption and levels are increased in some forms of osteoporosis (Canalis et al., 1991; Roodman, 1993). IL-1 was found to be involved in the inflammatory process of RA and in the release of IL-6. Both IL-1 and 6 have been reported to modulate expression of RANKL which is involved in ostoeclast bone resorption (Theoleyre et al., 2004; Feng, 2005a).

5.1.3 P2X$_7$ receptor-induced cytokine release

The P2X$_7$ receptor has been found to be important in immune and inflammatory responses, such as cytokine release (Di Virgilio et al., 2001; Bours et al., 2006). Ferrari et al., (1997) reported release of IL-1β by microglial cells stimulated with bacterial endotoxin. This effect was inhibited by the P2X$_7$ antagonist oATP which led to the receptor being described as the cytokine release pore (Ferrari et al., 1997). To prove the importance of the P2X$_7$ receptor in IL-1β release and signalling, P2X$_7$ receptor knockout mice were studied. In these mice, macrophages activated with ATP were unable to release IL-1β, and consequently the cytokine signalling cascade was impaired (Solle et al., 2001). A similar finding was reported by Labasi et al., (2002), who found that leukocytes from P2X$_7$ receptor-deficient mice lost their function including IL-1β production, and concluded that the receptor has an important role in inflammatory processes (Labasi et al., 2002). Furthermore, IL-1β release from macrophages stimulated with lipopolysaccharide (LPS) or lipopeptide was found to be 100x faster when ATP was added (Qu et al.,

2007). Additionally, $P2X_7$ receptors were found to be involved in the release of the anti-inflammatory protein, intracellular interleukin-1 receptor antagonist type-1 (icIL-1ra) from both macrophages and endothelium (Wilson et al., 2004). In addition to its release as a consequence of $P2X_7$ receptor stimulation, IL-1β may change $P2X_7$ receptor expression. In human fetal brain astrocytes, it was found to increase $P2X_7$ receptor-mediated Ca^{2+} influx and pore formation induced by the agonist, DBzATP (Narcisse et al., 2005).

Ferrari et al (2006) described the $P2X_7$ receptor as a key player in the release of the IL-1 family members (Ferrari et al., 2006). Short stimulation of $P2X_7$ receptor causes the release of the 17 kDa form of IL-1β, while long stimulation causes the release of the 31 kDa form (Ferrari et al., 2006). The need for LPS for $P2X_7$ receptor-induced IL-1β release is debatable (Ferrari et al., 2006). K^+ efflux was reported to be important for its release in response to $P2X_7$ receptor activation, but other intracellular ions such as Ca^{2+} might also be involved (Ferrari et al., 2006). IL-1β release was inhibited after a mutation of the $P2X_7$ receptor that prevented pore formation (Ferrari et al., 2006). Cell lysis is not necessary for IL-1β release as it was found that it occurred in the absence of cell lysis (Ferrari et al., 2006). While IL-1β and IL-10 release were found to be attenuated in knockout mice, IL-6 release was found to be enhanced, which is thought to be a potential compensatory mechanism (Hughes et al., 2007).

Murine mast cells were found to express the $P2X_7$ receptor and, following its stimulation, proinflammatory cytokines, such as IL-4, IL-6, IL-13, and TNF-α were released. This release was blocked by the $P2X_7$ antagonists KN62 and oATP (Bulanova et al., 2005).

In systemic lupus erythematosus (SLE), a polymorphism in the $P2X_7$ receptor gene was found in mice. Since the $P2X_7$ receptor gene in both mice and humans was shown to be located in the lupus susceptibility loci, it was hypothesized that a polymorphism in the human $P2X_7$ receptor gene may be involved in SLE pathogenesis. This was also based on the role of $P2X_7$ receptor in IL-1β secretion and apoptosis (Elliott et al., 2005).

Since $P2X_7$ receptor expression and function in human osteoblasts were demonstrated in the preceeding chapters, the current chapter investigates its relevance to bone formation and the release of markers associated with osteoblast function.

5.2 Aims

To study the effects of $P2X_7$ receptor activation and inhibition on aspects of osteoblast function using the SaOS2 and MG63 cell lines.

- To investigate the effect of $P2X_7$ receptor activation and inhibition on ALP release.
- To investigate the effect of $P2X_7$ receptor activation and inhibition on the release of the cytokines, IL-6 and IL-1β.
- To investigate the effect of $P2X_7$ receptor activation and inhibition on osteoblast mineralisation.

5.3 Experimental protocols

The protocols used to study the effect of activation and blocking of $P2X_7$ receptors on the physiological actions of osteoblasts were described in detail in Chapter 2.

In all experiments involving BBG, the cells were incubated with the antagonist for one hour then the agonists (ATP or DBzATP) were added. These experiments are described in detail below.

5.3.1 ALP assay

An experiment was performed to determine the effect of blocking the $P2X_7$ receptor with BBG on ALP activity induced by ATP and DBzATP. After 24 hours of plating, the cells were incubated with BBG at 37°C for one hour. Then the cells were treated with ATP or DBzATP, and ALP activity and cell number were measured after 2 days of treatment as described in chapter 2.

5.3.2 Cytokine release

SaOS2 cells were seeded in 24 well plates at 100,000 cells per well and incubated overnight. The next day, media was collected for baseline assay and cells were incubated with BBG at 37°C for one hour then the cells were treated with the vehicle, water, ATP or DBzATP. On day 4 after plating media was collected for assay, and fresh DMEM containing the different compounds (ATP & DBzATP ± BBG) was added. On day 7 after plating media was collected for the 6 day assay. Then, the IL-6 or IL-1β ELISA assay was performed as described in Chapter 2.

5.3.3 Mineralisation

SaOS2 cells were seeded in 6 well plates at 500,000 cells per well and incubated overnight. The next day, DMEM was changed to one containing ascorbic acid (50 μg/ml). On the next day, the cells were incubated at 37°C for one hour with DMEM containing ascorbic acid (50 μg/ml), β-glycerophosphate (8 mM), and BBG. Next the vehicle, water, ATP or DBzATP were added and the cells incubated at 37°C. This treatment was repeated twice weekly until day 15, when cells were stained with freshly prepared 1% alizarin red S as described in Chapter 2.

The mineralisation assay was not performed in MG63 cells because they do not form bone nodules in vitro (Hughes and Aubin, 1998).

5.4 Results

5.4.1 ALP

A ratio of alkaline buffer solution to phosphatase substrate of 9:1 was used in SaOS2 cells, while this ratio was 1:1 in MG63 cells. However, basal ALP levels were found to be significantly (p value<0.0001 for 2 day results) higher in SaOS2 cells (1.023 ± 0.021 OD corrected for cell number on day 2) compared to MG63 cells (0.052 ±0.012 OD corrected for cell number on day 2). In both cell lines these concentrations were found to decrease significantly from days 2 to 7. In SaOS2 cells basal ALP activity was found to decrease significantly by approximately 60 % and 75 % from day 2 to day 5 (p value<0.0001) and day 7 (p value<0.0001), respectively (Figure 5.1A). In MG63 cells ALP activity decreased significantly by approximately 73 % and 79 % from day 2 to day 5 (p value<0.01) and day 7 (p value<0.001), respectively(Figure 5.1B).

ATP $4x10^{-5}$ M significantly stimulated ALP activity in both cell lines. This increase in SaOS2 cells on days 2 and 5 was 52 and 30 %, respectively (Figure 5.2). In MG63 cells an increase of 21 and 15 % was observed on days 5 and 7, respectively (Figure 5.3). Significant increases or decreases in ALP activity were also seen with other ATP concentrations but were not consistent between the cell lines (Figures 5.1 and 5.2)

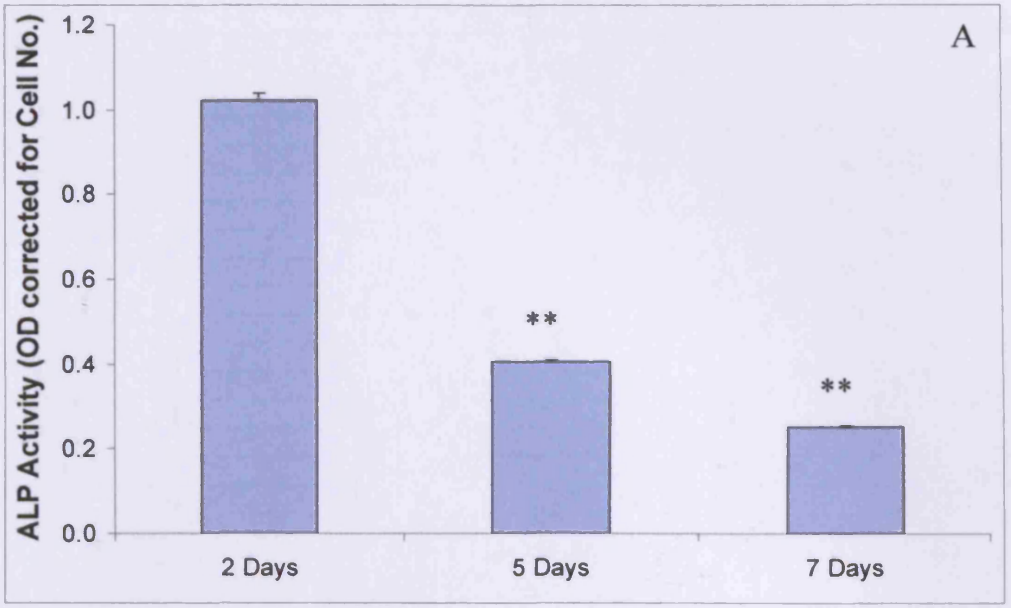
All the ATP concentrations used here were found to have no effect on the cell numbers except the $4x10^{-5}$ M concentration, which significantly reduced SaOS2 cell numbers on day 2, and MG63 cell numbers on days 5 and 7 (Figure 5.4).

Since the effect on ALP activity with ATP was most pronounced after 2 days of incubation in SaOS2 cells, this time point was used for all future studies. An increase in ALP activity was also found with DBzATP (Figure 5.5). At $1x10^{-6}$ M DBzATP significantly increased ALP activity by 20 % in SaOS2 cells, while no significant changes were seen in MG63 cells. The increase in ALP activity seen with DBzATP (20 % increase with $1x10^{-6}$ M) was significantly (p value=0.01) less than that seen with ATP (52 % increase by $4x10^{-5}$ M) in SaOS2 cells. Higher DBzATP concentrations caused massive cell death (data not shown) and therefore the effect on ALP could not be determined. The concentrations of DBzATP used here did not show any significant effect on cell number (Figure 5.6).

To find the effect of blocking the $P2X_7$ receptor on ALP activity, two antagonists, BBG and KN62 were studied. Preliminary experiments showed that KN62 could not be tested further because 10 % DMSO (KN62 vehicle) had a highly toxic effect on the cells, leading to cell

death. Hence only BBG was tested. BBG was used at a concentration of $5x10^{-6}$ M as this was the concentration which showed the maximum block in the YO-PRO 1 uptake assays (Chapter 4). Preliminary data showed that BBG at this concentration had no effect on ALP activity on its own (Figure 5.7).

In SaOS2 cells, BBG was found to decrease significantly the ALP activity induced by ATP by approximately 12 % (Figure 5.8A). ALP activity in response to the combination of DBzATP and BBG was significantly lower than DBzATP alone (Figure 5.8B). In MG63 cells, ATP significantly increased ALP activity by approximately 21 % (Figure 5.9A), but BBG had no effect on this increase. Neither DBzATP alone nor in combination with BBG had any effect on ALP activity (Figure 5.9B).



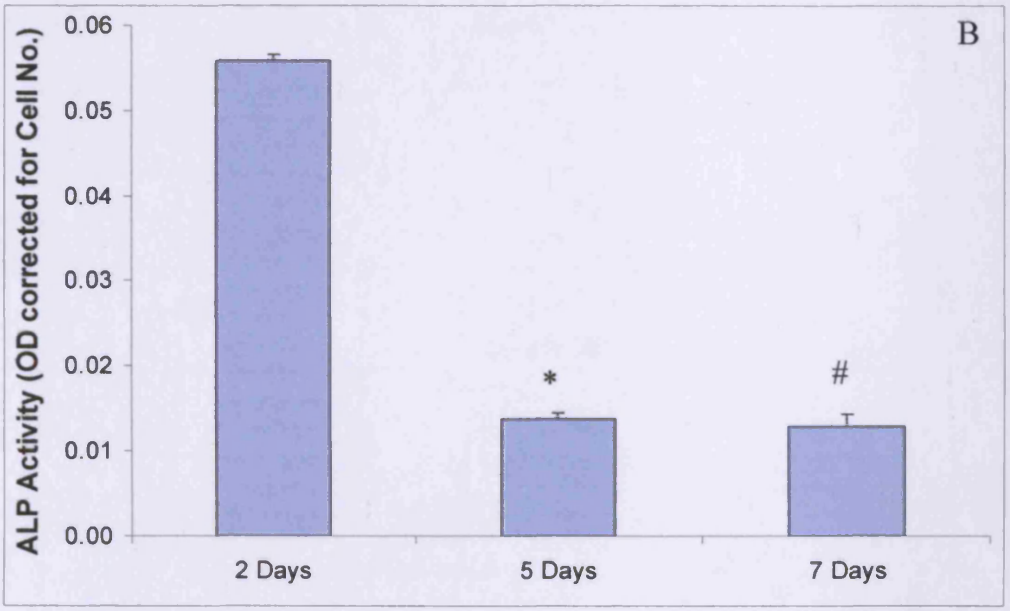


Figure 5.1: Basal ALP activity in SaOS2 and MG63 cells.

SaOS2 (A) and MG63 (B) cells were grown for 2, 5, and 7 days then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as mean ± SEM. Statistical analyses were performed using Student's t-Test. *P<0.01, #P< 0.001, **P<0.0001 vs. 2 days. (n=4-5)

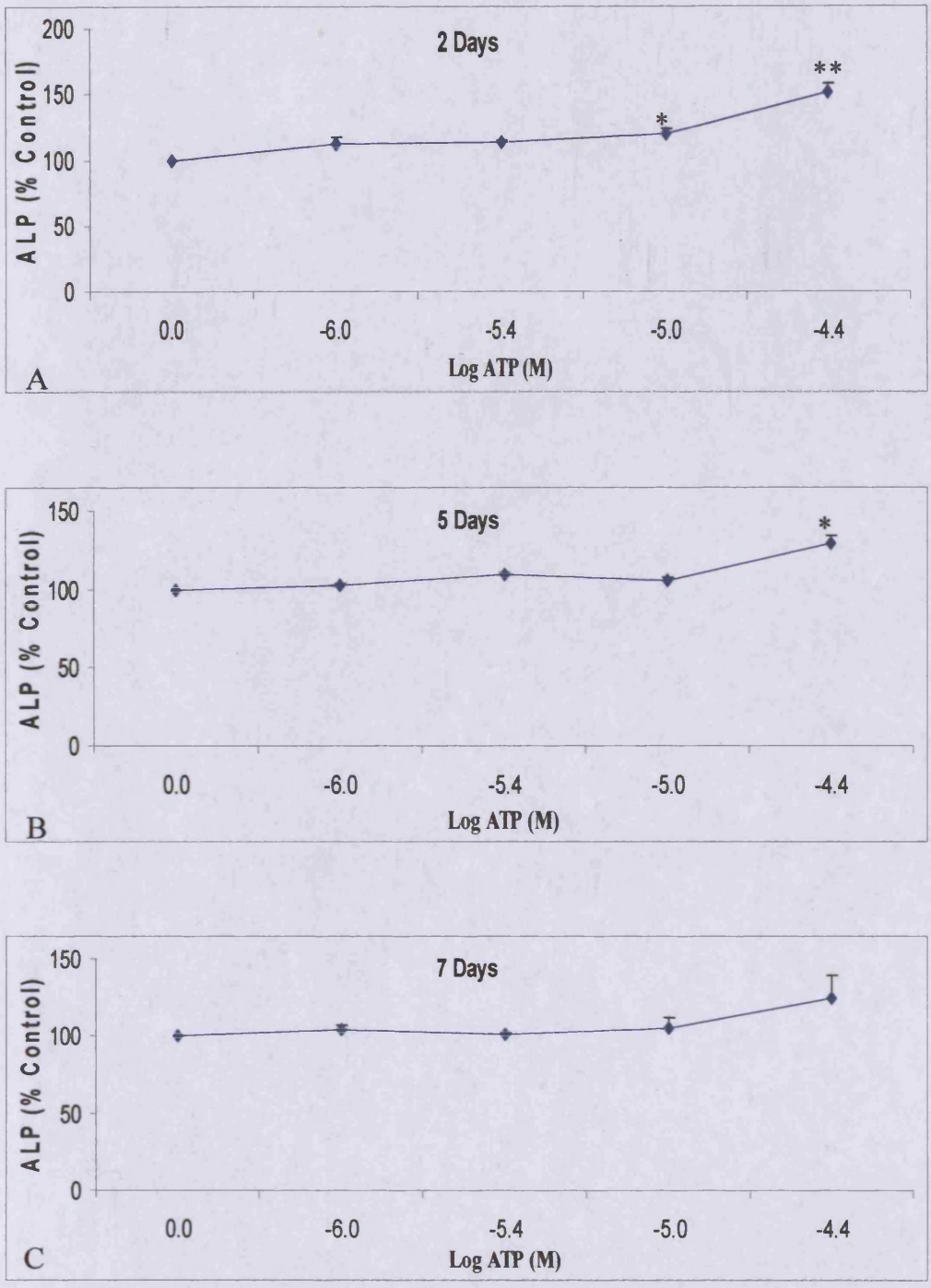


Figure 5.2: Effect of ATP on ALP activity in SaOS2 cells.

Cells were grown for 2(A), 5(B), and 7(C) days with ATP $1x10^{-6}$, $4x10^{-6}$, $1x10^{5}$, $4x10^{-5}$ M or water (ATP vehicle) then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05, **P<0.01 vs. control. (n=4)

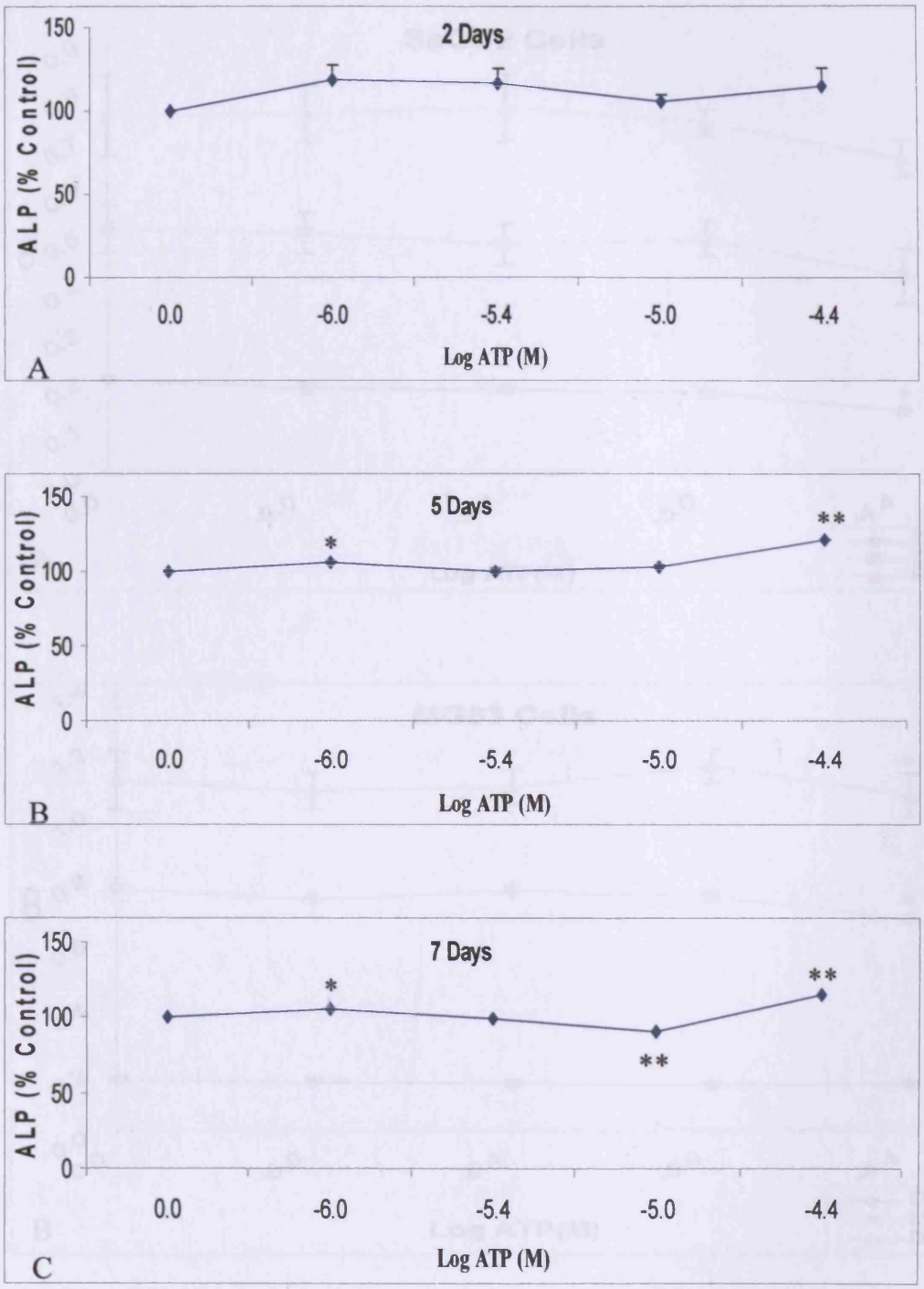


Figure 5.3: Effect of ATP on ALP activity in MG63 cells.

Cells were grown for 2(A), 5(B), and 7(C) days with ATP $1x10^{-6}$, $4x10^{-6}$, $1x10^{5}$, $4x10^{-5}$ M or water (ATP vehicle) then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05, **P<0.01 vs. control. (n=3-5)

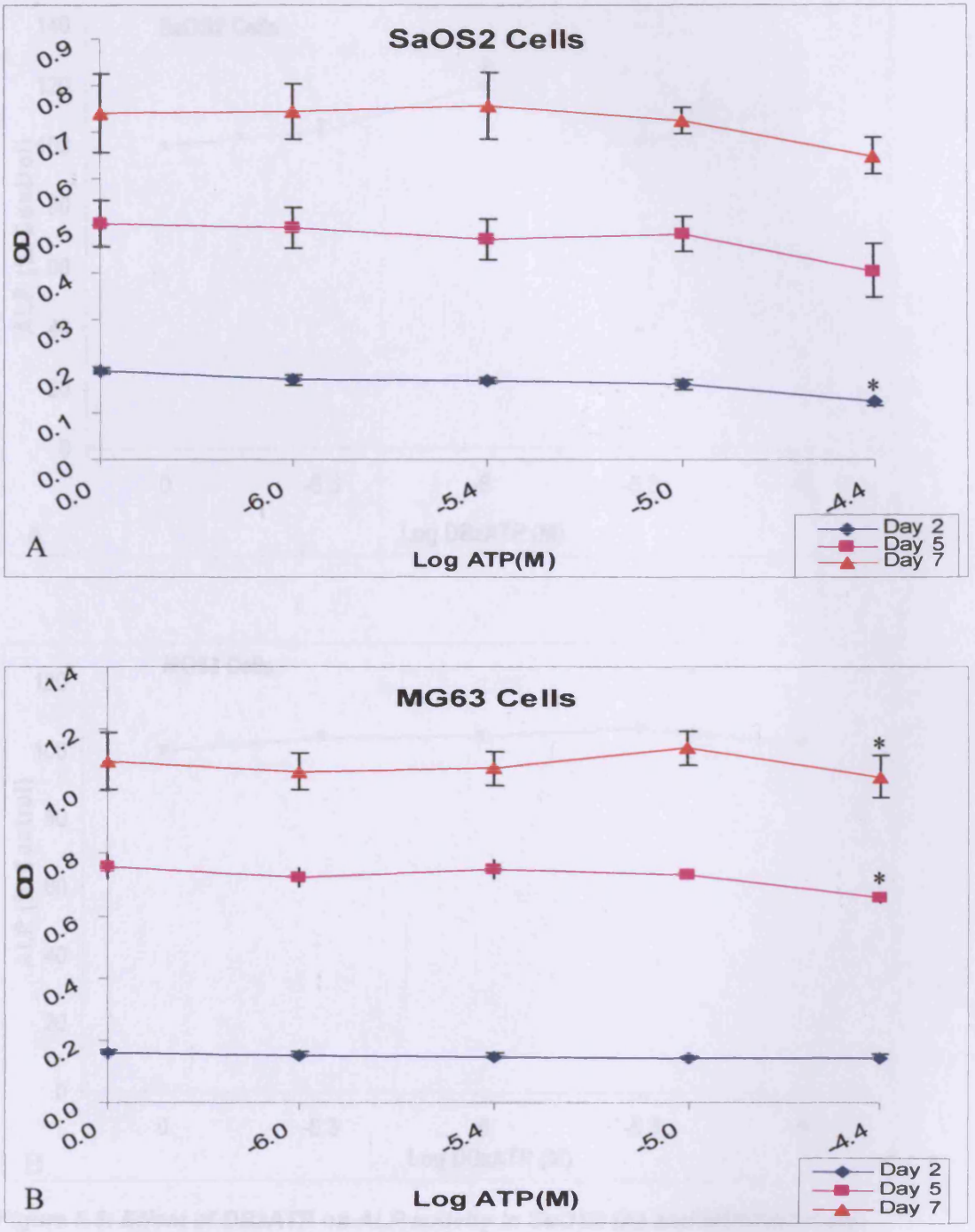
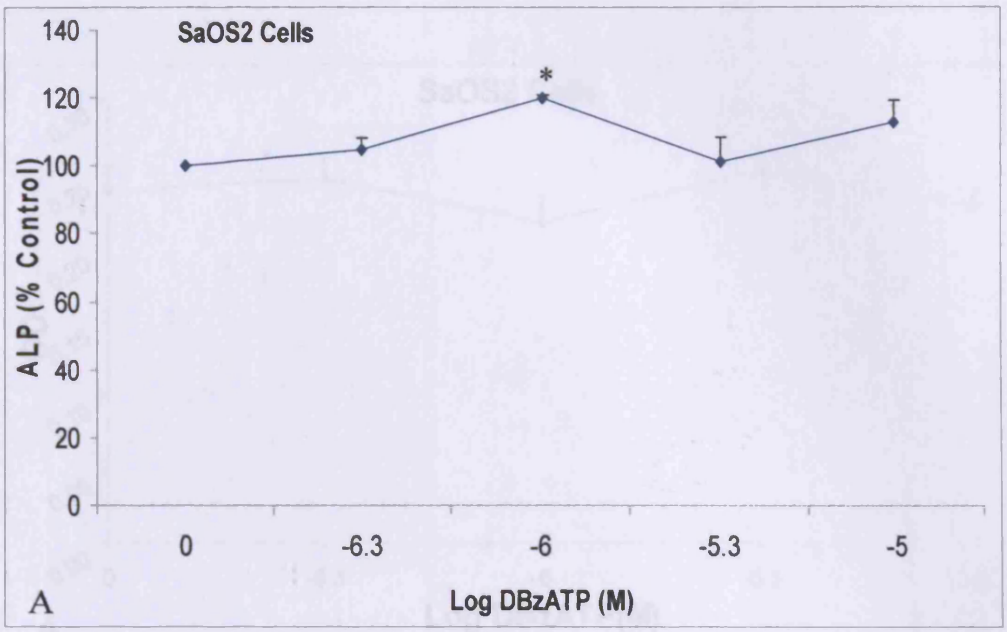


Figure 5.4: Effect of ATP on SaOS2 (A) and MG63 (B) cell number.

Cells were grown for 2, 5, and 7 days with ATP then cell numbers were determined by reading the absorbance at 490 nm (OD) using a Cell Titer 96 Non-Radioactive Cell proliferation assay. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. Data are expressed as mean ± SEM. *P<0.05 vs. control. (n=3).



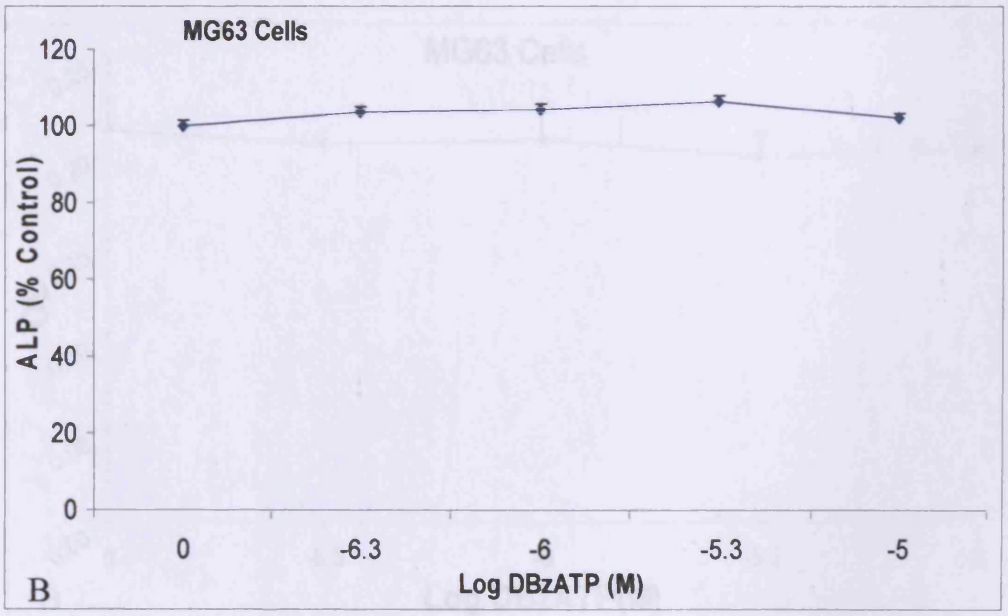


Figure 5.5: Effect of DBzATP on ALP activity in SaOS2 (A) and MG63 (B) cells. Cells were grown for 2 days with DBzATP $5x10^{-7}$, $1x10^{-6}$, $5x10^{-6}$, $1x10^{-5}$ M or water (DBzATP vehicle) then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. (n=4-5)

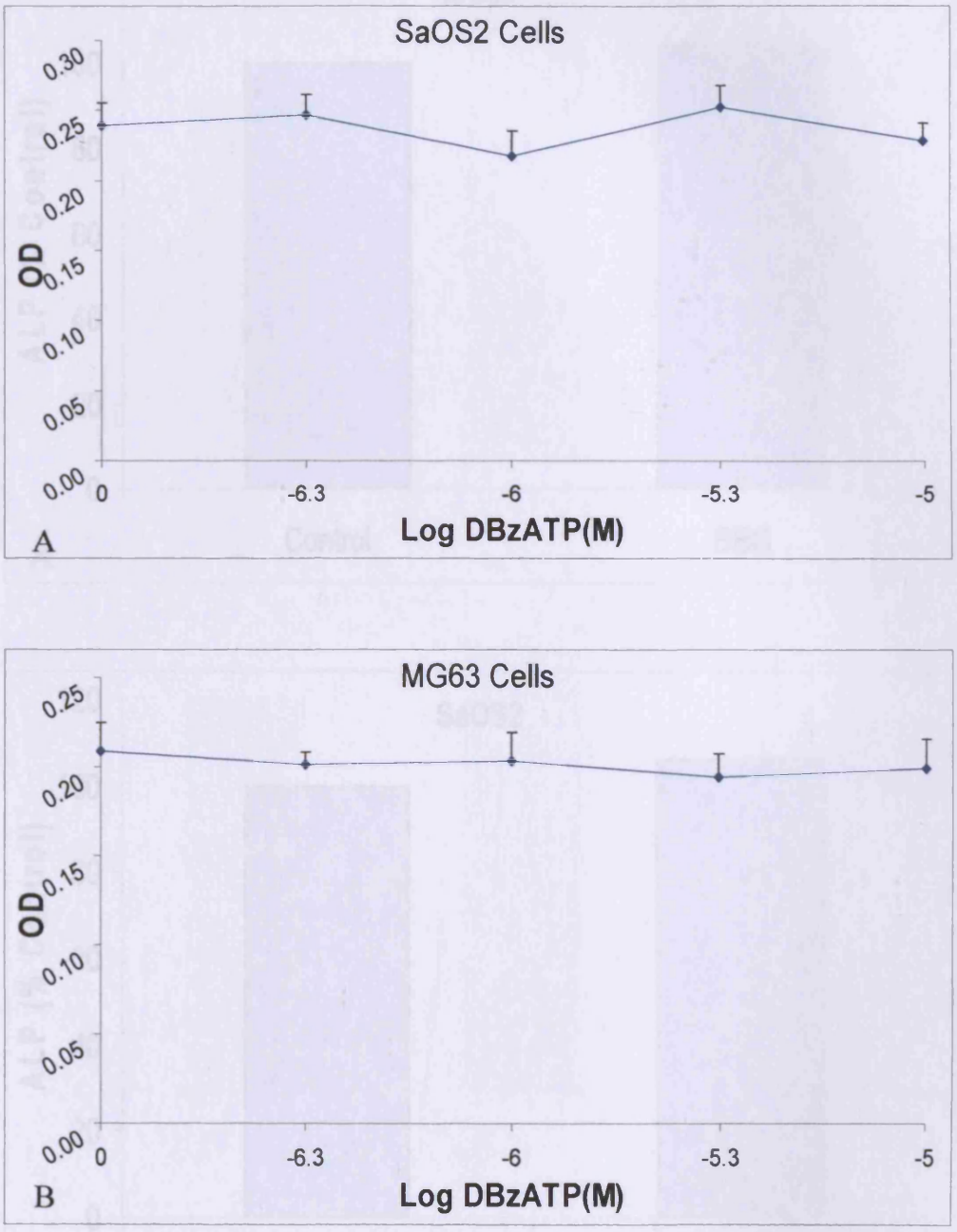


Figure 5.6: Effect of DBzATP on SaOS2 (A) and MG63 (B) cell number.

Cells were grown for 2 days with DBzATP then cell numbers were determined by reading absorbance at 490 nm (OD) using a Cell Titer 96 Non-Radioactive Cell proliferation assay. (n=3). Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. Data are expressed as mean ± SEM. *P<0.05 vs. control.

