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Molecular Analysis Of A_{2A} Adenosine Receptor Regulation Of NF-κB-Dependent Inflammatory Responses

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To Mum, with love.....

.....till we meet again.

Ps 23:1-6

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ABBREVIATIONS

A_1AR	A_1 Adenosine receptor
A_{2A}AR	A_{2A} Adenosine receptor
A_{2B}AR	A_{2B} Adenosine receptor
A_3AR	A_3 Adenosine receptor
ADA	Adenosine deaminase
AP-1	Activator protein-1
AR	Adenosine receptor
ARD	Ankyrin repeat domain
ASK	Apoptosis signal-regulating kinase
AV	Adenovirus
β_2AdR	β_2-adrenergic receptors
Blys/BAFF	B-lymphocyte stimulator/B-cell activating factor
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CaMKIV	Ca^{2+}/calmodulin kinase IV
CBP	CREB-binding protein
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CHUK	Conserved Helix-loop-helix ubiquitous kinase
cIAP	Cellular inhibitor of apoptosis
CKII	Casein kinase II
CNS	Central nervous system
COX	Cyclo-oxygenase
CREB	Cyclic AMP response element binding protein
CRM-1	Chromosome maintenance region-1
DAG	*sn*-1,2-diacylglycerol
DD	Death domain
DED	Death effector domain
DTT	Dithiothreitol
EBV	Epstein Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix

ECSIT	Evolutionarily conserved signalling intermediate in Toll pathways
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunoabsorbant assay
EMSA	Electrophoretic mobility shift assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ESL-1	E-selectin ligand-1
EST	Expressed sequence tag
FADD	Fas-associated death domain
FAF	Fas-associated factor
GAG	Glycosaminoglycans
GAS	γ-activated sequence
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinase
GST	Glutathione S-transferase
HA	Haemaglutinin epitope tag
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEK 293	Human embryonic kidney 293
HRP	Horseradish peroxidase
HTLV	Human T-cell leukaemia virus
HUVECs	Human umbilical vein endothelial cells
IBS	Irritable bowel syndrome
ICAM	Intracellular adhesion molecule
IFN γ	Interferon γ
IFN γ R	Interferon γ receptor
IκB	Inhibitor-κB
IKK	IκB kinase
IL-1	Interleukin-1

IL-1R	Interleukin-1 receptor
IL-1RAc-P	Interleukin-1 receptor accessory protein
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
JAK	Janus activated kinase
JNK	c-Jun-N-terminal kinase
LAD-II	Leukocyte adhesion deficiency II
LBP	LPS binding protein
LMP-1	Latent membrane protein-1
LPS	Lipopolysaccharide
LTβ	Lymphotoxin β
MadCAM-1	Mucosal addressin cell adhesion molecule
MAPK	Mitogen-activated protein kinase
Mal/TIRAP	MyD88 adaptor like protein/ TIR domain accessory protein
MCP-1	Monocyte chemoattractant protein-1
MEFs	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase /extracellular-signal regulated kinase kinase
MEKK	Mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase kinase
MIP	Macrophage inflammatory protein
MSK1	Mitogen- and stress-activated protein kinase
MTX	Methotrexate
MyD88	Myeloid differentiation gene 88
NIK	NF-κB inducing kinase
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear Factor-κB
NK	Natural killer cells
NLS	Nuclear localisation signal
nNOS	Neuronal nitric oxide synthase

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidinedithiocarbamate
PECAM	Platelet-endothelial cell adhesion molecule
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PKAc	Protein kinase A catalytic subunit
PKC	Protein kinase C
PKR	Protein Kinase R
PLC	Phospholipase C
PM	Plasma membrane
PMA	Phorbol 12-myristoyl 13-acetate
PMN	Polymorphonuclear leukocytes
PNS	Peripheral nervous system
PNAd	Peripheral node addressin
PP2A	Protein phosphatase 2A
PPARγ	Peroxisome proliferator-activated receptor-γ
PSGL	P-selectin glycoprotein ligand
RANK	Receptor activator of NF-κB
RHD	Rel homology domain
RIP	Receptor interacting protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src-homology region-2
SH3	Src-homology region-3
SHP	Src-homology region-2 containing protein tyrosine phosphatase
SOCS	Suppressor of cytokine signalling
SODD	Silencer of death domains
STAT	Signal transducer and activator of transcription

SUMO	Small ubiquitin-like modifier
T6BP	TRAF 6 binding protein
TAB	TAK-accessory binding protein
TACE	TNFα converting enzyme
TAK	TGFβ-activated kinase
TBK/NAK/ T2K	TANK-binding kinase/ NF-κB activating kinase/ TRAF 2-associated kinase
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	Transformation effector site
TGFβ	Transforming growth factor β
THP-1	Human monocytic leukemic cell line
TIR	Toll-like/interleukin-1 region
TIRP	TIR-domain-containing-adapter-protein
TLR	Toll-like receptor
TMB	3,3'5,5'-tetramethyl benzidine
TNFα	Tumour necrosis factor-α
TNFR	TNFα receptor
TOLLIP	Toll interacting protein
TPL-2	Tumour progression locus-2
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA	Very late antigen
WT	Wild type

All standard one and three letter amino acid codes have been used throughout.

Pharmacological Names

CGS21680	2-[*p*-(2-carboxyeth yl)phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine
NECA	5'-*N*-ethylcarboxamidoadenosine
ZM241385	4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl)phenol

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ABSTRACT

Adenosine is a potent inhibitor of inflammatory responses, and the A_{2A} adenosine receptor (A_{2A}AR) plays a key role in this process *in vivo*. This thesis has demonstrated that A_{2A}AR gene expression in human umbilical vein endothelial cells (HUVECs) and in C6 glioma cells could inhibit multiple inflammatory responses *in vitro* even in the absence of agonist. This is indicated by the reduced induction of the adhesion molecule E-selectin, by over 70% in HUVECs in response to either TNFα or LPS isolated from *E.coli*. In addition, the induction of inducible nitric oxide synthase (iNOS) was abolished in C6 glioma cells following treatment with interferon-γ (IFNγ) in combination with LPS or TNFα. This suggests that A_{2A}AR expression inhibits a common step in the induction of each of these pro-inflammatory genes. In agreement with this, A_{2A}AR expression was found to inhibit the activity of NF-κB, a key transcription factor in the expression of these pro-inflammatory genes. NF-κB binding to target DNA was severely inhibited in both cell types however, the mechanisms that mediated this were distinct. A_{2A}AR expression in HUVECs inhibited NF-κB translocation to the nucleus independent of any effect on the degradation of IκBα. In contrast, receptor expression in C6 glioma cells could block phosphorylation of IκBα resulting in its reduced degradation. In addition, receptor expression could also increase IFNγ-mediated degradation of STAT1, which may contribute to the complete loss of iNOS expression. Together, these results indicate that adenosine acting *via* the A_{2A}AR can inhibit pro-inflammatory responses by specifically inhibiting activation of the NF-κB and JAK-STAT signalling cascades at multiple steps.

Chapter 1
Introduction

1.1 General Inflammation

Inflammation is defined as "..a defensive response mediated by the immune system that begins after cellular injury to the vasculature" (Villarreal *et al.*, 2001). Many different pro-inflammatory stimuli lead to cellular injury; microbes, e.g. bacteria and viruses, physical agents, e.g. a burn or cut, chemicals, e.g. strong acids or alkalis and/or inappropriate immunological reactions to inflammatory stimuli or to self-antigens (auto-antigen; Sullivan *et al*, 2000). This variety of causes mean that inflammation plays a key role in the pathogenesis of several different disease states including, septic shock (Van Amersfoort *et al.*, 2003), ischaemia-reperfusion injury (Sullivan *et al*, 2000), atherosclerosis (Greaves and Channon, 2002), rheumatoid arthritis (Firestein, 2003), inflammatory bowel disease (IBS; Barbara *et al.*, 2002; Carty and Rampton 2003), asthma (Halayko and Amrani, 2003) and in neuro-inflammatory diseases such as multiple sclerosis (Bradl and Hohlfeld, 2003), stroke (Price *et al.*, 2003) and Alzheimer's disease (Akiyama *et al.*, 2000). Consequently, intensive research centres on the inflammatory process as effective anti-inflammatory therapeutic strategies may provide a wide-ranging solution to several different disease states.

Inflammatory processes can be divided into two types: acute or chronic, based on the duration of the response. This classification is quite arbitrary as the appearance of the lesion, if accessible, can often give a clearer indication of the type of inflammation (Villarreal *et al.*, 2001). Important differences that exist between acute and chronic inflammation are described in Table 1.

1.2 Acute Inflammation

Classical symptoms of acute inflammation include redness, heat, oedema and pain, which reflect a wide range of underlying cellular responses (Tortora and Grabowski, 2003). These include coagulation (Monroe and Hoffman, 2002; Weyrich *et al.*, 2003), vasodilatory increases in blood flow, increased vascular permeability at the site of infection (Suzuki *et al.*, 2003; Yuan, 2002), release of fluid (exudate) from the infected site, migration and accumulation of inflammatory leukocytes from blood vessels into the tissue (Muller, 2003), formation of granulomatous tissue and ultimately, tissue repair (Davis *et al.*, 2003). A comprehensive review of all aspects of inflammation is beyond the scope of this thesis, consequently, it will focus on the trafficking of leukocytes and the signalling pathways stimulated in response to pro-inflammatory mediators.

The recruitment of leukocytes to sites of inflammation comprises of three separate stages (Figure 1.1), 1) initial recognition and recruitment of leukocytes, 2) firm adhesion between leukocytes and the endothelium, and lastly 3) migration of leukocytes from the blood vessel to the inflamed area. Cell types involved in this process include platelets, neutrophils, monocytes and macrophages (Weyrich *et al.*, 2003). In addition, non-haematopoietic cells such as fibroblasts, smooth muscle cells and endothelial cells also play a key role in the inflammatory response. To provide an effective, appropriate response, a paracrine and autocrine communication network exists between these different cell types. This is mediated *via* soluble inflammatory mediators such as cytokines e.g. tumour necrosis factor α (TNFα) and interleukin-1 (IL-1), chemokines e.g. monocyte chemoattractant protein (MCP-1) and interleukin-8 (IL-8), and growth factors e.g. platelet-derived growth factor (PDGF) which will be discussed in Sections 1.4, 1.5 and 1.6 (Figarella-Branger *et al.*, 2003; Ulbrich *et al.*, 2003). The precise expression and secretion of these pro-inflammatory mediators is essential to all stages of the initial recognition and recruitment of leukocytes.

The expression and secretion of pro-inflammatory mediators is stimulated by the recognition of different pathogenic stimuli by receptors expressed at the cell surface. This leads predominately to the recruitment of neutrophils to the site of injury. Upon arrival, neutrophils roll along the endothelial cell boundary due to weak adhesive interactions between adhesion molecules present on both neutrophils and endothelial cells. Effective neutrophil recruitment requires the expression of P- and E-selectin, as knockout mice show impairment in neutrophil adhesion to the endothelium (Jung and Ley, 1999; Robinson *et al*, 1999). This demonstrates the key role selectins play in the adhesion of neutrophils and monocytes to the endothelium.

1.2.1 Selectins

Selectins are a family of three type 1 glycoproteins: E-, P-, and L-selectin (Figure 1.1). They range in size from 138-212 amino acids long and all have a similar domain structure. They consist of an N-terminal lectin-like domain, an epidermal growth factor (EGF) domain, 2-9 short consensus repeat domains, a short transmembrane region and a short cytoplasmic tail (Ley, 2003). The expression of each selectin is cell type-specific. Thus,

Table 1 **Acute *versus* Chronic Inflammation**

This table outlines the key differences between acute and chronic inflammation.

Table 1

	Acute Inflammation	Chronic Inflammation
Duration of response	Within seconds or minutes or at most hours.	Results after 6 weeks; Can last for months, years.
Possible Symptoms	Redness, Heat, Oedema, Pain.	Fever, Tiredness, Systemic wasting.
Predominant cell type	Neutrophil	Macrophage, Lymphocytes

L-selectin is constitutively expressed on leukocytes, some B and T lymphocytes and some natural killer (NK) cells, while P-selectin is stored in α-granules of platelets and in Weibel-Palade bodies of endothelial cells and is rapidly transferred to the cell surface upon cellular activation. In contrast, E-selectin is inducibly expressed in endothelial cells upon exposure to inflammatory stimuli. All selectins bind to fucosylated and sialylated glycoproteins, e.g. sialyl $Lewis_{(x)}$, which are expressed on inflammatory target cells. P-selectin weakly binds to P-selectin glycoprotein ligand (PSGL) which is found on infiltrating leukocytes (Ulbrich *et al.*, 2003). It has been reported that the weak interaction of P-selectin and PSGL-1, can be strengthened by its dimerisation with other P-selectin-PSGL-1 pairings (Zimmerman, 2001). PSGL-1 is also the major ligand for L-selectin in inflammation although L-selectin is more important in the homing of T lymphocytes to peripheral lymph nodes. This is mediated by L-selectin ligands such as peripheral node addressin molecules expressed on high endothelial venules of secondary lymph organs (Lowe, 2002). E-selectin binds to E-selectin-ligand-1 (ESL-1), a 150kDa high affinity sialoglycoprotein expressed on the surface of infiltrating leukocytes (Mourelatos *et al.*, 1996). LAD-II, a rare leukocyte deficiency disease is caused by a mutation in the gene that encodes a fucose transporter. This demonstrates the importance of fucosylation in the generation of selectin ligands, which are required for effective leukocyte adhesion. As a result, patients suffer from bacterial infections of the mucosal membranes and skin (Luhn *et al.*, 2001). The interaction of selectins on both the neutrophil and endothelial cell, in addition to the presence of cytokines and chemokines, leads to the expression of other types of adhesion molecule, which mediate the firm adhesion of the neutrophil to the endothelium. These are members of the immunoglobulin-like cell adhesion molecule (Ig-CAM) family and integrin adhesion molecules.

1.2.2 Immunoglobulin-like adhesion molecules (Ig-CAM)

Ig-CAMs are transmembrane glycoproteins that are characterised by the presence of several Ig-like binding extracellular domains (Figure 1.1). While there are many Ig-domain-containing proteins, those important in leukocyte-endothelial interactions are intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-2 (ICAM-2), vascular cell adhesion molecule (VCAM) and platelet endothelial cell adhesion molecule (PECAM; Bevilacqua *et al*, 1994). ICAM-1 contains five Ig-like domains and is expressed solely in leukocytes, fibroblasts, and epithelial cells in response to pro-inflammatory signals. Once expressed at the surface, ICAM-1 is able to bind to its cognate integrin-

binding partner LFA-1 ($\alpha_L\beta_2$) expressed on infiltrating leukocytes. By contrast, ICAM-2 which is constitutively expressed by endothelial cells, contains only two Ig-like domains, which share 35% amino acid identity with the last two ligand-binding domains of ICAM-1 (Lehmann *et al*, 2003). This results in a degree of redundancy between the two ligands, however, upon induction, ICAM-1 is predominately expressed in excess of ICAM-2. There are also family members ICAM-3-5 but although only ICAM-3 is minimally expressed in endothelial cells, it is thought not to be involved in leukocyte interactions (Patey *et al*., 1996).

There are two isoforms of VCAM-1, which are generated by alternative splicing. This results in either a full-length protein that contains seven Ig-like domains in the N-terminus or a protein that consists of all the domains except domain 4, which results in an adhesion molecule with reduced affinity (Figure 1.1; Cybulsky *et al*., 1991). Full length VCAM-1 is predominately expressed on endothelial cells in response to pro-inflammatory stimuli similar to ICAM-1, and will bind to the $\alpha_{IV}\beta_1$ integrin expressed on infiltrating leukocytes. VCAM-1 ligand binding stimulates NADPH oxidase-dependent production of reactive oxygen species (ROS) by endothelial cells, which are essential for lymphocyte migration into inflamed tissue (Matheny *et al*., 2000).

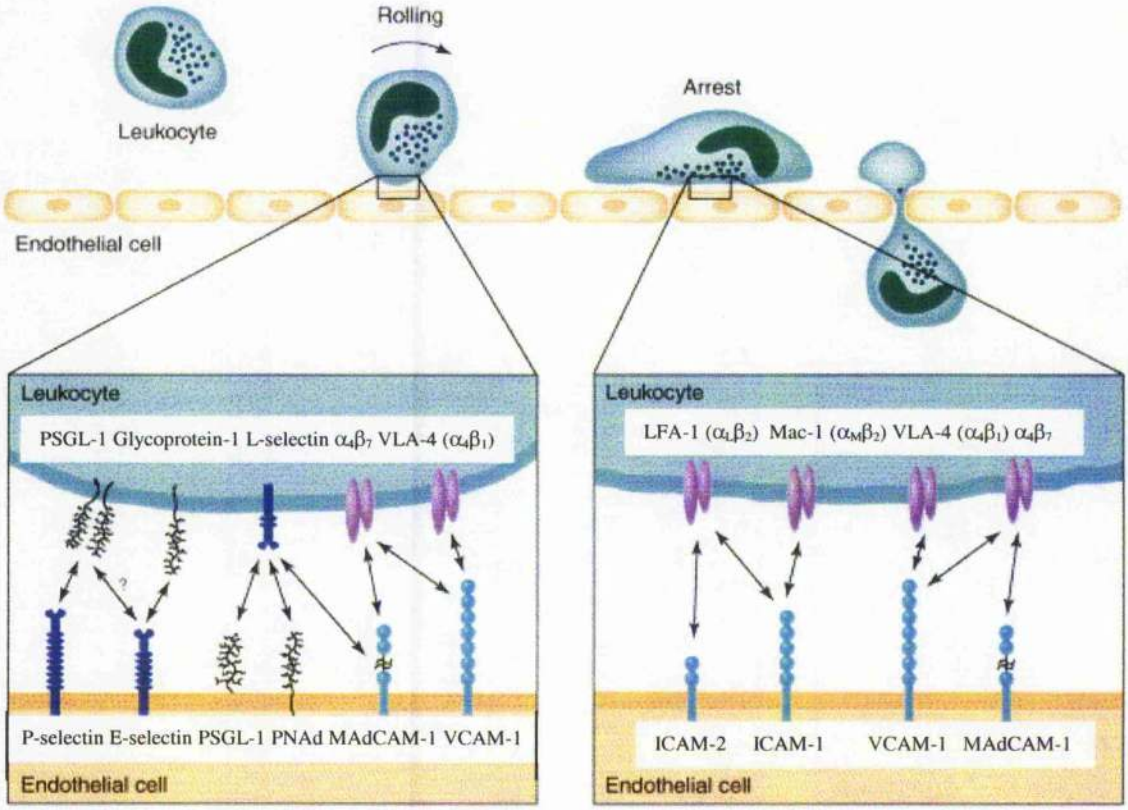
The primary transcript for PECAM is also subject to alternative splicing resulting in several domain-deficient proteins (Wang *et al*, 2003). These are constitutively expressed on endothelial cells, platelets, monocytes, neutrophils and specific subsets of T lymphocytes. Possibly because of its many isoforms, PECAM is able to bind several substrates: these include homophillic (Sun *et al*., 1996) as well as heterophillic interactions with proteoglycans (DeLisser *et al*., 1993) and integrins ($\alpha_v\beta_3$; Buckley., *et al*., 1996).

1.2.3 Integrins

Integrins each consist of a non-covalently associated heterodimer of α and β subunits comprising large ligand binding domains and short cytoplasmic domains (Figure 1.1). Binding of integrins to cognate ligands expressed on infiltrating leukocytes and to components of the ECM, cause changes in the cell cytoskeleton *via* a domain in their C-terminus. There are 19 different α and 8 different β subunits reported in vertebrates leading to many different possible integrin combinations (Shimaoka *et al*., 2002). The integrins involved in leukocyte-endothelial interactions are those that bind to the Ig-like

Figure 1.1 Adhesion molecules involved in leukocyte recruitment to inflammatory sites

Different adhesion molecules are involved in the recruitment of leukocytes to inflammatory sites. The left panel indicates the predominance of selectin-mediated adhesion during the 'rolling' phase of recruitment (Section 1.2.1). The right panel indicates the switch to Ig-like cell adhesion molecule-integrin interactions during the arrest and transendothelial migration of leukocytes (Section 1.2.2 & 1.2.3). (Taken from Ulbrich *et al.*, 2003)


Rolling
Leukocyte
Arrest
Endothelial cell
Leukocyte
PSGL-1 Glycoprotein-1 L-selectin $\alpha_4\beta_7$ VLA-4 ($\alpha_4\beta_1$)
P-selectin E-selectin PSGL-1 PNAd MAdCAM-1 VCAM-1
Endothelial cell
Leukocyte
LFA-1 ($\alpha_L\beta_2$) Mac-1 ($\alpha_M\beta_2$) VLA-4 ($\alpha_4\beta_1$) $\alpha_4\beta_7$
ICAM-2 ICAM-1 VCAM-1 MAdCAM-1
Endothelial cell

cell adhesion molecules. This includes the $\alpha_L\beta_2$ integrin for ICAM-1 and 2, $\alpha_{IV}\beta_1$ for VCAM-1 and the $\alpha_V\beta_3$ for PECAM. It is important to note that receptor activation by the binding of integrin and Ig-like domain of the cognate CAM, can trigger several signalling cascades including the JAK-STAT and MAPK pathways. In addition, integrins can also transmit signals out of the cell in a process known as 'inside-out' signalling (Miranti and Brugge, 2002).

Acute inflammation stimulates induction of adhesion molecule expression in a defined manner in response to pro-inflammatory stimuli. Firstly, selectin adhesion molecules are expressed at the surface within seconds. This is a transient response designed to recruit infiltrating neutrophils from peak luminal blood flow to the vascular wall, sometimes referred to as 'rolling'. Cytokine and chemokine-stimulated endothelial cells then express Ig-CAMs, which recognise and bind to integrins present on the neutrophil surface. This is a firmer adhesive interaction, which prevents further rolling and arrests the neutrophil at endothelial cell-cell junctions. Finally the sustained interaction of Ig-CAMs and integrins, in addition to the action of cytokines and chemokines, is required to ensure efficient neutrophil migration across the endothelial barrier. In this model of acute inflammation, the temporal nature of adhesion molecule expression is important to the effective recruitment of neutrophils. In contrast, this process occurs simultaneously in chronic inflammation leading to a much more complex lesion.

1.2.4 Other pro-inflammatory responses

In addition to effects on adhesion molecule expression, pro-inflammatory mediators (Section 1.4) can also lead to an increase in the generation of nitric oxide (NO), a potent vasodilatory molecule first identified as endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980; Janssens *et al.*, 1992). NO is enzymatically generated from the conversion of L-arginine to L-citrulline, by nitric oxide synthase (NOS) of which there are three isoforms: eNOS, nNOS and iNOS. eNOS is constitutively expressed on endothelial cells, platelets, and some astrocytes, and releases small amounts of NO that regulate vascular tone (Palmer *et al.*, 1987), inhibit platelet aggregation (Radomski *et al.*, 1987) and neuro-transmission (Garthwaite, 1991). nNOS is also constitutively expressed throughout the CNS and on nitrergic nerves in the PNS and is also important in neuro-transmission. iNOS, in contrast, is an inducible form of NOS and is expressed in many tissues in response to ischaemic, traumatic, neurotoxic, endotoxic or inflammatory insults (Lee *et al.*,

2003). iNOS is known to be regulated at the level of transcription by several transcription factors including NF-κB, AP-1, and STAT proteins (Nishiya *et al.*, 2000; Ganster *et al.*, 2001), and their upstream signalling cascades (Nishiya *et al.*, 2000; Xu and Malave, 2000; Dell'Albani *et al.*, 2001). iNOS also produces 1000-fold excess of NO in response to inflammatory mediators compared with cNOS and nNOS, which contributes to the anti-microbial effects in infections and tumour cells. The precise anti-microbial mechanism of NO is unclear although, in addition to the activation of soluble guanylyl cyclase leading to the generation of cGMP, NO can also inhibit cellular respiration *via* the inhibition of mitochondrial cytochrome c complex. At higher concentrations, it can also interact with superoxide anions (O_2^-) to form peroxynitrite, a strong oxidant that can promote lipid peroxidation and nitration of tyrosine residues on target proteins leading to cellular injury (Coeroli *et al.*, 1998; Moncada and Higgs, 2001). The production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), can also have non-cytotoxic effects, including the modulation of cytokine responses in lymphocytes and the regulation of immune cell apoptosis (Bogdan *et al.*, 2000; Garg and Aggarwal, 2002). The regulation of NO synthesis, and the subsequent generation of ROS/RNS is critical, as the cytotoxic effects of ROS/RNS, can lead to respiratory, neurodegenerative and chronic inflammatory diseases (Moncada and Higgs, 2001; Nevin and Bradley, 2002).

Failure of the acute inflammatory response to deal effectively with the pathogenic stimuli, leads to an inappropriately prolonged immune response termed chronic inflammation. This is also governed by the array of pro-inflammatory mediators released by the acute inflammatory response. Consequently, the accurate expression and secretion of these inflammatory mediators is essential to the resolution of inflammation and the restoration of normal tissue homeostasis.

1.3 Chronic inflammation

Chronic inflammation can be divided into two types dependent upon the degree of granuloma tissue formed within the lesion. Granuloma tissue consists of a localised collection of infiltrating leukocytes and networks of fibroblasts, which continue to grow and develop due to the continued presence of cytokines, chemokines and growth factors. It generally has systemic symptoms such as fatigue, weight loss and generalised wasting, examples of which are rheumatoid arthritis, and multiple sclerosis (Wakefield and Kumar,

2001). In contrast atherosclerosis, which exhibits none of these symptoms, is also an example of chronic inflammation because of the persistent low-level inflammatory response following injury to the endothelium, which is a prelude to atherosclerotic plaque formation and plaque rupture. This releases a wide array of thrombogenic agents including pro-inflammatory mediators that increase the inflammatory response. This systemic leakage also leads to a greater immune response by both the innate and adaptive immune system (Greaves and Channon, 2002). The variety of chronic inflammatory diseases is attributed to differences in recruited cells of the immune system, the cell-specific expression of pro-inflammatory mediators and the cellular responses generated. This underlies the importance of pro-inflammatory cytokines, chemokines and growth factors in chronic inflammation.

1.4 Cytokines

Cytokines are specific inflammatory mediators that are differentially regulated during inflammatory conditions. They have multiple functions and are released from several different sources, which act locally to control the inflammatory response. Key examples of cytokines involved in the inflammatory process are TNFα, IL-1 and interferon-γ (IFNγ), which will be explored in more detail.

1.4.1 Tumour Necrosis Factor α (TNFα)

TNFα is a homotrimer that is generated by the proteolytic cleavage of transmembrane TNFα by a metalloprotease TNFα-converting enzyme (TACE; Moss *et al.*, 1997). First recognised as a soluble factor responsible for the necrosis of tumours in mice, it is now known to be part of a wider TNF superfamily, members of which are ubiquitously expressed throughout the vasculature. Each TNF family member binds to a cognate receptor found expressed on its target cells, which mediates the downstream effects. There are two receptors that are selective for TNFα, namely TNFR1 (75kDa) and TNFR2 (55kDa). TNFR1 has been widely studied and comprises of an N-terminal TNFα binding domain, a short transmembrane region and an intracellular death domain (DD) in its C-terminus (Figure 1.2). TNFα binding results in receptor trimerisation causing the release of the protein, silencer of death domains (SODD; Chen and Goeddel, 2002). This exposes the DD of the receptor, which acts as a scaffold for other DD-containing proteins including the adaptor protein, TNFα receptor-associated death domain (TRADD). TRADD binds to

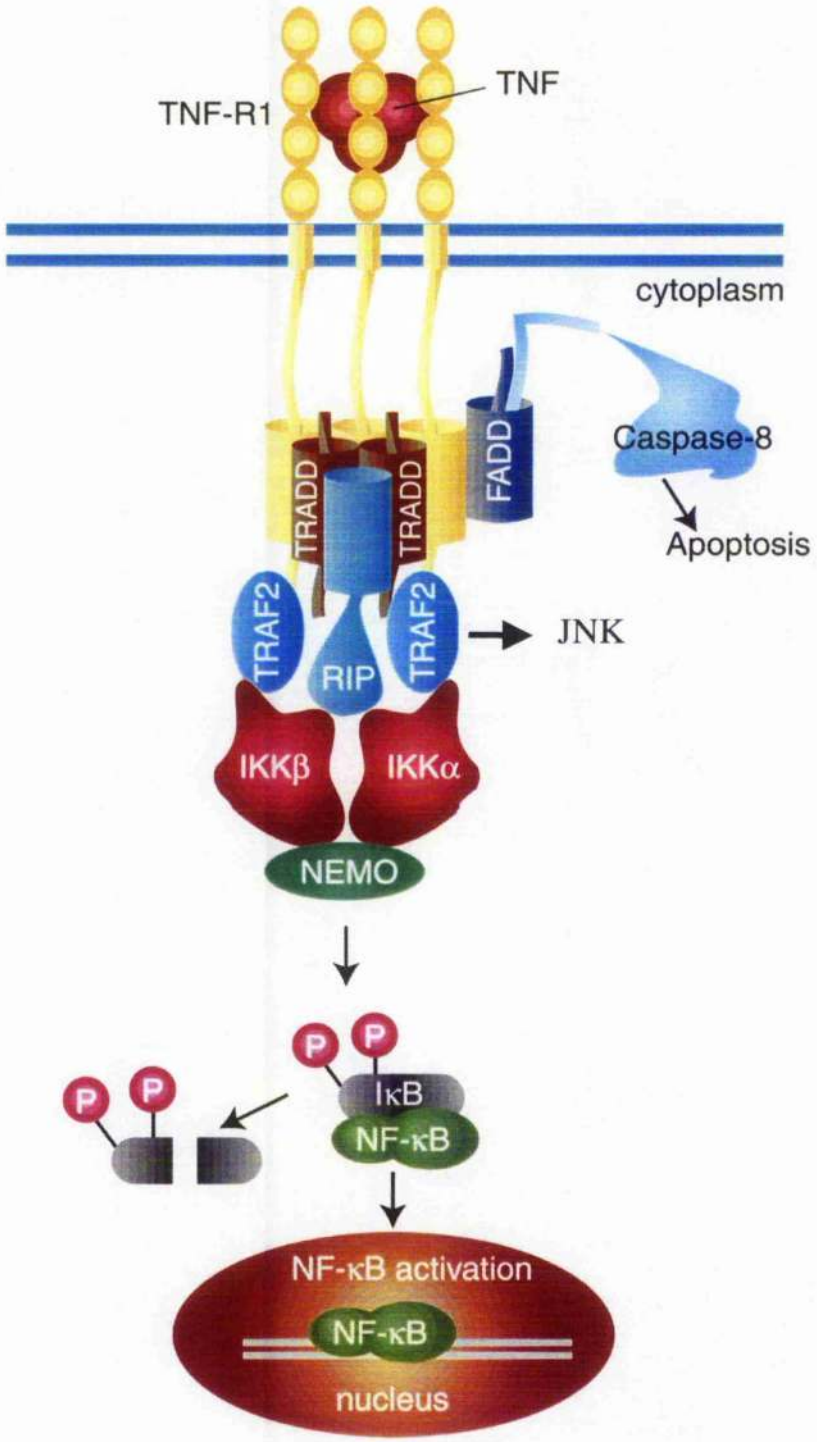
the receptor and is able to recruit other downstream signalling proteins to the receptor e.g. receptor-interacting protein (RIP) and TNFα receptor-associated factors (TRAFs). TRAFs are a family of six adaptor proteins (TRAF1-6), which are important in the downstream signalling pathways generated by pro-inflammatory receptors belonging to the TNF family and IL-1 receptor family. They consist of a C-terminal TRAF domain that is responsible for receptor recognition, five Zn binding domains and an N-terminal RING finger motif (Except TRAF-1), which mediates the signalling properties of TRAFs (Dempsey *et al.*, 2003). The TRAF domain is also required for the formation of homo- or hetero-trimers among specific TRAF family members (Pullen *et al.*, 1998; Park *et al.*, 1999). TNFR1-TRADD interactions lead to the recruitment of TRAF-2. This interacts with several other downstream signalling proteins including IκB kinase (IKK; Section 1.8), mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase kinase -1 (MEKK1; Baud *et al.*, 1999), apoptosis signal-regulating kinase-1 (ASK-1; Nishitoh *et al.*, 1998), germinal centre kinase (Yuasa *et al.*, 1998) and the germinal centre kinase-related kinase (Shi and Kehrl, 2003), which leads to the activation of nuclear factor-κB (NF-κB; Section 1.8), c-Jun-N-terminal kinase (JNK) and p38 MAPK (Song *et al.*, 1997; Saccani *et al.*, 2002; Platanias, 2003). Activation of JNK and p38 lead to the phosphorylation and activation of AP-1 heterodimers, which are important immuno-regulatory transcription factors. In addition to the activation of NF-κB, JNK and p38 MAPK, TNFα can also lead to the recruitment of the protein Fas-associated death domain (FADD). This protein can bind to TRADD and to the TNF receptor *via* its death domain. In contrast to TRADD, it also has a death effector domain (DED), which is a binding site for other DED containing proteins like procaspase-8. Upon recruitment to FADD, pro-caspase 8 undergoes proteolytic cleavage, which allows it to target other effector caspases such as caspase-3 and caspase-9 leading to apoptosis.

1.4.2 Interleukin-1

Two forms of interleukin-1 (α & β) were first cloned by March *et al.*, (1985) and were shown to act in concert with TNFα as a pro-inflammatory cytokine. IL-1α and IL-1β are synthesised as pro-IL-1 isoforms and are proteolytic cleaved by IL-1 converting enzyme to generate mature proteins of 17 kDa (Black *et al.*, 1989). IL-1 is synthesised and released by activated macrophages, monocytes and endothelial cells to act on target cells expressing interlekin-1 receptors (IL-1R; Figure 1.3; Sims *et al.*, 1988). The IL-1 receptor comprises

Figure 1.2 TNFα receptor signalling

TNFα binding induces receptor trimerisation leading to the release of SODD allowing the interaction of the receptor death domains. Receptor trimerisation generates a scaffold for the recruitment of adaptor proteins such as TRADD, FADD, TRAF2 and RIP, which are essential for downstream signalling. Recruitment of TRAF2 and RIP is necessary to activate the IKK complex leading to the phosphorylation-dependent polyubiquitination of IκB. This results in its degradation by the 26S proteosome, which allows NF-κB to translocate to the nucleus and activate transcription (Section 1.4.1). In addition, TRAF2 is also required for the TNF receptor-dependent activation of JNK. Recruitment of FADD to the TNFR causes the recruitment and activation of Caspase-8 leading to downstream signalling events that trigger apoptosis.


TNF-R1
TNF
cytoplasm
TRADD
TRADD
FADD
Caspase-8
Apoptosis
TRAF2
RIP
TRAF2
JNK
IKKβ
IKKα
NEMO
P
P
IκB
NF-κB
P
P
NF-κB activation
NF-κB
nucleus

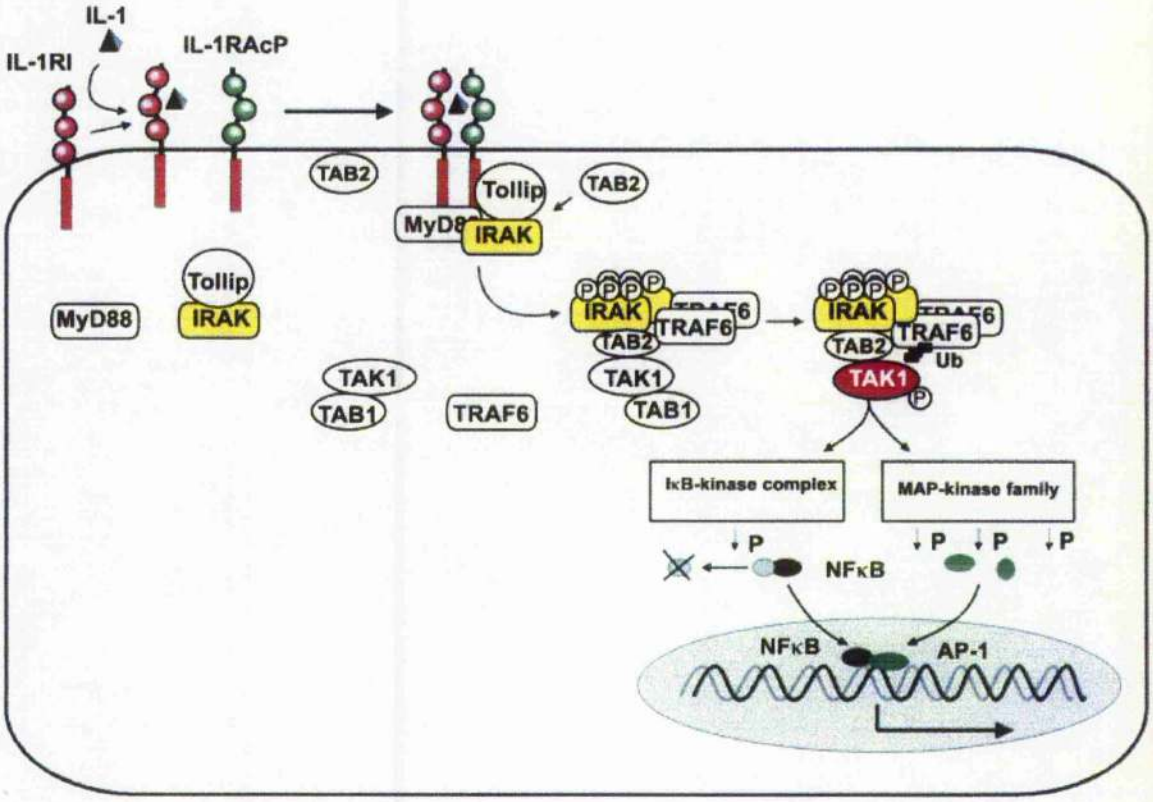
of a single transmembrane protein containing an N-terminal Ig-like domain and a C-terminal Toll-like/interleukin-1 (TIR) domain, which interacts with the transmembrane IL-1R accessory protein (IL-1RAc-P), upon ligand activation. This interaction recruits the adapter protein Myd88 *via* a distinct domain in the C-terminus. This domain is common to other inflammatory receptors of the Toll family (Section 1.4.4). Recruitment of Myd88 allows the subsequent recruitment of IL-1 receptor associated kinase (IRAK) whose subsequent phosphorylation, leads to the recruitment of TRAF 6. This protein acts as a scaffold for the recruitment of several kinases which signal to different downstream targets such as NF-κB and JNK. Several additional adapter proteins have recently been discovered whose role is as yet unclear including other IRAKs (Wesche *et al.*, 1999), Toll-interacting protein (Tollip; Burns *et al.*, 2000), TRAF 6 binding protein (T6BP; Ling and Goeddel, 2000), TIR-domain-containing-adapter-protein (TIRP; Bin *et al.*, 2003), TGFβ-activated kinase (TAK) and the accessory proteins TAB1 (TAK-binding protein) and TAB-2 (Takaesu *et al.*, 2000) and evolutionarily conserved signalling intermediate in Toll pathways (ECSIT; Kopp *et al.*, 1999). Activation of IL-1 signalling leads to the transcription of many pro-inflammatory genes such as adhesion molecules, TNF, Cyclo-oxygenase (COX-2), inducible nitric oxide synthase (iNOS) and collagenases which all participate in the immune response

1.4.3 Interferon gamma (IFNγ)

The potent anti-viral properties of interferons were first identified because of their ability to interfere with viral replication (Isaacs and Lindenmann, 1987). In addition, they now have several other physiological roles including a role in microbial defence (Shtrichman and Samuel, 2001). They are divided into two types; I and II, with IFNγ constituting the sole family member of the type II class. It is synthesised and released from natural killer (NK) cells, helper T lymphocytes (T_H1 cells) and cytotoxic T lymphocytes (T_c) (Young, 1996; Bach *et al.*, 1997), in response to other pro-inflammatory stimuli such as interleukin-12 (IL-12; Lammas *et al.*, 2000), IL-18 (Akira, 2000) and TNFα. IFNγ is 17kDa in size and functions as a dimer, which binds to the IFNγ receptor (IFNγR) expressed on nearly all cell surfaces (Ramana *et al.*, 2002). The IFNγR is composed of two heterologous subunits, the IFNγR-α and –β subunits. Receptor activation leads to heterologous dimerisation and the subsequent activation of Janus activated kinase (JAK) signal transduction pathway (Darnell *et al.*, 1994). JAK1 is recruited to IFNγR-α while JAK2 is recruited to IFNγR-β

Figure 1.3 IL-1 receptor signalling

IL-1-mediated binding to the IL-1R-IL-1RAcP complex recruits the adaptor protein MyD88 thus recruiting IRAK to the receptor. Autophosphorylation of IRAK leads to dissociation from the receptor and the activation of TRAF6. This leads to the formation of an IRAK/TRAF6/TAK1/TAB1/TAB2 complex leading to phosphorylation-dependent ubiquitination of TAK1 and TRAF 6. Active TAK1 can then phosphorylate further downstream kinases such as the IKK complex and MAP kinase, leading to the activation of NF-κB-and AP-1-dependent transcription.


IL-1
IL-1RI
IL-1RAcP
TAB2
Tollip
TAB2
MyD88
IRAK
Tollip
MyD88
IRAK
IRAK
TRAF6
TAB2
TAK1
TAB1
TAK1
TAB1
TRAF6
IRAK
TRAF6
TAB2
Ub
TAK1
P
IκB-kinase complex
MAP-kinase family
P
NFκB
P
P
P
NFκB
AP-1

(Shtrictmann and Samuel, 2001). JAK activation leads to tyrosine phosphorylation of signal transducers and activators of transcription (STAT) proteins. Phosphorylation of STAT1 induces homologous dimerisation and translocation to the nucleus, where it binds to target genes containing the gamma activation sequence (GAS; $TTCN_3GAA$) to initiate transcription. Target genes include interferon regulatory factor (IRF)-1, iNOS and suppressors of cytokine signalling-1 (SOCS-1). This simplistic model of JAK-STAT signalling is regulated in a complex manner by the antagonistic interactions of specific STAT family members. For example in endothelial cells, STAT3 can antagonise STAT1 activity. This interaction can be further regulated by the expression of SOCS-1 (Samuel, 2001).

1.4.4 Lipopolysaccharide (LPS)

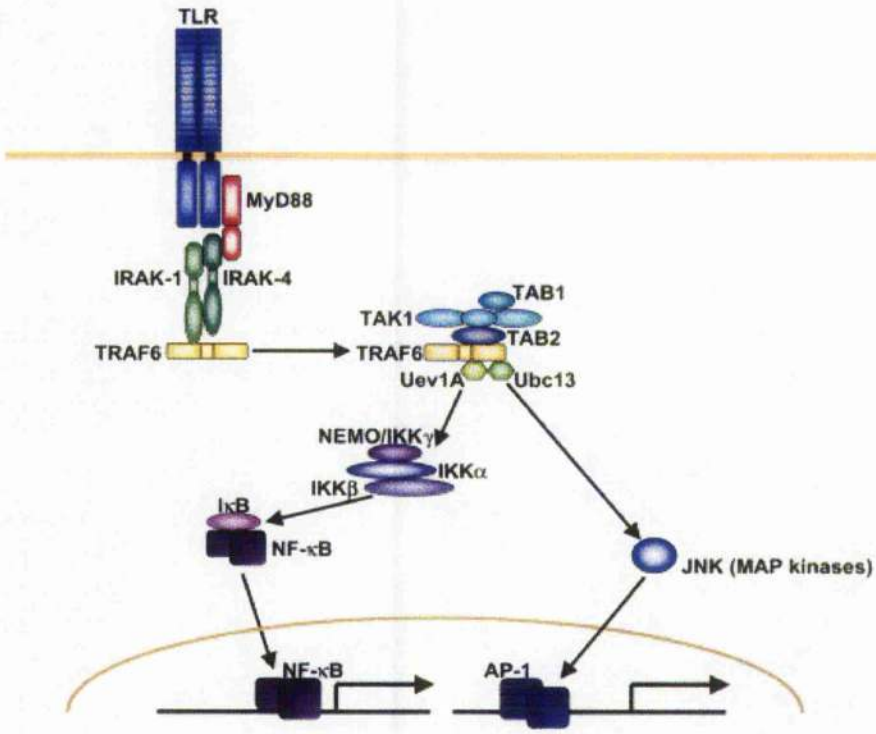
LPS is not a member of the cytokine family however it is a potent pro-inflammatory stimulus. It is a major structural component of the outer wall in Gram–negative bacteria and can activate monocytes, macrophages and endothelial cells to produce many pro-inflammatory cytokines, including TNFα, IL-1, IL-6, IL-8 and IL-12 (Cohen, 2002). LPS can initiate effects by binding to a cell-surface receptor-binding complex (Figure 1.4). This complex consists of CD14 (Schumann *et al.*, 1990; Fujihara *et al.*, 2003), a 55kDa transmembrane glycoprotein, Toll-like receptor-4 (TLR-4; Poltorak *et al.*, 1998a; Poltorak *et al.*, 1998b), a member of the Toll-like receptor family and myeloid differentiation protein-2 (MD-2), a co-receptor protein necessary for efficient TLR-4 expression and LPS recognition (Shimazu *et al.*, 1999; Silva Correia *et al.*, 2001).

1.4.4.1 Activation of LPS signalling

LPS binding protein (LBP) present in serum, transfers LPS to either membrane-bound or soluble CD14, as studies indicate that endothelial cells and epithelial cells lacking membrane-bound CD14, are still LPS-responsive (Pugin *et al.*, 1994). This complex then binds to the TLR-4-MD-2 receptor to initiate LPS responsive signal transduction pathways. TLR-4 is a member of the wider Toll-like receptor family, which were first characterised in *Drosophila,* and are important in mediating dorsal-ventral polarity in embryonic development (Morisato and Anderson, 1994) and in generating the anti-fungal response in *Drosophila* (Lemaitre *et al.*, 1996). There are ten human TLRs which all share a common (Toll-like/Interleukin-1 Receptor (TIR)-

Figure 1.4 **LPS signalling *via* TLR-4**

Activation of the TLR-4 receptor complex leads to the recruitment of MyD88 *via* the TIR domain. This leads to the phosphorylation of IRAK by IRAK-4, which recruits TRAF 6 to the complex. Upon activation, TRAF6 can interact with the TAK1/TAB1/TAB2 complex that also contains Uev1A and Ubc13. This leads to the polyubiquitination of TRAF6 and TAK1, which activates downstream signalling to IKK and JNK. Taken from Takeda and Akira, 2004.


TLR
MyD88
IRAK-1
IRAK-4
TRAF6
TRAF6
TAB1
TAK1
TAB2
Uev1A
Ubc13
NEMO/IKKγ
IKKα
IKKβ
IκB
NF-κB
JNK (MAP kinases)
NF-κB
AP-1

motif which generates the downstream signalling from TLRs. This motif is also common to the IL-1R family of receptors and consequently, downstream signalling events between TLR and IL-1R can share a common pathway (Imler and Hoffmann, 2001; Janeway and Medzhitov, 2002; Dunne and O'Neill, 2003; Takeda *et al.*, 2003).

Upon LPS binding to TLR-4, a multiprotein complex is recruited to and assembled at the cytoplasmic tail of the receptor mediated by the Toll-like/IL-1 receptor (TIR) domain of the TLR (Figure 1.4; Takeda *et al.*, 2003). Several different proteins have been identified as adaptor molecules for TLR-4. Myeloid differentiation gene 88 (MyD88) is a TIR containing TLR adaptor protein, which is recruited to and binds to the TIR domains of TLR-4 (Wesche *et al.*, 1997; Medzhitov *et al.*, 1998). It is also reported recently that a MyD88-adaptor like protein Mal/TIRAP can also bind to the TIR domain of TLR-4 (Horng *et al.*, 2001; Fitzgerald *et al.*, 2001). In addition to MyD88 and Mal, a further TIR interacting protein (Toll interacting protein) Tollip can also interact with TLRs 2, 4 and 6 (Bulut *et al.*, 2001). This interaction is reported to negatively regulate TLR signalling, as Tollip overexpression can block NF-κB activation in response to TLR-4 agonists (Zhang and Ghosh, 2002). The precise detail regarding the complex interactions between TLR-4 and MyD88, Mal and Tollip remain to be fully elucidated.

Following Myd88 recruitment to TLR-4, IL-1 receptor associated kinase (IRAK) is recruited to the receptor *via* an interaction between the DD present on both proteins, where it becomes phosphorylated by IRAK-4 (Suzuki *et al.*, 2002). The IRAK family consists of four members to date, IRAK-1, IRAK-2, IRAK-4 and IRAK-M which contain a conserved DD and kinase domain: (Li *et al.*, 2002). Each kinase is reported to interact with TLR-4 although the variety of upstream adaptor molecules may explain the non-specific nature of these interactions (Janssens and Beyaert, (2003). IRAK-4 has been reported as the most significant interaction as IRAK-4 deficient mice show almost no inflammatory responses to LPS or IL-1 (Suzuki *et al.*, 2002). Activated IRAK will then interact with TRAF6 a member of the TNF receptor associated factor family leading to the downstream activation of NF-κB and JNK. Activation of TLR-4 signalling leads to the transcription of many pro-inflammatory genes such as adhesion molecules, TNFα, cyclo-oxygenase (COX-2),

inducible nitric oxide synthase (iNOS) and collagenases which all participate in the anti-inflammatory response.

