The A_{2A} Adenosine Receptor: its role in suppressing vascular inflammation and its regulation by phosphorylation

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Abstract

Endothelial inflammation leading to vascular dysfunction is a major contributor to the development of atherosclerosis. The release of adenosine at sites of inflammation represents an endogenous mechanism for limiting excessive inflammation and tissue damage. The majority of the anti-inflammatory effects of adenosine are mediated by signalling through the A_{2A}AR and activation of the A_{2A}AR has been shown to be protective in numerous models of inflammatory disease. Little is known about the molecular mechanisms behind these effects. However, *in vitro* studies using cultured endothelial cells indicate that signalling through the A_{2A}AR can suppress activation of the NFκB and JAK/STAT proinflammatory signalling pathways. NFκB appears to be primed for activation in atherosclerosis-prone regions of the aorta indicating that suppression of NFκB signalling may protect against the development of atherosclerosis. In this study, the role of the A_{2A}AR in regulating NFκB and JAK/STAT signalling pathway activation in the aorta was studied using A_{2A}AR-deficient mice subjected to an LPS-induced model of septic shock. In response to LPS treatment, these mice displayed markedly elevated plasma levels of the pro-inflammatory cytokines TNFα, IL-6, IL-1β and GMCSF compared to wild-type mice. Consistent with this finding, heightened activation of the NFκB and JAK/STAT pathways was detected in aortic protein samples from A_{2A}AR-deficient mice as demonstrated by increased levels of the phosphorylated forms of IκBα and STAT1. However, expression of the NFκB and STAT1-regulated genes ICAM-1, VCAM-1 and TAP-1 was unaffected indicating the involvement of compensatory negative feedback mechanisms. These findings confirm a role for the A_{2A}AR in regulation of pro-inflammatory signalling in the aorta. Further analysis of mechanisms which mediate this response may reveal new targets for therapeutic intervention to suppress inflammation in inflammatory disorders such as atherosclerosis.

While the wide range of anti-inflammatory and tissue-protective responses elicited by the A_{2A}AR have been well documented, the molecular regulation of the A_{2A}AR has been less well studied. The presence of several serine and threonine residues in the extended C-terminal tail of the A_{2A}AR suggests that it may be regulated by phosphorylation events occurring in this region. Indeed, the canine A_{2A}AR is phosphorylated in response to PKC activation. Interestingly, several proteins have recently been identified as being able to interact with the C-terminal tail of the A_{2A}AR. However, how these interactions are regulated is not known. One of the aims of this study was to characterise phosphorylation

of the human A_{2A}AR and to determine whether this could provide a means for regulating the binding of C-terminal interacting proteins. This was examined using human umbilical vein endothelial cells infected with recombinant adenovirus encoding the human A_{2A}AR. It was found that phosphorylation of the human A_{2A}AR could be induced in HUVECs by treatment with PMA or by stimulation of endogenous histamine H1 receptors. This was due to activation of PKC, as phosphorylation was inhibited by the PKC inhibitor GF109203X and by depletion of PKC following chronic treatment with PMA. Treatment of cells with the calcium-chelating agent BAPTA/AM did not inhibit PMA-induced phosphorylation indicating that a calcium-insensitive isoform of PKC was responsible. Meanwhile an siRNA-mediated gene silencing approach confirmed that PKCε was not responsible indicating the involvement of either PKCδ or PKCθ. Previously reported interactions between the A_{2A}AR and TRAX and 14-3-3τ were confirmed *in vitro* by GST pull-down assay. Binding of 14-3-3τ to the A_{2A}AR appeared to be unaffected by treatment of HUVECs with PMA. However, A_{2A}AR complex formation with TRAX was significantly reduced in samples from PMA-stimulated cells. These findings indicate that PKC-mediated phosphorylation may represent a means of regulating which proteins can interact with the C-terminal tail of the A_{2A}AR. This may allow the A_{2A}AR to initiate distinct signalling pathways depending on the cellular context in order to achieve the appropriate response.

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Declaration

I hereby declare that the thesis which follows is my own composition, that it is a record of the work done myself, except where otherwise acknowledged, and that it has not been presented in any previous application for a Higher Degree.

Gillian Ruth Milne

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Abbreviations

β_2AR	β2 adrenergic receptor
A_{2A}AR	A_{2A} adenosine receptor
A_{2A}AR $^{-/-}$	A_{2A}AR-deficient
AC	Adenylyl cyclase
adA_{2A}AR	Adenovirus encoding the A_{2A}AR
adGFP	Adenovirus encoding GFP
ARF	ADP-ribosylation factor
ARNO	ARF nucleotide site opener
ATII	Angiotensin II
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CBP	CREB-binding protein
CHO	Chinese hamster ovary
CIS	Cytokine-inducible SH2 protein
CK1α	Casein kinase 1α
CK2	Casein kinase 2
CREB	cAMP-response-element-binding protein

CRP	C-reactive protein
DAB	Diaminobenzidine
DD	Death domain
DMEM	Dulbecco’s modified Eagle’s medium
DTT	Dithiothreitol
DUB	Deubiquitinating
EBM	Endothelial basal medium
ECL	Enhanced chemiluminescence
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
EVD	Enabled Vasp homology domains
FBS	Fetal bovine serum
GMCSF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GRK	G protein-coupled receptor kinase
HEK	Human embryonic kidney
HRP	Horseradish peroxidise
HUVEC	Human umbilical vein endothelial cell

ICAM-1	Intercellular adhesion molecule-1
IFNGR	IFNγ receptor
IKK	IκB kinase
IL-	Interleukin
IL-1R	Interleukin-1 receptor
IL-6Rα	IL-6 α receptor
iNKT	Invariant NK T cell
iNOS	Inducible nitric oxide synthase
IP3	Inositol-1,4,5-trisphosphate
IPTG	Isopropyl β-thiogalalactopyranoside
IRAK	IL-1R-associated kinase
IRF9	Interferon regulatory factor 9
IRI	Ischaemia reperfusion injury
ISRE	IFNα/β-response element
JAK	Janus kinase
JH	JAK homology domain
KIR	Kinase inhibitory region
LFA-1	Leukocyte function-associated antigen

LPS	Lipopolysaccahride
MCP-1	Macrophage chemotactic protein-1
MCSF	Macrophage colony-stimulating factor
mLDL	Modified low-density lipoprotein
myc-hA_{2A}AR	myc-tagged human A_{2A}AR
MyD88	Myeloid differentiation gene 88
NEMO	NFκB essential modifier
NES	Nuclear export sequence
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NHE3	Na^+/H^+ exchanger 3
NHERF	Na^+/H^+ exchanger regulatory factor
NK	Natural killer
NLS	Nuclear localisation sequence
NO	Nitric oxide
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PECAM-1	Platelet endothelial cell adhesion molecule-1

PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein inhibitors of activated STATs
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PMA	Phorbol 12-myristate 13-acetate
PSD	Postsynaptic density protein
PSGL-1	P-selectin glycoprotein-1
PTH1	Parathyroid hormone
PTP	Protein tyrosine phosphatases
RGS	Regulator of G-protein signalling
RHD	Rel homology domain
RIP	Receptor interacting protein
RT	Room temperature
SCF	Skp1-Cullin-F-Box
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src homology 2

sIL-6Rα	Soluble IL-6Rα
siRNA	Short interfering RNA
SLIM	STAT-interacting LIM protein
SOCS	Suppressors of cytokine signalling
SODD	Silencer of death domain
SOS	Son of sevenless
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
TAK1	Transforming growth factor β-activated kinase-1
TAP-1	Transporter of antigenic peptides 1
TIR	Toll/IL-1R motif
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptor
TM	Transmembrane domain
TNF-α	Tumour necrosis factor-α
TRAF	TNFR-associated factor
TRADD	TNFR-associated death domain protein
TRAM	TRIF-related adaptor molecule
TRAX	Translin-associated protein X

TRIF	TIR-containing adaptor inducing IFNβ
TRP	Transient receptor potential channel
UCH	Ubiquitin-carboxy-terminal-hydrolase domain
USP	Ubiquitin-specific protease
VCAM-1	Vascular cell adhesion molecule-1
VLA–4	Very late antigen-4

1 Introduction

1.1 Vascular inflammation

1.1.1 Inflammation

The process of inflammation is the immediate response of tissues to cellular injury or infection and is essential for the maintenance of tissue homeostasis and mediation of immune responses. Clinically, inflammation is characterised by heat, pain, swelling and redness, symptoms caused by increased blood flow to the affected area, leakage of fluid into tissues and the accumulation of activated leukocytes. The actions of cytotoxic lymphocytes and inflammatory mediators serve to remove pathogens and damaged tissues, clearing the way for healing and restoration of function (Murphy *et al.*, 2008).

Inflammation is initiated on activation of the innate immune system by microbes or damaged cells. Macrophages and neutrophils have germline-encoded cell surface receptors that recognise patterns of molecules that are common to many pathogens and the products released from damaged or dying cells. Receptor ligation triggers phagocytosis and induces changes in gene expression such as an increase in production of cytokines and other inflammatory mediators which recruit and activate neutrophils and other lymphocytes (Han and Ulevitch, 2005). Inflammation can also be triggered through activation of the complement cascade as the first component, C1q, can interact directly with the surface of certain pathogens. Complement is a series of proteases which act sequentially to produce fragments that are involved in clearing pathogens either through opsonisation or by direct lysis. The cleavage events also produce fragments including C3a, C4a and C5a which are inflammatory mediators (Tomlinson, 1993).

1.1.2 The role of the vascular endothelium

A crucial site in the development of the inflammatory response is the vascular endothelium, a single-celled layer that forms the lining of all blood vessels (Hurairah and Ferro, 2004; Michiels, 2003). In one capacity the vascular endothelium acts as a barrier between blood and neighbouring tissues, allowing the exchange of nutrients while in normal conditions, limiting the passage of blood cells and plasma proteins. In addition, the endothelium plays a critical regulatory role in many processes required for vascular

homeostasis including maintenance of vascular tone, humoral coagulation, angiogenesis and inflammation (Hurairah and Ferro, 2004; Michiels, 2003).

Endothelial cells of the microvasculature are the site of some of the first events in the inflammatory response (Muller, 2003). On activation by pathogens or damaged cells in tissues, mast cells, macrophages and neutrophils produce cytokines such as interleukin (IL) -1, IL-6 and tumour necrosis factor-α (TNFα) and other inflammatory mediators such as histamine and bradykinin which act on local blood vessels to stimulate vasodilatation, increase vessel wall permeability and activate the endothelium to express cell adhesion molecules. These bind reciprocal molecules on circulating lymphocytes, causing them to adhere to the endothelium before migrating to the site of injury attracted by an increasing concentration gradient of chemotactic cytokines such as macrophage chemotactic protein (MCP-1;McEver, 2001). As well as these local effects, cytokines produced by macrophages and neutrophils stimulate endothelial and fibroblast cells to trigger a secondary wave of cytokine secretion. These act systemically to activate the "acute phase response" with results such as induction of fever and changes in levels of proteins secreted by the liver such as C-reactive protein (CRP), complement proteins and fibrinogen which contribute to non-specific defences (Baumann and Gauldie, 1994).

1.1.3 Activation of the endothelium and leukocyte recruitment

The migration of leukocytes from the vascular system to a site of injury or infection is a key event in the process of inflammation. Inflammatory mediators including the cytokines, IL-1, IL-6 and tumour necrosis factor-α, play a central role in recruiting large numbers of neutrophils and monocytes to sites of infection and initiating innate and adaptive immune responses (Murphy *et al.*, 2008). The process of leukocyte recruitment follows a series of well-characterised steps which result in their adhesion to the endothelium, extravasation and migration into tissues. In response to inflammatory mediators, local small blood vessels become dilated which increases blood flow and slows the course of circulating lymphocytes, allowing them to make contacts with vascular endothelial cells before adhering to and crossing the endothelium (Muller, 2003; figure 1.1).

The endothelium is not normally adhesive and so for interactions between cells to occur, it must be activated to express adhesion molecules. The first to appear are the selectins

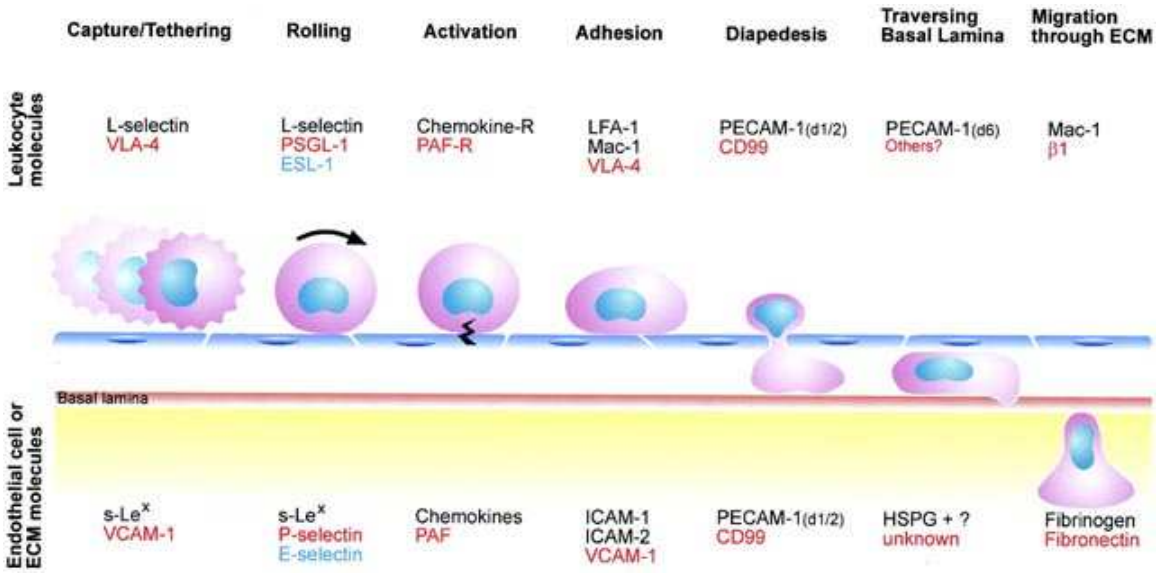

Figure 1.1 Recruitment of leukocytes to sites of inflammation

The process of leukocyte recruitment follows a series of well-characterised steps which result in their adhesion to the endothelium, extravasation and migration. Initially, leukocytes roll along the endothelium as a result of weak and reversible interactions between P-selectin and E-selectin on endothelium and sulphated sialyl-Lewis^x moieties of leukocyte glycoproteins such as P-selectin glycoprotein-1 (PSGL-1). Chemokines such as macrophage chemotactic protein-1 (MCP-1) and IL-8 then activate β-integrins including leukocyte function associated antigen (LFA-1) and Mac-1 on leukocytes, which allows them to make firmer contacts with receptors such as intracellular adhesion molecule-1 (ICAM-1) on the endothelium. Following adhesion, leukocytes transmigrate out of the blood vessel between or through endothelial cells with the help of other molecules such as platelet-endothelial-cell adhesion molecule-1 (PECAM-1) and CD99. In order to cross the basement membrane, leukocytes secrete matrix metalloproteinase enzymes and migrate following an increasing gradient of chemokines secreted by cells at the site of infection. (Figure from Muller, 2002)

(Carlos and Harlan, 1994). In response to mediators such as histamine (Geng *et al.*, 1990), P-selectin is rapidly translocated from intracellular Weibel-Palade bodies to the cell membrane while stimuli such as lipopolysaccahride (LPS), IL-1 and TNF-α subsequently induce the expression of E-selectin with maximal effect after 3-4 hours (Bevilacqua *et al.*, 1987). Selectins interact with sulphated sialyl-Lewis^x moieties of certain leukocyte glycoproteins, the major ligand for P- and L-selectin being P-selectin glycoprotein-1 (PSGL-1; McEver, 2001). This interaction is weak and reversible and so leukocytes appear to roll along the endothelium, pulled by the shearing force of the blood. This increases their probability of coming into contact with chemokines immobilised on the endothelial cell surface (Jung *et al.*, 1998). Leukocytes express β-integrins, for example, leukocyte function associated antigen (LFA-1) and Mac-1, which in response to chemokines such as IL-8 and macrophage chemotactic protein-1 (MCP-1), undergo a conformational change which increases their affinity for receptors such as intercellular adhesion molecule-1 (ICAM-1) on the endothelium (Harris *et al.*, 2000). Rolling is arrested and leukocytes are able to transmigrate out of the blood vessel between or through endothelial cells with the help of other molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1) and CD99 (Muller, 2003). In order to cross the basement membrane, leukocytes secrete matrix metalloproteinase enzymes and migrate following an increasing gradient of chemokines secreted by cells at the site of infection (McIntyre *et al.*, 2003).

1.2 The vascular endothelium and disease

Under normal conditions, the endothelium exhibits an anti-inflammatory, anti-coagulatory, anti-thrombotic state (Hurairah and Ferro, 2004; Michiels, 2003). This ensures that inflammatory responses are short-lived, resulting in the containment of infection and elimination of pathogens followed by reversion of the endothelium back to its resting phenotype. However, in certain circumstances, the endothelium becomes dysfunctional and the balance between the potentially damaging effects of the inflammatory response and the protective mechanisms exhibited by the endothelium is lost. In this way, endothelial inflammation contributes to the progression of numerous diseases including atherosclerosis (Anderson *et al.*, 1995), sepsis (Zimmerman *et al.*, 1999) and systemic lupus erythematosus (D'Cruz, 1998).