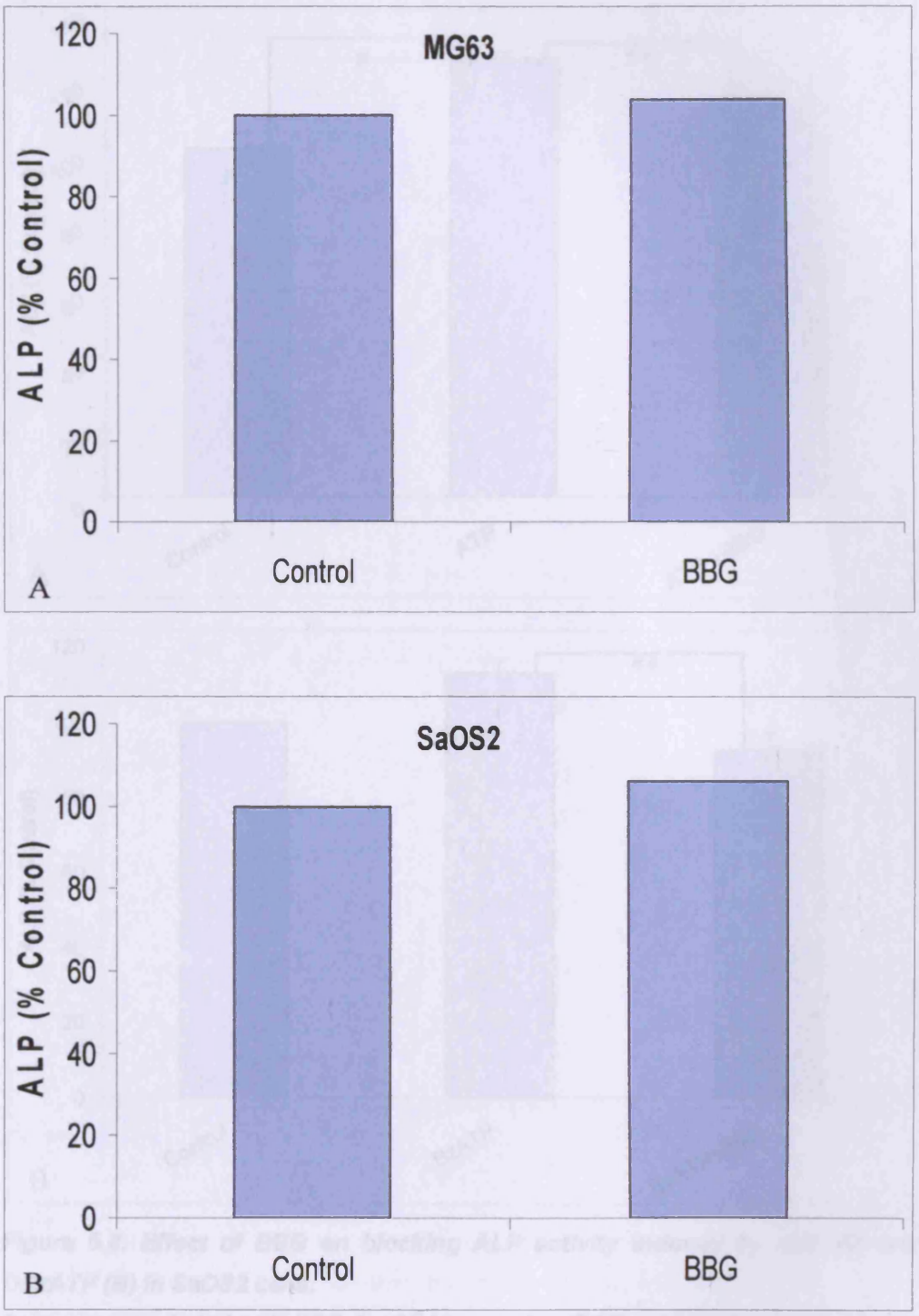


Figure 5.7: Effect of BBG on ALP activity in MG63 (A) and SaOS2 (B) cells.
Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 2 days then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as % of control containing vehicle. (n=1)

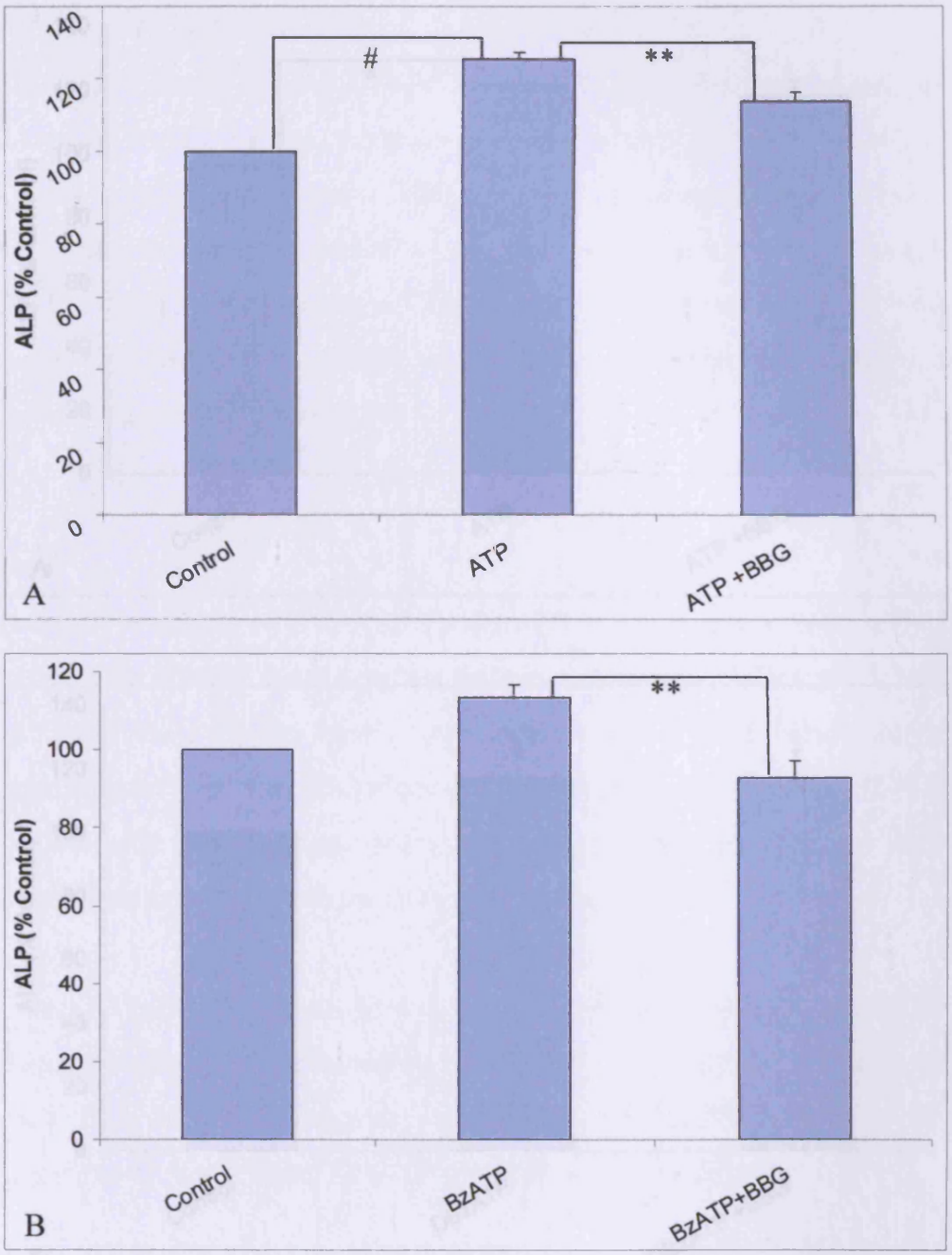


Figure 5.8: Effect of BBG on blocking ALP activity induced by ATP (A) and DBzATP (B) in SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 2 days with ATP $4x10^{-5}$M, DBzATP $1x10^{-6}$ M or water (ATP and DBzATP vehicle) then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. **P<0.01, #P<0.001. (n=3-4)

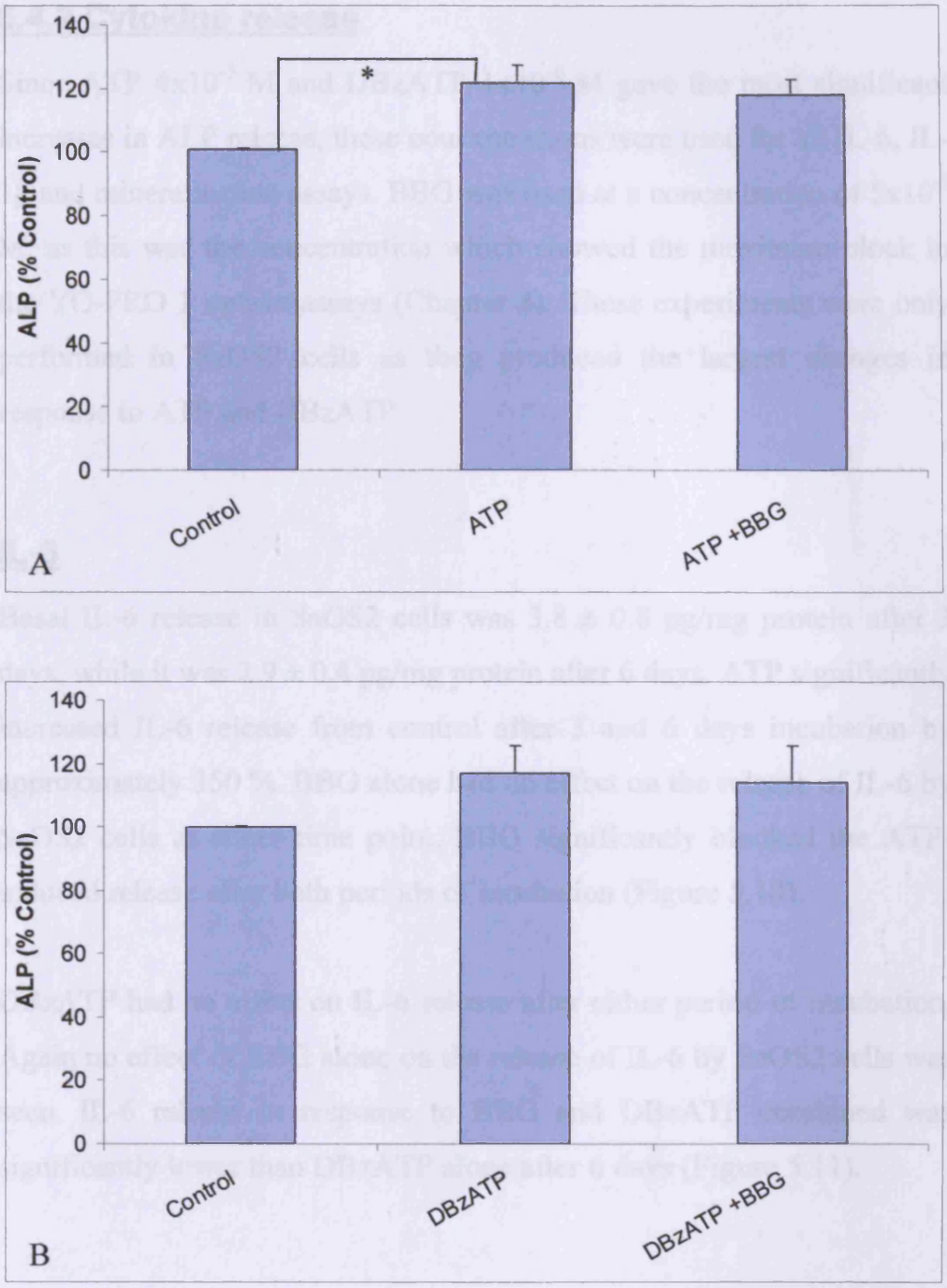


Figure 5.9: Effect of BBG on blocking ALP activity induced by ATP (A) and DBzATP (B) in MG63 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 2 days with ATP $4x10^{-5}$M, DBzATP $1x10^{-6}$ M or water (ATP and DBzATP vehicle) then ALP (OD) and cell number (OD) were determined. ALP activity was corrected for cell number. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. (n=3-4)

5.4.2 Cytokine release

Since ATP $4x10^{-5}$ M and DBzATP $1x10^{-6}$ M gave the most significant increases in ALP release, these concentrations were used for all IL-6, IL-1β and mineralisation assays. BBG was used at a concentration of $5x10^{-6}$ M, as this was the concentration which showed the maximum block in the YO-PRO 1 uptake assays (Chapter 4). These experiments were only performed in SaOS2 cells as they produced the largest changes in response to ATP and DBzATP.

IL-6

Basal IL-6 release in SaOS2 cells was 3.8 ± 0.8 pg/mg protein after 3 days, while it was 2.9 ± 0.4 pg/mg protein after 6 days. ATP significantly increased IL-6 release from control after 3 and 6 days incubation by approximately 350 %. BBG alone had no effect on the release of IL-6 by SaOS2 cells at either time point. BBG significantly blocked the ATP-induced release after both periods of incubation (Figure 5.10).

DBzATP had no effect on IL-6 release after either period of incubation. Again no effect of BBG alone on the release of IL-6 by SaOS2 cells was seen. IL-6 release in response to BBG and DBzATP combined was significantly lower than DBzATP alone after 6 days (Figure 5.11).

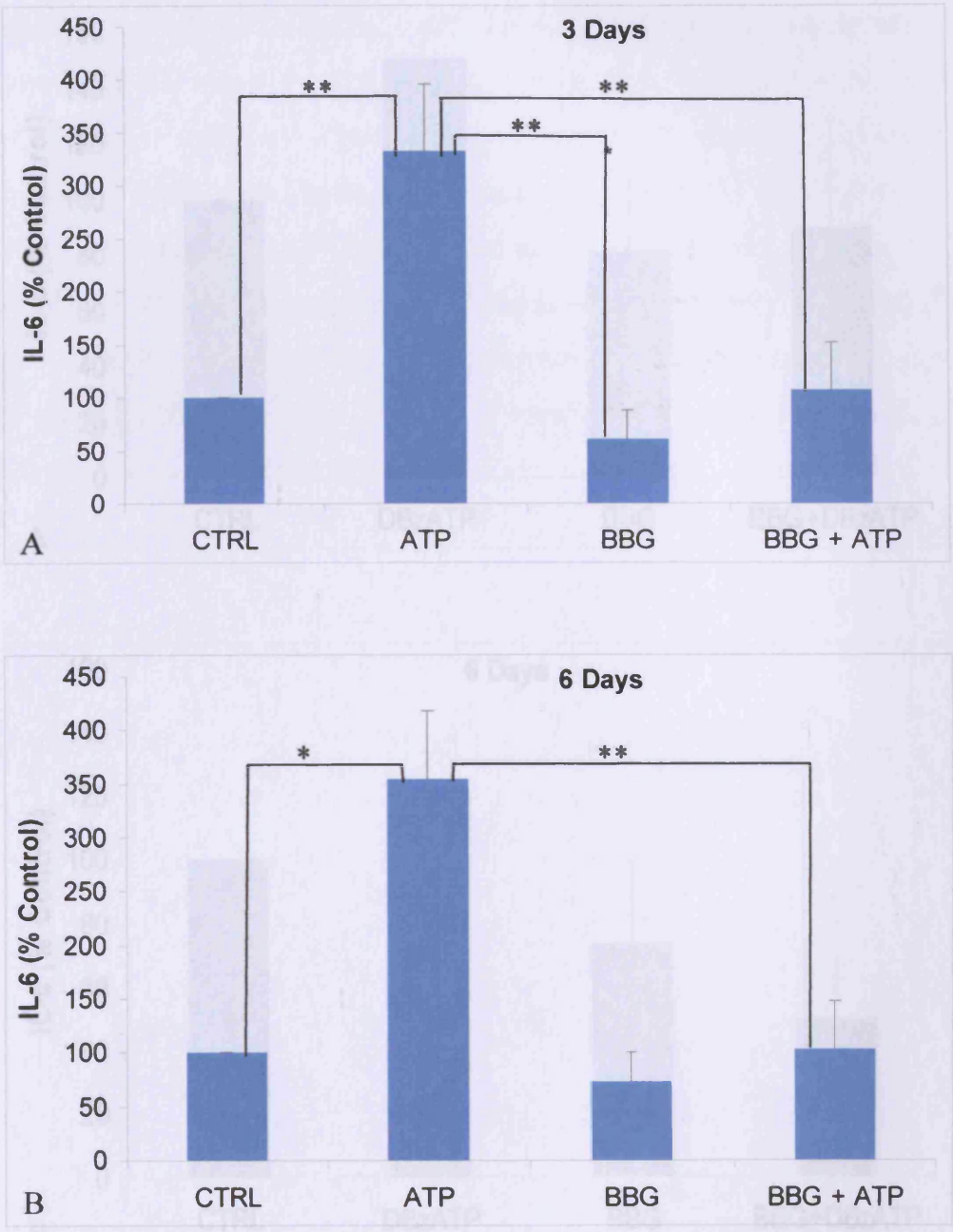
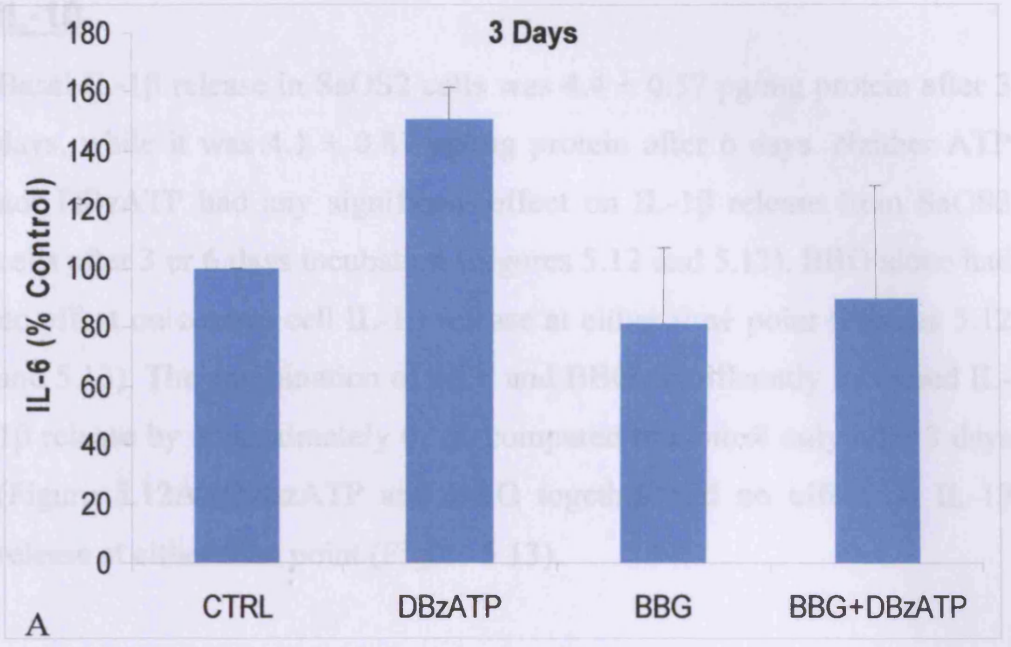


Figure 5.10: Effect of ATP and BBG on IL-6 release from SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 3 (A) and 6 (B) days with ATP $4x10^{-5}$M or water (ATP vehicle) then media and cells were collected for the IL-6 and protein assays. IL-6 data were corrected for protein concentrations. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05, **P<0.01. (n=3-4)



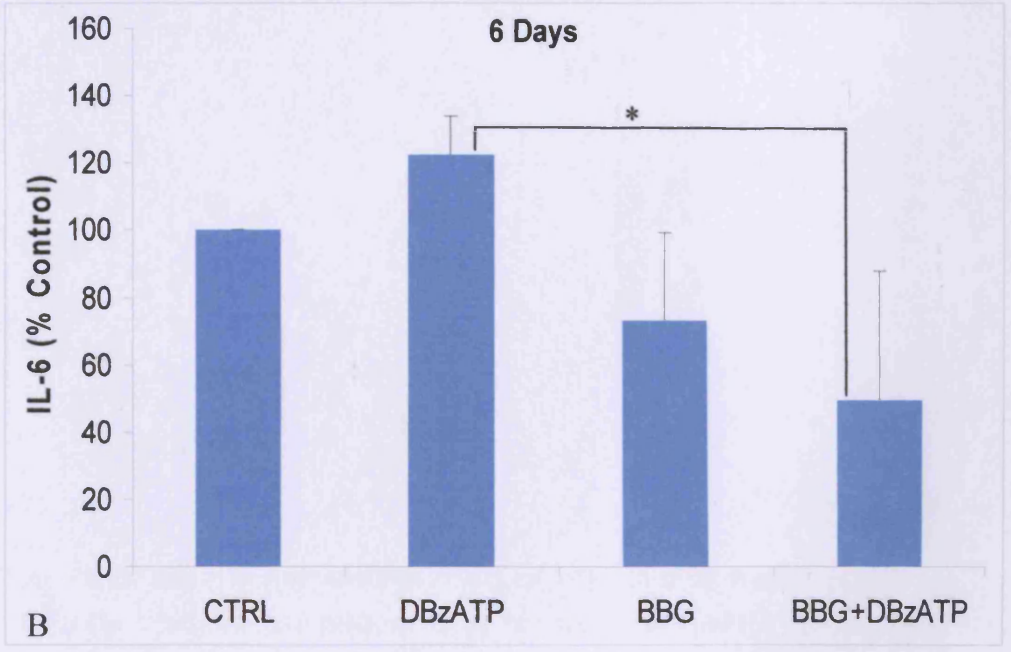


Figure 5.11: Effect of DBzATP and BBG on IL-6 release in SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 3 (A) and 6 (B) days with DBzATP $1x10^{-6}$ M or water (DBzATP vehicle) then media and cells were collected for the IL-6 and protein assays. IL-6 data were corrected for protein concentrations. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05 vs. control. (n=3-4)

IL-1β

Basal IL-1β release in SaOS2 cells was 4.4 ± 0.57 pg/mg protein after 3 days, while it was 4.1 ± 0.81 pg/mg protein after 6 days. Neither ATP nor DBzATP had any significant effect on IL-1β release from SaOS2 cells after 3 or 6 days incubation (Figures 5.12 and 5.13). BBG alone had no effect on control cell IL-1β release at either time point (Figures 5.12 and 5.13). The combination of ATP and BBG significantly increased IL-1β release by approximately 62 % compared to control only after 3 days (Figure 5.12A). DBzATP and BBG together had no effect on IL-1β release at either time point (Figure 5.13).

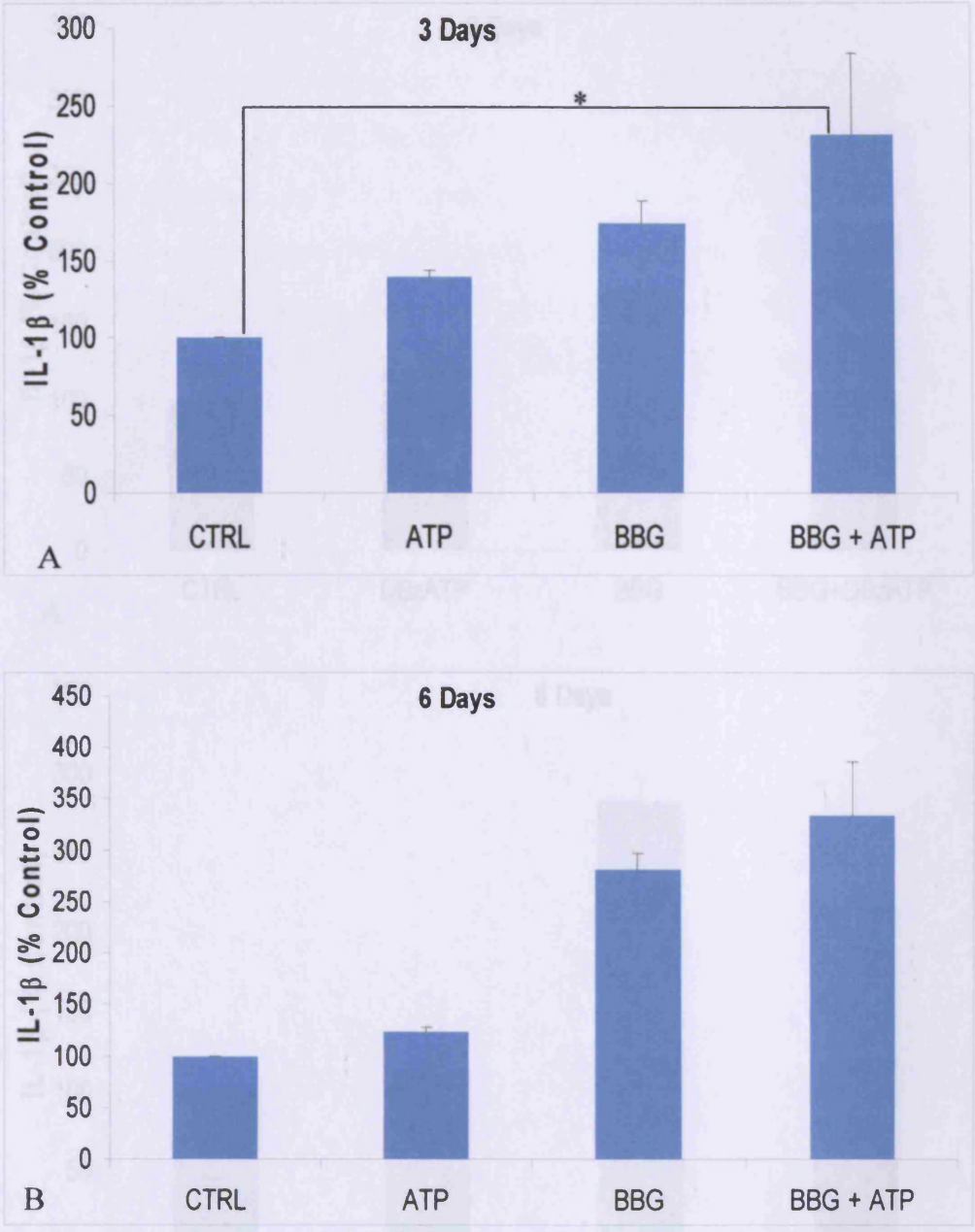


Figure 5.12: Effect of ATP and BBG on IL-1β release from SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 3 (A) and 6 (B) days with ATP $4x10^{-5}$M or water (ATP vehicle) then media and cells collected for IL-1β and protein assay. IL-1β data were corrected for protein concentrations. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05 vs. control. (n=3)

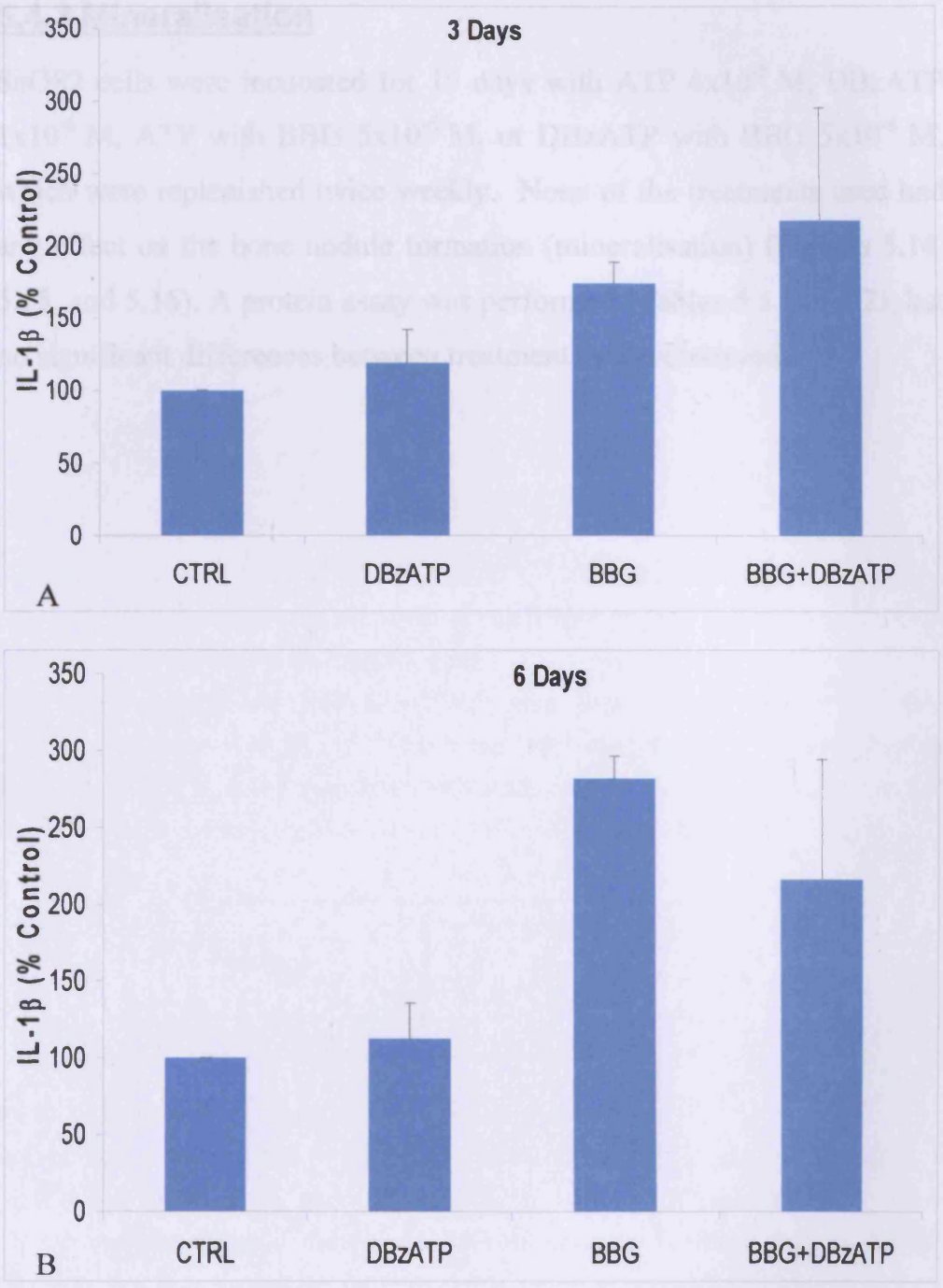


Figure 5.13: Effect of DBzATP and BBG on IL-1β release in SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 3 (A) and 6 (B) days with DBzATP $1x10^{-6}$M or water (DBzATP vehicle) then media and cells collected for IL-1β and protein assay. IL-1β data were corrected for protein concentrations. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test, but no significant differences were seen. (n=3)

5.4.3 Mineralisation

SaOS2 cells were incubated for 15 days with ATP $4x10^{-5}$ M, DBzATP $1x10^{-6}$ M, ATP with BBG $5x10^{-6}$ M, or DBzATP with BBG $5x10^{-6}$ M, which were replenished twice weekly. None of the treatments used had any effect on the bone nodule formation (mineralisation) (Figures 5.14, 5.15, and 5.16). A protein assay was performed (Tables 5.1 and 5.2), but no significant differences between treatments were observed.

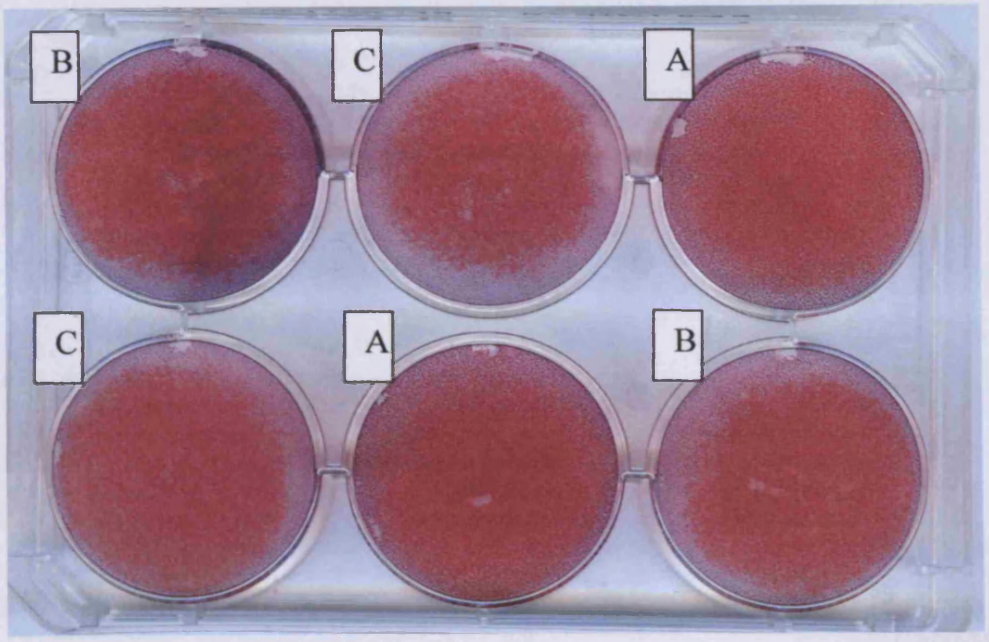


Figure 5.14: Representative example of the effect of ATP and BBG on nodule formation (mineralisation) in SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 15 days with ATP $4x10^{-5}$M or water (ATP vehicle), which were replenished twice weekly. Cells were then stained with alizarin red S. Another identical plate was used for a protein assay. (n=3) A: ATP, B: ATP + BBG, C: Control.

	Protein (mg/ml) ± SEM
Control	1.90 ± 0.37
ATP	1.72 ± 0.31
BBG + ATP	1.59 ± 0.30

Table 5.1: Effect of ATP and BBG protein concentration.