1.5 Chemokines

Chemokines are a large family of small (7-14 kDa) cysteine rich chemoattractant proteins that are central to the inflammatory response (Horuk, 2001). Secreted by leukocytes and other haematopoetic cells they are classified into four different groups according to the position of conserved cysteine residues within the N-terminus: C, CC, CXC, CX3C. Each chemokine binds to cognate receptors which belong to the G-protein coupled receptor (GPCR) family, a large family of transmembrane glycoproteins which will be discussed in detail later (Section 1.11). All chemokines contain a heparin-binding site defined by a highly basic helical region toward the C-terminal end of the protein. It is thought that glycosaminoglycans (GAGs) expressed on the surface of endothelial cells, bind to chemokines through an interaction with the heparin-binding site, a highly basic helical region located close to the C-terminal end of the chemokine. Experimental evidence for the formation of solid phase chemokine gradients was provided by Tanaka *et al.* (1993), who showed that macrophage inflammatory protein (MIP-1) could be immobilized by binding to proteoglycan, and that the immobilized chemokine could induce the binding of T cells to VCAM-1 *in vitro*. This can therefore generate a stable gradient for the effective recruitment of leukocytes. Two of the most important chemokines in leukocyte recruitment are MCP-1 (CCL2) and IL-8 (CXCL8) (Gerszten *et al.*, 1999).

1.5.1 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is prototypical CC family chemokine that was first isolated from LPS-stimulated human monocyte supernatants by Matsushima *et al.*, (1989). It conforms to a general chemokine structure comprising a flexible N-terminus, three anti-parallel β-sheets arranged in a 'Greek key' formation and an α-helix in the C-terminus (Mukaida *et al.*, 1998). It is secreted primarily by endothelial cells, which recruits monocytes and basophils to sites of inflammation. In addition to its chemotactic role, it can also stimulate respiratory burst, increases in β_2-integrin adhesion

Table 2 **Toll-like receptors and their ligands**

The table describes the array of ligands for members of the Toll-like receptor family.

TLR Family member	Some TLR Ligands
TLR-1	Tri-acyl lipoproteins,
TLR-2	Peptidoglycan, Bacterial lipoproteins
TLR-3	Double-stranded RNA
TLR-4	LPS (Gram negative)
TLR-5	Flagellin
TLR-6	Di-acyl lipoproteins
TLR-7	Imidazoquinoline (synthetic compound)
TLR-8	?
TLR-9	Unmethylated CpG DNA
TLR-10	?

molecule expression and the secretion of IL-1 and IL-6 (Rollins *et al.*, 1989; Jiang *et al.*, 1992). In basophils it can also stimulate leukotriene and histamine release. These effects are mediated by the CCR2 chemokine receptor, which is expressed by monocytes, B and T lymphocytes and is modulated by pro-inflammatory mediators LPS, TNFα, IL-1 and IL-6 (David and Mortari, 2000).

1.5.2 Interleukin-8 (IL-8)

IL-8 is a potent CXC chemokine, required for the recruitment of neutrophils to sites of inflammation. Produced *in vitro* by a wide variety of cell types including monocytes, neutrophils and vascular endothelial cells, in response to pro-inflammatory cytokines and LPS, IL-8 binds to two different receptors CXCR1 and CXCR2 expressed on neutrophils (Matsushima *et al.*, 1989; Baggiolini *et al.*, 1994). Different pro-inflammatory stimuli can also regulate receptor expression, for example, unlike CXCR1, CXCR2 expression is increased by neutrophil exposure to TNFα (Asagoe *et al.*, 1998). The active form of IL-8 is 79 amino acids long and conforms to the general chemokine structure. Ligand binding of IL-8 to CXCR1 and CXCR2 activates $G\alpha_{i2}$, which initiates several signal transduction pathways including the activation of phoshoinositide-specific phosphlipase C, protein kinase C (PKC), small GTPases, Src-related protein kinases and protein kinase B/Akt. The multitude of signalling pathways activated lead to the plethora of IL-8-stimulated effects including changes in cell morphology, the generation of ROS, the expression of bioactive lipids and the increase in adhesion molecule expression (Harada *et al.*, 1996; Mukaida *et al.*, 1998; David and Mortari, 2000).

The regulated expression of cytokines and chemokines co-ordinate the cellular response, which mediates the effective destruction and removal of the initial pathogenic stimuli. In the latter stages of inflammation, the secretion and expression of growth factors is essential to the efficient repair of damaged tissue and the restoration of homeostasis.

1.6 Growth factors

Growth factors such as platelet-derived growth factor (PDGF) are an essential requirement for tissue re-modelling that accompanies repair to damaged tissue,

following inflammation. They also play a role in switching off the pro-inflammatory immune response in order to prevent chronic activation.

1.6.1 Platelet-derived growth factor (PDGF)

PDGF is a potent stimulator of growth and migration of connective tissue cells including fibroblasts and smooth muscle cells and also of other cell types including neurons and endothelial cells. Homodimeric PDGF-AA and PDGF-BB and heterodimeric PDGF-AB are synthesised and released by many cell types including vascular endothelial cells, smooth muscle cells and platelets. All isoforms act at two distinct protein tyrosine kinase receptors, PDGF-α (170 kDa) and PDGF-β (180 kDa) which are expressed on a variety of epithelial, endothelial and neural cell types. Consistent with other receptor tyrosine kinase receptors, the PDGF receptors undergo autophosphorylation upon ligand binding. This recruits several Src-homology-2 (SH2) domain-containing proteins to the receptor including phosphoinositide-3-kinase (PI3K), phospholipase C (PLC), Grb2/SOS, SHP-2 and STAT proteins that activate downstream signalling pathways, which promote wound healing following inflammation (Heldin *et al.*, 1998; Heldin and Westermark, 1999).

Many of the actions of cytokines, chemokines and growth factors are controlled at the level of transcription by several transcription factors such as NF-κB, AP-1, and STAT proteins. NF-κB is a key transcription factor responsible for many pro-inflammatory signals which will be explored in more detail.

1.7 Activation of NF-κB

NF-κB plays a central role in controlling gene expression of proteins involved in cell survival and inflammatory responses. The inducibility of NF-κB target genes in response to a diverse range of stimuli is essential to this role. Different pathogenic stimuli activate NF-κB-dependent transcription of target genes, which include Bcl-2, cellular inhibitor of apoptosis (cIAP) proteins, E-selectin and ICAM-2 *via* different mechanisms. For example, NF-κB activation in response to TNFα, has been widely studied and many of the proteins involved in the cascade have been characterised. In contrast, stimuli such as oxidative stress and X-rays are believed to cause NF-κB

activation by a different mechanism *via* an as yet undetermined pathway (Imbert *et al*, 1996; Canty *et al*, 1999).

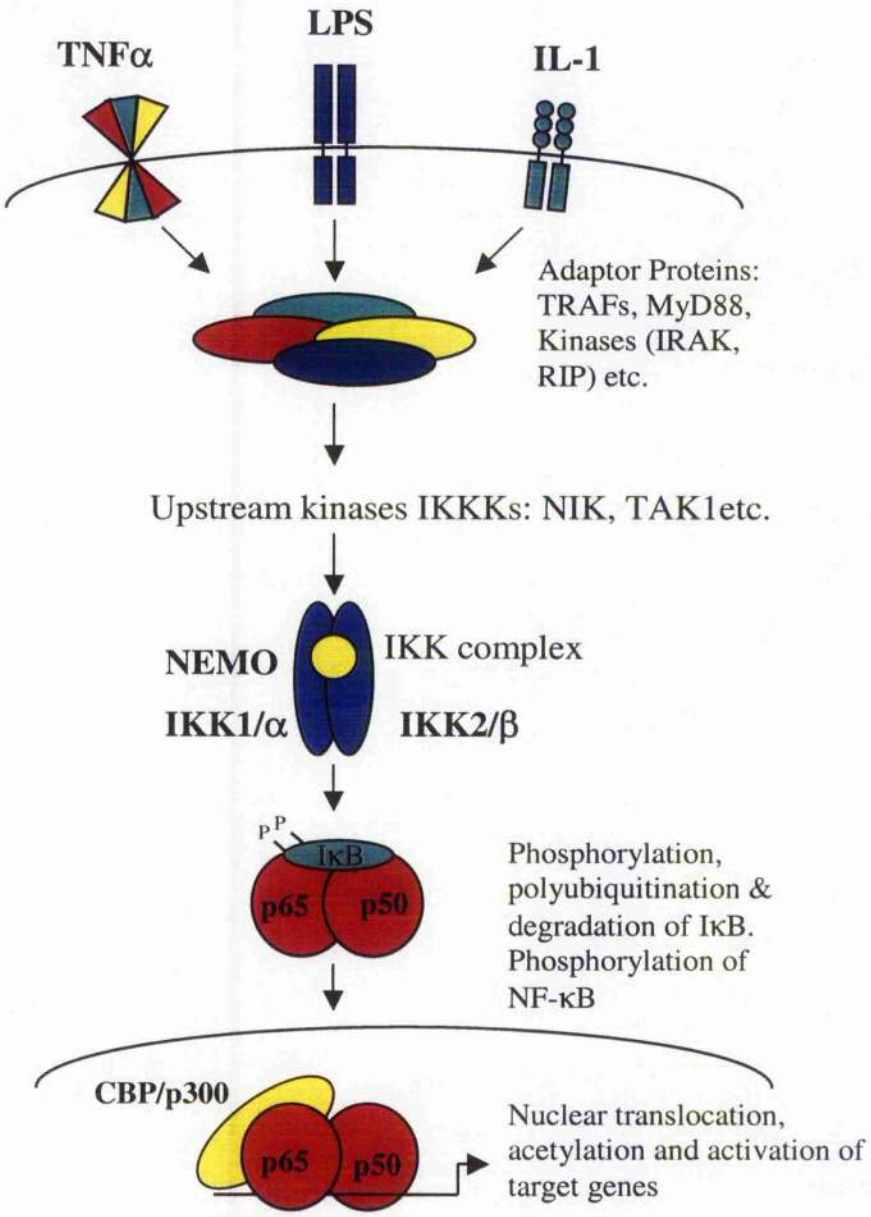
NF-κB is localised in the cytoplasm of unstimulated cells, in an inactive state bound to a member of the IκB family of inhibitory proteins (Ghosh *et al*, 1998; Figure 1.5, 1.6). The degradation of this inhibitor upon signal-induced activation has been regarded as the conventional model by which NF-κB is activated (Baeuerle *et al*, 1988). This exposes the NLS of NF-κB thereby allowing NF-κB to translocate to the nucleus to activate transcription. Many aspects of the signalling pathway that leads to the degradation of IκB family members have been elucidated. Briefly, stimuli-specific signalling pathways converge on the IκB kinase (IKK) which is a multi-protein complex comprising of IKKα/1, IKKβ/2 and IKKγ/NF-κB–essential modulator (NEMO). This specifically phosphorylates IκB at particular residues that prime IκB for polyubiquitination. This targets IκB for proteolytic degradation by the 26S proteosome such that NF-κB-dependent transcription can occur. This outline misrepresents the complexity of NF-κB signalling which involves multiple protein-protein interactions between the different members, which I will now explore in more detail.

1.7.1 IκB Kinase (IKK)

In 1997, five papers were published that used molecular cloning techniques to identify a novel cytokine-responsive IκB-kinase complex that was not dependent on ubiquitination to achieve IκB phosphorylation. Three of these papers described a 900 kDa complex that specifically phosphorylated IκBα at S32 and S36, and IκBβ at S19 and S23, upon TNFα stimulation (DiDonato *et al*, 1997; Mercurio *et al*, 1997; Zandi *et al*, 1997). This complex contained two polypeptides (85 kDa and 87 kDa in size) associated with kinase activities that were eluted on affinity columns (DiDonato *et al*, 1997). DiDonato *et al.* (1997) subjected the 85 kDa polypeptide to microsequencing and showed it to contain sequences identical to a human serine/threonine kinase named CHUK (Conserved Helix-loop-helix Ubiquitous Kinase). Its function was unknown, but with the identification of it now as an IκB-kinase, CHUK was renamed IKK (IκB kinase; Connelly *et al*, 1995). In addition,

Figure 1.5 Activation of NF-κB by different extracellular stimuli.

Many different extracellular stimuli can activate the NF-κB signalling cascade. (See sections 1.4.1, 1.4.2, and 1.4.4.) In general, receptor activation leads to the recruitment of several stimuli-dependent adaptor proteins e.g. TRADD, MyD88. This causes the recruitment of kinases that can activate the IKK complex. This complex specifically phosphorylates IκB resulting in its polyubiquitination-mediated degradation *via* the 26S proteosome. Several kinases can then phosphorylate NF-κB prior to its nuclear translocation. The phosphorylation of p65 directs the association with HATs that in concert with NF-κB, direct transcription of target genes. Adapted from Israel, 2000.


TNFα
LPS
IL-1
Adaptor Proteins:
TRAFs, MyD88,
Kinases (IRAK,
RIP) etc.
Upstream kinases IKKKs: NIK, TAK1etc.
NEMO
IKK complex
IKK1/α
IKK2/β
P P
IκB
p65
p50
Phosphorylation,
polyubiquitination &
degradation of IκB.
Phosphorylation of
NF-κB
CBP/p300
p65
p50
Nuclear translocation,
acetylation and activation of
target genes

Mercurio *et al*, (1997) and Zandi *et al*, (1997) showed that the 87 kDa polypeptide was also similar to CHUK, sharing 52% homology with the 85 kDa polypeptide. In addition, it was more efficient at phosphorylating IκBβ. Therefore, the two polypeptides were re-named IKKα (85 kDa) and IKKβ (87 kDa). Using a yeast two-hybrid screen for NF-κB-inducing kinase (NIK)-interacting proteins, Regnier *et al*, (1997) also identified IKKα. NIK was previously was reported to activate NF-κB in response to TNFα and IL-1β, whilst inactive NIK mutants inhibited NF-κB activation (Malinin *et al*., 1997), suggesting that NIK is a probable converging point of both signalling cascades. Woronicz *et al*, (1997) were also able to isolate IKKβ from a Jurkat T cell cDNA library, using an EST that shared 57% identity to residues 624-658 of IKKα in order to isolate full-length cDNAs. From these papers it became evident that phosphorylated IκB was located in the cytoplasm in a larger multi-protein complex with other unidentified proteins.

Further immunoprecipitation studies by Rothwarf *et al*, (1998), identified two extra polypeptides of 50 and 52kDa. Microsequencing identified both as different forms of the same protein, which were termed IKKγ1 and IKKγ2. Yamaoka *et al*, (1998) also separately discovered IKKγ using a complementation cloning approach, in which cDNAs thought to be involved in NF-κB signalling, were transfected into cells deficient in NF-κB activity and assayed for restoration of function. They termed this protein NEMO (NF-κB essential modulator; Yamaoka *et al*., 1998).

IKKγ/NEMO is a glutamine-rich protein, 419 amino acids in length containing no kinase catalytic domain. It contains coiled-coiled structural motifs, which facilitate binding to IKKα/β heterodimers. Removal of the C-terminus of NEMO prevents signal-induced activation by a variety of stimuli further indicating the significance of this regulatory subunit of the IKK complex (Rothwarf *et al*, 1998). These observations helped to establish that the integrity of the IKK complex was critical for phosphorylation of IκB leading to activation of NF-κB. IKKα, IKKβ and NEMO form a core signalling complex that leads to the phosphorylation of IκB family members. In addition to the core IKK complex, a novel IKK-related kinase has also been identified called IKKε/IKKi. This kinase is an LPS and PMA-inducible kinase

whose role in NF-κB activation is less well understood (Shimada *et al*, 1999; Peters *et al*, 2000; Nomura *et al*, 2000).

1.7.2 IKK activation

Presently, how various stimuli converge on the IKK complex is not yet clearly established. MEKK1, MEKK2, MEKK3 and NIK, members of the MAP3K (mitogen-activated protein kinase kinase kinase) family, have all been shown to phosphorylate IKK *in vitro* (Mercurio and Manning, 1999; Nakano *et al*, 1998). However, only MEKK3 has been identified as a physiologically relevant IKK kinase *in vivo*. MEKK1, which can preferentially bind to IKKβ, is also part of the JNK pathway. In contrast, NIK, preferentially activates IKKα which depends upon a TNFα pathway component, TNF receptor associated factor type 2 (TRAF 2) (Ling *et al*, 1998). However, the precise role of NIK in IKK activation is unclear as $NIK^{-/-}$ knockout mice are still responsive to TNFα and IL-1 but not to lymphotoxin-β (LTβ) (Yin *et al*, 2001). Nevertheless, when comparing mouse embryo fibroblasts (MEFs) from $NIK^{-/-}$ with those from WT, no differences were observed in NF-κB DNA binding activity, in response to either stimulus. This evidence suggests NIK may mediate NF-κB transcriptional activity in a receptor-specific manner *via* the IKKα subunit.

Other kinases known to activate the IKK complex include TBK/NAK/T2K (TANK binding kinase-1/NF-κB activating kinase/TRAF 2-associated kinase) in response to PDGF (platelet-derived growth factor) and the kinase subunit TPL-2 (Tumour Progression Locus 2) in response to T-cell signalling (Lin *et al*, 2000; Tojima *et al*, 2000). TPL 2 also performs a major role in p105 processing to p50 (Belich *et al*, 1999), although none of these have shown a direct association with IKK. Aside from protein kinases, other molecules can also activate the IKK complex. Latent membrane protein-1 (LMP1), a transmembrane protein expressed in Epstein-Barr virus (EBV)-associated carcinomas, results in NF-κB activation by utilising proteins in the TNFα signalling pathway (Sylla *et al*, 1998). It activates IKK by interacting with TRAF2 *via* its carboxy-terminal domain, known as the TES1 (Transformation Effector Site 1) domain, and with RIP and TRADD (TNFR-associated death domain) via a second site known as TES2. Another protein, TAX involved with the

Human T-cell leukaemia virus (HTLV) has been suggested to activate the IKK complex by directly binding to IKKγ/NEMO (Geleziunas *et al*, 1998).

Since proteins other than kinases can also activate the IKK complex directly, it has been postulated that protein-protein interaction rather than kinase activity induces IKK phosphorylation. For example, TNFα binding to TNFR1 recruits TRADD and TRAF 2 and TRAF 6, which then recruit the kinase complex to the receptor, where the protein kinase RIP1 can activate IKK1 *via* MEKK (Kelliher *et al*, 1998; Devin *et al*, 2000). However, IKK activation has been shown not to require RIP1 kinase activity suggesting kinase activity is not essential for this (Devin *et al*, 2000). In addition, IL-1 stimulation, which utilises IRAK1 to activate IKK, also does not require its kinase activity for activation purposes (Li *et al*, 1999). Another kinase that appears to activate IKK via protein-protein interactions is PKR, which responds to dsRNA, viral infection, and LPS from Gram-negative bacteria (Chu *et al*, 1999). To summarise it is possible that protein-protein interactions facilitate autophosphorylation within the IKK complex.

The integration of the many upstream signals leads to the phosphorylation of IKKα (S176, S180) and IKKβ (S177, S181; Mercurio *et al*, 1997). These are conserved serine residues present in the activation loops of either catalytic subunit. Gene targeting experiments have shown that although cytokines such as TNFα and IL-1β result in phosphorylation of IKKα and IKKβ, IKKβ is more necessary for NF-κB activation by most pro-inflammatory stimuli (Li *et al*, 1999; Tanaka *et al*, 1999). However, it is important to note a degree of specificity, IKKα is required for IKK activation in response to RANK (Receptor activator of NF-κB) protein and the B-lymphocyte stimulator Blys/BAFF (B-lymphocyte stimulator/B-cell activating factor; Cao *et al*, 2001; Claudio *et al*, 2002). Therefore, although certain signals can activate the kinase complex *via* IKKα, the majority of NF-κB inducers target the IKKβ subunit. In addition, the IKK complex, although phosphorylating both IκBα and IκBβ, it will preferentially target IκBβ subunits (Zandi *et al*, 1997). The complex regulation of IKK activity by upstream interactions result in the phosphorylation of the IκB family of proteins which we shall discuss in more detail.

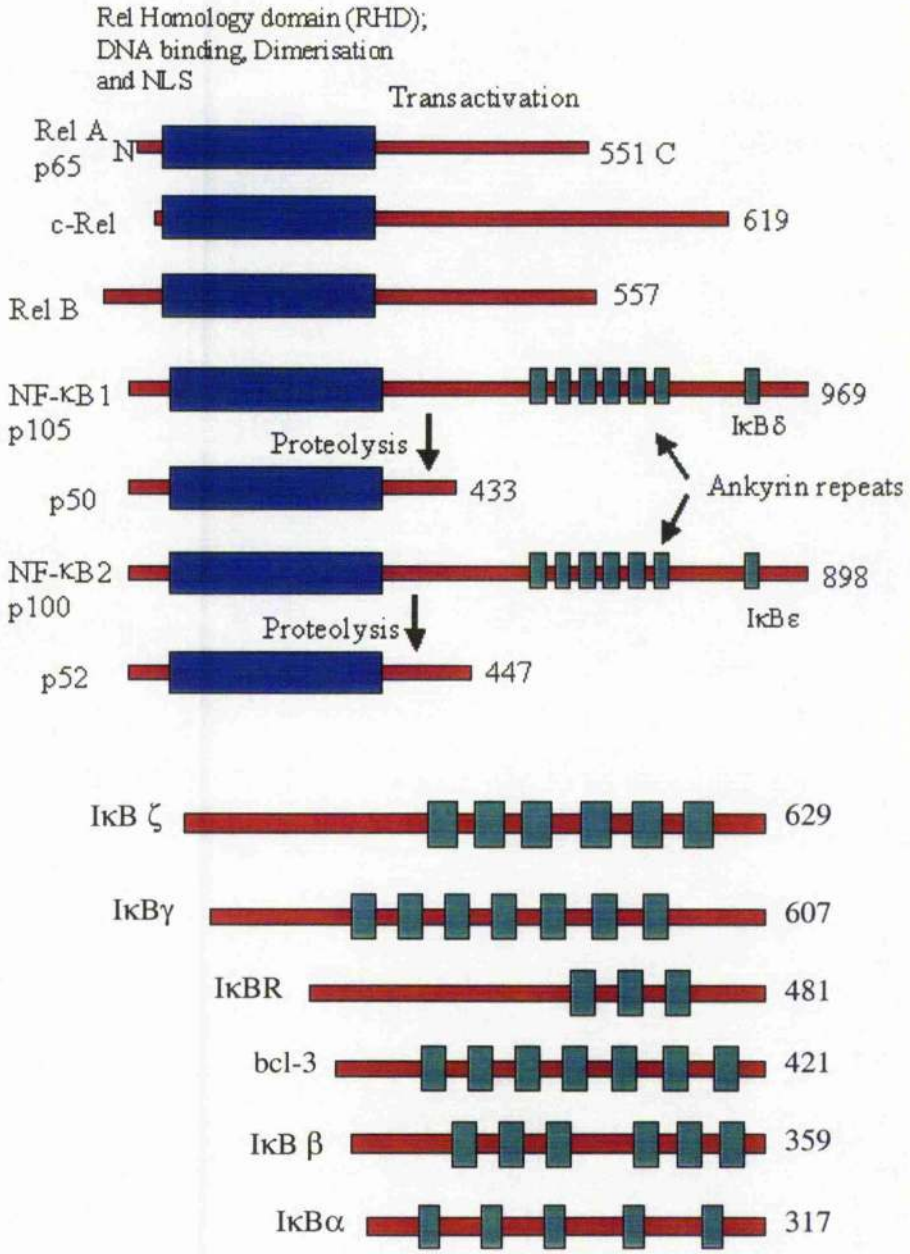
1.7.3 IκB proteins

The IκB family currently comprises eight members; IκBα, β, δ, γ, ε, ζ, R and bcl-3 (Figure 1.6; Ray *et al*, 1995; Ghosh, *et al*, 1998; Yamazaki *et al*., 2001). IκBδ and IκBε are the C-terminal products of p100 and p105 proteolytic processing, forming p50 and p52 respectively. All IκB proteins share a conserved domain comprising five to seven ankyrin repeats, which are responsible for interactions with NF-κB (Lux *et al*, 1990). Each IκB protein specifically binds to particular NF-κB subunits. For example, while IκBα and β, preferentially bind complexes containing RelA, and c-Rel, IκBγ binds to p50 and p52 homodimers (Thompson *et al*, 1995; Whiteside *et al*, 1997). p100 and p105 precursors complexes with p50, Rel A and c-Rel in the cytoplasm. However the IκB members, bcl-3 and IκBζ are unique in that they are expressed primarily in the nucleus. bcl-3 degradation causes p52-dependent repression of transcription (Bours *et al*, 1993), while IκBζ retains NF-κB proteins in the nucleus, instead of the cytoplasm (Yamazaki *et al*, 2001).

Presently, of the eight IκB proteins, only IκBα, IκBβ and IκBε are known to be involved in the signal-induced activation of NF-κB with IκBα being the most extensively studied, as it is rapidly degraded in response to most NF-κB-mobilising stimuli. It is a 37 kDa protein that can be divided into three parts; a 70 amino acid N-terminus containing a signal response domain, a central protease-resistant 250 amino acid domain consisting of six ARD and a final 42 amino acid C-terminus comprising of PEST (proline, glutamic acid, serine, threonine) rich sequences that confer rapid IκBα turnover (Lin *et al*, 1996; Brown *et al*, 1997; Kroll *et al*, 1997). Crystallographic studies have indicated that the ankyrin repeats, mask the NLS of NF-κB, thereby inhibiting NF-κB activity by impeding access to the nuclear import machinery. (Huxford *et al*, 1998; Jacobs *et al*, 1998). IκBα also contains a nuclear export sequence (NES), which is recognised by the nuclear export protein CRM1 (Malek *et al*, 2001). Transcription of IκB is NF-κB-dependent, consequently, newly synthesised IκB is able to enter the nucleus and sequester NF-κB from κB dependent promoters. This inhibits NF-κB-dependent transcription by facilitating export to the

Figure 1.6 The family of NF-κB and IκB proteins.

The vertebrate NF-κB family consists of five different members which all share a highly conserved Rel-homology domain (RHD). Rel A, c-Rel and Rel B also contain a transactivation domain necessary for activation of transcription. p50 and p52 are cleaved from larger precursors p105 and p100 respectively (Section 1.7.5). The C-terminal products of this cleavage are IκBδ and IκBε respectively, which are two members of the wider IκB family. There are eight IκB family members in total which all contain ankyrin repeat regions required for interactions with NF-κB (Section 1.7.4). Adapted from Karin and Ben-Neriah 2000


Rel Homology domain (RHD);
DNA binding, Dimerisation
and NLS
Transactivation
Rel A
p65
N
551 C
c-Rel
619
Rel B
557
NF-κB1
p105
969
IκBδ
Proteolysis
p50
433
Ankyrin repeats
NF-κB2
p100
898
IκBε
Proteolysis
p52
447
IκB ζ
629
IκBγ
607
IκBR
481
bcl-3
421
IκB β
359
IκBα
317

cytoplasm, using the NES present on IκBα (Arenzana-Seisdedos *et al*, 1995). In contrast, IκBβ and IκBε degrade less rapidly and this may account for slower activation of NF-κB responsive genes (Thompson *et al*, 1995; McKinsey *et al*, 1996). However, studies in IκBα knockout mice that express a transgene containing IκBβ under the control of an IκBα promoter, surprisingly indicate some degree of redundancy, as IκBβ can fully complement the lack of IκBα (Cheng *et al*, 1998).

The degradation of IκB proteins occurs *via* a process known as polyubiquitination. Ubiquitin is a highly conserved 76 amino acid protein that is added to target proteins in three stages. Firstly the C-terminal Gly residue of ubiquitin is activated in a step requiring ATP by the E1 activating enzyme. Next, the activated ubiquitin is transferred to a ubiquitin-carrier protein E2 on a specific Cys residue. Finally, ubiquitin is linked to a specific Lys residue of the target protein *via* an isopeptide linkage mediated by ubiquitin-ligase, E3. This pathway is repeated so that proteins become poly-ubiquitinated. This is then used to target the protein to the 26S proteosome where the protein is digested. As ubiquitin conjugation occurs at lysine residues, site-directed mutagenesis of IκBα identified K21 and K22 as the amino acids responsible for this modification (Scherer *et al*, 1995; Baldi *et al*, 1996; Rodriguez *et al*, 1996). The polyubiquitinated forms of IκBα were shown to rely on prior phosphorylation as the presence of a S32A/S36A mutation prevents phosphorylation or ubiquitination whilst the K21R/K22R mutant was able to undergo signal-induced phosphorylation but not ubiquitination, consequently neither mutant could be degraded preventing NF-κB activation.

There has been some debate about this model, as the precise location for ubiquitin-mediated proteolysis, resulting in IκB degradation, is not yet established. β-TrCP/E3RS, a component involved in the ubiquitination of IκB appears to be exclusively nuclear (Davis *et al*, 2002). Therefore further investigation is required to define the different cellular compartments of this pathway.

1.7.4 NF-κB

NF-κB was first discovered in 1986 by Sen and Baltimore as a constitutively active nuclear factor binding to a site in the kappa-light chain immunoglobulin enhancer in B cells (Sen and Baltimore, 1986). Since then it has been identified as a transcription factor existing in an inactive state in the cytoplasm of most cells (Liou and Baltimore, 1993). NF-κB transcription is induced by various stimuli, including cytokines (e.g. TNFα, IL−1β), bacteria, viruses, UV irradiation and hypoxia (Baldwin *et al*, 1996; Pahl, 1999). Activated NF-κB regulates the expression of numerous target genes involved in survival and inflammatory responses. These include genes critical for inhibiting apoptosis (e.g. bcl-2, cIAP 1 and 2), and genes involved in leukocyte trafficking such as adhesion molecules, VCAM and E-selectin (Ghosh *et al*, 2002). Consistent with its importance, dysregulation of NF-κB is intimately involved in the pathogenesis of rheumatoid arthritis, atherosclerosis, AIDS, and cancers like Hodgkin's lymphoma resulting in its intense study to date (Baldwin *et al*, 1996).

NF-κB transcription factors are a family of structurally related, evolutionary conserved proteins that form either homo or hetero-dimers (Ghosh *et al*, 2002). In vertebrates, they are composed from five distinct subunits that share a highly conserved 300 amino acid DNA-binding and dimerisation domain known as the Rel homology domain (RHD). These subunits can be subdivided into two functional groups; those that contain transcriptional activation domains and those that do not. The former include p65 (RelA), Rel B, and c-Rel, in which each possess at least one transcriptional activation domain in their C-terminus (Ryseck *et al*, 1992; Blair *et al*, 1994; Schmitz *et al*, 1994). The other members of the family are p100 and p105, which are precursors to the p50 and p52 subunit forms. The precursor forms both have large C-terminal regions containing 5-7 ankyrin repeat domains (ARD), which allow them to be retained in the cytoplasm. Proteolysis results in shorter proteins, which function by forming heterodimers with subunits from the other group, for example p50:p65. Indeed the p50 homodimer, lacking a transactivation domain, has been identified as a transcriptional repressor (Brown *et al*, 1994).

There are several different structural combinations of subunits possible in the cytoplasm, of which p50:p65 NF-κB is the most common. This is found in virtually all cell types bound to the inhibitory protein IκBα (Baeuerle *et al*, 1988). Following stimulation, degradation of IκBα allows NF-κB to translocate rapidly to the nucleus and bind to a κB-binding motif conforming to the sequence, 5'GGGACTTTCC 3' (Kunsch *et al*, 1992; Parry *et al*, 1994). Studies have shown that p50 will bind to the conserved 5' end, whilst p65 binds the less conserved 3' end (Urban and Baeurele, 1991). Other types of NF-κB dimers bind preferentially to different DNA sequence elements, contributing to the specificity of target genes that can be regulated by this family of transcription factors.

Crystal structures of NF-κB bound to DNA have been solved for p50, p52, RelA homodimers and the p50/p65 heterodimer (Ghosh *et al*, 1995; Muller *et al*, 1995; Chen *et al*, 1998). The crystal structures containing the RHDs of three Rel/NF-κB family polypeptides complexed with various DNA targets obtained indicate that the RHD is organised into three distinct sub-domains (Ghosh *et al*, 1995; Muller *et al*, 1995; Chen *et al*, 1998). The amino-terminal 180 amino acids fold into an Ig-like domain, connected by a short, 10 amino acid long, flexible linker region to a second Ig-like domain of approximately 100 amino acids in length. The amino-terminal Ig-like domain is involved in DNA recognition while the carboxy-terminal Ig-like fold is involved in dimerisation. Fourteen residues from each subunit participate in dimer formation, which is dominated by Van der Waals interactions. The C-terminal Ig-like fold also contains the 13 amino acid nuclear localisation sequence (NLS) required for translocation.

1.7.5 Regulation of NF-κB by post-translational modifications

In addition to regulation by IκB proteins, the localisation and transcriptional activity of p65 is regulated by phosphorylation-dependent acetylation (Chen *et al.*, 2002). Acetylation is a common post-translational modification, which was first associated with the regulation of histone packaging into chromatin fibres. Acetylated histones are associated with transcriptionally active segments of chromatin, whereas deacetylated histones are present in transcriptionally inactive segments (Marmorstein, 2001; Legube and Trouche, 2003). Further studies indicated a range

of transcriptional coactivator proteins that contained histone acetyltransferase (HAT) activity including CBP-associated factor (PCAF), p300/CREB binding protein (p300/CBP), TATA-box binding protein associated factor ($TAF_{II}250$) and steroid receptor coactivator (SRC-1; Marmorstein, 2001). Subsequently, proteins were also identified which contain histone deacetylase activity, termed 'HDACs', which actively repress transcription (de Ruijter *et al.*, 2003).

Phosphorylation of p65 occurs at different phosphorylation sites S276, S529 and S536, which are targeted by several upstream kinases including mitogen- and stress-activated protein kinase (MSK1; S276), protein kinase A catalytic subunit (PKAc; S276), casein kinase II (CKII; S529), IKK(S536), protein kinase Cζ (PKCζ; RHD) and Ca^{2+}/calmodulin kinase IV (CaMKIV; C-terminus; Vermeulen *et al.*, 2002). Phosphorylation status is also determined by the activity of phosphatases; protein phosphatase 2A (PP2A) is associated with p65 in unstimulated melanocytes and can dephosphorylate p65 following stimulation (Yang *et al.*, 2001). Phosphorylated p65 can then enter the nucleus where the specific phosphorylation pattern directs the interaction of NF-κB with either p300/CBP or HDACs (Ashburner *et al.*, 2001; Chen *et al.*, 2002; Kiernan *et al.*, 2003). Using truncated NF-κB mutants and site-directed mutagenesis, several possible Lys residues have been identified as sites for acetylation, K122, K123, (Kiernan *et al.*, 2003) and K218, K221, K310 (Chen *et al.*, 2002). The precise role for acetylation is still unclear as Chen *et al.*, (2002) report that acetylation at K221 enhances p65-DNA interactions. In contrast, Kiernan *et al.*, (2003) report that acetylation reduces binding of p65 to κB-containing DNA promoters and facilitates p65-IκB interactions that direct nuclear export of p65 *via* a CRM-1 dependent process. Further investigation will be required to identify the temporal nature of acetylation and its effects on transcription.

In conclusion, NF-κB is an essential transcription factor required for the effective transcription of pro-inflammatory genes. Up-regulation of these genes increases surface expression of proteins, which direct the recruitment of neutrophils, monocytes and macrophages to sites of inflammation. It responds to a diverse array of initial pathogenic stimuli *via* the integration of signals at the IKK complex, which leads to the degradation of IκB and the subsequent translocation of NF-κB to the

nucleus. Post-translational modifications such as phosphoryation of p65 and acetylation further regulate NF-κB activity.

1.8 Role of G-protein coupled receptors in inflammation

G-protein coupled receptors (GPCRs) play a key role in immune and cellular responses. They can bind to a diverse range of important inflammatory ligands that include, chemokines, serotonin (5HT), thrombin, histamine and adenosine. As such, they represent important sites for potential anti-inflammatory therapeutics. The general properties of GPCRs and the specific inflammatory properties of the adenosine family of receptors will be discussed here.

GPCRs are integral membrane proteins that make up the largest protein superfamily, consisting of ~ 750 members in human. They are single polypeptide chains that contain seven hydrophobic TM-spanning α-helical regions, a hydrophillic extracellular N-terminus and an intracellular C-terminus which vary in length. They can be divided into four different classes of receptor dependent upon amino acid sequence similarity. Class Ia receptors are termed "rhodopsin-like" owing to the similarity between family members and rhodopsin and include receptors for IL-8, prostaglandins, prostacyclins, histamine and adenosine. Class Ib receptors are termed "calcitonin and secretin-like", again owing to the similarity between family members, which include the glucagon receptor, parathyroid hormone receptor and growth hormone releasing-hormone receptor. Class Ic include the thyrotropin stimulating hormone and Class II GPCRs include metabotropic glutamate and pheromone receptors, which share similarities with other members including Ca^{2+}-sensing receptors and γ-amino butyric acid (GABA) receptors.

1.8.1 General GPCR signalling

The seven-transmembrane spanning domains of type Ia receptors are thought to form a central pore accessible to the extracellular surface where it can bind to its cognate ligand. Signal transduction across the plasma membrane is activated when the receptor binds to its cognate extracellular ligand. This causes a conformational change within the structure that is responsible for coupling to and activation of an associated heterotrimeric G-protein. This heterotrimeric G-protein can then in turn activate downstream effector molecules to initiate intracellular signalling. The

structure of these receptors was originally identified in studies involving the bovine rhodopsin and β_2-adrenergic receptors (β_2AdR; Dixon *et al.*, 1986; Henderson *et al.*, 1990) with further studies identifying key features that appear to be highly conserved throughout all GPCRs. Recently, Palczewski *et al.*, (2000) solved the crystal structure of rhodopsin, which allows a more accurate description of the structure.

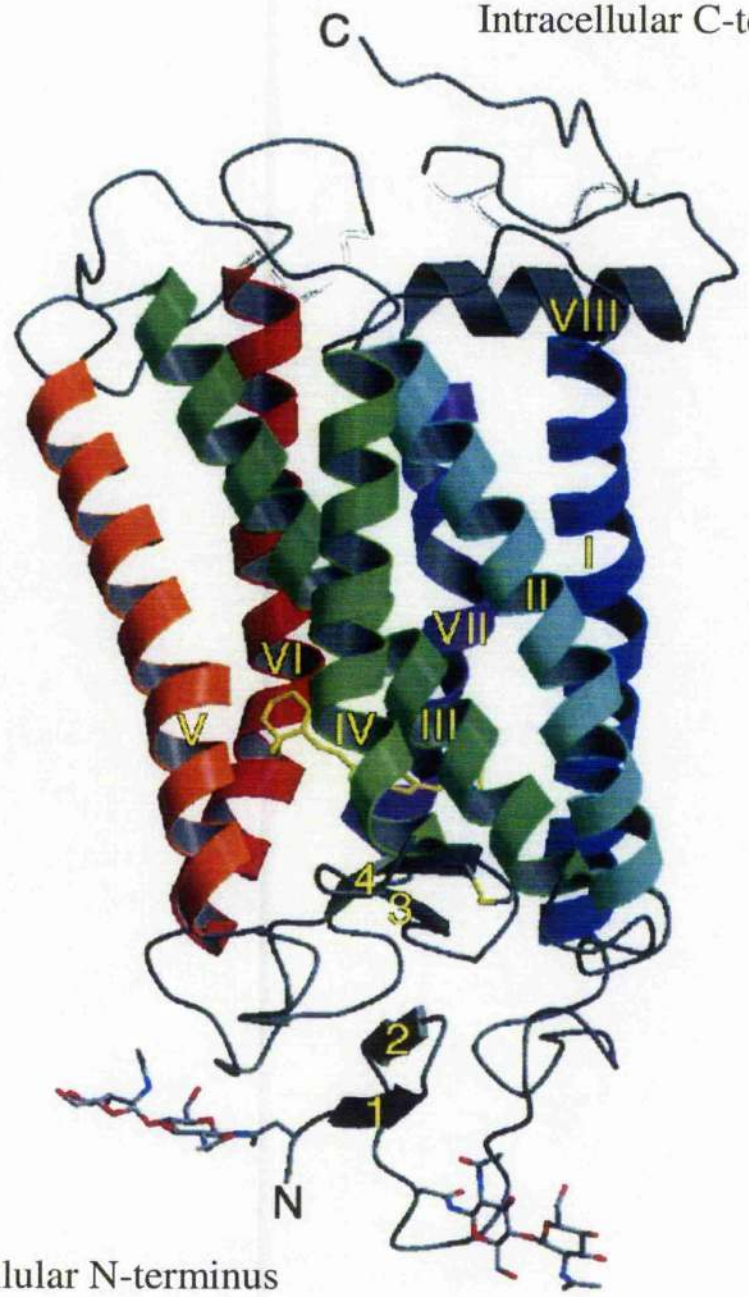
1.8.2 Structural features of Class Ia GPCRs

The TM domains of GPCRs display a relatively constant length with variation in the members of this superfamily seen primarily in the N-and C-termini and extracellular loop domains. The N-terminal domain has been shown to range from between 340-450 residues in mature glycoprotein hormone receptors to less than 10 residues in the A_{2A} adenosine receptor. Receptors also vary in the C-terminal domain, for example the metabotropic glutamate receptor (mGluR1a) contains 359 amino acid residues compared to 2 amino acids for the gonadotrophin releasing hormone receptor (GRHR) (Masu *et al.*, 1991; Brooks *et al.*, 1993). The third intracellular loop often displays a degree of variability as well with amino acid residues ranging from 239 in the m3 muscarinic acetylcholine receptor (mAchR) (Peralta *et al.*, 1987) to ~15 amino acids in the human complement C5a receptor (Gerard and Gerard, 1991; Figure 1.7).

In most receptors, the N-terminal domain contains potential sites of N-linked glycosylation, represented by the sequence Asn-X-Ser/Thr where X is any amino acid except proline (Hubbard and Ivatt, 1981). It has not yet been established whether all potential N-linked glycosylation sites are functional, but there is evidence of their requirement for receptor expression and ligand binding activity of GPCRs (Segaloff and Ascoli, 1993; Liu *et al.*, 1993). Class Ib GPCRs, such as receptors for endothelin A, B (ET_A, ET_B) and thrombin also contain an N-terminal hydrophobic leader sequence or signal peptide (Haendler *et al.*, 1992; Zhong *et al.*, 1992). This sequence directs newly synthesised receptor to the plasma membrane from the endoplasmic reticulum and is cleaved off to generate the mature receptor (Von Heijne, 1990). Almost all GPCRs contain Cys residues in the first and second extracellular loops that form a conserved disulphide bond. This is critical for maintaining the tertiary structure of the receptor as mutations of these residues in rhodopsin (Karnik *et al.*, 1998), β_2AdR (Dixon *et al.*, 1987) and the muscarinic

Figure 1.7 **X-ray crystallographic structure of rhodopsin, a prototypical Class1A GPCR**

GPCRs contain seven transmembrane α-helices (I-VII), an intracellular C-terminus and an extracellular N-terminus. The extracellular surface of the protein contains sites for N-linked glycosylation (Section 1.8.2). Rhodopsin also has a C-terminal α-helix (VIII) required for activation (Taken from Palczewski *et al.*, 2000)


C
Intracellular C-terminus
VIII
I
II
VII
VI
V
IV
III
4
3
2
1
N
Extracellular N-terminus
Sites of Glycosylation

acetylcholine receptor (mAchR) (Hulme, 1990) result in reduced expression levels, aberrant ligand binding properties and altered receptor activation in response to agonist. Consistent with a conserved role, Dohlman and colleagues (1990) showed that mutation of these Cys residues in other receptor families resulted in low levels of radioligand binding (Dohlman *et al.*, 1990). The C-terminal domain and third intracellular loop of GPCRs typically contain numerous Ser and Thr residues that are potential sites of regulation *via* phosphorylation. This is typically mediated by kinases such as G-protein-coupled receptor kinases (GRK) and/or second messenger kinases such as protein kinase A (PKA) and protein kinase C (PKC) (Dohlman *et al.*, 1987; Kobilka, 1992). These residues are particularly significant for desensitisation of receptor signalling.

Most GPCRs also contain a conserved Cys residue at the N-terminal region of the cytoplasmic tail. This has been shown in the β_2AdR (O'Dowd *et al.*, 1989), rhodopsin (Ovchinnikov *et al.*, 1998) and several other GPCRs (Quanbar and Bouvier, 2003) to be a site of palmitoylation. Palmitate is a saturated 16-carbon fatty acid chain that can attach to Cys residues through a labile, reversible thioester linkage, regulation of which is controlled by the activation state of the receptor (Wedegaertner *et al.*, 1995). Sites of palmitoylation are frequently found close to other lipid modifications, such as myristate or prenyl groups, or to stretches of hydrophobic amino acids such as those found in GPCR transmembrane domains (Mumby, 1997). This anchorage introduces an additional intracellular loop to the molecular structure of the receptor that may affect the interaction of the receptor with the G-protein (Milligan *et al.*, 1995). Chemical removal of the palmitate from rhodopsin has been reported to enhance coupling of the receptor to the G-protein transducin (Morrison *et al.*, 1991). In contrast, a β_2AdR with mutation of Cys 341 to Ala or Gly (Glycine) resulted in a receptor displaying a dramatic impairment in functional coupling to G_s (stimulatory) (O'Dowd *et al.*, 1989; Moffett *et al.*, 1993). $Cys^{341}Gly$ was also observed to have an increased phosphorylation level in the absence of agonist and displayed no increase in either phosphorylation levels or uncoupling from G_s upon agonist stimulation. The properties of the mutant β_2AdR resembled those of WT receptors that had undergone agonist-induced desensitisation, suggesting a role for palmitoylation in regulating the accessibility of the C-terminal

region to the action of protein kinases. This data implies that a universal role for palmitoylated cysteine residues is unlikely. However, it is possible that these Cys residues have varying functions in receptor regulation from interaction with G-proteins to receptor turnover, expression and subcellular localisation (Quanbar and Bouvier, 2003).

Class I GPCR TM regions contain the greatest degree of amino acid similarity among GPCRs with the greatest conservation observed between subtypes of receptor families (Probst *et al.*, 1992). Particularly well conserved throughout the GPCR superfamily are Pro residues occurring in TM regions 4, 5, 6 and 7. These residues are thought to be important for formation of the ligand-binding pocket in receptors such as the mAChR and also for expression of the β_2AdR at the cell surface (Strader, 1989). The sequence Asp-Arg-Tyr (DRY) occurring at the intracellular side of transmembrane region 3 is also very highly conserved. The Arg residue is invariant, with the Asp and Tyr replaced in very few receptors. For almost all GPCRs, this region along with the membrane proximal region of the second intracellular loop, is thought to be involved in receptor-G-protein coupling.

1.8.3 Receptor and G-protein interactions

Mutagenesis of TM helices and biochemical experiments using a variety of GPCRs suggest that receptor activation causes changes in the orientation of TM helices 3 and 6, which was confirmed by the crystal structure of rhodopsin. The activation of rhodopsin causes a 30° rotation in helix 6 causing the separation of TM helices 3 and 6. This forms a cleft for the binding of the Gα-subunit of the G-protein (Farrens *et al.*, 1996; Bourne, 1997). Deletion and site-directed mutagenesis studies have established that receptor-G-protein interaction relies on the receptors third cytoplasmic loop, and in particular, the regions of the loop lying immediately adjacent to TM regions 5 and 6 (Savarese and Fraser, 1992)

Although much is known about the structural features of GPCRs involved in ligand recognition and G-protein binding, the mechanisms underlying ligand-induced activation and subsequent G-protein coupling remain unclear. The DRY motif is a highly conserved triplet of amino acids located at the transmembrane region of TM helix 3 and the second intracellular loop (Probst *et al.*, 1992). With the Arg residue

being fully conserved among all Class I GPCRs, mutations of this residue in the M1 muscarinic and α_{1B}-adrenergic receptor (α_{1B}AdR) produced receptors with impaired signal transduction properties and it is therefore thought to be a key residue required for interaction of receptor with G-protein (Zhu *et al.*, 1994; Jones *et al.*, 1995). The importance of the DRY sequence has also been described for rhodopsin, angiotensin II (AngII) and the interleukin-8 (IL-8) receptors (Konig *et al.*, 1989; Ohyama *et al.*, 1992; Damaj *et al.*, 1996).

Analysis of several gain-of–function mutant receptors that result in constitutive receptor activity have shown that multiple regions of the receptor including the extracellular, intracellular and TM domains may be involved in keeping the receptor in a constrained state in the absence of agonist (Lefkowitz, 1993; Lefkowitz *et al.*, 1993; Leff, 1995). In the presence of agonist, conformational changes in the receptor lead to a disruption of complex intramolecular interactions that constrain the receptor in the inactive state. This causes rearrangement of the TM domains and exposure of the cytoplasmic regions to the G-protein to trigger signal transduction (Ulloa-Aguirre *et al.*, 1999).

1.8.4 Regulation of GPCR function

The precise control of receptor expression and function is critical for the role that GPCRs play in inflammation. It is essential that they can respond to rapid changes in agonist concentration in a precise manner. Consequently, they are regulated by phosphorylation of specific Serine, Threonine or Tyrosine residues by several different kinases. This allows multiple protein-protein interactions that lead to receptor desensitisation, sequestration from the cell surface, recycling of the receptor back to the surface and in some cases down-regulation of the receptor. One of the best characterised systems to study these processes is using the β_2AdR.

1.8.4.1 Desensitisation

Desensitisation has been defined as the process whereby a GPCR-initiated response reaches a plateau and then decreases despite the continued presence of agonist. It can be further sub-divided into homologous and heterologous desensitisation. Homologous desensitisation causes the desensitisation of only agonist-stimulated receptors. In contrast, heterologous desensitisation causes the desensitisation of both

agonist-induced and non-ligand bound receptors. Heterologous desensitisation is mediated by phosphorylation of specific residues by second messenger-activated kinases such as PKA and PKC, resulting in a decrease in the ability of the receptor to stimulate the associated G-protein (Benovic *et al.*, 1985; Pitcher *et al.*, 1992). In studies using the β_2AdR, Benovic *et al*, (1991) were able to purify a kinase from bovine brain responsible for homologous desensitisation, termed β-adrenergic receptor kinase (βARK), later renamed as G-protein coupled receptor kinase 2 (GRK2; Benovic *et al.*, 1991). Seven mammalian GRKs have subsequently been cloned to date, termed GRK1-7. GRKs 1 and 7 are expressed exclusively in the eye, while GRK 4 is solely expressed in the testis. GRKs 2, 3, 5 and 6 are ubiquitously expressed suggesting an important role for these kinases in mediating homologous desensitisation for a wide variety of GPCRs.