1.2.1 Vascular dysfunction

The normal, healthy endothelium regulates vascular homeostasis through maintenance of vascular tone and control of humoral coagulation, platelet function, smooth muscle growth and leukocyte invasion (Trepels *et al.*, 2006). Maintenance of vascular tone is achieved through synthesis and release of a balance of vasodilatory substances, such as nitric oxide (NO) and prostacyclin, and vasoconstrictory substances such as endothelin-1 (ET-1) and angiotensin II (ATII; Hurairah and Ferro, 2004). In addition to their roles in controlling vascular tone, many of the vasodilators produced also play protective roles while the vasoconstrictors often promote inflammatory and atherogenic responses. Therefore, any damage to the endothelium which alters the balance of these mediators results in vascular dysfunction. Vascular dysfunction is clinically defined as impairment of endothelium-dependent vasodilation but is also characterised by conversion of the endothelium to an "activated" phenotype associated with increased endothelial permeability and leukocyte adhesion and production of pro-inflammatory cytokines (Davignon and Ganz, 2004; Anderson, 1999). Many of these effects are due to the loss of NO activity. NO is the major vasodilatory substance in the endothelium. However, it also has other protective roles in inhibiting inflammatory responses. For example, NO opposes the actions of ATII to suppress adhesion molecule expression and inhibit leukocyte adhesion (Nabah *et al.*, 2005). In addition, NO works synergistically with prostacyclin to inhibit platelet aggregation (de Graaf *et al.*, 1992) and suppresses proliferation of smooth muscle cells (Garg and Hassid, 1989). Vascular dysfunction leads to the development of a pro-inflammatory, pro-thrombotic environment within the vascular system. Therefore, it is perhaps not surprising that vascular dysfunction is strongly implicated in the development of vascular diseases and in particular, atherosclerosis (Davignon and Ganz, 2004; Landemesser *et al.*, 2004; Anderson, 1999).

1.2.2 Atherosclerosis

Atherosclerosis can be considered to be a chronic inflammatory disease, characterised by the accumulation of macrophages, smooth muscle cells and lymphocytes in the arterial wall in response to pro-inflammatory stimuli. This process leads to the development of lipid-rich lesions known as atherosclerotic plaques. Over time, these plaques may evolve to occlude the artery lumen or alternatively they may rupture, triggering thrombosis which is often followed by myocardial infarction or stroke (Langheinrich and Bohle, 2005; Glass and Witzum, 2001).

Vascular dysfunction is generally accepted as the main predisposing factor towards atherosclerosis and is detected prior to the appearance of clinical symptoms (Anderson, 1999). Numerous different stimuli may activate the endothelium during the early stages of vascular dysfunction including modified low-density lipoproteins (mLDL), viruses, bacterial pathogens and free radicals. Initiation of atherosclerotic plaque formation is characterised by the infiltration of LDL into the artery wall and its modification through oxidation or enzymatic attack (Stoll and Bendszus, 2006). Macrophages recruited to the activated endothelium take up mLDL via toll-like receptors (TLRs) and scavenger receptors (Stoll and Bendszus, 2006). This initially serves a protective role. However, with continued accumulation of LDL, macrophages develop into lipid-lain foam cells and contribute to the formation of fatty streaks in the vessel wall which precede the development of atherosclerotic plaques (Stary *et al.*, 1994). The progression to a mature plaque occurs as a result of the immigration of smooth muscle cells into the subendothelial space. Here, smooth muscle cells may proliferate and take up modified lipoproteins to contribute to foam cell formation while they also secrete extracellular matrix proteins which leads to the production of a fibrous cap over the lesion (Hansson, 2005; Glass and Witzum, 2001).

Inflammatory processes are intricately involved at every stage of plaque development. This is perhaps not surprising as, of the numerous stimuli which may activate the endothelium in the early stages of vascular dysfunction, many have the capacity to activate the nuclear factor κB (NFκB) pathway which is the major signalling pathway involved in transcriptional control of inflammatory genes (de Winther *et al.*, 2005). In addition, the activated endothelium produces pro-inflammatory cytokines such as TNFα, IL-1 and IL-6. These are all NFκB-regulated gene products while TNFα and IL-1 also activate the NFκB pathway, thereby amplifying the inflammatory response. Another example of an NFκB-regulated gene is vascular cell adhesion molecule-1 (VCAM-1) which is believed to be the major adhesion molecule responsible for adhesion of monocytes to the endothelium during the early stages of lesion formation. In mouse models of atherosclerosis, VCAM-1 expression is upregulated specifically in areas prone to lesion formation (Cybulsky and Gimbrone, 1991). Furthermore, atherosclerosis-prone mice deficient in VCAM-1 expression have been found to display reduced lesion formation compared to mice expressing normal levels of VCAM-1 (Cybulsky *et al.*, 2001). Similarly, the adhesion molecules P-selectin and ICAM-1 have been shown to be important in mouse models of atherosclerosis at later stages of monocyte recruitment (Collins *et al.*, 2000; Cybulsky *et*

al., 2001). Following attachment to the endothelium, monocytes exit the vessel lumen by diapedesis, attracted by chemokines such as MCP-1, which is detected at elevated levels in both human and animal atherosclerotic lesions (Yla-Herttuala *et al.*, 1991). This is a crucial step in the development of atherosclerosis. Once resident in the vessel wall, monocytes are induced to differentiate to macrophages by macrophage colony-stimulating factor (MCSF) and granulocyte macrophage colony-stimulating factor (GMCSF). The roles of these cytokines in atherosclerosis progression is complex as demonstrated by the fact that administration and deficiency have been found to have similar effects in different studies (Hamilton, 2008). This is perhaps due to the nature of the macrophage-mediated response in that clearance of lipids by activated macrophages is initially protective but at the same time leads to foam cell formation, thereby promoting lesion formation. Activated macrophages also release pro-inflammatory cytokines that amplify local inflammation in the lesion. In addition to macrophages, other immune cells such as mast cells, dendritic cells and T cells are involved in the development of the mature plaque and interactions between all of these cell types contribute to the development of a chronic inflammatory state (Hansson, 2005; Glass and Witzum, 2001). T cells found in atherosclerotic plaques are generally $CD4^+$ T cells which are activated by antigens including mLDL presented by antigen presenting cells such as macrophages and dendritic cells. Cytokines expressed within the lesion, for example IL-12, promote differentiation into Th1 cells which produce the macrophage-activating cytokine IFNγ (Hansson, 2005).

As discussed above, many of the inflammatory mediators and adhesion molecules involved in development of atherosclerosis are regulated by signalling through the NFκB pathway which is activated either by exogenous stimuli or by the pro-inflammatory cytokines IL-1 and TNFα. However, many are also targets of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. The majority of cytokines signal through the JAK/STAT pathway to mediate effects on gene transcription that can be either pro- or anti-inflammatory. In the context of atherosclerosis, the pro-inflammatory role of IL-6 has been most extensively studied due to substantial evidence indicating its involvement in the disease process. For example, elevated levels of IL-6 and one of its target gene products, C-reactive protein (CRP), are associated with the increased risk of cardiovascular disease and events such as myocardial infarction (Tzoulaki *et al.*, 2005; Ridker *et al.*, 2000). In addition, both IL-6 and CRP have been detected in atherosclerotic lesions in humans and in animal models (Torzewski *et al.*, 2000; Sukovich *et al.*, 1998; Ikeda *et al.*, 1992). IL-6 is released by macrophages in the first steps of acute inflammation (Naka *et al.*, 2002) and at later stages of atherosclerotic plaque development by endothelial cells and smooth muscle

cells (Hansson, 2005). The endothelium is largely unresponsive to IL-6 in normal circumstances because it only expresses the gp130 subunit of the IL-6 receptor whilst initiation of IL-6 signalling requires the presence of both gp130 and an additional component termed the IL-6 α receptor (IL-6Rα; Kallen, 2002). However, a soluble form of the IL-6Rα, shed by neighbouring monocytes or macrophages, can bind IL-6 and form a complex with gp130 on endothelial cells to allow them to respond (Marin *et al.*, 2001). By similar means, cultured endothelial cells have been found to respond to a combination of IL-6 and sIL-6Rα resulting in the upregulation of VCAM-1, ICAM-1 and E-selectin and release of the chemokines MCP-1 and IL-8, indicating a potential role for IL-6 in promoting leukocyte recruitment during atherogenesis (Modur *et al.*, 1997; Romano *et al.*, 1997). IL-6 also has effects on monocytes and macrophages, which express both gp130 and the IL-6Rα, including stimulating their differentiation from monocytes to macrophages (Chomarat *et al.*, 2000). In addition, IL-6 stimulates smooth muscle cells both directly and by upregulating gp130 expression, thereby increasing their responsiveness to IL-6 (Klouche *et al.*, 1999). This induces smooth muscle cell proliferation, upregulates expression of ICAM-1 and MCP-1 and promotes foam cell formation (Klouche *et al.*, 1999), providing further evidence of the pro-atherogenic properties of IL-6.

1.3 Pro-inflammatory signalling

The process of atherogenesis is a complex multi-step process involving many different cell types which, directed by numerous pro-inflammatory mediators, contribute to the progression of disease. Traditionally, therapies for atherosclerosis have targeted hypercholesterolaemia owing to the central role of mLDL in initiating lesion formation. However, the recognition of atherosclerosis as an inflammatory disease indicates that modulation of inflammatory processes may provide a more useful means of limiting disease progression. As described above, many key molecules involved in the critical steps of lesion formation are regulated by two major pro-inflammatory signalling pathways: the NFκB pathway and the JAK/STAT pathway. Therefore, studying these signalling pathways and the mechanisms which regulate them will provide insights into the specific effector mechanisms which contribute to the development of atherosclerosis and allow identification of potential targets for novel therapies.

1.3.1 The NFκB pathway

NFκB is the collective name for a family of inducible, dimeric transcription factors found in the cytoplasm of most cell types. In resting cells, NFκB is retained in an inactive state through association with a family of inhibitory proteins known as IκBs which prevent its translocation to the nucleus (figure 1.2). NFκB activation occurs in response to a wide variety of stimuli including pro-inflammatory cytokines such as TNFα and IL-1, bacterial antigens such as LPS, viral proteins, double-stranded RNA and physical and chemical stresses (Karin and Ben-Neriah, 2000). These stimuli ligate a variety of receptors to initiate signalling pathways which converge on the IκB kinase complex (IKK). On activation, IKK phosphorylates IκB on two specific serine residues. Phosphorylated IκB is then recognised and ubiquitinated by members of the Skp1-Cullin-F-box (SCF) family of E3 ubiquitin ligases and targeted for degradation by the proteasome. This frees NFκB to enter the nucleus and bind to the promoter or enhancer regions of specific target genes (Hayden and Ghosh, 2004; Karin and Ben-Neriah, 2000).

1.3.1.1 NFκB

NFκB represents a family of structurally-related proteins which exist in resting cells as homo- or heterodimers bound to the inhibitory protein IκB. There are five members of this family in mammals: RelA (p65), RelB, NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100) and c-Rel. All of these proteins share an N-terminal 300 amino acid conserved region known as the Rel homology domain (RHD) which mediates their DNA-binding, dimerisation and interaction with IκB. A nuclear localisation sequence (NLS) is also contained within the RHD (Ghosh *et al.*, 1998; Verma *et al.*, 1995). Dimerisation is required for DNA binding and numerous combinations of Rel proteins have been described. These exert different effects on transcription of target genes by binding to κB sites with the consensus sequence GGGRNNYYCC (where R is purine and Y is pyrimidine) with different affinities (Ghosh *et al.*, 1998; Verma *et al.*, 1995). p65/RelA, RelB and c-Rel have transcriptional activation domains (TAD) which are required for transcriptional activity (Blair *et al.*, 1994; Rysek *et al.*, 1992; Schmitz *et al.*, 1994). However, dimers consisting only of Rel proteins that lack TADs may act to suppress transcription. For example, p50/p50 homodimers have been found to suppress expression of NFκB-regulated genes in unstimulated cells by binding histone deacetylase complexes which silence transcription (Zhong *et al.*, 2002). The most abundant and the

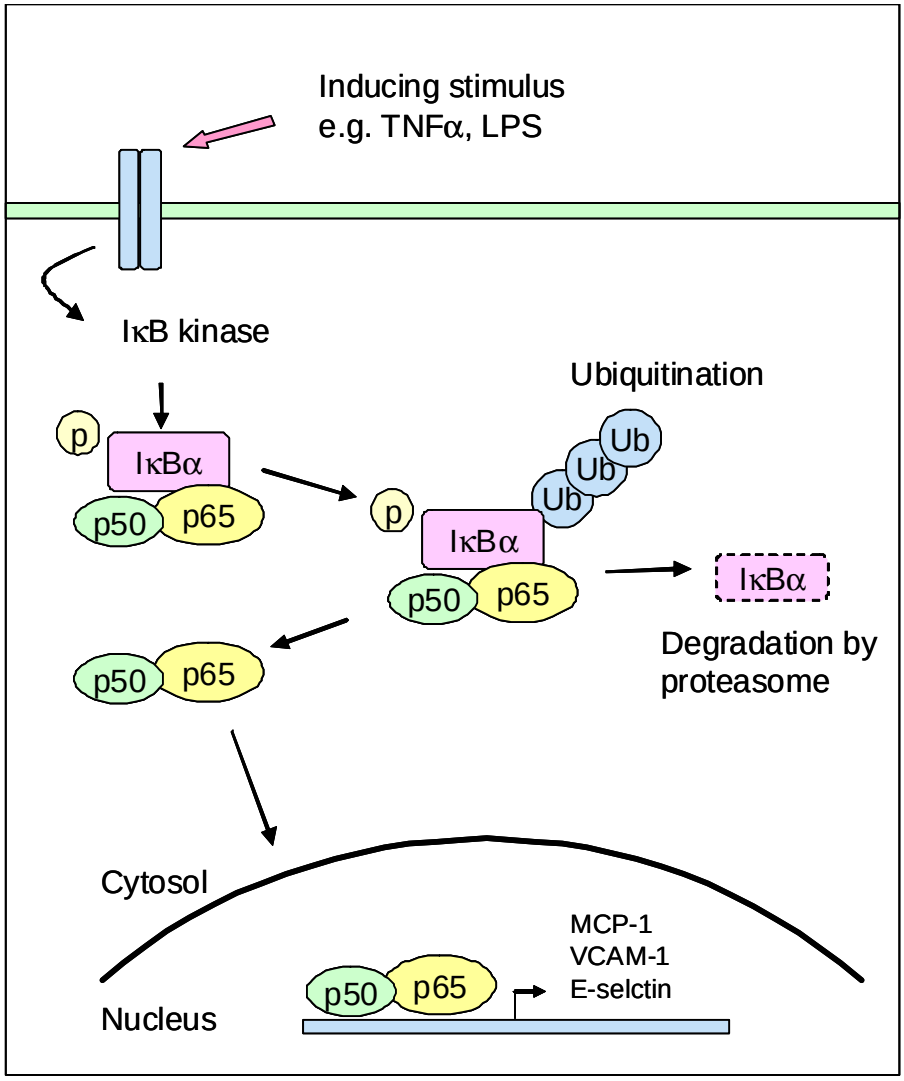

Figure 1.2 Schematic model of NFκB activation

In resting cells, NFκB is retained in an inactive state through association with IκBα. NFκB activation occurs in response to a wide variety of stimuli including pro-inflammatory cytokines such as TNFα and IL-1 which initiate signalling pathways that converge on the IκB kinase complex (IKK). On activation, IKK phosphorylates IκB on two specific serine residues (Ser32 and Ser36). Phosphorylated IκB is then recognised and ubiquitinated by members of the Skp1-Cullin-F-box (SCF) family of E3 ubiquitin ligases. Ubiquitinated IκBα is recognised by the 26S proteasome and degraded. This frees NFκB to enter the nucleus and bind to the promoter or enhancer regions of specific target genes.

best well characterised NFκB dimer is p65/p50. The term NFκB is therefore often used synonymously with p65/p50 and so far, mechanisms of regulation appear to be common to different complexes. However, dimers may show distinct preferences for binding particular IκBs (Karin and Ben-Neria, 2000; Ghosh *et al.*, 1998).

1.3.1.2 Regulation of NFκB by IκB

NFκB activity is regulated by its inhibitory protein IκB, which binds to NFκB and masks its nuclear localisation sequence (NLS) thereby inhibiting its translocation to the nucleus. The IκB family includes IκBα, IκBβ, IκBγ, IκBε, Bcl-3 and IκBζ in higher vertebrates and the Drosophila protein Cactus (Yamazaki *et al.*, 2001; Ghosh *et al.*, 1998). Two additional members are formed from the processing of the NFκB precursor proteins p105 and p100 (Rothwarf and Karin, 1999). IκB family members are characterised by the presence of either six or seven ankyrin repeats within their sequences. These are stretches of a 30-34 amino acids which form stacked helix-loop-helix structures, representing one of the most common protein-protein interaction domains in nature (Li *et al.*, 2006). Specific interactions occur between the ankyrin repeats of IκB proteins and the RHDs in NFκB, resulting in the masking of the NLS in NFκB.