SaOS2 cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 15 days with ATP $4x10^{-5}$M or water (ATP vehicle), which were replenished twice weekly. Cells were then removed for a protein assa. Another identical plate was stained with alizarin red S for mineralised nodule formation. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test but no significant differences were seen. (n=3)

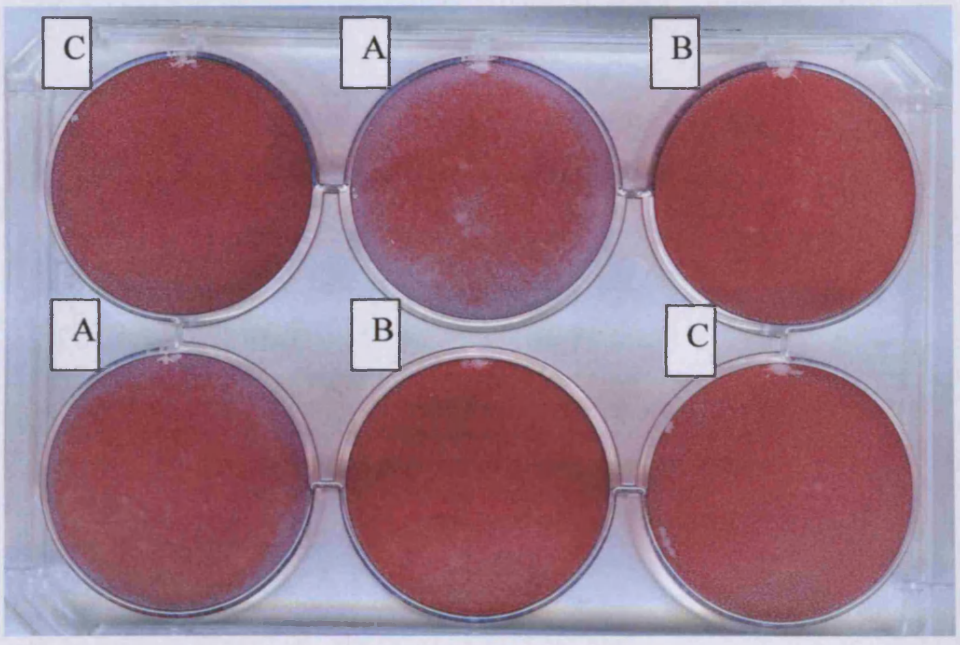


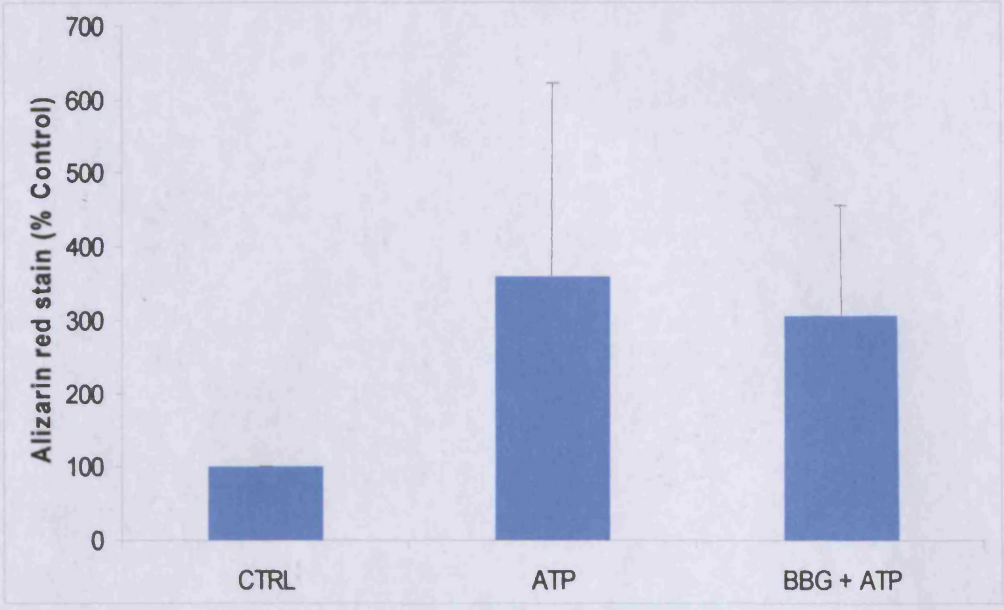
Figure 5.15: Effect of DBzATP and BBG on nodule formation (mineralisation) in SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 15 days with DBzATP$1x10^{-6}$ M or water (DBzATP vehicle), which were replenished twice weekly. Cells were then stained with alizarin red. Another identical plate was used for a protein assay. (n=3) A: DBzATP, B: DBzATP + BBG, C: Control.

	Protein (mg/ml) ± SEM
Control	1.89 ± 0.26
DBzATP	1.77 ± 0.20
BBG+DBzATP	1.80 ± 0.25

Table 5.2: Effect of DBzATP and BBG on protein concentration.

SaOS2 cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 15 days with DBzATP$1x10^{-6}$ M or water (DBzATP vehicle), which were replenished twice weekly. Cells were then removed for a protein assay for cell death. Another identical plate was stained with alizarin red S for mineralised nodule formation. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test but no significant differences were seen. (n=3)



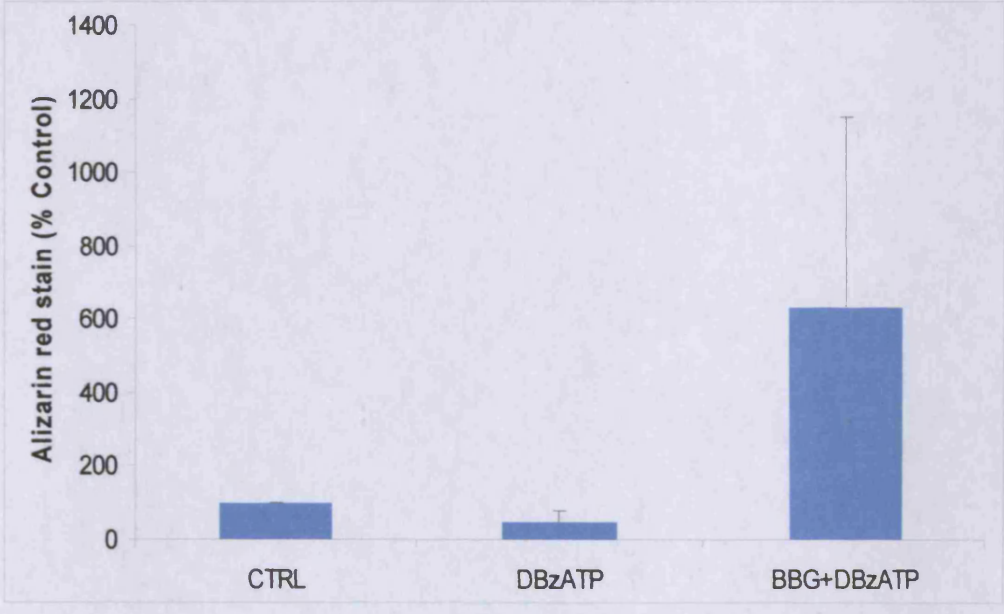


Figure 5.16: Effect of ATP, DBzATP and BBG on nodule formation (mineralisation) in SaOS2 cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then grown for 15 days with ATP $4x10^{-5}$M, DBzATP $1x10^{-6}$ M or water (ATP & DBzATP vehicle), which were replenished twice weekly. Cells were then stained with alizarin red. Data are expressed as mean percentage ± SEM of control containing vehicle. Another identical plate was used for a protein assay. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test but no significant differences were seen. (n=3)

5.5 Discussion

This chapter investigated the effect of $P2X_7$ receptor activation and inhibition on aspects of osteoblast physiological function i.e. ALP activity, cytokine release and mineralised nodule formation.

5.5.1 Effect of $P2X_7$ receptor activation and inhibition on ALP activity and mineralisation

The effect of ATP and DBzATP on ALP activity (corrected for cell number) in osteoblast-like cells was studied. It was found that ATP significantly increased ALP activity in a concentration-related manner after 2, 5 and 7 days incubation in SaOS2 and MG63 cells. SaOS2 cells are known to be more differentiated than MG63 cells (Hughes and Aubin, 1998), hence they have increased ALP production needed for bone formation, and this could explain why the effect of ATP was found to be greater in this cell line. ATP $4x10^{-5}$ M significantly increased ALP activity in SaOS2 by approximately 52% on day 2, but only a 21 % increase was seen in the MG63 cells on day 5, may be reflecting the difference in stage of osteoblast differentiation in these two cell lines (Hughes and Aubin, 1998). An interesting finding was that basal and stimulated ALP activity was found to decrease sequentially significantly from day 2 to 7 in both cell lines in all groups. This decrease was greater than 75 % in some cases, and might be explained by increased stress associated with increased cell number reducing the ability of the cells to produce ALP. Another reason might be that a production on need mechanism exists, where the release of ALP is dependent on the actual need for it i.e. when the need decreases activity decreases, and the release might be more in the first days of bone formation or in this case cells in growth.

DBzATP altered the ALP activity of SaOS2 cells as well, but this was found to be significantly less compared to ATP. At $1x10^{-6}$ M DBzATP significantly increased ALP activity by 20 % in SaOS2 cells only, while no effect was seen in MG63 cells. This suggests that the involvement of $P2X_7$ receptors in ALP activity might be small. However, there does appear to be a $P2X_7$ receptor component. BBG, a more potent antagonist on $P2X_7$ receptors than other P2 receptors (Hibell et al., 2000; Jiang et al., 2000; Khakh et al., 2001b; Sperlagh et al., 2006), was found to block the ATP-induced ALP release. Another more likely explanation is that some of the serum constituents available in the medium might be binding DBzATP and reducing its potency on ALP production, while ATP-induced ALP release was not affected. This has been shown previously for ATP and DBzATP on ethidium bromide uptake (Michel et al., 2001). The difference in $P2X_7$ receptor-induced ALP activity between MG63 and SaOS2 cells might be due to the different stages of differentiation of these two cell types (Hughes and Aubin, 1998). In a previous study, ATP inhibited ALP activity in rat osteoblasts, an effect claimed to be via $P2Y_2$ receptors (Orriss et al., 2007). The difference with the results seen here might be due to the receptor involved and it might be species-dependent. The involvement of $P2Y_{11}$ receptors in the increased ALP activity seen here might be possible taking into consideration that DBzATP is active on this receptor (Ziganshin et al., 2002; Burnstock, 2007). However, the possibility of $P2X_7$ receptor involvement is stronger as suggested by the inhibition of the ATP-induced ALP activity with BBG.

Since ALP is a bone formation marker (Bikle, 1997), the effect of $P2X_7$ receptor activation on its activity suggests a role for the $P2X_7$ receptor on bone formation. The findings in the current project are in accordance with the previous findings of the effect of $P2X_7$ receptor deletion on bone formation in mice (Ke et al., 2003). These $P2X_7$ receptor knockout mice

were found to have reduced bone formation compared to the wild-type (Ke et al., 2003).

Regarding the effect on cell number, ATP was used at concentrations that had a minimal effect on cell number in order to have a more accurate ALP assay. However, there was still a clear reduction in cell number, but only at one concentration. This reduction is most probably due to the action of ATP on $P2X_7$ receptors, as it has been described to be a death receptor, as cell death resulting from its stimulation by either necrosis or apoptosis has been reported (Di Virgilio et al., 1998; Sluyter and Wiley, 2002; Wang et al., 2004a; Elliott et al., 2005). The DBzATP concentrations used in this assay were lower than those of ATP (maximum concentration 40 μM) as DBzATP was found to be very toxic, causing death of almost all the cells at higher concentrations (data not shown). This might be an additional explanation for the small effect of DBzATP on ALP activity as its concentration (10 μM) may not have been high enough to activate $P2X_7$ receptors sufficiently to increase ALP activity. This is supported by the DBzATP EC_{50} values for YO-PRO 1 uptake which were found to be 0.5 mM ± 0.04 and 0.36 mM ± 0.04 for MG63 and SaOS2 cells, respectively, whilst they were 0.36 mM ± 0.03 and 0.43 mM ± 0.04 respectively for ATP (see Chapter 4).

Mineralised bone nodules can be formed in vitro by using β-glycerophosphate which acts as a substrate for ALP causing an increase in the concentration of inorganic phosphate and mineral deposition subsequently (Hughes and Aubin, 1998). Ascorbic acid is necessary for in vitro bone nodule formation as it promotes collagen maturation leading to extracellular matrix deposition (Hughes and Aubin, 1998). Neither ATP nor DBzATP were found to have any effect on mineralisation, an effect unlikely to be due to the assay as it was shown

to be working previously (Davies et al., 2003). In a previous study, ATP inhibited bone nodule formation by rat osteoblasts, an effect claimed to be via $P2Y_2$ receptors (Orriss et al., 2007). The difference with the results seen here might be due to the receptor involved and the species used. These findings suggest that the role of $P2X_7$ receptor on bone formation demonstrated previously (Ke et al., 2003) is not a direct action, but modulatory as indicated by the increase in ALP activity. Higher concentrations of ATP and DBzATP could not be tested due to their toxicity.

5.5.2 Effect of $P2X_7$ receptor activation and inhibition on cytokine release

Different cytokines (including IL-1β and 6) have been found to be involved in the RA inflammatory process (Andreakos et al., 2002; Boyce et al., 2005; Clark et al., 2005; Walsh et al., 2005). Additionally, IL-6 was reported to be involved in the communication between osteoblasts and osteoclasts and its upregulation is involved in the pathogenesis of osteoporosis (Manolagas, 2000; Fini et al., 2004). Furthermore, stimulation of the $P2X_7$ receptor has been reported to induce the release of many cytokines such as IL-1β, IL-4, IL-6, IL-13, and TNF-α (Ferrari et al., 1997; Bulanova et al., 2005; Di Virgilio, 2007; Hughes et al., 2007) which led to its description as the cytokine release pore (Ferrari et al., 1997).

The effect of $P2X_7$ receptor activation and inhibition on cytokine release was studied here and it was found that ATP significantly increased IL-6 release from SaOS2 cells after 3 and 6 days of incubation. The effect of BBG on blocking this ATP-induced IL-6 release suggests that it is most probably due to $P2X_7$ receptor activation. However, while DBzATP was

found to produce no significant increase alone, BBG significantly reduced IL-6 release compared to DBzATP alone after 6 days incubation. The small DBzATP effect compared to ATP might be due to its lower concentration used (to reduce cell death), or its binding to serum constituents. Since IL-6 was reported to be involved in osteoblast-osteoclast signalling, this increase in IL-6 release is likely to stimulate osteoclastogenesis and hence resorption.

Adenosine receptors were reported to be involved in the release of IL-6 from human osteoblast precursors (Evans et al., 2006) and osteoblastic cell lines (Russell et al., 2007). The involvement of adenosine receptors in the IL-6 release seen here from osteoblasts might be possible taking into consideration that adenosine is a metabolite of ATP (Lazarowski et al., 2003), and that any added ATP is degraded completely within a period of 2 hours (Buckley et al., 2003). Nonetheless, this involvement is most probably minimal due to the blocking effect of BBG on ATP-induced IL-6 release, since BBG has no effect on adenosine receptors (King and Townsend-Nicholson, 2003).

ATP was reported to have a concentration-(10–100 μM) related effect on IL-6 release from human osteoblastic SAM-1 cells (Ihara et al., 2005). This release was claimed to be due to P2Y receptors, as it was blocked by suramin and the $P2X_7$ receptor was thought not to be involved as it was not expressed by these cells (Ihara et al., 2005). Another study reported that ATP stimulated expression and release of IL-6 from human epidermal keratinocytes, an effect believed to be via P2Y receptors (Inoue et al., 2006). The involvement of P2Y receptors (if any) in the IL-6 release seen here is most probably very minimal due to the blocking effect of BBG on ATP-induced IL-6 release, since BBG has no effect on

P2Y receptors (Ziganshin et al., 2002; King and Townsend-Nicholson, 2003; Burnstock, 2006a; Burnstock, 2007).

$P2X_7$ receptor activation was found to be involved in the release of IL-1β from microglial cells (Ferrari et al., 1997), leucocytes (Labasi et al., 2002), and macrophages (Solle et al., 2001; Qu et al., 2007). IL-1 was reported to be involved in bone resorption (Canalis et al., 1991; Roodman, 1993) and it stimulates osteoblasts proliferation (Morioka et al., 2000). Additionally, IL-1β was found to have a role in the inflammation associated with RA (Andreakos et al., 2002; Boyce et al., 2005; Clark et al., 2005; Walsh et al., 2005). IL-1β was studied here and no effect on its release was observed with any of the drugs used except when the cells were incubated with ATP and BBG together. The mechanism for this is not clear, but the involvement of the $P2X_7$ receptor is unlikely as ATP and BBG alone had no effect on IL-1β release from osteoblasts. It is possible that the agonist concentrations used here are lower than those existing under physiological conditions needed to stimulate its release. Low extracellular concentrations of ATP are found naturally in tissues, but these may reach much higher levels after cell injuries such by hypoxia or ischemia (Liu and Rosenberg, 2001; Gartland et al., 2003a). Another potential reason might be the absence of LPS which was reported to be involved in IL-1β release from other cells (Ferrari et al., 1997), though this involvement is still debatable (Ferrari et al., 2006).

For ALP, IL-6, and IL-1β release, ATP and DBzATP were used at concentrations of $4x10^{-5}$ and $1x10^{-6}$ M, respectively. These concentrations are lower than those which induce pore formation (see Chapter 4) demonstrating that pore formation is not necessary for ALP activity and IL-6 release. These actions are probably therefore related to

opening of the ion channel formed by the $P2X_7$ receptor in response to activation with lower concentrations of ATP and DBzATP leading to an increase in intracellular calcium. Alternatively, an unknown second messenger might be involved.

5.6 Conclusion

ALP activity and IL-6 release were found to increase in response to ATP. This effect is most probably via $P2X_7$ receptor stimulation as suggested by the inhibition seen with BBG and the increase seen with DBzATP. No effect was seen on IL-1β release and mineralised nodule formation. These results suggest that the effect of the $P2X_7$ receptor on bone formation is likely to be modulatory rather than direct.

Chapter 6: Modulation of $P2X_7$ receptor expression and function by oestrogen and glucocorticoids

6.1 Introduction

6.1.1 Oestrogen receptors

Oestrogen receptors (ER) are widely distributed in the body (Moriarty et al., 2006). They exert either genomic effects through a nuclear receptor or non-genomic effects through a plasma membrane receptor (Deroo and Korach, 2006; Moriarty et al., 2006; Hammes and Levin, 2007). The nuclear receptor can enhance or repress genes either by direct binding to the target DNA or by interacting with co-activator or co-repressor proteins (Moriarty et al., 2006). The plasma membrane receptor can induce rapid responses (Moriarty et al., 2006; Wehling and Losel, 2006) via signalling pathways associated with G protein coupled-receptors such as inositol 1,4,5-triphosphate (IP_3) production and endothelial nitric oxide synthase (eNOS) stimulation (Jacob et al., 2006). Additionally, oestrogen plasma membrane rapid responses were also reported to be generated via ion channel signalling pathways such as increasing the intracellular calcium concentration (Jacob et al., 2006). Protein kinase signalling pathways such as MAPK were also reported to be one of the rapid response mechanisms of oestrogen plasma membrane receptors (Jacob et al., 2006). Oestrogen plasma membrane receptors have also been found to have genomic actions (Jacob et al., 2006; Hammes and Levin, 2007). ER have been found to be expressed by many tissues such as reproductive (breast, uterine, and ovarian), bone, neuronal and cardiovascular (Ciana et al., 2006; Moriarty et al., 2006). Oestrogens can have anti-inflammatory or pro-inflammatory actions depending on many factors such as the cell type involved, concentration of oestrogen, and ER isoform expressed (Straub, 2007).

The ER exists as two isoforms, ERα and ERβ, encoded by different genes (Bord et al., 2001; Deroo and Korach, 2006; Jacob et al., 2006;

Moriarty et al., 2006; Straub, 2007). Both ERα and ERβ are found in the plasma membrane in addition to the nucleus (Jacob et al., 2006; Hammes and Levin, 2007) and it is believed that ERβ can heterodimerize with and inhibit ERα (Denger et al., 2001; McDonnell, 2004; DiSilvio et al., 2006). The ERα consists of 595 amino acids and ERβ consists of 485 amino acids (Bord et al., 2001). A third plasma membrane receptor form named ER-X was also reported, but this is distinct from ERα and ERβ (Jacob et al., 2006).

In bone both isoforms of the receptors (ERα and ERβ) are expressed by osteoclasts and osteoblasts (Syed and Khosla, 2005; Deroo and Korach, 2006). Studies have shown that ER expression varies with age and bone type (cortical or cancellous) (Deroo and Korach, 2006). In osteoclasts oestrogen was found to suppress differentiation, inhibit activity and induce apoptosis, (Manolagas et al., 2002; Balasch, 2003; Cao et al., 2003; Syed and Khosla, 2005; Jacob et al., 2006) thus inhibiting bone resorption (Robinson et al., 1997; Balasch, 2003; Vural et al., 2006; Straub, 2007). The ERα and ERβ isoforms were both found to be expressed by osteoblasts (Bord et al., 2001; Denger et al., 2001; Waters et al., 2001; Wiren et al., 2002; Monroe et al., 2003). In cancellous bone ERα was found to be predominant compared to the β isoform, but the opposite was true in cortical bone (Bord et al., 2001). Oestrogen was shown to inhibit osteoblast apoptosis and hence increase their activity and lifespan (Manolagas et al., 2002; Balasch, 2003; Syed and Khosla, 2005; Jacob et al., 2006) via a non-genomic mechanism (Syed and Khosla, 2005). The effect of oestrogen on osteoblast proliferation and differentiation seems controversial as some studies have reported stimulation and others inhibition (Robinson et al., 1997; O'Shaughnessy et al., 2000; Syed and Khosla, 2005). The source of this controversy is believed to be the osteoblastic model system studied, the ER isoform

expressed and the oestrogen concentration. More recent studies of cells expressing ERα found an inhibitory action of oestrogen on osteoblast proliferation, but no effect was seen in cells expressing the ERβ isoform (Syed and Khosla, 2005; DiSilvio et al., 2006). Additionally, it was found that 17β-oestradiol-mediated regulation of mineralisation, ALP activity, and cytokine release was more via ERα than ERβ (Waters et al., 2001). MG63 and SaOS2 cell lines were found to express ERα (Penolazzi et al., 2004; Miki et al., 2007) and ERβ receptors (Miki et al., 2007).

Interestingly, 17β-oestradiol has been found to exert non-genomic inhibition of human $P2X_7$ receptor function in transfected CV-1 monkey kidney cells (Cario-Toumaniantz et al., 1998).

6.1.2 Glucocorticoid receptors

Glucocorticoids are the most potent anti-inflammatory agents and are used for the management of many inflammatory disorders such as asthma (Payne and Adcock, 2001; Valledor and Ricote, 2004; Barnes, 2006) and RA (Valledor and Ricote, 2004; Barnes, 2006). Their anti-inflammatory action arises from the suppression of many inflammatory mediators such as cytokines and chemokines by suppressing their genes. Furthermore, they activate certain genes responsible for the production of anti-inflammatory proteins. Additionally, glucocorticoids act at the cellular level by reducing the numbers of inflammatory cells such as eosinophils and T-lymphocytes (Barnes, 2006).

Glucocorticoid receptors (GR) are nuclear receptors (Bland, 2000; Yudt and Cidlowski, 2002; Valledor and Ricote, 2004; Duma et al., 2006) available in two isoforms GRα and GRβ (Bland, 2000; Valledor and

Ricote, 2004; Barnes, 2006; Duma et al., 2006; Morand, 2007). GRβ has much lower expression compared to GRα and is not activated by glucocorticoids (Bland, 2000; Barnes, 2006; Morand, 2007).

Glucocorticoids cross the cell membrane and bind to a cytoplasmic GR (Payne and Adcock, 2001; Yudt and Cidlowski, 2002; Yukawa et al., 2005; Barnes, 2006; Morand, 2007). After this binding, the GR conformation changes and the GR-glucocorticoid complex enters the nucleus and binds to glucocorticoid response elements (GRE) leading to changes in gene transcription (Payne and Adcock, 2001; Yudt and Cidlowski, 2002; Barnes, 2006; Morand, 2007). Some of the increase in gene transcription (trans-activation) causes the release of anti-inflammatory proteins (Payne and Adcock, 2001; Valledor and Ricote, 2004; Barnes, 2006). While some of the decrease in gene transcription (trans-repression) is related to the anti-inflammatory action of glucocorticoids (cytokine transcription) (Payne and Adcock, 2001; Valledor and Ricote, 2004; Barnes, 2006), part is related to the side effects of these drugs such as the effect on bone metabolism (Dovio et al., 2001; Barnes, 2006).

Many studies have reported that GR exist in the cell membrane exerting rapid non-genomic actions (Andrés et al., 1997; Borski, 2000; Evans et al., 2000), and it is believed that this is a GPCR (Borski, 2000; Evans et al., 2000).

Glucocorticoids were shown to inhibit ATP-induced intracellular calcium influx in mice neuroblastoma cell lines (Han et al., 2005). Conversely, another study found that glucocorticoids increase intracellular calcium in type I spiral ganglion neurons of the guinea-pig cochlea (Yukawa et al., 2005). It was believed that both effects were via

non-genomic mechanisms as they occurred rapidly (within 5-10 mniutes), and that the action of ATP was via P2X receptors (Han et al., 2005; Yukawa et al., 2005).

In bone, glucocorticoids have been documented to increase bone resorption due to increased osteoclast lifespan and decrease bone formation due to decreased osteoblastogenesis and decreased osteoblast lifespan (Bland, 2000; Manolagas, 2000; Boling, 2004; Gregório et al., 2006; Kanis et al., 2007). Excess glucocorticoids lead to a reduction in BMD and consequently development of osteoporosis (Bland, 2000; Gregório et al., 2006; Kanis et al., 2007). Expression of GRα mRNA and protein has been documented in human osteoblasts (Bland, 2000).

Since oestrogen and glucocorticoids are known to play a critical role in bone metabolism, and since 17β-oestradiol has been reported to have an antagonistic action on $P2X_7$ receptors in other cells, the modulation of $P2X_7$ receptor expression and function by 17β-oestradiol and dexamethasone has been studied as part of this thesis.

6.2 Aims

To study the effects of an oestrogen (17β-oestradiol) and a glucocorticoid (dexamethasone) which are known to affect bone formation and $P2X_7$ receptor function.

- To investigate the effect of 17β-oestradiol and dexamethasone on $P2X_7$ receptor protein expression
- To investigate the effect of 17β-oestradiol and dexamethasone on YO-PRO 1 uptake induced by ATP
- To investigate the effect of 17β-oestradiol and dexamethasone on ALP release upon $P2X_7$ receptor activation.
- To investigate the effect of 17β-oestradiol and dexamethasone on cytokine release (IL-6 and IL-1β) upon $P2X_7$ receptor activation.
- To investigate the effect of 17β-oestradiol and dexamethasone on bone mineralisation upon $P2X_7$ receptor activation.

6.3 Experimental protocols

The protocols used to study the effect of 17β-oestradiol and dexamethasone on the physiological actions of osteoblasts induced by $P2X_7$ receptor activation were described in detail in Chapter 2. 17β-oestradiol and dexamethasone are referred to as oest. and dex. in the diagrams.

6.3.1 Effect of 17β-oestradiol and dexamethasone on YO-PRO 1 uptake induced by $P2X_7$ receptor agonists

To determine the best incubation time for inhibition of YO-PRO 1 uptake by 17β-oestradiol and dexamethasone, approximately 100,000 SaOS2 cells / well were pre-incubated in Greiner 96-well plates with 17β-oestradiol $5x10^{-6}$ M, dexamethasone $1x10^{-6}$ M, or ethanol 0.5 % (vehicle) and 1 μm YO-PRO 1 in the assay buffer-A (see Chapter 2) at 37°C for 15-60 minutes followed by ATP $8x10^{-4}$ M for 5 minutes Then YO-PRO 1 uptake was measured. The concentrations of 17β-oestradiol and dexamethasone used were adapted from the literature (Cario-Toumaniantz et al., 1998; Yukawa et al., 2005).

To find if any effect was seen after short (30 minutes which might be a non-genomic effect) or long (2 days which might be a genomic effect) incubations, SaOS2 cells were grown in 25 cm^2 flasks until confluent, and then incubated with 17β-oestradiol and dexamethasone for 30 minutes or 2 days. The medium was then removed, cells trypsinized and counted. Cells were added to Greiner 96-well black plates (100,000 cells/well) containing ATP and 1 μM YO-PRO 1 in assay buffer-A, and then the plate was incubated at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data

were expressed as % of maximum uptake of ATP in vehicle in the absence of 17β-oestradiol or dexamethasone. When ATP alone was used in these experiments, ethanol (0.5 % v/v) was added for comparison. This differs from work in Chapter 5 as ethanol was not included with ATP.

Since 17β-oestradiol $1x10^{-5}$ and dexamethasone $5x10^{-6}$M gave the most significant block in the YO-PRO 1 uptake assays, these concentrations were used for all ALP, IL-6, IL-1β, and mineralisation assays.

6.3.2 Effect of 17β-oestradiol and dexamethasone on ALP activity induced by $P2X_7$ receptor agonists

An experiment was performed to determine the effect of modulating the $P2X_7$ receptor with 17β-oestradiol and dexamethasone on ALP activity induced by ATP and DBzATP. SaOS2 cells were seeded in 96 well plates at 1,500 cells per well and incubated overnight. Next day the medium was removed and cells were incubated with 17β-oestradiol, dexamethasone, or ethanol 0.5 % at 37°C for 30 minutes, and then with 17β-oestradiol, dexamethasone, or ethanol and ATP or DBzATP for 2 days. Next, ALP activity and cell number were measured as described in Chapter 2.

6.3.3 Effect of 17β-oestradiol and dexamethasone on cytokine release induced by $P2X_7$ receptor agonists

SaOS2 cells were seeded in 24 well plates at 100,000 cells per well and incubated overnight. Next day, cells were incubated with 17β-oestradiol, dexamethasone, or ethanol 0.5 % at 37°C for 30 minutes then water, ATP or DBzATP were added to the cells. On day 4 media were collected for

the 3 day assay, and DMEM was changed to one containing different treatments (17β-oestradiol, dexamethasone, or ethanol 0.5 % with or without ATP or DBzATP) as above. On day 7 media was collected for the 6 day assay. Then, the IL-6 or IL-1β ELISA assay was performed as described in Chapter 2.

6.3.4 Effect of 17β-oestradiol and dexamethasone on mineralisation induced by $P2X_7$ receptor agonists

SaOS2 cells were seeded in 6 well plates at 500,000 cells per well and incubated overnight. The next day, DMEM was changed to one containing ascorbic acid (50 μg/ml). On the next day, the cells were incubated at 37°C for 30 minutes with DMEM containing ascorbic acid (50 μg/ml), β-glycerophosphate (8 mM), and 17β-oestradiol, dexamethasone, or ethanol 0.5%. Next, water, ATP or DBzATP were added and the cells incubated at 37°C. This treatment was repeated twice weekly until day 15, when cells were stained with freshly prepared 1% alizarin red S as described in Chapter 2.

6.4 Results

6.4.1 Modulation of $P2X_7$ receptor protein expression by 17β-oestradiol and dexamethasone

To investigate the effect of 17β-oestradiol and dexamethasone on the expression of the $P2X_7$ receptor protein, SaOS2 cells were incubated with these agents for either 30 minutes or 2 days. As can be seen in Figures 6.1, 6.2, 6.3, and 6.4, the only apparent effect was on the 87 kDa (seen here at 92-103 kDa) band of the $P2X_7$ receptor in the cells incubated with 17β-oestradiol for 2 days, therefore these data were quantified. This

band, and the 65 kDa band were quantified using densitometry, but no significant differences were observed (Figure 6.2 B and C).

6.4.2 Effect of 17β-oestradiol and dexamethasone on YO-PRO 1 Uptake

To find the best short incubation time to see any effect of 17β-oestradiol and dexamethasone on YO-PRO 1 uptake induced by ATP, SaOS2 cells were pre-incubated for different periods (15-60 minutes) with these agents and then the assay was performed as shown in Figure 6.5. Up to 30 minutes incubation with either 17β-oestradiol or dexamethasone, inhibition of YO-PRO 1 uptake was seen, which was significant at 30 minutes. However, after 30 minutes an increase was seen which was not significant. Therefore, a 30 minute incubation period was used subsequently.

The effect of 17β-oestradiol on the concentration-effect curves of ATP after 30 minutes and 2 days are illustrated in Figures 6.6 and 6.7, respectively. After 30 minutes incubation, 17β-oestradiol at a concentration of 10 μM significantly inhibited the maximum YO-PRO 1 uptake induced by ATP by approximately 23 % (Figure 6.6). Additionally, after incubation for 2 days 17β-oestradiol was found to block the receptor to a greater extent compared to the 30 minutes incubation, as it significantly inhibited the maximum YO-PRO 1 uptake induced by ATP by approximately 37 % (Figure 6.7). However, the difference in inhibition between the two periods of incubation was not significant.

The effect of dexamethasone on the concentration-effect curves of ATP after 30 minutes and 2 days are illustrated in Figures 6.8 and 6.9,

respectively. Dexamethasone significantly reduced ATP-induced YO-PRO 1 uptake after 30 minutes incubation by approximately 18 % (Figure 6.8). No significant effect was seen on YO-PRO 1 uptake after 2 days incubation with dexamethasone (Figure 6.9).

EC_{50} values for ATP were not significantly altered by either 17β-oestradiol or dexamethasone (the values for the highest concentration of each compound used are shown in Table 6.1).

6.4.3 Effect of 17β-oestradiol and dexamethasone on ALP activity

A ratio of alkaline buffer solution to phosphatase substrate of 9:1 was used in SaOS2 cells, while this ratio was 1:1 in MG63 cells. However, basal ALP activity (OD corrected for cell number) in SaOS2 cells was significantly higher (1.44 ± 0.11, p value <0.01) compared to MG63 cells (0.026 ± 0.002). ATP significantly increased ALP activity by approximately 18 and 37 % compared to control in SaOS2 and MG63 cells, respectively (Figures 6.10-A and 6.12). 17β-oestradiol alone had no effect on ALP activity in either cell line. The combination of ATP and 17β-oestradiol significantly increased ALP activity by approximately 13 and 32 % compared to control in SaOS2 and MG63 cells, respectively. There were no significant differences between ATP alone and the combination of ATP and 17β-oestradiol (Figures 6.10 and 6.12). ATP-induced ALP activity was not significantly higher than control in the dexamethasone experiments (Figure 6.10B). Neither dexamethasone alone nor in combination with ATP had any significant effect on ALP activity in SaOS2 cells. The effect of dexamethasone on ATP-induced ALP activity was only studied in SaOS2 cells as they produced the largest changes in response to ATP and DBzATP (see Chapter 5). DBzATP increased ALP activity by approximately 10 % in SaOS2 cells,

but this was not significant (Figure 6.11). No significant effects on ALP activity were seen with 17β-oestradiol or dexamethasone alone or in combination with DBzATP (Figure 6.11).

6.4.4 Effect of 17β-oestradiol and dexamethasone on mineralisation

ATP alone and in combination with 17β-oestradiol significantly decreased mineralised bone nodule formation (Figures 6.13 and 6.15). In another set of experiments ATP alone and in combination with dexamethasone decreased mineralised bone nodule formation, but this effect was not significant (Figures 6.14 and 6.15). The combination of 17β-oestradiol and ATP reduced the amount of protein in the cells, but this was not significant (Table 6.2). Neither ATP alone nor dexamethasone plus ATP had any significant effect on protein content (Table 6.2).