1.8.4.2 Sequestration

Sequestration is the process whereby agonist stimulated GPCRs, having been phosphorylated by GRKs, are removed from the cell surface. This typically requires prior receptor phosphorylation and subsequent binding of an "arrestin" protein (Pippig *et al.*, 1993; Diviani *et al.*,1996; Ferguson *et al.*, 2002). There are four known mammalian arrestins, termed arrestin 1-4, which share 45% identity overall. Arrestins 1 and 4 are found only in the eye (Shinohara *et al.*, 1992; Murakami *et al.*, 1993) while arrestins 2 and 3 (Lohse *et al.*, 1990; Sterne-Marr *et al.*, 1993) are ubiquitously expressed and therefore play a general role in sequestration of many GPCRs. Binding of arrestin to phosphorylated GPCRs also results in the uncoupling of activated receptors from the G-proteins, which 'switch off' or arrest GPCR intracellular signalling (Bünemann and Hosey, 1999). Arrestins 2 and 3 are also able to simultaneously interact with clathrin *via* a clathrin-binding domain in the C-terminus. Clathrin is a ubiquitously expressed protein comprised of three 190kDa heavy chains, each with an associated light chain radiating from a central hub to form a triskelion structure (Pearse, 1976). The coat surrounding a phospholipid bilayer is made up of triskelions that form a network of pentagons and hexagonal structures enclosing the whole structure. Sequestration is due to the phosphorylation of GPCRs by GRKs, the recruitment of arrestin proteins and the recruitment and assembly of the clathrin complex around the internalised vesicle. Several other proteins are

involved in the process of internalisation including dynamin and the Rab GTPases, to which the reader is directed to for a more complete review of receptor internalisation (Seachrist and Ferguson, 2003).

In cells expressing β_2AdR following agonist stimulation, arrestins have also been shown to redistribute to the PM with activated Src (c-Src), a tyrosine kinase that phosphorylates Shc and Gab1, proteins involved in Ras dependent activation of ERK (Luttrell *et al.*, 1999). In the absence of arrestin, c-Src was no longer able to associate with the β_2AdR suggesting a role for arrestins in signalling by acting as a scaffold for the docking of other proteins.

1.8.4.3 Down-regulation

Prolonged agonist exposure over a period of hours results in receptor down-regulation, defined as an overall decrease in total receptor number (Gagnon *et al.*, 1998). Using the β_2AdR as a model, it has been suggested that sequestered receptors traffic from early endosomes to lysosomes where they undergo degradation. Many GPCRs including the thrombin (Hein *et al.*, 1994), thyrotropin (Petrou *et al.*, 1997) and cholecystokinin (Tarasova *et al.*, 1997) receptors have been shown to sort to lysosomes in an agonist-dependent manner.

After receptors and their ligands have been sequestered from the cell surface by clathrin coated vesicles, the vesicles are uncoated and the internalised molecules are delivered to 'early' sorting endosomes (Rothman and Schmid, 1986). Due to the acidic pH in early endosomes, the receptors and ligands are dissociated and the receptors are recycled back to the membrane for another round of agonist activation for receptors such as the transferrin receptor and the low-density lipoprotein receptor (LDL) (Dautry-Varsat *et al.*, 1983). Other receptors are degraded by the lysosome such as substance P receptor (Koenig and Edwardson, 1997; von Zastrow, 2003). The effective regulation of receptor surface expression maintains the sensitivity of GPCRs to extracellular concentrations of agonist which is important in the activation of signal transduction pathways *via* G-proteins.

1.9 G-proteins

The term "G-protein" refers to a heterotrimeric plasma membrane-associated GTP-binding protein which functions as an intermediary in transmembrane signalling pathways to transduce signals across the plasma membrane from activated receptors to effector enzymes or ion channels (Albert and Robillard, 2002). Evidence for the existence of G-proteins first came from Rodbell and Birnbaumer who described the stimulation of adenylyl cyclase to be dependent upon the presence of GTP (Rodbell and Birnbaumer, 1971). It was not until 1981 (Sternweis *et al.*, 1981) that the first G-protein was purified and initially described as a 'GTP-binding regulatory component of adenylyl cyclase'. This 45 kDa protein is now better known as the G_s G-protein α-subunit (Gilman, 1987). Consequently, another G-protein was soon described as an inhibitory regulatory component of adenylyl cyclase and termed G_i (Bokoch *et al.*, 1983). G-proteins are now known to consist of three distinct subunits, namely α, β, and γ. To date, 27 α, 6 β, and 14 γ genes have been cloned (Albert and Robillard, 2002). This variety allows a wide degree of specificity based on individual subunit composition.

Classically subunits of the heterotrimeric G-protein participate in a cycle of association and dissociation that is dependent upon the activation state of the G-protein. Upon receptor activation, the Gα-sububnit will dissociate from the heterotrimeric complex in response to the binding of GTP. The released α-subunit and βγ-subunits can each activate downstream effector proteins. The inherent GTPase function of the α-subunit will then hydrolyse GTP to GDP, which allows the G-protein to reassociate with the βγ--subunit in preparation for another cycle of activation. Conventional signalling by G-proteins was until recently thought to involve only one type of Gα subunit/receptor. This is now known not to be the case with several receptors activating more than one type of G-protein. This is termed "differential G-protein coupling" and is found in a range of different receptors, including the thyrotrophin receptor (Laugwitz *et al.*, 1996), A_{2B}AR (Gao *et al.*, 1999) and many others (Hur and Kim, 2002; Hermans, 2003). However, the precise mechanisms that determine receptor-G-protein coupling specificity remain unclear.

G-proteins are classified according to the function of the α-subunit (Hamm, 1998). G_s and G_i are so-named due to their ability to stimulate and inhibit adenylyl cyclase respectively. $G_{q/11}$ family proteins activates PLCβ (Offermans, 1994) and the $G_{12/13}$ family regulate the small GTP-binding protein Rho (Barac *et al.*, 2003). Activation of the G-protein generates both α and βγ subunits that can each interact with downstream effector targets to generate an intracellular response. The downstream effectors reportedly regulated by βγ–subunits include adenylyl cyclase isoforms, (PLCβ), inward rectifier G-protein-gated potassium channels, voltage-sensitive calcium channels, and PI3K. (Ford *et al.*, 1998; Hur and Kim, 2002)

1.9.1 G_s proteins

The G_s family of G-proteins is defined by its ability to stimulate adenylyl cyclase activity leading to the generation of cAMP. It has four isoforms, two of 45kDa and two of 47 kDa in size generated by alternative splicing of the same gene product (Sunahara *et al.*, 1996). G_s is ubiquitously expressed and is sensitive to cholera toxin which catalyses ADP-ribosylation of Arg 201 found within the GTP-binding site (Kassis *et al.*, 1982). This causes a block in the GTPase activity of the subunit rendering it constitutively active, leading to multiple cellular events that characterise the pathophysiology of cholera (Enomoto *et al.*, 1980.)

1.9.2 G_i/G_o proteins

The G_i/G_o family proteins comprise of $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, G_{o1} and G_{o2} and are classified based on differences in size (Gilman *et al.*, 1987; Hur and Kim, 2002). Defined by the inhibition of adenylyl cyclase activity they can also target other downstream signalling components including certain sodium, potassium and chloride channels and PLCβ (G_o; Moriarty *et al.*, 1990). They are all sensitive to the bacterial toxin from *Bordatella pertussis*. This toxin causes ADP-ribosylation of a conserved Cys residue in the C-terminus of the α-subunit. This prevents GDP/GTP exchange, leaving the subunit in a permanently inactive GDP-bound state. The retinal-specific G-proteins $G_{t1}\alpha$ and $G_{t2}\alpha$ are also members of this family. They couple stimulation of rhodopsin to the activation of retinal cGMP phosphodiesterases (PDEs; Berg *et al.*, 2002).

1.9.3 G_q family

This family has several members including $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, and $G_{16}\alpha$, which are insensitive to any of the toxins previously mentioned and are important in coupling GPCR activation to the activation of PLCβ (Rhee, 2001). PLCβ catalyses the hydrolysis of phosphatidyl inositol-4, 5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) which mediate the release of Ca^{2+} and activate PKC respectively. Interestingly with respect to inflammation, DAG can be metabolised into arachidonic acid by diacylglycerol lipase. This fatty acid is the precursor in the biosynthesis of eicosanoids, pro-inflammatory lipid molecules including prostaglandins, prostacyclins and thromboxanes (Berg *et al.*, 2002).

1.9.4 $G\alpha_{12}$ family

This family comprises of two family members G_{12} and G_{13}, each of which is 44kDa in size. The primary structure of this family diverges by more than 50% from other G-protein sequences. They couple GPCR activation to multiple downstream targets such as the Rho family of small GTP binding-proteins, JNK, phospholipase D, and non-receptor tyrosine kinases (Offermanns, 2003) which regulate a wide range of effects such as actin cytoskeleton remodelling, c-jun activation and transcription. Recently they have also been reported to interact with a class of adhesion molecules called "cadherins". This causes the release of the transcriptional activator β-catenin (Meigs *et al.*, 2001; Meigs *et al.*, 2002) to regulate transcription. They are ubiquitously expressed and are insensitive to cholera or pertussis toxin.

1.9.5 Gβγ subunits

In recent years it has been widely acknowledged that βγ-subunits also target specific effector molecules such as PLCβ and adenylyl cyclase (Kim *et al.*, 1989; Pang and Sternweis, 1990; Tang and Gilman, 1991; Vanderbild and Kelly; 2000). It has also been reported that there exists βγ-binding sites on GRK2. This allows regulation of GRK2 phosphorylation and inhibits Gβγ signalling (Lodowski et al., 2003; Eichmann *et al.*, 2003). With many different subunit combinations possible between family members, there exists a great potential for specificity, leading speculation that a specific combination of -β and -γ subunits, will activate a specific effector in response to specific GPCR activation. For example, $\beta_1\gamma_4$ subunits have been shown

to selectively couple the activation of muscarinic receptors to L-type calcium channels (Kleuss *et al.*, 1992; Garcia *et al.*, 1998). In contrast, studies by Quitterer and Lohse (1999) studied the synergy between G_i and G_q coupled receptors and reported no differences in inositol phosphate accumulation when co-transfected with different combinations of βγ-subunits (Quitterer and Lohse, 1999). Consequently, the specificity of βγ-subunits is not yet fully understood.

Several X-ray crystal structures of Gβγ subunits have been solved and show an overall seven-bladed β-propeller structure, with the γ-subunit interacting with the coiled-coil domain of the β-subunit (Gautam *et al.*, 1998). The γ-subunit also contains a CAAX motif on its C-terminal region that directs prenylation of the molecule (Resh, 1996; Higgins and Casey, 1996). Prenylation involves the direct attachment of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid group to the conserved Cys residue within the motif (Casey, 1994). Prenylation is known to facilitate Gβγ anchorage to the plasma membrane, as mutation of the conserved Cys to Ser resulted in cytosolic expression of Gβγ-subunits (Muntz *et al.*, 1992; Gelb *et al.*, 1998).

1.10 Inflammation and Adenosine

Several lines of evidence point to a role for adenosine in the regulation inflammation: 1) adenosine accumulation during ischemia and inflammation protect tissues from injury (Linden, 2001), 2) adenosine inhibits phagocytosis, the generation of reactive oxygen species, and adhesion to some surfaces, e.g. endothelial cells (Cronstein, 1994), 3) the anti-inflammatory effects of sulfasalazine and methotrexate involve an adenosine-dependent mechanism (Morabito *et al.*, 1998), 4) receptors for adenosine are found on cells throughout the immune system (Thiel *et al.*, 2003). Strong evidence therefore exists that adenosine has a general anti-inflammatory, however, how does it mediate these effects on inflammation?

1.10.1 Adenosine receptors

Adenosine receptors (ARs) belong to the Class IA receptor family and are known as purinergic receptors as they the bind the purine agonist adenosine (See Section 1.11.1 Figure 1.8). There are two main types of purinergic receptors: P_1 or adenosine

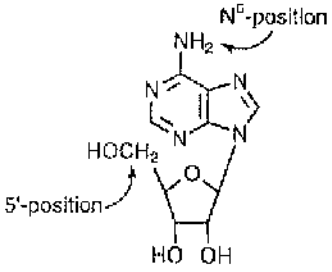
receptors, and P_2 receptors. The P_2 receptors recognise and bind to nucleotides such as ADP, ATP, UDP and UTP. P_1/ARs can be further subdivided into four distinct subtypes, namely A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR (Tucker and Linden, 1993; Figure 1.9), which all couple to G-proteins. P_2 receptors are divided into P_{2X} and P_{2Y} subgroups. The P_{2X} receptors are ligand-gated ion channels (Benham and Tsien, 1987), while the P_{2Y} receptors are GPCRs (Abbracchio and Burnstock, 1994; Fredholm *et al.*, 1994).

There are several structural components within ARs that are found within the general Class 1a GPCR family (Section 1.8.2). Each receptor has an extracellular N-terminus and intracellular C-terminus linked by 7 TM hydrophobic α-helices. Within the TM regions, helices 2, 3 and 5 show a large degree of sequence identity and residues within these regions are reported as being crucial for ligand binding and specificity. Studies by Klotz *et al.*, (1998) on the A_1AR reported that His residues were important in the binding specificity of agonist and antagonist. His residues have also been implicated in ligand binding for the $A_{2A}AR$ (Jacobson *et al.*, 1992). A_1AR, $A_{2A}AR$ and $A_{2B}AR$ receptors contain two such His residues within the TM regions and mutations of these residues to Leu, resulted in receptors having a decreased receptor affinity for agonist and antagonist ligands supporting a role for these residues in ligand binding (Olah *et al.*, 1992).

ARs also have consensus sites for N-linked glycosylation (N-X-S/T; Olah *et al.*, 1990; Barrington *et al.*, 1990; Palmer *et al.*, 1992) on their second extracellular loops, although the A_3ARs also possesses an additional site on its N-terminus. The precise role of glycosylation is relatively unclear, as it does not play a role in agonist binding (Piersen *et al.*, 1994), although it may be required for efficient receptor targeting to the plasma membrane. Conserved Cys residues in the second and third extracellular loops, which are predicted to form a disulphide bridge, are also a feature of ARs in common with other Class Ia GPCRs. This is reported as being essential to the pharmacology of the receptor, as treatment with increasing concentrations of dithiothreitol decreases agonist affinity (Mazzoni *et al.*, 1997).

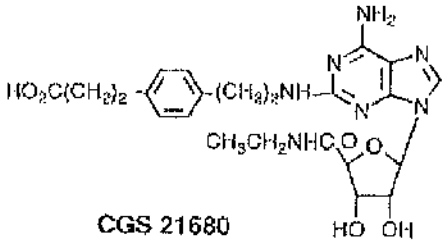
Figure 1.8 The structure of adenosine and the A_{2A}AR-selective drugs CGS21680, NECA and ZM241385.

The chemical structure of the endogenous ligand adenosine, the A_2AR-selective agonist NECA, the A_{2A}AR-selective agonist CGS21680 and the A_{2A}AR antagonist ZM241385 are shown. Both CGS21680 and NECA contain an adenine ring and ribose group similar to the endogenous adenosine, while ZM241385 is distinctly different in structure. (Taken from Olah and Stiles, 2000)

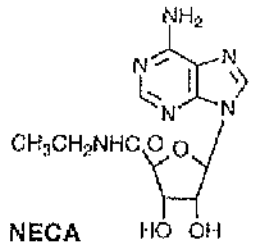


adenosine

Agonists

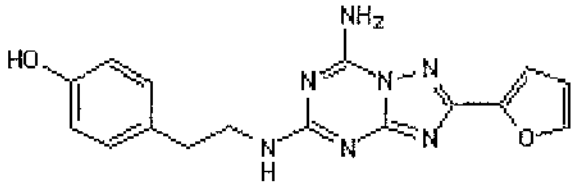


CGS 21680



NECA

Antagonist



ZM 241385

Specific cytoplasmic structural features involved in signalling from the ARs include the conserved DRY motif found at the C-terminal end of TM3. This is important in G-protein interactions and maintaining GPCR stability (Chung, 2002). In addition to G-protein binding sites, there is a common Cys residue present on the A_1AR (Cys^{309}), which has been shown to be the site of palmitoylation (Gao *et al.*, 1999). $A_{2B}AR$ and A_3AR also have predicted sites for palmitoylation in their C-termini. In contrast, $A_{2A}ARs$ do not possess a similar Cys residue suggesting that palmitoylation is not essential for downstream signalling by this receptor (Wedegaertner *et al.*, 1995).

As observed for other GPCRs the $A_{2A}AR$, $A_{2B}AR$ and A_3AR contain several potential sites of phosphorylation within the C-terminal tail, while the A_1AR contains some phosphorylation sites in the third intracellular loop. Palmer *et al.*, (1997) have reported that phosphorylation of the canine $A_{2A}AR$ on Thr^{298} is associated with the onset of rapid desensitisation in response to agonist (See Section 1.9.1). It is also reported that multiple isoforms of PKC can activate a downstream kinase responsible for the phosphorylation of the $A_{2A}AR$ in response to the phorbol ester PMA, although the significance of this is unclear (Palmer *et al.*, 1999).

1.10.2 Adenosine receptor subtypes

1.10.2.1 A_1ARs

The A_1AR receptor from several species including rat (Mahan *et al.*, 1991; Reppert *et al.*, 1991), cow (Olah *et al.*, 1992; Tucker *et al.*, 1992) and man (Libert *et al.*, 1992; Townsend-Nicholson *et al.*, 1992; Ren and Stiles, 1994) have been cloned, and found to encode proteins of 326 amino acids, with a corresponding size of ~37kDa. Overall sequence identity is 87% across the different species, rising to 92% within the TM regions. A_1AR mRNA has been found in brain, testis (Mahan *et al.*, 1991; Reppert *et al.*, 1991), heart and kidney (Olah *et al.*, 1992). Activation of A_1ARs leads to the inhibition of adenylyl cyclase activity *via* G_i-linked G-proteins. It also leads to the mobilisation of intracellular Ca^{2+} due to the activation of phospholipase C (Hansen *et al.*, 2003). It has been reported that the A_1AR will also activate PKCμ in a mechanism that involves MEK1 and PI3-K (Hill *et al.*, 2003). It has also been recently reported to dimerise with the dopaminergic D_1 receptor (Gines *et al.*, 2000).

In addition, the enzyme adenosine deaminase (ADA) can also interact with the A_1AR in CHO cells leading to increased receptor binding of the AR agonist NECA (Ciruela *et al.*, 1996).

1.10.2.2 A_{2A}ARs

A_{2A}ARs have been cloned from several species, including dog (Libert *et al.*, 1990) rat (Fink *et al.*, 1992) and man (Furlong *et al.*, 1992) and show 84% sequence identity between rat and human forms. They encode proteins that are 410-412 amino acids long, and ~45 kDa in size, with over 120 amino acids comprising the C-terminal tail of the receptor (Barrington *et al.*, 1990; Olah *et al.*, 1997). The A_{2A}AR is expressed in several cell types including the immune system, platelets, vascular smooth muscle cells and endothelial cells. It is highly expressed in specific regions of the brain (nucleus accumbens, olfactory tubercle, striatum) that are also enriched in D_2 dopamine receptors. The A_{2A}AR classically signals through the G_s family of G-proteins leading to the activation of adenylyl cyclase and an increase in cAMP levels (Daly *et al.*, 1983). However, it has also been shown to promote $p21^{ras}$-dependent activation of ERK in endothelial cells independent of G_s activation (Scxl *et al.*, 1997).

1.10.2.3 A_{2B}ARs

A_{2B}ARs have been cloned from several species including human (Pierce *et al.*, 1992), rat (Rivkees and Reppert, 1992) and mouse (Marquardt *et al.*, 1994). cDNAs encode for a protein of 322 residues with a molecular weight of ~36kDa (Lynge *et al.*, 2003). It is expressed at low levels on many cell types in most species thus far examined (Ralevic and Burnstock, 1998). It was first shown to couple to G_s proteins to activate adenylyl cyclase activity like the A_{2A}AR (Linden *et al.*, 1998), but has since been shown to also couple *via* $G_{q/11}$ proteins to activate PLCβ leading to an IP_3-dependent increase in intracellular calcium (Feoktistov and Biaggioni, 1995; Linden *et al.*, 1999). Recently it was also reported that A_{2B}AR activation leads to ERK activation in a $p21^{ras}$-dependent mechanism. This mechanism is regulated by PLC as U73122, a PLC-selective inhibitor, could decrease ERK phosphorylation by ~50% (Gao *et al.*, 1999). A_{2B}ARs have also been found associated with ADA in CHO cells and Jurkat T Lymphocytes. The presence of ADA increases the affinity of A_{2B}AR for NECA and increases cAMP production (Herrera *et al.*, 2001).

1.10.2.4 A_3ARs

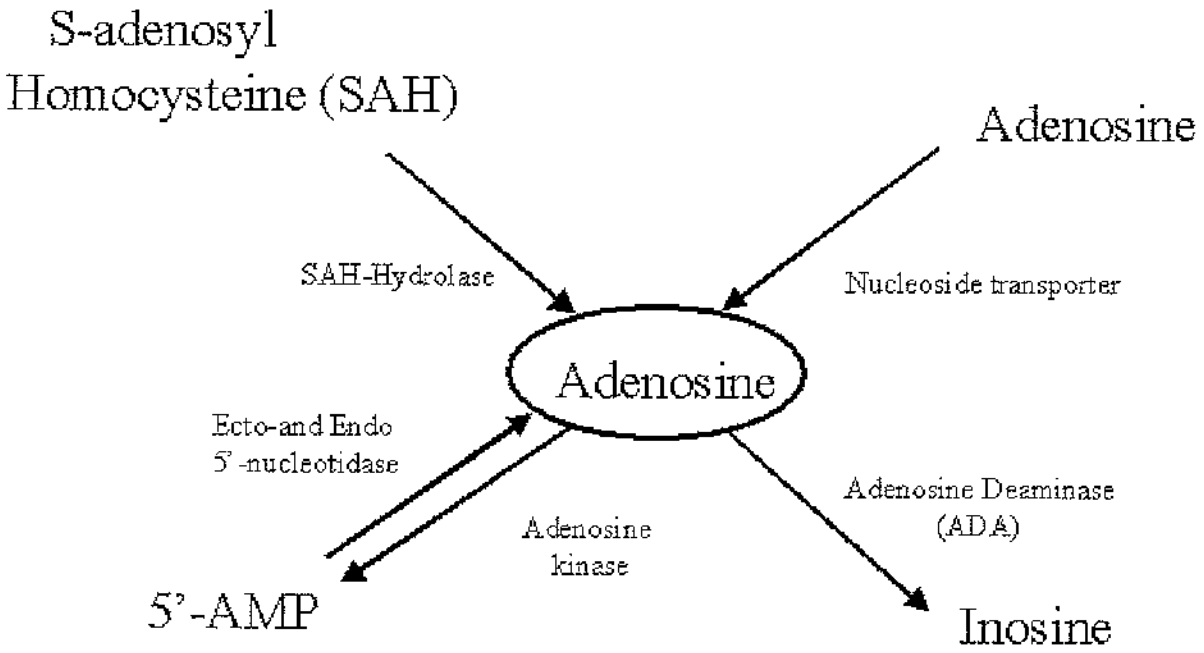
The most recently discovered of the ARs, the A_3AR has also been cloned from multiple species including rat, dog, sheep, rabbit and human (Salvatore *et al.*, 2000; Fredholm *et al.*, 2001). The human A_3AR is 318 residues long encoding a protein of ~36kDa. The A_3AR displays the greatest degree of difference between species, with the rat and human sharing only 72% sequence identity reflecting marked differences in pharmacology for example, all non-nucleoside antagonists e.g. MRS 1220 display high affinity for the human A_3AR but low affinity for the rat A_3AR. Recent studies indicate a small N^6-subsitiuent is important in the species differences (Gao *et al.*, 2002). In rodents it is expressed primarily on mast cells with some expression in the testis, and in the brain (Fredholm *et al.*, 2001; Hammerbeg *et al.*, 2003). In contrast, in humans and sheep it is expressed on eosinophils and not on mast cells. It is known to couple to the activation of G_i proteins leading to the inhibition of adenylyl cyclase activity. It is also known to activate pathways leading to ERK phosphorylation. This pathway is dependent upon $G\beta\gamma$ release from G_i proteins, $p21^{ras}$-dependent activation of PI3K and MEK1 but is independent of calcium, PKC, and Src activation (Schulte and Fredholm, 2002). Signalling has also been demonstrated in astroglia cells from the A_3AR to the activation of Rho proteins involved in actin cytoskeletal re-arrangement (Abbracchio *et al.*, 2001).

1.11 Evidence for a role of ARs in inflammation

Inflammatory agents, such as those previously mentioned and inflamed conditions, such as asthma, cause a net increase in the concentration of adenosine by several potential mechanisms: increased catabolism of 5'AMP to adenosine by ecto- and endo- 5'nucleotidases, inhibition of adenosine kinase and adenosine deaminase activity, and activation of S-adenosylhomocysteine hydrolase, responsible for S-adenosylhomocysteine hydrolysis into adenosine (Haskó *et al.*, 2004; Figure 1.9). These mechanisms generate adenosine, which can act at the four adenosine receptor subtypes, each of which play distinct roles in inflammation. In general, several studies suggest that the A_1AR, A_{2B}AR and A_3AR have both pro- and anti-inflammatory properties dependent upon the model system used, as described below.

Figure 1.9 The synthesis and catabolism of adenosine.

Adenosine is synthesized by the action of several ecto- and endo-5'nucleotidases, which degrade 5' AMP to adenosine. In addition, the production of adenosine is catalysed by S-adenosylhomocysteine hydrolase using S-adenosylhomocysteine as a substrate. Intracellular concentrations of adenosine can also increase due to the action of the nucleoside transporter. Catabolism of adenosine involves the phosphorylation of adenosine into 5'AMP by adenosine kinase and the deamination of adenosine to inosine.


S-adenosyl
Homocysteine (SAH)
Adenosine
SAH-Hydrolase
Nucleoside transporter
Adenosine
Ecto-and Endo
5'-nucleotidase
Adenosine Deaminase
(ADA)
Adenosine
kinase
5'-AMP
Inosine

1.11.1 The inflammatory effects of A_1AR activation

A_1AR activation is known to have a prominent cardio-protective effect in animal models of ischaemia by the activation of K_{ATP}-channels (Vinten-Johansen *et al.*, 1999). In addition to its role in cardioprotection, it also has both pro- and anti-inflammatory effects. In rodent models of inflammation and nerve-injury pain, A_1AR activation reduces hypersensitivity to acute heat or acute paw pressure (Li *et al.*, 2003). In addition, rat adjuvant-induced models of arthritis show improvement when treated intrathecally with A_1AR agonist (Boyle *et al.*, 2002). A protective effect of the A_1AR is also suggested by studies of renal ischaemia-reperfusion injury in A_1AR knockout mice. Mice lacking the A_1AR, exhibit increased renal injury as determined by renal tubular neutrophil infiltration, ICAM-1, TNFα, and IL-1 mRNA expression, compared with their WT littermate controls (Lee *et al.*, 2004). In contrast, animal models of asthma suggest that the A_1AR is involved in mediating bronchospasm (Meade *et al.*, 2001) *via* a neural mechanism independent of mast cell degranulation. The different roles of A_1AR within each animal model suggest alternative signalling pathways that mediate the different effects.

1.11.2 The inflammatory effects of A_{2B}AR activation

A_{2B}ARs also display both pro- and anti-inflammatory properties within different model systems. In rodent models of Alzheimers disease, chronic infusion of LPS into the fourth ventricle, induced neuro-inflammation that corresponded to a reduced expression of A_{2B}ARs. Treatment with a NO-releasing non-steroidal anti-inflammatory drug prevented the down-regulation of A_{2B}ARs suggesting the potential involvement of A_{2B}ARs in the pathogenesis of the disease (Rosi *et al.*, 2003). *In vitro* studies of murine bone marrow-derived macrophages show that specific A_{2B}AR agonists induce cAMP production, which inhibits macrophage proliferation through a mechanism involving the induction of $p27^{kip1}$ expression (Xaus *et al.*, 1999). Pro-inflammatory effects of the A_{2B}AR are also reported in different models of inflammation. The A_{2B}AR is important in mast cell degranulation, which triggers bronchospasm in rodent models of asthma (Meade *et al.*, 2001). In addition, *in vitro* studies of intestinal inflammation indicate that adenosine acting at A_{2B}ARs increases the secretion of IL-6, a pro-inflammatory cytokine involved in neutrophil degranulation and lymphocyte differentiation (Sitaraman *et al.*, 2001).

1.11.3 The inflammatory effects of A_3AR activation

Like the A_1AR and $A_{2B}AR$, the A_3AR exhibits both pro- and anti-inflammatory effects. In two murine models of colitis, an A_3AR agonist, N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA), decreased inflammatory cell infiltration into the colon and decreased cytokine and chemokine levels (Mabley *et al.*, 2003). *In vivo* studies on $A_3AR^{-/-}$ mice indicate that the A_3AR activation can inhibit LPS-induced TNFα production (Salvatore *et al.*, 2000). In addition, Szabo *et al.*, (1998) reported that A_3AR activation can reduce macrophage inflammatory protein (MIP)-1α production and collagen-induced arthritis by reducing the production of IL-12, IL-6 and NO production (to a lesser extent; Szabo *et al.*, 1998). In addition, A_3AR agonists act as cardio- and neuroprotective agents and attenuate ischaemic damage (Fishman and Bar-Yehuda, 2003). In contrast, A_3AR activation in basophils and mast cells, stimulates degranulation and consequently bronchospasm leading to asthma (Nagano *et al.*, 2000; Tilley *et al.*, 2003). Additionally, studies of carrageenan-induced inflammation in A_3AR knockout mice indicate a pro-inflammatory role for the A_3AR, as knockout mice had significantly reduced inflammation when compared to WT litter mates (Wu *et al.*, 2002).

1.11.4 The anti-inflammatory role of $A_{2A}AR$ activation

In contrast to other adenosine receptors, multiple *in* vivo and *in* vitro studies suggest a potent anti-inflammatory effect of the $A_{2A}AR$. Early reports indicated that A_2AR activation could inhibit superoxide release from guinea pig eosinophils stimulated with opsonised zymosan and from human eosinophils (Yukawa *et al.*, 1989). Further studies by Salmon and Cronstein (1990), reported that NECA, an A_2AR selective agonist could inhibit Fc-γ R-mediated phagocytosis and superoxide generation in polymorphonuclear leukocytes (PMN). This effect was blocked by the AR antagonist, 8-*p*-sulfophenyltheophylline. In further studies, Cronstein and colleagues also reported that at concentrations of adenosine and NECA sufficient to inhibit the generation of ROS, they could detect a decrease in the adhesion of fMLP-induced PMN to endothelial cells (Cronstein *et al.*, 1992). Later studies in neutrophils indicated that this reduction was due to the inhibition of $\alpha_M\beta_2$ integrin expression, which is required for adhesion. (See Section 1.2.3; Wollner *et al.*, 1993).

The potent anti-inflammatory role of the folate antagonist methotrexate (MTX) was also shown to be dependent upon adenosine. When used at low concentrations, injection of MTX could reduce leukocyte accumulation in carrageenan-inflamed air pouches, due to the accumulated presence of adenosine following up-regulation of the adenosine transporter AICAR, in splenocytes. This effect was completely reversed by an A_2AR antagonist DMPX suggesting that adenosine acting at an A_2AR subtype mediated the protective effect. In additional studies, inhibition of adenosine kinase could also reduce leukocyte accumulation in the same model. This effect could also be completely reversed by co-injection of ADA further implicating a role for adenosine acting at A_2ARs (Cronstein *et al.*, 1994).

With the cloning of the A_{2B}AR, studies suggested a more potent anti-inflammatory role for the A_{2A}AR. In models of ischaemia-reperfusion injury in the rat kidney, an A_{2A}AR-selective agonist could reduce serum creatinine levels, blood urea nitrogen levels and reduce tubular epithelial necrosis. This effect was blocked by the A_{2A}AR-selective antagonist, ZM241385 (Okusa *et al.*, 1999). *In vivo* rat models of meningitis have also indicted that A_{2A}AR activation could decrease neutrophil accumulation in the sub-arachnoid space, reduce cytokine-enhanced neutrophil adherence and superoxide release. These effects were also blocked by the A_{2A}AR antagonist ZM241385 (Sullivan *et al.*, 1999). Furthermore in rodent studies of atherosclerosis, A_{2A}AR activation could reduce endothelial activation and neo-intimal growth in sections of carotid artery.

Studies have also been carried out on the role of the A_{2A}AR in allergen-induced pulmonary airway inflammation. In ovalbumin-sensitised rats, CGS21680 treatment given intrathecally, dose-dependently inhibited broncheolar lavage fluid leukocyte numbers, protein content and myeloperoxidase and eosinophil peroxidase activities measured 24hr post-challenge. This effect was blocked when the A_{2A}AR-selective antagonist ZM241385 was administered 1h prior to agonist (Fozard *et al.*, 2002). In addition to the anti-inflammatory role of the A_{2A}AR, it has also been shown that activation of the A_{2A}AR can promote wound healing and mediate angiogenesis in response to tissue injury (Montesinos *et al.*, 2002). Further, systemic administration of ATL-146e during reperfusion following spinal cord ischaemia, decreased neuronal apoptosis leading to improved preservation of hind limb function. (Cassada *et al.*,

2001). Animal models of acute intracerebral brain hemorrhage also indicate a protective effect of CGS21680 when administered directly into the striatum immediately prior to the induction of haemorrhaging (Mayne *et al.*, 2001).

In vivo studies also indicate that in mouse models of ischaemia/reperfusion injury, co-reperfusion of the A_{2A}AR-selective agonist ATL146e, decreases liver injury by 90%. Co-administration of the A_{2A}AR-selective antagonist ZM241385, attenuates this effect indicating the receptor specificity of the response (Day *et al.*, 2004). In addition, A_{2A}ARs were also recently shown to protect from concanavilin A-induced liver injury *in vivo* (Ohta and Sitkovsky, 2001). Further studies *in vitro*, suggest the anti-inflammatory response is due to a reduction in the expression of cytokine and chemokines from cells expressing A_{2A}ARs. THP-1 cells treated with LPS increased the expression of A_{2A}ARs and adenosine. Further treatment of the cells with an A_{2A}AR selective agonist CGS21680, decreased LPS-stimulated TNFα production in a time- and dose-dependent manner. This inhibitory pathway involves A_{2A}AR stimulation of adenylyl cyclase generating cAMP that results in the activation of PKA and phosphorylation of cAMP response element binding protein (CREB; Bshesh *et al.*, 2002).

The consistent anti-inflammatory role for A_{2A}AR receptor signalling in a variety of different animal models of inflammation, indicates a possible common mechanism of action. Initial studies showed that A_{2A}AR activation can inhibit the expression of pro-inflammatory cytokines and chemokines, key components in the inflammatory response, suggest that this inhibition may occur at the level of transcription. Transcription factors important in the expression of many pro-inflammatory genes include NF-κB and AP-1. The aim of my project was to determine the molecular mechanism by which the A_{2A}AR can modulate pro-inflammatory gene expression, by studying the upstream transcription factors and signalling components. The dissection of the pathways involved could lead to more specific drug design that may benefit a wide range of disease states.

Chapter 2
Materials and Methods

2.1 Chemicals and Suppliers

All reagents were of the highest grade commercially available and obtained by the following suppliers:

Affinity Bioreagents, Golden, CO, U.S.A.

Anti-$A_{2A}AR$ specific antibody

Alexis Corporation, San Diego, CA, U.S.A.

Dithiothreitol (DTT)

Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire

[^{125}I] Sodium iodide

Bio-Rad Laboratories Ltd, Maylands Avenue, Hemel Hempstead

Bradford's Reagent

BD Biosciences, Pharmingen, San Ljose, CA, U.S.A.

Anti-iNOS antibody

BDH Chemicals Ltd., Poole

Acrylamide

Cambrex Bio Science Wokingham Ltd. BioWhittaker House, Wokingham, Berkshire

Endothelial Basal Media-2™, Singlequots™ (foetal bovine serum (FBS), hydrocortisone, fibroblast growth factor-B (hFGF-B), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), ascorbic acid, epidermal growth factor (hEGF), gentamicin sulphate and amphotericin-B (GA-1000) and heparin), human umbilical vein endothelial cells (HUVECs)

Calbiochem-Novabiochem (UK) Ltd., Nottingham

Forskolin, phorbol-12-myristate-13-acetate (PMA), SB 203580, PD98059, pyrrolidinedithiocarbamate (PDTC), N-acetyl cysteine, H-89

Cell Signalling Technology Inc., Beverly, MA U.S.A.

Anti-$Ser^{32}Ser^{36}$-phosphorylated IκBα, anti-Ser^{63}-phosphorylated c-jun, anti $Thr^{180}Tyr^{182}$-phosphorylated p38

CN Biosciences, Merck Biosciences Ltd., Beeston, Nottingham

GeneJuice transfection reagent

European Collection of Cell Cultures, CAMR, Porton Down, Salisbury, Wiltshire

C6 glioma cells

Fisher Scientific, Loughborough, Leicestershire

N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) HEPES, sodium dodecyl sulphate (SDS), ethylenediaminotetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), ethidium bromide solution, glacial acetic acid, methanol, ethanol, concentrated hydrochloric acid (HCl), sodium fluoride

GIBCO BRL Life Technologies, Paisley

Phenol:chloroform:isopropanol, Lipofectamine, new born calf serum, Optimem, phosphate-free Dulbecco's modified Eagle's medium (DMEM)

Interactiva-Thermo Hybaid, Thermo Biosciences GmbH, Ulm, Germany

Oligonucleotides

Inverclyde Biologicals, Strathclyde Business Park, Bellshill, Lanarkshire

Protran nitrocellulose Schleicher and Schuell membrane

Melford, Chelsworth, Ipswich, Suffolk

Kanamycin

Merck, Darmstadt, Germany

Bactotryptone, agar

Molecular Probes,Poortgebouw, Leiden, The Netherlands

Alexa^{TM}594-conjugated goat anti-mouse IgG, Alexa^{TM}594-conjugated goat anti-rabbit IgG

New England Biolabs Inc., Beverley, MA U.S.A.

Pre-stained protein molecular weight marker, restriction enzymes

NEN Life Science Products Inc., Boston, U.S.A.

ECL reagents, [^{32}P]-orthophosphate

PAA Laboratories, Linz, Austria

G-418 disulphate sodium salt

Perkin-Elmer Life Sciences, Monza, Italy

[γ-^{32}P] ATP

Pierce, Rockford, IL, U.S.A.

EZ-Link^{TM} biotin-LC-hydrazide, horseradish peroxidase (HRP)-conjugated streptavidin, Western Blot Stripping Solution

Promega, Southhampton

T4 DNA Ligase, SV DNA mini-prep kit, G-418 disulphate sodium salt, restriction enzymes, dNTPs

Qiagen, Crawley, West Sussex

DNA gel purification kit, DNA plasmid maxi-prep kit

Research Biochemicals International, Natick, MA, U.S.A.

NECA, CGS 21680

Roche Molecular Biochemicals/ Boehringer-Mannheim, Mannheim Germany

Tris[hydroxymethyl]aminomethane; (Tris), DNA molecular size marker, restriction enzymes

Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.

Mouse anti-hemagglutinin(HA) epitope (YPYDVPDYA) antibody, mouse anti-p65 (A) antibody, rabbit anti-IκBα (C-21) antibody

Serotec Ltd., Kidlington, Oxford

Mouse anti-human E-selectin/CD62E (CL2/6) antibody

Sigma-Aldrich Company Ltd., Poole, Dorset

Triton X-100, soybean trypsin inhibitor, benzamidine, bovine serum albumin, protein A-Sepharose, sodium periodate, bisacrylamide, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG, horseradish peroxidase (HRP)-conjugated streptavidin, thimerosal, bromophenol blue, sodium azide, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), agarose, deoxycholic acid sodium salt, polyethylenimine, ampicillin, adenosine deaminase, paraformaldehyde, N,N,N',N'-tetramethylethylenediamine (TEMED), phenylmethylsulfonyl fluoride (PMSF), Ham's F-12 media, Dulbecco's modified Eagle's medium (DMEM), sterile phosphate buffered saline (PBS), foetal bovine serum, trypsin, penicillin/streptomycin, L-glutamine, recombinant human tumour necrosis factorα (TNFα), recombinant murine TNFα, lipopolysaccharide (LPS), 30:1 acrylamide: bisacrylamide solution, tetracycline, protein G-Sepharose, rat interferon-γ (IFN-γ), sulphanilamide, *N*-1-naphthylenediamine dihydrochloride, bicinchoninic acid, 3,3'5,5'-tetramethylbenzidine (TMB)

Stratagene

Pfu Turbo DNA polymerase

Whatman International Ltd., Maidstone, Kent

GF/B glass fibre filters, filter paper.

9E10 monoclonal antibody specific to the *myc* epitope EQKLISEEDL, was prepared in-house at Duke University, Durham, NC, USA by Dr. Tim Palmer.

ZM241385 was a kind gift from Dr. Simon Parker, Astra-Zeneca, Macclesfield, Cheshire and was iodinated according to Palmer & Stiles, (1999)

Rec $^{+}$ BJ5183 *E.coli* bacterial cells were a generous gift from Prof. B. Vogelstein, John Hopkins University, MD, USA.

C6 glioma cells stably expressing the canine A_{2A}AR were prepared in-house at Duke University, Durham, NC, USA by Dr. Tim Palmer (Palmer and Stiles, 1999)

2.2 Cell Culture & Transfection Methods

2.2.1 Cell maintenance

All cell types were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

HUVECs were maintained in endothelial basal medium supplemented with 2% (v/v) foetal bovine serum, 0.04% (v/v) hydrocortisone, 0.4% (v/v) human fibroblast growth factor-B (hFGF-B), 0.1% (v/v) vascular endothelial growth factor (VEGF), 0.1% (v/v) insulin-like growth factor-1 (IGF-1), 0.1% (v/v) ascorbic acid, 0.1% (v/v) human epidermal growth factor (hEGF), 0.1% (v/v) gentamicin sulphate and amphotericin-B (GA-1000) and 0.1% (v/v) heparin. Cells were routinely sub-cultured until passage 6 as HUVECs will adapt to cell culture conditions over time, by regulating the expression of different cellular proteins such as adhesion molecules (Muller *et al.*, 2002). When confluent, cells were washed in 3 ml of HBSS (30 mM HEPES Buffered Salt Solution) and then treated briefly with 3 ml endothelial grade trypsin in order to detach the cells. The trypsin was then neutralised with 3 ml trypsin-neutralising solution and the contents transferred to a 13 ml centrifuge tube. Cells were centrifuged for 5 min at 1000g and the supernatant was discarded. The cell pellet was gently resuspended in medium at a dilution factor suitable to establish a cell density that could be reliably counted within a standard haemocytometer, which was typically 1:10 dilution. Wells were then seeded at an appropriate level according to the analysis performed as indicated in the Figure Legends. Typically a 6-well plate would be seeded with 5×10^5 cells/well ready for infection the following day, or 5×10^6 cells if a confluent monolayer of cells was required for the next day. A minimum of 1×10^4 cells was used to maintain the cell line in a fresh 75-cm^2 tissue culture flask to which 9 ml of fresh medium was added.

Human Embryonic Kidney 293 cells (HEK 293) were maintained in DMEM supplemented with 10% (v/v) FBS, 1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Confluent monolayers were washed once in 5 ml of sterile PBS and then treated with 1 ml trypsin. Cells were then returned to the incubator for a few minutes before the flask was disrupted to dislodge the cells. Cells were then typically diluted 1:8, 7 ml was used in experimental analysis and 1 ml was used to maintain the cells to which 9 ml of fresh DMEM was added. C6 cells were similarly

passaged and maintained in DMEM supplemented with 10% (v/v) FBS, 1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Chinese Hamster Ovary cells were maintained in Ham's F-12 media supplemented with 10% (v/v) FBS, 1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin and were subcultured in an analogous fashion to HEK 293 cells. Stable cell lines were also were supplemented with 0.0016mg/ml G418 disulphate salt in order to ensure a constant selection pressure. U937 cells were maintained in RPMI medium also supplemented with 10% (v/v) FBS, 1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. As these cells grow in suspension, they were maintained by dilution of the cell population into fresh medium.

2.2.2 Transfection with Lipofectamine

The following transfection method is based on one well of a six-well plate. In a sterile microfuge tube, 120 µl of Optimem and 2 µg of plasmid DNA were gently mixed/per well to be transfected. In a separate sterile microfuge tube, 120 µl of Optimem and 4 µl of Lipofectamine transfection reagent/well were also gently mixed and then added to the microfuge containing the plasmid DNA/Opitmem mix. This was then incubated at room temperature in the dark for 45 min. While the mix was incubating, 70-80% confluent cells were washed once with 2 ml/well of Optimem and the medium replaced with 760 µl of Optimem. The DNA-Lipofectamine mix was then added dropwise to the cells and incubated for 3 hr. The transfection was then removed and the medium replaced. For experiments, cells were analysed 24 hr after transfection.

2.2.3 Transfection with GeneJuice

For transfection into C6 glioma cells, GeneJuice yielded greater transfection efficiency compared with Lipofectamine, therefore GeneJuice was the preferred choice. In a sterile microfuge 1µg of plasmid DNA was incubated with 100 µl of Optimem and vortexed briefly. In a different microfuge tube 6µl of GeneJuice transfection reagent was also incubated with 100µl of Optimem. After 5 min, the GeneJuice was added to the plasmid DNA, mixed gently, and incubated for 30 min at room temperature in the dark. The DNA-GeneJuice mix was then added to each well containing 3 ml of fresh medium and left to incubate for 3-5 hr then replaced with fresh DMEM. Cells were then analysed 24 hr post-transfection.

2.3 Molecular Biology

2.3.1 Preparation of antibiotic agar plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 1.5% (w/v) agar) was prepared, autoclaved and allowed to cool before addition of antibiotic at the following final concentrations, (ampicillin 50 μg/ml kanamycin 30 μg/ml. The liquid LB agar was then poured into 90 mm-diameter Petri dishes, allowed to solidify and then allowed to sweat overnight at room temperature to get rid of excess moisture. Plates were then stored at 4°C until required for a maximum period of 2 weeks.

2.3.2 Preparation of competent XL1 Blue *E.Coli*

An overnight culture of XL1 Blue *E.Coli* was grown in 3 ml of LB broth containing 50 μg/ml tetracycline. The following day, this was used to inoculate 250 ml of LB broth, which was then grown with aeration in a 37°C shaking incubator at 200 rpm, until the growth rate reached log phase as determined by the culture reaching an optical density at 600 nm of 0.35-0.375. Bacteria were transferred to two chilled 250 ml centrifuge tubes and left for one hour on ice. Bacteria were then sedimented by centrifugation at 3,500 g for 20 min at 4°C and the supernatant discarded. The bacterial pellet was then washed and resuspended in 62.5 ml of ice-cold 0.1M magnesium chloride. Following another 20 min centrifugation at 3,500g, the bacteria were resuspended in 62.5ml of ice-cold 15% (v/v) glycerol with 0.1M calcium chloride. 250 μl of bacteria were aliquoted into sterile microfuge tubes in a dry ice/methanol bath to induce rapid freezing, and stored at –80°C until required.

2.3.3 Transformation of competent XL1 Blue *E.Coli*

Approximately 30-50 ng of plasmid DNA was added to a plastic 13 ml Falcon round-bottom tube on ice. Once thawed 50 μl/tube of competent *E.coli* was immediately added and the mix incubated on ice for 10 min. The tubes were incubated for 5 min at 37°C before 0.5 ml of LB per tube was added. Tubes were then shaken at 37°C for 45 min. 0.2 ml of the transformation mix was then plated out onto LB agar plates containing the appropriate selection antibiotic and incubated overnight at 37°C.

2.3.4 Preparation of plasmid DNA

Transformed colonies picked from agar plates using sterile pipette tips, were used to inoculate 10 ml of LB broth supplemented with the appropriate selection antibiotic, and placed in the shaking incubator at 37°C overnight. Plasmid DNA was then isolated using the Promega™ Wizard Plus SV miniprep purification system as per the manufacturer's instructions. For larger quantities of plasmid DNA, the initial 10 ml culture was then used to inoculate a 500 ml culture containing the appropriate antibiotic and grown overnight. Plasmid DNA was isolated using the Qiagen Maxi Kit system as directed by the manufacturer's instructions. The concentration of double stranded DNA obtained was calculated based on the assumption that 1 absorbance unit (A_{260}) is equivalent to 50 μg/ml of double stranded DNA. An absorbance ratio (A_{260}/A_{280}) equal to 1 indicated good quality DNA.

2.3.5 Digestion of plasmid DNA

1-2 μg of purified plasmid DNA was digested in a sterile microfuge tube containing the appropriate enzyme buffer and 2-4 units of enzyme as per the manufacturer's instructions. In certain cases it was necessary, due to incompatible buffer restrictions, to purify the linearised plasmid DNA from the first digestion, before digestion with the second enzyme and buffer. This was achieved by ethanol precipitation. Briefly 1/10 volume of 3M sodium acetate and 3 volumes of ice-cold absolute ethanol were added to the sample. Following 30 min incubation on ice, samples were centrifuged at 4°C for 30 min at 13,000 g and the supernatant removed. The DNA pellet was washed once in 50 μl of 70% (v/v) ethanol, centrifuged at 4°C for 5 min at 13,000g and allowed to air-dry. It was then resuspended in 15 μl of the second enzyme buffer before the addition of 2-4 units of the second enzyme as per the manufacturer instructions. Restriction fragments were typically resolved on a 1% (w/v) agarose gel containing 2.5 μg/ml ethidium bromide run at 75 mV for 20-30 min in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, and 0.1% (v/v) glacial acetic acid). Purification of DNA from agarose gels was by Qiagen QIAquick gel purification kit as per manufacturer's instructions.