Only IκBα, IκBβ and IκBε have N-term regulatory regions which are required for stimuli-induced degradation (Karin and Ben-Neriah, 2000). IκBα has been most well characterised. It shares a common domain structure with IκBβ consisting of an N-terminal regulatory region, which is phosphorylated in response to stimuli, a central ankyrin repeat domain and a C-terminal PEST sequence which is involved in regulation of protein turnover (Ghosh *et al.*, 1998; Verma *et al.*, 1995). In addition, IκBα also contains leucine-rich nuclear export sequences (NES) which interact with the nuclear export receptor CRM1 (Huang *et al.*, 2000; Johnson *et al.*, 1999). Originally, masking of the NFκB NLS by IκBα was thought to be solely responsible for its cytoplasmic localisation. However, crystallographic structures of NFκB in complex with IκBα show that IκBα only masks the NLS of p65 while the p50 NLS remains exposed (Malek *et al.*, 2003; Huxford *et al.*, 1998). A role for an NES was revealed by the finding that NFκB/IκBα complexes are almost completely redistributed to the nucleus following inhibition of CRM1 (Huang *et al.*, 2000) or deletion of an N-terminal NES in IκBα (Johnson *et al.*, 1999). The presence of both the NLS on p50 and the NES on IκBα results in constant shuttling of the complex between the nucleus and cytoplasm although a dominant effect of the NES favours

cytoplasmic localisation (Huang *et al.*, 2000; Johnson *et al.*, 1999). The ability of IκBα to shuttle between the cytoplasm and the nucleus allows it to play a critical role in terminating NFκB activity. Unlike other members of the IκB family, IκBα expression is induced by NFκB as part of an autoregulatory feedback loop (de Martin *et al.*, 1993; Le Bail *et al.*, 1993). Newly synthesised IκBα enters the nucleus, removes NFκB from DNA and transports it back into the cytoplasm thereby terminating its activity (Rodriguez *et al.*, 1999; Arenzana-Seisdedos *et al.*, 1997).

1.3.1.2.1 Stimulus-induced degradation of IκB

The defining event in NFκB activation is stimulus-induced degradation of IκB. This is a multi-step process requiring the phosphorylation, polyubiquitination and 26S proteasome-mediated degradation of IκBα (Hayden and Ghosh, 2004; Karin and Ben-Neriah, 2000). Protein modification by ubiquitination involves the formation of an isopeptide linkage between the C-terminal Gly76 of ubiquitin and ε-amino groups on lysine residues in the substrate protein. Similar linkages between lysine residues in ubiquitin molecules can allow formation of polyubiquitin chains which have different effects on the fate of the substrate protein depending on the particular lysine residue utilised. For example, Lys48-linked chains target proteins for degradation by the 26S proteasome while Lys63-linked chains mediate protein-protein interactions and have been shown to have various effects including activation of protein kinases such as the transforming growth factor β activated kinase-1 (TAK1) complex which phosphorylates and activates IKK (Fang and Weissman, 2004). The process of ubiquitination requires the sequential action of three enzymes: E1, a ubiquitin activating enzyme, E2, a ubiquitin-conjugating enzyme and E3, a ubiquitin ligase. The E3 component confers specificity on the system as it interacts directly and specifically with the substrate protein to bring it together with ubiquitin-loaded E2 (Liu, 2004). The E3 for IκBα belongs to the Skp1-Cullin-F-box (SCF) family of E3 ubiquitin ligases which generally recognise phosphorylated target proteins (Karin and Ben-Neriah, 2000; Hatakeyama *et al.*, 1999; Yaron *et al.*, 1998).

In response to NFκB-inducing stimuli, IκBα is phosphorylated by IKK on the N-terminal residues Ser32 and Ser36 which triggers its ubiquitination and degradation (Chen *et al.*, 1995, Brockman *et al.*, 1995; Brown *et al.*, 1995). The βTrCP receptor subunit of the SCF family binds specifically to the phosphorylated form of IκB via the E3 recognition sequence (DpSGXXpS) which incorporates Ser32 and Ser36 in IκBα (Yaron *et al.*, 1998;

Yaron *et al.*, 1997). This leads to its polyubiquitination on Lys21 and Lys22 and targets IκBα for degradation by the 26S proteasome (Di Donato *et al.*, 1996; Chen *et al.*, 1995; Scherer *et al.*, 1995).

1.3.1.3 IκB kinases

The IKK complex comprises three subunits: IKKα, IKKβ and IKKγ (also known as NFκB essential modifier (NEMO)). IKKα and IKKβ are highly homologous proteins sharing 50 % amino acid identity (Karin and Ben-Neriah, 2000). These are the catalytic subunits of the complex and have similar functional domains including an N-terminal catalytic domain, a central leucine zipper motif and a C-terminal helix-loop-helix domain (Yamamoto and Gaynor, 2004). IKKγ has no catalytic activity but is essential for IKK activity as demonstrated by the fact that TNFα, IL-1 or LPS fail to induce NFκB activation in IKKγ-deficient fibroblasts (Rudolph *et al.*, 2000). IKKγ has two coiled coli domains, a leucine zipper domain and a zinc finger motif which are known to mediate protein-protein interactions. The leucine zipper domain and a novel ubiquitin-binding domain have been found to be important for mediating interactions between the IKK complex and upstream signalling proteins that are essential for its activation (Ea *et al.*, 2006; Devin *et al.*, 2001).

1.3.1.3.1 Activation by TNFα

TNFα activates NFκB signalling through ligation of the TNF receptor, TNFR1 (figure 1.3). TNFα exists as a trimer and binding of the cytokine to its receptor results in receptor aggregation which also induces dissociation of the endogenous TNFR inhibitory protein, silencer of death domain (SODD). This exposes an intracellular domain of the receptor called the death domain (DD) which is recognised by the adaptor protein TNFR-associated death domain (TRADD). TRADD then recruits additional adaptor proteins such as members of the TNFR-associated factor (TRAF) family and the serine/threonine kinase receptor interacting protein (RIP1; Hayden and Ghosh, 2004; Chen and Goeddel, 2002). TRAF2 and TRAF5 are RING domain proteins and are thought to act as ubiquitin ligases, mediating the Lys63-polyubiquitination of RIP1 (Ea *et al.*, 2006). The IKK complex has been reported to be recruited to the receptor complex by two different mechanisms. One study showed that IKK recruitment is dependent on an interaction between the RING domain of TRAF2 and the leucine zipper motif in either IKKα or IKKβ (Devin *et al.*, 2001). However, another group identified an interaction between IKKγ and Lys63-polyubiquitin chains on RIP1 as being necessary for IKK recruitment and NFκB activation

Figure 1.3 TNFR1 and TLR4 signalling to IKK

Activation of the TNFR1 induces association with the adaptor protein TNFR-associated death domain (TRADD). TRADD then recruits additional adaptor proteins such as members of the TNFR-associated factor (TRAF) family and the serine/threonine kinase receptor interacting protein, RIP1. TRAF2 and TRAF5 are ubiquitin ligases which mediate Lys63-polyubiquitination of RIP1. IKK and the TAK1 complex are recruited to the signalling complex via interactions with Lys63 polyubiquitin chains on RIP1. TAK1 is thus activated and phosphorylates IKK either directly or via MEKK. Activation of TLR4 by LPS results in recruitment of TIR domain-containing adaptor proteins such as myeloid differentiation gene 88 (MyD88), TIR-containing adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). MyD88 interacts with members of the IL-1R-associated kinase (IRAK) family. Following interaction with MyD88, IRAKs are phosphorylated which leads to the dissociation of IRAK-1 from MyD88 and its interaction with TRAF6. TRAF6 Lys63-linked polyubiquitinates several target proteins including TRAF6 itself and the IKK complex. The TAK1 complex binds to TRAF6 via its Lys63 polyubiquitin chains and is activated which allows it to phosphorylate IKKβ. Deubiquitinating enzymes such as A20 and CYLD regulate NFκB pathway activation by removing Lys63 polyubiquitin chains from TRAFs, RIP1 and IKK. (Figure from Silverman and Fitzgerald, 2004)

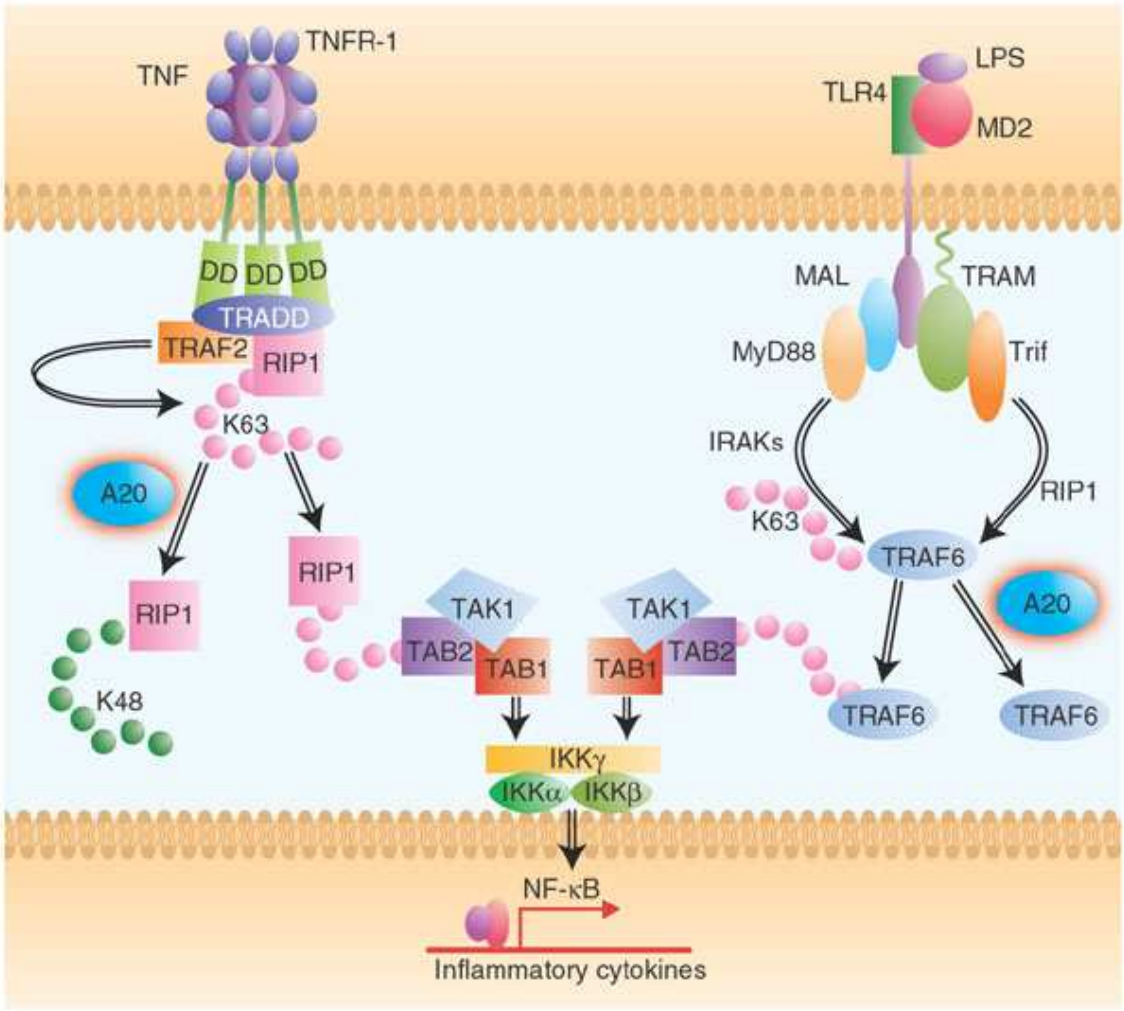

Figure 1.3 TNFR1 and TLR4 signalling to IKK

in response to TNFα (Ea *et al.*, 2006). RIP1 has also been found to interact with a protein complex involving TAK1, which is a member of the MAP3K family, and its regulatory subunits TAB2 and TAB3. This depends on an interaction between a highly conserved zinc finger domain in TAB2 and TAB3 and the polyubiquitin chains of RIP1 (Kanayama *et al.*, 2004; Ea *et al.*, 2006). This results in activation of TAK1 which then phosphorylates and activates IKK either directly or via another MAP3K, MEKK (Kovalenko and Wallach, 2006).

1.3.1.3.2 Activation by LPS/IL-1

Pathogen-associated molecules such as bacterial LPS are recognised by TLRs which are expressed on various immune cells such as macrophages, dendritic cells and neutrophils as well as non-immune cells such as fibroblasts and epithelial cells (Kawai and Akira, 2007). TLRs and IL-1Rs share many of the same signalling components owing to the presence of a conserved Toll/IL-1R (TIR) motif within their intracellular domains which mediates oligomerisation with downstream signalling molecules which also express TIR domains (Martin and Wesche, 2002). Activation of TLR4 by LPS results in recruitment of TIR domain-containing adaptor proteins such as myeloid differentiation gene 88 (MyD88), TIR-containing adaptor protein (TIRAP), TIR-containing adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM; figure 1.3). MyD88 was the first TIR domain-containing protein to be characterised and has been shown to be necessary for normal activation of NFκB by IL-1 and LPS (Kawai *et al.*, 1999). MyD88 contains an N-terminal DD which allows it to interact with the DDs of members of the IL-1R-associated kinase (IRAK) family including IRAK-1, IRAK-2, IRAK-4 and IRAK-M. Following interaction with MyD88, IRAK-4 and IRAK-1 are sequentially phosphorylated which leads to the dissociation of IRAK-1 from MyD88 and its interaction with TRAF6 (Kawai and Akira, 2007). TRAF6 is a RING domain ubiquitin ligase which facilitates Lys63-linked polyubiquitination of target proteins including TRAF6 itself and the IKKγ subunit of the IKK complex (Chen *et al.*, 2006). TAB2 or TAB3 can then recruit the TAK1 complex to TRAF6 by interacting with its Lys63 polyubiquitin chains (Kanayama *et al.*, 2004). Activated TAK1 has been reported to phosphorylate and activate IKKβ (Wang *et al.*, 2001; Deng *et al.* 2000).

1.3.1.4 Downregulation of NFκB signalling

IKK activation represents a crucial step in the pathway leading to activation of NFκB by practically all stimuli and therefore represents a key point for regulation. IKK is activated following a complex series of protein-protein interactions and activation steps, many of which depend upon Lys63-linked polyubiquitination of NFκB signalling proteins as described above (Kawai and Akira, 2007; Hayden and Ghosh, 2004). As ubiquitin ligases, TRAFs are key mediators of this process and their substrates include TRAFs themselves, IKKγ and the TAB2 and TAB3 components of the TAK1 complex (Adhikari and Chen, 2007). Therefore, regulation of the ubiquitination status of these proteins represents an important means of controlling NFκB activity. This is mediated by specific deubiquitinating enzymes (DUBs). Two of the best studied DUBs involved in regulating NFκB activity are A20 and CYLD. These proteins are encoded by NFκB-regulated genes and so induction of A20 and CYLD expression provides a negative feedback loop to suppress NFκB activity (Sun, 2008).

CYLD is a member of the ubiquitin-specific protease (USP) family of deubiquitinating enzymes (Sun, 2008). It is proposed that CYLD suppresses NFκB activity by binding to signalling components upstream of IKK and de-conjugating Lys63 ubiquitin chains via a ubiquitin-carboxy-terminal-hydrolase domain (UCH) present in its C-terminal region (Adhikari and Chen, 2007). In support of this, CYLD has been found to bind to several upstream mediators of IKK activation including NEMO, TRAF2 and TRAF6 (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003 Trompouki *et al.*, 2003). Furthermore, while polyubiquitination of IKKγ, TRAF2 and TRAF6 has been observed when these proteins are overexpressed in conjunction with HA-ubiquitin, co-transfection with CYLD prevented detection of the polyubiquitinated forms. In contrast, co-transfection with catalytically inactive CYLD had no effect on the ubiquitin status of these proteins indicating that wild-type CYLD actively deubiquitinates IKKγ, TRAF2 and TRAF6 (Brummelkamp *et al.*, 2003). The role of CYLD in regulating NFκB activity has been demonstrated in several ways. For example, suppression of CYLD expression using short hairpin RNAs has been shown to lead to increased activation of IKK in response to TNFα treatment (Brummelkamp *et al.*, 2003). A similar effect was achieved by transfecting cells with a catalytically inactive CYLD mutant lacking the active site cysteine residue indicating that this was due to the loss of the deubiquitinating activity of CYLD (Brummelkamp *et al.*, 2003). In agreement with this finding, overexpression of CYLD has

been found to inhibit activation of NFκB in response to a wide variety of stimuli indicating that it may represent a general mechanism for regulating the NFκB pathway (Kovalenko *et al.*, 2003).

A20 is another DUB which has been shown to potently inhibit NFκB activity (Wertz *et al.*, 2004; Boone *et al.*, 2004). The critical nature of A20 in regulating NFκB activation is clear from studies using A20 knock-out mice (Lee *et al.*, 2000). These mice suffered severe inflammation and increased sensitivity to TNFα and LPS. Examination of this effect in A20-deficient fibroblasts revealed that this was due to persistent activation of the NFκB pathway as demonstrated by rapid degradation of IκBα and a failure to reaccumulate newly synthesised IκBα following stimulation with TNFα (Lee *et al.*, 2000). A20 contains an N-terminal ovarian tumour (OTU) - type domain which has DUB activity and seven C-terminal zinc finger domains which are reported to have E3 ligase activity (Wertz *et al.*, 2006; Evans *et al.* 2004). Therefore, A20 has the unique ability to act as both a DUB and an E3 ligase. A20 has been found to act as a DUB, removing Lys63-linked ubiquitin chains from several NFκB signalling proteins including TRAF6, RIP1 and IKKγ (Boone *et al.*, 2004, Wertz *et al.*, 2004; Mauro *et al.*, 2006). In addition, A20 has been reported to mediate a second level of regulation by catalysing the Lys48-linked ubiquitination of RIP1 and targeting it for degradation (Wertz *et al.*, 2004).