6.4.4 Effect of 17β-oestradiol and dexamethasone on IL-6 release

Basal IL-6 release in SaOS2 cells was 2.04 ± 0.38 pg/mg protein after 3 days, while it was 2.59 ± 0.67 pg/mg protein after 6 days. After 3 days of incubation of ATP ($4x10^{-5}$ M) with SaOS2 cells, there was a significant increase in IL-6 release by approximately 530 % compared to control, while the release increased significantly by approximately 760 % compared to control when ATP was combined with 17β-oestradiol (Figure 6.16A). 17β-oestradiol alone had no effect on IL-6 release compared to control. IL-6 release in response to 17β-oestradiol alone was significantly less than ATP alone, while the release in response to the

combination of 17β-oestradiol and ATP was significantly higher than 17β-oestradiol alone.

After 6 days of cells in growth, ATP increased IL-6 release by approximately 450 % compared to control, but this effect was not significant, while the release increased significantly by approximately 1500 % compared to control when ATP was combined with 17β-oestradiol (Figure 6.16B). The release in response to the combination of 17β-oestradiol and ATP was significantly higher than ATP and, 17β-oestradiol. 17β-oestradiol alone had no effect on IL-6 release compared to control.

DBzATP and 17β-oestradiol alone or in combination had no significant effect on IL-6 release (Figure 6.17).

In another series of experiments, after 3 days of cells in growth, ATP significantly increased IL-6 release by approximately 500 % when compared with control, while the release in response to the combination of ATP and dexamethasone decreased significantly by approximately 250 % when compared with ATP alone (Figures 6.18-A). The release in response to dexamethasone alone was significantly less than ATP but not different to control.

After 6 days of cells in growth, ATP increased IL-6 release by approximately 400 % compared to control, but this effect was not significant, while the release increased significantly by approximately 640 % compared to control when ATP was combined with dexamethasone (Figure 6.18-B). The release in response to the combination of dexamethasone and ATP was significantly higher than

dexamethasone alone. The release in response to dexamethasone alone was not different to control.

6.4.3 Effect of 17β-oestradiol and dexamethasone on IL-1β release

Basal IL-1β release in SaOS2 cells was 4.2 ± 0.79 pg/mg protein after 3 days, while it was 7.1 ± 2.8 pg/mg protein after 6 days. After 3 days of SaOS2 cells in growth, ATP and 17β-oestradiol alone or in combination had no significant effect on IL-1β release (Figure 6.19A). DBzATP alone had no effect on IL -1β release, but 17β-oestradiol alone significantly increased the release by approximately 18 % from control (Figure 6.20A). The release in response to 17β-oestradiol alone and to its combination with DBzATP was significantly higher than DBzATP alone.

After 6 days of cells in growth, ATP significantly decreased IL-1β release by approximately 60 % from control (Figure 6.19B). The release in response to 17β-oestradiol alone and to its combination with ATP was significantly higher than ATP alone, but not different to control release. No significant effect was seen with DBzATP and 17β-oestradiol alone or in combination on IL-1β release (Figure 6.20B).

After 3 days of cells in growth, neither dexamethasone nor ATP alone or in combination had any significant effect on the release of IL-1β (Figure 6.21A).

After 6 days of cells in growth ATP alone and dexamethasone alone significantly decreased IL-1β release by approximately 64 % and 54 %, respectively from control (Figure 6.21-B). IL-1β release in response to

the combination of dexamethasone and ATP was significantly higher than ATP alone and dexamethasone alone, but not different from control.

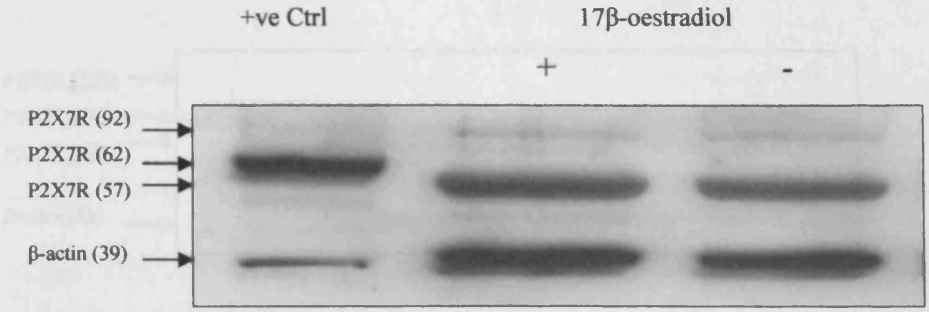


Figure 6.1: Representative example of Western blotting of the effect of incubating SaOS2 cells with 17β-oestradiol for 30 minutes on $P2X_7$ receptor expression.

SaOS2 cells were incubated with medium containing 17β-oestradiol 1×10^{-5} M or 0.5 % ethanol (17β-oestradiol vehicle) at 37°C for 30 minutes, then trypsinized and lysed. Positive control (10 µg of HEK7 cell membrane in lane 1), and 25 µg of SaOS2 cell lysates were separated on 10 % gels. $P2X_7$ receptor bands were seen at 57, 62 and 92 kDa, while the β-actin band was seen at 39 kDa. [n=3]

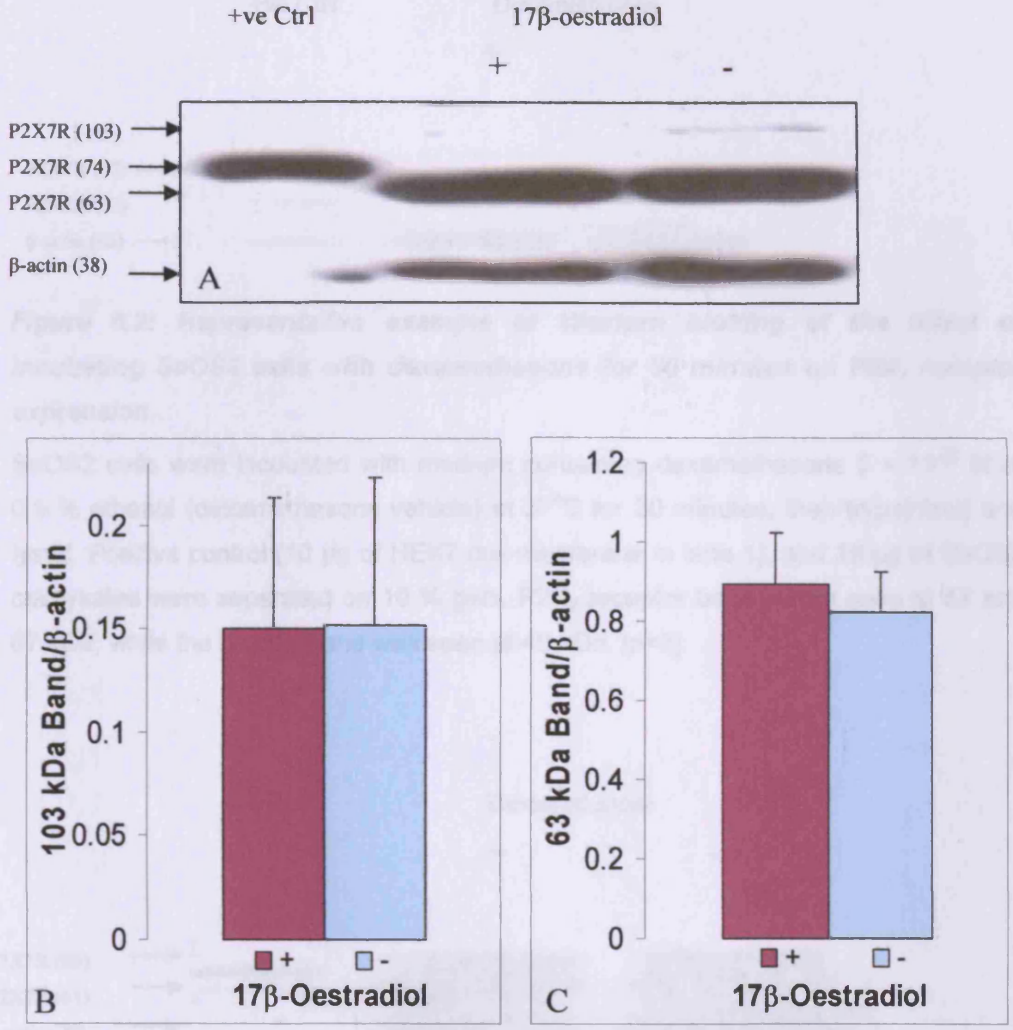


Figure 6.2: Representative example of Western blotting of the effect of incubating SaOS2 cells with 17β-oestradiol for 2 days on P2X$_7$ receptor expression.

SaOS2 cells were incubated with medium containing 17β-oestradiol 1 × 10^{-5} M or 0.5 % ethanol (17β-oestradiol vehicle) at 37°C for 2 days, then trypsinized and lysed. Positive control (10 µg of HEK7 cell membrane in lane 1), and 25 µg of SaOS2 cell lysates were separated on 10 % gels. P2X$_7$ receptor bands were seen at 63, 74, and 103 kDa, while the β-actin band was seen at 38 kDa. These bands were quantified using densitometry and the ratios of P2X$_7$ receptor to β-actin bands are shown in B (90 kDa) and C (70 kDa) [n=3]

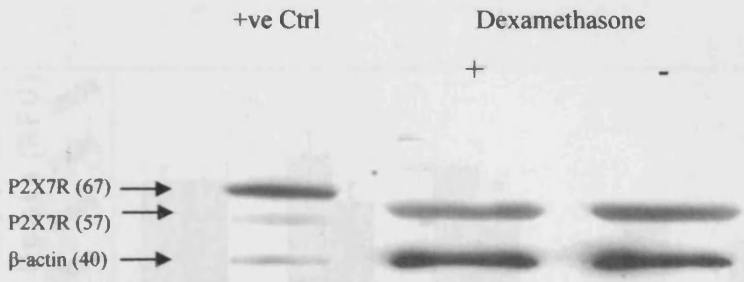


Figure 6.3: Representative example of Western blotting of the effect of incubating SaOS2 cells with dexamethasone for 30 minutes on $P2X_7$ receptor expression.

SaOS2 cells were incubated with medium containing dexamethasone 5×10^{-6} M or 0.5 % ethanol (dexamethasone vehicle) at 37°C for 30 minutes, then trypsinized and lysed. Positive control (10 µg of HEK7 cell membrane in lane 1), and 25 µg of SaOS2 cell lysates were separated on 10 % gels. $P2X_7$ receptor bands were seen at 57 and 67 kDa, while the β-actin band was seen at 40 kDa. [n=3]

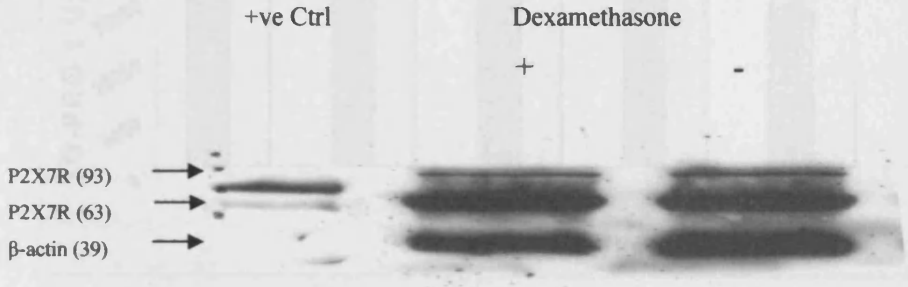
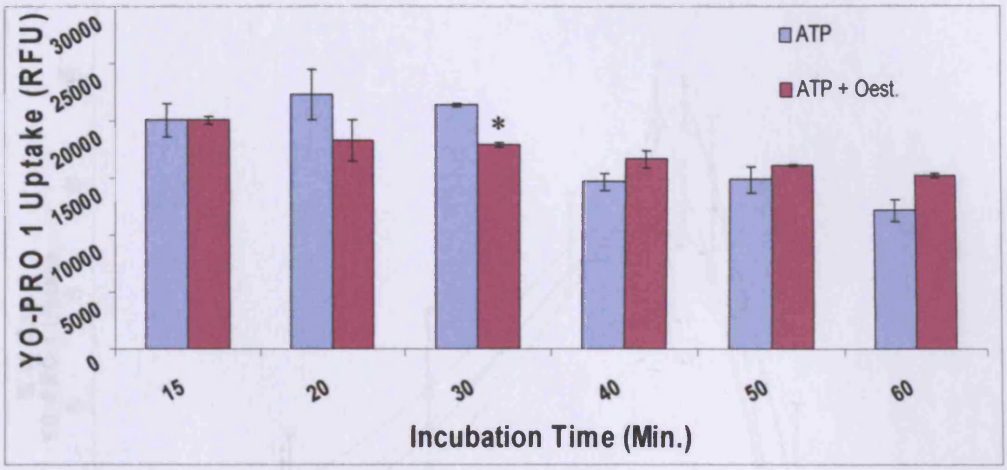


Figure 6.4: Representative example of Western blotting of the effect of incubating SaOS2 cells with dexamethasone for 2 days on $P2X_7$ receptor expression.

SaOS2 cells were incubated with medium containing dexamethasone 5×10^{-6} M or 0.5 % ethanol (dexamethasone vehicle) at 37°C for 2 days, then trypsinized and lysed. Positive control (10 µg of HEK7 cell membrane in lane 1), and 25 µg of SaOS2 cell lysates were separated on 10 % gels. $P2X_7$ receptor bands were seen at 63 and 93 kDa, while the β-actin band was seen at 39 kDa. [n=3]



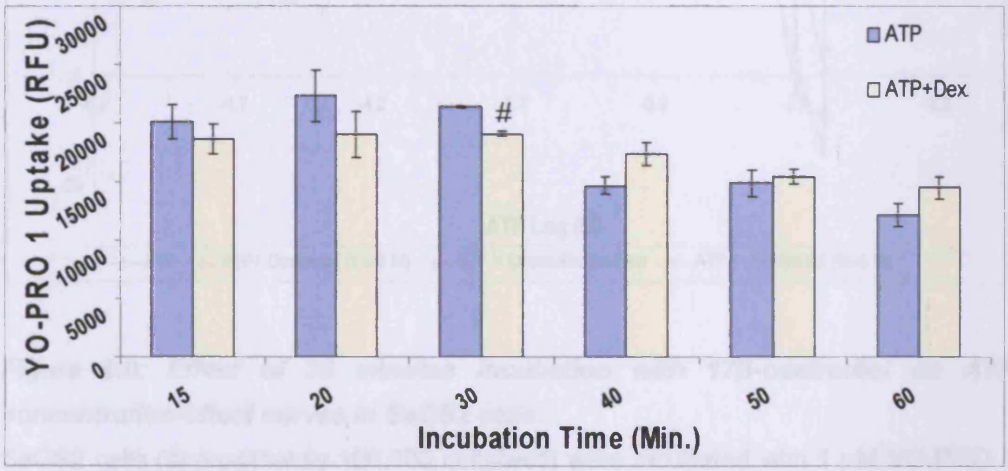


Figure 6.5: Effect of incubation time with 17β-oestradiol and dexamethasone on ATP-induced YO-PRO 1 uptake in SaOS2 cells.

SaOS2 cells (approximately 100,000 cells / well) were incubated with 17β-oestradiol $5x10^{-6}$ M, dexamethasone $1x10^{-6}$ M, or 0.5 % ethanol (17β-oestradiol and dexamethasone vehicle) and 1 μM YO-PRO 1 in the assay buffer at 37°C for 15-60 minutes followed by ATP $8x10^{-4}$ M for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. The only significant inhibition of the ATP-induced YO-PRO 1 uptake was found to be with a 30 minutes incubation. Statistical analyses were performed using Student's t-test. *P<0.0005 vs. ATP, # P<0.005 vs. ATP. [n=3]. RFU: relative fluorescence unit.

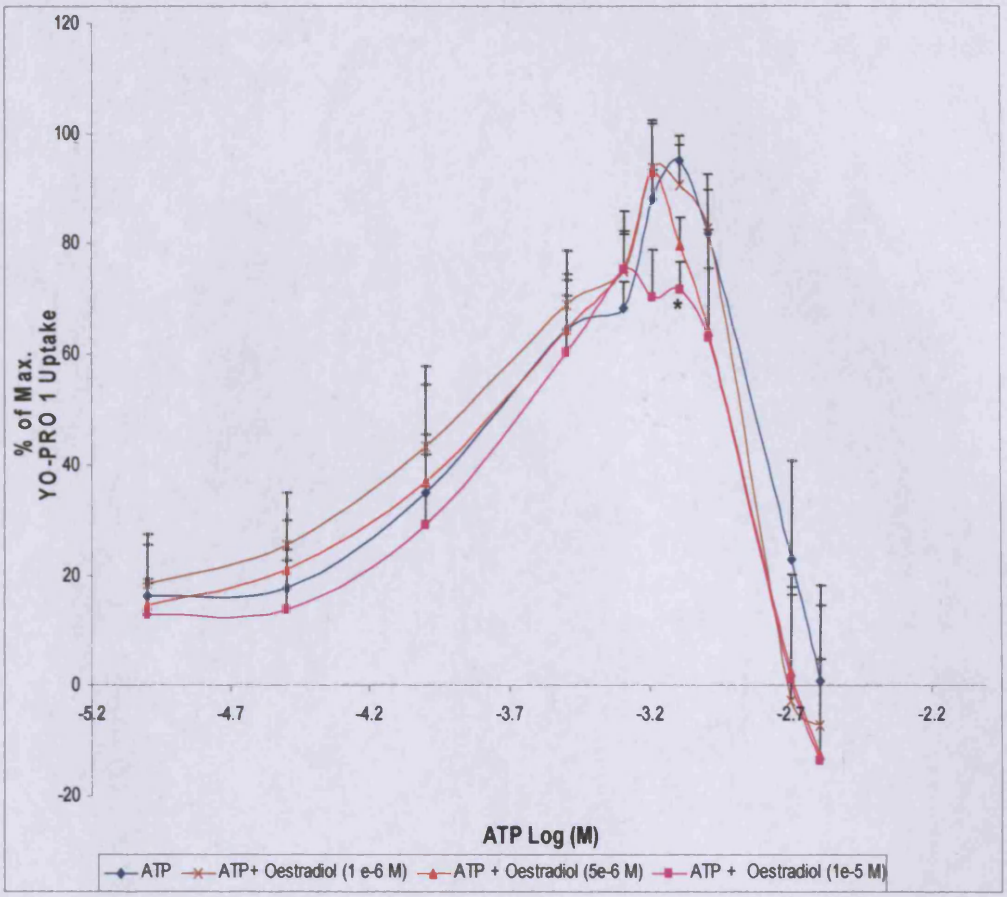


Figure 6.6: Effect of 30 minutes incubation with 17β-oestradiol on ATP concentration-effect curves in SaOS2 cells.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of 0.5 % ethanol (vehicle for 17β-oestradiol), $1x10^{-6}$M, $5x10^{-6}$M, or $1x10^{-5}$M of 17β-oestradiol at 37°C for 30 minutes followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the absence of 17β-oestradiol. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. [n=5]

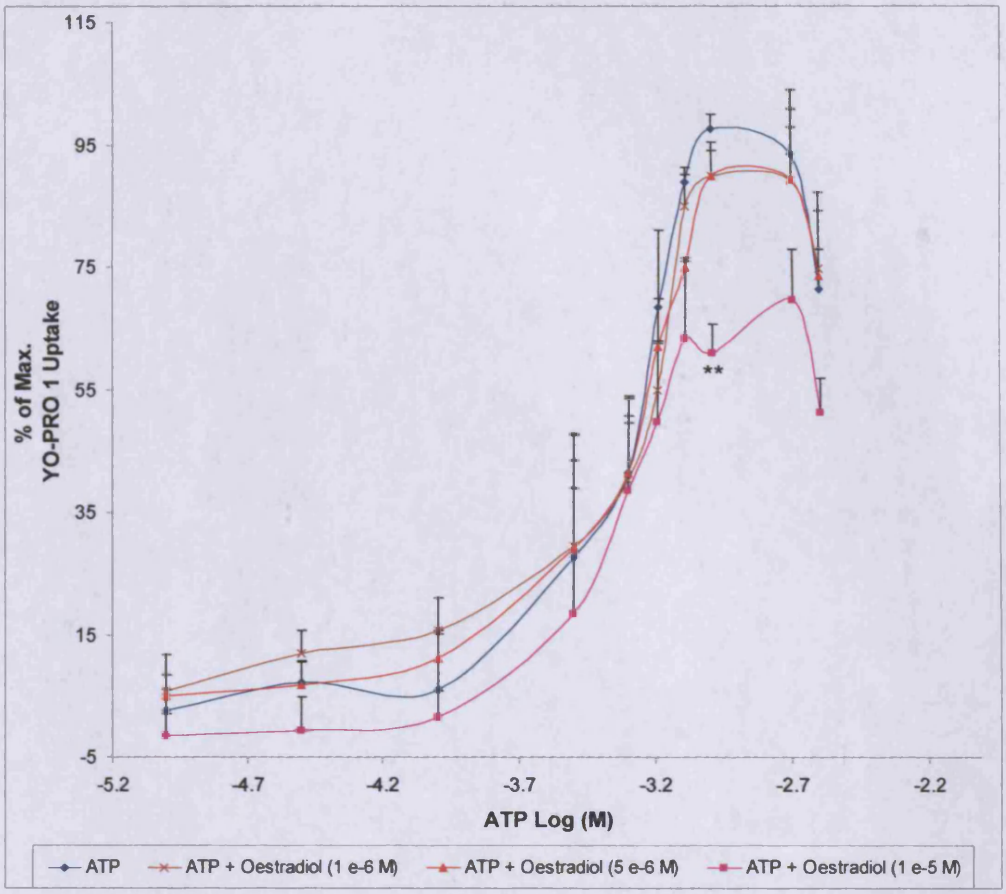


Figure 6.7: Effect of 2 days incubation with 17β-oestradiol on ATP concentration-effect curves in SaOS2 cells.

SaOS2 cells were incubated with 0.5 % ethanol (vehicle for 17β-oestradiol), $1x10^{-6}$M, $5x10^{-6}$M, or $1x10^{-5}$M of 17β-oestradiol at 37°C for 2 days. The YO-PRO 1 uptake assay was performed where the cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer and ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the absence of 17β-oestradiol. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. **P<0.01 vs. control. [n=3]

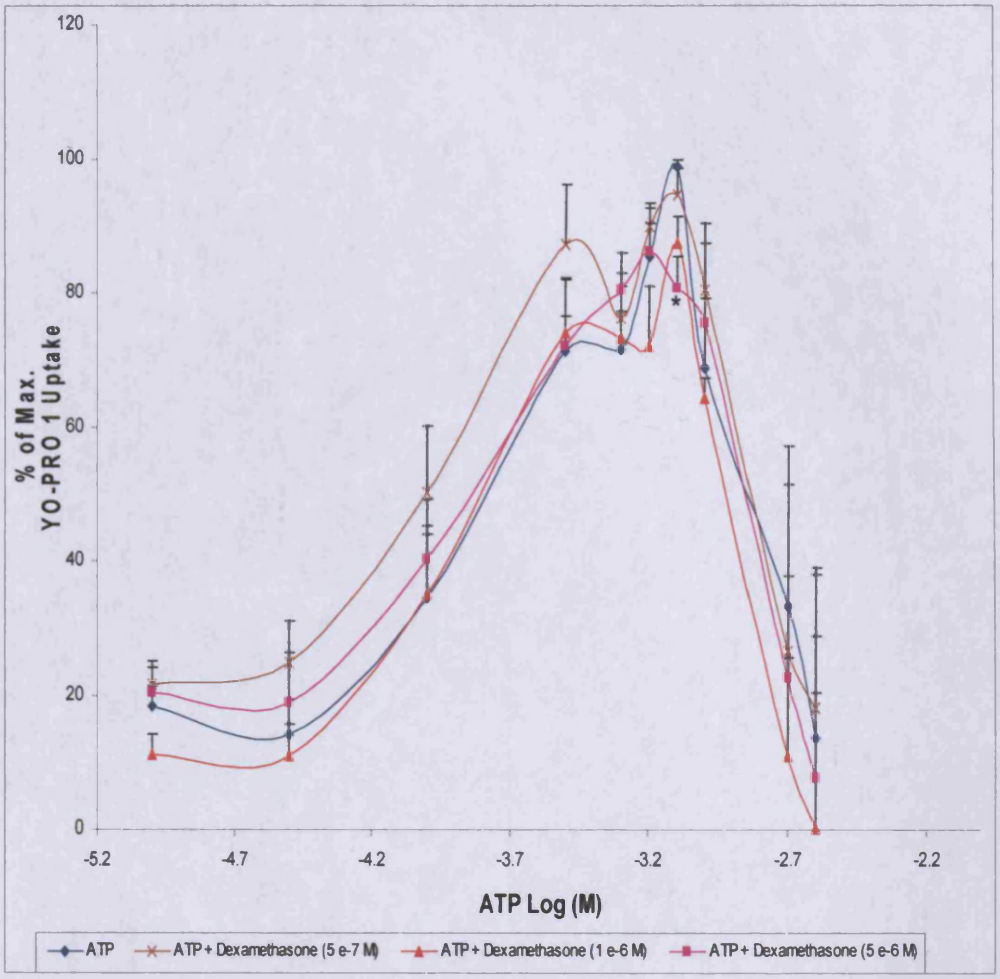


Figure 6.8: Effect of 30 minutes incubation with dexamethasone on ATP concentration-effect curves in SaOS2 cells.

SaOS2 cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer in the presence of 0.5 % ethanol (vehicle for dexamethasone), $5x10^{-7}$M, $1x10^{-6}$M, or $5x10^{-6}$M of dexamethasone at 37°C for 30 minutes followed by ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the absence of dexamethasone. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. [n=4]

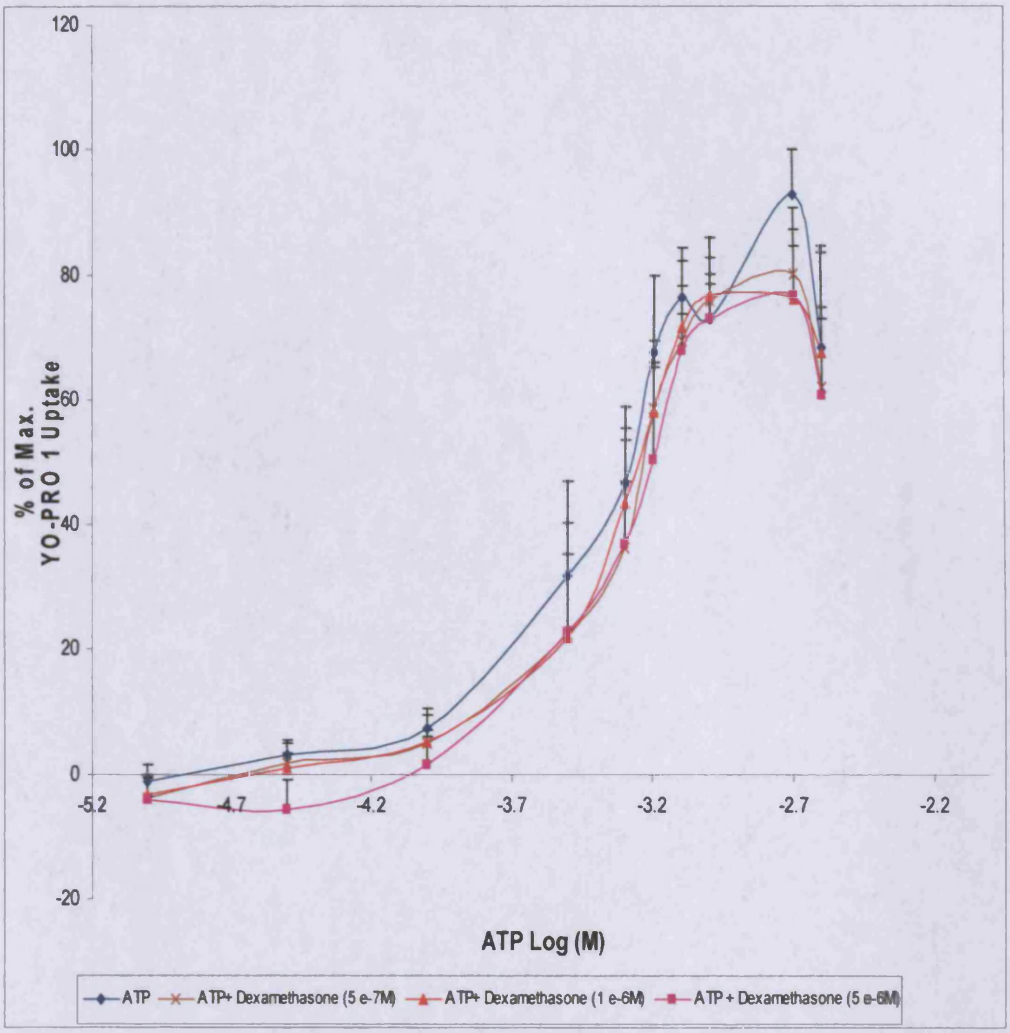


Figure 6.9: Effect of 2 days incubation with dexamethasone on ATP concentration-effect curves in SaOS2 cells.

SaOS2 cells were incubated with 0.5 % ethanol (vehicle for dexamethasone), $5x10^{-7}$M, $1x10^{-6}$M, or $5x10^{-6}$M of dexamethasone at 37°C for 2 days. The YO-PRO 1 uptake assay was performed where the cells (approximately 100,000 cells/well) were incubated with 1 μM YO-PRO 1 in the assay buffer and ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data are expressed as % of maximum uptake of ATP in the absence of dexamethasone. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test but no significant differences were seen. [n=4]

30 Minutes incubation	ATP	0.38 mM
	ATP+ 17β-oestradiol $1x10^{-5}$ M	0.13 mM
	ATP	0.43 mM
	ATP+ Dexamethasone $5x10^{-6}$ M	0.23 mM
2 Days incubation	ATP	0.51 mM
	ATP+ 17β-oestradiol $1x10^{-5}$ M	0.46 mM
	ATP	0.48 mM
	ATP+ Dexamethasone $5x10^{-6}$ M	0.5 M

Table 6.1: EC_{50} values for ATP in the presence or absence of 17β-oestradiol and dexamethasone.

SaOS2 cells (approximately 100,000 cells/well) were pre-incubated with 0.5 % ethanol (vehicle), 17β-oestradiol $1x10^{-5}$ M, or dexamethasone $5x10^{-6}$ M at 37°C for 30 minutes or 2 days followed by 1 μM YO-PRO 1 in the assay buffer in the presence of ATP for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. EC_{50} values were calculated using Prism. The data for the highest concentrations of 17β-oestradiol and dexamethasone are shown here, no differences were seen with the lower concentrations either. Statistical analyses were performed using Student's t-tests, but no significant differences were seen. n=3-5

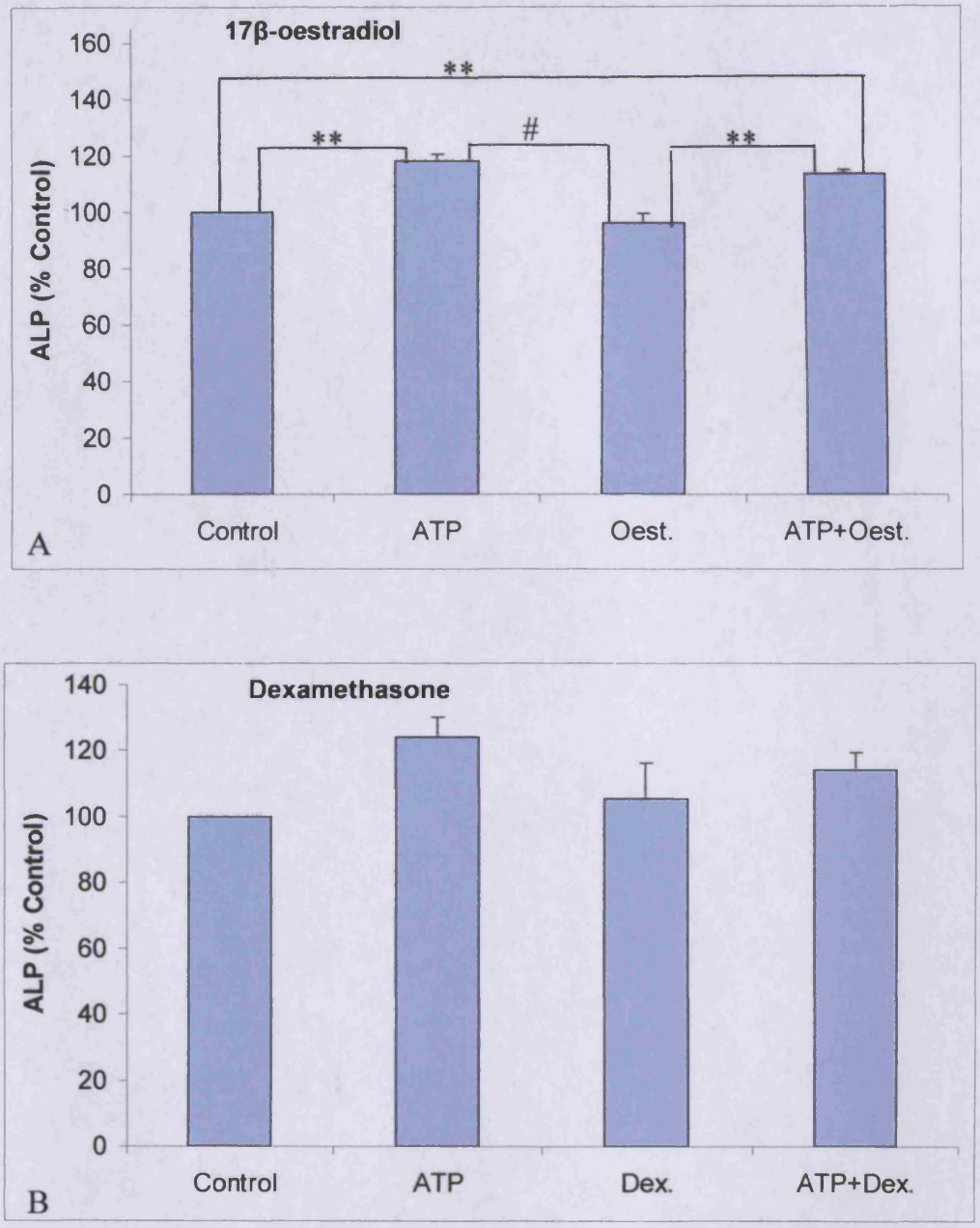
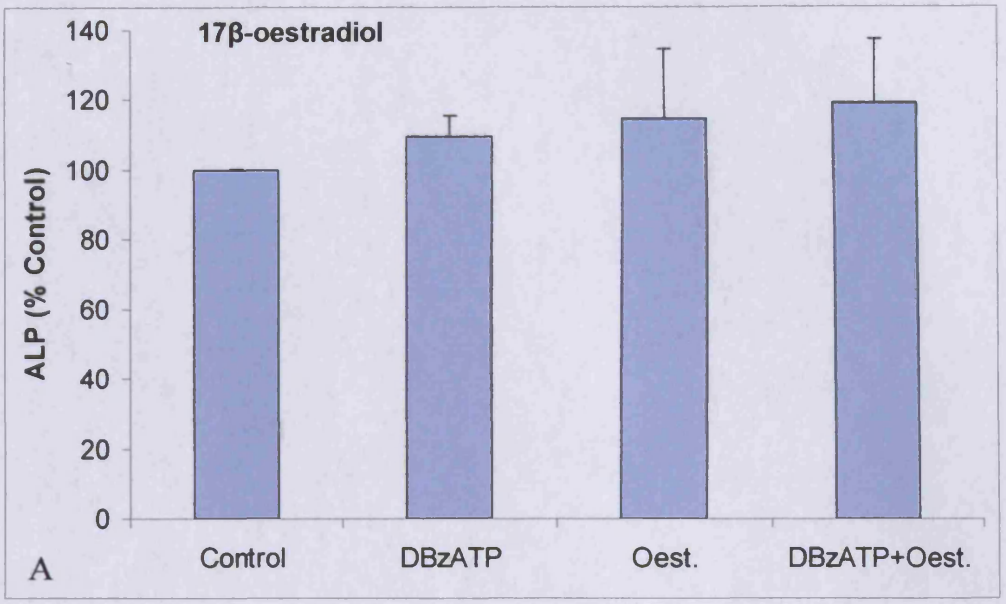


Figure 6.10: Effect of 17β-oestradiol (A) and dexamethasone (B) on ALP activity induced by ATP in SaOS2 cells.