2.3.6 Ligation of DNA fragments

Ligation of DNA fragments into vectors was carried out overnight at 4°C using Promega™ T4 DNA ligase. A typical ligation contained 1.5 μl 10X Ligation buffer

(30 mM Tris-HCl pH 7.8, 10 mM magnesium chloride, 10 mM dithiothreitol (DTT), 1 mM ATP, a 4:1 ratio of cut insert DNA to the relevant cut vector DNA, and 10 units of T4 DNA Ligase in a final reaction volume of 15µl. Ligation reactions were then used to transform competent XL1 Blue *E.coli* as described in Section 2.3.3.

2.3.7 Construction of a *myc*-His tagged human A_{2A} adenosine receptor mammalian expression construct.

The *myc*-His tagged human A_{2A}AR was constructed using a polymerase chain reaction (PCR) approach. The oligonucleotide primers used in the PCR were designed to amplify the human A_{2A}AR coding sequence, remove the stop codon, and add an *Xba*I site (underlined) at the C-terminus using pCMV5/human-A_{2A}AR cDNA as a template. This was carried out using the following primers:

5'-TAGCAGAGCTCGTTTAGT-3' (sense)

and 5'-TGATTTCTAGAGGACACTCCTGCTCCATCCTG-3' (antisense).

This was used to amplify a 1.8 kbp PCR product in a reaction containing 100 ng template cDNA, 100 µM dNTPs, 50 pmol sense/antisense primers, 0.002 units of *Pfu* Turbo polymerase, 10% (v/v) *Pfu* Turbo amplification buffer and 5% (v/v) DMSO in a final volume of 100 µl. The reaction was then subject to 25 cycles of 95°C for 1 min, 45°C for 1 min, 72°C for 1.5 min followed by one cycle of 95°C for 1 min, 55°C for 1 min and 72°C for 10 min. The PCR product was extracted with phenol:chloroform:isopropanol (25:24:1,v/v) and further purified by ethanol precipitation. The resulting product was then digested using the restriction enzymes *Hind*III and *Xba*I as described in section 2.3.5. Products were then resolved on a 1% (w/v) agarose gel in parallel with *HindIII/XbaI* digests of pcDNA3/*myc*HisA vector (Invitrogen). The products were then gel purified using the Qiagen Gel Extraction kit as per the manufacturer's instructions. Purified, digested DNA products were then ligated overnight as described in Section 2.3.6 and used to transform competent XL1 Blue *E.coli* as descried in Section 2.3.3. Ampicillin-resistant colonies were screened for successful ligation, by *HindIII/XbaI* digestion of minipreped cDNA prepared from 10 ml LB medium cultures supplemented with 50 µg/ml ampicillin and grown overnight at 37°C in a shaking incubator. The construction of the A_{2A}AR truncated mutants was carried out in precisely the same fashion. The same PCR conditions were used except using the following antisense primers.

A_{2A}AR Δ55 5'-TGATTTCTAGAGCCATTGGGCCTCCGCTC -3'

A_{2A}AR Δ101 5'- TGATTTCTAGATTGCTGCCTCAGGACGTGGCT-3'

Both primers were designed to truncate the wild-type receptor and add an *Xba*I site at the C-terminus. The A_{2A}AR Δ55 truncation removed 55 amino acids from the C-terminus containing six potential phosphorylation sites, five serine and one threonine residue. The A_{2A}AR Δ101 truncation removed 101 amino acids, removing a further five serine residues and one threonine residue. Correct sequences were confirmed using dideoxynucleotide sequencing.

2.4 Propagation and purification of human A_{2A} adenosine receptor adenovirus

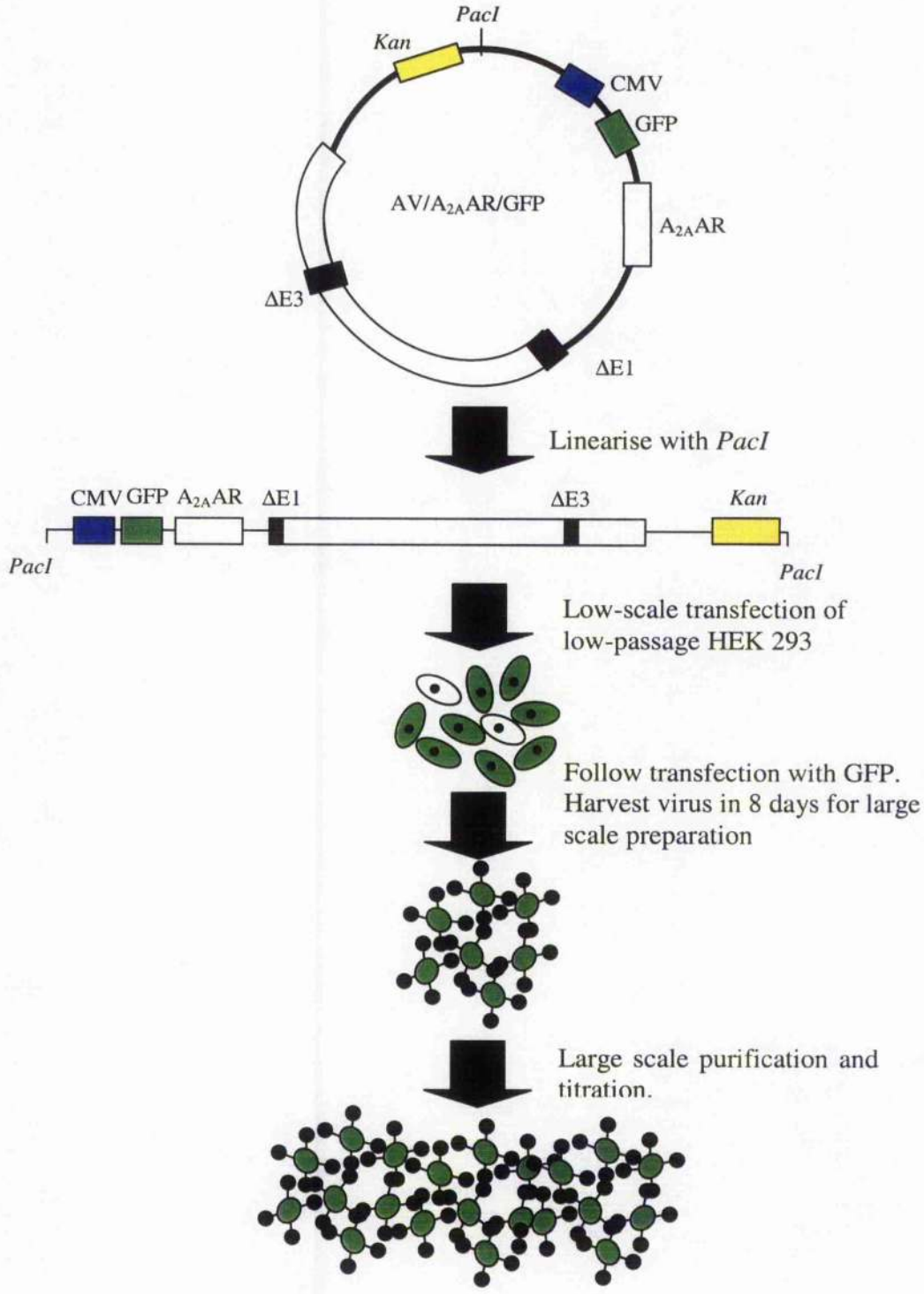
In order to express *myc*-tagged human A_{2A}AR in HUVECs, an adenoviral gene transfer approach was used. This is because it achieved much greater expression when compared with commercially available transfection reagents, electroporation or calcium phosphate-mediated transfection methods. The propagation and purification of the AV-A_{2A}AR/GFP and AV-GFP were carried out in accordance with the protocols of Nicklin and Baker, (2001).

The pAdEasy™ system from Qbiogene was used as per the manufacturer's instructions in order to generate adenoviral cDNA stocks which would express AV-A_{2A}AR (See Figure 2.1). Briefly the first step required excision of the *myc*-tagged human A_{2A}AR from pcDNA3/*myc*HisA using *Hind*III/*Pme*I and the subsequent subcloning into a *Hind*III/*EcoR*V-digested shuttle vector pAdTrackCMV, destroying the *EcoR*V and *Pme*I sites. The resulting pAdTrackCMV/*myc*A_{2A}AR construct was then cut at the *Pme*I site within the vector in order to expose the inverted terminal repeats, and co-transformed with the viral DNA backbone construct pAdEasy1 into *E. coli rec*+ strain BJ5183 by electroporation (2 kV, 25 μF, 200 Ω. Recombination-positive colonies were screened by digestion with *Pme*I and PCR using *myc*A_{2A}AR-specific primers. These colonies were then expanded and the cDNA isolated. The plasmid cDNA was then linearised with *Pac*I and 10 μg was transfected using 30 μl Lipofectamine into low-passage HEK 293 cells in 10 cm tissue culture dishes. The presence of the gene encoding GFP in a separate open reading frame allowed us monitor viral expression by fluorescence microscopy. 8 days post-transfection, cells were scraped and collected in a sterile centrifuge tube and pelleted by a 5 min

centrifugation step at 400 g at room temperature. The pellet was washed in 1 ml PBS, re-centrifuged and the supernatant discarded. Cells were then disrupted by three freeze/thaw cycles in a dry ice/methanol bath and the pellet resuspended in 10 ml of serum-free medium. This was then used to infect two T-150 flasks of 50-60% confluent HEK 293 cells, grown in medium containing 2% (v/v) FBS. Virus particles from these cells were then prepared 3 days later, and used to infect a larger scale culture of 20 x T-150 flasks of HEK 293 cells. From this large scale preparation virus was isolated as per the method of Nicklin and Baker (2001). Briefly, dislodged cells were pooled and pelleted by a brief centrifugation at 250 g for 10 min at room temp. The supernatant was then discarded and the pellet resuspended in 1 ml of sterile PBS. The pellet was then washed four times with 1 ml of sterile PBS and the supernatant discarded. This was then subjected to three freeze/thaw cycles in order to lyse the cells and release the virus particles. The preparation was then added to a discontinuous caesium chloride gradient set up in a 13ml sterile ultracentrifuge tube, in order to separate intact virus particles from other cellular debris and empty viral capsids. Turbulence caused by friction under braking would disrupt the separated adenoviral layer so the samples were subject to centrifugation at 100,000g for 18 hr with zero deceleration. The opaque adenovirus band was then isolated by syringe extraction and added into a Slide-A-Lyser that allowed efficient overnight dialysis at 4°C in 1L of dialysis buffer (135 mM sodium chloride, 10 mM Tris-HCl (pH 7.5), 1 mM magnesium chloride) which was changed three times. This was then diluted in a 1:1 (v/v) ratio with sterile virus storage buffer (10 mM Tris-HCl, pH 8, 100 mM sodium chloride, 0.1% (w/v) BSA, 10% (v/v) glycerol) and stored at -80°C in 30µl aliquots.

Figure 2.1 Generation of a recombinant adenovirus.

Plasmid cDNA from kanamycin-resistant A_{2A}AR/GFP-positive colonies was isolated and linearised with *Pac*I. 10 μg of cDNA was then transfected into low-passage HEK 293 cells in 10 cm tissue culture dishes. 8-days later, virus particles were then harvested from infected cells and used to infect 20 x T150 flasks of low-passage HEK293 cells. Virus prepared from these cells, was then titred, aliquoted and used in further experiments. (Adapted from www.qbiogene.com)


PacI
Kan
CMV
GFP
AV/A2AAR/GFP
A2AAR
ΔE3
ΔE1
Linearise with PacI
CMV GFP A2AAR ΔE1 ΔE3 Kan
PacI
PacI
Low-scale transfection of low-passage HEK 293
Follow transfection with GFP. Harvest virus in 8 days for large scale preparation
Large scale purification and titration.

Once isolated, the virus was subject to titration by the end-point dilution method (Nicklin and Baker, 2001) using GFP as a marker for positive colonies. 1 x 10^4 HEK 293 cells/well were subcultured into 80 wells of a 96-well plate in order to reach approximately 50-60% confluency after 24 hr growth. 0.1 ml of virus was then used to infect the wells over a range of serial dilutions (10^{-2}-10^{-11}). This was incubated for 18 hr before the virus was removed and the medium replaced. The medium was replaced every 3 days up to 10 days post-infection until the well showed evidence of viral infection as assessed by GFP expression. After 10 days the number of positive colonies was counted for each concentration of virus and the virus titre was calculated. An example of this calculation is found in Appendix 1.

2.5 Laboratory techniques

2.5.1 Discontinuous SDS-PAGE and Immunoblotting

Samples prepared for SDS-PAGE were equalised for protein using the bicinchoninic acid assay described in Section 2.5.2. Pre-stained protein markers (Invitrogen Rainbow Markers, range 6.5-175kDa) in sample buffer (50 mM Tris (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001% (w/v) of bromophenol blue, 6 μM DTT) were also prepared in the electrophoresis in order to determine protein molecular mass. 30 μl of sample, equalised for protein and volume, was then subjected to discontinuous SDS-PAGE using a 6 cm 10% (w/v) polyacrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH 8.8), 0.1% (w/v) SDS, 3% (v/v)glycerol, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and a 2 cm 3% (w/v) stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED). Electrophoresis was carried out using Biorad Mini-Protean II or III gel electrophoresis systems in running buffer (27.4 mM Tris, 0.19M glycine, 0.1% (w/v) SDS) at 150 V until the bromophenol blue dye front reached the end of the gel. Proteins were then transferred electrophoretically onto a nitrocellulose membrane at 400mA for 45 min in transfer buffer (24.7 mM Tris, 0.19M glycine in 20% (v/v) methanol). The nitrocellulose membrane was then removed, trimmed and briefly washed in PBS before incubating for 1 hr at room temperature in Blotto (5% (w/v) skimmed milk, 0.2% (v/v) Triton X-100 in PBS) to block non-specific binding of antibody. The membrane was then briefly washed in PBS before being transferred to a plastic bag containing 2 ml of the relevant primary

antibody, diluted in Blotto as indicated in the Figure Legends. This was incubated in the cold room overnight on a rotating platform. The membranes were rinsed briefly in PBS before being washed three times in Blotto for 10 min. Membranes were then washed again briefly in PBS before being transferred to a bag containing 2 ml of the appropriate secondary antibody conjugated to HRP in High-Detergent Blotto (10% (v/v) Blotto in PBS supplemented with 1.02% (v/v) Triton X-100 and 0.1% (w/v) SDS). This was then placed on a rotating platform at room temperature and incubated for 1 hr. The membrane was then washed three times for 10 min in Blotto and then washed a further twice for 10 minutes in PBS. Membranes were then exposed to an enhanced chemiluminescent procedure. HRP-specific oxidative degeneration of luminal causes emission of light at 428 nm, detected by Kodak X-OMAT Blue X-ray film in to visualise immunoreactive proteins.

2.5.2 Protein concentration determination using the bicinchoninic acid (BCA) protein assay.

Duplicate 0.01 ml samples of known BSA standards in the range 0-2 mg/ml and unknown protein samples were added to a 96-well plate. 0.2 ml BCA solution (1% (w/v) 4,4 dicarboxy-2,2 biquinoline disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium bicarbonate pH 11.25, 0.08% (w/v) copper (II) sulphate) was then added to each well. Protein concentration-specific reduction of Cu^{2+} to Cu^{1+} allows the bicinchoninic acid sodium salt to bind the Cu^{1+} ion forming an intense purple colour allowing measurement of the absorbance at 492 nm. Colour was therefore allowed to develop at room temperature for 30 min and the absorbance of the standards was used to determine a straight line from which unknown protein concentrations could be calculated (Smith *et al*, 1985).

2.5.3 Protein concentration determination using Bradford's reagent

This assay was based on the method of Bradford (Bradford, 1976). Duplicate 0.01 ml of known BSA standards in the range 0-2 mg/ml and unknown protein samples were added to a 96-well plate. 0.01 ml of Bradford's reagent was then added to each well. Binding of protein to the dye component of the Bradford reagent, Brilliant Blue G, causes a shift in the absorption spectra of the dye from 465nm to 595nm. Colour was therefore allowed to develop for 10 min and the OD_{600} of the standards and

samples measured. A straight line was produced based on the absorbance of known standards from which, unknown protein concentrations were calculated.

2.5.4 Cell surface biotin-labelling of A_{2A}ARs

Chinese Hamster Ovary (CHO) cells transiently transfected with cDNA encoding the *myc*His-tagged human A_{2A}AR were used to analyse receptor cell-surface expression. Following treatment, as indicated in the Figure Legends, cells were placed on ice and washed gently three times with 3 ml ice-cold PBS-CM (PBS supplemented with 1 mM magnesium chloride and 0.1 mM calcium chloride). All further steps were carried out at 4°C unless otherwise stated. In subdued lighting conditions, washed cells were treated with 10 mM sodium periodate in PBS-CM for 30 min, to oxidise carbohydrate groups to aldehydes. Cells were then washed once with 3 ml PBS-CM and then twice with 3 ml 0.1M sodium acetate-CM (0.1M sodium acetate pH 5.5 supplemented with 1 mM magnesium chloride and 0.1 mM calcium chloride). In subdued lighting, cells were labelled for 30 min with 0.75 ml/well of cell non-permeable 1 mM biotin-LC-hydrazide in sodium acetate-CM. The reactive aldehyde groups bind to the hydrazide group forming a stable hydrazone linkage that is stable and allows for the identification of the cell-surface expressed proteins. Labelling of the cells was terminated by the removal of biotin and washing three times with 3 ml PBS without magnesium chloride and calcium chloride. Cells were then solubilised with 0.25 ml/well of RIPA+ buffer (50 mM HEPES pH 7.5, 150 mM sodium chloride, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS supplemented with 10 mM sodium fluoride, 5 mM EDTA pH 8, 0.01M sodium phosphate, 0.1 mM PMSF and 10 μg/ml each of soybean trypsin inhibitor and benzamidine) and transferred to a chilled microfuge tube on ice. Wells were then rinsed with 0.25 ml of RIPA+ buffer which was added to the samples. Samples were then solubilised for 1 hr on a rotating wheel at 4°C. Solubilised samples were centrifuged for 15 min at 14,000g to pellet insoluble material from the cell lysate and 450 μl of supernatant was removed to a fresh pre-chilled microfuge tube. Duplicate 10 μl aliquots were used to determine the protein concentration using the BCA assay (Section 2.5.2). 400 μl of sample, normalised for protein concentration and volume, were then added to pre-chilled microfuge tubes containing 20 μl of protein G-Sepharose beads and 5μl ascites fluid/tube generated against the *myc*-epitope.

Samples were then immunoprecipitated overnight in the cold room on a rotating wheel. Samples were subsequently removed and the beads pelleted by microcentrifugation at 13,000 g for 30 sec. Supernatants were removed and beads washed three times with 1 ml of RIPA+ buffer. 50 µl of sample buffer was then added and samples were placed at 37°C for 1 hr to elute immunoprecipitated proteins. Samples were vortexed briefly every 15 min to aid in the elution. Following elution, samples were centrifuged to pellet the beads and the supernatant removed with a Hamilton syringe prior to loading on SDS-PAGE. These samples were then fractionated and transferred to a nitrocellulose membrane (Section 2.5.1). Membranes were then washed briefly in PBS and non-specific binding sites were blocked for 1 hr using Blotto. Biotinylated receptor proteins were detected by incubation with 1 µg/ml HRP-streptavidin for 1 hr in Blotto on a rotating platform at room temperature. Following washes as described in the immunoblotting procedure (Section 2.5.1), biotinylated immunoprecipitated receptors were identified by an enhanced chemiluminescent procedure.

2.5.5 Saturation radioligand binding studies with ^{125}I-ZM241385

A confluent T-75 flask containing CHO or COS-P cells, transiently expressing the constructs as indicated in the Figure Legends, was placed on ice. After three washes with 10 ml ice-cold PBS, cells were scraped in 4 ml of PBS and transferred to a pre-chilled 13 ml centrifuge tube on ice. Cells were then pelleted by centrifugation at 3,000 g for 10 min at 4°C. The supernatant was then discarded and the cell pellet resuspended in 1 ml of cell lysis buffer (10 mM Tris, 5 mM EDTA, pH 7.5). Cells were then transferred to a chilled 8 ml tight-fitting glass-on-glass Dounce homogeniser on ice and lysed with 20 up-and-down strokes. The homogenate was then removed to a chilled microfuge tube and membranes pelleted by centrifugation at 14,000g for 15 min at 4°C. The membrane pellet was then resuspended in 0.3 ml of radioligand binding buffer (50 mM Tris, 10 mM magnesium chloride (pH 6.8)) from which 30 µl was removed for protein concentration analysis using the BCA assay (Section 2.5.2). 3.7 ml of radioligand binding buffer was then added to the membrane preparation and transferred to the chilled Dounce homogeniser. 1µl of stock ADA was added to the homogenate giving a final concentration of 0.47units/ml and the membranes were lysed again with 20 up-and-down strokes.

Duplicate assay tubes were set up containing iodinated radioligand ranging from 0.25–8 nM to which 150 µl of membrane preparation was added. One set of duplicate tubes contained 50 µl of water, in order to determine total binding of ^{125}I-ZM241385, while in the other set of tubes, 50 µl NECA at a final concentration of 10 µM was included to determine non-specific binding. Assay tubes were then placed in a shaking water bath at 37°C for 45 min to allow the assay to reach equilibrium. The binding assays were washed through GF/B filters, pre-soaked in 300 ml of distilled water supplemented with 0.03% (v/v) polyethylenimine, using a Brandel cell harvester. Assay binding tubes were rinsed three times in 5 ml of assay wash buffer (binding buffer supplemented with 0.01%(w/v) CHAPS) to ensure efficient collection of membranes. Filter discs representing each assay tube were then counted for 30 sec in a gamma counter. Duplicate 10 µl aliquots of the ^{125}I-ZM241385 radioligand dilutions used in the assay were also counted to determine the exact concentration of ^{125}I-ZM241385 present in each tube.

Following subtraction of non-specific counts, the total amount of specific receptor-bound radioligand was calculated using a graph of specific counts *versus* radioligand concentration. This graph was fitted to a non-linear one-site hyperbolic curve using GraphPad Prism. From this, the total number of receptors (B_{max}) and the equilibrium dissociation constant (K_d) were calculated. An example of this calculation is found in Appendix 2.

2.5.6 Competition radioligand binding with ^{125}I-ZM241385 in isolated membranes.

Membranes were prepared as for the saturation binding assay and 0.15 ml of resuspended membrane was added to each tube. In competition binding assays, duplicate tubes were set up containing 0.05 ml of ^{125}I-ZM241385 and 0.05 ml of unlabelled competing ligand between 10^{-12} and 10^{-5}M. Competition binding assays were then placed in a shaking water bath at 37°C for 60 min to reach equilibrium. Assays were then washed through pre-soaked GF/B filters, treated as above, and the radioactivity on each filter counted in a gamma counter. The IC_{50} (the concentration at which 50% of specific binding was inhibited) was determined by plotting specific

binding (cpm) against log [competing ligand (M)] and fitted to a one-site competition curve using GraphPad Prism. The equilibrium dissociation constant (K_i) of the competing ligand was then calculated using the Cheng-Prusoff equation (Lazareno and Birdsall, 1993) and expressed as a μM concentration. This equation is found in Appendix 3.

2.5.7 Determination of cell surface E-selectin expression by enzyme-linked immunoabsorbant assay (ELISA).

$1x10^5$ HUVECs/well were seeded in a 96-well plate. The following day, cells were treated with the appropriate concentration of cytokine/lipopolysaccharide (LPS) for the time indicated in the Figure Legends. The assay was stopped by transferring the plate to ice and cells were washed gently three times with 200 μl PBS. Cells were then treated with 0.1 ml of 4% (w/v) paraformaldhehyde in 5% sucrose (w/v) in PBS (pH 7.2), covered and incubated overnight in the cold room. The next day, cells were washed gently three times with 200 μl PBS and then incubated for 1 hr at room temperature in PBS containing 0.1% (w/v) BSA in order to block non-specific antibody binding sites. Cells were then washed gently three times with 200 μl PBS before incubation with mouse anti-human E-selectin antibody at 1:1000 dilution in PBS/0.1% (w/v) BSA for 2 hr at room temperature. A mouse anti-haemagglutinin (HA) epitope antibody at 1:1000 dilution was also used, in wells treated in parallel, to determine non-specific binding. Cells were then gently washed three times with 200 μl PBS before the addition of HRP-conjugated anti-mouse IgG for 1 hr at room temperature. Cells were then gently washed five times with 200 μl PBS, before the addition of 100 μl, stock, 3,3'5,5'-tetramethylbenzidine (TMB). The colour was allowed to develop for 5-10 min at room temperature and the A_{600} determined using a plate reader.

2.5.8 Electrophoretic Mobility Shift Assay (EMSA)

Confluent cells in a 6-well plate were incubated with the appropriate treatment for the time indicated in the Figure Legends. Cells were then washed gently twice in 3 ml ice-cold PBS and transferred to a sterile microfuge tube on ice in a final volume of 1 ml PBS. Cells were then pelleted by centrifugation at 4,000g for 5 min at 4°C,

and resuspended in 0.4 ml of buffer A (10 mM Tris, pH 7.5, 10 mM potassium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 1 μg/ml each of soybean trypsin inhibitor and benzamidine). Cells were then allowed to swell for 10 min before NP-40 was added to a final concentration of 0.06% (v/v). Cells were then vortexed briefly and lysates pelleted by centrifugation at 13000g at 4°C for 30 sec. The supernatant was removed and the pellet resuspended in 0.025 ml of buffer B (10 mM Tris pH7.5, 450 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 1 μg/ml each of soybean trypsin inhibitor and benzamidine). This was left to incubate on ice for 15 min on a shaking platform. Nuclei were then pelleted by a 5 min centrifugation at 13,000g at 4°C. The supernatant was then assayed for protein concentration using a Bradford assay as DTT present in the buffer interferes with the BCA assay.

A ssDNA probe (5 pmol/μl) containing the NF-κB consensus sequence GGGGACTTTCCC was used to detect any induced NF-κB transcription factors. Radiolabelled using [γ–^{32}P] ATP and T4 polynucleotide kinase, the labelling reaction was performed at 37°C for 30 min in a total volume of 20 μl. The reaction was then incubated at 95°C for 2 min following the addition of the complementary strand (5 pmol/μl) and 0.5M sodium chloride. The reaction was then allowed to cool over the next few hours. The probe was then purified by centrifugation in a spin column (Pharmingen) to isolate the radiolabelled DNA probe. The radiolabelled DNA probe was then incubated for 30 min at room temperature in a binding reaction containing 5-10 μg of sample, 1 μl radiolabelled DNA probe in binding buffer (100 mM HEPES pH 7.9, 5 mM magnesium chloride, 0.5 mM EDTA, 250 mM sodium chloride, 50% (v/v) glycerol, 100 mM DTT, 10 mg/ml BSA, 1 mg/ml sodium polydeoxyinosinicdeoxycytidylic salt in a total volume of 22 μl. The binding reaction was then loaded on a non-reducing 6% (w/v) acrylamide (60:1 acrylamide:bisacrylamide) gel in 0.5X TBE (45 mM Tris-borate, pH 8.3, 1mM EDTA). The gel was then run at 5 mA overnight until the dye reached the end of the gel. The gel was then dried down under vacuum and bands were identified using autoradiography.

2.5.9 Monocyte adhesion assay

$1x10^4$ HUVECs/well were seeded into a 24-well plate and grown to 70% confluence. The cells were then infected for 24 hours at a m.o.i. of 25:1 with recombinant adenoviruses encoding either the *myc*A_{2A}AR and GFP or GFP alone. Following treatment as indicated in the Figure Legends, the medium was removed and the HUVEC monolayers overlayed with $1x10^5$ U937 cells/well, a pre-monocytic cell line, and allowed to adhere for 1 hr at 37°C. U937 cells were then removed and the monolayers washed three times with 1 ml/well of serum-free DMEM to remove any non-adherent U937 cells. HUVEC monolayers were then fixed in 0.5 ml/well 4% (w/v) paraformaldehyde in 5% (w/v) sucrose/PBS, pH 7.2, and analysed using a combination of fluorescent and brightfield microscopy to determine the number of U937 cells that were adherent to GFP-expressing HUVECs. At least 300 GFP-expressing cells were counted in 3-5 separate fields to calculate the average number of adherent cells per 100 GFP-expressing HUVECs for any given treatment.

2.5.10 Confocal Laser Scanning Microscopy

$1x10^5$ cells were split onto coverslips and then treated as described in the Figure Legends. Following cell treatments, cell monolayers were washed three times in 1.5 ml PBS and then fixed in 1.5 ml of 4% (w/v) paraformaldehyde in 5% sucrose/PBS as previously described for 20 min. Cells were then washed again in 1.5 ml PBS and permeabilised in 0.4% (v/v) Triton X-100 in PBS for 3 min. Antibody dilutions were prepared in 0.1% (v/v) new born calf serum (NBCS), 0.2% (w/v) gelatin, in PBS. The primary antibody anti-p65 was used at a 1:200 dilution and the Alexa 594-conjugated anti-mouse IgG was used at 1:400 dilution. 100µl of antibody solution/coverslip was then spotted onto Nescofilm and the coverslip placed down onto the antibody dilution and incubated for 1 hr at room temperature. Cells were then washed twice in NBCS/PBS/gelatin and placed onto Nescofilm containing 100 µl of the relevant secondary antibody. This was again left to incubate for 1 hr at room temperature. Coverslips were then washed again twice in NBCS/PBS/gelatin before a final wash in PBS prior to being mounted on microscope slides with 40% (v/v) Glycerol in PBS.

Cells were observed using a Zeiss Axiovert 500 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) using a Zeiss Plan-Apo 63 x 1.4 NA oil immersion

lens, pinhole of 20 and zoom set between 1 and 4. GFP was excited using a 488nm argon/krypton laser and detected with 515-540 nm band pass filter. Alexa-594 conjugated antibodies were excited at 543nm and detected with a long pass band filter at 590nm. The images were adjusted with Zeiss LSM software.

2.5.11 Nitric oxide measurements

$1x10^5$ C6 glioma cells were plated out into a 96-well plate and left overnight to adhere. Cells were then treated as indicated in the Figure Legends and the cell culture medium removed. NO production was monitored by measuring nitrite accumulation in culture medium. This was performed by mixing 50 µl of sample in a 1:1 ratio with Greiss reagent, (1% (w/v) sulphanilamide, 0.1% (w/v) *N*-1-naphthylenediamine dihydrochloride and 2.5% (v/v) phosphoric acid). Known standards of sodium nitrite (0-10 mM) were analysed in parallel to quantify nitrite levels. Absorbance was measured at 492 nm after 10 min incubation at room temperature.

2.5.12 Analysis of NF-κB transcription using a luciferase reporter assay.

$1x10^5$ cells/well were seeded into a 6 well dish. The following day, cells were transfected with 0.5 µg of a β-galactosidase reporter plasmid, 0.5 µg NF-κB Luciferase reporter plasmid and 1 µg of receptor cDNA using 6 µl Gene Juice (See section 2.2.3). The cells were then subject to the treatments stated in the Figure Legends. Incubations were stopped by transferring the plate to ice and washing the cells gently three times in 3 ml of ice-cold PBS. Cells were then solubilised in 50 µl luciferase lysis buffer according to the manufacturer's protocol (β–Galactosidase Enzyme Assay System with Reporter Lysis Buffer, Promega). 20 µl of lysate was used to determine luciferase activity, 20 µl of the lysate, was used to determine β–galactosidase enzyme activity and 10 µl was used to assay the protein concentration. Luciferase activities were determined using a luminometer coupled to WinGlo software. β–Galactosidase enzyme activity was determined as per the manufacturer's instructions while protein concentration was measured using a Bradford's assay. Luciferase activities were then normalised to β–galactosidase enzyme activity and protein concentration.

2.5.13 Pull-down assay of c-Jun N-terminal kinase (JNK) activation

Confluent C6 cells in 6-well dishes were treated as indicated in the Figure Legends. Incubations were terminated by placing the cells on ice and washing in ice-cold PBS. All subsequent procedures were performed at 4°C unless indicated otherwise. Cells were solubilised by scraping into 0.3 ml/well JNK lysis buffer (25 mM Hepes-KOH, pH 7.7, 20 mM β-glycerophosphate, 0.3 M sodium chloride, 1.5 mM magnesium chloride, 10 mM sodium fluoride, 0.1 mM sodium vanadate, 0.1 mM PMSF and 10 μg/ml each of soybean trypsin inhibitor and benzamidine) and incubated on ice for 30 min. Following isolation of soluble fractions by microcentrifugation, samples equalised for protein content and volume were incubated overnight with rotation with 10 μg recombinant c-Jun(1-73) immobilised to glutathione-Sepharose beads. Complexes were isolated by brief microcentrifugation, washed three times with 1 ml wash buffer (20 mM Hepes-KOH, 50 mM sodium chloride, 2.5 mM magnesium chloride, 0.1 mM EDTA and 0.05 % (v/v) Triton X-100) and then resuspended in 30 μl kinase assay buffer (25 mM Hepes, pH 7.5, 10 mM magnesium chloride, 20 mM β-glycerophosphate, 75 mM sodium vanadate) supplemented with 100 μM ATP and 2 mM dithiothreitol. Kinase assays were performed at 30°C for 30 min and terminated by the addition of 10 μl electrophoresis sample buffer containing 12% SDS followed by incubation at 95°C for 5 min. Samples were fractionated by SDS-PAGE using 12% (w/v) polyacrylamide resolving gels and transferred to nitrocellulose for immunoblotting with anti-phospho-Ser^{63} c-Jun antibody for visualisation of GST-c-Jun(1-73) phosphorylation by immobilised cellular JNK.

2.5.14 Statistical Analysis

Statistical analysis was carried out using Student t-test using GraphPad Prism 3.0 as indicated in the Figure Legends. Significance assessed as $p<0.05$.

Chapter 3

Characterisation Of A *Myc*-Tagged WT $A_{2A}AR$

3.1 Introduction

The potent anti-inflammatory properties of the A_{2A}AR have been demonstrated in a variety of different animal models (Section 1.11.4). However, the precise molecular mechanism that results in this general property is not yet fully understood. To investigate this property further, a *myc*-epitope was added to the C-terminus of the human A_{2A}AR, which could be specifically identified by a high-affinity, low-background monoclonal anti-*myc* (EQKLISEEDL) 9E10 antibody, as reported for other *myc*-tagged receptors (Watterson *et al.*, 2002). This was used in further experiments to distinguish recombinant A_{2A}ARs from endogenously expressed WT receptors. (For future purification purposes, the construct also contained a His-tag; HHHHHH). To be a useful tool, it was essential that this tagged receptor behaved similarly to the WT receptor. Therefore, it was necessary to characterise the *myc*-His/A_{2A}AR and compare it with the WT human A_{2A}AR.

3.2 Results and Discussion

A cDNA plasmid was generated which would express a *myc*-His-tagged human A_{2A}AR using the strategy and protocol outlined in Methods Section 2.3.7. A full nucleotide and protein sequence of the receptor is shown in Figure 3.1. To determine whether the anti-A_{2A}AR antibody could specifically identify the recombinant *myc*-His/A_{2A}AR, CHO cells were transiently transfected with cDNA plasmids that would express canine A_1AR, and A_{2A}ARs. Western blotting was then performed as described in Section 2.5.1 of the Materials and Methods, using a commercially available A_{2A}AR antibody raised against residues E^{373}-K^{391}, which are highly conserved between human and canine receptors. As shown in Figure 3.2, the anti-A_{2A}AR specifically identified only the A_{2A}AR, which migrates as a smear centred at 45kDa. Higher molecular weight immunoreactive bands are consistent with the formation of dimers, which has been recently reported (Ciruela *et al.*, 1995; Canals *et al.*, 2004). Over-exposure of the western blot reveals the absence of any A_1AR cross-reactivity suggesting the high specificity for the A_{2A}AR.

Having confirmed the specificity of the anti-A_{2A}AR antibody, CHO cells transiently transfected with *myc*-His/A_{2A}AR cDNA plasmids, were used to confirm the specificity of the anti-*myc* antibody. Cells were then solubilised and duplicate samples were fractionated by SDS-PAGE. Following transfer of the proteins to each

nitrocellulose membrane, immunoblotting was performed using either the anti-*myc* (*left panel)* or the anti-A_{2A}AR antibody (*right panel*; Figure 3.3). A *myc*-epitope tagged LacZ construct was also transfected into cells to act as a positive control for the antibody. Lanes 3, 4, and 5 indicate that the anti-*myc* antibody displays a high level of affinity for the receptor as a band is still present when cells are transfected with low amounts of cDNA. Figure 3.3 also indicates that there are three specific immunoreactive bands of ~49kDa, ~45 kDa and 43kDa which correspond to the A_{2A}AR. The right panel in Figure 3.3 indicates that the anti-A_{2A}AR antibody specifically recognises the same three immunoreactive bands. This suggests that the anti-*myc* antibody could be used in future experiment to specifically identify the *myc*-His/A_{2A}AR. The increase in size of the receptor is consistent with the tagging of the receptor while the lower molecular weight bands might represent partial and fully glycosylated receptors.

To determine if the recombinant *myc*-His-tagged human A_{2A}AR was accurately expressed at the cell surface, biotin-labelling experiments were performed as described in Section 2.5.4 of the Materials and Methods. Briefly, *myc*-His/A_{2A}AR transfected CHO cells were incubated with biotin hydrazide and were subject to immunoprecipitation with the anti-*myc* antibody. Immunoprecipitated proteins were fractionated on SDS-PAGE and transferred to a nitrocellulose membrane. Following incubation with HRP-streptavidin, biotinylated cell-surface expressed A_{2A}ARs were visualised using enhanced chemiluminescence techniques. Figure 3.4 indicates that the *myc*-His/A_{2A}AR is expressed at the cell surface. In addition, the presence of only one A_{2A}AR-specific band in transfected cells confirms the earlier conclusion that lower molecular weight bands in Figure 3.3 represent incompletely processed receptors.

To investigate whether the addition of the *myc* epitope interfered with the pharmacological properties of the A_{2A}AR, tagged and WT A_{2A}ARs were transfected into COS-P cells from which membranes were prepared for saturation radioligand binding assays, using the A_{2A}AR-selective antagonist ^{125}I-ZM241385 as described in Section 2.5.5. Figure 3.5 shows typical saturation binding curves for both receptors. B_{max} values for both receptors were similar and K_d values were slightly different although within a range reported for the A_{2A}AR (Palmer *et al.*, 1995). A summary of

saturation binding data is found in Table 3.1. As addition of the *myc* epitope conferred minimal differences in the binding of antagonist to the recombinant receptor, it was important to investigate if the presence of the *myc*-epitope altered any agonist-binding properties. Therefore, agonist competitive binding assays were performed using NECA (See Figure 3.6 and Table 3.2) as described in Section 2.5.6 of the Materials and Methods. K_i values for tagged versus untagged receptors (53.7 ± 0.9 nM versus 63.1 ± 0.9 nM; n=3) indicate that there was a slight change in agonist binding properties. Thus, from both saturation radioligand binding and competitive binding studies, addition of the *myc* epitiope did not dramatically alter any of the pharmacological properties of the receptor. In addition, the K_d and K_i values obtained are consistent with published values for the A_{2A}AR (Fredholm *et al.*, 2001). In conclusion, addition of the *myc*-epitope confers no significant difference on the cell surface expression and pharmacological profile of the A_{2A}AR.

As previously stated the A_{2A}AR has a large C-terminal domain containing two clusters of potential phosphorylation sites; Cluster 1, S^{320}-G^{360} and Cluster 2, Y^{361}-S^{412} (See Figure 3.1). One ultimate aim of these studies was to determine the effect of receptor phosphorylation on the A_{2A}AR-mediated anti-inflammatory effects. Therefore, two truncated mutant A_{2A}ARs were generated. A_{2A}ARΔ55 and A_{2A}ARΔ101 had 55 amino acids and 101 amino acids respectively removed from the C-terminal domain of the receptor. This would allow us to determine which if any possible phosphorylation sites were important for any anti-inflammatory effect. The truncated receptors were generated from the tagged receptor using a similar approach outlined in Section 2.3.8 of the Methods. Figure 3.7 shows a schematic diagram of the truncated receptors.

To ensure the efficient expression of the truncated receptors, CHO cells were transfected with cDNA plasmids encoding full-length *myc*-His/A_{2A}AR, A_{2A}ARΔ55 and A_{2A}ARΔ101. 24 hr post-transfection, cell lysates were made which were fractionated on SDS-PAGE and transferred to nitrocellulose membrane. Figure 3.8 shows western blotting of the truncated receptors using the anti-*myc* antibody. The truncated receptors produced bands of ~48kDa and ~45kDa consistent with the truncation of the C-terminus. In addition, biotinylation experiments were performed

to investigate the effect of truncation on the cell surface expression of the receptor. Figure 3.9 indicates that truncation of 55 amino acids makes little difference on the cell surface expression of the receptor. In contrast, removal of 101 residues makes a significant impact on the cell surface expression of the receptor.

The pharmacological profile of the truncated constructs was also assessed using saturation binding studies and competition binding analysis as previously described (See Figure 3.5 and 3.6). Figure 3.10 and Table 3.3 indicate that neither truncation made any significant difference to the K_d of the receptors however, the B_{max} value for the $A_{2A}AR$ Δ101, was significantly decreased when compared with the full-length receptor, which is consistent with the immunoblotting and biotinylation data (Figure 3.8, 3.9). In addition, competitive binding assays using NECA and CGS21680 were also performed with the full-length $A_{2A}AR$ and the truncated receptor proteins. Results indicated that there was no significant difference in K_i values between each of the receptors treated with either NECA or CGS21680. Figure 3.11 shows a typical competition curve and Table 3.4 shows a summary of the data. In conclusion, the loss of 55 amino acids makes little difference on the cell surface expression or pharmacological profile of the receptor. In contrast, truncation of 101 amino acids had a prominent effect on the cell surface expression and pharmacology of the receptor.

Figure 3.1 **Schematic diagram of the *myc*-His tagged $A_{2A}AR$**

The *myc*-His tagged $hA_{2A}AR$ was constructed using a polymerase chain reaction (PCR) approach. A *myc*-His tag was added to the C-terminus of the $A_{2A}AR$. The C-terminus of the protein is indicated by an asterisk and the *myc*-epitope tag is underlined opposite.

```
ATGCCCATCATGGGCTCCTCGGTGTACATCACGGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAAT 72
 M  P  I  M  G  S  S  V  Y  I  T  V  E  L  A  I  A  V  L  A  I  L  G  N

GTGCTGGTGTGCTGGGCCGTGTGGCTCAACAGCAACCTGCAGAACGTCACCAACTACTTTGTGGTGTCACTG 144
 V  L  V  C  W  A  V  W  L  N  S  N  L  Q  N  V  T  N  Y  F  V  V  S  L

GCGGCGGCCGACATCGCAGTGGGTGTGCTCGCCATCCCCTTTGCCATCACCATCAGCACCGGGTTCTGCGCT 216
 A  A  A  D  I  A  V  G  V  L  A  I  P  F  A  I  T  I  S  T  G  F  C  A

GCCTGCCACGGCTGCCTCTTCATTGCCTGCTTCGTCCTGGTCCTCACGCAGAGCTCCATCTTCAGTCTCCTG 288
 A  C  H  G  C  L  F  I  A  C  F  V  L  V  L  T  Q  S  S  I  F  S  L  L

GCCATCGCCATTGACCGCTACATTGCCATCCGCATCCCGCTCCGGTACAATGGCTTGGTGACCGGCACGAGG 360
 A  I  A  I  D  R  Y  I  A  I  R  I  P  L  R  Y  N  G  L  V  T  G  T  R

GCTAAGGGCATCATTGCCATCTGCTGGGTGCTGTCGTTTGCCATCGGCCTGACTCCCATGCTAGGTTGGAAC 432
 A  K  G  I  I  A  I  C  W  V  L  S  F  A  I  G  L  T  P  M  L  G  W  N

AACTGCGGTCAGCCAAAGGAGGGCAAGAACCACTCCCAGGGCTGCGGGGAGGGCCAAGTGGCCTGTCTCTTT 504
 N  C  G  Q  P  K  E  G  K  N  H  S  Q  G  C  G  E  G  Q  V  A  C  L  F

GAGGATGTGGTCCCCATGAACTACATGGTGTACTTCAACTTCTTTGCCTGTGTGCTGGTGCCCCTGCTGCTC 576
 E  D  V  V  P  M  N  Y  M  V  Y  F  N  F  F  A  C  V  L  V  P  L  L  L

ATGCTGGGTGTCTATTTGCGGATCTTCCTGGCGGCGCGACGACAGCTGAAGCAGATGGAGAGCCAGCCTCTG 648
 M  L  G  V  Y  L  R  I  F  L  A  A  R  R  Q  L  K  Q  M  E  S  Q  P  L

CCGGGGGAGCGGGCACGGTCCACACTGCAGAAGGAGGTCCATGCTGCCAAGTCACTGGCCATCATTGTGGGG 720
 P  G  E  R  A  R  S  T  L  Q  K  E  V  H  A  A  K  S  L  A  I  I  V  G

CTCTTTGCCCTCTGCTGGCTGCCCCTACACATCATCAACTGCTTCACTTTCTTCTGCCCCGACTGCAGCCAC 792
 L  F  A  L  C  W  L  P  L  H  I  I  N  C  F  T  F  F  C  P  D  C  S  H

GCCCCTCTCTGGCTCATGTACCTGGCCATCGTCCTCTCCCACACCAATTCGGTTGTGAATCCCTTCATCTAC 864
 A  P  L  W  L  M  Y  L  A  I  V  L  S  H  T  N  S  V  V  N  P  F  I  Y

GCCTACCGTATCCGCGAGTTCCGCCAGACCTTCCGCAAGATCATTCGCAGCCACGTCCTGAGGCAGCAAGAA 936
 A  Y  R  I  R  E  F  R  Q  T  F  R  K  I  I  R  S  H  V  L  R  Q  Q  E

CCTTTCAAGGCAGCTGGCACCAGTGCCCGGGTCTTGGCAGCTCATGGCAGTGACGGAGAGCAGGTCAGCCTC 1008
 P  F  K  A  A  G  T  S  A  R  V  L  A  A  H  G  S  D  G  E  Q  V  S  L

CGTCTCAACGGCCACCCGCCAGGAGTGTGGGCCAACGGCAGTGCTCCCCACCCTGAGCGGAGGCCCAATGGC 1080
 R  L  N  G  H  P  P  G  V  W  A  N  G  S  A  P  H  P  E  R  R  P  N  G

TATGCCCTGGGGCTGGTGAGTGGAGGGAGTGCCCAAGAGTCCCAGGGGAACACGGGCCTCCCAGACGTGGAG 1152
 Y  A  L  G  L  V  S  G  G  S  A  Q  E  S  Q  G  N  T  G  L  P  D  V  E

CTCCTTAGCCATGAGCTCAAGGGAGTGTGCCCAGAGCCCCCTGGCCTAGATGACCCCCTGGCCCAGGATGGA 1224
 L  L  S  H  E  L  K  G  V  C  P  E  P  P  G  L  D  D  P  L  A  Q  D  G

GCAGGAGTGTCCTCTAGAGGGCCCTTCGAACAAAAACTCATCTCAGAAGAGGATCTGAATATGCATACCGGT 1296
 A  G  V  S  S  R  G  P  F  E  Q  K  L  I  S  E  E  D  L  N  M  H  T  G
          *

CATCATCACCATCACCATTGA 1317
 H  H  H  H  H  H  .
```

Figure 3.2 **Immunodetection of the canine A_{2A}AR using an anti-A_{2A}AR antibody**

CHO cells were transiently transfected with cDNAs encoding the canine A_1AR and canine A_{2A}AR. Cells were then solubilised and fractionated on SDS-PAGE. Proteins were then transferred to nitrocellulose and immunoblotted for the presence of the A_1AR (*left panel)* and the A_{2A}AR (*right panel)* as described in Section 2.5.1 in the Materials and Methods. This western blot represents one of multiple experiments.