1.3.2 The JAK/STAT Pathway

In general, cytokines mediate their effects through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway (figure 1.4). This pathway is initiated on binding of a cytokine to its receptor, which results in either receptor multimerisation or stabilisation of preformed dimers. Activation of the receptor induces a conformational change which allows auto- and trans-phosphorylation of constitutively associated JAKs. The active JAKs are then able to phosphorylate key tyrosine residues on the receptor, which then act as docking sites for the Src homology 2 (SH2) domains of STATs and other signalling proteins. STATs are then themselves phosphorylated which enables them to dimerise and translocate to the nucleus where they modulate transcription of specific STAT-responsive genes (Rawlings *et al.*, 2004; O'Shea *et al.*, 2002, Kisseleva *et al.*, 2002).

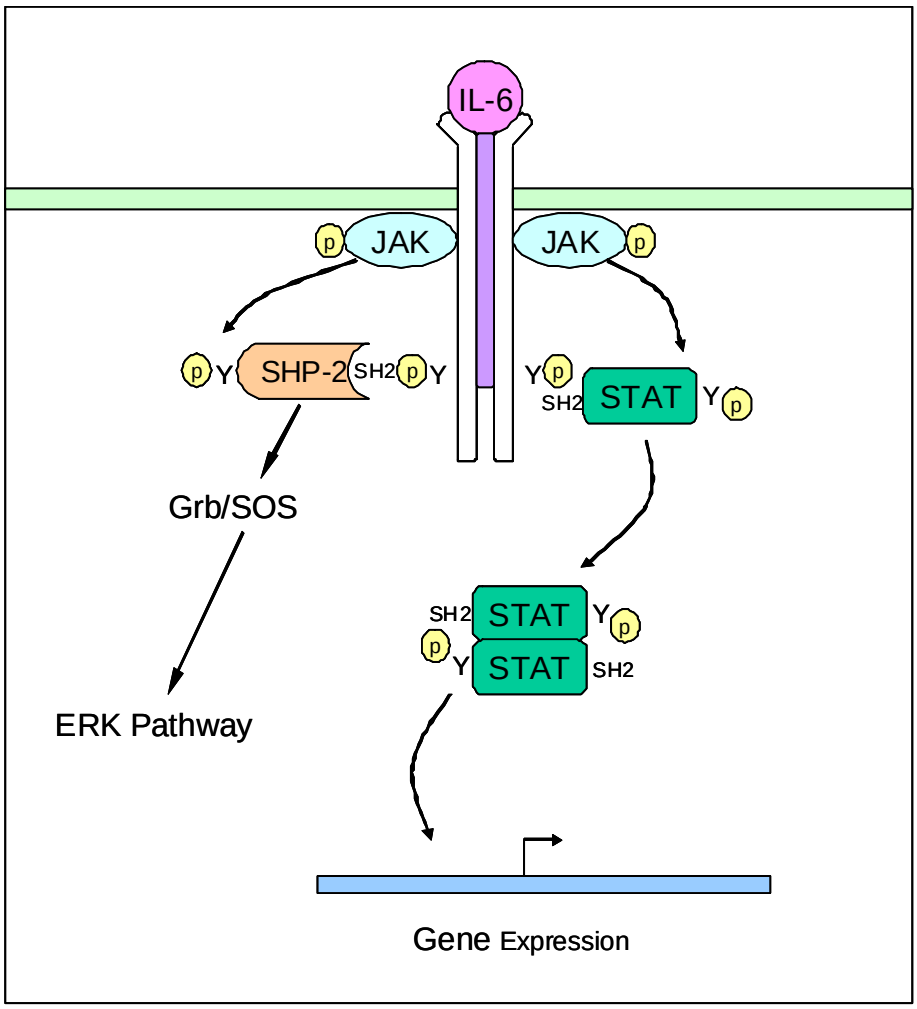

Figure 1.4 Activation of the JAK/STAT pathway by IL-6

The IL-6 receptor is composed of two different subunits, an 80 kDa IL-6-binding protein (IL-6Rα) and a 130 kDa signal-transducing subunit (gp130) Binding of IL-6 to IL-6Rα induces dimerisation of the gp130 subunit and the receptor undergoes a conformational change which allows auto- and trans-phosphoryhlation of constitutively associated Janus kinases (JAK1, JAK2 and Tyk2). JAKs phosphorylate gp130 on specific tyrosine residues which then act as docking sites for the SH2 domain-containing proteins, signal transducer and activator of transcription 1 (STAT1) and STAT3. STAT1 and STAT3 are then themselves phosphorylated, following which they form homo- or heterodimers and are translocated to the nucleus where they can modulate transcription of specific STAT-responsive genes. Activation of the IL-6 receptor also leads to activation of the ERK pathway via the SH2 domain-containing tyrosine phosphatase, SHP2 which binds to pTyr^{759} on gp130 and is activated by phosphorylation by JAK1. Activated SHP2 interacts with the growth-factor-receptor-bound protein 2 (Grb2) which is constitutively associated with the Ras-GTP-exchange factor, Son of Sevenless (SOS).

1.3.2.1 JAKs

In mammals, four different members of the JAK family have been identified: JAK1, JAK2, JAK3 and Tyk2. JAK1, JAK2 and Tyk2 are ubiquitously expressed while JAK3 is present only in myeloid and lymphoid cells (Kisseleva *et al.*, 2002). JAKs associate with the membrane-proximal region of cytokine receptors. In the case of many cytokine receptors, this region contains two conserved sequence elements termed box 1 and box 2. Box 1 is proline-rich and has been found to be required for the binding of JAKs to receptors for IL-2 (Howard *et al.*, 1995) and the IL-6 family of cytokines (Radtke *et al.*, 2002; Murakami *et al.*, 1991). Box 2 is rich in hydrophobic amino acids and is important for JAK association with only certain receptors (Heinrich *et al.*, 1998). Receptor ligation triggers a conformational change in receptor dimers that brings associated JAKs into close proximity, permitting autophosphorylation (Remy *et al.*, 1999).

JAKs are composed of seven conserved JAK homology domains (JH1-7; Kisseleva *et al*, 2002). JH1 at the carboxyl terminus is the kinase domain and has considerable homology to other kinases, displaying features such as an activation loop identified as being important in regulating activity (Hubbard and Till, 2000). Mutational analysis has identified tyrosine residues in JAK2, JAK3 and Tyk2 which are critical components of this activation loop; Y1007/Y1008 in JAK2 (Feng *et al.*, 1997), Y980/Y981 in JAK3 (Zhou *et al.*, 1997) and Y1054/Y1055 in Tyk2 (Gauzzi *et al.*, 1996). JAK1 also has two conserved tyrosine residues, Y1022/Y1023, which are important for activation (Leonard and O'Shea, 1998). JH2 is termed the kinase-like domain, which although catalytically inactive, has a regulatory function. Natural mutations in this region of JAK3 result in an inactive enzyme and severe combined immunodeficiency (SCID; Chen *et al.*, 2000). Conversely, experimentally-induced mutations in the kinase-like domain of the *Drosophila* JAK homologue, *hopscotch*, results in a constitutively active enzyme and causes leukaemia (Luo *et al.*, 1997). The JAK carboxyl terminus (JH3-JH7) contains an SH2-like domain and a Band-4.1 ezrin, radixin, moesin (FERM) homology domain that is involved in receptor association. For example, binding of JAK2 to IFNγ receptor 2 which has no box 1/box 2 motif is mediated by the JH6 and JH7 domains (Kohlhuber *et al.*, 1997). These regions are also important for binding of JAK3 to the γ chain of the IL-2 receptor (Chen *et al.*, 1997). The FERM domain has also been found to associate with the kinase domain to enhance activity (Zhou *et al.*, 2001).

1.3.2.2 STATs

There are seven mammalian STAT family members designated STAT1, 2, 3, 4, 5a, 5b and 6 (Kisseleva *et al.*, 2002). They are ubiquitously expressed with the exception of STAT4 which is mainly found in the testis, thymus and spleen (Zhong *et al.*, 1994). STATs are composed of several structurally and functionally conserved domains including an amino-terminal oligomerisation domain, a coiled-coil domain, a DNA binding domain, a linker region, an SH2 domain and a carboxyl-terminal transactivation domain (Becker *et al.*, 1998; Chen *et al.*, 1998; Vinkemeier *et al.*, 1998).

1.3.2.2.1 The SH2 domain

The SH2 domain is highly conserved and is involved in the recruitment of STATs to activated cytokine receptors (Heim *et al.*, 1995; Stahl *et al.*, 1995; Greenlund *et al.*, 1994) and the formation of STAT dimers (Shuai *et al.*, 1994) through recognition of specific phosphorylated tyrosine motifs. Different receptor motifs determine which STATs are recruited, for example, STAT3 will bind to phospho (p)YXXQ (Stahl *et al.*, 1995) while STAT1 will only bind to pYXPQ (Gerhartz *et al.*, 1996). This difference has been shown to be due to the SH2 domain through the creation of a chimaeric STAT3 molecule. Hemmann and co-workers (1996) found that substituting the SH2 domain of STAT3 with a STAT1 SH2 domain resulted in a molecule that showed the receptor motif binding preference of STAT1. On recruitment to an activated cytokine receptor, STATs are phosphorylated by JAKs on a single tyrosine residue at the carboxyl end of the SH2 domain (Tyr^{701} in STAT1 (Shuai *et al.*, 1994) and Tyr^{705} in STAT3 (Kaptein *et al.*, 1996)). This enables them to form dimers through an interaction of the phosphorylated tyrosine on one STAT with the SH2 domain of another.

1.3.2.2.2 Gene Regulation

STAT dimers translocate to the nucleus and bind DNA motifs known as GAS (γ activated sequence) elements ($TTN_{5-6}AA$) except in the case of the IFNα/β response, where complexes formed between STAT1, STAT2 and IRF9 (interferon regulatory factor 9) bind to the IFNα/β-response element (ISRE), $AGTTN_3TTTC$ (O'Shea *et al.*, 2002). The STAT transcriptional activation domain (TAD) is proposed to participate in modulation of transcription through interaction with additional transcription factors and co-activators such as c-Jun, BRCA1 and the cAMP-response-element-binding (CREB)-binding protein (CBP)/p300 family of histone acetyltransferases (Horvath, 2000). Phosphorylation of a

conserved serine residue within the TAD (Ser^{727} in STAT1, STAT3 and STAT4) is believed to regulate these interactions to provide full transcriptional activity (Horvath, 2000).

1.3.2.3 IL-6 signalling

The IL-6 receptor is composed of two different subunits, an 80 kDa IL-6-binding protein (IL-6Rα) and a 130 kDa signal-transducing subunit (gp130), which is shared by all IL-6-type cytokines (Heinrich *et al.*, 2003). The gp130 subunit is ubiquitously expressed while IL-6Rα expression is restricted to hepatocytes, monocytes, neutrophils and some B and T cells (Kallen, 2002). However, IL-6 can also bind to a soluble form of the receptor (sIL-6Rα) which is either shed from cell membranes (Mullberg *et al.*, 1993) or created by alternative splicing of mRNA (Lust *et al.*, 1992). This complex can associate with gp130 on cells that do not express the membrane-bound IL-6R thereby widening the spectrum of IL-6-responsive cells. For example, vascular endothelial cells which express only the gp130 subunit of the IL-6 receptor become responsive to IL-6 in the presence of sIL-6Rα shed from the membranes of activated neutrophils (Marin *et al.*, 2001).

Binding of IL-6 to IL-6Rα induces dimerisation of the gp130 subunit and formation of a fully functional receptor complex (Murakami *et al.*, 1993; figure 1.4). JAK1, JAK2 and Tyk2 are activated upon receptor stimulation (Stahl *et al.*, 1994; Narazaki *et al.*, 1994) and phosphorylate gp130 on tyrosine residues 683, 759, 767, 814, 905 and 915 (Stahl *et al.*, 1994; Hirano *et al.*, 1997). Studies using mutant cell lines lacking JAK1, JAK2 or Tyk2 have revealed that signalling absolutely depends on the presence of JAK1 whereas JAK2 and Tyk2 may be interchangeable (Guschin *et al.*, 1995). Phosphorylation of gp130 was greatly reduced in the absence of JAK1 but was unimpaired in the absence of JAK2 or Tyk2.

STAT1 and STAT3 are recruited to the phosphorylated receptor through recognition of consensus sequences pY^{905}LPQ and pY^{915}MPQ. In addition, STAT3 also recognises pY^{767}RHQ and pY^{814}FKQ, (Stahl *et al.*, 1995; Gerhartz *et al.*, 1996). These sites are not equivalent, as has been demonstrated by Schmitz *et al.* (2000a) using mutant gp130 constructs lacking each of the cytoplasmic tyrosine residues present in wild-type gp130. Tyr^{905} and Tyr^{915} were found to be more potent than Y^{767} and Y^{814} in terms of their ability to activate STATs and STAT-mediated transcription. Upon binding to the receptor,

STAT1 and STAT3 are phosphorylated, following which they form homo- or heterodimers and are translocated to the nucleus to modulate transcription.

STATs are not the only proteins that are recruited to the activated IL-6 receptor. The SH2-domain-containing tyrosine phosphatase, SHP2 binds to $pTyr^{759}$ on gp130 and is phosphorylated by JAK1 (Schaper *et al.*, 1998). Activated SHP2 can then activate the extracellular signal-regulated kinase (ERK) pathway through interaction with the growth factor receptor-bound protein 2 (Grb2) which is constitutively associated with the Ras-GTP-exchange factor, Son of Sevenless (SOS) (Li *et al*., 1994).

1.3.2.4 IFNγ signalling

The functional IFNγ receptor (IFNGR) comprises two 90 kDa IFNGR1 and two 62 kDa IFNGR2 chains. IFNGR1 is involved in ligand binding and signal transduction while IFNGR2 plays only a small role in ligand binding but is essential for signalling (Stark *et al.*, 1998). Originally, these subunits were not thought to interact in unstimulated cells (Bach *et al.*, 1996) but advances in spectroscopic techniques using intact cells have shown that the receptor is preassembled and ligand binding results in a conformational change to allow signalling to occur (Krause *et al.*, 2002). IFNGR1 and IFNGR2 have binding motifs for JAK1 and JAK2 respectively. JAK1 binds to a membrane proximal sequence, LPKS at residues 266-269 on IFNGR1 (Kaplan *et al.*, 1996) while JAK2 binds a proline-rich non-contiguous motif, ^{263}PPSIP^{267} followed by ^{270}IEEYL^{274} on IFNGR2 (Bach *et al.*, 1996). On activation of the receptor, JAK2 autophosphorylates and is then able to phosphorylate JAK1 (Briscoe *et al.*, 1996). The activated JAKs phosphorylate each IFNGR1 chain on tyrosine residue 440 in the sequence ^{440}YDKPH^{444} and this creates a pair of docking sites for STAT1 molecules. STAT1 is thus phosphorylated and dissociates from the receptor, forms homodimers and is translocated to the nucleus.

Activation of the phosphatidylinositol 3-kinase (PI3K) pathway also appears to play a role in IFNγ-induced STAT1-mediated transcriptional regulation. Inhibition of PI3K or one of its downstream effectors, protein kinase C δ (PKCδ), blocks phosphorylation of STAT1 on Ser727 and reduces its transcriptional activity. IFNγ has been shown to activate PKCδ in a PI3K dependent manner and so it is proposed that PKCδ is a serine kinase for STAT1 (Nguyen *et al.*, 2001; Deb *et al.*, 2003).

1.3.2.5 Regulation of the JAK/STAT pathway

JAK/STAT signalling is central to many biological processes and so numerous mechanisms exist to modulate the pathway at different stages.

1.3.2.5.1 Protein tyrosine phosphatases

Tyrosine phosphorylation by kinases is a key event in the JAK/STAT pathway. For controlled signalling, it is clear that this rapid, reversible process must be balanced by the action of protein tyrosine phosphatases (PTPs). Several PTPs have been implicated in the regulation of JAK/STAT signalling, for example, SHP1, SHP2, and PTPε (Shuai and Lui, 2003). SHP1 is a non-transmembrane phosphatase expressed mainly in haematopoietic cells. Its role in regulation of JAK/STAT signalling is apparent on consideration of the naturally occurring *motheaten* mouse strain which lacks SHP1 activity. Bone-marrow derived macrophages from these mice show dramatically increased levels of JAK1 and STAT1 phosphorylation following stimulation with IFNα (David *et al.*, 1995). SHP1 has been shown to bind to receptors for erythropoietin (Epo; Klingmuller *et al.*, 1995) and IL-3 (Wheadon *et al.*, 2002) to suppress phosphorylation of JAKs and receptors respectively.

SHP2 is highly homologous to SHP1 but is ubiquitously expressed. It is involved in both positive and negative regulation of signalling for a variety of cytokines including IL-6 and IFNγ (Qu, 2002). SHP2 is rapidly recruited to Tyr^{759} in gp130 following IL-6 stimulation (Stahl *et al.*, 1995). It has a positive role in activating the ERK pathway but an inhibitory effect on JAK/STAT signalling. Mutation of Tyr^{759} in gp130 impairs SHP-2 recruitment and phosphorylation (Stahl *et al.*, 1995) and leads to enhanced JAK/STAT signalling but reduced ERK activation (Schaper *et al.*, 1998). In addition, it has been found that overexpression of dominant negative SHP-2 mutants leads to increased phosphorylation of receptors, JAKs and STATs in murine fibroblasts stimulated with IL-6 (Lehmann *et al.*, 2003). SHP2 is constitutively associated with the IFNα/β and IFNγ receptors (David *et al.*, 1996). The relevance of this is apparent in $SHP2^{-/-}$ mouse fibroblasts. These cells show increased STAT1 and STAT2 activity as measured by DNA binding in response to IFNα and IFNγ which is diminished on reintroduction of SHP2 (You *et al.*, 1999). Furthermore, SHP2 has been described as a dual-specificity phosphatase that dephosphorylates STAT1 at both Tyr^{701} and Ser^{727} (Wu *et al.*, 2002).