Cells were incubated with 17β-oestradiol $1x10^{-5}$ M, dexamethasone $5x10^{-6}$ M or 0.5 % ethanol (17β-oestradiol and dexamethasone vehicle) for 30 minutes then grown for 2 days with ATP $4x10^{-5}$M or water (ATP vehicle) then ALP and cell number were determined. ALP activity was corrected for cell number. Data are expressed as percentage of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. **P<0.01, #P<0.001. (n=3)



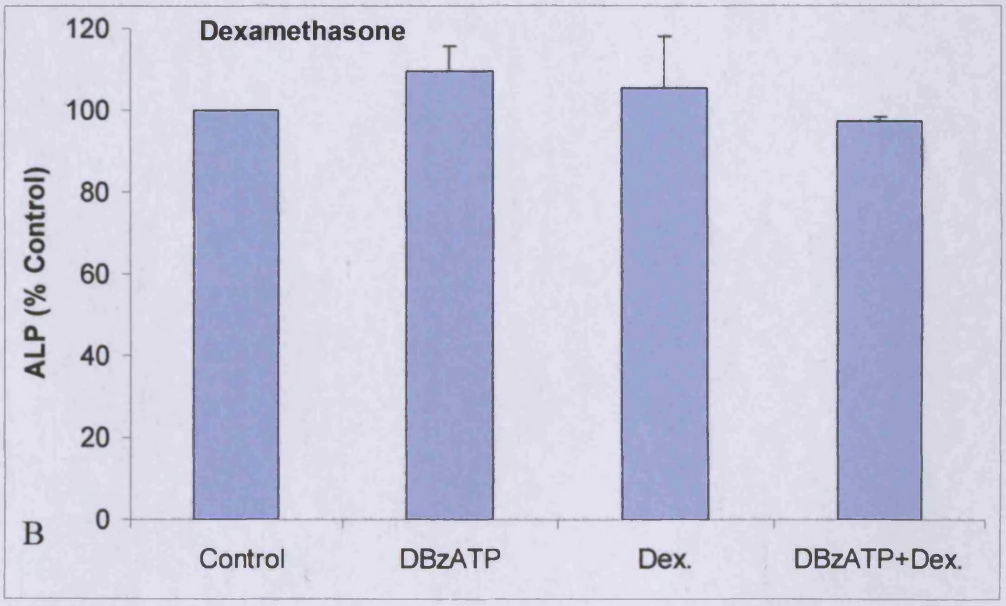


Figure 6.11: Effect of 17β-oestradiol (A) and dexamethasone (B) on ALP activity induced by DBzATP in SaOS2 cells.

Cells were incubated with 17β-oestradiol $1x10^{-5}$ M, dexamethasone $5x10^{-6}$ M or 0.5 % ethanol (17β-oestradiol and dexamethasone vehicle) for 30 minutes then grown for 2 days with DBzATP $1x10^{-6}$ M or water (DBzATP vehicle) then ALP and cell number were determined. ALP activity was corrected for cell number. Data are expressed as percentage of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test, but no significant differences were seen. (n=3)

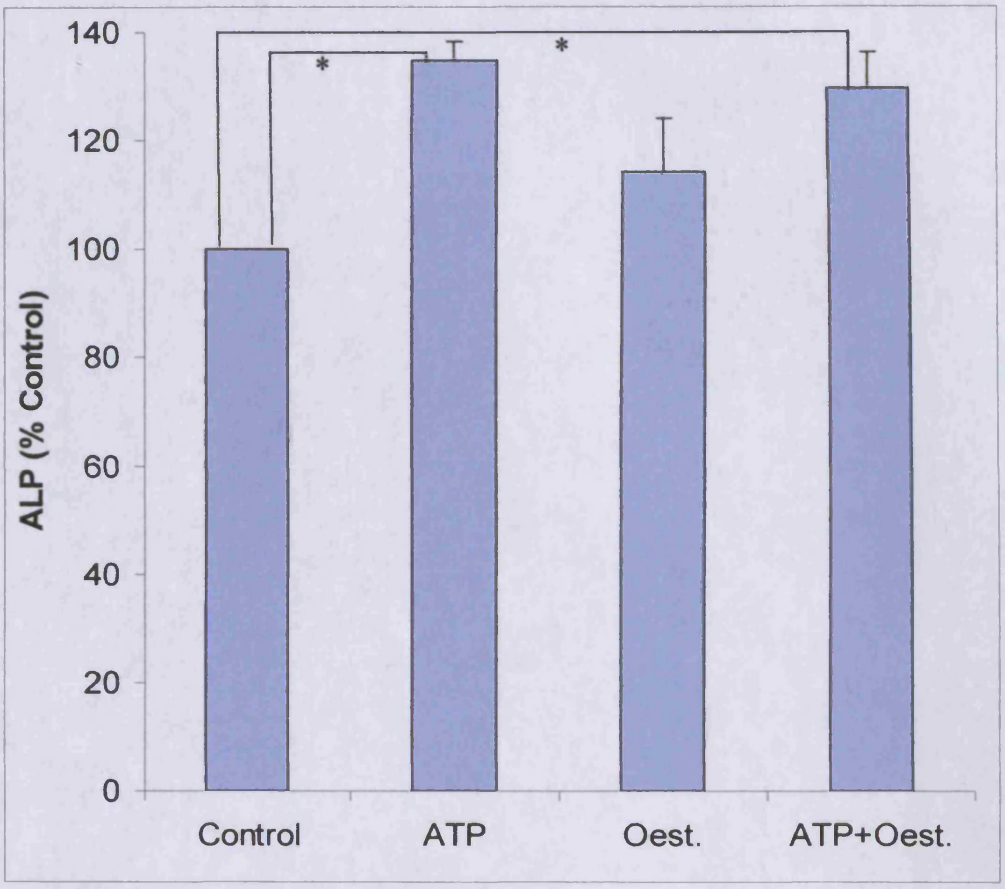


Figure 6.12: Effect of 17β-oestradiol on ALP activity induced by ATP in MG63 cells.

Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then grown for 2 days with ATP $4x10^{-5}$M or water (ATP vehicle) then ALP and cell number were determined. ALP activity was corrected for cell number. Data are expressed as percentage of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. control. (n=3)

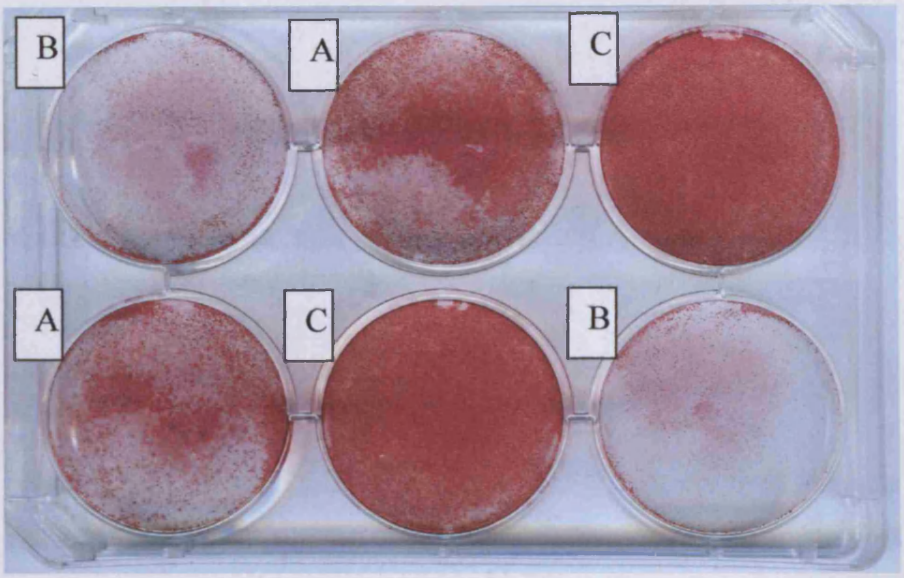


Figure 6.13: Representative example of the effect of ATP and 17β-oestradiol on nodule formation (mineralisation) in SaOS2 cells.

Cells were incubated with 17β-oestradiol 1x10^{-5} M, or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then grown for 15 days with ATP 4x10^{-5}M or water (ATP vehicle), which were replenished twice weekly. Cells were then stained with alizarin red. Another identical plate was used for a protein assay. A: Ethanol + ATP, B: ATP + 17β-Oestradiol, C: Control (Ethanol+Water). (n=3)

	Protein (mg/ml) ± SEM
Control (Ethanol+Water)	1.81 ± 0.49
ATP	1.63 ± 0.93
17β-oestradiol + *ATP*	1.00 ± 0.74

	Protein (mg/ml) ± SEM
Control (Ethanol+Water)	1.69 ± 0.58
ATP	1.38 ± 0.89
Dexamethasone + ATP	1.71 ± 0.82

Table 6.2: Effect of ATP, DBzATP and BBG on SaOS2 cell protein concentration.

Cells were incubated with 17β-oestradiol 1x10^{-5} M, dexamethasone 5x10^{-6} M or 0.5 % ethanol (17β-oestradiol or dexamethasone vehicle) for 30 minutes then grown for 15 days with ATP 4x10^{-5}M or water (ATP vehicle), which were replenished twice weekly. Cells were then removed for a protein assay. Another identical plate was stained with alizarin red for mineralised nodule formation. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test but no significant differences were seen. (n=3)

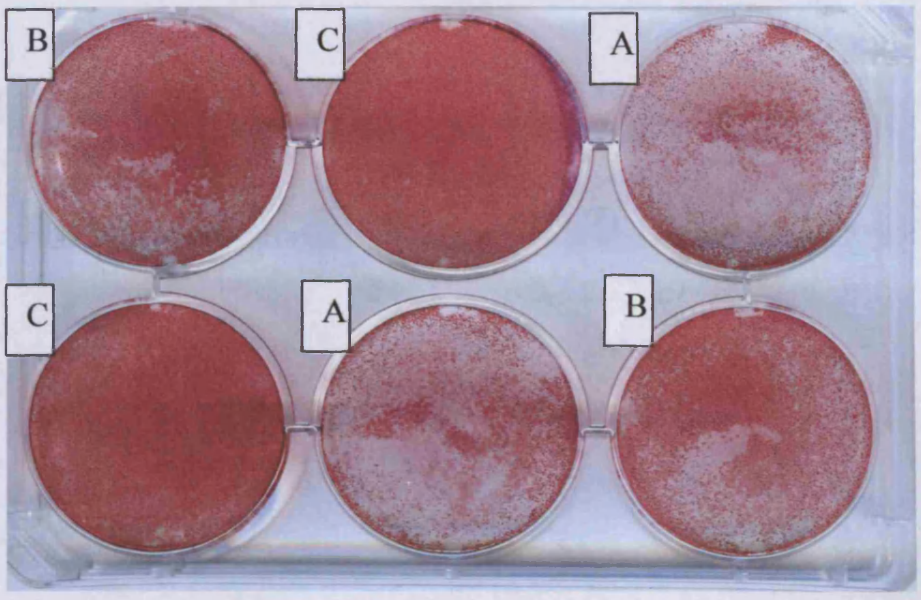


Figure 6.14: Representative example of the effect of ATP and dexamethasone on nodule formation (mineralisation) in SaOS2 cells.

Cells were incubated with dexamethasone $5x10^{-6}$ M or 0.5 % ethanol (dexamethasone vehicle) for 30 minutes then grown for 15 days with ATP $4x10^{-5}$M or water (ATP vehicle), which were replenished twice weekly. Cells were then stained with alizarin red. Another identical plate was used for a protein assay. A: Ethanol + ATP, B: ATP + dexamethasone, C: Control (Ethanol+Water). (n=3)

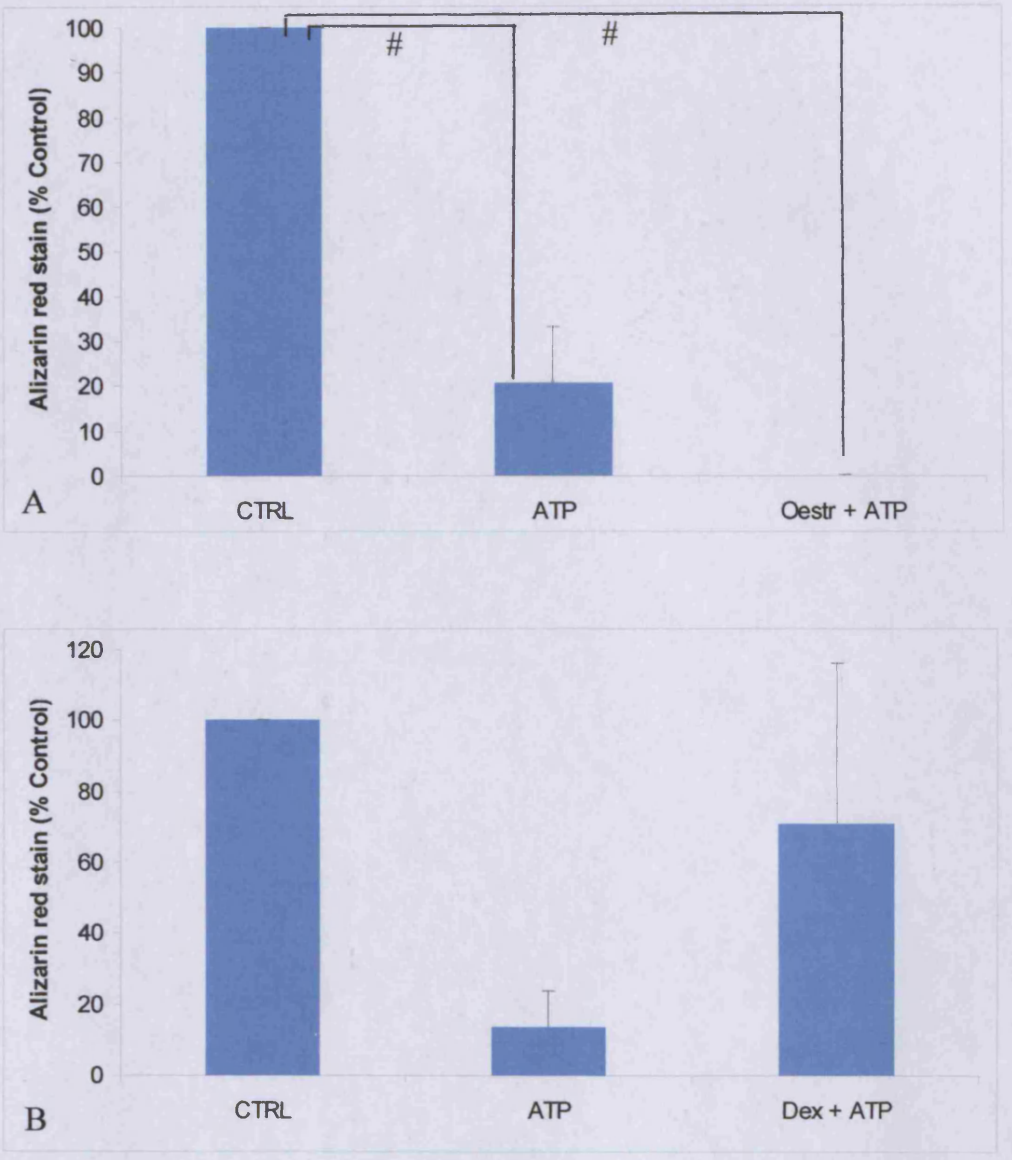
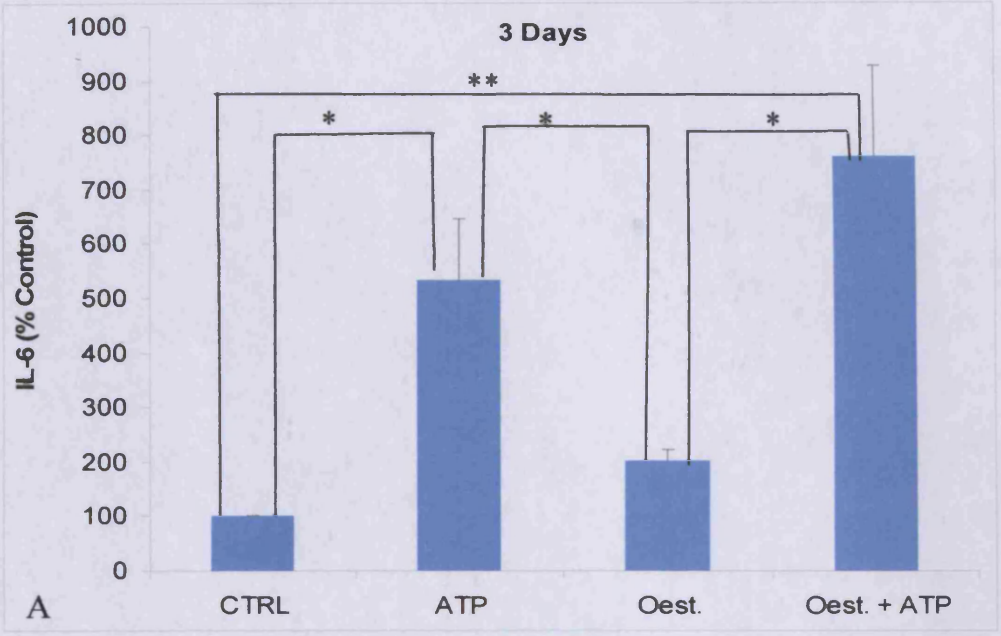


Figure 6.15: Effect of ATP, 17β-oestradiol and dexamethasone on nodule formation (mineralisation) in SaOS2 cells.

Cells were incubated with 17β-oestradiol $1x10^{-5}$ M (A), dexamethasone $5x10^{-6}$ M (B) or 0.5 % ethanol (17β-oestradiol and dexamethasone vehicle) for 30 minutes then grown for 15 days with ATP $4x10^{-5}$M or water (ATP vehicle), which were replenished twice weekly. Cells were then stained with alizarin red. Another identical plate was used for a protein assay for cell number. Data are expressed as mean percentage ± SEM of control containing vehicle. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. #P<0.001 vs. control. (n=3)



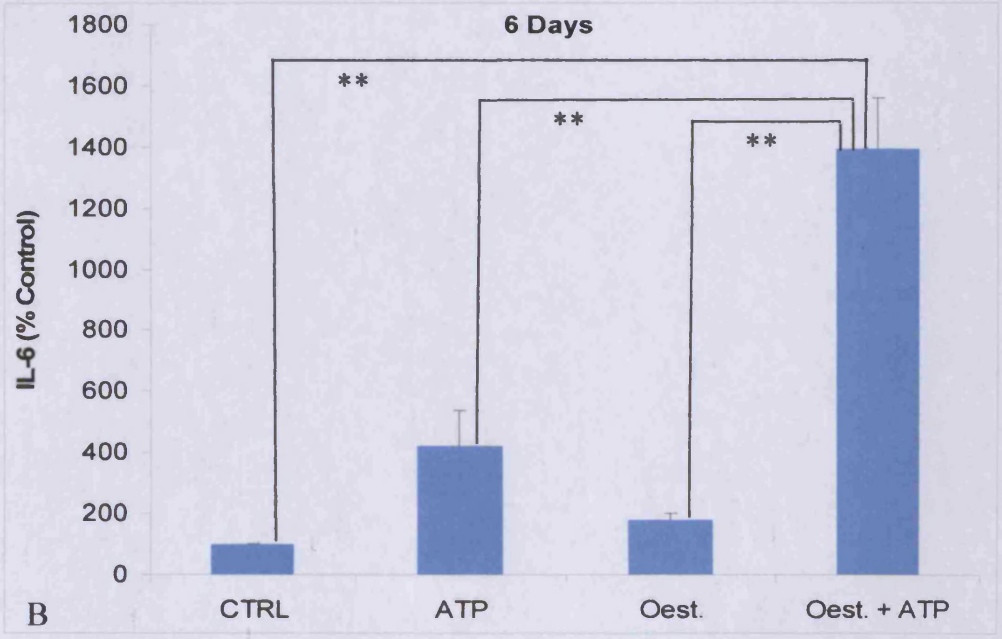


Figure 6.16: Effect of ATP and 17β-oestradiol on IL-6 release in SaOS2 cells.
Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then grown for 3 (A) and 6 (B) days with ATP $4x10^{-5}$M or water (ATP vehicle) then media and cells were collected for IL-6 and protein assays. IL-6 data were corrected for protein. Data are expressed as percentage of control. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05, **P<0.01. (n=3)

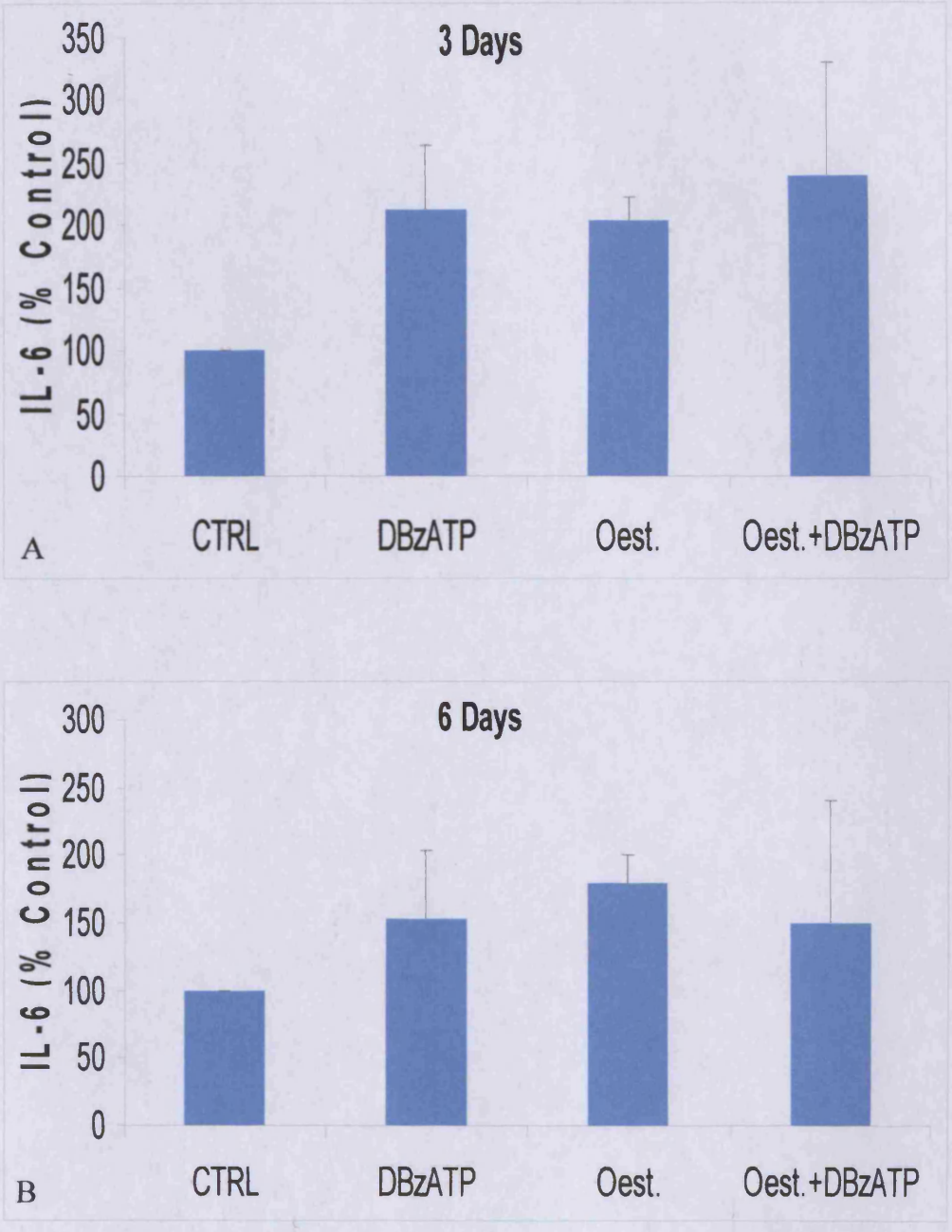


Figure 6.17: Effect of DBzATP and 17β-oestradiol on IL-6 release in SaOS2 cells. Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then grown for 3 (A) and 6 (B) days with DBzATP $1x10^{-6}$M or water (DBzATP vehicle) then media and cells were collected for IL-6 and protein assays. IL-6 data were corrected for protein. Data are expressed as percentage of control. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test, but no significant differences were seen. (n=3)

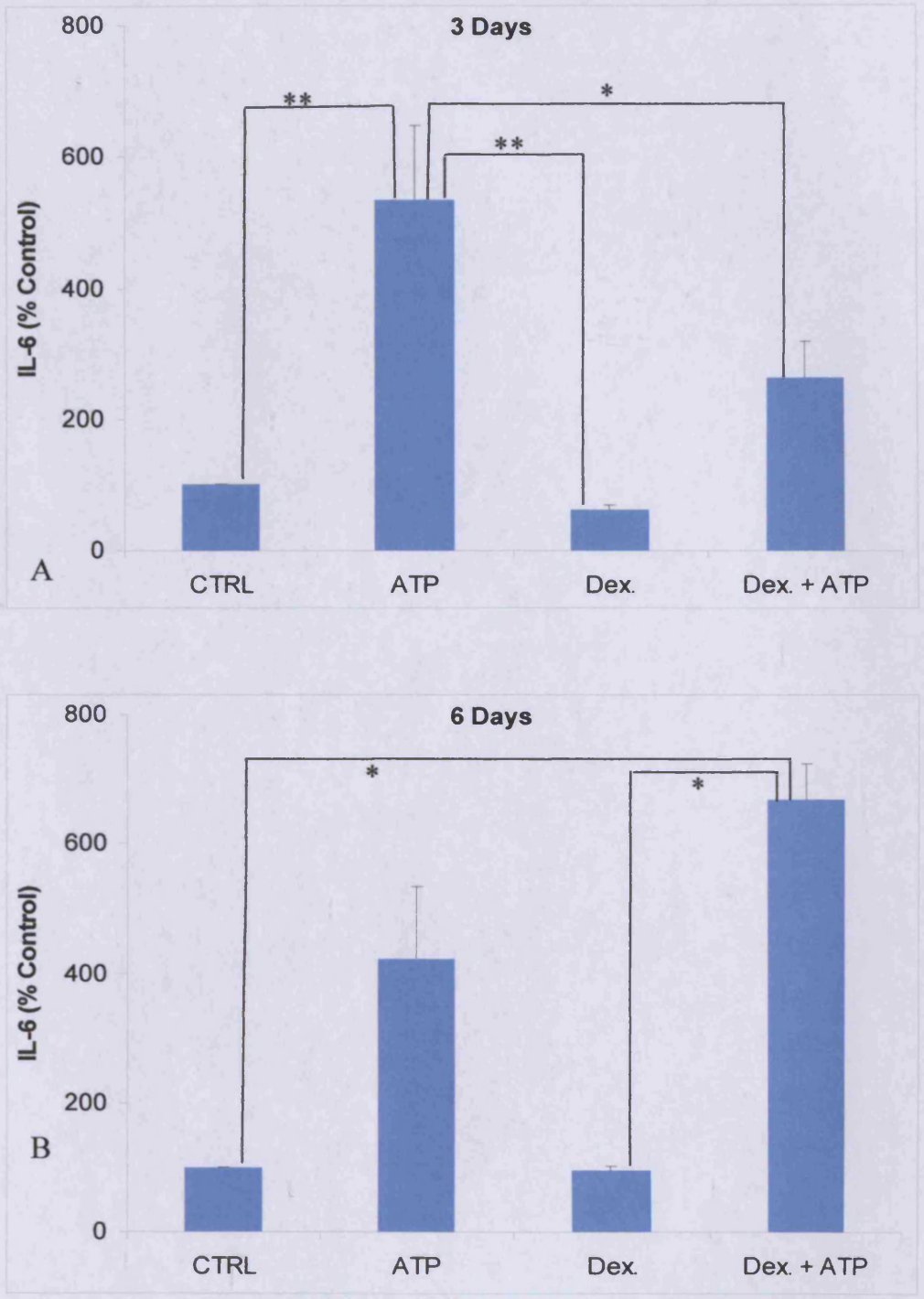


Figure 6.18: Effect of ATP and dexamethasone on IL-6 release in SaOS2 cells. Cells were incubated with dexamethasone $5x10^{-6}$ M or 0.5 % ethanol (dexamethasone vehicle) for 30 minutes then grown for 3 (A) and 6 (B) days with ATP $4x10^{-5}$M or water (ATP vehicle) then media and cells were collected for IL-6 and protein assays. IL-6 data were corrected for protein. Data are expressed as percentage of control. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05, **P<0.01. (n=3)

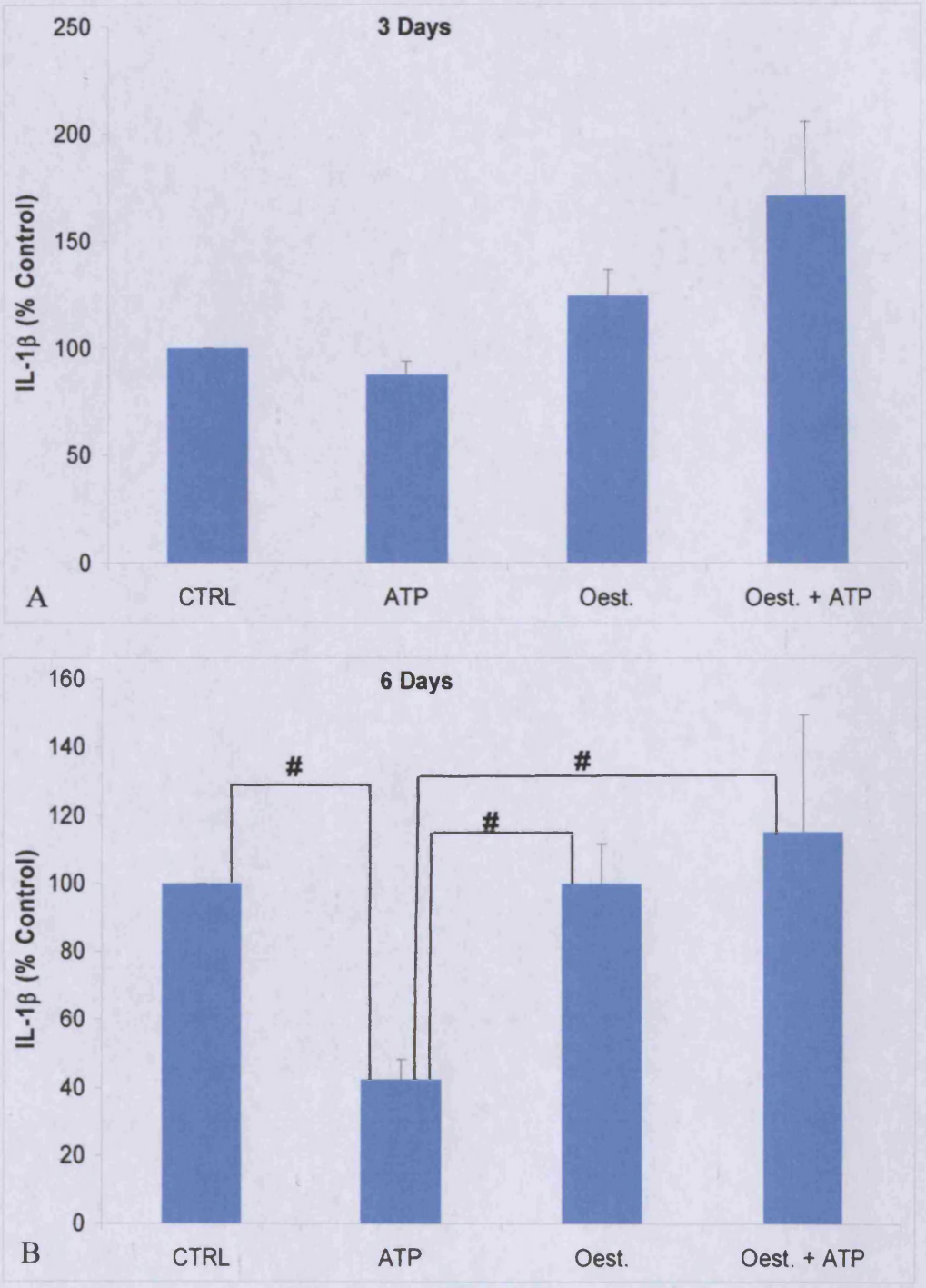


Figure 6.19: Effect of ATP and 17β-oestradiol on IL-1β release in SaOS2 cells.
Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then grown for 3 (A) and 6 (B) days with ATP $4x10^{-5}$M or water (ATP vehicle) then media and cells were collected for IL-1β and protein assays. IL-1β data were corrected for protein. Data are expressed as percentage of control. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. #P<0.001. (n=3)