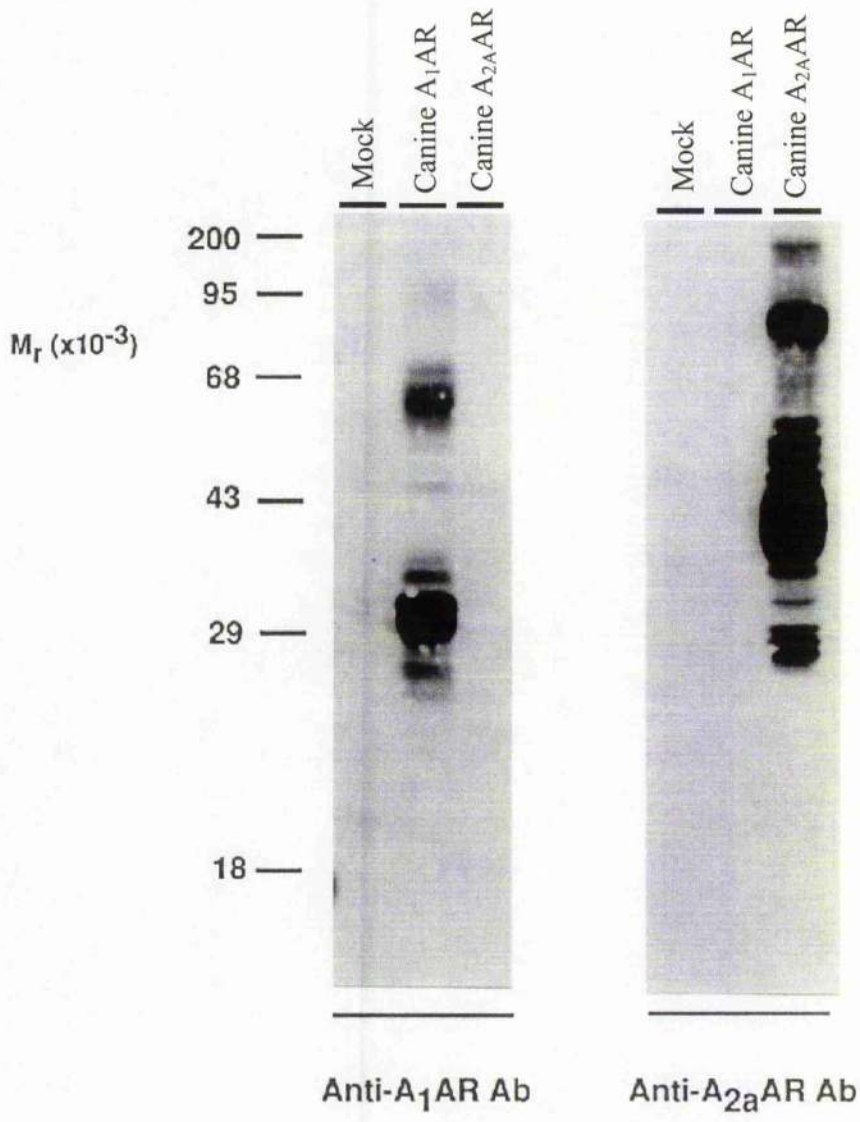

Mock
Canine A1AR
Canine A2AAR
Mock
Canine A1AR
Canine A2AAR
200
95
68
43
29
18
Mr (x10-3)
Anti-A1AR Ab
Anti-A2aAR Ab
Immunoblotting Abs

Figure 3.3 **Immunodetection of the human A_{2A}AR using an anti-*myc* antibody**

CHO cells were transiently transfected with the following different cDNAs; pCMV5 vector, *myc*-tagged Lac Z and increasing concentrations of *myc*-A_{2A}AR in μg, as indicated. Cells were then solubilised and equalised for protein. Duplicate samples were then fractionated on SDS-PAGE and transferred to nitrocellulose. One blot was immunoblotted with the anti-9E10 antibody, as indicated in the left panel while the other was immunoblotted with an anti-A_{2A}AR antibody (*right panel*). These blots represent one of multiple experiments.

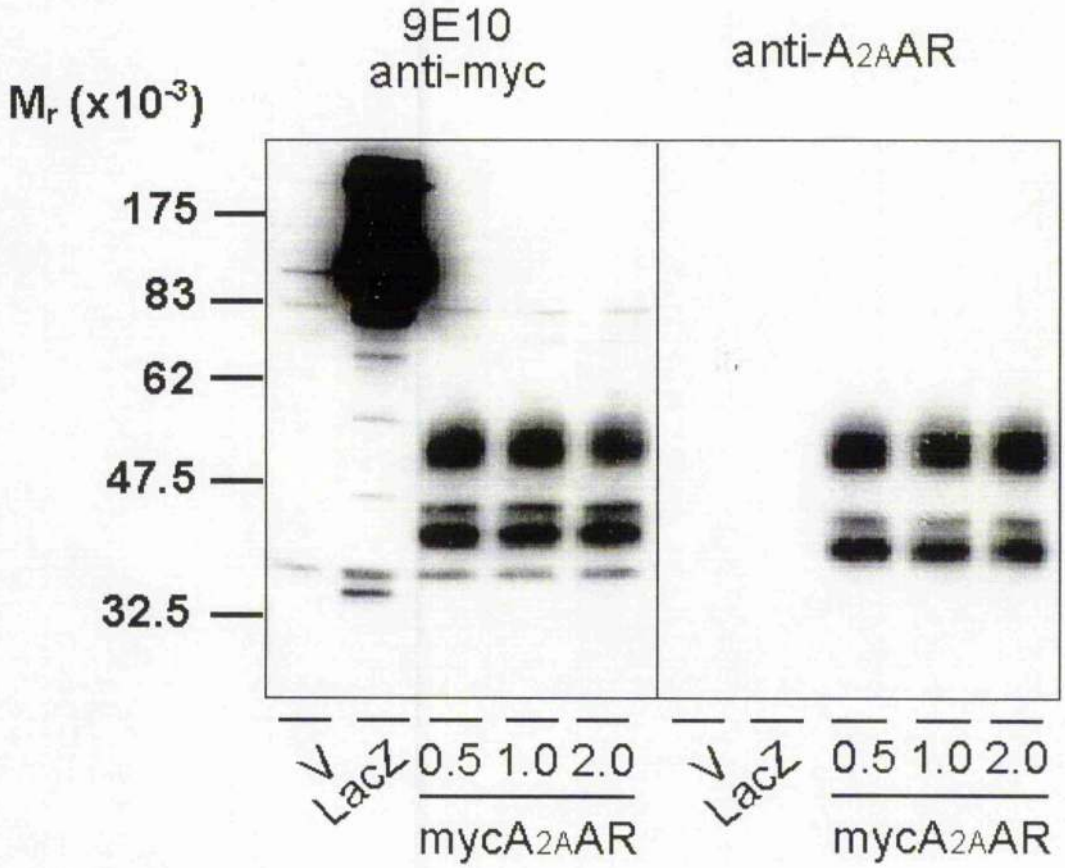

9E10
anti-myc
anti-A2AAR
Mr (x10-3)
175
83
62
47.5
32.5
V
LacZ
0.5
1.0
2.0
mycA2AAR
V
LacZ
0.5
1.0
2.0
mycA2AAR

Figure 3.4 Biotinylation of cell-surface A_{2A}ARs

CHO cells transiently expressing the *myc*-His-tagged A_{2A}AR were labelled with biotin-LC-hydrazide according to the Methods in Section 2.5.3. Cell lysates were then prepared, equalised for protein content, and immunoprecipitated with anti-*myc* (9E10). Immunoprecipitates were then fractionated by SDS-PAGE and transferred to nitrocellulose. Biotinylated A_{2A}ARs were detected by incubation with HRP-conjugated streptavidin as described in Section 2.5.3. Biotin-labelled proteins were then visualised using enhanced chemiluminescence. This represents one of several experiments.

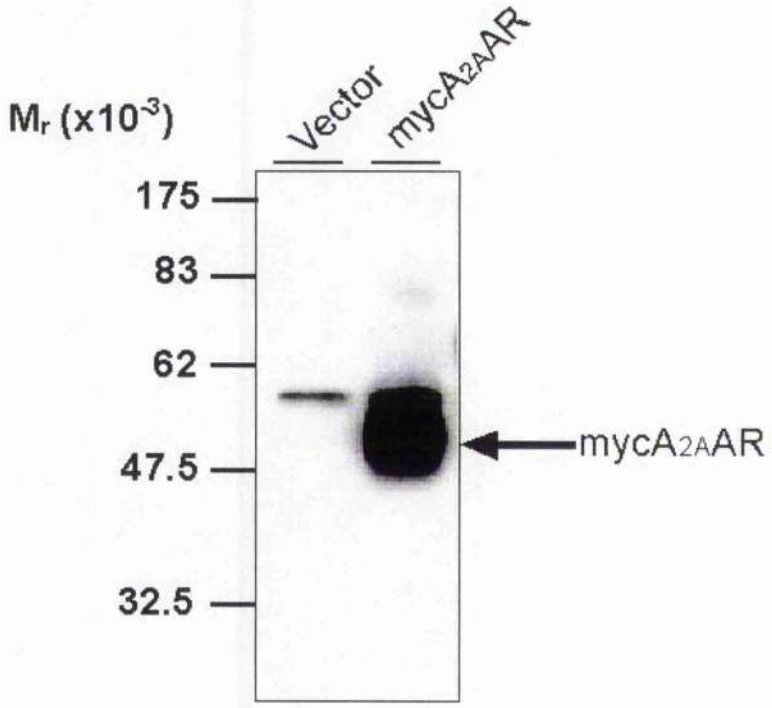

Mr (x10-3)
Vector
mycA2AAR
175
83
62
47.5
32.5
mycA2AAR

Figure 3.5 **^{125}I-ZM241385 saturation binding analysis of WT and *myc*-His tagged human A_{2A}ARs**

Saturation binding studies were carried out on COS-P cells transiently transfected with the WT A_{2A}AR and *myc*-His tagged A_{2A}AR using the A_{2A}AR-selective antagonist ^{125}I-ZM241385. Membrane preparation and radioligand binding procedures are detailed in the Methods Section 2.5.4. The graph shown here represents one typical saturation binding curve. Composite data from three experiments is shown in Table 3.1.± S.E.M.

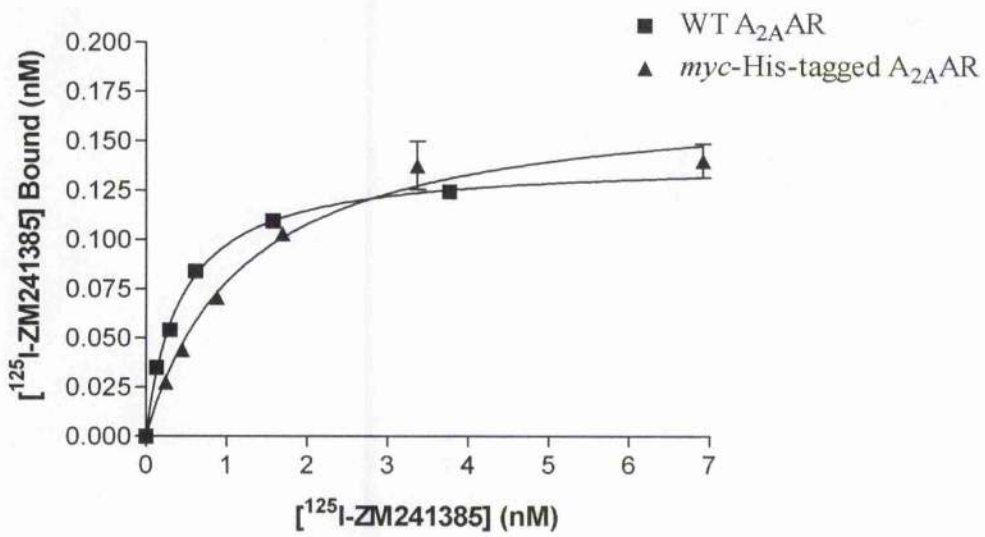

WT $A_{2A}AR$
myc-His-tagged $A_{2A}AR$
[^{125}I-ZM241385] Bound (nM)
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
0
1
2
3
4
5
6
7
[^{125}I-ZM241385] (nM)

Table 3.1 **^{125}I-ZM241385 saturation binding analysis of WT and *myc*-His tagged human A_{2A}ARs**

Membranes prepared from COS-P cells transiently transfected with WT A_{2A}AR and *myc*-His tagged A_{2A}ARs were used for saturation binding analysis using the A_{2A}AR-selective antagonist ^{125}I-ZM241385 as described in Section 2.5.4 of the Materials and Methods. Results are presented as means ± standard error of the mean for three experiments.

Receptor	K_d (nM)	B_{max} (pmol/mg protein)
WT $A_{2A}AR$	0.50 ± 0.07	1.45 ± 0.51
Myc-His-tagged $A_{2A}AR$	1.00 ± 0.11	3.38 ± 0.47

Figure 3.6 **Agonist competition radioligand binding assay at WT and *myc*-His tagged A_{2A}ARs using the AR agonist NECA**

Membranes were prepared from COS-P cells transiently transfected with the WT A_{2A}AR and the *myc*-His tagged A_{2A}AR. Competitive binding assays were set up containing 1nM ^{125}I-ZM241385 and increasing concentrations of NECA as indicated (Section 2.5.6). The IC_{50} (the concentration at which 50% of specific binding was inhibited) was determined by plotting cpm against log {[NECA] (M)} and fitted to a one-site competition curve using non-linear regression in GraphPad Prism. The K_i was then calculated using the Cheng-Prusoff equation. The graph shown here represents one typical competition curve. Composite data from three experiments is shown in Table 3.2 ± S.E.M.

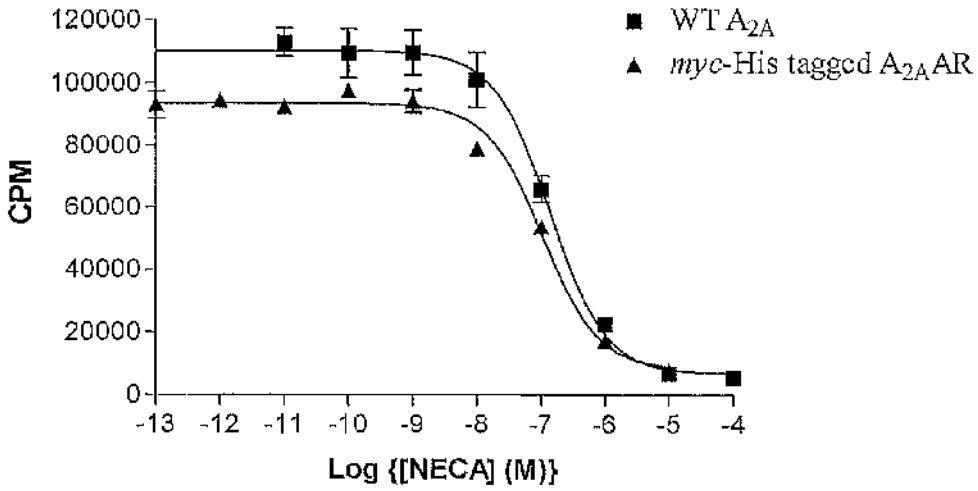

120000
100000
80000
60000
40000
20000
0
CPM
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log {[NECA] (M)}
WT A_{2A}
myc-His tagged A_{2A}AR

Table 3.2 **Agonist competition radioligand binding assay at WT and *myc*-His tagged A_{2A}ARs using the AR agonist NECA**

Membranes prepared from COS-P cells transiently transfected with WT A_{2A}AR and *myc*-His tagged A_{2A}ARs, were used for competition radioligand binding analysis using ^{125}I-ZM241385 and increasing concentrations of NECA as described in Section 2.5.6 of the Materials and Methods. The IC_{50} (the concentration at which 50% of specific binding was inhibited) was determined by plotting cpm against log {[NECA] M} and fitted to a one-site competition curve using non-linear regression in GraphPad Prism. The K_i was then calculated using the Cheng-Prusoff equation. Results are presented as means ± standard error of the mean for three experiments.

Receptor	pK_i NECA
WT $A_{2A}AR$	7.27 ± 0.04
Myc-His-tagged $A_{2A}AR$	7.20 ± 0.03

Figure 3.7 Schematic diagram of A_{2A}AR Δ55 and A_{2A}AR Δ101

The *myc*-His tagged A_{2A}AR was used as a template to generate truncated constructs at the positions indicated (*), according to the procedure outlined in Section 2.3.7 of the Materials and Methods. The mutants contained deletions of two clusters of potential phosphorylation sites, on the C-terminal tail of the receptor indicated by either — (Cluster 1) or ‾ (Cluster 2).

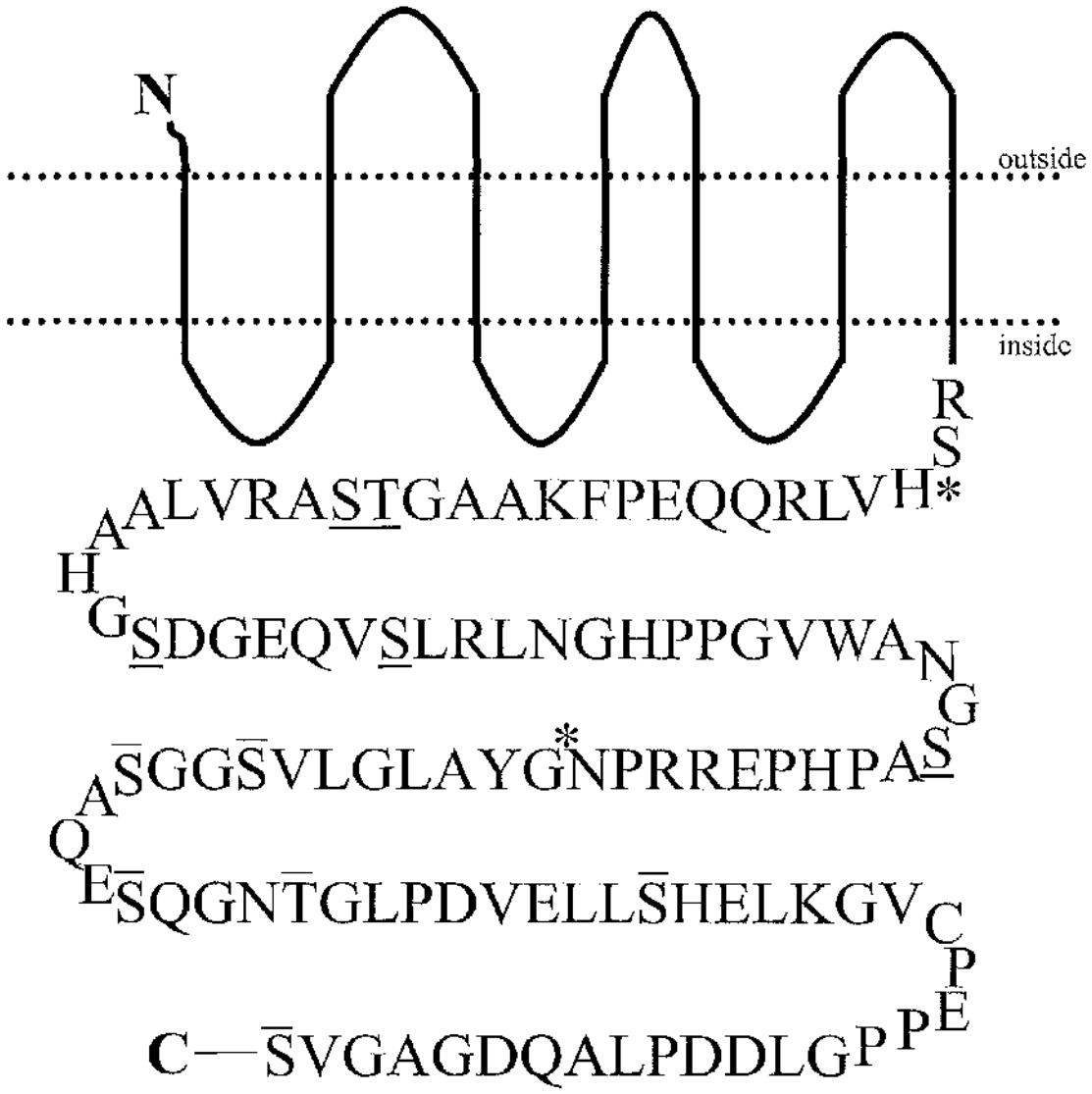

N
outside
inside
R
S
AALVRASTGAAKFPEQQRLVH*
H
GSDGEQVSLRLNGHPPGVWAN
G
ASGGSVLGLAYG*NPRREPHPAS
Q
ESQGNTGLPDVELLSHELKGVC
P
C—SVGAGDQALPDDLGPPE

Figure 3.8 Immunodetection of truncated receptor constructs using an anti-*myc* antibody

CHO cells were transiently transfected with cDNAs encoding the full-length $A_{2A}AR$, $A_{2A}AR$ Δ55 and $A_{2A}AR$ Δ101. Cells were then solubilised and fractionated on SDS-PAGE. Proteins were then transferred to nitrocellulose and immunoblotted for the presence of the $A_{2A}AR$ using an anti-*myc* antibody. This blot represents one of multiple experiments performed.

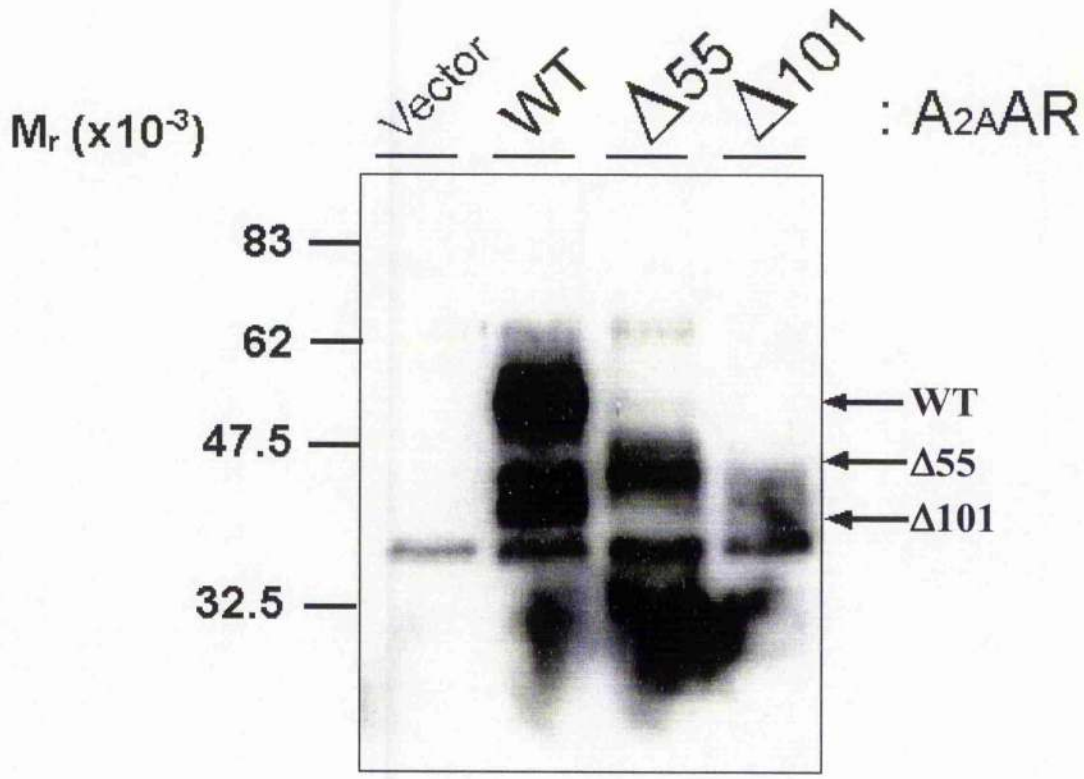

Vector
WT
Δ55
Δ101
: A2AAR
Mr (x10-3)
83
62
47.5
32.5
WT
Δ55
Δ101

Figure 3.9 Biotinylation of cell surface expressed truncated receptor constructs

CHO cells were transiently transfected with cDNAs encoding the full-length A_{2A}AR, A_{2A}AR Δ55and A_{2A}AR Δ101. The next day cells were labelled with biotin-LC-hydrazide according to the Methods in Section 2.5.3. Cell lysates were then prepared, equalised for protein content, and immunoprecipitated with anti-*myc* (9E10). Immunoprecipitates were then fractionated by SDS-PAGE and transferred to nitrocellulose. Biotinylated A_{2A}ARs were detected by incubation with HRP-conjugated streptavidin as described in Section 2.5.3. Biotin-labelled proteins were then visualised using enhanced chemiluminescence. This represents one of several experiments.

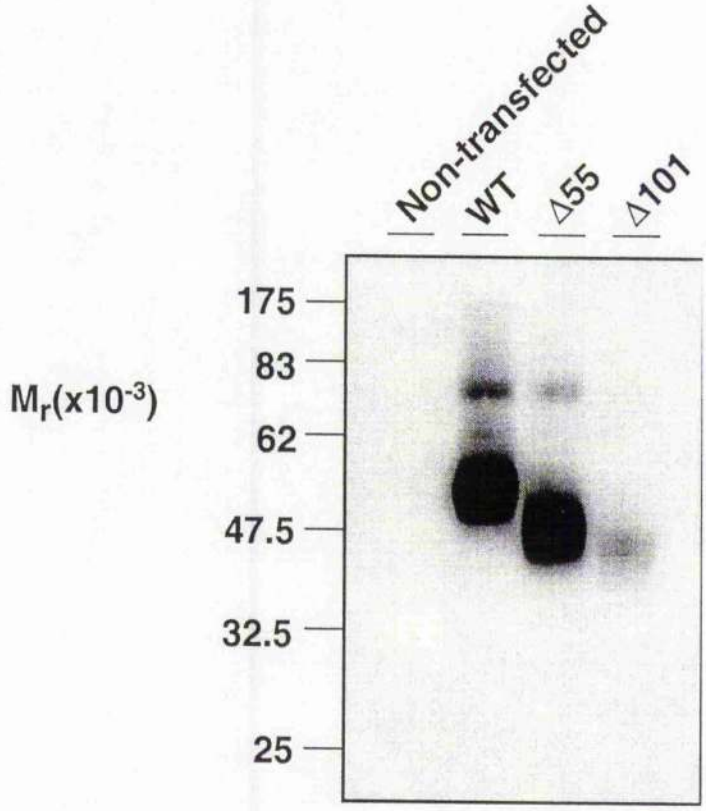

Non-transfected
WT
Δ55
Δ101
M_r(x10^{-3})
175
83
62
47.5
32.5
25

Fig 3.10 **^{125}I-ZM241385 saturation binding analysis of WT and truncated *myc*-His tagged A_{2A}AR receptors**

Saturation binding studies were carried out on COS-P cells transiently transfected with the *myc*-His tagged A_{2A}AR, A_{2A}AR Δ55 and A_{2A}AR Δ101 using the A_{2A}AR-selective antagonist ^{125}I-ZM241385. Membrane preparation and radioligand binding procedures are detailed in the Section 2.5.4. of the Materials and Methods. The graph shown opposite represents one typical experiment. Composite data from three experiments is presented in Table 3.3 ± S.E.M.

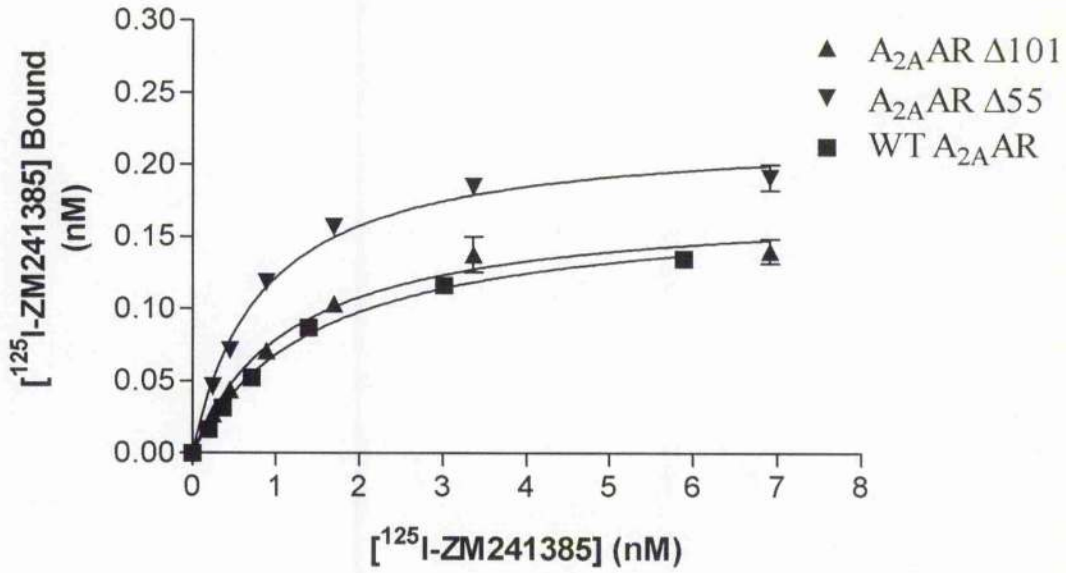

0.30
0.25
0.20
0.15
0.10
0.05
0.00
[125I-ZM241385] Bound (nM)
0
1
2
3
4
5
6
7
8
[125I-ZM241385] (nM)
A2AAR Δ101
A2AAR Δ55
WT A2AAR

Table 3.3 **^{125}I-ZM241385 saturation binding analysis of WT and truncated *myc*-His tagged A_{2A}AR receptors**

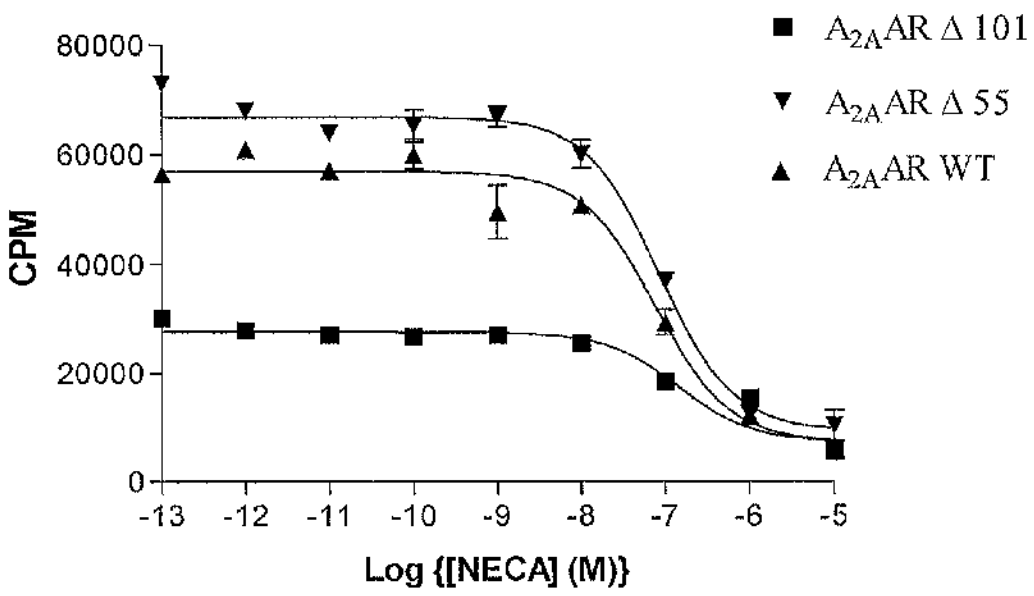
Membranes prepared from COS-P cells transiently transfected with *myc*-His tagged A_{2A}AR, A_{2A}AR Δ55and A_{2A}AR Δ101 were used for saturation binding analysis using the A_{2A}AR-selective antagonist ^{125}I-ZM241385 as described in Section 2.5.4 of the Materials and Methods. Results are presented as means ± standard error of the mean for three experiments.

Receptor	K_d (nM)	B_{max} (pmol/mg protein)
$A_{2A}AR$	1.00 ± 0.11	3.38 ± 0.47
$A_{2A}AR\Delta 55$	0.98 ± 0.11	5.40 ± 0.85
$A_{2A}AR\Delta 101$	1.27 ± 0.24	1.61 ± 0.03

Figure 3.11 Agonist competition radioligand binding assay at WT and *myc*-His tagged A_{2A}ARs using the AR agonists NECA and CGS21680

Membranes were prepared from COS-P cells transiently transfected with *myc*-His tagged A_{2A}AR, A_{2A}AR Δ55 and A_{2A}AR Δ101. Competitive binding assays were set up containing 1nM ^{125}I-ZM241385 and increasing concentrations of NECA as indicated (Section 2.5.6). The IC_{50} (the concentration at which 50% of specific binding was inhibited) was determined by plotting cpm against log {[competing ligand] (M)} and fitted to a one-site competition curve using non-linear regression in GraphPad Prism. The K_i was then calculated using the Cheng-Prusoff equation. The graph shown here represents one typical competition curve. Composite data from three experiments is shown in Table 3.4 ± S.E.M.

A


80000
60000
40000
20000
0
CPM
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log {[NECA] (M)}
A2AAR Δ 101
A2AAR Δ 55
A2AAR WT

B

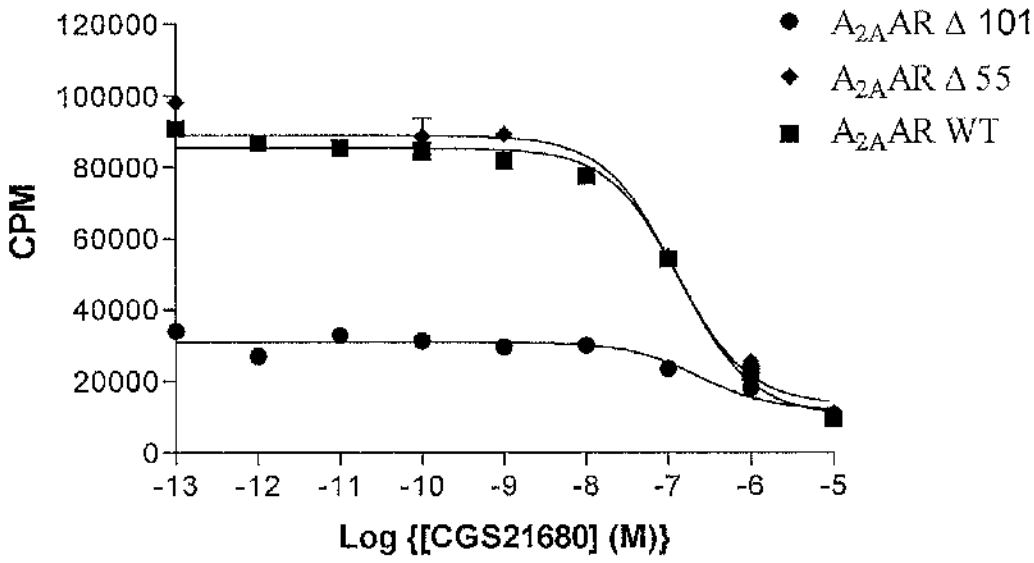

120000
100000
80000
60000
40000
20000
0
CPM
-13
-12
-11
-10
-9
-8
-7
-6
-5
Log {[CGS21680] (M)}
A2AAR Δ 101
A2AAR Δ 55
A2AAR WT

Table 3.4 **Agonist competition radioligand binding assay WT and truncated *myc*-His tagged $A_{2A}AR$ receptors using the AR agonist NECA**

Membranes prepared from COS-P cells transiently transfected with *myc*-His tagged $A_{2A}AR$, $A_{2A}AR$ Δ55and $A_{2A}AR$ Δ101, were used for competition radioligand binding analysis using ^{125}I-ZM241385 and increasing concentrations of NECA as described in Section 2.5.6 of the Materials and Methods. The IC_{50} (the concentration at which 50% of specific binding was inhibited) was determined by plotting cpm against log {[NECA] M} and fitted to a one-site competition curve using non-linear regression in GraphPad Prism. The K_i was then calculated using the Cheng-Prusoff equation. Results are presented as means ± standard error of the mean for three experiments.

Receptor	pK_i NECA	pK_i CGS21680
$A_{2A}AR$	7.18 ± 0.06	7.10 + 0.07
$A_{2A}AR\Delta 55$	7.17 ± 0.03	7.12 ± 0.07
$A_{2A}AR\Delta 101$	6.76 ± 0.125	6.82 ± 0.154

Chapter 4

Effect Of $A_{2A}AR$ Gene Transfer On The Regulation Of NF-κB-Dependent Inflammatory Responses In HUVECs

4.1 Introduction

Inflammation is a key factor in the progression of several disease states such as ischaemia-reperfusion injury (Sullivan *et al*, 2000) and atherosclerosis (Greaves and Channon, 2002). The regulated interactions between endothelial cells and leukocytes are fundamental to the inflammatory response as they trigger further downstream events including cytokine, chemokine and growth factor release, surface expression of adhesion molecules and expression of other pro-inflammatory proteins such as iNOS, COX-2 and others (See Chapter 1). Thus, the suppression of endothelial cell-leukocyte interactions in chronic inflammation could provide therapeutic benefit. In several studies of inflammation, activation of the A_{2A}AR has an anti-inflammatory effect *in vivo* (McPherson *et al.*, 2001; Cassada *et al.*, 2002; Section 1.11.4). However, the precise mechanism that underlies this important property is not yet fully understood. In order to investigate this property, it was necessary to establish a suitable model of inflammation *in vitro*. HUVECS are a commonly used endothelial cell type used in studies of inflammation (Carman *et al.*, 2003; Ahn *et al.*, 2003; Mo *et al.*, 2003), as they grow more easily in cell culture compared to other human endothelial cell types. However, a disadvantage of using primary endothelial cells is the difficulty with which new cDNAs can be introduced. This was important as any inhibitory effect of the A_{2A}AR activation in transfected cells would be undetected if transfection efficiencies were poor. Generally standard transfection methods yield poor transfection efficiencies (2-10%) in endothelial cells (Teifel *et al.*, 1997). Therefore, in order to bypass this problem, a replication-deficient recombinant adenovirus encoding a *myc*-His-tagged human A_{2A}AR, was used to obtain higher levels of A_{2A}AR transfection. The aim of this chapter was to determine what effect adenoviral gene transfer of the *myc*-His-tagged human A_{2A}AR would have on the inflammatory response in HUVECs, and what A_{2A}AR-stimulated molecular mechanisms were involved.

4.2 Results

To investigate the role of endogenous A_{2A}ARs *in vitro*, confluent HUVEC monolayers were treated with increasing concentrations of TNFα for 6 hours in the presence or absence of an A_{2A}AR-selective agonist CGS21680, and a monocyte adhesion assay was performed. Figure 4.1 indicates that there is a saturable dose-dependent increase in the number of adherent U937 cells to TNFα-stimulated HUVEC monolayers. Co-administration of CGS21680, reduces the monocyte adhesion suggesting that endogenous

$A_{2A}AR$ activation has an anti-inflammatory role *in vitro*. The observation that some U937 cells still adhere to TNFα-stimulated HUVECs in the presence of CGS21680, suggested that potentiation of the $A_{2A}AR$ effect might further reduce the number of adherent U937 cells. Comparative studies by Armstrong *et al.*, (2001) in $A_{2A}AR^{+/-}$ and $A_{2A}AR^{-/-}$ cells revealed the absence of any receptor reserve in murine T-lymphocytes suggesting that increasing agonist concentration would have no effect in increasing the reduction in monocyte adhesion. Taking this into consideration, one way of potentiating the $A_{2A}AR$ effect was to increase $A_{2A}AR$ expression. In order to achieve this efficiently, a replication-deficient recombinant adenovirus expressing the *myc*-His-tagged-human $A_{2A}AR$ (AV/*myc*-His $A_{2A}AR$) was used to drive $A_{2A}AR$ gene expression in HUVECs. A schematic outline of the isolation and propogation of the AV/*myc*-His $A_{2A}AR$ is described in detail in Section 2.4 of the Materials and Methods. One advantage of the AV/*myc*-His $A_{2A}AR$ construct was the co-expression from a separate open reading frame of GFP, thus allowing us to easily detect and monitor recombinant protein expression in AV-infected cells by fluorescence microscopy.

To confirm the efficient AV-directed expression of the $A_{2A}AR$, confluent HUVEC monolayers were infected with increasing concentrations of virus (pfu/ml; Figure 4.2). The next day cells were analysed by fluorescence microscopy and immunoblotting with the anti-*myc* antibody 9E10. Figure 4.2 indicates that there is a titration dependent increase in the percentage of cells infected with $A_{2A}AR$, which is maximal at 25 pfu/ml. In addition, there is also a titration-dependent increase in $A_{2A}AR$ expression of the *myc*-His $A_{2A}AR$ which peaks at 25 pfu/ml as determined by Western blotting. The immunoreactive bands present at ~49 kDa and ~46 kDa correspond with that of $A_{2A}AR$-transfected cells (See Figure 3.2) indicating that expression of AV/*myc*-His $A_{2A}AR$ in HUVECs, does not alter the $A_{2A}AR$ protein. It was then necessary to show that the $A_{2A}AR$ was fully processed and expressed at the cell surface. Cell-surface biotinylation assays were performed on AV/*myc*-His $A_{2A}AR$ infected HUVECs as described in the Materials and Methods Section 2.5.4. Figure 4.3 shows that only one reactive band is present which is consistent with the molecular weight of the major $A_{2A}AR$ immunoreactive band in Figure 4.2. This suggests that lower molecular weight bands most likely correspond to non-glycosylated receptors. The detection of *myc*-His $A_{2A}AR$ expression in HUVECs confirmed the use of the AV/*myc*-His $A_{2A}AR$ as an efficient means of gene transfer.

^{125}I-ZM-241385 saturation binding analysis was also performed upon membranes isolated from AV/*myc*-His A_{2A}AR-infected HUVECs in order to quantify the expression of exogenously expressed *myc*-His A_{2A}AR. Figure 4.4 shows a typical saturation binding curve for one such experiment. The B_{max} of receptor expression is similar to levels found in the human striatum ~0.3 pmol/mg (Ji *et al.*, 1992) and the K_d is similar to published values obtained in human neutrophils (Sullivan *et al.*, 1999). No specific binding was detected in control AV-infected cells.

Confirmation of efficient AV/*myc*-His A_{2A}AR expression in HUVECs, facilitated the investigation into the effect of A_{2A}AR expression on monocyte adhesion to inflamed HUVECs. Therefore, confluent HUVEC monolayers infected with either AV/A_{2A}AR or control virus were treated with TNFα for 6 hours and subjected to a monocyte adhesion assay as described in Section 2.5.9. Consistent with Figure 4.1, Figure 4.5 indicates that in control infected HUVEC monolayers there is an increase in the number of adherent U937 cells, when monolayers are treated with TNFα. This effect is not significantly affected when treated with the AR agonist NECA. In contrast, infection of HUVECs with AV/*myc*-His A_{2A}AR significantly reduces the number of adherent U937 cells when compared with control virus even in the absence of agonist. This indicates that the expression of the A_{2A}AR alone can constitutively inhibit U937 monocyte adhesion to TNFα-treated HUVECs in the absence of agonist (84 ± 8%, n=3, $p < 0.05$ *versus* TNFα-treated HUVECs infected with control AV/GFP).

Since the adhesion of monocytes to endothelial cells is dependent upon the expression of adhesion molecules (See Figure 1.1), the monocyte adhesion data suggests that the A_{2A}AR may decrease adhesion molecule expression. One adhesion molecule important in the initial rolling and tethering of monocytes to endothelial cells, under laminar flow, is E-selectin (Ulbrich *et al.*, 2003; Section 1.2.1). Therefore to determine if cell surface E-selectin was expressed, cell surface ELISAs were performed as described in Section 2.5.7 of the Materials and Methods. In agreement with other studies (Mutin *et al.*, 1997; Cohen, 2002), a dose response curve to TNFα when treated for 6 hr indicates that E-selectin was maximally induced by 10 ng/ml (Figure 4.6). In addition, a time-course experiment in response to 10 ng/ml TNFα indicates that E-selectin was maximally induced at 6 hr which then decreases to approximately half maximal expression where it remains elevated for up

to 24 hr (Figure 4.7). Thus, E-selectin expression was maximally induced in HUVECs using 10 ng/ml TNFα for 6 hr. The efficient identification of cell surface E-selectin expression allowed its characterisation in AV/*myc*-His A_{2A}AR and control virus-infected TNFα-stimulated HUVECs. Figure 4.8 indicates that there is a 76 ± 5% reduction (n=3, $p<0.05$ versus TNFα-treated AV/GFP infected HUVECs) in the expression of E-selectin in response to TNFα. These data are consistent with the monocyte adhesion assays and indicates that A_{2A}AR expression can inhibit E-selectin expression leading to a decrease in the adhesion of monocytes to TNFα-stimulated HUVECs. Treatment of A_{2A}AR-infected HUVEC monolayers with purified LPS from *E.coli* was also used to determine if the A_{2A}AR-mediated effects on E-selectin expression were specific to TNFα. Figure 4.9 shows that in control virus-infected HUVECs, treatment with LPS for 6 hr increases the expression of E-selectin similar to TNFα. There is no difference in the presence of the AR selective agonist NECA. In contrast, in AV/*myc*-His A_{2A}AR infected HUVECs, there is no detectable increase in E-selectin expression indicating that A_{2A}AR activation can also inhibit LPS-stimulated E-selectin expression in HUVECs. One possible reason for this is that A_{2A}AR activation can inhibit a shared component of both TNFα-stimulated and LPS-stimulated pathways. One such component is NF-κB, suggesting its potential involvement in the A_{2A}AR-mediated inhibition of E-selectin expression.

The regulation of E-selectin expression is controlled primarily at the level of transcription (Meacock *et al.*, 1994). Analysis of the human E-selectin promoter indicates that there are four positive regulatory domains (PD), which regulate the inducible transcription of E-selectin. PDs I, III and IV are NF-κB binding sites while PD II is an AP-1 site at which an ATF2/c-jun heterodimer is constitutively bound (Read *et al.*, 1997). Therefore induction of E-selectin is dependent upon the activation of both NF-κB as well as the p38- and JNK-mediated phosphorylation of ATF2 and c-jun respectively. Therefore A_{2A}AR activation may inhibit one or more of the transcription factors or upstream signalling cascades responsible for E-selectin activation. Therefore different signalling cascade inhibitors, which target NF-κB and ATF2 were used to inhibit E-selectin expression in TNFα-stimulated HUVECs. Figure 4.10 shows clearly the induction of E-selectin following TNFα treatment. Treatment of the cells with the p38 MAPK inhibitor SB203580, an upstream kinase of ATF2, failed to inhibit the expression of E-selectin. This suggests that inhibition of ATF2-dependent transcription by A_{2A}AR activation would not inhibit E-

selectin expression. In addition, A_{2A}AR activation in endothelial cells can also activate MEK1 (Sexl *et al.*, 1997) in a G_s-independent manner, leading to ERK phosphorylation. Cells were therefore treated with the MEK1 inhibitor PD98059, however it failed to inhibit E-selectin expression suggesting that ERK activity is not important for the A_{2A}AR inhibition of E-selectin expression. In contrast, treatment with PDTC, an antioxidant that selectively blocks the dissociation of IκB from the cytoplasmic NF-κB, thus preventing the activation and nuclear translocation of NF-κB (Schreck *et al.*, 1992), shows a marked decrease in E-selectin expression. Cells were also incubated with the anti-oxidant N-acetyl-cysteine as a control for the anti-oxidant effects of PDTC. This result indicates that A_{2A}AR activation in AV/*myc*-His A_{2A}AR-infected HUVECs may inhibit NF-κB activity. In addition, to the activation of ERK, other A_{2A}AR-activated signalling pathways might mediate the inhibition of E-selectin. A_{2A}AR activation classically stimulates the production of cAMP by G_s-coupled activation of adenylyl cyclase. Treatment with the adenylyl cyclase activator forskolin, which mimics receptor activation however, failed to inhibit E-selectin expression suggesting that the inhibition of E-selectin, is not cAMP-dependent. Overall the results suggest that the inhibition of E-selectin expression in AV/*myc*-His A_{2A}AR-infected HUVECs, occurs at the level of NF-κB *via* an unidentified A_{2A}AR-stimulated mechanism.

The marked effects of PDTC on monocyte adhesion and E-selectin expression in TNFα-stimulated HUVECs, which mimic the A_{2A}AR-mediated responses, indicate that inhibition of NF-κB (Section 1.7.5) may be responsible for the A_{2A}AR anti-inflammatory response. Therefore, to determine any differences in the DNA binding activity of NF-κB in AV/A_{2A}AR–infected cells, nuclear extracts were prepared for EMSA analysis using a κB-specific-radiolabelled probe, from TNFα-stimulated HUVECs infected with either control virus or AV/*myc*-His A_{2A}AR as detailed in Section 2.5.9. Figure 4.12 indicates that in control virus-infected cells, treatment with TNFα led to an increase in the level of NF-κB bound to the DNA probe. Use of subunit specific antibodies in supershift analysis indicated that this complex consists of p50 and p65 subunits. In contrast, in AV/*myc*-His A_{2A}AR infected cells, the level of NF-κB bound to the DNA probe was dramatically reduced following TNFα treatment.

The reduction in NF-κB binding to DNA may represent one of two possibilities, one in which there is a reduction in NF-κB activity or another in which there is a reduction in translocation of NF-κB from the cytoplasm to the nucleus. To discriminate between these possibilities, confocal laser scanning microscopy of HUVECs infected with either control virus or AV/*myc*-His A_{2A}AR, in the presence or absence of TNFα was performed as indicated in Section 2.5.11. Figure 4.13 indicates that in unstimulated AV/GFP infected cells, p65 (red) is predominantly located outside the nucleus. Upon stimulation with TNFα, p65 translocates into the nucleus in cells infected with control virus. In contrast, in cells expressing the AV/*myc*-His A_{2A}AR, p65 is still predominantly located outside the nucleus. Notably, some uninfected cells present within the same field also show p65 nuclear translocation similar to control virus-infected cells.

The nuclear import of p65 is tightly regulated by the interaction NF-κB with the cytoplasmic partner IκB (Section 1.7.4). This is due to masking of the NLS present on p65. However, stimulus-dependent degradation of IκB unmasks this sequence and NF-κB can then translocate to the nucleus to drive NF-κB-dependent transcription (Section 1.7 of Introduction). Therefore having demonstrated an inhibitory effect of AV/*myc*-His A_{2A}AR on nuclear translocation, it was important to ascertain if AV/*myc*-His A_{2A}AR had any effect upon IκB degradation. Thus immunoblotting analysis using anti-*myc* and anti-IκBα antibodies were performed on TNFα-stimulated virus infected HUVECs. Figure 4.14 indicates that in control virus-infected HUVECs, IκBα was degraded by 15 min and returned by 60 min due to the fact that NF-κB regulates the transcription of IκBα (Section 1.7.4). Surprisingly, in AV/*myc*-His A_{2A}AR-infected cells, IκBα was degraded with the same kinetics of degradation and re-appearance, although reappearance at 60 min was reduced. Western blots were stripped and re-probed to indicate the presence of the *myc*-His A_{2A}AR. Thus AV/*myc*-His A_{2A}AR directed gene transfer does not impair the degradation of IκBα.