A cytoplasmic form of transmembrane PTPε (PTPε C) is involved in regulation of IL-6 signalling. Tanuma *et al.* (2000) found that overexpression of PTPε C inhibited

phosphorylation of gp130, JAK1, Tyk2, and STAT3 while overexpression of a dominant negative form potentiated the IL-6 response. This inhibitory effect is thought to be specific to certain cytokines as PTPε C does not affect IFNγ-induced STAT1 phosphorylation (Tanuma *et al.*, 2001). A phosphatase that does appear to regulate STAT1 activation in response to IFNγ is a nuclear form of the T cell PTP, PTP TC45 (ten Hoeve *et al.*, 2002). Murine embryonic fibroblasts and primary thymocytes expressing defective PTP TC45 fail to dephosphorylate STAT1 in the nucleus following IFNγ stimulation.

1.3.2.5.2 Protein inhibitors activated STATs

The protein inhibitors of activated STATs (PIAS) are a family of constitutively expressed transcriptional regulators (Chen et al., 2004). PIAS1 was identified as a STAT-interacting protein in a yeast two-hybrid screen and subsequently, four other members of the family, PIAS3, PIASy, PIASxα and PIASxβ were recognised based on their high sequence homology to PIAS1 (Liu et al., 1998). Following IFNγ stimulation, PIAS1 binds to activated STAT1, inhibiting DNA binding and therefore gene activation (Liu et al., 1998). PIAS3 on the other hand, shows specificity for STAT3 and suppresses IL-6 induced gene expression (Chung et al., 1997).

The effect of PIAS on STAT-mediated transcription cannot be fully explained by suppression of STAT DNA-binding activity as PIASy, which also inhibits STAT1-dependent gene induction, does not prevent STAT-DNA interactions (Liu *et al.*, 2001). An alternative method of regulation that has been suggested is PIAS-directed sumoylation of STATs. SUMO is a small ubiquitin-like protein modifier which has roles in the regulation of protein-protein interactions and protein stability, localisation and activation (Schmidt and Muller, 2002; Kotaja *et al.*, 2002). PIAS family members all have SUMO ligase activity (Wormald and Hilton, 2004) and both PIAS1 and PIASxα have been found to promote sumoylation of STAT1 on lysine residue 703 following stimulation of fibroblasts with IFNγ (Rogers *et al.*, 2003). However, the functional relevance of this is unclear as there have been conflicting reports on the effect of mutating Lys^{703} on the expression of IFNγ-induced genes (Rogers *et al.*, 2003; Ungureanu *et al.*, 2003). Ungureanu *et al.* (2003) found that this potentiated transcription of a GAS-luciferase reporter gene while Rogers *et al.* (2003) reported no effect. More recent studies indicate that sumoylation has a selective inhibitory effect on STAT1-mediated transcription. Using quantitative PCR, Ungureanu *et al.* (2005) showed that mutation of alternative STAT1 residues that are also required for sumoylation (Ile^{702} and Glu^{705}) resulted in increased transcription of some but not all IFNγ

target genes tested. How sumoylation inhibits STAT1 activity is unclear but it has been suggested that it may act as a targeting signal (Wormald and Hilton, 2004), a theory supported by the fact that STAT1 mutants which cannot be sumoylated show prolonged nuclear localisation (Ungureanu *et al.*, 2005).

1.3.2.5.3 Suppressors of cytokine signalling

SOCS (suppressors of cytokine signalling) are a family of inducible inhibitors of cytokine signalling (Starr *et al.*, 1997). The expression of several SOCS proteins is induced by activation of the JAK/STAT pathway by cytokines such as IL-6, IFNγ, granulocyte colony-stimulating factor and IL-11. They act as classical negative feedback inhibitors by inhibiting the phosphorylation of JAKs, which in turn prevents STAT activation (Chen *et al.*, 2004). There are eight SOCS family members (CIS (cytokine-inducible SH2 protein) and SOCS1-7). Genetic studies have revealed that SOCS1 is particularly important in IFNγ signalling while SOCS3 has specificity for IL-6 signalling. Generation of mice with a conditional deletion of SOCS3 in hepatocytes or macrophages has revealed the importance of SOCS3 in the IL-6 response. These mice exhibit hyperresponsiveness to IL-6 as shown by sustained activation of STAT1 and STAT3 and an increase in the number of IL-6-responsive genes while IFNγ responses are normal (Croker *et al.*, 2003; Lang *et al.*, 2003). SOCS1-deficient mice in contrast, show prolonged IFNγ-induced STAT1 activation but normal STAT activation in response to IL-6 (Croker *et al.*, 2003).

SOCS proteins are characterised by a central SH2 domain and a carboxyl-terminal motif termed the SOCS box (Starr *et al.*, 1997) but their inhibitory effects are mediated by several different mechanisms. SOCS1 has been found to bind directly to the kinase domain of JAK2 via its SH2 domain (Endo *et al.*, 1997). Inhibition is thought to be mediated by a second SOCS1 domain, the kinase inhibitory region (KIR), which binds to and blocks the substrate-binding site of the JAK kinase domain (Yasukawa *et al.*, 1999). However, this model is further complicated by the recent finding that in the case of IFNγ signalling, SOCS1 appears to interact directly with a phosphorylated Tyr^{441} in subunit 1 of the IFNγ receptor before binding to JAK2 (Qing *et al.*, 2005). Inhibition of IL-6 signalling by SOCS3, like SHP-2, is dependent upon interaction with phosphorylated Tyr^{759} on activated gp130 (Schmitz *et al.*, 2000b; Nicholson *et al.*, 2000). However, SOCS3 can also bind to JAKs and has a KIR motif which may contribute to inhibition (Sasaki et al., 1999).

Another aspect of negative regulation by SOCS may involve the SOCS box motif. Zhang and co-workers (1999) found that the SOCS box binds to elongins B and C which are known to participate in the targeting of proteins to the proteasome for degradation. By this means, excess SOCS proteins and their binding partners could be removed from the signalling system.

1.3.2.5.4 Targeted degradation of STATs

Ubiquitin-proteasome-dependent degradation has also been suggested to regulate cellular levels of STATs. Kim and Maniatis (1996) found that inhibition of the proteasome led to accumulation of ubiquitinated STAT1 molecules in HeLa cells stimulated with IFNγ and that this effect was dependent on STAT phosphorylation. Haspel *et al.* (1996) also observed sustained levels of STAT1 in response to IFNγ when Bud-8 fibroblasts were pre-incubated with proteasome inhibitors. However, this was determined to be due to reduced receptor turnover and preservation of the signal rather than STAT levels as the effect was lost in the presence of a kinase inhibitor. It is possible that more than one mechanism of STAT regulation exists. In a later study, it was found that proteasome inhibitors prevented downregulation of phosphorylated STAT4, STAT5 and STAT6 but not phosphorylated STAT1, STAT2 or STAT3 in several cell lines (Wang *et al.*, 2000). This effect was maintained in the presence of a kinase inhibitor. This group also identified the carboxyl-terminal transcriptional activation domain of STAT5 as the region involved in regulation by the proteasome.

Certain viruses evade the antiviral activities of interferons by targeting STATs for degradation through the ubiquitin-proteasome pathway. Paramyoxaviruses of the *Rubulavirus* genus express "V" proteins which co-ordinate the assembly of STAT-ubiquitinating enzyme complexes. Viral V proteins are E3 ligases with high species-dependent specificity for different STATs, for example, simian virus 5 targets STAT1 for degradation (Didcock *et al.*, 1999) while type II human parainfluenza virus targets STAT2 (Parisien *et al.*, 2001) and mumps virus targets STAT1 and STAT3 (Ulane *et al.*, 2003).

New evidence for the role of STAT degradation in regulation of cytokine signalling comes from the identification of a mammalian STAT-specific E3 ligase. STAT-interacting LIM protein (SLIM) was identified in a yeast two-hybrid screen and subsequently was found to bind phosphorylated STAT1 and STAT4 in the nucleus and to inhibit their transcriptional activity (Tanaka *et al.*, 2005). SLIM has a LIM domain which forms a Zn finger structure that resembles RING and PHD structures found in E3 ligases (Capili *et al.*, 2001; Liu,

2004). This suggested a role for SLIM in ubiquitination which was confirmed using transfection studies where the presence of SLIM promoted ubiquitination and degradation of STAT1 and STAT4 (Tanaka *et al.*, 2005).

1.4 Adenosine

The production of adenosine represents a means of limiting inflammation and tissue damage that is in addition to the many specific mechanisms that have evolved to control cytokine signalling. Adenosine is a ubiquitous purine nucleoside that accumulates in many tissues in response to metabolic stress such as hypoxia during inflammation (Sitkovsky *et al.*, 2004).

1.4.1 Production

Under normal conditions, adenosine is continuously produced by cells through the dephosphorylation of AMP by cytosolic 5'-nucleotidases or through hydrolysis of S-adenosyl-homocysteine. In hypoxic conditions, ATP synthesis is inhibited and AMP levels rise which causes a large increase in adenosine production. Substantial amounts of adenosine are also produced by the hydrolysis of adenine nucleotides released from the granules of neutrophils, mast cells and endothelial cells as a result of cellular damage (Sitkovsky *et al.*, 2004; Ramkumar *et al.*, 2001; Linden, 2001). Ecto-apyrases such as CD39 hydrolyse ATP or ADP to AMP which is then converted to adenosine by the extracellular 5'-ectonucleotidase CD73 (Zimmermann, 2000). Both of these enzymes are induced during hypoxia to enhance adenosine production. In addition, CD73 expression on endothelial cells can be upregulated by IFNα (Niemela *et al.*, 2004) and adenosine itself (Narravula *et al.*, 2000). Extracellular accumulation of adenosine is further enhanced during hypoxia by inhibition of the enzyme adenosine kinase which converts excess adenosine back into AMP (Sitkovsky *et al.*, 2004).

1.4.2 Anti-inflammatory effects of the A_{2A} adenosine receptor

Adenosine exerts its effects through four different G-protein coupled receptor subtypes termed A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2007). These differ in their distribution patterns and the type of G-protein with which they associate but all have been ascribed roles in tissue protection (Linden, 2001). The A_{2A} adenosine receptor (A_{2A}AR) is coupled to G_s which stimulates adenylyl cyclase to raise intracellular levels of cAMP. However,

A_{2A}AR stimulation also results in activation of the ERK pathway and in addition, some G protein-independent effects have been reported (Fredholm *et al.*, 2007). The A_{2A}AR is expressed on many lymphoid cells including neutrophils, monocytes, macrophages, T cells and natural killer (NK) cells and its activation by adenosine or adenosine analogues results in a wide range of anti-inflammatory responses (Haskó *et al.*, 2008; Palmer and Trevethick; 2008). Activation of the A_{2A}AR on neutrophils has long been know to have suppressive effects on their cytotoxic functions by inhibiting phagocytosis (Salmon and Cronstein, 1990) and production of reactive oxygen metabolites (Cronstein *et al.*, 1992). In addition, A_{2A}AR activation results in reduced neutrophil recruitment to inflammatory sites by downregulating expression of the neutrophil adhesion molecule, very late antigen 4 (VLA–4) which is required for adherence to the endothelium (Sullivan *et al.*, 2004; Zhao *et al.*, 1996). The A_{2A}AR plays a more general role in suppressing inflammation by regulating cytokine production by macrophages. For example, adenosine inhibits release of TNFα and IL-12 from macrophages predominantly through activation of A_{2A}ARs (Kreckler *et al.*, 2006; Haskó *et al.*, 2000). In addition to suppressing pro-inflammatory cytokine production, adenosine and the A_{2A}AR-selective agonist CGS21680 have been found to potentiate production of the anti-inflammatory cytokine, IL-10 (Haskó *et al.*, 2000). This effect has been confirmed using macrophages from A_{2A}AR-deficient mice which fail to produce IL-10 in response to treatment with *E. coli* and adenosine while the same treatment induces a dramatic increase in IL-10 production by wild-type macrophages (Csóka *et al.*, 2007). A_{2A}AR-selective agonists have also been found to produce effects which may limit the course of inflammation through ligation of A_{2A}ARs expressed by the endothelium. For example, ATL-146E inhibits neutrophil and macrophage adhesion to the endothelium through down-regulation of VCAM-1, ICAM-1 and P-selectin on the activated endothelium (McPherson *et al.*, 2001). The anti-inflammatory effects of A_{2A}AR stimulation on endothelial cells are discussed further in chapter 3.

In addition to these effects on cells of the innate immune system, the A_{2A}AR also plays key roles in regulating T lymphocyte function. For example, the A_{2A}AR-selective agonist ATL313 has been shown to suppress proliferation of naïve $CD4^+$ T cells by inhibiting production of IL-2 and expression of the IL-2 receptor, CD25 (Sevigny *et al.*, 2007). In this study, ATL313 also reduced expression of the co-stimulatory molecule CD40L (Sevigny *et al.*, 2007) which binds to CD40 on macrophages and in conjunction with IFNγ, induces their activation (Stout *et al.*, 1996). In addition, stimulation of the A_{2A}AR in activated $CD4^+$ T cells inhibits IL-4 and IFNγ production (Naganuma *et al.*, 2006; Lappas *et al.*, 2006). Another small subset of T cells more recently recognised to be regulated by

the A_{2A}AR is formed by the invariant NK (iNKT) cells, so called because they express an invariant form of the T cell receptor alongside NK cell markers such as NK1.1 (Haskó *et al.*, 2008, Kronenberg, 2005). Unlike conventional T cells, iNKT cells can be rapidly activated early in inflammatory responses and produce copious amounts of cytokines shortly after TCR engagement. iNKT cells function in innate immunity by recognising self or foreign lipid antigens presented by APCs via an MHC1-related molecule called CD1d (Kronenberg, 2005). iNKT cells also express the A_{2A}AR and activation has been shown to suppress iNKT production of IFNγ induced by the marine sponge glycolipid α-galactoceramide (Lappas *et al.*, 2006).

The role of the A_{2A}AR in regulating inflammatory responses has been demonstrated in several *in vivo* models of inflammation and tissue injury and has been particularly well studied in ischaemia reperfusion injury (IRI). For example, in rats subjected to IRI, the A_{2A}AR agonist DWH-146e causes a dramatic reduction in tissue injury that is associated with reduced neutrophil accumulation and adhesion molecule expression (Okusa *et al.*, 2000). Similarly, Day *et al.* (2004) found that ischaemic liver injury and chemokine production were suppressed in mice treated with ATL-146E. Meanwhile, A_{2A}AR-deficient mice failed to respond to ATL-146E and liver injury was exacerbated compared with wild-type mice indicating that endogenously produced adenosine had a protective role (Day *et al.*, 2004). More recently, A_{2A}AR activation has been shown to be beneficial in protecting against hepatic IRI due to effects on iNKT cells. Lappas *et al.* (2006) found that treatment with ATL146e immediately after reperfusion resulted in reduced injury compared to untreated mice. A similar reduced response was observed in RAG-1-deficient mice which lack mature lymphocytes and in mice treated with antibodies against either NK1.1 to deplete NK cells or CD1d to block iNKT cell activation. Meanwhile, liver injury in RAG-1-deficient mice following IRI could be reconstituted to wild-type levels by adoptive transfer of $NK1.1^+$ cells from wild-type mice. These findings suggested that iNKT cells play a critical role in mediating IRI. Adoptive transfer of NK cells from A_{2A}-deficient mice was also able to reconstitute liver injury in RAG-1-deficient mice but IRI could not be attenuated by ATL146e indicating that ATL146e exerts its effects by activating receptors on NKT cells. Similarly, $NK1.1^+$ cells from IFNγ-deficient mice could not reconstitute the response showing that injury was dependent on production of IFNγ by NKT cells. Taken together, these finding indicate that hepatic IRI is initiated by activation of iNKT cells and that activation of the A_{2A}AR inhibits this response (Lappas *et al.*, 2006).

1.5 G protein-coupled receptors

G protein-coupled receptors (GPCRs) constitute the largest single family of signalling receptors, estimated to represent 1 % of the human genome (Takeda *et al.*, 2002; Bockaert and Pin, 1999). GPCRs are extremely diverse in their functions, transducing signals from a vast array of extracellular stimuli including photons of light, ions, odorants, amino acids, nucleotides, peptides and phospholipids (Kristiansen, 2004). GPCRs signal predominantly via interaction with and activation of heterotrimeric G-proteins which in turn modulate the activity of numerous effector proteins resulting in a wide range of physiological responses. The significance of GPCRs is further demonstrated by the fact that GPCRs represent over 25 % of current drug targets (Overington *et al.*, 2006).