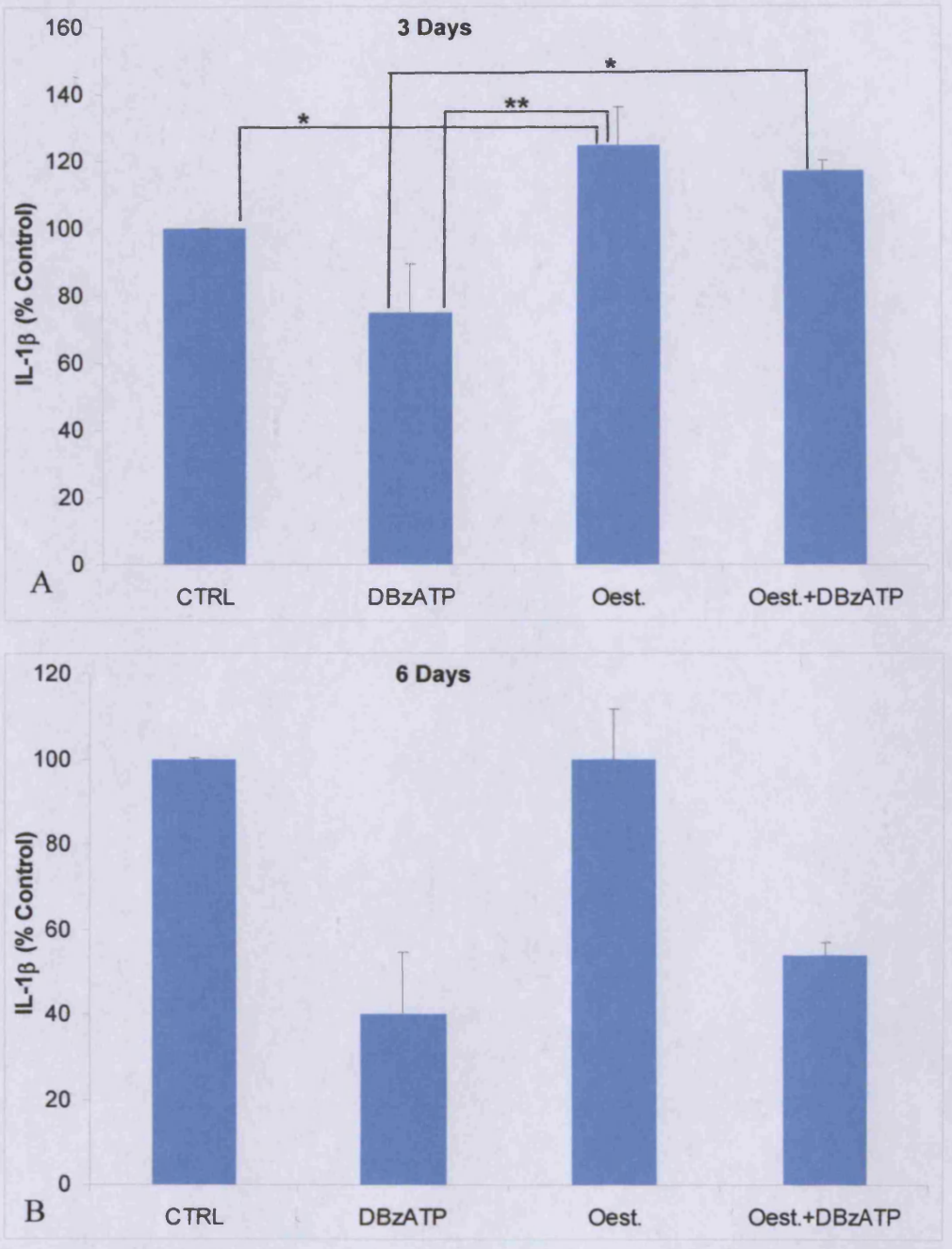
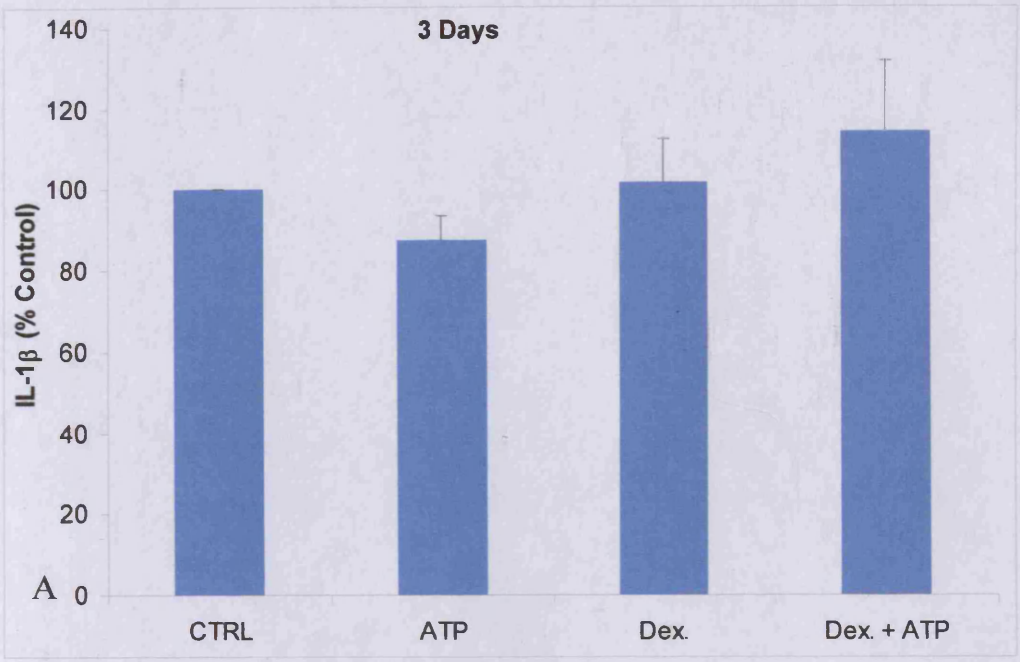


Figure 6.20: Effect of DBzATP and 17β-oestradiol on IL-1β release in SaOS2 cells.

Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then grown for 3 (A) and 6 (B) days with ATP $1x10^{-6}$M or water (DBzATP vehicle) then media and cells were collected for IL-1β and protein assays. IL-1β data were corrected for protein. Data are expressed as percentage of control. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05, **P<0.01. (n=3)



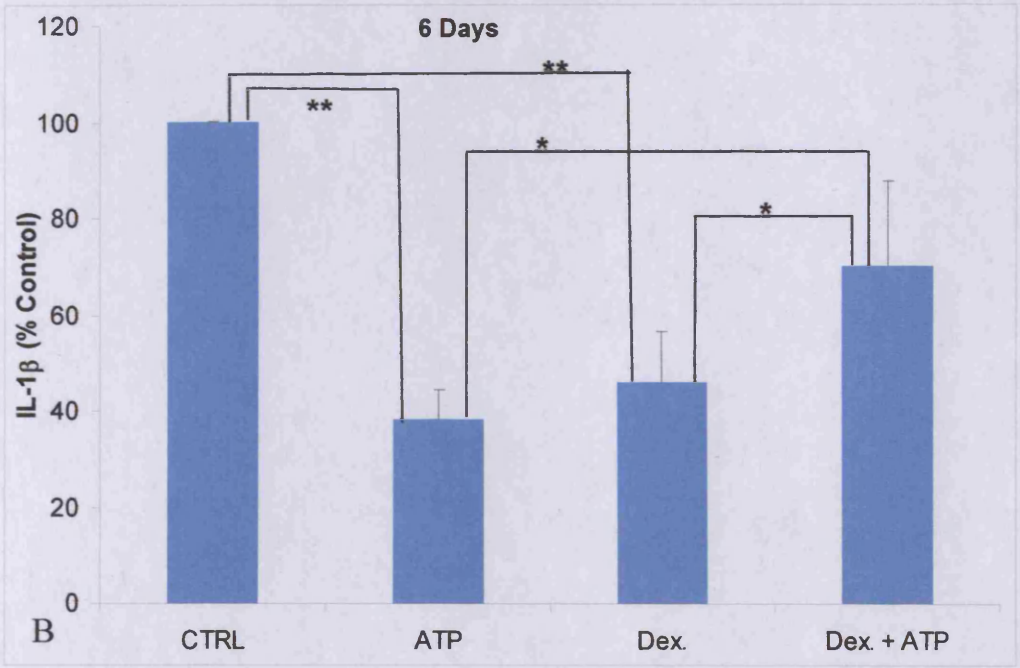


Figure 6.21: Effect of ATP and dexamethasone on IL-1β release in SaOS2 cells. Cells were incubated with dexamethasone $5x10^{-6}$ M or 0.5 % ethanol (dexamethasone vehicle) for 30 minutes then grown for 3 (A) and 6 (B) days with ATP $4x10^{-5}$M or water (ATP vehicle) then media and cells were collected for IL-1β and protein assays. IL-1β data were corrected for protein. Data are expressed as percentage of control. Statistical analyses were performed using one-way ANOVA with Newman-Keuls Post-Hoc test. *P<0.05, **P<0.01. (n=3)

6.5 Discussion

This chapter investigated the modulation of expression and function of $P2X_7$ receptors by 17β-oestradiol and dexamethasone in human osteoblasts. Additionally, the action of 17β-oestradiol and dexamethasone on some of the effects of ATP on osteoblast function were also studied.

6.5.1 Modulation of $P2X_7$ receptor expression and function (YO-PRO 1 uptake) by 17β-oestradiol and dexamethasone

The results show that $P2X_7$ receptor expression at the protein level is not modulated by 17β-oestradiol or dexamethasone. Although the 87 kDa band seen in Western blotting appeared to be downregulated by 17β-oestradiol after a 2 day incubation, the densitometric analysis did not show a significant difference. Therefore, any effect of 17β-oestradiol and dexamethasone is not due to alterations in $P2X_7$ receptor expression.

In relation to YO-PRO 1 uptake, efforts to generate concentration-effect curves for 17β-oestradiol and dexamethasone in the presence of ATP failed, which may be due to the atypical pharmacology of the receptor in these cells (discussed in detail in Chapter 4). Therefore, concentration-effect curves for ATP in the presence of different concentrations of 17β-oestradiol and dexamethasone were studied. The concentration-effect curves showed that the main action of these agents was to reduce the maximum YO-PRO 1 uptake induced by ATP, while there was no shift in the EC_{50} values, suggesting they could be behaving as weak non-competitive antagonists.

The results obtained in this project confirm the finding of Cario-Toumaniantz et al. (1998) showing an effect of 17β-oestradiol on blocking the $P2X_7$ receptor after a 30 minute incubation. This was claimed to be due to non-genomic ER inhibition of the $P2X_7$ receptor.

Although glucocorticoids have been reported to have rapid non-genomic effect on ATP-induced intracellular calcium influx in guinea-pig and mouse, results have been conflicting (Han et al., 2005; Yukawa et al., 2005). One explanation for this effect might be that it is species-dependent, especially since the homology in amino acid sequence between P2X receptors was found to be quite variable between species (North, 2002).

Both the effects of dexamethasone and 17β-oestradiol after 30 minutes of incubation must be mediated by a non-genomic mechanism as they occurred too quickly to be the result of a change in protein synthesis (Cario-Toumaniantz et al., 1998).

The inhibition of ATP-induced YO-PRO 1 uptake after a 2 day incubation with 17β-oestradiol might be through a genomic mechanism, although no evidence exists to support this hypothesis. However, as there was no significant difference between results obtained for the 30 minute and 2 day incubation, another mechanism may be involved.

$P2X_7$ receptor-induced pore formation is usually followed by cell death (Di Virgilio et al., 1998; Sluyter and Wiley, 2002; Wang et al., 2004a), therefore inhibition of pore formation by 17β-oestradiol and dexamethasone might inhibit the cell death induced by $P2X_7$ receptor activation. This might be another mechanism for the effect of these agents on osteoblasts.

6.5.2 Effect of 17β-oestradiol and dexamethasone on ATP- and DBzATP-induced ALP activity and mineralisation

The only significant effect of 17β-oestradiol on ALP activity was observed when it was used in combination with ATP. 17β-oestradiol in combination with ATP significantly increased ALP activity compared to 17β-oestradiol alone, suggesting that this effect is probably due to ATP.

No studies have been found investigating the effect of 17β-oestradiol or dexamethasone on ATP- or DBzATP-induced ALP activity, but one study has reported an increase in activity with 17β-oestradiol alone in a human foetal osteoblastic cell line after 2 days of cells in growth (Robinson et al., 1997). Another study reported that no effect on ALP activity was seen when SaOS2 cells were treated with 17β-oestradiol alone for 3 days, but in combination with PTH the activity increased significantly (Nasu et al., 2000). 17β-oestradiol added intermittently but not continuously was found to increase ALP activity in SaOS2 cells (Rao et al., 2003). In post-menopausal women ALP activity was observed to increase in the first 4 weeks of treatment with 17β-oestradiol implants, but it dropped significantly after that (Pereda et al., 2002). Other studies reported an increase in ALP activity with 17β-oestradiol when the cells were grown for longer periods (O'Shaughnessy et al., 2000; Kanno et al., 2004). One explanation for the difference in ALP activity in response to 17β-oestradiol seen here could be that it depends on the type of osteoblasts used (cell line or primary). In addition, 2 days of cells in growth might not be enough to see the effect of 17β-oestradiol.

The absence of effect of 17β-oestradiol on ATP-induced ALP activity supports the suggestion that its effect is not on osteoblast activity, but on

their life span (Moriarty et al., 2006). This is supported by the observation that 17β-oestradiol blocks ATP-induced pore formation (as measured here by YO-PRO 1 uptake) which is reported to be followed by cell death (Virginio et al., 1999).

ATP alone and in combination with 17β-oestradiol showed a significant reduction in mineralisation by SaOS2 cells. Previous studies showed contradictory results with one study showing an increase in mineralisation in mouse osteoblasts (Kanno et al., 2004), while another study showed inhibition in a human foetal osteoblastic cell line (Waters et al., 2001). In another study mineralisation was found to be higher in SaOS2 cells treated intermittently with 17β-oestradiol rather than continuously (Rao et al., 2003). Hence the difference in mineralisation might be species-dependent and timing of treatment might also be important. The significant decrease in mineralisation in response to ATP alone or in combination with 17β-oestradiol is unlikely to be due to cell death resulting from $P2X_7$ receptor activation as no significant differences in protein concentration were seen between different treatments. The data presented in Chapter 5 showed that ATP had no significant effect on mineralisation in SaOS2 cells, which are different to the results in this Chapter. An explanation might be the vehicle used here (ethanol for 17β-oestradiol). Ethanol was reported to inhibit or potentiate ATP-induced currents in $P2X_4$ and $P2X_3$ receptors, respectively, in a concentration-related manner (Davies et al., 2006). Overall, these results indicate that SaOS2 cells are probably not a good model for mineralisation assays.

Dexamethasone had no effect on ALP activity, either alone, or in combination with ATP or DBzATP. Previous studies reported a significant increase in ALP activity with dexamethasone alone following

in vitro culture of human osteoblasts for 1 - 3 weeks (Yang et al., 2003; Jorgensen et al., 2004; Eijken et al., 2006). Again, a possible explanation for the discrepancy in ALP activity in this project compared to the literature might be due to insufficient period of treatment or type of osteoblast used (primary or cell line).

ATP alone or in combination with dexamethasone had no effect on mineralisation. A significant increase in mineralisation with dexamethasone alone was reported in a study in a human foetal osteoblastic cell line (Eijken et al., 2006) and in another study in HOB cells (Jorgensen et al., 2004). The difference in the results seen here to those reported in the literature might be due to an insufficient period of treatment here or the type of osteoblast used (primary or cell line). The lack of effect of ATP and dexamethasone on mineralisation suggests that the role of the $P2X_7$ receptor on mineralisation in these cells is minor and that dexamethasone does not have a direct effect on bone formation.

6.5.3 Effect of 17β-oestradiol and dexamethasone on ATP- and DBzATP-induced cytokine release

ATP alone significantly increased IL-6 release, a similar effect to that presented in Chapter 5. Although many studies have reported inhibition of IL-6 release in response to 17β-oestradiol (Waters et al., 2001; Manolagas et al., 2002; Straub, 2007), another study reported that there was no effect in MG63 and SaOS2 cells (Dovio et al., 2001). In the current study, I observed that 17β-oestradiol had no effect on release after 3 and 6 days of cells in growth, but it enhanced ATP-induced release compared to control after 3 days and compared to control and ATP alone after 6 days of cells in growth. There is no clear explanation for this enhancement, especially since 17β-oestradiol was observed to

block ATP-induced YO-PRO 1 uptake. One possible explanation might be that a genomic effect of 17β-oestradiol is involved as the cells are incubated for a relatively long period which would be enough for alteration of protein synthesis. Therefore, it is possible that pore formation is not necessary for ALP production and IL-6 release from osteoblasts as these effects were observed with lower concentrations of ATP than those which induce YO-PRO 1 uptake.

Neither DBzATP alone or in combination with 17β-oestradiol had any effect on IL-6 release. Similar to earlier results seen with DBzATP (see Chapter 5), the absence of a DBzATP effect compared to ATP might be due to its lower concentration, or its binding by serum constituents.

No significant effect in comparison to control was observed on IL-6 release when dexamethasone alone was used, but in combination with ATP the release was decreased significantly after 3 days. This decrease is expected due to the potent anti-inflammatory action of dexamethasone (Payne and Adcock, 2001; Valledor and Ricote, 2004; Barnes, 2006), and IL-6 is one of the cytokines reported to be suppressed by glucocorticoids (Dovio et al., 2001; Payne and Adcock, 2001; Chen et al., 2005; Barnes, 2006). After 6 days dexamethasone significantly enhanced ATP-induced IL-6 release compared to control. This enhancement was unexpected, since dexamethasone is one of the most potent anti-inflammatory agents known, and the reason is not clear.

ATP significantly reduced IL-1β release after 6 days. This was significantly reversed when ATP was used in combination with 17β-oestradiol. Similarly, in combination with DBzATP the release was significantly higher than DBzATP alone after 3 days, but decreased after 6 days, but this was not significant. A study found that IL-1β release

increased in post-menopausal women, but this had decreased significantly after two months of treatment with hormone replacement therapy (HRT) (Vural et al., 2006). The results obtained here and those reported in the literature suggest that ATP could inhibit IL-1β release post-menopause, and that pre-menopause ATP and oestrogen should balance each other.

The above results suggest that the IL-6 and IL-1β release induced by $P2X_7$ receptor activation could be modulated by 17β-oestradiol and dexamethasone. The effect of dexamethasone might be due to it being anti-inflammatory, in which case this should be seen for both periods of incubation, but it was not. Therefore another unknown mechanism is probably involved. The absence of an effect of DBzATP compared to ATP might be due to its lower concentration, or its binding by serum constituents. The difference between the results obtained here and those reported in the literature, might be due to the cell type used, ER isoform expressed (α or β), and their density.

YO-PRO 1 uptake (see Chapter 4) is related to the formation of a pore in response to $P2X_7$ receptor activation with high concentrations of agonists, but cytokine release is most probably due to channel opening as lower concentrations of agonists were used. It is likely to be due to a signalling pathway coupled to cation influx. Alternatively, an unknown second messenger might be involved.

The results presented in this chapter suggest that oestrogen and glucocorticoids may have a direct action on increasing osteoblast lifespan via inhibition of $P2X_7$ receptor-induced cell death. Additionally, they may have a modulatory action on some of the signalling pathways involving the $P2X_7$ receptor.

6.6 Conclusion

Both 17β-oestradiol and dexamethasone were found to have an inhibitory action on $P2X_7$ receptor function. A non-classical, non-genomic mechanism might be responsible for the action of dexamethasone and 17β-oestradiol, although a genomic mechanism might be responsible for some of the results.

Since the $P2X_7$ receptor is known to be a death receptor, its blockade might be another mechanism through which 17β-oestradiol inhibits osteoblast apoptosis, and hence prolongs their life span. Apparently, as a result of 17β-oestradiol deficiency in menopause, osteoblast apoptosis increases, which predisposes to osteoporosis (Manolagas, 2000; Fini et al., 2004).

ALP activity and IL-6 and IL-1β release in response to $P2X_7$ receptor activation were found to be modulated by 17β-oestradiol and dexamethasone. This modulation might play a role in the actions of oestrogen and glucocorticoids on bone cells and osteoporosis pathophysiology.

Chapter 7:

Characterisation of the expression and function of $P2X_7$ receptors in primary human osteoblasts

7.1 Introduction

When I started my project and in order to characterize $P2X_7$ receptors in osteoblasts, the options were: animal (mouse, rat, or guinea pig) osteoblasts, HOB cells, or human osteoblast-like cell lines. The animal option was very unfavourable due to the species differences in the amino acid sequence of the $P2X_7$ receptor and receptor function (Hibell et al., 2000; North and Suprenant, 2000b; Hibell et al., 2001a; North, 2002; Michel et al., 2006a; Burnstock, 2007). Therefore, I decided to concentrate on human osteoblasts. HOB cells were not preferred to start with for many reasons. These included limited availability of human primary cells due to the difficulties in obtaining them for ethical reasons, the cost if obtained from commercial sources, and the problems associated with only being able to use a limited number of passages. Additionally, it was reported in the literature that $P2X_7$ receptor function in primary human osteoblasts might be different between one population and another (different individuals or sites) (Gartland et al., 2001). Based on the above I decided to start my project with two human osteoblast-like cell lines, MG63 and SaOS2. An SaOS2 cell is closer to a well-differentiated osteoblast and has many of its features (Hughes and Aubin, 1998). However, subsequently I decided to study primary human osteoblasts as a population of these cells became available near the end of my PhD. I thought it possible that primary cells could behave differently to cell lines and they have the advantage of being more comparable physiologically to the in vivo situation (Hughes and Aubin, 1998).

Due to the limited availability of the primary cells, a detailed characterisation of $P2X_7$ receptor expression and function in HOB cells

could not be done. Therefore, key experiments were identified based on previous results with the MG63 and SaOS2 cells.

7.2 Aims

To characterise $P2X_7$ receptor expression and function in HOB.

- To study $P2X_7$ receptor expression by HOB cells at the mRNA and protein levels.
- To compare the receptor expression at the protein level with that of SaOS2 and MG63 cells.
- To investigate the expression of other pore-forming P2X receptors ($P2X_2$, $P2X_4$, and $P2X_5$) by HOB cells at the mRNA and protein levels.
- To study the effect of $P2X_7$ receptor agonists on YO-PRO 1 uptake by HOB cells.
- To study the effect of $P2X_7$ receptor agonists on IL-6 release in HOB cells.
- To study the effect of $P2X_7$ receptor agonists on mineralisation (nodule formation) in HOB cells.

7.3 Experimental protocols

The protocols used to study the expression and function of $P2X_7$ receptors in HOB cells were described in detail in Chapter 2.

7.3.1 Cell culture

Human primary osteoblasts referred to here as HOB were obtained from the knee of a 64 year-old Caucasian female. HOB cells (characterised by osteocalcin production) and all the solutions used for their growth were obtained from PromoCell (Heidelberg, Germany). The cells were grown in 25 cm^3 flasks in osteoblast growth medium (containing 50 µg/ml gentamicin sulphate, 50 ng/ml amphotericin B and 0.62 ng/ml phenol red) supplemented with 10 % FCS. To passage the cells, they were washed with HepesBSS solution (Hepes, D-glucose, NaCl, KCl, sodium phosphate, phenol red), incubated with 1 ml detach kit (Trypsin/EDTA solution 0.04% / 0.03%) for 5 minutes at room temperature, then 2 ml trypsin neutralizing solution obtained from soybean (0.05%) was added, and the cells were counted and seeded at approximately 300,000 cells/25 cm^2 flask. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and the medium changed every three or four days.

7.3.2 RT-PCR

To study the expression of $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_7$ receptors at the mRNA level, RT-PCR was done using the primers described in Chapter 3. MG63 cell cDNA was used as a positive control for $P2X_4$, $P2X_5$, and $P2X_7$ receptors, while human pancreas cell (BON-1) cDNA was used as a positive control for $P2X_2$ receptors.

7.3.3 Western Blotting

To study the expression of $P2X_2$, $P2X_4$, and $P2X_7$ receptors at the protein level, Western blotting was carried out using the method and antibodies described in Chapter 2. Membranes of HEK7 cells were used as a positive control for $P2X_7$ receptors, while rat brain membranes were used as a positive control for $P2X_2$ and $P2X_4$ receptors.

In order to quantify the relative expression of $P2X_4$, and $P2X_7$ receptors in HOB cells and to compare it to those of MG63 and SAOS2 cells, the OD of the bands for these receptors and a housekeeping gene (β-actin) were analysed using densitometry in NIH Image software (see Chapters 4 and 6).

It is important to note here that the batch of the $P2X_7$ receptor antibody used for work in this chapter was different to that used in Chapter 3.

7.3.4 YO-PRO 1 uptake

The protocol described in Chapter 2 was followed exactly but using approximately 40,000 HOB cells/well.

7.3.5 IL-6 release

HOB cells were seeded in 96 well plates at 3,000 cells per well and incubated overnight. Next day, cells were incubated at 37°C with BBG for one hour or 17β-oestradiol or dexamethasone for 30 minutes. Next, water, ATP or DBzATP were added to the cells. On day 4 media were collected for the assay. Then, the IL-6 ELISA assay was performed as described in Chapter 2. The vehicle for 17β-oestradiol and dexamethasone, ethanol, was used at 0.5 % v/v.

7.3.6 Mineralisation

HOB cells (passages 10 and 12) were seeded in 24 well plates at 40,000 cells per well and incubated overnight. The next day, alpha-MEM was changed to media containing ascorbic acid (50 μg/ml), β-glycerophosphate (2 mM), dexamethasone (0.1 μM in 0.5% ethanol) and either water, ATP or DBzATP and the cells were incubated at 37°C. The solutions were replenished twice weekly for 3-4 weeks, then the cells were stained with freshly prepared 1% alizarin red S as described in Chapter 2.

7.4 Results:

7.4.1 Expression

RT-PCR for $P2X_4$, $P2X_5$, and $P2X_7$ receptors (Figure 7.1) in MG63 and HOB cell mRNA samples gave bands of the expected size (200 bp for the $P2X_4$ and $P2X_5$ receptors and 100 bp for the $P2X_7$ receptor). Control samples (no AMV in the RT reaction) did not show any bands. No bands were seen using the $P2X_2$ receptor primers in either MG63 or HOB cells, while in human pancreatic cell line mRNA (BON-1 cells), used as a positive control, the primers did amplify the expected product (200bp, Figure 7.1A).

In Western blotting membranes of HEK7 cells were used as a positive control for the $P2X_7$ receptor, while rat brain membranes were used as a positive control for the $P2X_4$ receptor. Using the $rP2X_7$ receptor antibody, bands were seen in the positive control, HOB, MG63 and SaOS2 cell lysate samples at 53 ± 1.5 kDa (Figure 7.2). Figure 7.3 shows bands in the positive control, HOB, MG63 and SaOS2 cell lysate samples at 55 ± 1.4 kDa for the $P2X_4$ receptor. Using the β-actin

antibody, bands were seen in all samples at 39 ± 1.7 kDa. Using the $P2X_2$ receptor antibody, a band was only seen in the positive control, Rat brain cell membranes (data not shown).

In order to quantify $P2X_4$ and $P2X_7$ receptor expression, the bands were compared to those for β-actin using densitometric analysis as shown in Figures 7.2-B and 7.3-B. Expression of both receptors was found to be significantly higher in HOB cells compared to MG63 cells, but compared to SaOS2 cells the expression was significantly higher only in the case of the $P2X_7$ receptor.

7.4.2 YO-PRO 1 Uptake

Due to the limited availability of the cells, concentration-effect curves for ATP and DBzATP were constructed using fewer concentrations than for the SaOS2 and MG63 cells (see Chapter 4).

ATP and DBzATP were found to induce YO-PRO 1 uptake in HOB cells and their concentration-effect curves (Figure 7.4) gave EC_{50} values of 0.53 mM ± 0.06 and 0.54 mM ± 0.04, respectively. Table 7.1 compares the EC_{50} values of ATP and DBzATP in HOB cells to those in MG63 and SaOS2 cells. No significant differences were seen.

The results obtained in this project were compared with those reported by Gartland et al (2001) on HOB cells using the same concentrations of ATP and DBzATP. Figure 7.5A shows the YO-PRO 1 uptake obtained with $1x10^{-3}$ M and $5x10^{-4}$ M ATP and DBzATP by Gartland et al (2001). ATP and DBzATP at the same concentrations produced similar YO-PRO 1 uptake in my HOB cells (Figure 7.5B).

To compare YO-PRO 1 uptake in HOB cells with that in MG63 and SaOS2 cells, Figure 7.6 shows the YO-PRO 1 uptake obtained with $1x10^{-3}$ M and $5x10^{-4}$ M ATP and DBzATP in these cells corrected for cell number. The YO-PRO 1 uptake was found to be significantly higher (p value <0.001) in HOB cells compared to both MG63 and SaOS2 cells.

7.4.3 IL-6 Release

Basal IL-6 release in HOB cells was 10.277 ± 4.309 ng/mg protein after 3 days of cells culture. The basal IL-6 release from HOB cells was found to be significantly higher than that in SaOS2 cells (3.8 ± 0.8 pg/mg protein, p value<0.05). ATP and DBzATP had no significant effect on IL-6 release, either alone or in combination with BBG or 17β-oestradiol (Figures 7.7-7.10). BBG and 17β-oestradiol alone had no effect on IL-6 release.

In another series of experiments basal IL-6 release in HOB cells was 11.292 ± 6.256 ng/mg protein. ATP significantly increased IL-6 release by approximately 40 % compared to control while dexamethasone significantly decreased release by 95 % compared to control. The effect of ATP was completely reversed by dexamethasone (Figure 7.11).

7.4.4 Mineralisation

Due to limited availability of the HOB cells, a qualitative experiment was performed to investigate the effect of $P2X_7$ receptor activation on mineralised nodule formation. Both ATP and DBzATP were found to reduce mineralised nodule formation compared to control cells (Figure 7.12). Analysis of the results could not be done as the experiment was

only carried out twice. No data were obtained for protein content due to the limited availability of the cells.

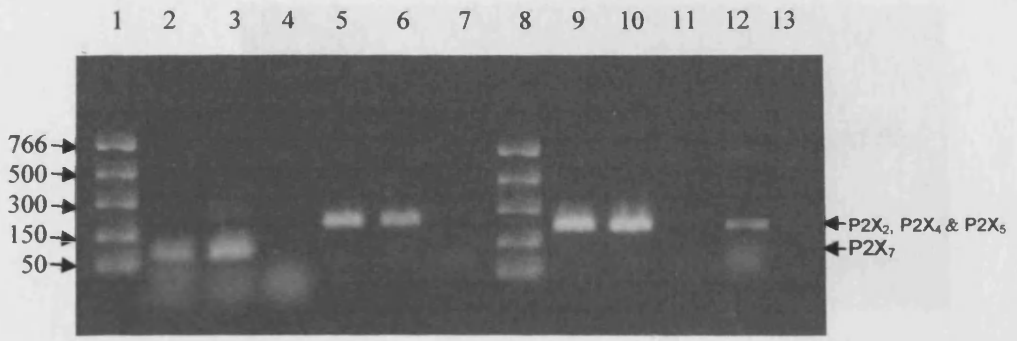
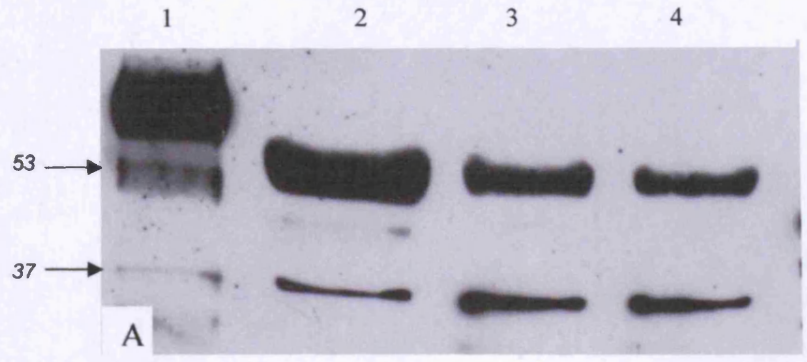


Figure 7.1: Representative example on RT-PCR of $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_7$ receptor mRNA in HOB cells.

RT-PCR was performed on RNA extracted from HOB cells using primers for the human $P2X_7$ (lanes 3 and 4), $P2X_5$ (lanes 6 and 7), $P2X_4$ (lanes 10 and 11) and $P2X_2$ (lane 13) receptors. The marker ladder is shown in lanes 1 and 8, and $P2X_7$, $P2X_5$, and $P2X_4$ receptor positive controls (MG63 cell cDNA) are shown in lanes 2, 5, and 9, respectively. The $P2X_2$ receptor positive control used was pancreas cell cDNA and is shown in lane 12. A band of 100 bp was seen in lanes 2 and 3 for MG63 and HOB cells, respectively corresponding to the $P2X_7$ receptor. Bands of 200 bp were seen in lanes 5 and 6 for MG63 and HOB cells corresponding to the $P2X_5$ receptor and in lanes 9 and 10 for MG63 and HOB cells corresponding to the $P2X_4$ receptor. For $P2X_2$ receptors the positive control shown in lane 12 gave a band of 200 bp, while no band was seen in the HOB cell sample (lane 13). No bands were seen in lanes 4, 7, and 11 when HOB cell RNA samples were incubated without the RT enzyme. [n=3]



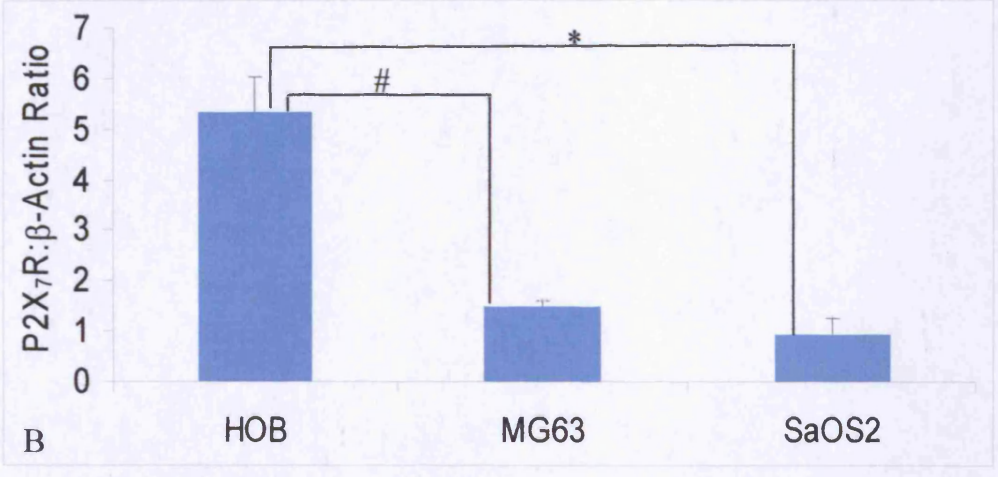


Figure 7.2: P2X$_7$ receptor Western blotting in HOB, MG63, and SaOS2 cells.