4.3 Discussion

The anti-inflammatory effects of the nucleoside adenosine have been documented in several animal models of inflammation (See Section 1.11.4 of Introduction). However, the precise molecular details are incompletely understood. The results presented here demonstrate that activation of endogenous A_{2A}ARs can decrease monocyte adhesion to

TNFα stimulated HUVECs *in vitro*. The number of A_{2A}ARs is rate-limiting in murine T lymphocytes as previously reported by Armstrong *et al.*, (2001), therefore one way to potentiate the anti-inflammatory effect of the A_{2A}AR was to increase receptor number. Infection of low-passage HUVECs with AV/A_{2A}AR, could significantly inhibit adhesion of U937 monocytes to TNFα-treated confluent HUVEC monolayers. This A_{2A}AR-mediated effect correlated with a significant decrease in cell surface expression of E-selectin, an adhesion molecule important in leukocyte-endothelial cell interactions (See Section 1.2.1). The decrease in E-selectin expression was associated with a decrease in the DNA-binding ability of the transcription factor NF-κB. This decreased DNA binding ability correlated with a reduction of signal-induced p65-dependent nuclear import, which is an essential step in NF-κB-dependent transcription. As p65 nuclear import is dependent upon the signal-induced degradation of IκBα, Western blot analysis of IκBα surprisingly revealed that there is no inhibition of IκBα degradation in response to TNFα. This suggests that the A_{2A}AR-mediated mechanism of inhibition occurs downstream of IκBα degradation. The results presented suggest that A_{2A}AR activation can inhibit NF-κB activity at the level of nuclear translocation, although the precise mechanism is still unknown.

The results of this investigation are in agreement with several other investigations of NF-κB inhibition which occurs at the level of nuclear transport, including inhibition by synthetic compounds (Wong *et al.*, 2003; Ariga *et al.*, 2002; Loewe *et al.*, 2002) or by naturally occurring anti-oxidants (Schubert *et al.*, 2002), although the role of anti-oxidants remains unclear as N-acetyl cysteine showed no anti-inflammatory effect in endothelial cells in this study. One report by Majumdar and Aggarwal (2003), concludes that adenosine can inhibit NF-κB activation in human myeloid KBM-5 cells in an A_2AR-dependent manner. This effect was not limited to KBM-5 cells as adenosine could also inhibit NF-κB activation in other lymphocytic and epithelial cell types. The A_2AR-dependent effects were determined by EMSA analysis and reporter gene assays suggesting that adenosine inhibits NF-κB binding to DNA and consequent reporter gene activity. This decrease was not due to inhibition of TNFα-induced IκBα phosphorylation, degradation or IKK activity. They proposed a mechanism whereby A_2AR activation modulates the amount of adenosine in the cell resulting in the direct interaction of adenosine with NF-κB, which inhibits NF-κB-DNA binding. It is unknown in this study if intracellular adenosine

can directly inhibit NF-κB, however, in contrast to their study, results presented in this chapter indicate that A_{2A}AR expression blocked NF-κB-mediated induction of E-selectin by both TNFα and LPS suggesting that A_{2A}AR expression can affect a common component in TNFα and LPS/TLR4 signalling cascades. One such possibility is the stimulus-induced translocation of p65 to the nucleus.

The current model of NF-κB activation (Figure 1.5), although useful, does not adequately explain all aspects of NF-κB activation. For example, Huang *et al* (2000) demonstrated that in unstimulated HEK293 cells, treatment with the nuclear export inhibitor leptomycin B resulted in the nuclear accumulation of inactive p65:p50:IκBα complexes. This suggests that inactive complexes constitutively shuttle between the nucleus and the cytoplasm. Further structural studies have indicated that this is due to incomplete masking of the NLS present on p50, and the NES present upon IκBα and p65 (Arenzana-Seisdedos *et al.*, 1997; Rodriguez *et al.*, 1999; Haraj and Sun, 1999; Ghosh and Karin, 2002). However, the cytoplasmic location of these complexes, suggest that nuclear export of the complex predominates over the nuclear import. The absence of a NES on IκBβ or IκBγ suggests that only IκBα can shuttle in this way (Malek *et al.*, 2001; Tam and Sen, 2001). In addition, nucleocytoplasmic shuttling has already been reported for other transcription factors e.g. STAT3 (Pranada *et al.*, 2004) and E2F4 (Deschenes *et al.*, 2004).

The possibility of nucleocytoplasmic shuttling between the nucleus and cytoplasm raises the question of where cytokine-induced IκBα phosphorylation and degradation occurs. Cytosolic phosphorylation and degradation is supported by several findings, which include the observation that the IKK complex, which leads to the cytokine-induced phosphorylation of IκBα, is mainly found in the cytoplasm. This is despite recent evidence supporting an additional role for IKKα in the nucleus where nuclear IKKα activity phosphorylates Ser 10 on histone-3, an event known to correlate to active gene expression, however this is not required for cytokine-induced degradation of IκBα (Anest *et al.*, 2003; Yamamoto *et al.*, 2003)). In addition, the degradation of IκBα is blocked in leptomycin B-treated cells, suggesting that phosphorylation and degradation occur in the cytoplasm (Rodriguez *et al.*, 1999). In contrast, studies by Renard *et al.*, (2000) indicated that treatment of cells with leptomycin B for 40 min, resulted in the TNFα- and IL-1-stimulated degradation of IκBα suggesting that phosphorylation and degradation were still

possibly supporting a role for nuclear degradation. This is supported by evidence that shows that β-TrCP, a component of the IκBα ubiquitination pathway required for IκBα degradation is exclusively located in the nucleus *via* an association with hnRNP-u. Thus, the precise mechanism leading to the phosphorylation and degradation of IκBα is still unclear.

Applying these observations to the A_{2A}AR-mediated inhibition of p65-dependent translocation, two models can be proposed. If the existing model of NF-κB activation is correct, *ie* non-cytoplasmic shuttling, the results suggest that A_{2A}AR activation inhibits nuclear import. If in contrast, nucleocytoplasmic shuttling does occur, then A_{2A}AR activation could facilitate p65 nuclear export in addition to the inhibition of nuclear import. Furthermore, if IκBα phosphorylation and degradation occurs in the nucleus, then A_{2A}AR activity would facilitate the rapid nuclear export of p65 to the cytoplasm prior to gene activation. Irrespective of the location of IκBα phosphorylation and degradation, each theoretical model relies upon either the inhibition of nuclear import or the rapid nuclear export of p65. This could be achieved in a number of different ways which will be outlined below.

One possible mechanism to inhibit nuclear import of active p65 into the nucleus would be the binding of another protein to p65, such that following IκBα degradation, p65 is retained in the cytosol in an analogous fashion to IκBα. Examples might include other IκB proteins e.g. IκBβ or IκBε (Tam and Sen, 2001) or perhaps with other cytosolic proteins e.g. Fas-associated factor (FAF-1). FAF-1 was identified in a yeast-two hybrid screen for Fas-interacting proteins and has been shown to inhibit NF-κB activation by directly binding to p65 to suppress nuclear translocation (Park *et al.*, 2004). This suggests that A_{2A}AR activation may promote the binding of p65 to other cytosolic proteins thereby inhibiting p65 nuclear translocation.

In addition to the interaction with FAF-1, p65 has also been shown to interact with Pin-1. Pin-1 is a peptidyl-prolyl isomerase, which binds to phosphorylated serine or threonine residues which precede proline residues on target proteins to induce conformational changes that modulate the activity, phosphorylation status, subcellular localization and protein stability of target proteins. Investigations reveal that following cytokine treatment, Pin-1 specifically binds to the phospho-Thr^{254}-Pro motif on p65, which inhibits the

interaction with IκBα but has no effect on the interaction with p50. This results in enhanced nuclear accumulation and increased protein stability of p65 and therefore enhances NF-κB activity (Ryo *et al.*, 2003). Thus in this model, $A_{2A}AR$ activation would inhibit the binding of Pin-1 to p65 resulting in a decrease in nuclear translocation.

Other potential mechanisms that may also inhibit the nuclear import of p65 include post-translational modification of p65 such that p65 is no longer recognised by the nuclear pore complex. Several studies have shown that p65 is phosphorylated by a number of upstream kinases including IKK, MSK1, CKII and cPKA (Vermeulen *et al.*, 2002). This post-translational modification has been shown to alter NF-κB activity by directing the association of p65 with CBP or HDAC. The interaction with CBP leads to the acetylation of p65, which enhances p65-dependent transcription. However, neither of these modifications show a clear role in nuclear import. In contrast, studies in *Drosophila* of the Rel-family transcription factor *Dorsal,* indicate that phosphorylation of S317 is necessary for nuclear import. As this residue is conserved amongst all Rel-family transcription factors it is possible that phosphorylation at this site in other family members may also regulate NF-κB activity (Drier *et al.*, 1999). Further studies by Maier *et al.*, (2003) indicate that mutation of Ser 281, the corresponding residue in p65, resulted in a decrease in DNA-binding activity and a decrease in NF-κB-dependent reporter gene transcription, but they did not analyse nuclear translocation (Maier *et al.*, (2003). However, investigations of the corresponding residue in Rel B showed that mutation of Ser 368 is not critical for nuclear import but is instead important for dimerisation with other members of the family.

Finally, one other potential mechanism that would impede nuclear import of p65, is the direct alteration of nuclear pore complex components such as importin α or β resulting in the cytoplasmic retention of p65. Studies in dividing and quiescent nuclei suggest that soluble transport factors such as RanGTP present in the cytoplasm are required for nuclear transport. Despite the relative constant levels of RanGTP during the cell cycle however, nuclear pore complex composition and conformation may change depending upon cellular demands. Thus the state of the cell may dictate nuclear pore complex formation (Gasiorowski and Dean, 2003). However, the most common regulation of nucelocytoplasmic movement results from post-translational modifications of the cargo

proteins themselves, which have been discussed above. Therefore alteration of the nuclear pore complex is unlikely to account for the pattern of p65 expression observed in this investigation.

In contrast to the inhibition of p65 nuclear import, rapid activation of nuclear export could also account for the sustained cytosolic localisation of p65. This could be facilitated by the NES present on p65 (Haraj *et al.*, 1999). In this model, p65 nuclear import would take place however, in contrast to its sustained nuclear localisation for NF-κB-dependent gene transcription, it would be rapidly exported out of the nucleus leading to the observed cytoplasmic localisation. This model suggests that p65 interaction with a nuclear protein, for example CBP or HDAC (Zhong *et al.*, 2002) is essential for the continued presence of p65 within the nucleus. Thus the A_{2A}AR activation could inhibit this interaction leading to rapid export.

In addition to direct p65 nuclear export *via* the NES, it has also been recently reported that in Caco-2 cells, treatment with the non-pathogenic bacteria *B. thetaiotaomicron* can selectively antagonise NF-κB activity by enhancing its nuclear export by a peroxisome proliferator-activated receptor-γ (PPARγ)-dependent, Crm-1-independent mechanism (Kelly *et al.*, 2004). PPARs are a family of three ligand-activated nuclear receptors that are widely expressed in the body, although their relative levels differ greatly between tissues. PPARs are present in cells of the vascular wall and the immune system and are activated by natural ligands such as fatty acids, eicosanoids and oxidized fatty acids. They have previously been shown to regulate genes involved in lipid and lipoprotein metabolism, glucose and energy homeostasis, and as cellular differentiation (Chinetti *et al.*, 2003). However, several investigations suggest they can also regulate the inflammatory response (Devchand *et al.*, 1996; Delerive *et al.*, 2001; Wang *et al.*, 2002). The precise mechanism by which PPARs control the inflammatory response is poorly characterised although it can antagonise a number of transcription factors which are important in activating the immune response such as NF-κB, AP-1, STAT and NF-AT. Further investigations indicate that PPAR-α and -γ can inhibit NF-κB activation by a direct interaction with p65 *via* its RHD. In addition PPAR-α can also induce IκBα transcription. Thus the mechanisms of PPAR-mediated inhibition suggest that PPAR activation leads to the transcription of IκBα, which may bind nuclear p65 and transport it out the nucleus.

This model is however dependent upon Crm-1, therefore it is unlikely that this mechanism is present in the investigations by Kelly *et al.*, (2004). Therefore potentially p65 is exported *via* an as yet unidentified mechanism that is dependent upon PPAR activity. Interestingly, Crm-1-independent nuclear export has already been identified for the thyroid hormone nuclear receptor (Bunn *et al.*, 2001), suggesting that this mechanism might represent an additional nuclear export pathway. The interaction of PPARs with p65 would therefore represent a piggy-back mechanism of nuclear export, as has been suggested for the nuclear import of IκBα following resynthesis (Turpin *et al.*, 1999). Thus in this situation, A_{2A}AR activation would promote the binding of p65 to PPAR which would facilitate nuclear export. Further investigation of p65 nucleocytoplasmic movements by fluorescence microscopy in live cells might allow further characterisation of the subcellular distribution of p65.

One other important observation from this study indicates that treatment of HUVECs with the adenylyl cyclase activator forskolin failed to inhibit the induction of E-selectin. This suggests that activation of the classical A_{2A}AR signalling pathway, resulting in increased cAMP synthesis, may not be required for the anti-inflammatory effect of AV/*myc*-His A_{2A}AR gene transfer. This result is in contrast to other studies suggesting that cAMP mediates a potent anti-inflammatory effect in different cell types (Focy *et al.*, 2003; Wuyts *et al.*, 2003). Recent studies by Sullivan *et al.*, (2004), demonstrated that treatment of neutrophils with TNFα or LPS followed by fMLP treatment, results in a four-five fold induction of the very late antigen (VLA-4; $\alpha_4\beta_1$ integrin). A_{2A}AR activation with the selective agonist ATL146e reduced expression of VLA-4, an effect mediated by cAMP. In contrast, observations in this chapter suggest the improbable involvement of cAMP, as treatment with forskolin could not mimic the A_{2A}AR-mediated inhibition of E-selectin expression. One possible cAMP-independent signalling mechanism activated by the A_{2A}AR in freshly isolated HUVECs, has been reported by Sexl *et al.*, (1997). This involves the $p21^{ras}$ /MEK1-dependent activation of ERK. This possibility is however unlikely as the MEK1 inhibitor, PD98059 failed to inhibit E-selectin expression in TNFα-stimulated HUVECs. Further investigations of the A_{2A}AR signalling cascade including use of the A_{2A}AR truncated constructs may provide more information. In conclusion, the anti-inflammatory effect of the A_{2A}AR in HUVECs may not be mediated by either cAMP or ERK activation.

In conclusion, expression of the $A_{2A}AR$ led to the inhibition of monocyte adhesion in TNFα treated HUVECs. This correlated with a decrease in E-selectin expression in response to TNFα. In addition, $A_{2A}AR$ expression could also inhibit the induction of E-selectin in response to LPS suggesting that receptor expression can inhibit a common component of the TNFα and LPS/TLR4 signalling cascades. This effect is specific to the inhibition of the transcription factor NF-κB as analysis of p38 and JNK activity indicated that $A_{2A}AR$ expression made little or no difference. Further analysis of the NF-κB signalling cascade indicated that receptor expression led to an inhibition in p65-DNA binding and transcriptional activity, which correlated with the reduced entry of p65 to the nucleus following TNFα stimulation, despite the signal-induced degradation of IκBα.

Figure 4.1 Effect of endogenous $A_{2A}AR$ expression on U937 monocyte adhesion to TNFα stimulated HUVECs

$1x10^6$ HUVECs were seeded into a 24-well plate and grown to confluence. The following day, cells were pre-treated for 30 min with 5 μM CGS21680 prior to incubation with increasing concentrations of TNFα for 6 hr as indicated. Following treatment, the medium was removed and the HUVEC monolayers were overlayed with U937 cells, which were allowed to adhere for 1 hr at 37°C. Cells were then washed gently to remove non-adherent cells and fixed in paraformaldehyde. The number of U937 cells attached per 100 HUVECs was counted by microscopy. This graph represents one of multiple experiments.

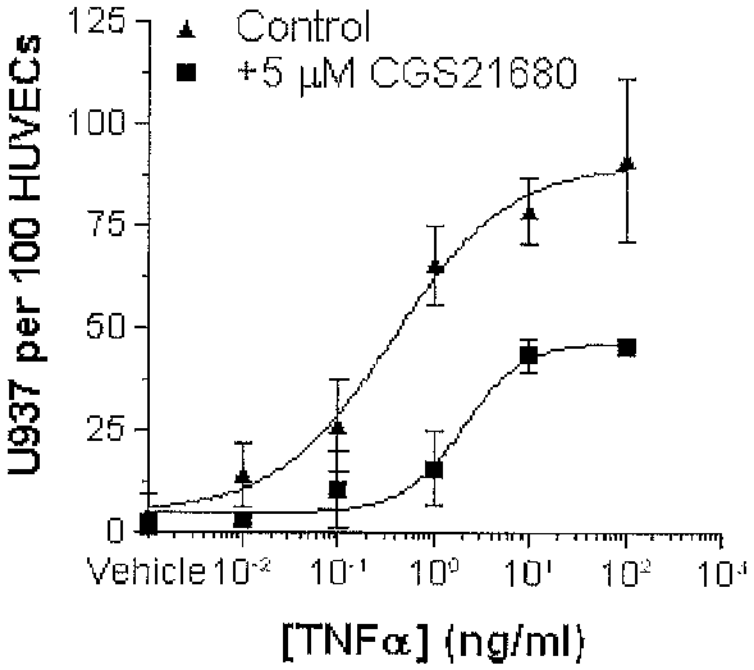

U937 per 100 HUVECs
125
100
75
50
25
0
Control
+5 μM CGS21680
Vehicle
10⁻²
10⁻¹
10⁰
10¹
10²
10³
[TNFα] (ng/ml)

Figure 4.2 Titration-dependent expression of AV/*myc*-His $A_{2A}AR$

$1x10^6$ HUVECs were infected with increasing multiplicities of infection (m.o.i.) as indicated. 24hr post-infection, cells were monitored for the expression of GFP and the percentage of infected cells was determined by calculating the percentage of GFP-positive cells in five random fields of view. Cells were then transferred to ice, solubilised, equalised for protein concentration and fractionated on SDS-PAGE. Proteins were then transferred to nitrocellulose and immunoblotted for the presence of the $A_{2A}AR$ using the anti-myc antibody as described in Section 2.5.1 in the Materials and Methods. Blots were then stripped and reprobed for the presence of $G_{i\alpha 2}$ as a loading control for protein equalisation.

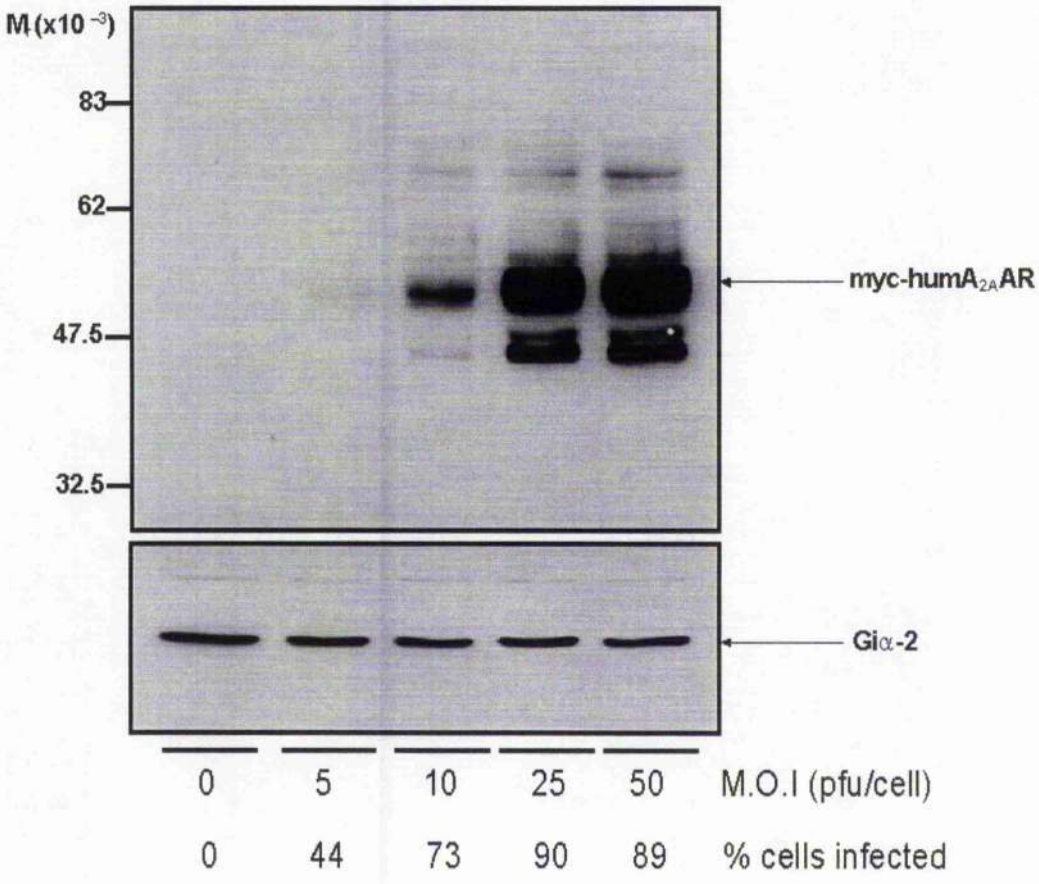

M (x10 -3)
83
62
47.5
32.5
myc-humA2AAR
Giα-2
0
5
10
25
50
M.O.I (pfu/cell)
0
44
73
90
89
% cells infected

Figure 4.3 Biotinylation of cell surface expressed AV/*myc*-His A_{2A}AR

$1x10^6$ HUVECs were infected with increasing multiplicities of infection (m.o.i.) as indicated. 24hr post-infection HUVECs were labelled with biotin-LC-hydrazide according to the Methods in Section 2.5.3. Cell lysates were then prepared, equalised for protein content, and immunoprecipitated with anti-*myc* (9E10). Immunoprecipitates were then fractionated by SDS-PAGE and transferred to nitrocellulose. Biotinylated A_{2A}ARs were detected by incubation with HRP-conjugated streptavidin as described in Section 2.5.3. Biotin-labelled proteins were then visualised using enhanced chemiluminescence. This represents one of several experiments.

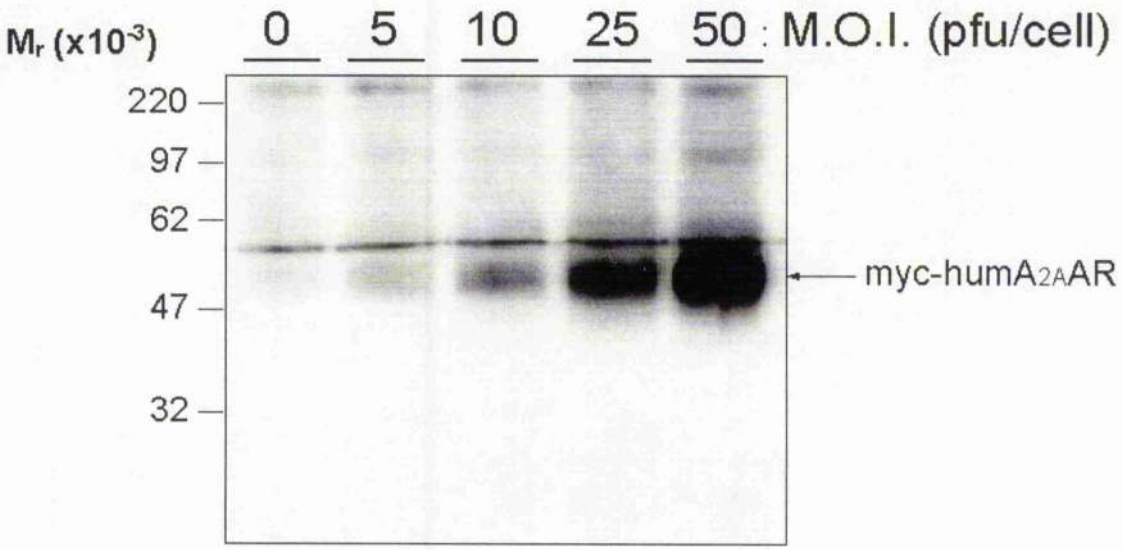

Mr (x10-3)
0
5
10
25
50 : M.O.I. (pfu/cell)
220
97
62
47
32
myc-humA2AAR

Figure 4.4 **^{125}I-ZM241385 saturation binding analysis of *myc*-His A_{2A}AR in HUVECs**

Saturation binding studies were carried out on membranes prepared from HUVECs infected with 25 pfu/cell using the A_{2A}AR-selective antagonist ^{125}I-ZM241385. Membrane preparation and radioligand binding procedures are detailed in the Methods Section 2.5.4. The graph shown here represents one typical saturation-binding curve.

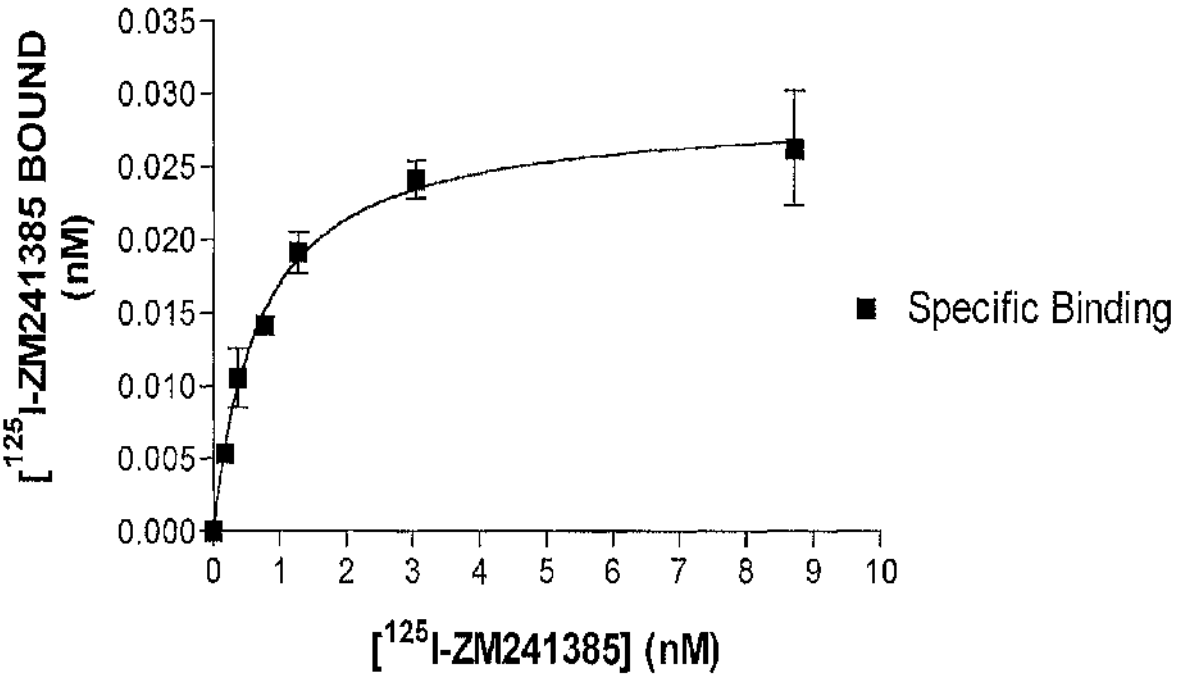

0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
[125I-ZM241385 BOUND (nM)
0
1
2
3
4
5
6
7
8
9
10
[125I-ZM241385] (nM)
Specific Binding

Figure 4.5 Effect of AV/*myc*-His A_{2A}AR expression on TNFα-stimulated U937 monocyte adhesion to HUVECs

$1x10^6$ HUVECs were seeded into a 24-well plate and grown to 70% confluence. 24hr after seeding, cells were infected with 25 pfu/cell of recombinant AV/*myc*-His A_{2A}AR or AV/GFP. 24hr post-infection, cells were treated with 10 ng/ml TNFα for 6hr in the presence or absence of the AR-agonist NECA. Following treatment, the medium was removed and the HUVEC monolayers were overlayed with U937 cells, which were allowed to adhere for 1 hr at 37°C. Cells were then washed gently to remove non-adherent cells and fixed in paraformaldehyde. The number of U937 cells attached per 100 HUVECs was counted using a combination of bright-field and fluorescence microscopy. This result represents one of multiple experiments performed.

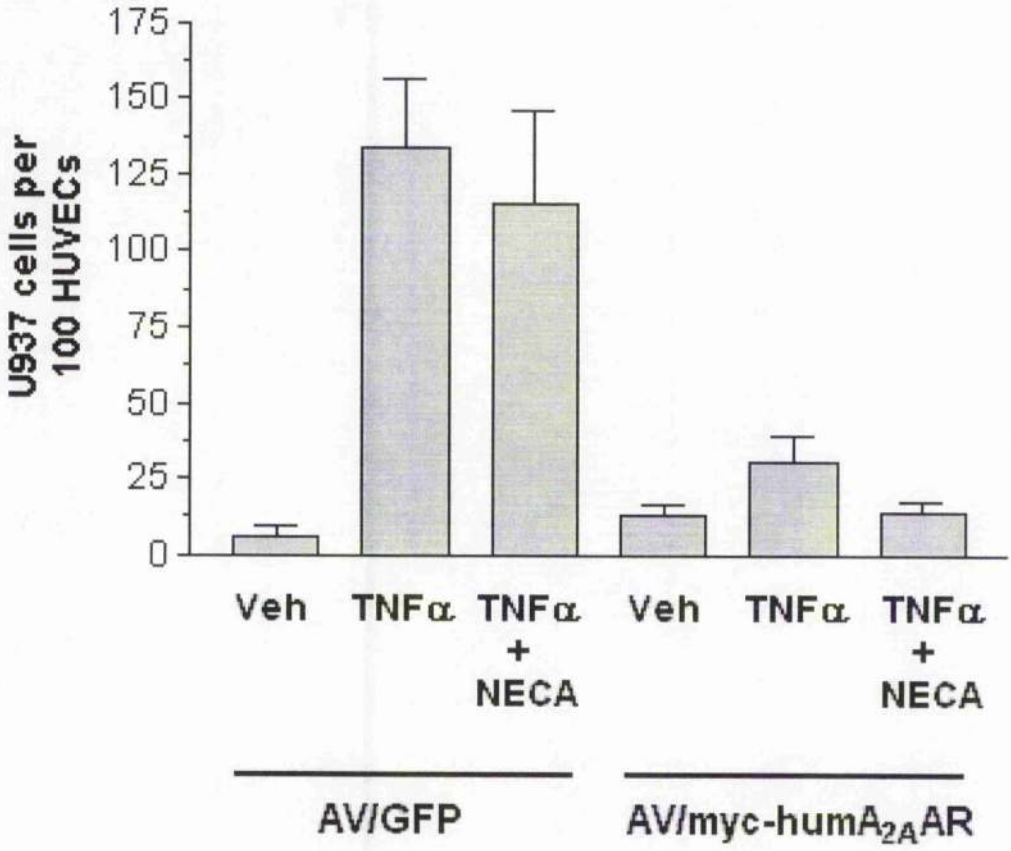

U937 cells per 100 HUVECs
175
150
125
100
75
50
25
0
Veh
TNFα
TNFα + NECA
Veh
TNFα
TNFα + NECA
AV/GFP
AV/myc-humA2AAR

Figure 4.6 Effect of increasing concentrations of TNFα on the induction of E-selectin in HUVECs

$1x10^5$ HUVECs were seeded in triplicate wells and in parallel into a 96-well plate. 24hr after seeding, cells were treated with increasing concentrations of TNFα for 6hr as indicated. The induction of E-selectin was determined by cell-surface ELISA as described in Section 2.5.7 of the Materials and Methods. Following subtraction of non-specific binding, the mean OD_{600} ± S.E.M. was calculated and fitted to a non-linear regression sigmoidal dose response curve using GraphPad Prism. This represents an average of several experiments log EC_{50} = 0.563 ng/ml

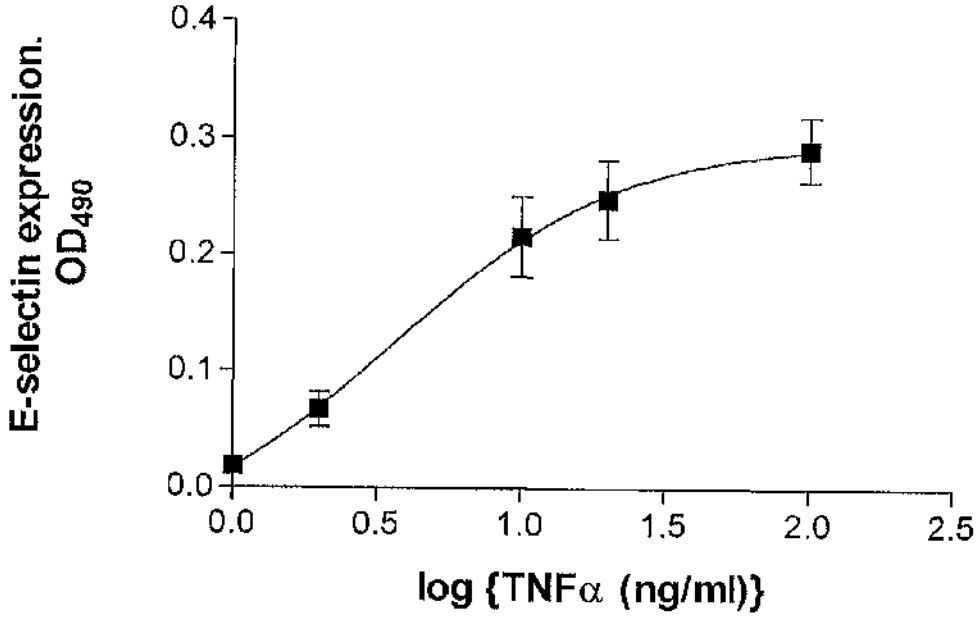

E-selectin expression.
OD_{490}
0.4
0.3
0.2
0.1
0.0
0.0
0.5
1.0
1.5
2.0
2.5
log {TNFα (ng/ml)}

Figure 4.7 Time course of E-selectin induction following treatment with TNFα

$1x10^5$ HUVECs were seeded in triplicate wells and in parallel into a 96-well plate. 24hr after seeding, cells were treated for 0, 3, 6, 9, 12 and 24 hr with 10ng/ml TNFα. The induction of E-selectin was determined by cell-surface ELISA as described in Section 2.5.7 of the Materials and Methods. Following subtraction of non-specific binding, the mean OD_{600} ± S.E.M. was calculated. This represents an average of several experiments.

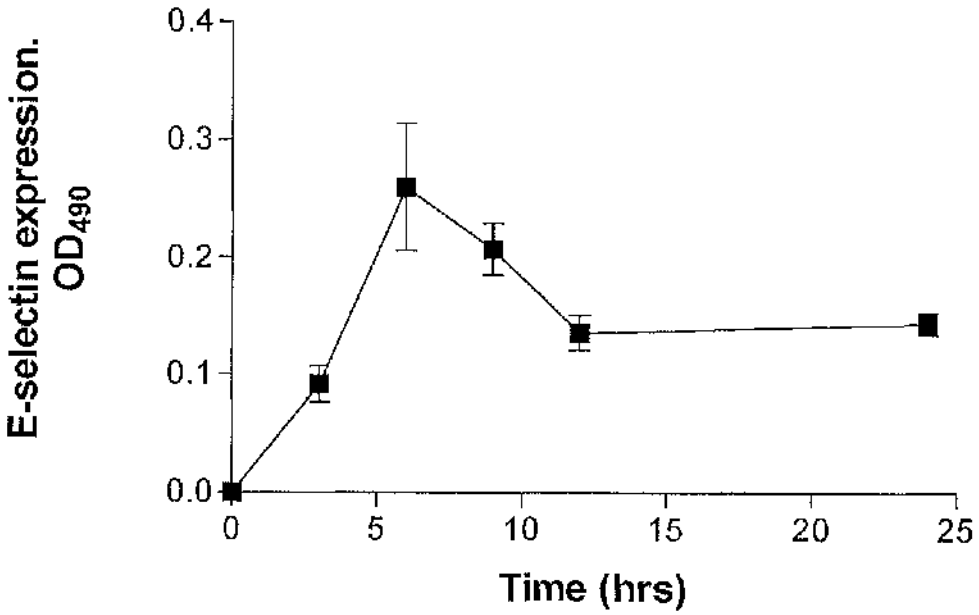

E-selectin expression.
OD_{490}
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
Time (hrs)

Figure 4.8 **Effect of AV/*myc*-His A_{2A}AR gene transfer on E-selectin induction in HUVECs when exposed to TNFα**

$1x10^5$ HUVECs were seeded in triplicate wells and in parallel into a 96-well plate. The next day, cells were infected with 25 pfu/cell of AV/*myc*-His A_{2A}AR and control AV/GFP. 24hr post-infection, cells were treated for 6hr with 10ng/ml TNFα. E-selectin expression was then determined by cell-surface ELISA as described in Section 2.5.7 of the Materials and Methods. Following subtraction of non-specific binding, data were plotted as mean OD_{600} ± S.E.M.

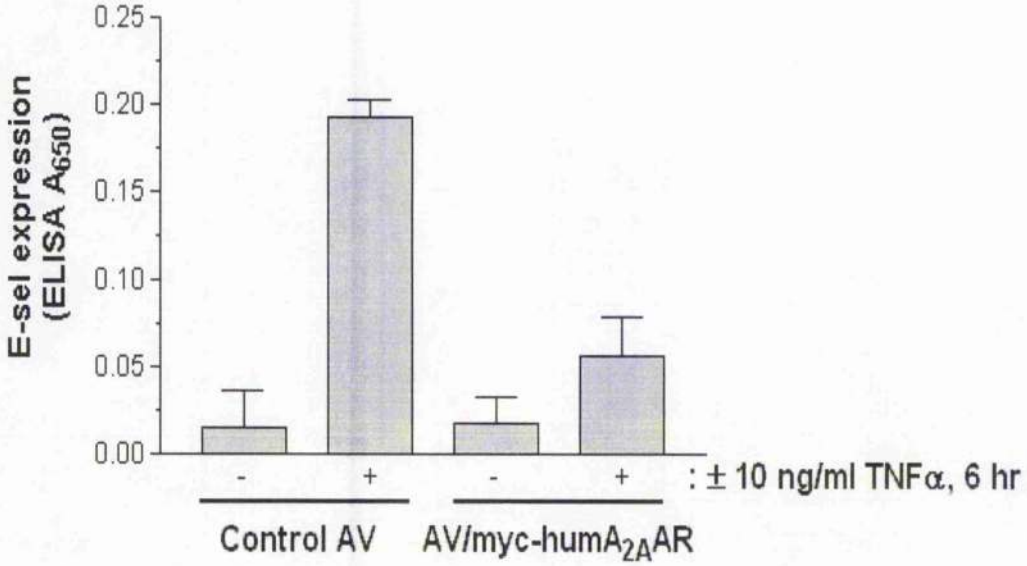

E-sel expression
(ELISA A_{650})
0.25
0.20
0.15
0.10
0.05
0.00
-
+
-
+
: ± 10 ng/ml TNFα, 6 hr
Control AV
AV/myc-humA_{2A}AR

Figure 4.9 Effect of AV/*myc*-His A_{2A}AR gene transfer on E-selectin induction in HUVECs when exposed to LPS

$1x10^5$ HUVECs were seeded into a 6-well plate overnight. The next day, cells were infected with 25 pfu/cell of AV/*myc*-His A_{2A}AR and control AV/GFP. The following day, cells were treated for 6hr with 1 μg/ml LPS in the presence or absence of 5 μM NECA (N). Cells were then transferred to ice, solubilised, equalised for protein concentration and fractionated on SDS-PAGE. Proteins were then transferred to nitrocellulose and immunoblotted for the presence of the E-selectin using the anti-E-selectin antibody as described in Section 2.5.1 in the Materials and Methods.

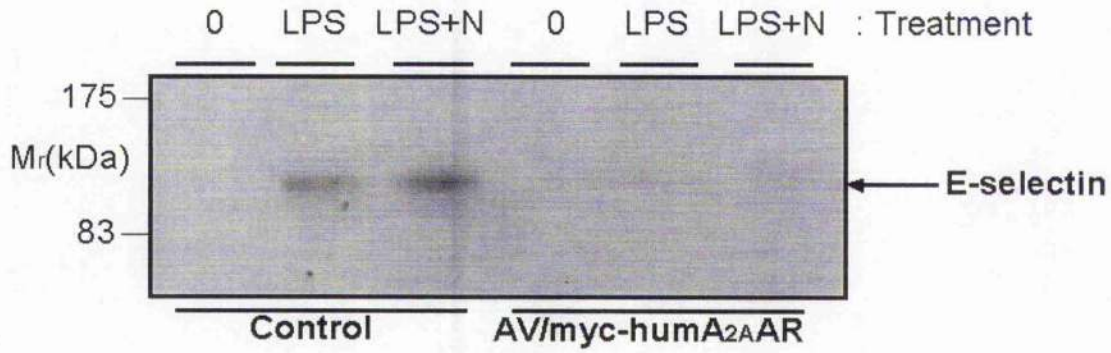

0
LPS
LPS+N
0
LPS
LPS+N
: Treatment
175
Mr(kDa)
83
E-selectin
Control
AV/myc-humA2AAR

Figure 4.10 Analysis of the signalling mechanisms that regulate E-selectin expression

$1x10^6$ HUVECs were plated out in 6-well plates and pre-treated for 30 min with the p38 MAPK inhibitor SB203580 (10 μM), the MEK1 inhibitor PD98059 (10 μM), the NF-κB inhibitor PDTC (100 μM), N-acetyl-cysteine (100 μM), and the adenylyl cyclase activator forskolin (50 μM). Cells were then treated with 10 ng/ml TNFα for 6 hr. Cells were then solubilised, equalised for protein prior to fractionation by SDS-PAGE and immuoblotted using an anti-E-selectin antibody as described in Section 2.5.1. This represents one of several experiments.

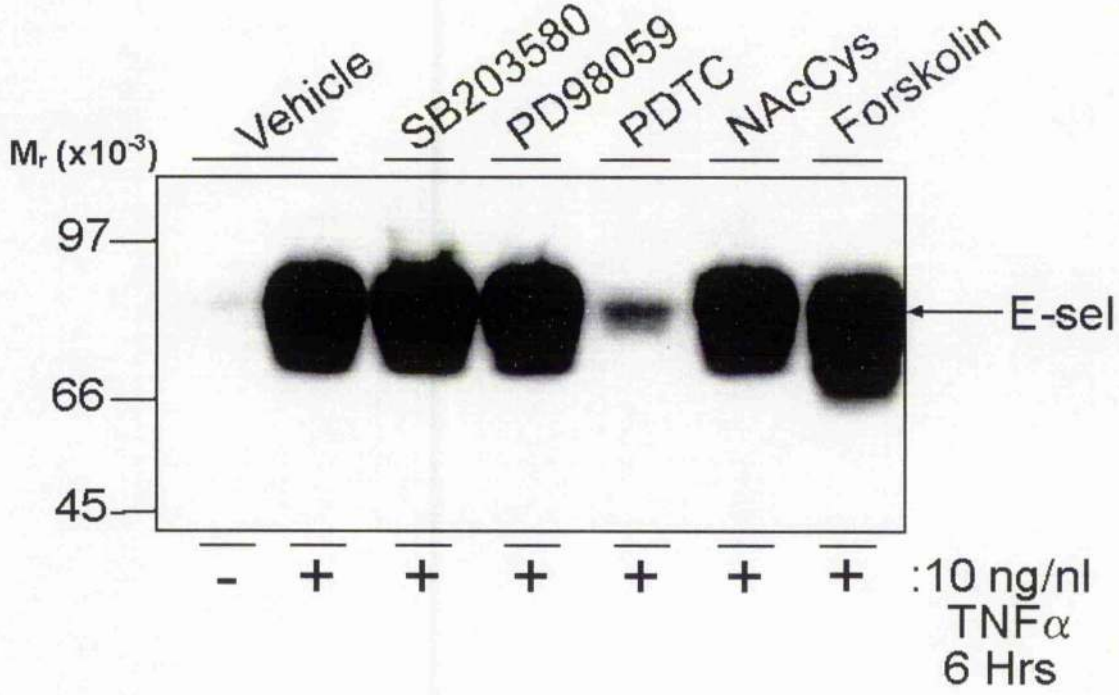

Vehicle
SB203580
PD98059
PDTC
NAcCys
Forskolin
Mr (x10-3)
97
66
45
E-sel
- + + + + + +
:10 ng/nl
TNFα
6 Hrs

Figure 4.11 Effect of the NF-κB inhibitor PDTC on TNFα-stimulated adhesion of U937 monocytes to HUVECs

$1x10^6$ HUVECs were seeded into a 24-well plate and grown to 70% confluence. 24 hr after seeding, cells were pre-treated for 30 min with 100 μM PDTC or 100 μM N-acetyl cysteine as indicated. Cells were then treated with 10 ng/ml TNFα for 6 hours. Following treatment, the medium was removed and the HUVEC monolayers were overlayed with U937 cells, which were allowed to adhere for 1 hr at 37°C. Cells were then washed gently to remove non-adherent cells and fixed in paraformaldehyde. The number of U937 cells attached per 100 HUVECs was counted by microscopy. This bar chart represents the average of several experiments.

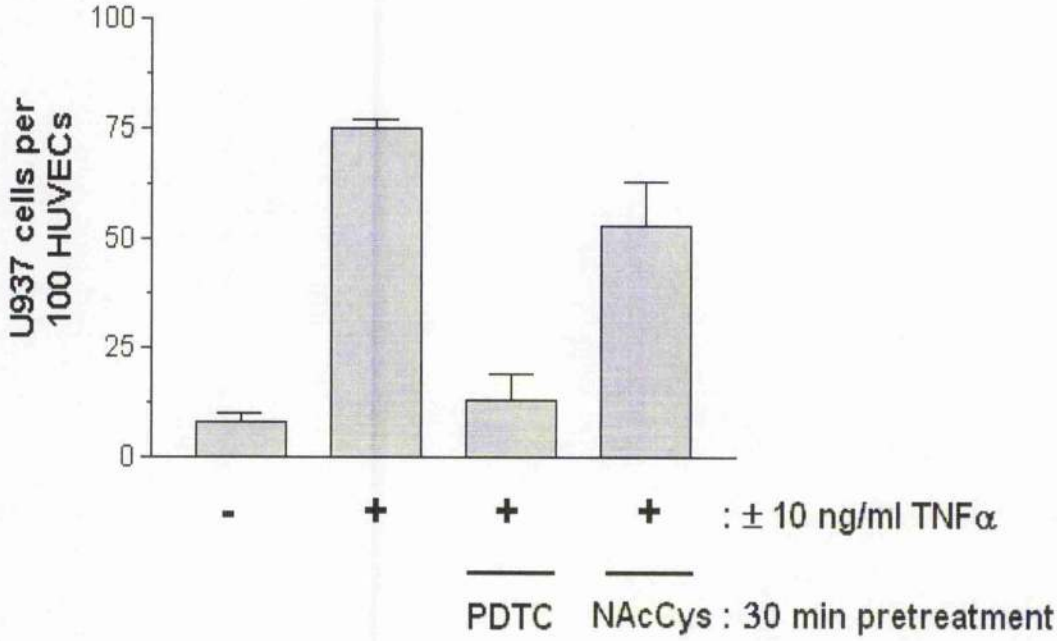

U937 cells per 100 HUVECs
100
75
50
25
0
-
+
+
+
: ± 10 ng/ml TNFα
PDTC
NAcCys : 30 min pretreatment

Figure 4.12 Effect of AV/*myc*-His A_{2A}AR expression on NF-κB binding to target DNA in HUVECs

$1x10^6$ HUVECs were seeded into a 6-well plate and grown to 70% confluence. 24hr after seeding, cells were infected with 25 pfu/cell of recombinant AV/*myc*-His A_{2A}AR or AV/GFP. 24hr post-infection, cells were treated with 10 ng/ml TNFα for 6hr and the nuclei isolated according to the protocol in Section 2.5.9 of the Materials and Methods. Nuclear extracts were then incubated in a binding assay containing ^{32}P-labelled NF-κB target oligonucleotide and the relevant supershift antibody as indicated. Binding reactions were then loaded onto a non-reducing 6% (w/v) acrylamide gel. The gel was then dried down under vacuum and bands were visualised using autoradiography. This figure represents one of several experiments.