Phylogenetic analysis has revealed that GPCRs can be divided into five main families, termed Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin (Fredriksson *et al.*, 2003). The Rhodopsin family is by far the largest family comprising approximately 670 receptors which bind a vast array of ligands including odorants and small endogenous agonists like adenosine, histamine and adrenaline (Fredriksson *et al.*, 2003; Kristiansen, 2004). While overall sequence homology is low between members of this family, they do share several conserved sequence motifs. The Secretin family is a small family (15 members) that in contrast to the Rhodopsin family, has significant sequence identity (21 – 67%) between members. These receptors bind peptide hormones such as secretin, glucagon and vasoactive intestinal peptide. With 33 members, the Adhesion family is the second largest family of GPCRs. These share some sequence similarities with Secretin receptors but bind extracellular matrix molecules such as glycosaminoglycan chondroitin sulphate via their long, diverse N-terminal regions (Lagerström and Schiöth, 2008; Fredriksson *et al.*, 2003). The Glutamate family consists of 22 members made up of metabotropic glutamate receptors, γ-aminobutyric acid receptors, calcium-sensing and taste receptors (Fredriksson *et al.*, 2003). Meanwhile, the Frizzled/Taste2 family comprises 10 frizzled receptors, one smoothened receptor and 25 taste2 receptors. There is not much overall similarity between the frizzled/smooth receptors and the taste2 receptors. However, they do share certain sequence motifs that are not found in other GPCR families. Frizzled/smoothened receptors bind Wnt glycoproteins and so participate in regulation of cell fate and proliferation during development while taste2 receptors are less well characterised but appear to act as bitter taste receptors (Lagerström and Schiöth, 2008; Fredriksson *et al.*, 2003).

1.5.1 Structure

There is no overall sequence similarity between GPCRs from different families. However, they do all share one common feature which is the presence of seven stretches of 25-35 amino acids that are mostly hydrophobic in nature. These regions are predicted to form alpha helices in the plasma membrane to give GPCRs their characteristic seven-transmembrane configuration (Ulloa-Aguirre *et al.*, 1999). Of the five families described above, GPCRs of the Rhodopsin family have been most well studied. Until recently, the only crystal structure information available for any GPCR came from studies on rhodopsin and this was used to predict a general structural model for other GPCRs (Gether, 2000; Baldwin, 1993). However, rhodopsin is unique among GPCRs in that it constitutively binds its ligand, 11-*cis*-retinal, via a covalent interaction which maintains the receptor in a stable conformation (Filipek *et al.*, 2003). All other GPCRs bind diffusible ligands and exist in much more flexible conformations. Therefore, the recent solving of structures for the β_2 adrenergic receptor (β_2AR) has been an important step in allowing more detailed analysis of structural features that may be conserved amongst GPCRs in general (Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007; Rasmussen *et al.*, 2007).

All GPCR structures obtained so far conform to a general model where the seven hydrophobic regions identified by sequence analysis form seven α-helical transmembrane domains (TM I-VII; figure 1.5). These are linked by alternating intracellular and extracellular loops of varying lengths which extend on either side of the membrane (Bockaert and Pin, 1999). The transmembrane domains form a barrel shape orientated perpendicular to the membrane with the helices tightly packed on the intracellular side but more openly arranged on the extracellular side to form a binding pocket (Unger *et al.*, 1997). The barrel conformation is stabilised by numerous hydrogen bonds and hydrophobic interactions, mostly between residues which are highly conserved between GPCRs. The majority of Rhodopsin family receptors (72 %) have an E/DRY triplet sequence within TM III (Kobilka and Deupi, 2007). In rhodopsin, Glu134 and Arg135 on TM III interact via a salt bridge while Arg135 also interacts with Thr251 and Glu247 on TM VI. This is thought to stabilise rhodopsin in an inactive conformation and prevent constitutive activity in the absence of light, which is essential for vision (Palczewski *et al.*, 2000). In the β_2AR, although the corresponding residues (Asp130-Arg131-Tyr132) are present, they do not form the same electrostatic interactions in the crystal structure and the transmembrane helices are arranged in a more open conformation (Rosenbaum *et al.*, 2007;

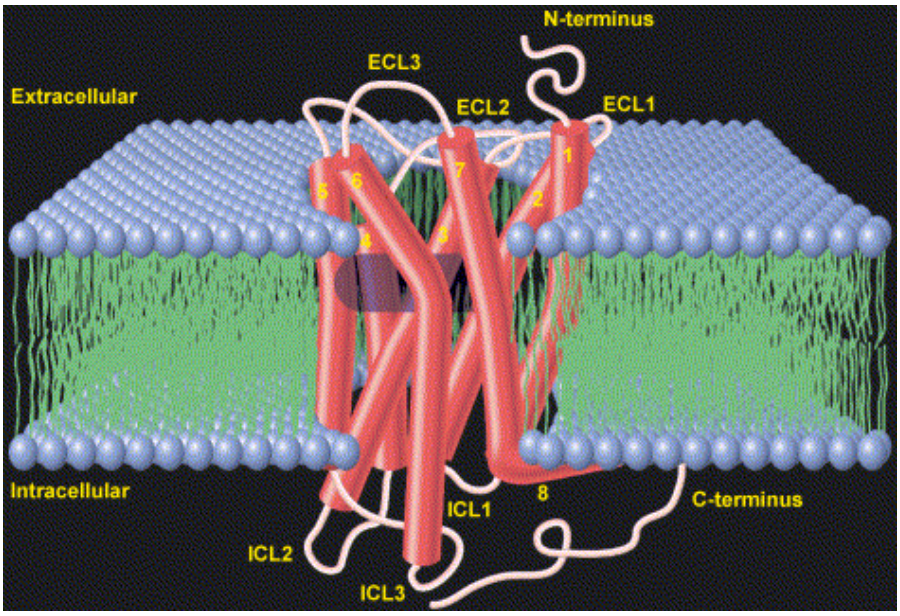

Figure 1.5 Schematic representation of a family A G protein-coupled receptor

Seven transmembrane domains form a barrel shape in the plasma membrane. These domains are linked by alternating intracellular and extracellular loops. The N-terminus is on the extracellular side of the membrane and typically contains sites for *N*-linked glycosylation. Meanwhile the C-terminus is intracellular and usually contains several serine and threonine residues representing sites for phosphorylation by kinases involved in desensitisation. This region is also the site of interactions between GPCRs and an increasing number of proteins reported to modulate GPCR signalling. (Figure from Kristiansen, 2004)

Rasmussen *et al.*, 2007; Cherezov *et al.*, 2007). This is proposed to reflect the ability of the β_2AR to isomerise between active and inactive conformations leading to constitutive activity in the absence of agonist (Audet and Bouvier, 2008; Rosenbaum *et al.*, 2007). Another difference between the crystal structures of rhodopsin and the β_2AR lies in the second extracellular loop. In rhodopsin, this loop folds deep into the rhodopsin molecule in contact with 11-*cis*-retinal and restricts access to the binding pocket from the extracellular side (Filipek *et al.*, 2003). However, in the β_2AR, the loop forms an α-helix on the periphery of the receptor which appears to be stabilised by disulphide bonds therefore leaving the binding pocket exposed and accessible to diffusible ligands (Cherezov *et al.*, 2007). The N- and C- terminal domains of GPCRs vary considerably in size and sequence. Most GPCRs contain consensus sites for *N*-linked glycosylation (N-X-S/T) in their extracellular N-terminal regions. The role of glycosylation is unclear but may be important for correct folding and trafficking as prevention of glycosylation has been found to reduce cell-surface expression of some GPCRs (Ulloa-Aguirre *et al.*, 1999). The C-terminus is intracellular and alongside the other intracellular portions of GPCRs is important for G protein recognition and activation. The C-terminal tails of most GPCRs contain several Ser/Thr residues representing potential sites for phosphorylation by kinases involved in desensitisation but this region is also the site of interactions between GPCRs and an increasing number of proteins reported to modulate GPCR signalling (Kristiansen, 2004; Hall and Lefkowitz, 2002).

1.5.2 Receptor activation

Activation of GPCRs is a dynamic process. In a simplified model, receptors exist in equilibrium between an inactive state and an active state that differ in their ability to activate G proteins (Samama *et al.*, 1993). In the absence of agonist, the receptor is maintained mostly in the inactive state by intramolecular interactions between the transmembrane domains. Agonist binding stabilises the active state in which the receptor is able to interact with G proteins and initiate downstream signalling (Maudsley *et al.*, 2005). However, it is now known that GPCRs can exist in multiple conformations and distinct states of activation that are influenced by the binding of ligands with different efficacies (Kobilka and Deupi, 2007).

Activation of the receptor initiates a change in conformation resulting from disruption of interhelical interactions including those mediated by the E/DRY motif. This causes TM III and TM VI to move apart and exposes key sites in intracellular loops 2 and 3 that allow the

receptor to interact with G proteins (Wess, 1997; Ulloa-Aguirre *et al.*, 1999; Bhattacharya *et al.*, 2008). The activated receptor acts as a guanine nucleotide exchange factor for the associated G protein, catalysing the exchange of GDP for GTP. The activated heterotrimeric G protein dissociates into an α subunit and a βγ dimer which independently activate downstream effector proteins (Ulloa-Aguirre *et al.*, 1999; figure 1.6).

1.5.3 G proteins

G-proteins are composed of three subunits termed α, β and γ. The alpha subunit contains two domains, a GTPase domain which binds and hydrolyses GTP to GDP, and a helical domain that buries GTP within the core of the protein (Noel *et al.*, 1993). The βγ subunits form a single functional unit which associates with a hydrophobic pocket in the GDP-bound form of the α subunit. On GTP binding, the hydrophobic pocket is lost and βγ dissociates (Lambright *et al.*, 1996).

G proteins can be divided into four groups based on sequence similarities between their α subunits and the distinct sets of downstream effector proteins with which they interact (Cabrera-Vera *et al.*, 2003, Neves *et al.*, 2002). Active $G\alpha_s$ subunits classically stimulate, while $G\alpha_{i/o}$ subunits inhibit, adenylyl cyclase (AC) activity, thereby altering intracellular levels of cAMP. Elevation of intracellular levels of cAMP results in activation of downstream effector proteins such as PKA, exchange proteins directly activated by cAMP (Epacs) and cyclic nucleotide-gated ion channels (Beavo and Brunton, 2002; figure 1.6). $G\alpha_{q/11}$ subunits activate different PLCβ isoforms leading to the generation of the second messengers inositol-1,4,5-trisphosphate (IP_3) and sn1,2-diacylglycerol (DAG; Neves *et al.*, 2002; Wess, 1998). The $G\alpha_{12/13}$ subunits have been found to interact with a number of effector proteins, the most well-characterised being guanine nucleotide exchange factors for the RhoA family of monomeric small G proteins (Kelly *et al.*, 2007).

Free βγ subunits generated on dissociation of the active α subunit also have important roles in downstream signalling with the ability to interact with a large number of effectors including PLCβ, AC, G protein-coupled receptor kinase (GRK) 2, components of MAPK pathways and Ca^{2+} and K^+ channels (Cabrera-Vera *et al.*, 2003). Some of these targets are shared by α subunits and βγ subunits can have effects that are either synergistic or opposing to the activity of the α subunit (Wess, 1998). Free α subunits also have the ability to modulate βγ activity. This is because the structure of the βγ subunit is not altered

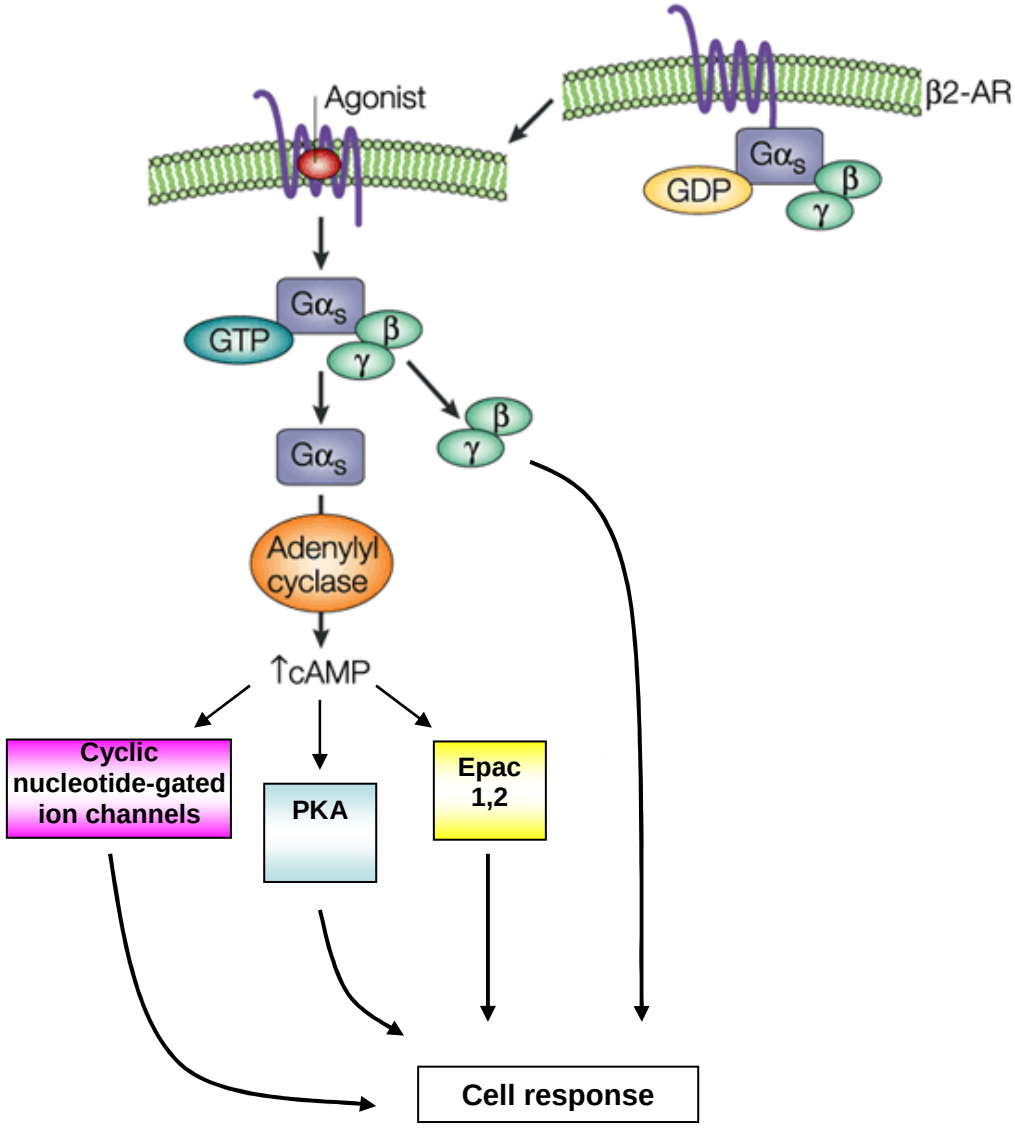

Figure 1.6 Classical G-protein-dependent signalling

Agonist binding to the receptor initiates a conformational change that allows the receptor to interact with G proteins. The activated receptor acts as a guanine nucleotide exchange factor for the associated G protein, catalysing the exchange of GDP for GTP. The activated heterotrimeric G protein dissociates into an α subunit and a βγ dimer which independently activate downstream effector proteins. $G_{\alpha s}$ activates adenylyl cyclase (AC) resulting in an elevation in the intracellular levels of cAMP which then activates downstream effectors PKA, exchange proteins directly activated by cAMP (Epacs) and cyclic nucleotide-gated ion channels. Meanwhile, free βγ subunits have the ability to interact with a large number of effectors including PLCβ, AC and components of MAPK pathways. (Adapted from Pierce *et al.*, 2002)

on dissociation from active GTP-bound α meaning that following GTP hydrolysis, free GDP-bound α subunits can bind and reform the original inactive trimeric G protein (Cabrera-Vera *et al.*, 2003; Wess, 1998). Hydrolysis of GTP to GDP by the α subunit marks termination of the GPCR activation cycle and is regulated by regulator of G-protein signalling (RGS) proteins. These proteins act as GTPase activating proteins for the α subunit promoting the hydrolysis of GTP and accelerating the deactivation of the pathway (De Vries *et al.*, 2000).

1.5.4 Regulation

Many mechanisms have evolved to regulate GPCR signalling. One of the best-studied is the phenomenon of receptor desensitisation which is crucial in protecting the cell from over-stimulation. The process of desensitisation results in the dampening of receptor responsiveness despite the continued presence of agonist. Desensitisation is mediated by several different mechanisms including phosphorylation of receptors resulting in uncoupling from G proteins, sequestration of receptors by internalisation, receptor degradation and downregulation of receptor gene expression (Kristiansen, 2004; Ferguson, 2001). The importance of this process is demonstrated in diseases involving mutations in genes encoding proteins which regulate desensitisation. For example, patients with Oguchi's disease suffer night blindness and retinal degeneration as a result of over-stimulation of rhodopsin (Métayé *et al.*, 2005). Desensitisation may be described as homologous or heterologous. Homologous desensitisation is initiated on binding of agonist to a receptor and results in dampening of signalling from the same receptor. Meanwhile during heterologous desensitisation, agonist activation of one receptor leads to dampening of signalling from other unrelated receptors even if they are not occupied by agonist (Kelly *et al.*, 2008; Pierce *et al.*, 2002).

1.5.4.1 Phosphorylation

Typically, agonist stimulation leads to rapid desensitisation (seconds to minutes) as a result of receptor phosphorylation. This is mediated by both second messenger-dependent kinases, such as PKC and PKA, and a distinct family of seven G protein-coupled receptor kinases (GRKs). Phosphorylation mediated by PKC or PKA results in the direct uncoupling of receptors from their respective G protein. Meanwhile, GRK phosphorylation promotes the binding of cytosolic inhibitory proteins called arrestins, which sterically inhibit further interactions between the receptor and the G protein and

therefore terminate downstream signalling (Kristiansen, 2004; Krupnick and Benovic, 1998; figure 1.7).