A representative example of a Western blot for P2X$_7$ receptors. Positive control (10 µg of HEK7 cell membrane on lane 1), and 25 µg of HOB (lane 2), MG63 (lane 3), and SaOS2 (lane 4) cell lysates were separated on 10% gels. The nitrocellulose membrane was probed with the rP2X$_7$ antibody (1:1000). All the samples gave a P2X$_7$ receptor band at 53 kDa, and a β-actin band at 37 kDa. These bands were quantified using densitometry and the ratio of P2X$_7$ receptor expression to β-actin expression is shown in B. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.01, #P<0.001 vs. HOB samples. [n=4]

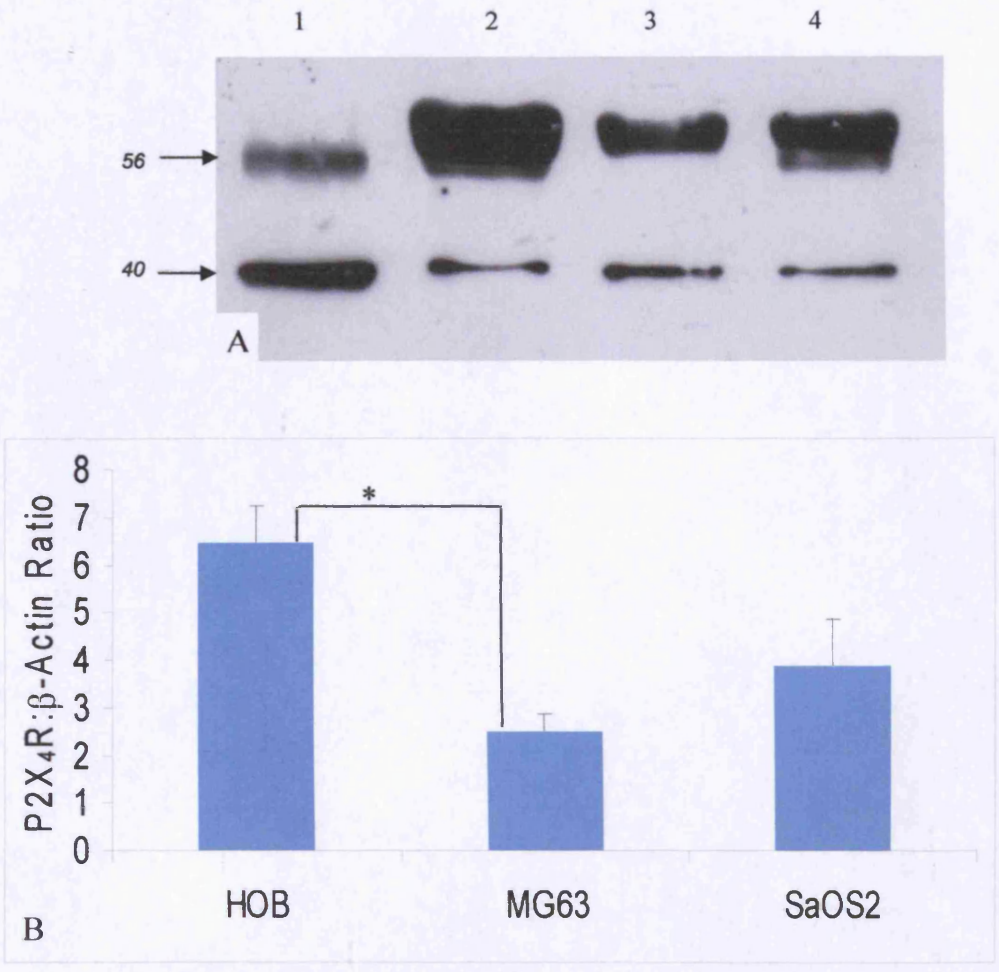


Figure 7.3: $P2X_4$ receptor Western blotting in HOB, MG63, and SaOS2 cells.

A representative example of a Western blot for $P2X_4$ receptors. Positive control (60 µg of rat brain on lane 1), and 25 µg of HOB (lane 2), MG63 (lane 3), and SaOS2 (lane 4) cell lysates were separated on 10% gels. The nitrocellulose membrane was probed with the r$P2X_4$ antibody (1:1000). All the samples gave a $P2X_4$ receptor band at 56 kDa, and a β-actin band at 40 kDa. These bands were quantified using densitometry and the ratio of $P2X_4$ receptor expression to β-actin expression is shown in B. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. *P<0.05 vs. HOB samples. [n=3]

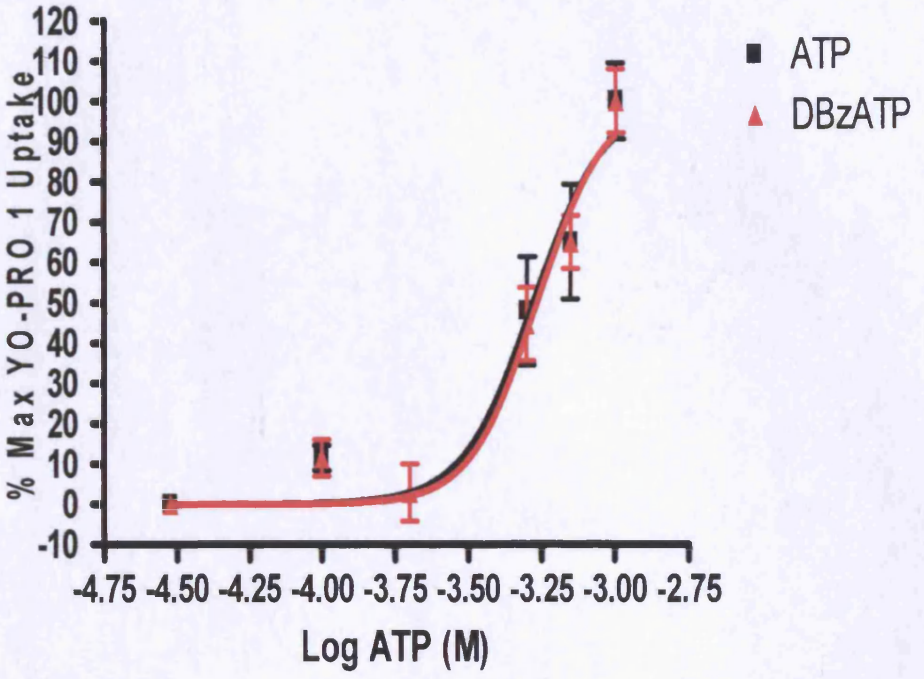


Figure 7.4: Concentration-effect curves for ATP and DBzATP in HOB cells.
HOB cells (approximately 40,000 cells/well) were incubated with each agonist and 1 μM YO-PRO 1 in assay buffer-A at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data were plotted as % maximum ± SEM YO-PRO 1 uptake for each agonist. [n= 4].

Agonist	***MG63 EC_{50} (mM) ±SEM***	***SaOS2 EC_{50} (mM) ±SEM***	***HOB EC_{50} (mM)±SEM***
ATP	0.36 ± 0.03	0.43 ± 0.02	0.53 ± 0.03
DBzATP	0.5± 0.04	0.36± 0.04	0.54± 0.02

Table 7.1: EC_{50} values for ATP and DBzATP in MG63, SaOS2, and HOB cells.
Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test, but no significant differences were seen.

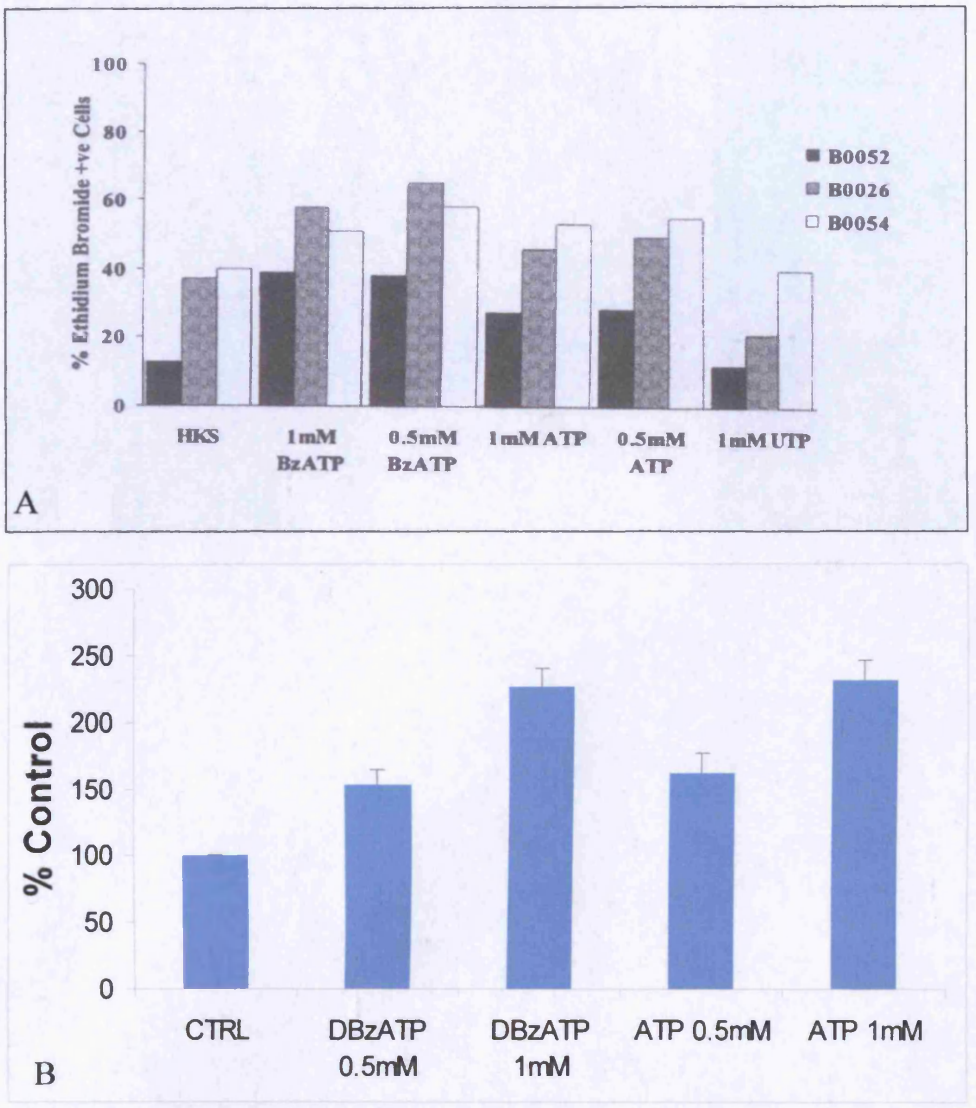


Figure 7.5: YO-PRO 1 uptake induced by 1x10^{-3} M and 5x10^{-4} M ATP and DBzATP in HOB cells.

A comparison between the results obtained by Gartland et al (2001) for several populations of HOB cells using ethidium bromide and an hour incubation with the agonists (A) and the results obtained in this project (B) with the same concentrations of ATP and DBzATP. Data are expressed as % of control ± SEM. Statistical analyses were performed using Student's t-test between each concentration of ATP and DBzATP for the data obtained here. No significant differences were seen. [n=4]. A adapted from Gartland et al (2001).

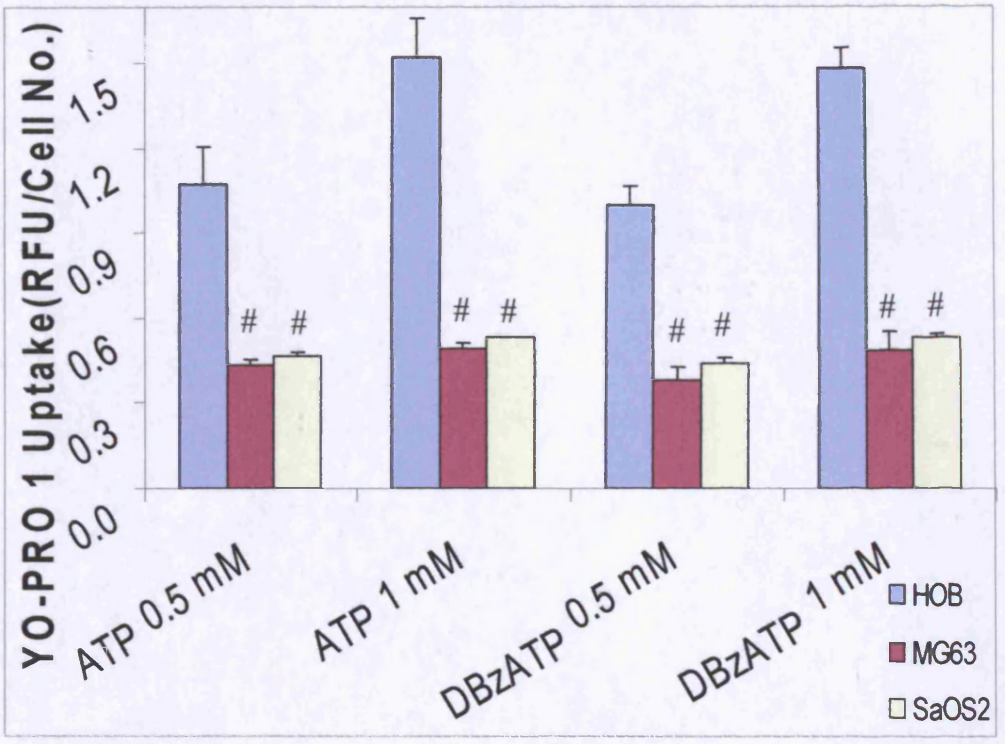


Figure 7.6: Comparsiosn of YO-PRO 1 uptake induced by 1x10^{-3} M and 5x10^{-4} M ATP and DBzATP in HOB, MG63, and SaOS2 cells.

Approximately 40,000 HOB cells/well and 100,000 MG63 and SaOS2 cells/well were incubated with each agonist and 1 μM YO-PRO 1 in 0.5 mM assay buffer-A at 37°C for 5 minutes. YO-PRO 1 fluorescence was monitored in a 96-well plate reader using an excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Data were corrected for cell number. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. # $P<0.001$ vs. HOB cells. [n=4 - 45].

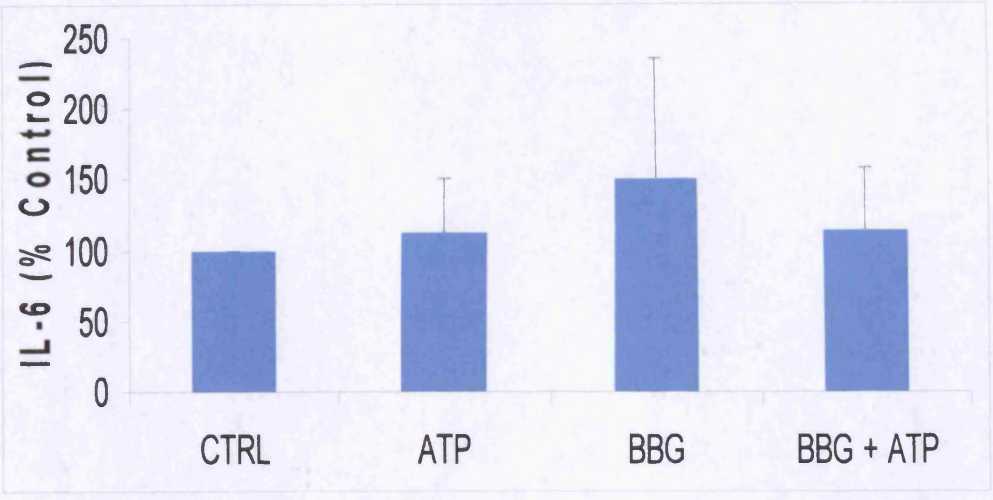


Figure 7.7: Effect of ATP and BBG on IL-6 release in HOB cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then ATP $4x10^{-5}$M or water (ATP vehicle) were added and the cells were grown for 3 days. Media and cells were then collected for IL-6 and protein assays. IL-6 data were corrected for protein content. Data are expressed as percentage of control ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. No significant differences were seen.(n=3)

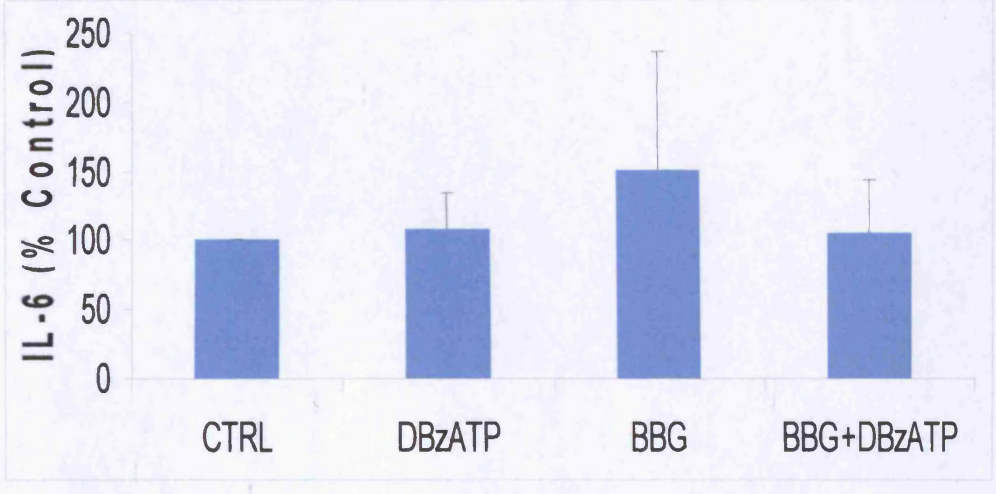


Figure 7.8: Effect of DBzATP and BBG on IL-6 release in HOB cells.

Cells were incubated with BBG $5x10^{-6}$ M or water (BBG vehicle) for one hour then DBzATP $1x10^{-6}$ M or water (DBzATP vehicle) were added and the cells were grown for 3 days. Media and cells were then collected for IL-6 and protein assays. IL-6 data were corrected for protein content. Data are expressed as percentage of control ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. No significant differences were seen. (n=3)

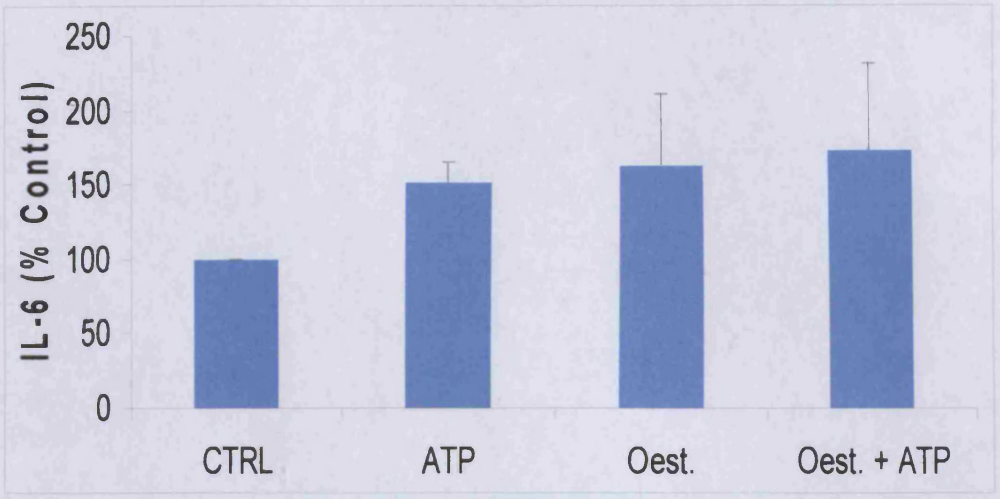


Figure 7.9: Effect of ATP and 17β-oestradiol on IL-6 release in HOB cells.
Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then ATP $4x10^{-5}$M or water (ATP vehicle) were added and the cells were grown for 3 days. Media and cells were then collected for IL-6 and protein assays. IL-6 data were corrected for protein content. Data are expressed as percentage of control ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. No significant differences were seen. (n=3)

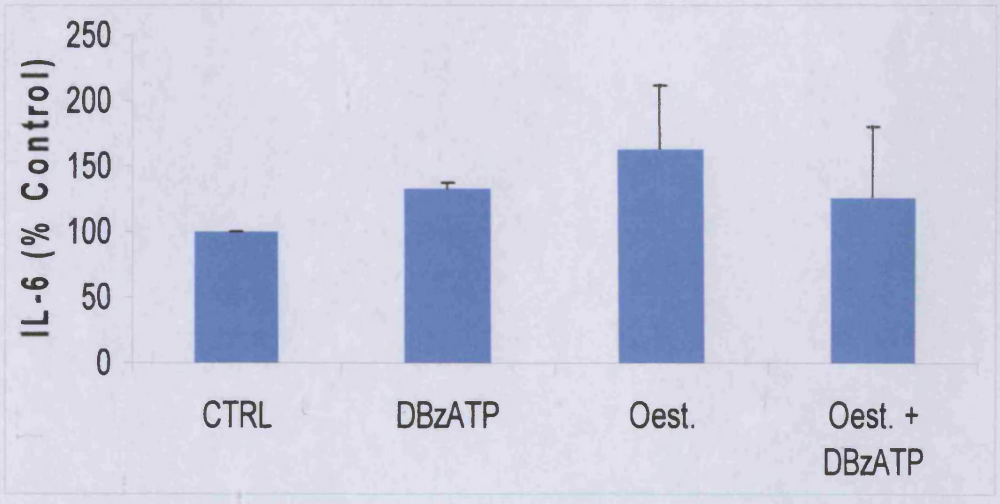


Figure 7.10: Effect of DBzATP and 17β-oestradiol on IL-6 release in HOB cells.
Cells were incubated with 17β-oestradiol $1x10^{-5}$ M or 0.5 % ethanol (17β-oestradiol vehicle) for 30 minutes then ATP $4x10^{-5}$M or water (ATP vehicle) were added and the cells were grown for 3 days. Media and cells were then collected for IL-6 and protein assays. IL-6 data were corrected for protein content. Data are expressed as percentage of control ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. No significant differences were seen. (n=3)

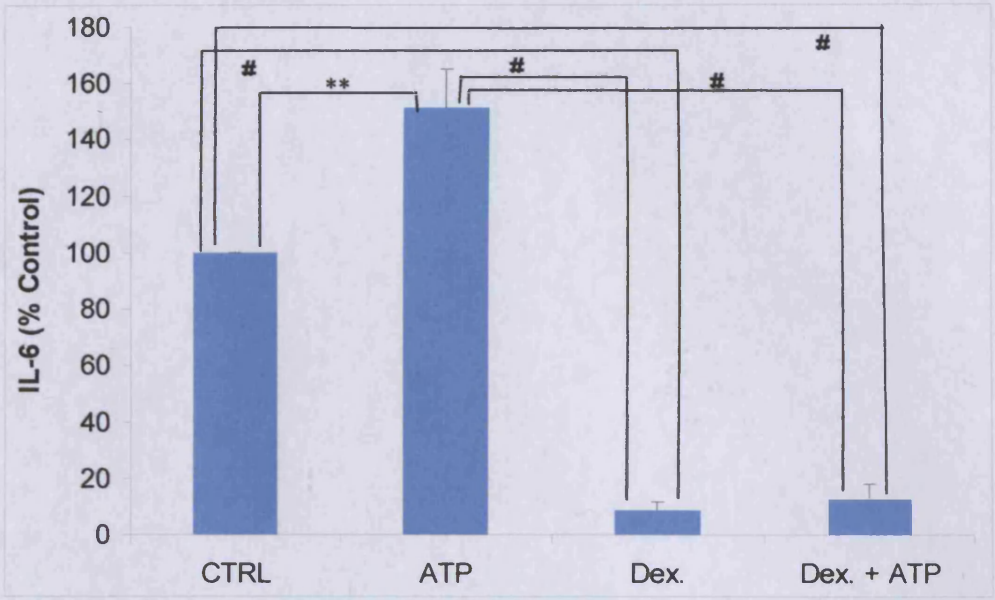
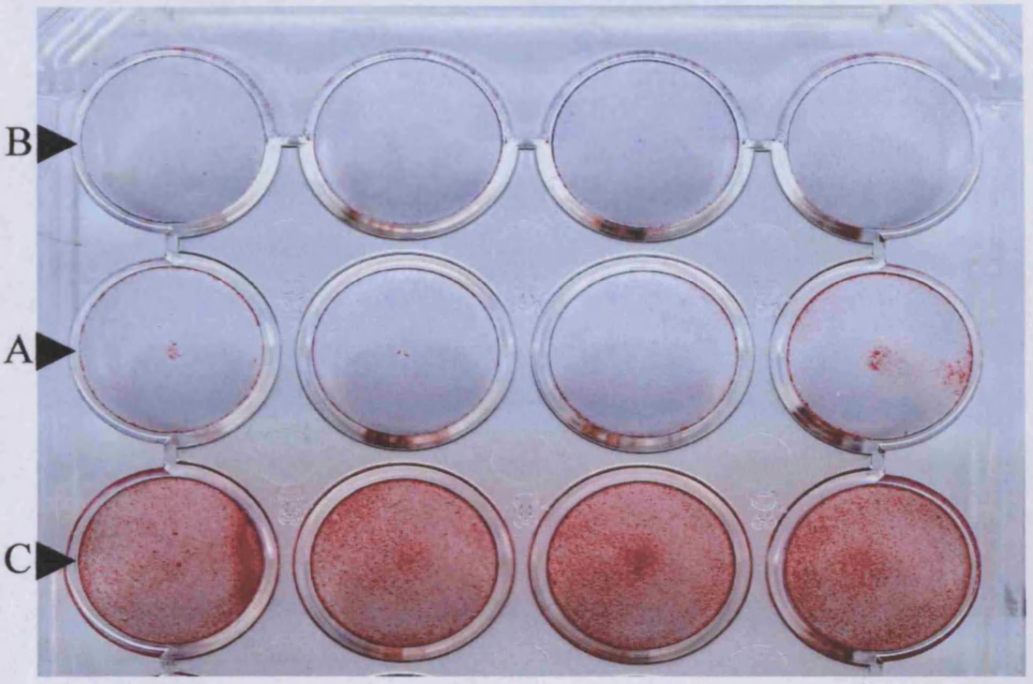


Figure 7.11: Effect of ATP and dexamethasone on IL-6 release in HOB cells.
Cells were incubated with dexamethasone $5x10^{-6}$ M or 0.5 % ethanol (dexamethasone vehicle) for 30 minutes then ATP $4x10^{-5}$M or water (ATP vehicle) were added and the cells were grown for 3 days. Media and cells were then collected for IL-6 and protein assays. IL-6 data were corrected for protein content. Data are expressed as percentage of control ± SEM. Statistical analyses were performed using one-way ANOVA with Newman-Keuls post-hoc test. **P<0.01, #P<0.001 vs. control or ATP. (n=3)

Experiment 1



Experiment 2

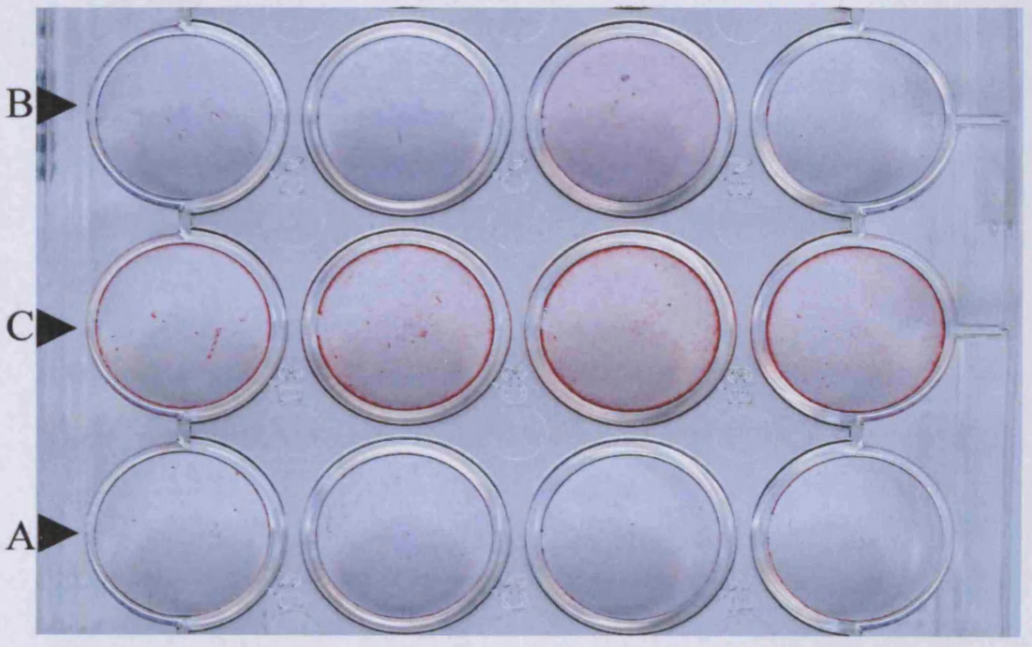


Figure 7.12: Effect of ATP and DBzATP on mineralised bone nodule formation in HOB cells.

Cells were grown for 3-4 weeks with ATP $4x10^{-5}$M, DBzATP $1x10^{-6}$ M or water (ATP and DBzATP vehicle) replenished twice weekly, then cells were stained with alizarin red. (n=2) A: ATP, B: DBzATP, C: Control.

7.5 Discussion

This chapter investigated the expression of $P2X_7$ receptors in HOB cells at the mRNA and protein levels. Additionally, the effect of $P2X_7$ receptor activation on YO-PRO 1 uptake, IL-6 release, and bone nodule formation were also studied.

7.5.1 $P2X_7$ receptor expression

The results described in this chapter demonstrated expression of $P2X_7$ receptors by HOB cells at the mRNA and protein levels. Additionally, expression of two other pore-forming P2X receptors, i.e. $P2X_4$ and $P2X_5$, was also seen.

In Western blotting bands were seen at 53 ± 1.5 kDa and 55 ± 1.4 kDa corresponding to $P2X_4$ and $P2X_7$ receptors, respectively. The 85 kDa band claimed by some studies to be the mature and functional form of the receptor was absent in all the experiments performed. However, the 60 kDa band, believed to be the mature and functional form of the receptor in MG63 and SaOS2 cells, was labelled clearly. These results suggest that the 60 kDa band of the $P2X_7$ receptor is the functional form in the human primary osteoblasts in addition to the osteoblastic-like cell lines tested (MG63 and SaOS2 cells). The $P2X_7$ receptor band was seen previously in MG63 and SaOS2 cells samples at 68 kDa and 54kDa. The 54 kDa band corresponds to the deglycosylated form of the receptor and, due to a change in the batch of the primary antibody used, it only appeared to recognise this form of the receptor.

Densitometric analysis of the Western blotting bands when normalised using β-actin revealed that the expression of $P2X_7$ receptors by HOB cells was significantly higher than in MG63 and SaOS2 cells. The reason

for the difference in expression between these cell lines and primary cells is not clear, but may be due to higher synthesis of the receptor by the primary cells. Due to limited availability of the HOB cells, the subcellular localisation of the receptor could not be studied.

Neither $P2X_2$ receptor mRNA nor protein (data not shown) were detected in HOB cells suggesting that the receptor is not expressed in these cells. Positive controls for the PCR primers and antibody demonstrated that the $P2X_2$ receptor mRNA and protein could be detected by both methods. Similar results were found in MG63 and SaOS2 cells. However, $P2X_4$ receptor mRNA and protein were identified in HOB cells. Densitometric analysis of the Western blotting bands revealed that the expression of $P2X_4$ receptors by HOB cells is also significantly higher than MG63 cells, which might again due to greater synthesis of this receptor by the primary cells compared to cell lines. Additionally, expression of $P2X_5$ receptors was also examined following a study claiming it can also form a pore (Bo et al., 2003). Using RT-PCR, the presence of $P2X_5$ receptor mRNA was identified.

Thus, to summarize, the results presented here confirm earlier findings in MG63 and SaOS2 cells that human osteoblasts do not express $P2X_2$ receptors. Therefore, primary human osteoblasts express three potential pore-forming purinergic receptors, $P2X_4$, $P2X_5$ and $P2X_7$. Additionally, the increased expression in the HOB cells compared to MG63 and SaOS2 cells, suggests that $P2X_4$ and $P2X_7$ receptors have a role in these HOB cells.

7.5.1 $P2X_7$ receptor functional studies

Significantly higher expression of $P2X_7$ receptors by HOB cells compared to MG63 and SaOS2 cells allowed the use of fewer HOB cells for the YO-PRO 1 uptake, IL-6 release, and mineralised bone nodule formation studies.

The YO-PRO 1 results demonstrated that ATP and DBzATP were weak agonists (EC_{50} values in the millimolar level). These results were similar to those obtained in MG63 and SaOS2 cells where DBzATP showed similar effects in opening the pore (Table 7.1) and was equipotent to ATP. A likely explanation for this is that the $P2X_7$ receptor is behaving differently in cells expressing endogenous receptors compared to transfected cells in which most of the previous studies have been done. Interestingly, the similar EC_{50} values for the agonists in HOB cells and cell lines makes the possibility of the influence of high receptor number on potency less likely. This is based on the significantly higher expression of the receptor in the HOB cells which allowed the use of a lower number of cells for this assay. Alternatively, a $P2X_7$ receptor heterodimer with other P2X receptors could be involved, a possibility that very recently been reported in other cells for $P2X_4$ and $P2X_7$ receptors (Guo et al., 2007). This would not occur in transfected cells. Additionally, Gartland et al. (2001) described inconsistencies in ethidium uptake between ATP and DBzATP in some populations of primary human osteoblasts. The atypical pharmacology of the $P2X_7$ receptor identified in MG63 and SaOS2 cells (discussed in detail in Chapter 4) based on the low potency of DBzATP, also occurs in HOB cells. The data obtained here were similar to those obtained by Gartland et al (2001) in several populations of HOB cells, strengthening the hypothesis about the atypical pharmacology of $P2X_7$ receptors in osteoblasts. Thus, results in HOB cells confirm earlier findings in MG63 and SaOS2 cells.