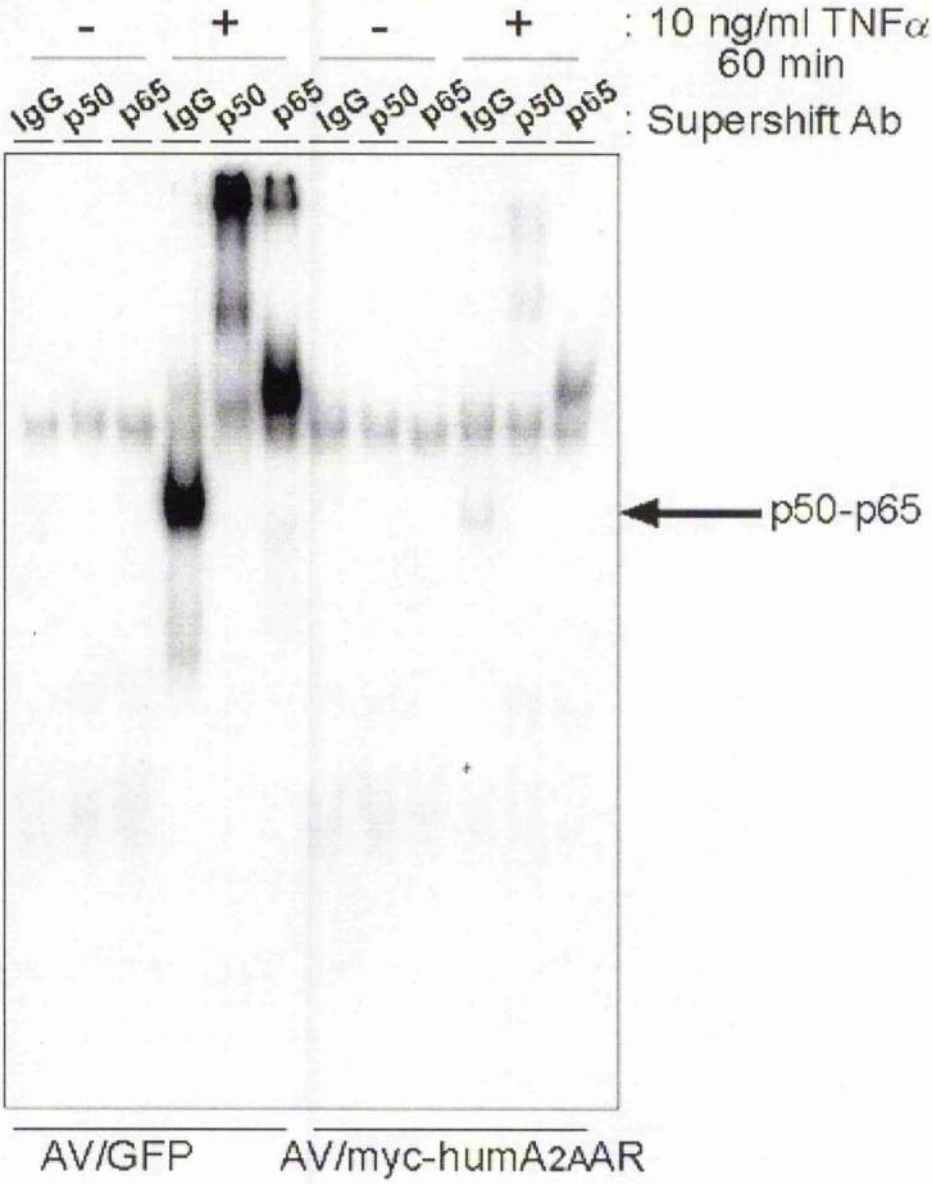

- + - + : 10 ng/ml TNFα
60 min
IgG p50 p65 IgG p50 p65 IgG p50 p65 IgG p50 p65 : Supershift Ab
p50-p65
AV/GFP
AV/myc-humA2AAR

Figure 4.13 Effect of AV/*myc*-His A_{2A}AR gene transfer on the nuclear translocation of p65 in TNFα stimulated HUVECs

$1x10^6$ HUVECs were seeded into 6-well plates onto sterile coverslips. 24 hr after seeding, cells were infected with 25 pfu/cell of recombinant AV/*myc*-His A_{2A}AR or AV/GFP. 24hr post-infection cells were treated were then treated with (lower panels) and without (upper panels) 10 ng/ml TNFα for 60min. Following treatment cells were washed and fixed in paraformaldehyde. Cells were then semi-permeabilised and incubated with anti-p65 antibody (1:200 dilution) for 1hr at room temperature. Cells were then rinsed and incubated with the Alexa 594-conjugated secondary antibody (1:400) for 1hr at room temperature. Following several rinses, coverslips were mounted onto microscope slides and analysed using a Zeiss Axiovert 500 laser scanning confocal microscope as indicated in Section 2.5.11 of the Materials and Methods. Images were taken at 40x magnification.

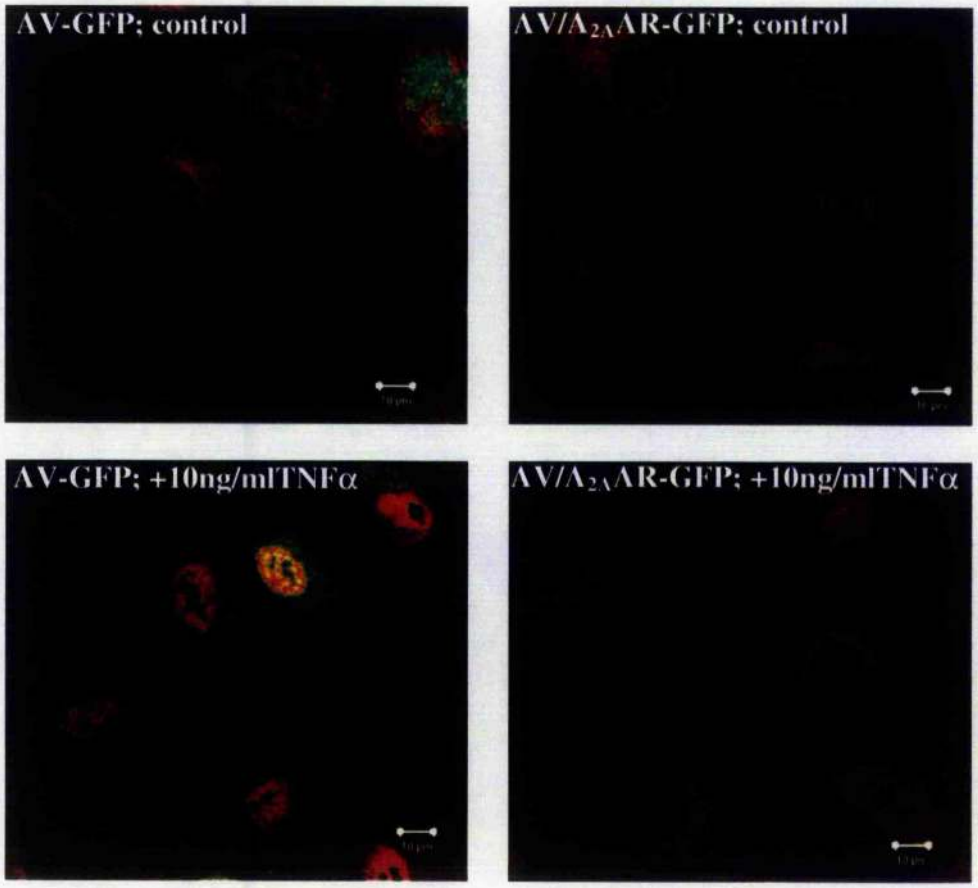
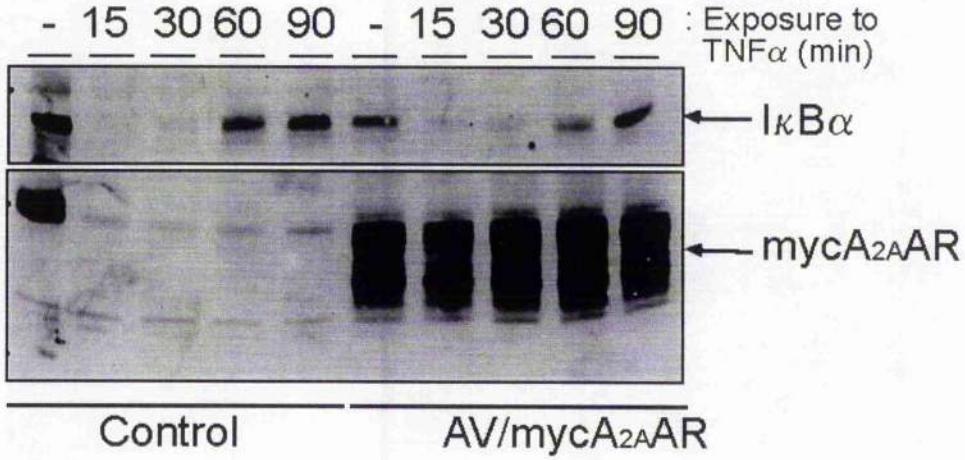

AV-GFP; control
AV/A2AAR-GFP; control
AV-GFP; +10ng/mlTNFα
AV/A2AAR-GFP; +10ng/mlTNFα

Figure 4.14 Effect of A_{2A}AR expression on the degradation of IκBα

$1x10^6$ HUVECs were seeded into a 6-well plate and grown to confluence. 24hr after seeding, cells were infected with 25 pfu/cell of recombinant AV/*myc*-His A_{2A}AR or AV/GFP. 24hr post-infection, cells were treated with 10ng/ml TNFα for the indicated time periods. Cells were then washed, solubilised, and fresh lysates were prepared. Proteins were then fractionated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was carried out using the anti-*myc* antibody (1:500) and the anti-IκBα antibody (1:1000) according to the protocols in Section 2.5.1. This figure represents one of several experiments.


- 15 30 60 90 - 15 30 60 90 : Exposure to TNFα (min)
IκBα
mycA2AAR
Control
AV/mycA2AAR

Chapter 5

Effect Of $cA_{2A}AR$ Expression On The Regulation Of NF-κB-Dependent Inflammatory Responses In C6 Glioma Cells

5.1 Introduction

The potent anti-inflammatory effects of the $A_{2A}AR$ have been demonstrated in a variety of different cell types (Section 1.11.4). One possible reason for this is that $A_{2A}AR$ activation can activate common mechanisms that lead to the suppression of inflammation. The results in Chapter 4 suggest that $A_{2A}AR$ expression can inhibit the activation of the transcription factor NF-κB. Therefore to determine whether this represents a common anti-inflammatory signalling mechanism employed by this GPCR, C6 glioma cells stably expressing the canine $A_{2A}AR$ ($cA_{2A}AR$) were used to identify anti-inflammatory pathways present in glial cells. Glial cell inflammation plays a key role in disease states such as multiple sclerosis (Bradl and Hohlfeld, 2003), stroke (Price *et al.*, 2003) and Alzheimer's disease (Akiyama *et al.*, 2000), therefore any anti-inflammatory properties of the $A_{2A}AR$ agonism could be therapeutically beneficial in a number of diseases. In addition, C6 glioma cells represent a tractable model for the in-depth analysis of the mechanisms that regulate the inflammatory response. One important consideration was that in contrast to HUVECs, C6 glioma cells do not express the NF-κB-regulated gene E-selectin, therefore the induction of another NF-κB-regulated gene, iNOS was used as a marker for inflammation, as iNOS-derived nitric oxide has been shown to contribute to the ability of activated glial cells to enhance neuronal cell death (Brown and Bal-Price, 2003). The use of the canine receptor in this study also allowed us to determine if the anti-inflammatory effects of the human receptor represents a general feature of $A_{2A}ARs$ or if it is specific only to the human form. Figure 5.1 shows a primary sequence alignment of human and canine receptors and indicates that they share 87% sequence identity, with the greatest degree of divergence occurring in the C-terminus, which is thought to be important in regulating the efficiency of signalling in downstream pathways (Klinger *et al.*, 2002).

5.2 Results

Before determining whether $cA_{2A}AR$ expression could mediate similar anti-inflammatory effects in C6 glioma cells as in HUVECs, it was important to confirm that functional $cA_{2A}AR$ was efficiently expressed at the cell surface in C6 glioma cells. Therefore freshly prepared cell lysates from untransfected C6 and C6/$cA_{2A}AR$ glioma cells were subject to SDS-PAGE followed by immunoblotting with an anti-$A_{2A}AR$ antibody as described in Section 2.5.1 of the Materials and Methods. Figure 5.2 shows clearly the presence of the $cA_{2A}AR$ at ~47 kDa in transfected cells compared to control cells. To verify that the

receptor was fully processed and expressed at the cell surface, cell-surface biotinylation assays were performed on untransfected C6 and C6/cA_{2A}AR glioma cells as described in Section 2.5.4 of the Materials and Methods. Figure 5.3 shows a smear of biotinylated protein centred at ~47 kDa indicating that cA_{2A}ARs are expressed at the surface of the cell. In addition, there is a higher molecular weight species evident that is also expressed at the surface, which is consistent with the formation of multimers as reported by Canals *et al.*, (2004). Finally to quantify the level of cA_{2A}AR expression in glioma cells, ^{125}I-ZM241385 saturation binding analysis was performed upon membranes isolated from untransfected C6 glioma cells and C6/cA_{2A}AR cells. Figure 5.4 shows a typical saturation binding curve for one such experiment. In C6/cA_{2A}AR glioma cells the B_{max} for ^{125}I-ZM241385 ranged between 2.57 and 6.02 pmol/mg of membrane protein, with a K_d value of 1.10 ± 0.29 nM (n=5). No specific binding was observed in untransfected C6 glioma cells suggesting that there is very little or no endogenous A_{2A}AR expression in C6 glioma cells. Taken together, these results confirm the cell surface expression of a functional fully processed cA_{2A}AR in stably transfected C6 glioma cells.

To investigate if cA_{2A}AR expression could regulate the release of nitric oxide in C6 glioma cells, untransfected C6 and C6/cA_{2A}AR glioma cells were treated with maximally effective concentrations of LPS/TNFα/IFNγ either alone or in combination for 24 hr. Supernatants were then removed and the level of nitric oxide released was quantified by measuring the accumulation of NO-derived nitrite in the supernatant using the Greiss reaction according to the protocol in Section 2.5.12. Figure 5.5 indicates that none of TNFα, LPS or IFNγ alone could stimulate a detectable increase in nitrite accumulation. However, when treated with IFNγ plus either TNFα or LPS, or when treated with LPS/TNFα/IFNγ, there was a detectable increase in nitrite accumulation that was maximal at 20 μmol/well in untransfected C6 glioma cells. This effect however was completely abolished in C6/cA_{2A}AR cells even in the absence of agonist, indicating that A_{2A}AR expression can suppress nitric oxide production in response to LPS/TNFα/IFNγ. The effect of A_{2A}AR expression upon nitrite accumulation is not due to the presence of extracellular adenosine acting at the receptor, as experiments were performed in the presence of 3 units/ml of ADA, which converts adenosine to inosine, which is inactive at the A_{2A}AR (Jin *et al.*, 1997). In addition, treatment of the cells with the A_{2A}AR-selective neutral antagonist ZM241385 could not reverse the inhibition of nitrite accumulation. Together, this suggests

that the receptor is constitutively active, and can therefore constitutively inhibit nitrite accumulation. Further characterisation of the A_{2A}AR-mediated effect indicates that in control cells, treatment with LPS/TNFα/IFNγ produced a time-dependent accumulation of nitrite in the medium (4.13±0.08 fold stimulation over control at 24 hr, n=4) as shown in Figure 5.6. In contrast, expression of the A_{2A}AR could suppress nitrite accumulation throughout the time course suggesting that cA_{2A}AR expression produces a sustained suppression of nitrite accumulation. Composite data from these experiments is summarised in Figure 5.7.

The release of nitric oxide is due to the activity of iNOS whose expression is largely regulated by gene transcription in response to extracellular stimuli (Section 1.2.4). Therefore, one possible mechanism that might explain the synergistic effect of LPS/TNFα/IFNγ on the release of nitric oxide is that LPS/TNFα/IFNγ exposure can activate multiple transcription factors which synergistically enhance iNOS expression. Analysis of the rat iNOS promoter indicates the presence of transcription factor binding sites for NF-κB, and STAT proteins (Niwa *et al.*, 1997; Taylor *et al.*, 1998; Ganster *et al.*, 2001). As these transcription factors are downstream targets of the pro-inflammatory signalling cascades initiated by TNFα, LPS and IFNγ, this mechanism seems most likely (Sections 1.4.1, 1.4.3, 1.4.4). Therefore, to determine the effect of A_{2A}AR expression upon the induction of iNOS expression, freshly prepared cell lysates from untransfected C6 and C6/cA_{2A}AR glioma cells, treated with maximally effective concentrations of LPS/TNFα/IFNγ were subject to SDS-PAGE followed by immunoblotting with an anti-iNOS antibody. Figure 5.8 shows that cytokine treatment of untransfected C6 glioma cells could markedly increase iNOS expression consistent with the data on nitrite accumulation. However, in C6/cA_{2A}AR cells, this effect was completely abolished. Further treatment with the AR-agonist NECA did not further affect iNOS expression in untransfected C6 or C6/cA_{2A}AR glioma cells, further indicating that the effects are agonist-independent. These results indicate that A_{2A}AR expression can completely attenuate the induction of iNOS in C6 glioma cells.

As detailed above the A_{2A}AR is most likely to attenuate iNOS induction at the level of transcription. Two observations suggest that a likely target for the A_{2A}AR-mediated effect is the transcription factor NF-κB. Firstly, the rat iNOS promoter contains two transcription

factor binding sites for NF-κB, which are necessary for iNOS induction (Xie *et al.*, 1994). Secondly, results from Chapter 4 indicate that A_{2A}AR expression can inhibit NF-κB activity. This suggests that inhibition of NF-κB might mimic the A_{2A}AR-mediated effects on iNOS induction. To test this hypothesis, nitrite accumulation assays were performed on untransfected C6 glioma cells stimulated with LPS/TNFα/IFNγ, in the presence or absence of the NF-κB inhibitor PDTC. N-acetyl-cysteine was also included in this study to control for the possible anti-oxidant side effects of PDTC. Pre-treatment with 100 μM PDTC could attenuate nitrite accumulation by 55±7%, ($p<0.05$, n=3). This was not due to any anti-oxidant side effects of PDTC as parallel pre-treatment with 100 μM N-acetyl cysteine failed to inhibit nitrite accumulation (Figure 5.9). This demonstrates that NF-κB plays an important role in the induction of iNOS expression in C6 glioma cells and suggests that inhibition of NF-κB can partly suppress iNOS induction.

The results from Figure 5.9 suggest that A_{2A}AR expression may inhibit the activity of NF-κB leading in part to the suppression of iNOS. Therefore, to determine what effect A_{2A}AR expression had on NF-κB, EMSA analysis was performed in C6 and C6/cA_{2A}AR cells. Figure 5.10 indicates that there is an increase in the DNA-binding capacity of NF-κB in untransfected C6 glioma cells when treated with LPS or TNFα but not with IFNγ. This effect is markedly decreased in C6/cA_{2A}AR cells suggesting that cA_{2A}AR expression inhibits the capacity of NF-κB to bind to target DNA. Both bands present in Figure 5.10 represent p65/p50 heterodimers as both bands are retarded when co-incubated with anti-p65 or p50 antibodies in supershift experiments (W.Sands, personal communication). To test the functional consequences of decreased DNA binding, reporter gene analysis was performed and indicates that treatment of untransfected C6 glioma cells with TNFα induced a 53.4±3.6-fold stimulation of luciferase expression (n=4), *versus* 4.3±2.7-fold stimulation in C6/cA_{2A}AR cells ($p<0.05$, n=4, Figure 5.11). Together these results suggest that A_{2A}AR expression can inhibit DNA-binding activity of NF-κB and subsequently inhibit the ability of NF-κB to activate target gene transcription.

Exposure to TNFα and LPS leads to the activation of the p38 MAPK and JNK signalling cascades in addition to NF-κB (Section 1.4.1 and 1.4.4; Manning and Davis, 2003; Karin *et al.*, 2004). Therefore, to determine if A_{2A}AR expression had other anti-inflammatory

effects, experiments were performed to determine the activity of p38 MAPK and JNK. Figure 5.12 indicates that in C6 cells, treatment with TNFα induced a transient increase in p38 phosphorylation that peaks at 5 min and returned to basal levels after 30 min. In C6/cA$_{2A}$AR cells, p38 phosphorylation at 5 min was reduced by 25±6% ($p<0.05$,n=3), but similarly returned to basal levels after 30 min. Therefore A$_{2A}$AR expression can decrease the maximal extent to which p38 was activated. In addition, Figure 5.13 indicates that there is no significant difference in the capacity of JNK to phosphorylate c-jun in response to TNFα between C6/cA$_{2A}$AR and control cells (maximal activation reduced by 9±6% at 15 min, NS, n=3). However, maximal activation was typically reached after 5 min exposure in C6/cA$_{2A}$AR cells, as compared with 15 min in control cells. These results suggest that A$_{2A}$AR expression has minimal effects on p38 and JNK activation and that the main anti-inflammatory effects of A$_{2A}$AR expression on TNFα and LPS/TLR4 signalling is at the level of NF-κB activation.

As identified in Figure 5.10, A$_{2A}$AR expression can inhibit the DNA-binding capacity of NF-κB to target DNA. However, as there are several different key upstream signalling events required for NF-κB activation by multiple stimuli, it is unclear how A$_{2A}$AR expression can lead to the inhibition of DNA-binding. One essential step necessary for cytokine-induced NF-κB activation is the phosphorylation-dependent polyubiquitination and degradation of IκBα (Section 1.4.1). Therefore, to determine what effect A$_{2A}$AR expression had on IκBα degradation, Western blotting was performed using anti-IκBα antibodies in cell lysates made from C6/A$_{2A}$ARs and control cells following treatment with TNFα (Figure 5.14) and LPS (Figure 5.15). Treatment of untransfected C6 cells with TNFα or LPS led to a time-dependent degradation of IκBα that peaked at 15 min. In addition, Figure 5.15 indicates that there is a detectable rise in the levels of IκBα at 45 min, consistent with the fact that IκBα transcription is regulated by NF-κB. In contrast, there was no detectable degradation of IκBα in response to either TNFα or LPS in A$_{2A}$AR expressing cells suggesting that A$_{2A}$AR expression can inhibit the degradation of IκBα.

As highlighted above, one step essential for IκB degradation is its prior phosphorylation that specifically occurs at Ser^{32} and Ser ^{36}. Therefore, to determine the effects of A$_{2A}$AR expression on the phosphorylation of IκBα in response to TNFα or LPS, Western blotting

was performed using anti-phospho Ser^{32}, Ser^{36} antibodies. Results from Figure 15.4 and 15.5 indicate that in control cells, phosphorylated IκBα was detectable after 5 min and remained evident up to 30 min after stimulation although at a reduced levels. In contrast, there was no significant increase in IκBα phosphorylation in C6/cA_{2A}AR cells. Together, these results indicate that A_{2A}AR expression can inhibit the phosphorylation and degradation of IκBα in response to TNFα and LPS. As both of these stimuli activate NF-κB *via* distinct signalling mechanisms (Sections 1.4.1; 1.4.4), A_{2A}AR expression must inhibit a component of the pathway that is common to both signalling cascades such as the IKK complex.

The ability of A_{2A}AR expression to efficiently suppress iNOS induction is therefore partly explained due to the inhibition of IκBα phosphorylation and degradation. However, as treatment with the NF-κB inhibitor PDTC, could attenuate only 55% of nitrite accumulation in C6 cells, other regulatory mechanisms must play a role in iNOS induction. One such mechanism is suggested by the presence of 5 GAS sites present on the rat iNOS promoter. GAS sites are targets for tyrosine phosphorylated dimeric STAT transcription factors, which are induced by a variety of cytokines, including IFNγ (Section 1.4.3). This suggested that the cA_{2A}AR could potentially inhibit iNOS induction by inhibiting IFNγ activation of the JAK-STAT signalling cascade. Therefore, to determine the effects of A_{2A}AR expression on STAT1 activation, Western blotting using anti-STAT1 and anti-phospho-Tyr^{701} STAT1 antibodies was performed on freshly prepared cell lysates from untransfected C6 and C6/cA_{2A}AR glioma cells. Figure 5.16 indicates that in response to IFNγ in untransfected C6 cells, there is a time-dependent increase in STAT1 phosphorylation that peaks at 60 min, which is markedly reduced in C6/cA_{2A}AR cells. In addition the overall levels of STAT1 protein are reduced at later time points in C6/cA_{2A}AR expressing cells indicating that A_{2A}AR activation may play a role in the degradation of STAT1, resulting in a decrease in transcriptional activity. Thus the decrease in iNOS expression is likely due to at least two separate A_{2A}AR-mediated effects; inhibition of IκBα phosphorylation resulting in a decreased NF-κB activation and degradation of STAT1.

One possible mechanism for the cA_{2A}AR-mediated anti-inflammatory response on both NF-κB and STAT1 is that activation of the A_{2A}AR causes the release of anti-inflammatory

factors, which could act in an autocrine or paracrine fashion to inhibit both NF-κB and STAT1 activation. To test this hypothesis, conditioned media from untransfected C6 glioma cells and C6/cA$_{2A}$AR cells were used to treat untransfected C6 cells in the presence and absence of LPS/TNFα/IFNγ. Figure 5.17 indicates that conditioned media from C6 or C6 cA$_{2A}$AR cells could not prevent the induction of iNOS in response to LPS/TNFα/IFNγ. This suggests that the A$_{2A}$AR-mediated effect is intracellular and does not involve the regulated release of any autocrine or paracrine anti-inflammatory cytokines.

These results collectively predict a model whereby A$_{2A}$AR expression can directly inhibit both the phosphorylation-dependent degradation of IκBα in response to TNFα and LPS. This in turn prevents the nuclear translocation of NF-κB, resulting in a decrease in iNOS induction and a subsequent reduction in the accumulation of nitrite in cell supernatants. In addition, A$_{2A}$AR expression can prime STAT1for INFγ-mediated degradation, which is also required for iNOS gene transcription.

5.3 Discussion

The anti-inflammatory effects of the A$_{2A}$AR have been widely demonstrated in a number of different cell types and in Chapter 4 one potential anti-inflammatory mechanism was identified. To determine if this mechanism was generally applicable among different cell types, C6 glioma cells stably expressing the A$_{2A}$AR, were investigated in a rodent model of glial cell inflammation. Glial cell inflammation contributes to the pathogenesis of several neurological disease states including multiple sclerosis (Bradl and Hohlfeld, 2003), stroke (Price *et al.*, 2003) and Alzheimer's disease (Akiyama *et al.*, 2000). Therefore, therapies that mediate any decrease in inflammation could prove beneficial in a wide range of neurological disease states.

Our results demonstrate that efficient cA$_{2A}$AR expression could inhibit the release of nitric oxide which correlated with the inhibition of iNOS expression. As the expression of iNOS is regulated by the transcription factor NF-κB, investigations were performed to determine if A$_{2A}$AR expression could modulate its transcriptional activity and activation. NF-κB reporter gene assays, EMSA analysis and nitrite accumulation assays in the presence of the NF-κB specific inhibitor PDTC, all indicate that cA$_{2A}$AR expression can inhibit DNA-binding and reporter gene activity upstream of gene expression. Further investigation of

the NF-κB signal transduction pathway, revealed that $cA_{2A}AR$ activity can inhibit the phosphorylation of IκBα which prevents the signal-induced degradation of IκBα, an important step in the activation of NF-κB by TNFα. In addition to the effects on NF-κB, $cA_{2A}AR$ activation can also decrease the phosphorylation of STAT resulting in an increased rate of STAT degradation. This suggests that $cA_{2A}AR$ activity can also inhibit STAT activity leading to the reduction in iNOS activity.

As phosphorylation of IκBα is an essential step in the activation of NF-κB one possible mechanism leading to the inhibition of IκBα phosphorylation is the $cA_{2A}AR$-dependent inhibition of the IKK complex. This could be mediated in several ways including inhibition of IKK activation by upstream kinases, post-translational modifications of IKK resulting in a decrease in activity or by reduction in IKK expression levels. Activation of the IKK complex leading to phosphorylation of IκBα occurs by phosphorylation of S176 and 180 in IKKα and S177 and S181 in IKKβ. However, studies in IKKα knockout mice reveal that in TNFα stimulation, IKKβ is the dominant kinase (Hu *et al.*, 1999). Several studies report a diverse array of upstream protein kinases that can phosphorylate IKK, including different PKC isoenzymes (Lallena *et al.*, 1999), NIK (Woronicz *et al.*, 1997), Protein Kinase B (PKB; Ozes *et al.*, 1999), MEKK1 (Lee *et al.*, 1998), MEKK3 (Yang *et al.*, 2001), COT/TPL-2 (Lin *et al.*, 1999) and TGF-β activating kinase (TAK1; Sakurai *et al.*, 1999). However, the precise kinase required for activation varies according to which NF-κB-activating stimulus is used for example, TNFα stimulation of IKK requires the MAP3K RIP1, however its catalytic activity is not required for this activation (Devin *et al.*, 2000) suggesting that $A_{2A}AR$ activity may inhibit RIP-IKK interaction preventing activation.

One further possible mechanism of $A_{2A}AR$-mediated inhibition of IKK activity is the activation of an IKK phosphatase. PP2Cβ is one such phosphatase which is reported to interact with IKKβ , however this interaction only occurs at later time points following TNFα stimulation and therefore more likely represents a mechanism to decrease IKK activity (Prajapati *et al.*, 2004). Furthermore, PP2A has also recently been identified as interacting with IKKγ. This interaction is reported to be responsible for the rapid deactivation of IKK and that inhibition of PP2A by the human T-lymphotropic retrovirus type-I trans-activator, Tax, leads to the constitutive activation of IKK and NF-κB activity.

Thus A_{2A}AR receptor activation may also stimulate PP2A activity resulting in decreased IKK activity (Fu *et al.*, 2003). One way to address the issue of A_{2A}-mediated inhibition of upstream kinase activity or phosphatase activation would be to use Western blotting with phospho-specific antibodies for each IKK subunit. This would allow detection of any defects in IKK phosphorylation.

In addition to the regulation of IKK activation by phosphorylation, IKK activity might also be controlled by further post-translational modification such as ubiquitination or sumoylation. Recent studies have identified that NEMO/IKKγ can be ubiquitinated in a manner which does not target it for degradation. This mechanism is directed by Bcl10, a common component of the pathway leading from T cell and B cell activation to NF-κB activation (Zhou *et al.*, 2004). In addition, TNFα stimulation also results in the ubiquitination of NEMO in its zinc finger domain by cellular inhibitor of apoptosis (cIAP; Tang *et al.*, 2003) in HeLa cells suggesting that this mechanism may play a role in different cell types. Ubiquitination of NEMO therefore likely plays a key role in the activation of NF-κB signalling by certain stimuli including TNFα. To further support a role for ubiquitination in the activation of IKK, the tumour suppressor protein CYLD, which contains deubiquitinating activity, was recently reported to interact with TRAF 2 and NEMO where it decreased IKK activity. Truncations of CYLD resulted in a marked decrease in enzyme activity and therefore restoration of IKK activity (Kovalenko *et al.*, 2003). Thus A_{2A}AR-activation could lead to the deubiquitination of NEMO resulting in a decrease in IKK activity. More recently, IKKγ/NEMO was reported to be subject to modification by sumoylation. Sumoylation is the attachment of SUMO (small ubiquitin like modifier) to target proteins which is thought to regulate protein stability by competition with ubiquitin thereby preventing proteolysis (Desterro *et al.*, 1998). It may also modulate protein-protein interactions to alter subcellular localization. Huang *et al.*, (2003) report that NEMO becomes sumoylated in response to genotoxic stress. This modification mediates nuclear translocation of 'free NEMO' into the nucleus, where it becomes ubiquitinated in an ATM-dependent manner leading to the activation of the IKK complex and NF-κB. Therefore SUMO-modification of the IKK complex may also occur in response to certain stimuli, however, this may not play a role in cytokine stimulated activation of NF-κB signalling.

The identification of 'free IKKγ/NEMO' suggests that one further $A_{2A}AR$ mediated mechanism of inhibition may be in the regulation of the individual IKK complex components. Since its initial characterization as a complex of ~900 kDa several investigators have tried to identify the total composition of the IKK complex. Stoichiometric analysis of reconstituted components *in vitro* and in yeast suggested a 1:1:2 stoichiometry of IKKα:IKKβ:IKKγ (Miller and Zandi, 2001). However recent observations suggest a tetrameric head-to-head arrangement of IKKγ in which each dimer can bind to IKKα/β (Tegethoff *et al.*, 2003). Recently it has been reported that IKKγ can constitutively shuttle between the cytoplasm and the nucleus where it interacts with the nuclear coactivator cAMP-responsive element-binding protein-binding protein (CBP; Verma *et al.*, 2004). Further investigation shows that IKKγ/NEMO competed with p65 and IKK alpha for binding to the N terminus of CBP, inhibiting CBP-dependent transcriptional activation by p65. Thus, regulation of the IKK component levels and there cellular localization may also alter IKK activity. In an analogous fashion, $A_{2A}AR$ activation leads to the phosphorylation of cAMP response element binding protein (CREB) *via* PKA, which can also inhibit NF-κB-dependent transcription by recruiting CBP thus preventing p65:CBP interactions (Parry and Mackman, 1997). However, in this study $A_{2A}AR$ inhibits p65–DNA binding by the inhibition of IκBα phosphorylation suggesting that p65 is unlikely to enter the nucleus, this further implies that co-activator competitive inhibition of NF-κB-dependent genes does not play a role in this model.

One final mechanism that might control the activity of the IKK complex is the interaction of the IKK complex with other proteins. Several proteins have been identified as possible negative regulators of IKK signalling in this manner. Hong *et al.*, (2001) using a yeast two hybrid system for IKKγ interacting proteins, identified a component of the COP9 signalosome, CSN3 as an inhibitor of TNFα-dependent signalling but not IL-1 signalling. In addition, Zetoune *et al.*, (2001) showed that A20, a NF-κB-inducible gene can effectively inhibit NF-κB activity by inhibiting IKKβ activity. Further yeast two hybrid analysis has also revealed the interaction of IKKγ with ZNF216, an A20-like protein which can bind to IKKγ, TRAF6 and RIP. This interaction negatively regulates activation of NF-κB at the level of the IKK as overexpression of p65 could restore NF-κB activity. Thus $A_{2A}AR$ activation may inhibit IKK activation by promoting the association of the IKK complex with negatively regulating proteins. Further investigation, for example by cross-

linking and immunoprecipitation studies, may shed more light upon the interaction of the IKK complex with other negatively regulating proteins.

The reduction by 50% of iNOS induction when exposed to the NF-κB inhibitor PDTC is in contrast to the total attenuation of iNOS expression in A_{2A}AR expressing cells, therefore it is evident that in addition to the cA_{2A}AR-mediated inhibition of the IκBα phosphorylation resulting in the decrease in iNOS activity, cA_{2A}AR activation must also inhibit iNOS induction by inhibiting other iNOS-inducing signaling pathways e.g. JAK-STAT. Results indicate that this effect is not due to the activation of JNK or p38 as A_{2A}AR expression had a little or no effect on the activation of either signaling cascade. In contrast, A_{2A}AR expression led to the more rapid degradation of STAT1 in the JAK-STAT signaling cascade. Cross-talk between cAMP-mediated signalling pathways and STAT activation has been previously reported (Sengupta *et al.*, 1996), and suggests that cA_{2A}AR mediated inhibition of STAT is cAMP-dependent. In addition, studies by Ivashkiv *et al.*, (1996) have shown that in T cell activation, cAMP can inhibit STAT1 activity by inhibiting DNA-binding and down-regulating the levels of STAT1 mRNA and protein. Thus it is likely that the slight inhibition of STAT phosphorylation and down-regulation of STAT1 protein levels is mediated by the A_{2A}AR-dependent activation of cAMP.

Thus, it is apparent that cA_{2A}AR activation can inhibit the induction of iNOS *via* two possible mechanisms. One mechanism involves the inhibition of IκBα phosphorylation by several possible mechanisms that are outlined above. The second mechanism involves the inhibition of STAT activity most likely *via* the generation of the second messenger cAMP. Both of these mechanism act to coordinately inhibit the expression of iNOS and the consequent synthesis of nitric oxide.

Figure 5.1 Comparison of human and canine A_{2A}AR primary sequence

The primary sequences of human and canine A_{2A}AR receptors are aligned opposite indicating specific residue differences between both receptors (blue). Note that most differences occur in the C-terminal tail.


CANINE 1 MSSMGSSVYITVELAIAVLAILGNVLVCWA
HUMAN 1 MSIMGSSVYITVELAIAVLAILGNVLVCWA
31 VWLNSNLQNVTNYFVVSLAAADIAVGVLAI
31 VWLNSNLQNVTNYFVVSLAAADIAVGVLAI
61 PFAITISTGFCAACHSCLFSACFVLVLTQS
61 PFAITISTGFCAACHGCLFIACFVLVLTQS
91 SIFSLLAIAIDRYIAIRIPLRYNGLVTGTR
91 SIFSLLAIAIDRYIAIRIPLRYNGLVTGTR
121 AKGIIAVCWVLSFAIGLTPMLGWNNCSQPK
121 AKGIIASCWVLSFAIGLTPMLGWNNCGQPR
151 EGSNSSQGCGEGQVACLFEDVVPMNYMVY
151 EGKNHSQGCGEGQVACLFEDVVPMNYMVYF
181 NFFAFVLVPLLLMLGVYLRIFLAARRQLKQ
181 NFFATVLVPLLLMLGVYLRIFLAARRQLKQ
211 MESQPLPGERARSTLQKEVHAAKSLAIIVG
211 MESQPLPGERARSTLQKEVHAAKSLAIIVG
241 LFALCWLPLHIINCFTFFCPSCSHAPLWLM
241 LFALCWLPLHIINCFTFFCPDCSHAPLWLM
271 YLSIVLSHTNSVVNPFIYAYRIREFRQTFR
271 YLATVLSHTNSVVNPFIYAYRIREFRQTFR
301 KIIRSHVLRSSEPFKAGGTSARALAAHGSD
301 KIIRSHVLRQQEPFKASGTSARSLAAHGSD
331 GEQSSLRLNGHPPGVWANGSAPHPERRPNG
331 GEQVSLRLNGHPPGVWANGSAPHPERRPNG
361 YSLGLVSGGIASESSGDSGLPDVELLSHEL
361 YALGLVSGGSAQESQGSTGLPDVELLSHEL
391 KGACPESPGLSGPLAQDGAGVS
391 KGSCPESPGLDSPLAQDGAGVS

Figure 5.2 Immunodetection of the canine A_{2A}AR in C6 glioma cells

Plain C6 glioma cells and C6 glioma cells stably transfected with the HA-epitope-tagged canine A_{2A}AR were plated out overnight in 6-well plates. The next day, cells were washed, solubilised, and then fresh lysates were prepared. Proteins were then equalised for protein concentration prior to fractionation by SDS-PAGE. Proteins were then transferred to nitrocellulose cells followed by immunoblotting with an anti-A_{2A}AR antibody, as described in Section 2.5.1 in the Materials and Methods. This Western blot represents one of multiple experiments.

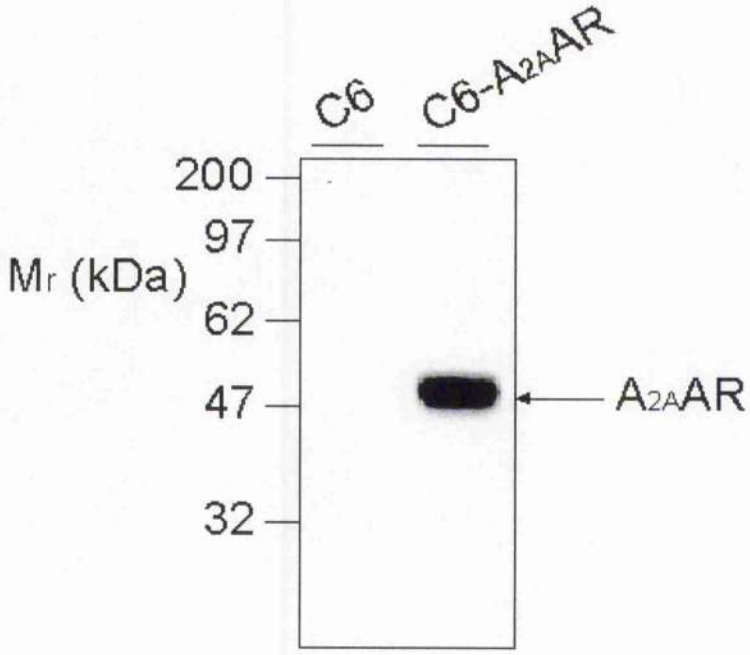

C6
C6-A2AAR
200
97
Mr (kDa)
62
47
32
A2AAR

Figure 5.3 Biotinylation assay of cell surface expressed canine A_{2A}ARs

Plain C6 glioma cells and C6 glioma cells stably expressing the HA-tagged-canine A_{2A}AR were plated into 6-well plates overnight. The next day, cells were labelled with biotin-LC-hydrazide according to the Methods in Section 2.5.3. Cell lysates were then prepared, equalised for protein content, and immunoprecipitated with anti-HA (12CA5). Immunoprecipitates were then fractionated by SDS-PAGE and transferred to nitrocellulose. Biotinylated A_{2A}ARs were detected by incubation with HRP-conjugated streptavidin as described in Section 2.5.3. Biotin-labelled proteins were then visualised using enhanced chemiluminescence. This represents one of several experiments.

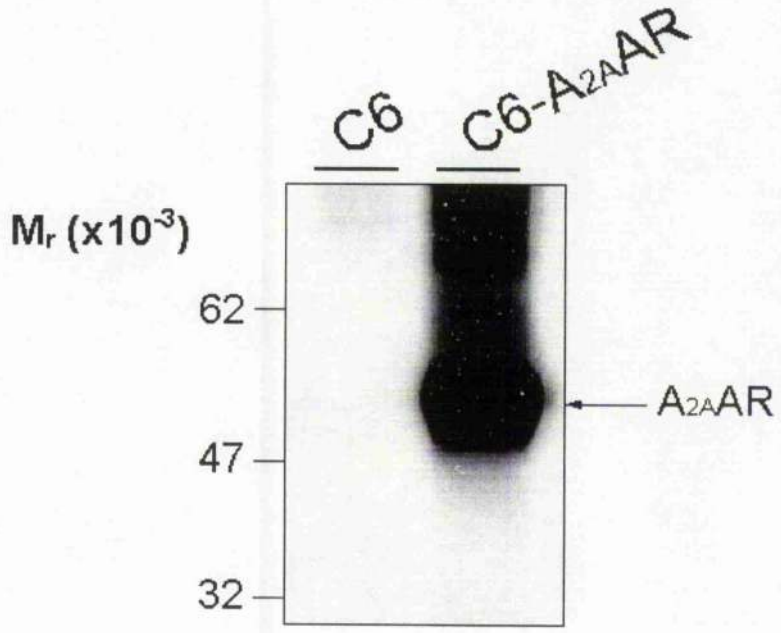

C6
C6-A2AAR
Mr (x10-3)
62
47
32
A2AAR

Figure 5.4 **^{125}I-ZM241385 saturation binding analysis for HA-tagged-canine A_{2A}ARs in C6 glioma cells**

Saturation binding studies were carried out on membranes isolated from plain C6 and C6/cA_{2A}AR glioma cells using the A_{2A}AR-selective antagonist ^{125}I-ZM241385 according to the protocol in Section 2.5.4 of the Materials and Methods. This experiment represents one of five different experiments.

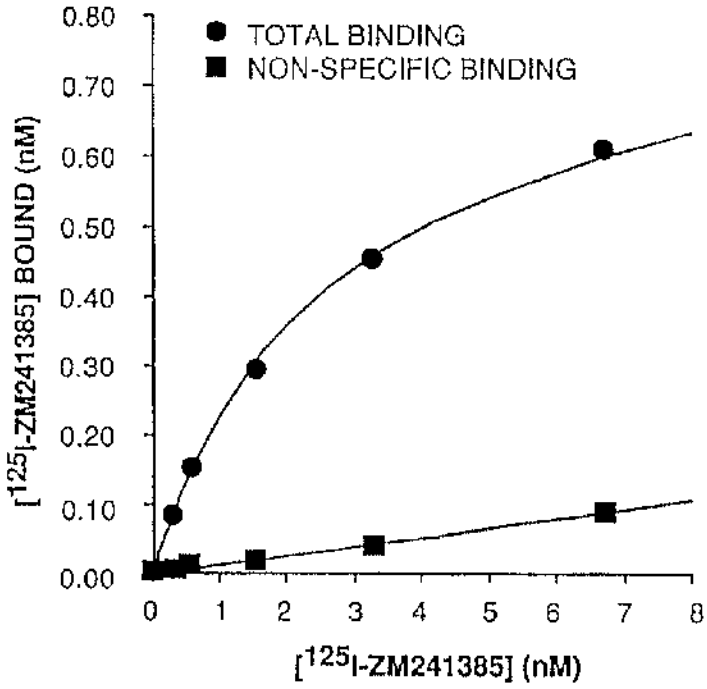

TOTAL BINDING
NON-SPECIFIC BINDING
[125I-ZM241385] BOUND (nM)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
1
2
3
4
5
6
7
8
[125I-ZM241385] (nM)

Figure 5.5 Effect of $A_{2A}AR$ expression on the pro-inflammatory stimuli-induced accumulation of nitrite in C6 glioma cells

C6/cA_{2A}AR and control glioma cells were plated out in 96-well plates overnight. The next day cells were treated with either 10 ng/ml TNFα (T), 1 μg/ml LPS (L) or 10 units/ml IFNγ (I) alone or in combination as indicated in the presence of 3 units/ml ADA for a further 24 hours. In addition, cells were also treated with the A_{2A}-selective neutral antagonist ZM241385 either alone (Z) or in combination with TNFα/LPS/IFNγ (ZM) Medium from the cells was then removed and analysed for nitrite accumulation as described in Section 2.5.12 of the Materials and Methods.

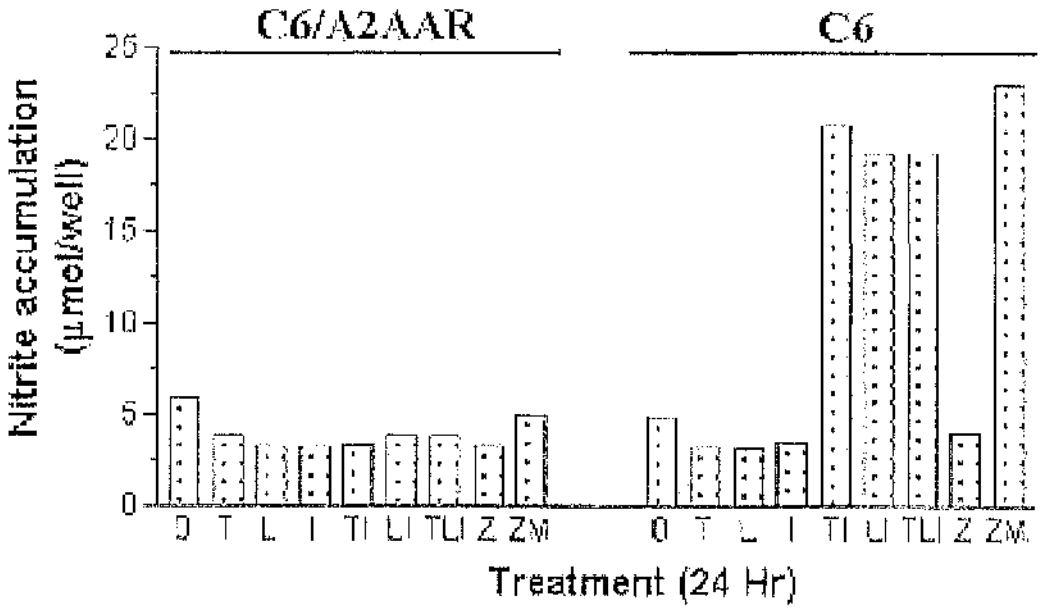

C6/A2AAR
C6
Nitrite accumulation (µmol/well)
25
20
15
10
5
0
D T L I TI LI TLI Z ZM
D T L I TI LI TLI Z ZM
Treatment (24 Hr)

Figure 5.6 **Effect of A_{2A}AR expression on pro-inflammatory stimuli-induced accumulation of nitrite over time**

Plain C6 glioma cells and HA-tagged cA_{2A}AR expressing C6 glioma cells were incubated in the presence of LPS (1 μg/ml)/TNFα (10 ng/ml)/ IFNγ (10 units/ml) for the times indicated. Cell supernatants were then removed and nitrite accumulation was determined using the Greiss reaction described in Section 2.5.12 of the Materials and Methods.

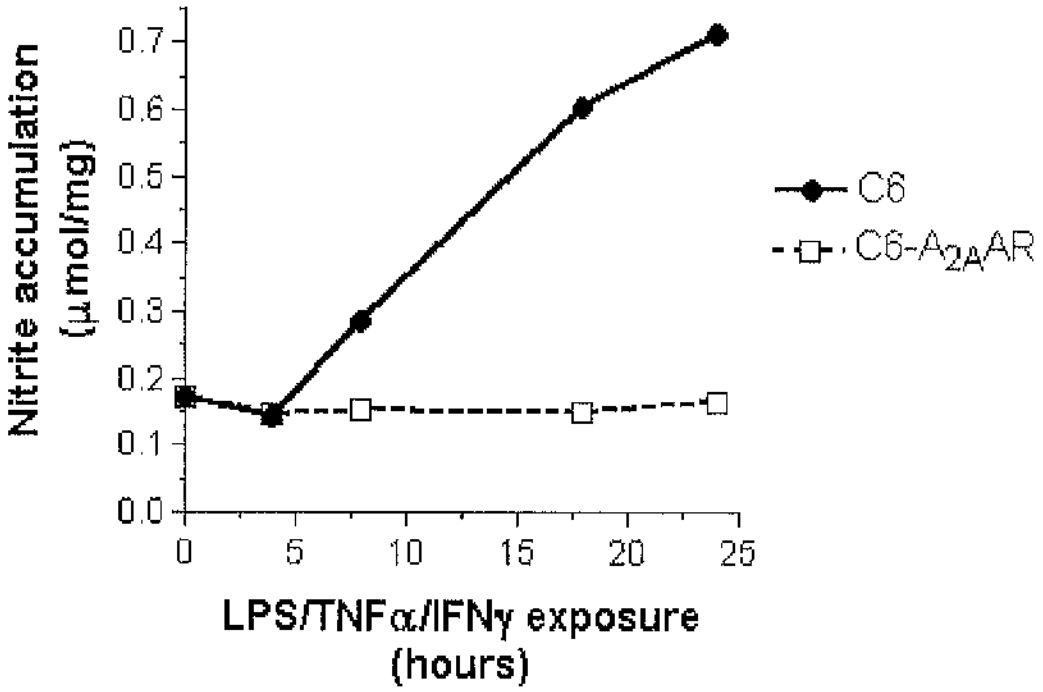

Nitrite accumulation
(μmol/mg)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
LPS/TNFα/IFNγ exposure
(hours)
C6
C6-A2AAR

Figure 5.7 **Effect of A_{2A}AR expression on nitrite accumulation in C6 glioma cells**

Plain C6 glioma cells and HA-tagged cA_{2A}AR expressing C6 glioma cells were incubated in the presence of 1 μg/ml LPS, 10 ng/ml TNFα and 10 units/ml IFNγ for 24 hr. Cell supernatants were then removed and nitrite accumulation was determined using the Greiss reaction described in Section 2.5.12 of the Materials and Methods.