1.5.4.1.1 G protein-coupled receptor kinases (GRKs)

GRKs mediate a very general mechanism of desensitisation that is homologous in nature owing to the fact that GRKs selectively phosphorylate agonist-occupied receptors. There are seven GRK family members, termed GRK1-7. GRK1 and 7 are primarily expressed in the retina where they regulate the opsin light receptors while GRK4 is mainly found in the testis. GRKs 2, 3, 5 and 6 however, are widely expressed and phosphorylate a wide range of receptors with overlapping preferences (Premont and Gainetdinov, 2007). GRK family members share a common functional domain structure with an N-terminal substrate recognition domain, a central catalytic domain, and a C-terminal domain that is involved in membrane targeting (Ferguson, 2001). Following receptor stimulation, GRKs 1, 2 and 3 are translocated to the plasma membrane while GRKs 4, 5 and 6 are found primarily at the membrane even in the absence of agonist. GRKs bind to activated receptors and phosphorylate specific serine and threonine residues in their cytoplasmic regions (Premont *et al.*, 1995). In the case of the β_2AR, GRK1, GRK2 and GRK5 phosphorylate sites in the C-terminal tail of the receptor (Premont *et al.*, 1994) while the M_2 muscarinic receptor, which has a short C-terminal tail, is phosphorylated on residues in its third intracellular loop (Nakata *et al.*, 1994). A specific consensus sequence for phosphorylation by GRKs has not been defined. However, in studies using synthetic peptides, GRK1, GRK2 and by extension GRK3 have been found to preferentially phosphorylate serine and threonine residues present in an acidic environment (Onorato *et al.*, 1991; Benovic *et al.*, 1990).

GRK-mediated phosphorylation in itself has little effect on receptor function but it triggers the desensitisation process by increasing the affinity of the receptor for arrestin proteins. There are four arrestin family members. Visual and cone arrestin are expressed exclusively in the retina while β-arrestin 1 and β-arrestin 2 are ubiquitously expressed (Luttrell and Lefokowitz, 2002). Arrestins bind to regions of GRK-phosphorylated receptors involved in G-protein coupling thereby mediating desensitisation by sterically blocking interactions with G proteins. In addition, the β-arrestins further contribute to desensitisation by facilitating agonist-induced internalisation as described below (Luttrell and Lefokowitz, 2002).

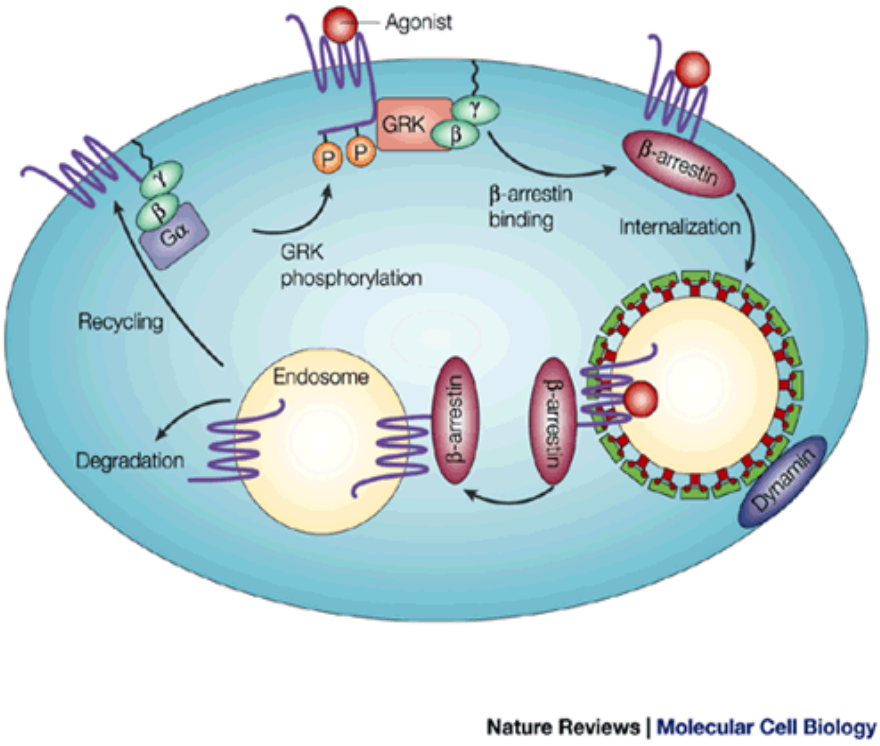

Figure 1.7 The role of GRKs and β-arrestin in desensitisation and internalisation

Following agonist-stimulation, GPCRs undergo a conformational change which allows them to act as a guanine nucleotide exchange factor for G proteins. The activated GPCR is recognised by members of the G protein-coupled receptor kinase (GRK) family which phosphorylate the receptor at specific sites on the intracellular loops and C-terminal tail. GRK phosphorylation promotes the binding of cytosolic inhibitory proteins called arrestins, which sterically inhibit further interactions between the receptor and the G protein and therefore terminate downstream signalling. β-arrestins also mediate internalisation of the receptor by interacting with components of the endocytic machinery required for formation of clathrin-coated pits, including the heavy chain of clathrin itself and the clathrin adapter protein AP-2. Newly formed vesicles are pinched off from the plasma membrane by the GTPase dynamin to form endosomes. Receptors may be dephosphorylated by phosphatases present in endosomes and recycled back to the cell surface or they may be targeted to lysosomes for degradation. (Figure from Pierce *et al.*, 2002)

1.5.4.1.2 2nd messenger dependent kinases

GPCR stimulation results in elevation of cellular levels of second messengers such as cAMP and DAG which activate the second messenger-dependent kinases PKA and PKC respectively. PKA and PKC have the potential to phosphorylate a multitude of downstream targets but they can also feedback and phosphorylate GPCRs to regulate their activity in either a homologous or heterologous manner. For example, in response to agonist treatment, the β_2AR is phosphorylated by PKA on serine262 within the PKA consensus sequence RRSSK[263] (Yuan *et al.*, 1994; Clark *et al.*, 1989). This sequence is in the third intracellular loop of the receptor adjacent to sites required for coupling to G_s (O'Dowd *et al.*, 1988; Strader *et al.*, 1987) and so it is likely that desensitisation occurs as a result of phosphorylation inhibiting the receptor/G protein interaction. Phosphorylation of the β_2AR by PKA appears to occur independently of GRK-mediated phosphorylation (Vaughan *et al.*, 2006) and is believed to be an important mechanism of desensitisation at low agonist concentrations when GRK activity is low. Phosphorylation at the PKA site occurs at low agonist concentrations (EC_{50} = 20-40 pM epinephrine) because only small changes in cAMP are required to activate PKA while phosphorylation by GRKs requires higher concentrations (EC_{50} = 200 nM epinephrine) as receptors must be occupied (Tran *et al.*, 2004). Interestingly, phosphorylation by PKA not only results in desensitisation by uncoupling the receptor from G_s, it also increases receptor affinity for G_i, thereby converting the stimulatory effect on AC to an inhibitory one. In addition, coupling to G_i allows activation of the ERK pathway that does not occur through G_s, indicating a role for receptor phosphorylation in initiating new signalling events (Daaka *et al.*, 1997). Several other receptors have been reported to undergo desensitisation in response to phosphorylation by PKC. For example, desensitisation of the α_{2A}AR is regulated by phosphorylation by PKC on serine 360 in the third intracellular loop of the receptor (Liang *et al.*, 2002) while in the case of the α_{1B}AR (Diviani *et al.*, 1997) and the type 1A angiotensin II receptor (Tang *et al.*, 1998), phosphorylation of serine residues in the C-terminal tail is required for PKC-mediated desensitisation.

Other serine/threonine kinases implicated in the regulation of GPCR activity are casein kinase 1α (CK1α) and casein kinase 2 (CK2). Agonist mediated phosphorylation of the third intracellular loop of the G_q coupled muscarinic M_3 acetylcholine receptor is at least in part mediated by CK1α (Budd *et al.*, 2001, Tobin *et al.*, 1997). The functional significance of CK1α-mediated phosphorylation on receptor activity has been

demonstrated. M_3 activation of the ERK pathway is compromised in CHO cells expressing either a mutant M_3 receptor lacking the third intracellular loop or a dominant negative mutant of CK1α (Budd *et al.*, 2001). CK1α phosphorylates serine residues within consensus sequences commonly found in GPCRs indicating potential for regulating phosphorylation of GPCRs in general (Tobin, 2002). CK2 has also been shown to phosphorylate the M_3 receptor. This has no effect on internalisation of the receptor or agonist-mediated ERK activation but does affect Jun kinase MAPK activation demonstrating that phosphorylation by different kinases can modulate receptor signalling in different cell types (Torrecilla *et al.*, 2007).

1.5.4.2 Internalisation

Following agonist-stimulation, many receptors undergo internalisation into endocytic vesicles. This contributes to desensitisation by sequestering receptors away from the cell surface but also promotes receptor resensitisation through dephosphorylation and recycling to the plasma membrane (Ferguson, 2001). The best well characterised mechanism of internalisation involves β-arrestin-mediated targeting of receptors to clathrin-coated pits (Luttrell and Lefkowitz, 2002, Ferguson, 2001; figure 1.7). This is facilitated by the ability of β-arrestins to interact with components of the endocytic machinery required for formation of clathrin-coated pits, including the heavy chain of clathrin itself and the clathrin adapter protein AP-2 (Luttrell and Lefkowitz, 2002; Claing *et al.*, 2002). Newly formed vesicles are pinched off from the plasma membrane by the GTPase dynamin to form endosomes. Receptors may be dephosphorylated by phosphatases present in endosomes and recycled back to the cell surface or they may be targeted to lysosomes and degraded (Luttrell and Lefkowitz, 2002; Claing *et al.*, 2002).

An alternative mechanism for receptor internalisation appears to involve cholesterol-rich plasma membrane structures called caveolae (Claing *et al.* 2002). It is not entirely clear how receptors are targeted for internalisation by caveolae but numerous GPCRs, probably due to their palmitoylated nature, have been found to be localised to caveolae including the M_2 muscarinic receptor (Feron *et al.*, 1997), the β_2-adrenergic receptor (Dupree *et al.*, 1993) and the endothelin ET_B receptor (Teixeira *et al.*, 1999). Furthermore, agents which disrupt the structure of caveolae have been shown to inhibit internalisation of receptors such as the ET_B endothelin receptor (Okamoto *et al.*, 2000).

1.5.5 GPCR-interacting proteins

Classically, signalling by GPCRs relies on their ability to interact with heterotrimeric G proteins. However, this is a very simplistic model as GPCRs are able to interact with a large number of proteins other than G proteins and the kinases and arrestins which mediate desensitisation and internalisation (Bockaert *et al.*, 2004; Heuss and Gerber, 2000). Many GPCRs have specific sequence motifs in their C-terminal tails or in their third intracellular loop which enable interactions with proteins which have particular protein-protein interaction domains within their structures (Kristiansen, 2004; Heuss and Gerber, 2000). For example, PDZ domains (named after their discovery in postsynaptic density protein (PSD) 95, the Drosophila septate junction protein Discs-large, and the epithelial tight junction protein ZO-1) which bind specifically to short sequences at the very C-terminus of target proteins (Sheng and Sala, 2001). Other proteins interact via Src homology 2 (SH2), SH3 or enabled Vasp homology domains (EVD) or are recruited by arrestins. Proteins recruited to the GPCR in this manner may directly initiate alternative signalling pathways independently of G proteins and/or may act as adaptor or scaffold proteins which allow recruitment and spatial organisation of additional signalling components to improve the specificity and efficiency of downstream signalling (Bockaert *et al.*, 2004; Hall and Lefkowitz, 2002; Heuss and Gerber, 2000).

1.5.5.1 PDZ

A clear example of a GPCR-binding protein which promotes efficient signalling through scaffolding interactions is the large cytoplasmic protein INAD which interacts with *Drosophila* rhodopsin (Xu *et al.*, 1998; Chevesich *et al.*, 1997). INAD has five PDZ domains, two of which mediate binding to rhodopsin while the other three are involved in interactions with a number of proteins required for visual signalling in *Drosphila* (Xu *et al.*, 1998). Light activation of rhodopsin results in G_q-mediated stimulation of PLCβ which results in elevation of intracellular calcium levels, activation of PKC and opening of calcium-regulated transient receptor potential (TRP) channels (Tsunoda and Zuker, 1999). INAD has been found to interact with most of the components of this signalling pathway including PLCβ, PKC and TRP thereby creating a highly organised “transducisome” to allow extremely rapid signalling (Tsunoda *et al.*, 1997). The importance of these interactions has been demonstrated using *Drosophila* mutants lacking functional INAD. In INAD-null cells, TRP, PLCβ and PKC are mislocalised and photoreceptors have profound signalling defects, only responding to the highest level of stimuli (Tsunoda *et al.*, 1997).

In addition to scaffolding roles, the association of some PDZ-containing proteins with GPCRs has been shown to regulate the nature of downstream signalling events. For example, agonist-induced association of the β_2AR with the Na^+/H^+ exchanger regulatory factor (NHERF) 1 confers the ability to positively regulate renal Na^+/H^+ exchange by Na^+/H^+ exchanger 3 (NHE3; Hall *et al.*, 1998). Normally, activation of a G_s coupled receptor such as the β_2AR would not be expected to have this effect as increasing cAMP usually leads to PKA-mediated association of NHERF1 with NHE3 which inhibits its activity (Weinman and Shenolikar, 1993). The stimulatory effect of the β_2AR on NHE3 function can be blocked by mutating the final residue in the receptor so that it cannot bind NHERF1. Therefore, it is proposed that the agonist-induced association of NHERF1 with β_2AR displaces NHERF1 from NHE3 and removes its inhibitory effect leading to an increase in NHE3 activity (Hall *et al.*, 1998).

The closely-related NHERF2 has been found to modulate downstream signalling from the parathyroid hormone (PTH1) receptor. NHERF2 interacts simultaneously with the parathyroid hormone 1 (PTH1) receptor and PLCβ through two separate PDZ domains (Mahon *et al.*, 2002). The PTH1 receptor can couple to G_s, G_q and G_i although in most cells, signalling occurs predominantly through activation of AC (Abou-Samra *et al.*, 1992). When the PTH1 receptor is expressed in cells lacking NHERF, signalling occurs almost exclusively through AC (Mahon *et al.*, 2002). However, when wild-type PTH1 and NHERF are coexpressed, only small increases in cAMP are observed following receptor stimulation and signalling occurs predominantly through activation of PLC. This response can be reversed and AC signalling partially restored by treatment with pertussis toxin indicating that NHERF binding acts like a molecular switch to promote PTH1 receptor signalling through G_i rather than G_s (Mahon *et al.*, 2002; Weinman *et al.*, 2006). NHERF2 also plays a scaffolding role not dissimilar to INAD by bringing the PTH1 receptor and PLCβ into close proximity thereby promoting more efficient signalling through G_i (Mahon *et al.*, 2002).

1.5.5.2 Non-PDZ

While PDZ-containing proteins bind predominantly to the sequences at the very C-terminus of GPCRs, other proteins bind to sequences in other regions of the receptor tail. Certain proteins have been found to bind via their SH2 domains to phosphorylated tyrosine residues in the tail of GPCRs to enable organisation of signalling complexes in a manner similar to that seen for growth factor receptors (Hall *et al.*, 1999). This is believed to be

the underlying mechanism by which stimulation of the angiotensin II AT_{1A} receptor results in activation of the JAK/STAT pathway which is otherwise generally regarded as a cytokine/growth factor-regulated pathway (Godeny *et al.*, 2007; Marrero *et al.*, 1998). JAK2 associates with the AT_{1A} receptor via a specific phosphorylated YIPP motif in the C-terminal tail of the receptor (Ali *et al.*, 1997) but since JAK2 does not contain an SH2 domain, it was not clear initially how this was achieved. It is now apparent that this association depends upon the SH2 domain-containing protein SHP2 which appears to act as an adaptor for JAK2 (Godeny *et al.* 2007, Marrero *et al.*, 1998). JAK2 then recruits and phosphorylates STAT1 leading to activation of the JAK/STAT pathway (Ali *et al.*, 2000).

Another class of proteins which interacts with phosphorylated motifs in target proteins is the 14-3-3 family which comprises seven isoforms termed β, ε, γ, η, σ, τ and ζ (Fu *et al.*, 2000). Numerous biological activities have been attributed to 14-3-3 proteins including cell signalling, regulation of cell cycle progression, intracellular trafficking and transcription (Aitken, 2006). Many of these functions involve the regulation of interactions between proteins with 14-3-3 proteins often acting as scaffold or adapter proteins (Tzivion *et al.*, 2001). This is facilitated by the fact that 14-3-3 proteins exist as dimers and so can bind to two interaction partners simultaneously (Jones *et al.*, 1995). 14-3-3 proteins bind to proteins containing phosphorylated serine residues within either R-S-X-pS-X-P or R-X-ϕ-X-pS-X-P motifs (Yaffe *et al.*, 1997; Muslin *et al.*, 1996) although some interacting proteins do not contain these sequences indicating that other modes of binding do occur (Aitken *et al.*, 2006). There are several reports of 14-3-3 proteins interacting with GPCRs with varying functional consequences. For example, 14-3-3ε interacts with the β_1AR with effects on regulation of cardiac repolarisation by the voltage-gated potassium channel Kv11.1 (Tutor *et al.*, 2006). This was found to occur in a PKA-dependent manner and required the presence of two PKA phosphorylation motifs in the third intracellular loop and tail of the receptor, suggesting that 14-3-3ε binds at these sites. The functional effects of this interaction were demonstrated by co-transfecting Kv11.1 and 14-3-3ε in the presence or absence of the β_1AR. It was found that in cells lacking the β_1AR, Kv11.1 bound to 14-3-3ε, an interaction which enhances Kv11.1 activity (Kagan *et al.*, 2002), while coexpression of the β_1AR disrupted this interaction. It was therefore proposed that β_1AR competing for 14-3-3ε binding represents a novel mechanism by which the β_1AR regulates the Kv11.1 channel (Tutor *et al.*, 2006). In further examples of 14-3-3 proteins interacting with GPCRs, 14-3-3ζ is reported to bind to the α_{2A}, α_{2B} and α_{2C} ARs (Prezeau *et al.*, 1999) and to the thromboxane TPα and TPβ receptors (Yan *et al.*,

2006). This is proposed to facilitate Ras-mediated activation of the ERK pathway owing to the ability of 14-3-3 proteins to bind and aid in the activation of Raf (Tzivion *et al.*, 1998; Luo *et al.*, 1996). This is discussed further in Section 3.3.