Although the results of $P2X_7$ receptor activation on IL-6 release from HOB cells are preliminary, they suggest that the $P2X_7$ receptor possibly does not play a role in IL-6 release from these cells. These results are opposite to those from SaOS2 cells, where ATP was found to play an important role in IL-6 release (see Chapters 5 and 6). The basal IL-6 release from HOB cells was found to be significantly higher than SaOS2 cells. An increase in IL-6 release from HOB cells with ATP was not seen when water was used as a vehicle (Figure 7.7), but an increase was seen when ethanol was used as a vehicle (Figure 7.11). Therefore the increase in IL-6 release might be due to an effect of ethanol on the cells. An effect of ethanol on ATP-induced currents in other P2X receptors was reported previously in the literature (Davies et al., 2006). A possible reason for the absence of an effect of ATP on IL-6 release from HOB cells is that the release from these cells is already maximal and can not be increased further. Further work is needed to confirm this.

The suppression of IL-6 release by dexamethasone was expected due to its potent anti-inflammatory action. The apparent reversal of the ATP effect is likely to be due to the strong dexamethasone anti-inflammatory effect (Payne and Adcock, 2001; Valledor and Ricote, 2004; Barnes, 2006) and not direct inhibition. 17β-oestradiol did not show any effect on IL-6 release either alone or in combination with ATP in HOB cells. However, ATP-induced IL-6 release was enhanced in SaOS2 cells by 17β-oestradiol. Again, the high basal release of IL-6 by HOB cells may explain these differences.

The effect of $P2X_7$ activation on IL-1β release from HOB cells could not be performed due to limited availability of the cells, but preliminary experiments showed that the basal release of IL-1β from these cells is approximately 1-fold lower than SaOS2 cells (data not shown).

The results of $P2X_7$ receptor activation on mineralised nodule formation in HOB cells are preliminary, but very interesting as they suggest that the $P2X_7$ receptor plays an important role in this physiological action of primary osteoblasts. Due to limited availability of the HOB cells, protein assays could not be performed in these experiments, but no toxicity of ATP and DBzATP was observed by visual examination of the cells. Inhibition of mineralised bone nodule formation in response to ATP has been observed previously in rat osteoblasts, but it was claimed to be via $P2Y_2$ receptors (Hoebertz et al., 2002). This possibility can not be excluded here although the DBzATP results suggest it to be unlikely as DBzATP is not an agonist at $P2Y_2$ receptors (Ziganshin et al., 2002; Burnstock, 2007). Again, the presence of ethanol as a vehicle raises a question as to its involvement in mineralization inhibition by ATP, which needs to be clarified.

The absence of any effect of DBzATP on IL-6 release might be due to its lower concentration, or its binding by the serum constituents. Conversely, the effect of DBzATP on mineralisation was clear which might be due to the increased length of treatment.

The IL-6 release and mineralised bone nodule formation data in HOB cells are different than those in SaOS2 cells. These differences suggest that $P2X_7$ receptors modulate IL-6 release in SaOS2 cells but not mineralisation and vice versa in HOB cells. Therefore, SaOS2 cells do not appear to be a good model for some aspects of osteoblast function.

7.6 Conclusion

The results presented in this chapter demonstrate that the $P2X_7$ receptor is expressed and functional in HOB cells. The 60 kDa form of the receptor appears to be the mature and functional form of the receptor in the human osteoblast. Additionally, $P2X_7$ receptor protein expression was found to be significantly higher in HOB cells compared to the cell lines MG63 and SaOS2, and this was translated into significantly higher YO-PRO 1 uptake. However, the EC_{50} values for ATP and DBzATP were found to be similar in the HOB cells and the cell lines, confirming the atypical pharmacology. Similarly to the MG63 and SaOS2 cell lines, HOB cells also express $P2X_4$ and $P2X_5$ receptors, but not $P2X_2$ receptors.

The $P2X_7$ receptor appears to control IL-6 release in SaOS2 cells and mineralised bone nodule formation in HOB cells. However, my results generally suggest SaOS2 cells to be a reasonable model for HOB for the pharmacological assays of $P2X_7$ receptors but not for the physiological functions.

Chapter 8: General Discussion

8.1 General discussion of results

The general aim of this project was to characterise the expression and function of $P2X_7$ receptors in human osteoblasts. Additionally, the effects of $P2X_7$ receptor activation on several physiological functions of osteoblasts were studied. The results obtained in this project indicate that human osteoblasts express $P2X_7$ receptors. Furthermore, they are functional and probably play an important role in osteoblast function by increasing ALP activity and IL-6 release and decreasing mineralisaion.

8.1.1 Expression of $P2X_7$ receptors by human osteoblasts

In this study I have confirmed the expression of the $P2X_7$ receptor in human osteoblasts both in primary human osteoblasts and in the MG63 and SaOS2 osteosarcoma cell lines. I showed receptor expression at the mRNA level using RT-PCR and at the protein level using Western blotting and immunocytochemistry. With immunocytochemistry I found that the immunolabelling was stronger on the nucleus compared to the cytoplasm and cell membrane when the $P2X_7$ receptor antibodies was used. A similar finding was observed in Western blotting as the band seen in the nuclear samples was stronger than that seen in the cell membranes or lysate. However, quantification could not be performed due to alterations in house-keeping gene protein (β-actin and GAPDH) expression with cell density.

The fully functional form of all P2X receptors is believed to be the glycosylated form (North, 2002). The functional $P2X_7$ receptor was claimed to be the 85 kDa form as it was reported to be the fully glycosylated form, and the 67 kDa form was said to be a deglycosylated form (Wang et al., 2005). However, all the results obtained in this project indicate that the 67 kDa form of the receptor is the functional state, and

that the 85 kDa band was either absent or a minor state as it gave a faint band in the experiments performed. Deglycosylation of the $P2X_7$ receptor was carried out in membrane samples of MG63 and SaOS2 cells and it was found that the receptor band size (67 kDa) decreased by approximately 9 kDa to 58 kDa. It should be noted that all three bands observed (85, 67 and 58 kDa) disappeared when the primary antibody was incubated with peptide used to raise it, suggesting that they are indeed different forms of the $P2X_7$ receptor. The 85 kDa band might be the extra protein needed for pore formation according to a hypothesis suggested in the literature (North, 2002; Faria et al., 2005; Liang and Schwiebert, 2005). An alternative explanation for this 85 kDa band might be that it is a $P2X_4/P2X_7$ heteromer, which was suggested in a recent study (Guo et al., 2007). Another possible explanation might be that the functional form of the receptor differs from one cell type to another and it is the 67 kDa form in human osteoblasts, but the 85 kDa form in CaSki cells (transformed cervical epithelial cells) (Wang et al., 2005).

The expression of other pore-forming P2X receptors ($P2X_2$, $P2X_4$ and $P2X_5$) was studied to see whether other receptor subtypes could be responsible for the results obtained. Expression of $P2X_4$ and $P2X_5$, but not $P2X_2$ receptors was demonstrated in human primary osteoblasts, MG63 and SaOS2 cell lines. Presence of $P2X_4$ receptor supports the possibility of existence of $P2X_4/P2X_7$ heteromer that very recently been reported in other cells (Guo et al., 2007). Therefore, it is possible that these receptors could mediate some of the effects of the P2X receptor agonists described below.

Interestingly the expression of the $P2X_7$ receptor protein by human primary osteoblasts was found to be significantly greater than MG63 and

SaOS2 cells, suggesting that the primary cells synthesize higher amounts of the receptor compared to the cell lines. Similarly, expression of $P2X_4$ receptor protein by human primary osteoblasts was also found to be significantly greater than MG63 cells only. These results suggest that both these receptor types play a role in human primary osteoblast functions.

8.1.2 Pharmacological characterisation of $P2X_7$ receptors in human osteoblasts

Once $P2X_7$ receptor expression had been confirmed in human osteoblasts, the pharmacological characterisation of the receptor was studied using the YO-PRO 1 uptake method. This method utilizes the pore-forming characteristic of the $P2X_7$ receptor and YO-PRO 1, a dye which fluoresces after entering the cell through the pore and binding to nucleic acid. This assay is well known for the pharmacological study of the $P2X_7$ receptor (Michel et al., 1999; Gartland et al., 2001; Michel et al., 2006b). Pore formation in human osteoblasts was found to occur within 5 minutes of incubating the cells with the agonist, in contrast to results from Gartland et al (2001) showing that an hour was needed for pore formation. These findings led these authors to suggest an atypical pharmacology of the $P2X_7$ receptor in human osteoblasts. Based on different data, atypical pharmacology can also be applied to the $P2X_7$ receptors in this project as DBzATP was found to be equipotent to ATP. DBzATP is thought to be the best $P2X_7$ receptor agonist based on results in the literature showing it to be at least 10-fold more potent compared to ATP on $P2X_7$ receptors (Chessell et al., 1998; Michel et al., 1999; Lee et al., 2006). However, these studies were performed in transfected cells. Other studies performed in native cells have found DBzATP to be inactive (Cario-Toumaniantz et al., 1998) or equipotent to ATP

(Hickman et al., 1994; Gartland et al., 2001) at $P2X_7$ receptors. Other agonists, 2Me-S-ATP and ATP-γ-S were also tested and their EC_{50} values were found to be similar to those of ATP and DBzATP. The EC_{50} values obtained suggest that the receptor involved in the YO-PRO 1 uptake is $P2X_7$ and not $P2X_2$, $P2X_4$, or $P2X_5$ receptors. The reason for the equipotency of these agonists might be the atypical pharmacology of the receptor in human osteoblasts as stated earlier. Another explanation might be a $P2X_4/P2X_7$ receptor heteromer, suggested to exist in a recent study (Guo et al., 2007). However, the overall affinities of the agonists used in inducing pore formation and the lack of receptor desensitisation seen with ATP ($3x10^{-3}$ M) indicate that the $P2X_7$ receptor is most likely to be responsible for YO-PRO 1 uptake.

This hypothesized atypical pharmacology of the osteoblast $P2X_7$ receptor may also explain the results obtained with the antagonists BBG, KN62, oATP and PPADS on ATP- and DBzATP-induced YO-PRO 1 uptake. Although these antagonists were found to behave in a non-competitive manner, similar to data reported in the literature in other cell lines (Michel et al., 2000), their antagonist action in human osteoblasts was found to be weak. The effect of BBG here is most probably on $P2X_7$ receptors as it was reported to be more potent at $P2X_7$ receptors compared to the other P2X receptor subtypes (Hibell et al., 2000; Jiang et al., 2000; Khakh et al., 2001b; Sperlagh et al., 2006). Although KN62 is reported in the literature to be the most potent human $P2X_7$ receptor antagonist (Sperlagh et al., 2006), that was not found here as it was very weakly active. Although the atypical pharmacology of the osteoblast $P2X_7$ receptor is the most likely explanation for the data with the antagonists, a $P2X_4/P2X_7$ receptor heteromer could also be involved.

8.1.3 Effect of $P2X_7$ receptor activation on human osteoblast function

$P2X_7$ receptor activation was found to increase ALP production and IL-6 release from the SaOS2 cell line and these effects were blocked by BBG. The effect of DBzATP on ALP production and IL-6 release was either absent or weak. The absence of an effect with DBzATP on ALP and IL-6 raises a question as to the involvement of the $P2X_7$ receptor. However, the BBG inhibition of ATP-induced ALP production and IL-6 release suggests that the ATP effect is most likely to be via $P2X_7$ receptors and not adenosine or P2Y receptors as BBG has no effect on these receptors. A possible explanation for the weak action of DBzATP might be the hypothesized atypical pharmacology of $P2X_7$ receptors in human osteoblasts. However, low concentrations of DBzATP, below the EC_{50} value obtained in the YO-PRO 1 assay which had to be used to avoid cell death might be a reason also. Another explanation might be that some of the serum constituents available in the medium might be binding DBzATP and reducing its potency, an effect reported previously for DBzATP on ethidium bromide uptake (Michel et al., 2001).

In contrast to previous studies reporting the involvement of $P2X_7$ receptors in IL-1β release in other cells (Ferrari et al., 1997; Solle et al., 2001; Labasi et al., 2002; Qu et al., 2007), no effect was seen here. This suggests either that the $P2X_7$ receptor is not involved in IL-1β release from osteoblasts, or that the agonist concentrations used here are lower than those found under physiological conditions which are needed to stimulate its release. However, the latter reason is unlikely as the agonist concentrations used here caused IL-6 release, unless IL-1β and IL-6 are released by different mechanisms. A more likely explanation might be that lipo-polysaccharide is needed for $P2X_7$ receptor-induced IL-1β release, an effect which is still debatable (Ferrari et al., 2006). An effect

on bone nodule formation was only observed with ATP when ethanol was used as the vehicle suggesting that this effect is probably not due to $P2X_7$ receptor activation.

8.1.4 $P2X_7$ receptor channel versus pore formation

Activation of the $P2X_7$ receptor passes through two stages, channel opening and pore formation (North, 2002). The channel is permeable to small cations such as calcium (North, 2002; Dubyak, 2007) and opened by low concentrations of agonists (North, 2002; Burnstock, 2007; Dubyak, 2007). The pore, however, is opened by higher concentrations of agonists, is permeable to large cations (<900Da) and is the end stage leading to cell death unless the agonist is removed (Ferrari et al., 1997; North, 2002; Burnstock, 2007). Effects on the physiological actions (ALP production and IL-6 release) of osteoblasts were observed here with ATP at concentrations lower than those which induce YO-PRO 1 uptake suggesting that pore formation is not necessary for these effects. Therefore, these actions are most probably related to the $P2X_7$ receptor channel opening in response to lower concentrations of ATP and an increase in intracellular calcium. Alternatively, another unknown second messenger might be involved.

8.1.5 Effect of 17β-oestradiol and dexamethasone on $P2X_7$ receptor expression and function in human osteoblast

17β-oestradiol and dexamethasone were found to affect $P2X_7$ receptor function in the SaOS2 cell line, an effect likely to be via a non-genomic mechanism. They could be acting as weak, non-competitive antagonists in blocking YO-PRO 1 uptake with no effect on $P2X_7$ receptor

expression. Dexamethsone had no effect on ALP release, but decreased ATP-induced IL-6 release after 3 days incubation, probably as a consequence of its potent anti-inflammatory action. The unexpected increase in ATP-induced IL-6 release by dexamethasone after 6 days incubation might be due to an upregulation of $P2X_7$ receptors (although this was not tested). The effect of dexamethasone on $P2X_7$ receptors in osteoblasts is not conclusive and more work is needed. No effect of $P2X_7$ receptor activation on IL-1β release was seen except when ethanol was used as a vehicle with the agonists, demonstrating that this action is not $P2X_7$ receptor induced. 17β-oestradiol was reported in the literature to increase ALP production (O'Shaughnessy et al., 2000; Rao et al., 2003; Kanno et al., 2004) and to decrease (Waters et al., 2001; Manolagas et al., 2002; Straub, 2007), or have no effect (Dovio et al., 2001) on IL-6 release. Dexamethasone was reported to increase ALP production (Yang et al., 2003; Jorgensen et al., 2004; Eijken et al., 2006) and decrease IL-6 release (Dovio et al., 2001; Payne and Adcock, 2001; Chen et al., 2005; Barnes, 2006). The results obtained in this project for 17β-oestradiol and dexamethasone are contradictory to those reported in the literature, which might be due to the cell type used (primary cells or cell lines), oestrogen receptor isoform expressed (α or β), and receptor density.

8.1.6 Expression and function of $P2X_7$ receptors in primary human osteoblast

Altough preliminary, the results obtained with the primary human osteoblast cells supported the earlier findings with the SaOS2 and MG63 cell lines in that the primary osteoblasts expressed functional $P2X_7$ receptors involved in many of the physiological actions of osteoblasts (IL-6 release and nodule formation). Additionally, the YO-PRO 1 assay results confirmed the hypothesis of atypical pharmacology of $P2X_7$

receptors in human osteoblasts. The $P2X_7$ receptor-induced decrease in bone nodule formation in the primary cells was particularly interesting and unexpected.

8.1.7 Osteoblast model for the study of $P2X_7$ receptors

At the end of this project, it is possible to suggest which might be the best osteoblast model for studying $P2X_7$ receptors. The MG63 cell line would be a good model for the pharmacological characterization of $P2X_7$ receptors using the YO-PRO 1 assay. However, these cells are not suitable for studying $P2X_7$ receptor-mediated effects on ALP activity or bone nodule formation as it was reported that they don't produce ALP or form nodules (Hughes and Aubin, 1998). Conversely, the SaOS2 cell line is considered to be the closest to the mature osteoblast (Hughes and Aubin, 1998) and most of the results I obtained with them were then confirmed in primary human osteoblasts, though the results with the latter were preliminary. I believe that the SaOS2 cell line represents a good model for the study of $P2X_7$ receptors in human osteoblasts, although primary cells would clearly be the ideal choice. This is based on the following factors: the ease and low cost of growing the SaOS2 cells, and the possibility of growing them for a higher number of passages compared to the primary human osteoblasts and the similarity of my results in the SaOS2 and primary cell,

8.2 Role of $P2X_7$ receptors in bone formation

Figure 8.1 shows a summary of the effect of $P2X_7$ receptor activation and inhibition on several physiological actions of osteoblasts. Activation of the $P2X_7$ receptor with low concentrations of agonist opens a channel

permeable to small cations such as calcium, which I believe results in the release of IL-6 and the production of ALP seen in this project. These two proteins are important factors in the pathophysiology of osteoporosis and RA. IL-6 is an inflammatory mediator involved in the pathophysiology of RA and in communication between osteoblasts and osteoclasts, where an imbalance results in osteoporosis. The increase in ALP production and release results in an increase in osteoblastic bone formation and a disturbance in ALP activity predisposes to osteoporosis. Activation of the $P2X_7$ receptor with high concentrations of agonist or prolonged activation with low agonist concentrations leads to the formation of a pore permeable to large cations (<900 Da) such as YO-PRO 1 or ethidium bromide. The consequence of pore formation is usually cell death either by apotosis or necrosis, which led to the $P2X_7$ receptor being described as a death receptor. All the above actions of $P2X_7$ receptor activation are inhibited with BBG, an antagonist more potent on $P2X_7$ receptors compared to the other P2X receptors.

The results revealed in this project confirm the earlier findings in knockout mice reported in the literature on the role of the $P2X_7$ receptor in bone formation (Ke et al., 2003). Bone formation in these knockout mice was found to be reduced compared to the wild-type animals (Ke et al., 2003). The increase in ALP release, a known bone formation marker (Bikle, 1997), seen here as a result of $P2X_7$ receptor activation, supports the role proposed for $P2X_7$ receptors in bone formation (Ke et al., 2003). The effect of $P2X_7$ receptor activation on bone formation is probably indirect and this is supported by lack of clear effect on mineralisation in SaOS2 cells. The mineralisation results might be due to involvement of another purinergic receptor or a vehicle effect. However, interestingly in HOB cells, preliminary data showed a decrease in nodule formation, opposite to the results with knockout mice and my ALP results, so the

question remains: are $P2X_7$ receptors involved in bone formation? The answer is that they most probably are involved, but have a modulatory role in bone formation capable of both increasing and decreasing it depending on the environment. Additionally, species variations may also occur as Ke et al. (2003) worked in mice and my project used human cells, but more work is needed to confirm this hypothesis.

17β-oestradiol blocked $P2X_7$ receptor-induced pore formation, which I believe to be via a non-genomic mechanism as reported in a previous study in other cells (Cario-Toumaniantz et al., 1998). In the literature, the effect of oestrogen was reported to increase the activity and lifespan of osteoblasts by inhibiting their apoptosis (Manolagas et al., 2002; Balasch, 2003; Syed and Khosla, 2005; Jacob et al., 2006) via a non-genomic mechanism (Syed and Khosla, 2005). Therefore the reduction in pore formation seen here might be another mechanism underlying the action of 17β-oestradiol on osteoblast bone formation as pore formation is often followed by cell death (Virginio et al., 1999). In the menopause bone formation is affected due to oestrogen deficiency and the loss of its effect on inhibiting $P2X_7$ receptor-induced osteoblast apoptosis which could contribute to osteoporosis.

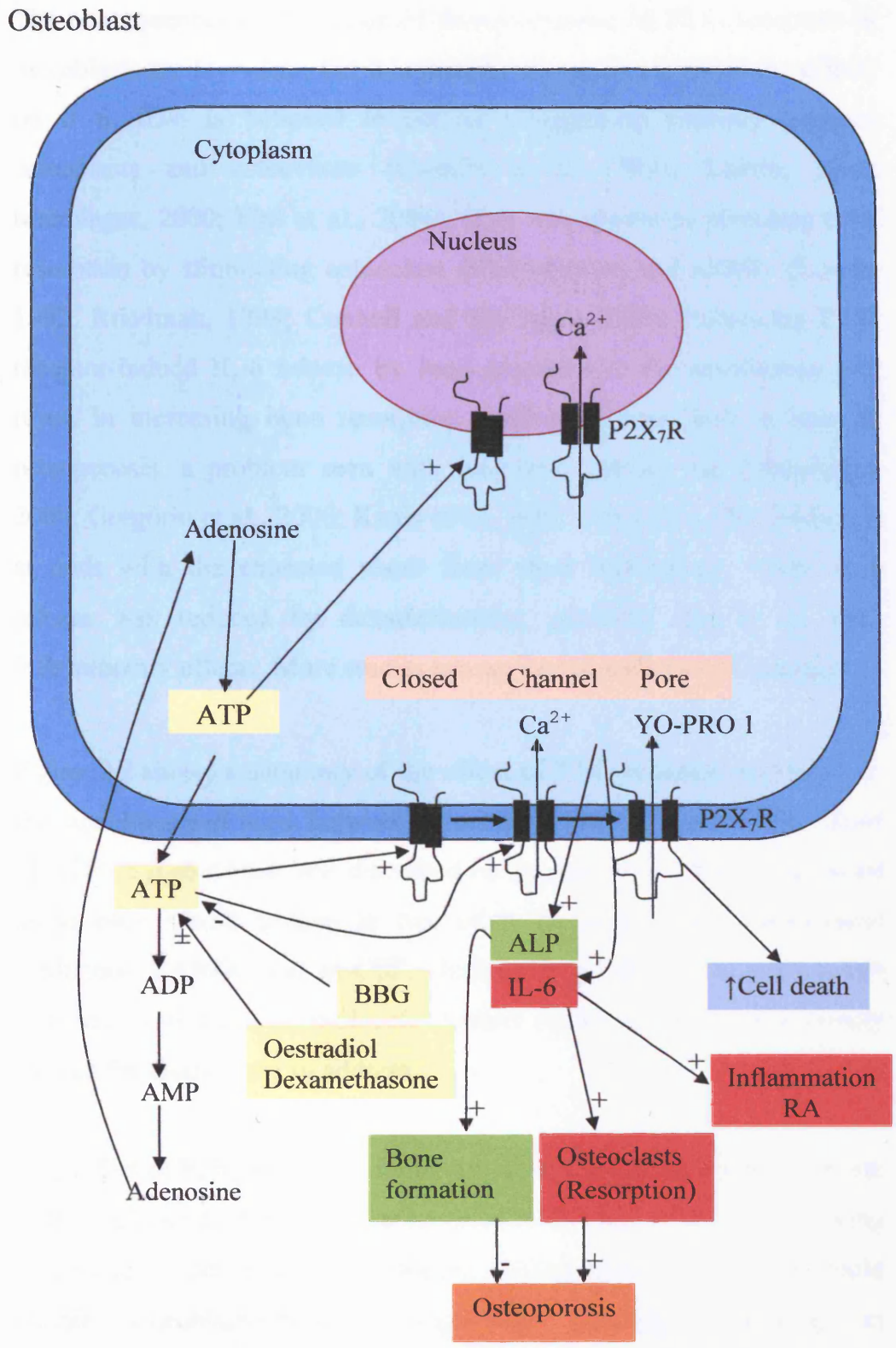


Figure 8.1: A diagram summarising the results obtained in this thesis showing the effect of P2X$_7$ receptor activation and inhibition on the physiological actions of osteoblasts and bone formation. Extracellular ATP represents the exogenous and endogenous nucleotide. + Activation, - inhibition, ↑ increase. RA: rheumatoid arthritis.

The consequences of the action of dexamethasone on $P2X_7$ receptors in osteoblasts are less clear, but it is possible to explain some of the effects on IL-6. IL-6 is believed to act as a signalling pathway between osteoblasts and osteoclasts (Canalis et al., 1991; Lowik, 1992; Manolagas, 2000; Fini et al., 2004). IL-6 was shown to stimulate bone resorption by stimulating osteoclast differentiation and activity (Lowik, 1992; Roodman, 1993; Connell and McInnes, 2006). Enhancing $P2X_7$ receptor-inducd IL-6 release by long exposure to dexamethasone will result in increasing bone resorption leading to bone loss as seen in osteoporosis, a problem seen with long-term steroid use (Manolagas, 2000; Gregório et al., 2006; Kanis et al., 2007). However, this finding is at odds with the expected result from short incubations where IL-6 release was reduced by dexamethasone, probably due to its anti-inflammatory effects. More studies are needed to address this question.

Figure 8.2 shows a summary of the effect of $P2X_7$ receptor activation on the signalling pathways between osteoblasts and osteoclasts. The effect of ATP on IL-6 release was discussed earlier, and this raised the question as to what would happen to two other important osteoblast-derived mediators, RANKL and M-CSF. Unfortunately, there was not enough time to investigate these molecules further in this project. This is clearly an area for future work to address.

Any effect of $P2X_7$ receptor stimulation on RA might be via the increase in IL-6 release. IL-6 release was reported to increase in RA and blocking its release would improve the disease. Inhibition of IL-6 release would inhibit osteoblast-osteoclast signalling pathways resulting in prevention/reduction of bone loss arising from osteoclast activity. Additionally, the increase in oseoblast apoptosis caused by $P2X_7$ receptor activation would result in decreased bone formation. The net result of

$P2X_7$ receptor activation in RA would be an increase in bone damage resulting from increased osteoclastic resorption and decreased osteoblastic bone formation. These effects are definitely possible as there is a large increase in ATP release caused by inflammatory processes (Bours et al., 2006) such as in RA .

Overall, the results presented in this project suggest that oestrogen and glucocorticoids may have a direct action on increasing osteoblast lifespan via inhibition of $P2X_7$ receptor-induced cell death. Additionally, they may have a modulatory action on some of the signalling pathways involving the $P2X_7$ receptor.

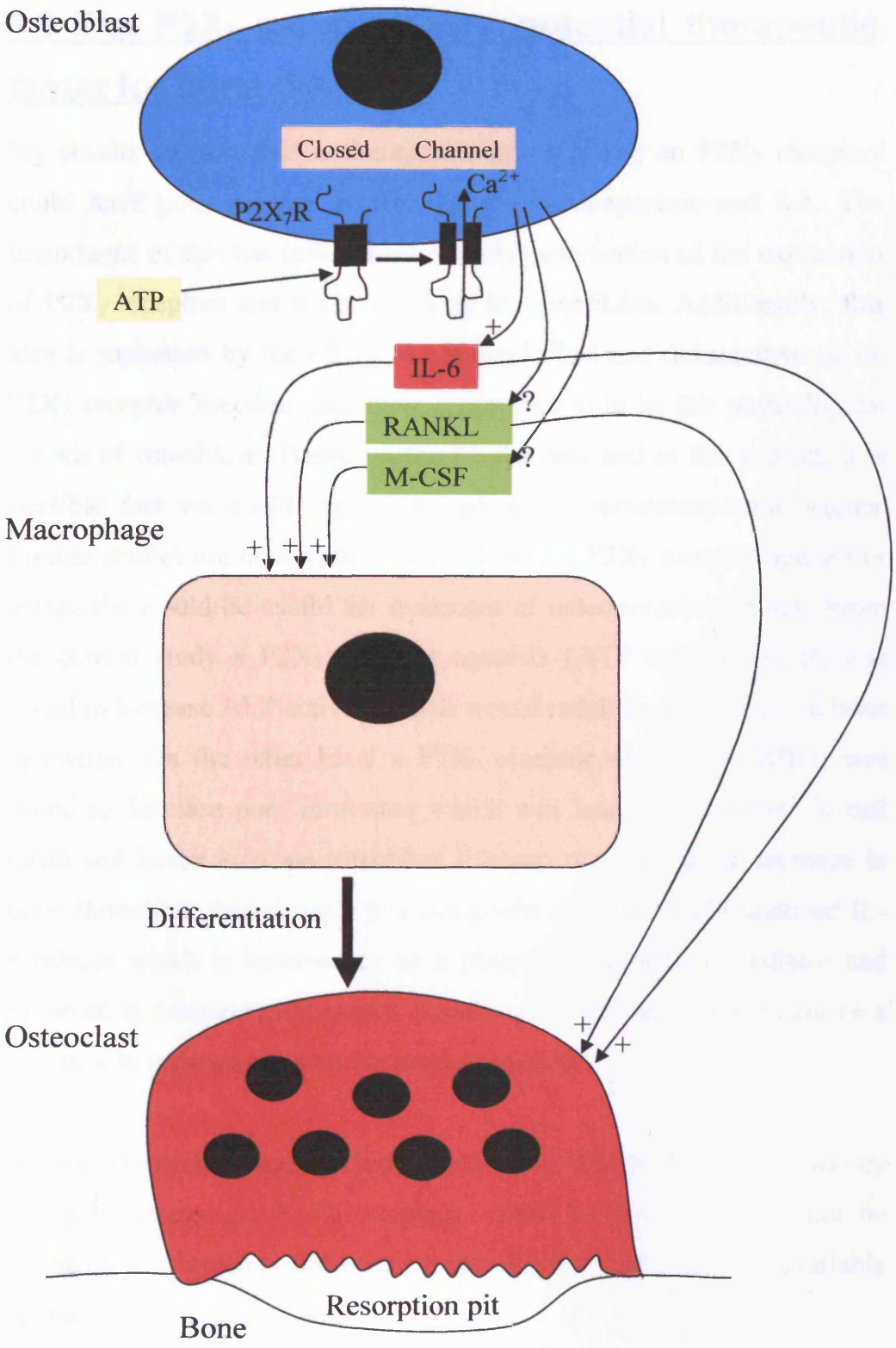


Figure 8.2: A diagram showing the consequences of P2X$_7$ receptor activation on IL-6 release and the relationship between osteoblasts and osteoclasts. *+ Activation, ? Not studied in this project.*

8.3 The $P2X_7$ receptor as a potential therapeutic target for bone diseases

My results suggest that a therapeutic agent acting on $P2X_7$ receptors could have potential for the treatment of osteoporosis and RA. The importance of this has arisen from the characterisation of the expression of $P2X_7$ receptors and their functions in osteoblasts. Additionally, this idea is supported by the effect of 17β-oestradiol and dexamethasone on $P2X_7$ receptor function and their speculated role in the physiological actions of osteoblasts. Based on the results obtained in this project, it is possible that we could see a new group of anti-osteoporotic agents. Further studies are needed to decide whether a $P2X_7$ receptor agonist or antagonist would be useful for treatment of osteoporosis and RA. From the current study a $P2X_7$ receptor agonists (ATP and DBzATP) was found to increase ALP activity which would result in an increase in bone formation. On the other hand a $P2X_7$ receptor antagonist (BBG) was found to decrease pore formation which will lead to a decrease in cell death and hence increase osteoblast lifespan resulting in an increase in bone formation. Additionally this antagonist decreased ATP-induced IL-6 release which is believed to be a potent inflammatory mediator and involved in osteoblast-osteoclast signalling, so the net effect would be a decrease in inflammation and osteoclastogenesis.

An anti-osteoporotic agent which induces osteoblastic bone formation by acting on purinergic $P2X_7$ receptors would be novel, as it would be acting by a completely different mechanism than the currently available agents.

8.4 Recommendations for future work

The results obtained in this thesis are preliminary in some areas, so need to be investigated further. These results also suggest further work to learn more about $P2X_7$ receptors in osteoblasts.

- Study a time course for the effect of $P2X_7$ receptor activation on IL-6 release in HOB cells.
- Generate concentration-effect curves for $P2X_7$ receptor agonists on IL-6 release in HOB cells.
- Investigate the effect of BBG and other P2X antagonists (PPADS and oATP) on $P2X_7$ receptor-induced IL-6 release in HOB cells.
- Study the effect of $P2X_7$ receptor activation and inhibition on ALP production in HOB cells.
- Investigate further the effect of 17β-oestradiol and dexamethasone on $P2X_7$ receptor-induced IL-6 release and ALP production in HOB cells.
- Investigate the effect of $P2X_7$ receptor activation and inhibition on LPS-induced IL-6 and IL-1β release from SaOS2 and HOB cells.
- Investigate further the effect of $P2X_7$ receptor activation and inhibition on mineralised bone nodule formation in HOB cells.
- Study the effect of $P2X_7$ receptor activation and inhibition on RANKL and M-CSF release from SaOS2 and HOB cells.
- Study the expression and involvement of $P2X_4/P2X_7$ heteromer in osteoblast function

Longer-term future studies could include: characterisation of $P2X_7$ receptors in human osteoporotic osteoblasts, $P2X_7$ receptor-coupled signal transduction mechanisms, and electrophysiology of $P2X_7$ receptor channels.

8.5 Conclusion

Bone diseases such as osteoporosis and RA are major risk factors for morbidity and mortality, therefore bone research has expanded recently as reflected by the many new potential treatments for these bone diseases.

In this project expression of functional $P2X_7$ receptors in human osteoblasts was confirmed, and 17β-oestradiol and dexamethasone were found to inhibit $P2X_7$ receptor function probably via a non-genomic mechanism. Additionally, I found that $P2X_7$ receptor activation increases ALP production and IL-6 release, an effect found to be modulated by both 17β-oestradiol and dexamethasone. These results suggest that the $P2X_7$ receptor is playing a role in bone formation.

$P2X_7$ receptors as targets look to be promising in the bone field generally and in the treatment of osteoporosis and RA in particular. The importance of this depended on the identification of $P2X_7$ receptor expression and function in osteoblasts and the role of the agents known to have an effect in osteoporosis. Based on the results obtained in this project, the potential for $P2X_7$ receptors as a target for a new group of anti-osteoporotic agents has been highlighted.

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