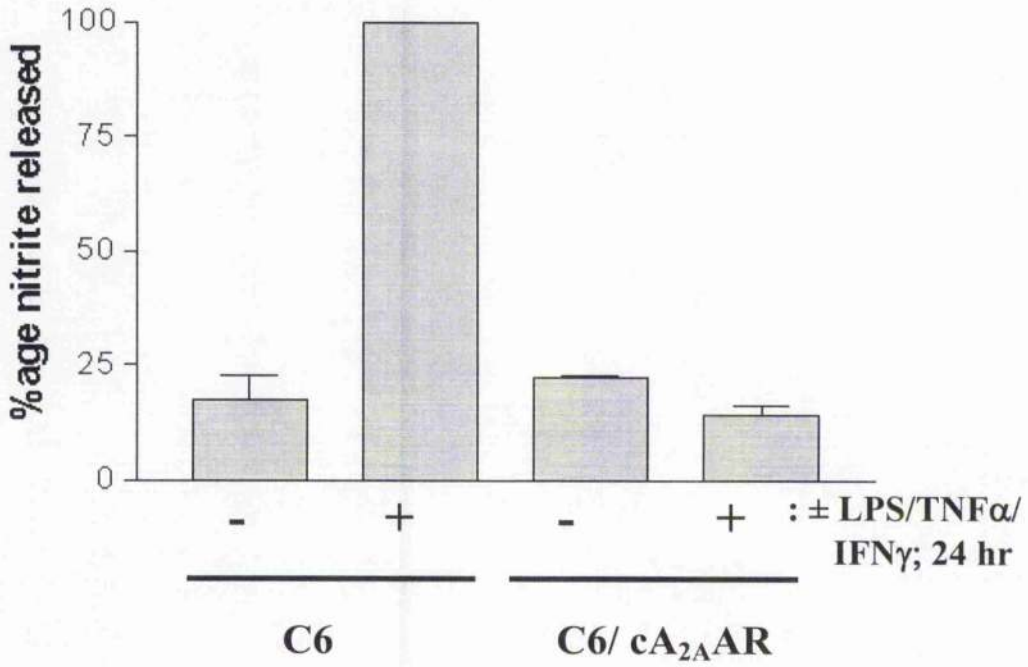

%age nitrite released
100
75
50
25
0
-
+
-
+
: ± LPS/TNFα/
IFNγ; 24 hr
C6
C6/ cA2AAR

Figure 5.8 Effect of A_{2A}AR expression on the pro-inflammatory stimuli-induced induction of iNOS

Plain C6 glioma cells and C6 cells stably expressing HA-tagged cA_{2A}ARs were treated for 24 hr with 1 μg/ml LPS, 10 ng/ml TNFα and 10 units/ml IFNγ. Prior to cytokine treatment, those cells indicated were pre-treated for 30 min with 5 μM NECA. Cell lysates were then subject to SDS-PAGE followed by immunoblotting with anti-A_{2A}AR and anti-iNOS antibodies as described in Section 2.5.1 of the Materials and Methods. This blot represents one of several experiments

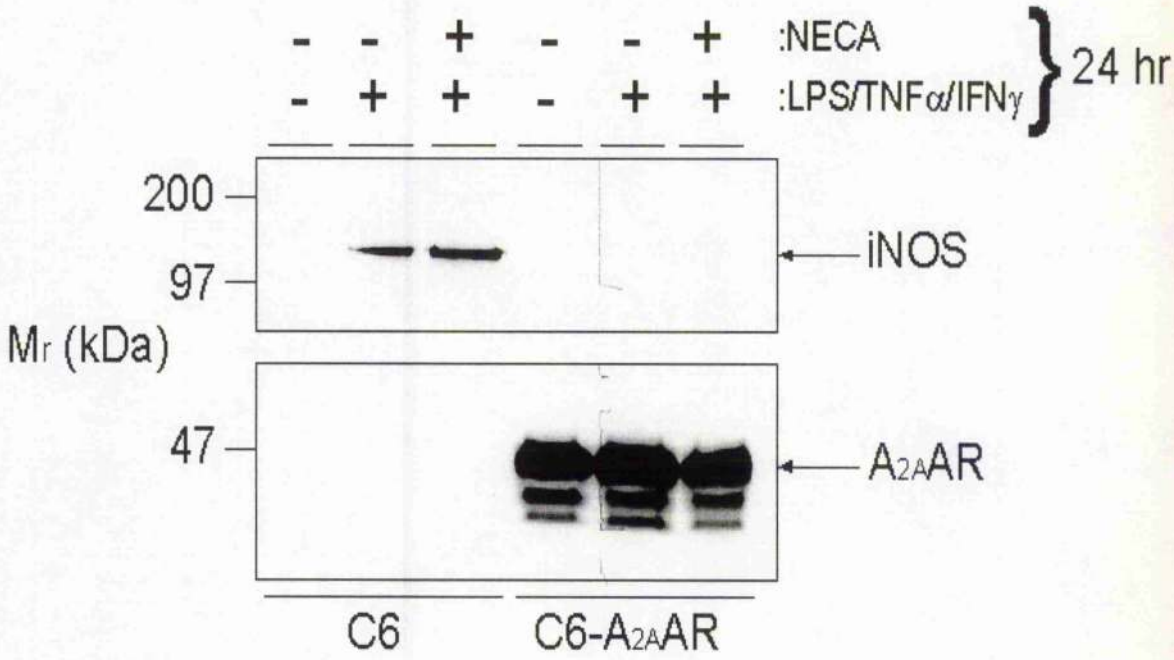

- - + - - + :NECA
- + + - + + :LPS/TNFα/IFNγ
24 hr
200
97
Mr (kDa)
47
iNOS
A2AAR
C6
C6-A2AAR

Figure 5.9 Effect of PDTC treatment on nitrite accumulation in HUVECs

Plain C6 glioma cells were pre-treated with or without 100 μM PDTC or 100 μM N-acetyl cysteine for 30 min prior to the addition of vehicle or 1 μg/ml LPS, 10 ng/ml TNFα and 10 units/ml IFNγ for a further 24 hr. Medium from treated cells was then removed and analysed for nitrite accumulation as described in Section 2.5.12 of the Materials and Methods.

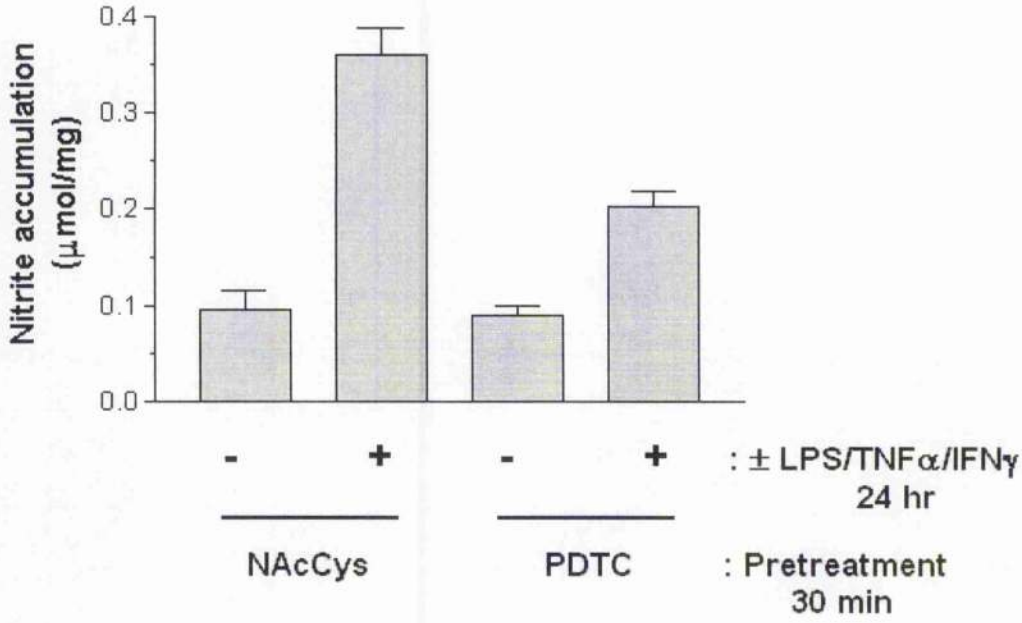

Nitrite accumulation
(μmol/mg)
0.4
0.3
0.2
0.1
0.0
- + - + : ± LPS/TNFα/IFNγ
24 hr
NAcCys
PDTC
: Pretreatment
30 min

Figure 5.10 Effect of A_{2A}AR expression on the DNA-binding activity of NF-κB

Plain C6 glioma cells and C6 cells stably expressing HA-tagged cA_{2A}ARs were incubated with 10ng/ml TNFα, 1 μg/ml LPS or 10 units/ml IFNγ for 6 hr. Nuclear extracts were then prepared and subject to analysis bc EMSA as described in Section 2.5.9 of the Materials and Methods. Thc idcntity of NF-κB subunits was determined by supershift analysis. This figure represents one of several experiments

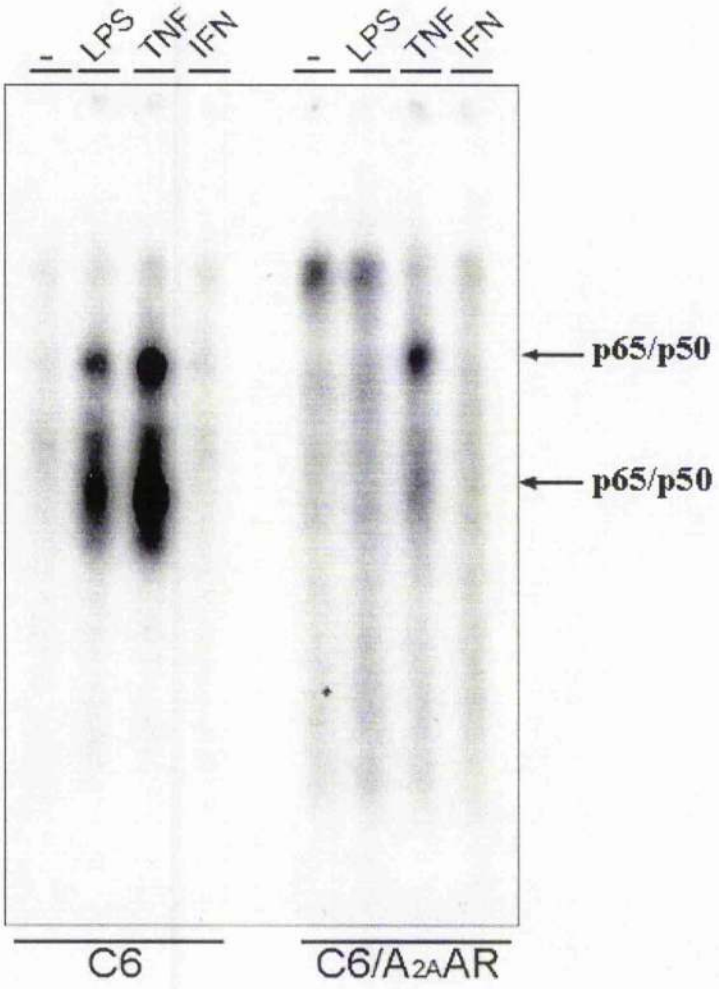

-
LPS
TNF
IFN
-
LPS
TNF
IFN
p65/p50
p65/p50
C6
C6/A2AAR

Figure 5.11 Effect of A_{2A}AR expression on TNFα-stimulated NF-κB reporter gene activity

$1x10^5$ plain C6 glioma cells and C6 cells stably expressing HA-tagged cA_{2A}ARs were transiently transfected with cDNA plasmids encoding for β-galactosidase and an NF-κB-luciferase reporter plasmid. Following stimulation with TNFα as indicated, cell lysates were prepared for luciferase analysis as described in Section 2.5.13 of the Materials and Methods. This experiment represents one of several experiments.

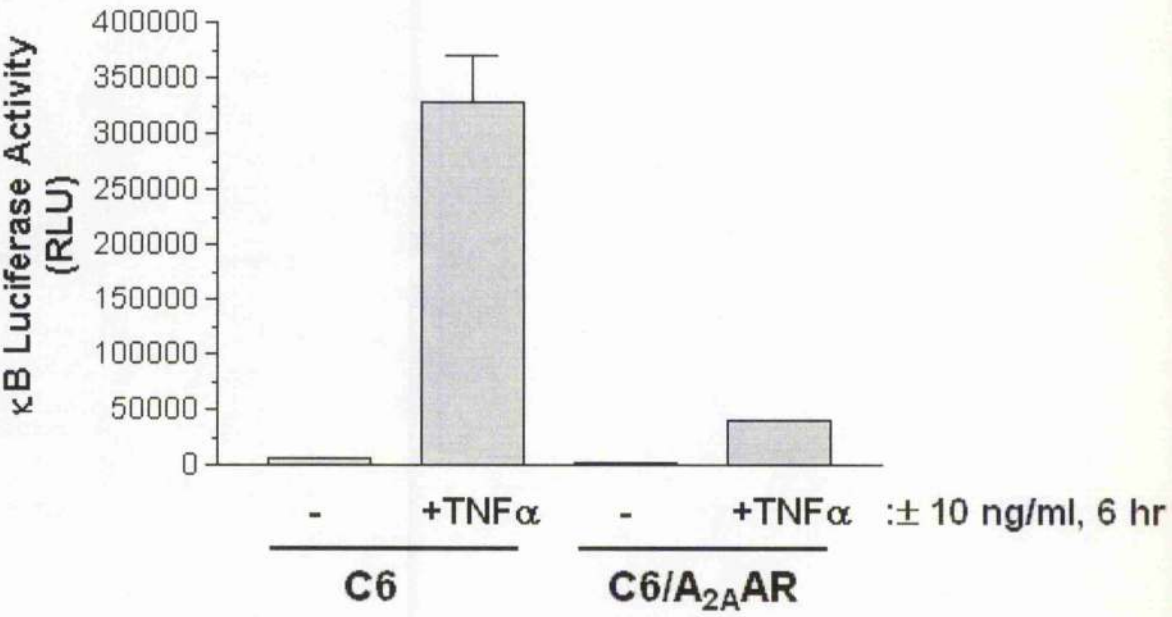

κB Luciferase Activity (RLU)
400000
350000
300000
250000
200000
150000
100000
50000
0
-
+TNFα
-
+TNFα
:± 10 ng/ml, 6 hr
C6
C6/A2AAR

Figure 5.12 Effect of A_{2A}AR expression on p38 MAPK activity

$1x10^6$ plain C6 glioma cells and C6 cells stably expressing HA-tagged cA_{2A}ARs were treated with 10ng/ml TNFα for the times indicated. Cells were also treated for 30 min with 5 μM anisomycin as a positive control (+) for p38 activation. Cell lysates were subject to SDS-PAGE followed by immunoblotting with anti-phospho-p38 antibodies as described in Section 2.5.1 of the Materials and Methods. This blot represents one of several experiments

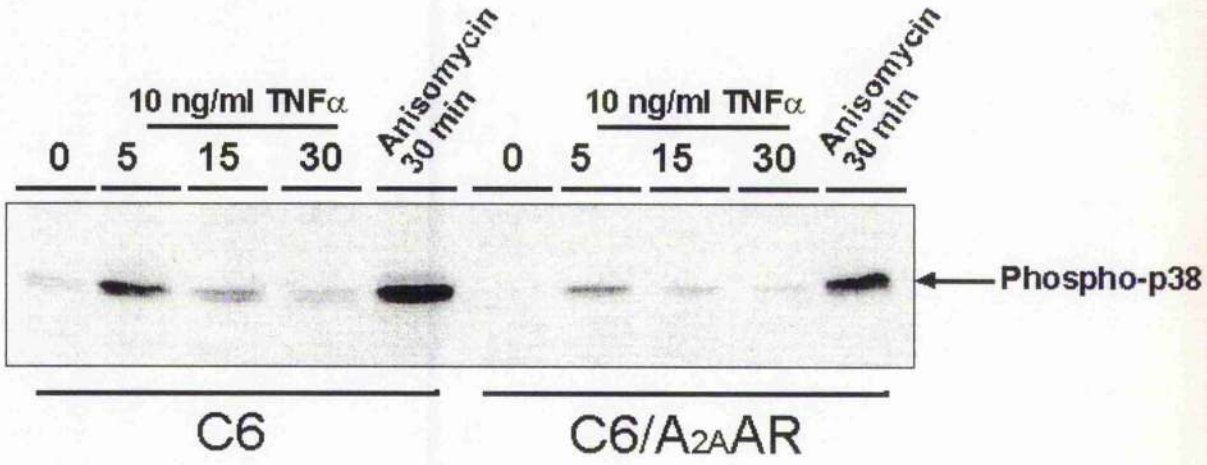

10 ng/ml TNFα
Anisomycin 30 min
10 ng/ml TNFα
Anisomycin 30 min
0
5
15
30
0
5
15
30
Phospho-p38
C6
C6/A2AAR

Figure 5.13 Effect of A_{2A}AR expression on JNK activity

$1x10^6$ plain C6 glioma cells and C6 glioma cells stably expressing HA-tagged cA_{2A}ARs were treated with 10ng/ml TNFα for the times indicated. Cells were also treated for 30 min with 5μM anisomycin as a positive control (+) for JNK activity. Cell lysates were then incubated with GST-c-jun (1-73) on glutathione-Sepharose beads and the precipitates used for *in vitro* kinase assays as described in Section 2.5.14 of the Materials and Methods. Following termination of the assay, samples were subject to SDS-PAGE followed by immunoblotting with anti-Ser^{63}phospho c-jun antibodies as described in Section 2.5.1 of the Materials and Methods. This blot represents one of several experiments.

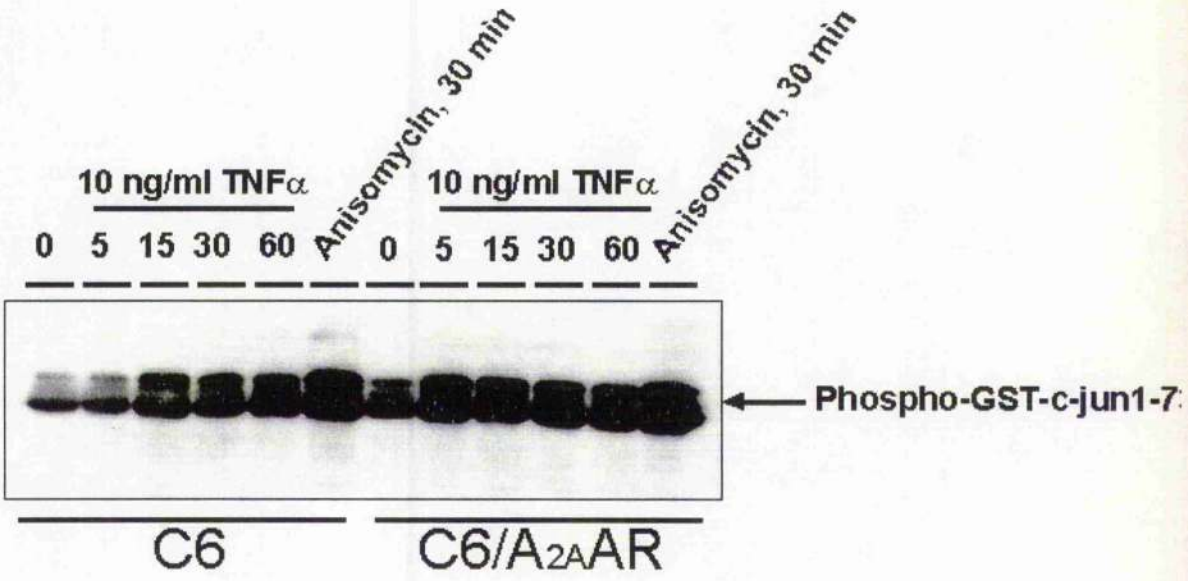

10 ng/ml TNFα
Anisomycin, 30 min
10 ng/ml TNFα
Anisomycin, 30 min
0 5 15 30 60 0 5 15 30 60
Phospho-GST-c-jun1-7
C6
C6/A2AAR

Figure 5.14 Effect of A_{2A}AR expression on TNFα-stimulated IκBα phosphorylation and degradation

$1x10^6$ plain C6 glioma cells and C6 cells stably expressing HA-tagged cA_{2A}ARs were incubated with 10ng/ml TNFα for the times indicated in min. Cell lysates were prepared and subject to SDS-PAGE followed by immunoblotting with anti-IκBα antibodies as described in Section 2.5.1 of the Materials and Methods. Blots were then stripped and re-probed for the presence of phospho-IκBα. This blot represents one of several experiments.

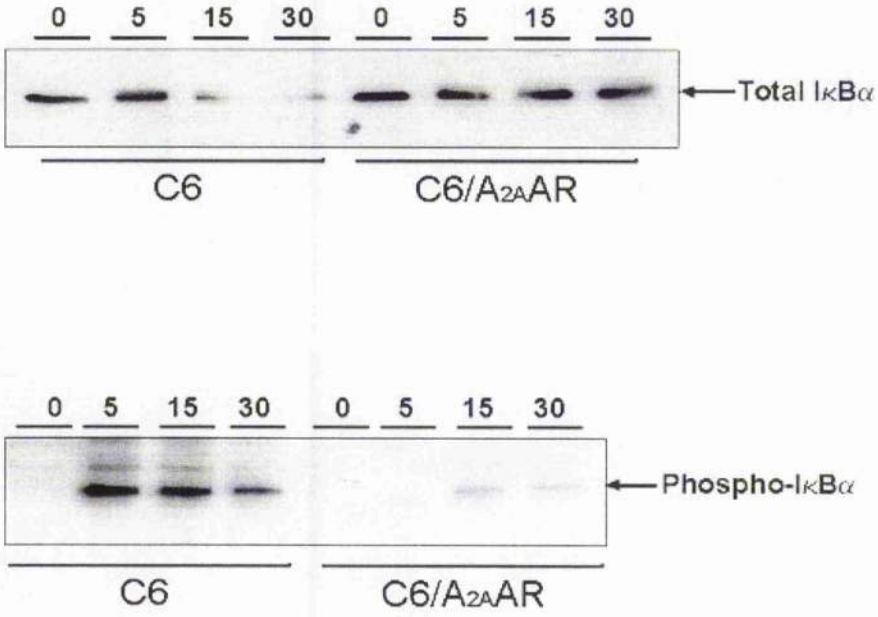

0
5
15
30
0
5
15
30
Total IκBα
C6
C6/A2AAR
0
5
15
30
0
5
15
30
Phospho-IκBα
C6
C6/A2AAR

Figure 5.15 Effect of A_{2A}AR expression on LPS-stimulated IκBα phosphorylation and degradation

$1x10^6$ plain C6 glioma cells and C6 cells stably expressing HA-tagged cA_{2A}ARs were incubated with LPS for the times indicated in min. Cell lysates were prepared and subject to SDS-PAGE followed by immunoblotting with anti-IκBα antibodies as described in Section 2.5.1 of the Materials and Methods. Blots were then stripped and re-probed for the presence of phospho-IκBα. This blot represents one of several experiments.

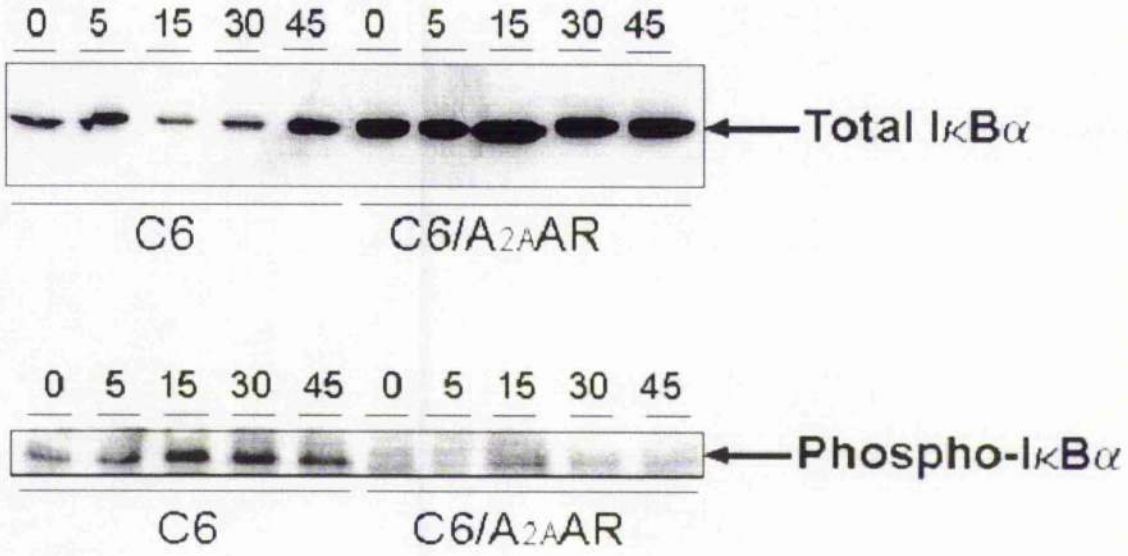

0 5 15 30 45 0 5 15 30 45
Total IκBα
C6
C6/A2AAR
0 5 15 30 45 0 5 15 30 45
Phospho-IκBα
C6
C6/A2AAR

Figure 5.16 Effect of $A_{2A}AR$ expression on IFNγ stimulated phosphorylation and degradation of STAT1

Confluent plain C6 and C6/cA_{2A}AR glioma cells were treated with 10ng/ml TNFα,1 μg/ml LPS or 10 units/ml IFNγ for the times indicated in min. Cells were then washed, solubilised, and fresh lysates were prepared. Proteins were then equalised for protein concentration prior to fractionation by SDS-PAGE. Proteins were then transferred to nitrocellulose cells followed by immunoblotting with STAT1 and anti-phospho STAT1 antibodies as described in Section 2.5.1 of the Materials and Methods.

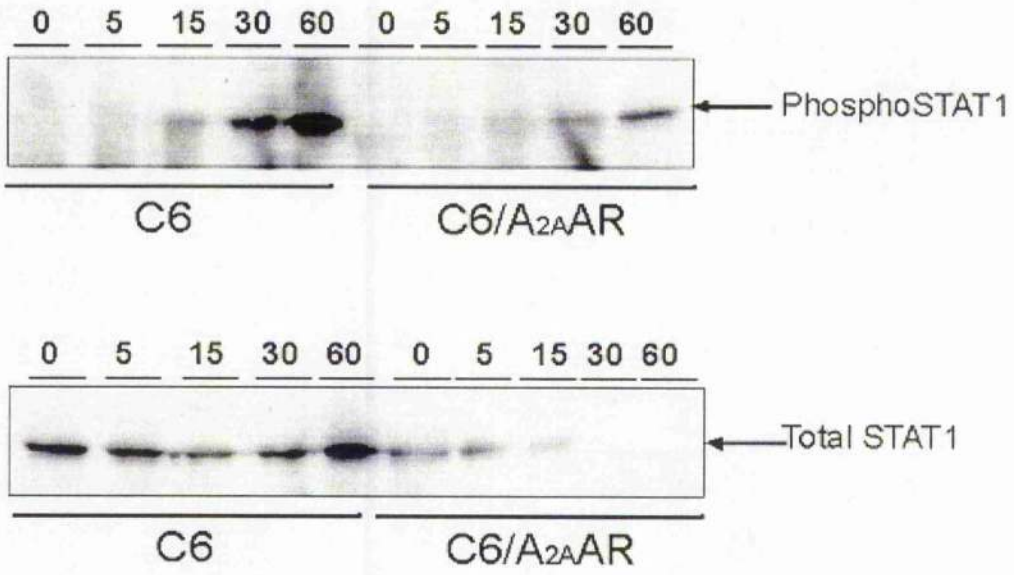
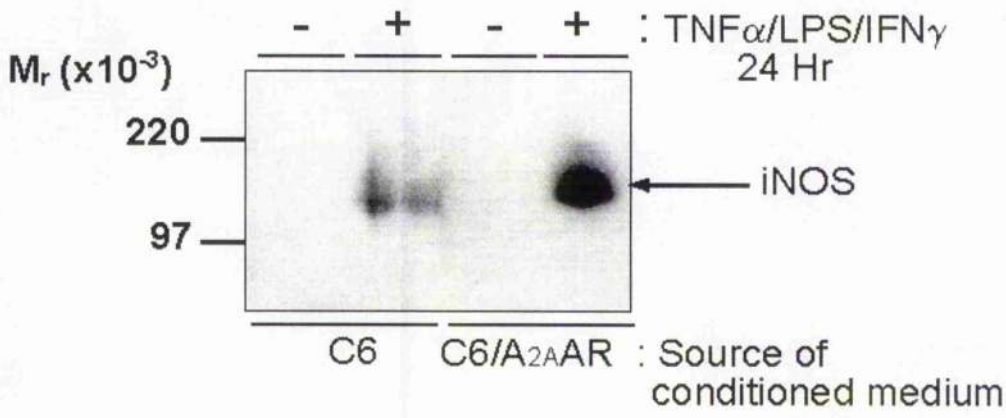

0 5 15 30 60 0 5 15 30 60
PhosphoSTAT1
C6
C6/A2AAR
0 5 15 30 60 0 5 15 30 60
Total STAT1
C6
C6/A2AAR

Figure 5.17 Effect of conditioned media upon iNOS induction in C6 glioma cells

Fresh medium was added to confluent dishes of plain C6 glioma cells and C6/cA$_{2A}$ARs. The next day, the medium was removed, supplemented with or without 10ng/ml TNFα, 1 μg/ml LPS and 10 ng/ml IFNγ and added to plain C6 glioma cells overnight. The following day cells were solubilised, equalised for protein concentration and fractionated by SDS-PAGE. Proteins were then transferred to nitrocellulose and immunoblotted with anti-iNOS antibodies as described in Section 2.5.1 of the Materials and Methods.


- + - + : TNFα/LPS/IFNγ
24 Hr
Mr (x10-3)
220
97
iNOS
C6
C6/A2AAR
: Source of
conditioned medium

Chapter 6
Final Discussion

Inflammation plays a key role in the pathogenesis of several disease states and consequently the recent demonstration that adenosine acting *via* the A_{2A}AR represents the natural 'brakes' of the immune system (Ohta and Sitkovsky, 2001), suggests that any therapy which can improve A_{2A}AR-dependent signalling could help resolve chronic inflammatory responses. However, the precise mechanism that underlies this important property of the A_{2A}AR is poorly understood. Results presented in this thesis have demonstrated that A_{2A}AR expression can inhibit the activation of the key inflammatory transcription factor NF-κB *via* at least two distinct cell-specific mechanisms. In HUVECs, A_{2A}AR expression can inhibit the translocation of p65 to the nucleus in an IκBα-degradation-independent manner. In contrast, A_{2A}AR expression in C6 glioma cells inhibits phosphorylation of IκBα preventing its degradation, thereby blocking NF-κB activation. In addition, A_{2A}AR expression also has NF-κB-independent anti-inflammatory effects *via* the degradation of STAT1 in C6 glioma cells. Thus, the A_{2A}AR represents a good anti-inflammatory therapeutic target that could inhibit excessive or inappropriate inflammation.

The key finding that A_{2A}AR expression can inhibit NF-κB activity raises several important issues. One important feature of this study is the distinct cell-specific mechanisms employed by the A_{2A}AR to inhibit NF-κB activity. In HUVECs, A_{2A}AR expression can inhibit the translocation of p65 to the nucleus independent of IκBα degradation. This suggests that under certain circumstances, an additional signal is required for the translocation of p65 into the nucleus. One possibility is phosphorylation, which has been reported by a number of investigators (Vermeulen *et al.*, 2002). It should be noted however, that phosphorylation is thought to direct the interaction of p65 with co-activator proteins such as CBP, consequently the role of phosphorylation in regulating p65 nuclear translocation is unclear at present. One further possibility is acetylation, which is also reported for p65. The precise role of acetylation is however unclear at present as acetylation of p65 at positions Lys^{221} and Lys^{310} is reported to enhance transactivation by the NF-κB complex (Chen *et al.*, 2002). In contrast, acetylation of Lys^{221} alone or in combination with Lys^{218} impairs the assembly of p65 and IκBα following gene activation (Chen *et al.*, 2002). The role of p65 acetylation is therefore more likely to determine transcriptional activation and turn-off rather than impair nuclear import (Kiernan *et al.*, 2003). Future studies using epitope-tagged p65 could be used to determine the addition of

any post-translational modification and the functional consequences of such a modification.

In contrast to HUVECs, the inhibition of NF-κB-dependent transcription in C6 cells occurs at the level of IκBα phosphorylation. As A_{2A}AR expression could inhibit IκBα phosphorylation when stimulated with LPS and TNFα, this suggests that A_{2A}AR expression can inhibit a common component of the pathway leading to IκBα phosphorylation. One such possibility is the IKK complex, which integrates the signals coming from both LPS/TLR4 signalling and TNFα signalling. The complex architecture of the IKK complex suggests that there are many possible ways to regulate the activity of the complex. One potential mechanism to inhibit the IKK complex is to inhibit one or more steps prior to its activation. IKK activation occurs by phosphorylation of residues Ser^{176} and Ser^{180} on IKKα and Ser^{177} and Ser^{181} of IKKβ (Section 1.7.2) although IKKα is not essential to cytokine induced activation of NF-κB. In contrast to the range of possible upstream kinases identified *in vitro*, MEKK3 is the only upstream kinase that can directly phosphorylate IKK following TNFα stimulation *in vivo* (Yang *et al.*, 2001). It has also been demonstrated that the catalytic activity of RIP is not required for the activation of IKK in response to TNFα (Devin *et al.*, 2000). This has led to the suggestion that IKK activation can occur by autophosphorylation by either IKKα or IKKβ (Ghosh and Karin, 2002). Therefore one possible way for the A_{2A}AR to inhibit IκBα phosphorylation would be to inhibit IKK activation by upstream activators.

In addition to the stimulus-induced activation by upstream activators, A_{2A}AR expression may also alter the components of the IKK complex leading to the suppression of IKK kinase activity. Recent studies indicate that IKKα and IKKγ may have extra roles within the nucleus in regulating NF-κB-dependent transcription. IKKα has been shown to translocate into the nucleus and lead to the phosphorylation-dependent acetylation of histone H3 at Ser^{10}, which is important in the activation of NF-κB responsive gene expression (Yamamoto *et al.*, 2003). The role of IKKγ is still relatively unclear however it is reported to translocate to the nucleus and compete with p65 for limiting amounts of the co-activator protein CBP, thereby leading to repression of p65-dependent transcription (Verma *et al.*, 2004). The extra-nuclear role of some of the IKK components implies that there may be dynamic interactions occurring between individual IKK members. This

suggests that one possible mechanism of inhibition could be the impairment of IKK assembly necessary for IKK kinase activity. To identify if this is the case, confocal laser scanning microscopy using differently–tagged fluorescent IKKβ and IKKγ might be employed to study the dynamic interactions between different proteins.

One issue not fully addressed in this thesis is if the A_{2A}AR-mediated effects on NF-κB p65/p50 heterodimers can also be found for other NF-κB subunit combinations. The specific subunit composition of NF-κB is thought to be one mechanism of determining the specificity of gene transcription for NF-κB responsive genes. Therefore it is possible that A_{2A}AR expression can only inhibit the expression of a particular subset of NF-κB responsive genes. To specifically identify which genes are regulated by which subunit, Kunsch *et al.*, (1992) identified preferred DNA-binding motifs specific for p50, Rel A and c-Rel homodimers. This specificity can be found *in vivo*, for example several genes have been reported to selectively bind Rel A homodimers (ICAM-1; Ledebur and Parks, 1995 and IL-4; Casolaro *et al.*, 1995). This degree of specificity may be relevant for other NF-κB-inducing stimuli, since in this investigation TNFα and LPS only activate p50/p65 heterodimers. Further analysis of different NF-κB-regulated genes induced by various stimuli using EMSA and reporter gene assays would be useful in assessing the ability of the A_{2A}AR to affect genes that are activated by different NF-κB subunits.

The multiple distinct mechanisms employed by the A_{2A}AR to inhibit NF-κB activity raise an important question, how does the A_{2A}AR affect NF-κB activity by differing mechanisms? One possibility is that A_{2A}AR expression leads to the activation of multiple distinct signalling molecules, which affect the NF-κB cascade by different mechanisms. One key feature of the A_{2A}AR is the long C-terminal tail, which plays a role in regulating the levels of constitutive activity of the receptor (Klinger *et al.*, 2002). In addition it is reported to be involved in the formation of heterodimers with the dopamine D_2 receptors within the rat striatum (Canals *et al.*, 2003), however it is not required for the formation of A_{2A}AR homodimers (Canals *et al.*, 2004), therefore its precise role in the formation of oligomers is still unclear at present. The presence of several proline rich domains within the receptor, may represent SH3 domains which are important for protein-protein interactions suggesting the activation of other SH3 domain containing proteins may be important in mediating the downstream effects.

The $A_{2A}AR$ couples predominantly to G_s although it is also reported to couple to G_{olf} and G_{16} (Offermanns and Simon, 1995; Kull *et al.*, 2000). G-protein coupling leads to the activation of adenylyl cyclase resulting in the generation of cAMP. cAMP has a clear role in the inhibition of STAT activity as previously described (Section 5.3) however, its precise role in the modulation of NF-κB-dependent responses is unclear. In addition to the inhibition of NF-κB-dependent responses *via* competition for limiting amounts of CBP between cAMP-stimulated phospho-CREB and p65 (Parry and Mackman, 1997), cAMP can also inhibit TNFα-stimulated phosphorylation of IκBα in human aortic endothelial cells pretreated with adiponectin (Ouchi *et al.*, 2000). Therefore, cAMP may regulate NF-κB-dependent responses by multiple mechanisms. In this investigation, $A_{2A}AR$ expression in C6 cells also inhibits the phosphorylation of IκBα suggesting that at least in this model, cAMP may play a part although this was not tested. In contrast, $A_{2A}AR$ expression in HUVECs inhibited the nuclear translocation of p65 independent of IκBα degradation suggesting that this mechanism represents a novel pathway for the inhibition of NF-κB-dependent gene transcription.

In addition to the activation of adenylyl cyclase leading to the generation of cAMP, $A_{2A}AR$ activation has also been reported to activate the pathway in a $p21^{ras}$-, MEK1-dependent manner independent of its ability to stimulate G_s in HUVECs. There are contrasting reports about the effect of ERK activation on NF-κB dependent gene expression. Overexpression of either MEK1 or ERK caused the constitutive nuclear translocation of NF-κB to the nucleus in a lymphoblastoid cell line suggesting that ERK would increase NF-κB-dependent gene transcription (Briant *et al.*, 1998). In addition, ERK is also reported to enhance NF-κB-dependent IL-6 expression in response to okadaic acid in human monocytes (Tuyt *et al.*, 1999). In contrast, it has also been reported that constitutive activation of the MEK-ERK pathway negatively regulates NF-κB dependent gene expression. However, this is due to the inhibition of phosphorylation of TATA-box binding protein (TBP), an essential component of the basal transcription machinery resulting in a reduced interaction with p65 (Carter and Hunninghake, 2000). Therefore, the precise role of ERK activity in the regulation of NF-κB-dependent gene transcription is still unclear. In the present study, PD98059 a MEK1 inhibitor failed to decrease the

expression of the E-selectin when treated with TNFα. This suggests that A_{2A}AR-mediated activation of ERK does not play any role in the NF-κB regulation of E-selectin expression.

A_{2A}AR activation has also been reported to activate PKCζ in rat pheochromocytoma cells leading to the down-regulation of VEGF expression although the precise mechanism from receptor to PKCζ is not precisely known (Gardner and Olah, 2003). PKCζ activity can also directly phosphorylate p65 at Ser^{311} in response to TNFα (Duran *et al.*, 2003). In addition, PKCζ activity is required for the transcription of matrix-metalloproteinase-9 in C6 glioma cells when treated with TNFα or IL-1 (Esteve *et al.*, 2002). Therefore it is possible that A_{2A}AR activation of PKCζ may have a role in regulating the activity of NF-κB. However, in this study IκBα was not degraded and therefore p65 would remain bound to the complex suggesting that A_{2A}AR expression does not mimic PKCζ in this fashion. However, future work using PKC inhibitors such as PKCζ pseudosubstrate could be used to clarify if PKC plays any role in the A_{2A}AR mediated response in C6 cells in this system.

The A_{2A}AR displays two desirable features that would make it suitable to act as a brake of the inflammatory response. One important feature is the level of constitutive activity displayed by the receptor, which is associated with the C-terminal tail of the receptor as its removal can inhibit the level of constitutive activity (Klinger *et al.*, 2002). The constitutive activity of the receptor would allow the receptor to mediate its anti-inflammatory effects even in the absence of agonist as described in this thesis. The constitutive activity of the receptor suggests that regulation of A_{2A}AR expression would be critical in the inhibition of inflammatory responses. Studies by Lukashev *et al* (2003) demonstrate that in $A_{2A}AR^{-/-}$ cells, there is no significant compensatory increase in the expression of any other AR suggesting that the anti-inflammatory events triggered by A_{2A}AR expression are very specific for this receptor. In addition, the demonstration by Armstrong *et al.*, (2001) that there is no receptor reserve in murine T-cells, suggests that A_{2A}AR expression is tightly regulated. The regulation of A_{2A}AR expression is supported by the reported alterations in expression in response to T_H1 cytokines (Nguyen *et al.*, 2003). In addition, in mature plasmacytoid dendritic cells, A_{2A}ARs become up-regulated in response to CpG oligodeoxynucleotide activation of TLR-9 leading to a decrease in IL-6, IL-12 and IFNα (Schnurr *et al.*, 2004) production. Therefore, there is precedent for the

regulation of $A_{2A}AR$ expression by different inflammatory stimuli resulting in the inhibition of inflammatory responses.

One final important issue raised in this thesis is the anti-inflammatory NF-κB independent effects of $A_{2A}AR$ expression. Results presented in this thesis suggest that in C6 cells, an NF-κB-independent mechanism accounts for ~50% of nitric oxide production in response to TNFα/LPS/IFNγ. However, $A_{2A}AR$ expression can completely attenuate the expression of iNOS suggesting other $A_{2A}AR$-mediated anti-inflammatory mechanisms regulate the expression of iNOS. Analysis of p38 and JNK activity, two important signalling cascades in inflammatory responses, reveals that $A_{2A}AR$ expression has minimal effects on either signalling mechanism, suggesting that neither plays a significant role in the $A_{2A}AR$-mediated NF-κB-independent attenuation of iNOS expression. In contrast, $A_{2A}AR$ expression leads to the accelerated degradation of STAT1. The JAK-STAT signalling cascade plays an important role in the activation of several different inflammatory genes including iNOS (Section 1.4.3). It is unclear at present how STAT1 degradation is accelerated although cAMP has been reported to inhibit STAT1 activation (Section 5.3; Sengupta *et al.*, 1996). In addition, STAT1 activation is negatively regulated by SOCS proteins. Thus, $A_{2A}AR$ expression may up-regulate SOCS expression leading to accelerated STAT1 degradation. SOCS proteins were originally identified as cytokine-inducible SH2-domain-containing proteins (CIS). They are known to inhibit STAT activation by at least two distinct mechanisms that differ between family members SOCS1 and SOCS3 (Fujimoto et al., 2003). Expression of SOCS1 but not SOCS3 promotes the degradation of ubiquitinylated JAK2 (Shuai and Liu, 2003) leading to the reduction of STAT activation. In addition, STAT1 is known to be regulated by phosphorylation-dependent polyubiquitination in response to IFNγ (Kim and Maniatis, 1996). Given that SOCS1 can promote the degradation of JAK2, it is also possible that SOCS1 can target the degradation of associated STAT1. Therefore $A_{2A}AR$ expression may negatively regulate STAT1 protein levels by increasing the levels of SOCS1 expression. To further identify the precise role for $A_{2A}AR$-mediated NF-κB-independent anti-inflammatory effects, immunoblotting using anti-SOCS antibodies could be used to determine the effects of the $A_{2A}AR$ on SOCS expression. In addition, it would be interesting to determine if this represented a common mechanism of action. For example, STAT activation is also stimulated by other cytokines like IL-6 and IL-11, which utilise a common gp130 receptor

component. Similar analysis of STAT1 activation and SOCS1 expression could be used to determine if $A_{2A}AR$ is important in this way.

While it is important to analyse the precise role of $A_{2A}AR$ regulation of NF-κB activity with respect to inflammation, NF-κB is also emerging as an important protein involved in cancer. This can partly be explained by its ability to regulate the expression of a number of genes which are involved in the regulation of apoptosis including cIAPs, caspase-8-FLICE inhibitory protein (c-FLIP), A20 and Bcl-X_L. The best studied NF-κB-induced anti-apoptotic proteins are the cIAPs. These proteins directly bind to effector caspases such as caspase-3 and caspase-7 as well as inhibiting the activation of pro-caspase-6 and caspase-9, which are activated in response to both intrinsic and extrinsic apoptotic pathways (Karin and Lin, 2002). Another example of an anti-apoptotic protein that is regulated by NF-κB is c-FLIP. This was first identified as a cellular homolog of viral FLIP (Thome *et al.*, 1997) and can inhibit apoptosis by directly binding to FADD and pro-caspase-8 *via* the DED (Irmler *et al.*, 1997). Thus inhibition of NF-κB by $A_{2A}AR$ activation would also possibly sensitise cells to apoptosis. Sensitisation to apoptosis would be of great benefit in treating tumours, however it could also lead to undesirable side effects such as premature apoptosis of immune cells leading to an immunocompromised situation. However, it is important to note that NF-κB has recently been reported to actively repress anti-apoptotic gene expression when activated by UV-C and chemotherapeutic drugs such as daunorubicin. This inhibition is mediated by the association of p65 with HDAC and therefore indicates that NF-κB can act both as a transcriptional activator or repressor dependent upon the manner in which it is induced (Campbell *et al.*, 2004).

In conclusion, adenosine released in the cellular response to stress, acting at the $A_{2A}AR$ can dramatically inhibit the pro-inflammatory response of NF-κB *via* the activation of a distinct signalling mechanism, which can inhibit the phosphorylation of IκBα and the nuclear translocation of NF-κB. This leads to the decrease in monocyte recruitment to sites of inflammation and therefore to a reduced leukocyte infiltration. This suggests that the $A_{2A}AR$ may represent a good therapeutic target for the inhibition of chronic inflammation *in vivo*. Further investigation is required however to determine where exactly within the NF-κB signalling pathway the $A_{2A}AR$-mediated response has its effect. Results presented in this study suggest that this occurs at least in two places, i.e. at the

level of nuclear translocation and at the level of IKK activation. The differences may be cell-type specific or stimulus dependent and therefore a rigorous investigation of the A_{2A}AR-mediated effects in further cell-types and with the same stimuli might help clarify the role of the A_{2A}AR. Further investigations may also centre upon if this effect is specific to a particular Rel family member and which specific genes are therefore affected. Finally it will be essential to understand what the functional consequences of this are, not only with respect to inflammation but also with respect to apoptosis as this could potentially lead to severe side effects for any potential therapy.

Appendix I

Calculation of adenovirus titre

Readings from plate

Virus dilution	No. of positive wells
10-4	10/10
10-6	10/10
10-7	10/10
10-8	9/10
10-9	3/10
10-10	0/10
10-11	0/10

In order to calculate the titre, the above results are fitted into the equation below:

The proportionate distance =

$$\frac{\%\text{ positive above }50\% - 50\%}{\%\text{positive above }50\% - \%\text{ positive below }50\%}$$

$$\therefore \frac{90\% - 50\%}{90\% - 30\%} = 0.67$$

Now log ID_{50} (infectivity dose) =

Log dilution above 50% + (proportionate distance x –1) x dilution factor

$\therefore$ log $ID_{50} = -8 + (0.67 \times -1) = -8.76$

$ID_{50} = 10^{-8.76}$

Now $TCID_{50}$ (Tissue culture infectivity dose 50) = $\frac{1}{10^{-8.76}}$

$\therefore TCID_{50} = 10^{8.76}$ / 100 µl x dilution factor (10)

$\therefore TCID_{50}/ml = 10^{9.86}$

$= 4.67 \times 10^{9}$

Now 1 TCID50 ≡ 0.7 pfu $\therefore$ final titre = $0.7 \times 4.67 \times 10^{9}$

$= 3.27 \times 10^{9}$ pfu/ml

Appendix 2

Sample calculation for B_{max} :

B_{max} = 0.2102 nM

Since Molarity = $\frac{\text{Moles}}{\text{Volume}}$

$\therefore$ Moles = Molarity (M) x Volume (L)

$\therefore$ Moles = $(0.2102 \times 10^{-9}) \times (250 \times 10^{-6})$

= 52.55 fmol

Actual protein conc. = $\frac{1.303 \text{ mg/ml (from protein assay)}}{14.7 \quad \text{(Dilution factor)}}$

= 88.6 µg/ml

Since 0.15 ml of membrane prep. was added per tube this makes total protein concentration per tube:

= 88.6 mg/ml x 0.15 ml/tube

= **13.29 µg protein/tube**

$\therefore$ 52.55 fmol in 13.29 µg of protein = $\frac{52.55}{13.29}$

= 3.95 fmol/µg (≅ pmol/ mg)

Appendix 3

The Cheng-Prusoff Equation

$$K_i = \frac{EC_{50}}{1 + \frac{[Ligand]}{K_d}}$$

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