1.5.5.3 Arrestins

More generalised scaffold formation is mediated by arrestins which do not require specific motifs in order to interact with GPCRs. Following agonist activation, most GPCRs are phosphorylated by GRKs which leads to receptor association with β arrestins and uncoupling from G proteins (Ferguson, 2001). In addition to interactions with proteins involved in receptor endocytosis during desensitisation, β arrestins also bind a variety of other proteins such as Src family tyrosine kinases and components of MAPK pathways and recruit them to agonist-occupied receptors (Luttrell and Lefkowitz, 2002). For example, agonist stimulation of the β_2AR promotes its association with Src via β-arrestin 1. As a consequence of binding to β-arrestin 1, Src is activated resulting in Ras-dependent ERK signalling (Luttrell *et al.*, 1999). β-arrestins further influence MAPK signalling by scaffolding together the appropriate kinases of the ERK and JNK MAPK pathways to allow specificity in signalling (Reiter and Lefkowitz, 2006).

β-arrestins have also been found to bind to members of the phosphodiesterase (PDE) family which degrade cAMP (Perry *et al.*, 2002). Following agonist stimulation of the β_2AR, the PDE isoform PDE4D5 is translocated to the receptor concomitantly with β-arrestin 1 or β-arrestin 2. By this means, arrestins not only dampen signalling from the receptor to AC, they also increase the rate of cAMP degradation, thereby reducing activation of PKA at the plasma membrane (Perry *et al.*, 2002). Phosphorylation of the β_2AR by membrane-localised PKA switches G protein coupling of the receptor from G_s to G_i leading to activation of the ERK pathway (Daaka *et al.*, 1997). The importance of β-arrestin-mediated recruitment of PDE4D5 to the receptor in regulating this switch has been demonstrated using a dominant negative form of PDE4D5 which cannot bind β-arrestins. Overexpression of this construct in HEK 293 cells resulted in enhanced agonist-induced phosphorylation of the β_2AR and a marked potentiation in ERK activation (Baillie *et al.*, 2003).

Other important binding partners of arrestins are the ubiquitin E3 ligases Mdm2 and Nedd4, which have been shown to have distinct roles in regulating intracellular trafficking

of the β_2AR. β-arrestin 2 is ubiquitinated by Mdm2 and this modification is required for rapid agonist-induced internalisation of the β_2AR (Shenoy *et al.*, 2001). Meanwhile, recruitment of Nedd4 to the β_2AR via β-arrestin 2 facilitates ubiquitination of the receptor and is required for targeting receptors to lysosomes for degradation following long-term stimulation (Shenoy *et al.*, 2008).

1.6 The A_{2A}AR

In common with the other AR subtypes, the A_{2A}AR assumes a protein structure typical of GPCRs with TM5, 6 and 7 forming a hydrophobic pocket where adenosine binds (Kim *et al.*, 1995). The recent solving of the crystal structure of the A_{2A}AR in complex with high affinity anatagonist ZM241385 has revealed that the extracellular loops of the receptor are arranged in a different manner to those of the previously characterised β1AR, β2AR and rhodopsin (Jaakola *et al.*, 2008). In particular, a network of disulphide bridges contributes to the formation of a rigid open structure that exposes the ligand-binding cavity to solvent. ZM241385 binds within this cavity in an extendend conformation perpendicular to the membrane in quite a different manner to ligands of the βARs and rhodopsin (Jaakola *et al.*, 2008). Another finding from this study was that an eighth helical segment known as helix 8 which is found in the cytoplasmic tail of the receptor is stabilised by interactions with helix 1. Many GPCRs are palmitoylated in this region (Kristiansen, 2004) and in rhodopsin, helix 8 is stabilised via interactions with the plasma membrane (Moench *et al.*, 1994). However, this cannot occur in the case of the A_{2A}AR as it does not contain sites for palmitoylation (Palmer and Stiles, 1995).

In common with other GPCRs, the A_{2A}AR has the conserved DRY sequence which is believed to be important for G protein activation present in its second intracellular loop (Palmer and Stiles, 1995). Two N-linked glycosylation consensus sequences are present in the second extracellular loop of all adenosine receptors (Palmer and Stiles, 1995). In the case of the A_{2A}AR, immunoblotting analysis suggests that only one of these sites is used (Palmer *et al.*, 1992). The significance of this modification is unclear but it does not appear to be important for ligand binding as inhibition of N-linked glycosylation has been shown to have no effect on the agonist binding characteristics of the A_{2A}AR (Piersen *et al.*, 1994). Structurally, the A_{2A}AR differs most noticeably from other adenosine receptors in its size. While the genes for human A_1, A_{2B} and A_3 receptors encode proteins of 326 (Libert *et al.*, 1992), 328 (Pierce *et al.*, 1992) and 318 residues respectively (Salvatore *et*

al., 1993), the A_{2A}AR is 412 amino acids long, the additional 84-94 amino acids of its sequence constituting an extended C-terminal tail (Furlong *et al.*, 1992; figure 1.8, figure 1.9).

The A_{2A}AR, like many GPCRs can form dimeric or possibly oligomeric complexes and studies using cell-surface biotinylation and of proteins and fluorescence resonance energy transfer (FRET) have indicated that homodimers may be the predominant form on the cell surface (Canals *et al.*, 2004). In addition, the A_{2A}AR has also been found to form heterodimers with the A_1AR and the dopamine D_2 receptor in striatal tissues (Fredholm *et al.*, 2007). The interaction of the A_{2A}AR with the A_1AR is thought to allow different signalling responses depending on the concentration of adenosine present. At low concentrations, the high-affinity A_1AR is activated preferentially leading to decreases in levels of cAMP while at higher concentrations, the A_{2A}AR is activated and levels of cAMP rise (Schiffmann *et al.*, 2007). Meanwhile, the interaction of the A_{2A}AR with the dopamine D_2 receptor is antagonistic with A_{2A}AR agonists such as CGS21680 reducing the affinity of the D_2 receptor binding site (Fuxe *et al.*, 2005).

1.6.1.1 G protein coupling

The region of the A_{2A}AR responsible for G protein coupling has not been fully described. Studies in other GPCRs indicate that several different regions may be important for interactions with G proteins, particularly regions in intracellular loops 2 and 3 and the membrane proximal portion of the C-terminal tail (Wess, 1997; Ulloa-Aguirre *et al.*, 1999). The importance of the third intracellular loop of the A_{2A}AR in mediating coupling to G_s has been shown in a study using a series of chimaeric A_1/A_{2A} receptors. Olah (1997) found that replacement of the third intracellular loop of the canine A_{2A}AR with corresponding sequences from the human A_1AR drastically reduced agonist-stimulated activation of AC. Through the use of more restricted chimaeras, a stretch of 15 amino acids in the N-terminal portion of intracellular loop 3 and in particular Lys219 and Glu212 were identified as being important for coupling to G_s (Figure 1.8). Similar examination of the second intracellular loop revealed that Gly118 and Thr119 at the junction of this loop and TMIV were required for G_s coupling. However, since individual substitutions or substitution with alanine residues had no effect on AC activation, it was suggested that these residues play a role in directing protein conformation to allow interaction with G proteins rather than directly activating G_s. Importantly, none of the more restricted chimaeras or individual mutations used in this study fully mimicked the effect of

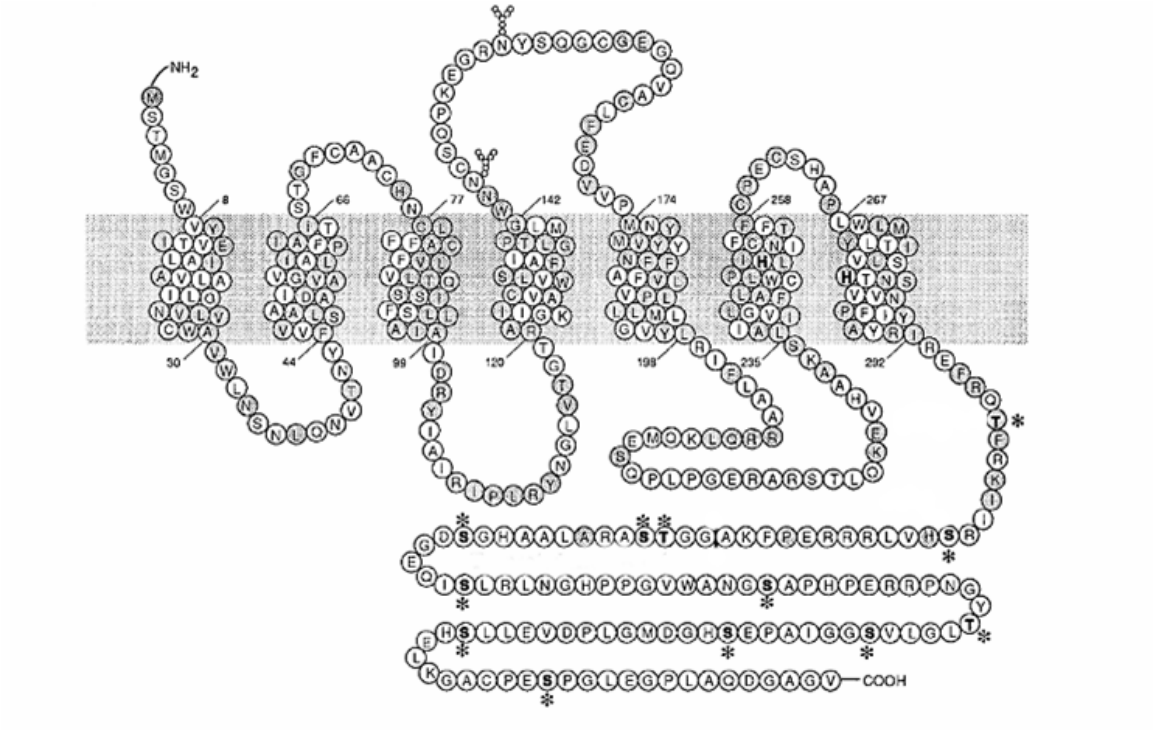

Figure 1.8 Schematic representation of the membrane topology of the canine A_{2A}AR

The A_{2A}AR has an extended C-terminal tail. Serine and threonine residues representing potential phosphorylation sites are marked by asterisks. The last ~100 amino acids of the receptor appear to be dispensable for agonist binding, G protein coupling and agonist-induced desensitisation. However, several proteins are reported to interact with the A_{2A}AR in this region. (Figure adapted from Palmer and Stiles, 1997)

```
                                                  ICL1
A2AAR_HUMAN      MPIMGSSVYITVELAIAVLAILGNVLVCWAVWLNSNLQNVTNYFVVSLAA 50
A2AR_CAN         MSTMGSWVYITVELAIAVLAILGNVLVCWAVWLNSNLQNVTNYFVVSLAA 50
                 *. *** ********************************************

A2AR_HUMAN       ADIAVGVLAIPFAITISTGFCAACHGCLFIACFVLVLTQSSIFSLLAIAI 100
A2AR_CAN         ADIAVGVLAIPFAITISTGFCAACHNCLFFACFVLVLTQSSIFSLLAIAI 100
                 ************************.***:******************

                           ICL2
A2AR_HUMAN       DRYIAIRIPLRYNGLVTGTRAKGIIAICWVLSFAIGLTPMLGWNNCGQPK 150
A2AR_CAN         DRYIAIRIPLRYNGLVTGTRAKGIIAVCWVLSFAIGLTPMLGWNNCSQPK 150
                 **********************:******************.***

A2AR_HUMAN       EGKNHSQGCGEGQVACLFEDVVPMNYMVYFNFFACVLVPLLLMLGVYLRI 200
A2AR_CAN         EGRNYSQGCGEGQVACLFEDVVPMNYMVYYNFFAFVLVPLLLMLGVYLRI 200
                 **:*:************************:**** ***************

                            ICL3
A2AR_HUMAN       FLAARRQLKQMESQPLPGERARSTLQKEVHAAKSLAIIVGLFALCWLPLH 250
A2AR_CAN         FLAARRQLKQMESQPLPGERARSTLQKEVHAAKSLAIIVGLFALCWLPLH 250
                 **********************************************

A2AR_HUMAN       IINCFTFFCPDCSHAPLWLMYLAIVLSHTNSVVNPFIYAYRIREFRQTFR 300
A2AR_CAN         IINCFTFFCPECSHAPLWLMYLTIVLSHTNSVVNPFIYAYRIREFRQTFR 300
                 **********:**********:****************************

A2AR_HUMAN       KIIRSHVLRQQEPFKAAGTSARVLAAHGSDGEQVSLRLNGHPPGVWANGS 350
A2AR_CAN         KIIRSHVLRRREPFKAGGTSARALAAHGSDGEQISLRLNGHPPGVWANGS 350
                 *********::*****.*****.**********:****************

A2AR_HUMAN       APHPERRPNGYALGLVSGGSAQESQGNTGLPDVELLSHELKGVCPEPPGL 400
A2AR_CAN         APHPERRPNGYTLGLVSGGIAPESHGDMGLPDVELLSHELKGACPESPGL 400
                 **********:******* * **:*: **************.***.***

A2AR_HUMAN       DDPLAQDGAGVS 412
A2AR_CAN         EGPLAQDGAGVS 412
                 :.**********
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Figure 1.9 Sequence alignment of human and canine A_{2A}ARs

Sequences defining intracellular loop 1 (ICL1; Leu33-Val40), ICL2 (Ile108-Gly118) and ICL3 (Leu208-Ala221) as identified in the crystal structure of the human A_{2A}AR (Jaakola *et al.*, 2008) are shown in blue. The C-terminal tail of the receptor is shown in green. Identical residues are marked with an asterisk while (:) denotes a conserved substitution and (.) denotes a semi-conserved substitution. Residues with no similarity are unmarked.

substituting larger portions of the receptor suggesting that multiple amino acids are likely to be involved in selective coupling of the A_{2A}AR to G_s (Olah, 1997).

1.6.1.2 A_{2A}AR signalling

Classically, signalling through the A_{2A}AR relies on its coupling to G_s and stimulation of AC (Linden, 2001). This results in elevation of intracellular levels of cAMP which then activate downstream effectors including PKA, cyclic nucleotide-gated ion channels and exchange proteins directly activated by cAMP (Epacs; Beavo and Brunton, 2002). Stimulation of the A_{2A}AR also results in activation of the ERK signalling cascade. This can occur via G_s-dependent or G_s-independent mechanisms. For example, in CHO cells heterologously expressing the A_{2A}AR and in PC12 cells, G_s stimulation of AC has been shown to result in PKA-mediated activation of ERK via Src kinases (Klinger *et al.*, 2002a). Meanwhile, in endothelial cells, ERK can be activated independently of cAMP elevation and requires Ras (Sexl *et al.*, 1997).

1.6.1.3 Regulation of the A_{2A}AR

1.6.1.3.1 *Desensitisation*

The A_{2A}AR has been shown to undergo rapid functional desensitisation after short-term agonist treatment in a variety of cell types which express endogenous receptors including rat aortic vascular smooth muscle cells (Anand-Srivastava *et al.*, 1989), NG108-15 neuroblastoma × glioma hybrid cells (Mundell and Kelly, 1998), hamster smooth muscle DDT_1 MF-2 cells (Ramkumar *et al.*, 1991) and the PC12 rat adrenal tumour cell line (Chern *et al.*, 1993).

This effect and the various mechanisms responsible have been further characterised using CHO cells stably expressing the canine A_{2A}AR. In these cells, short-term exposure to agonist (30 minutes) resulted in a rapid reduction in subsequent agonist-induced AC activation (Palmer *et al.*, 1994). This was associated with receptor phosphorylation and reduced coupling to G_s. However, this effect could not be mimicked by treatment with forskolin or PMA indicating that phosphorylation by PKA or PKC was not responsible. Long-term agonist treatment (24 hours) produced a comparable reduction in AC activity but much slower recovery and this was associated with receptor downregulation (Palmer *et al.*, 1994). The structural requirements for desensitisation of the A_{2A}AR have been examined using mutated forms of the canine A_{2A}AR expressed in CHO cells (Palmer and

Stiles, 1997). Palmer and Stiles (1997) found that truncation of the receptor removing 95 amino acids from the C-terminal tail did not inhibit desensitisation. In fact the mutant receptor desensitised faster than the wild-type form (T. Palmer; personal communication). This was surprising as numerous serine and threonine residues are present in this region representing potential sites for phosphorylation by regulatory kinases. Truncation of other GPCRs such as the α_{1B}AR (Lattion *et al.*, 1994) and the β_2AR (Bouvier *et al.*, 1988) which have relatively long C-terminal tails rich in serine and threonine residues abolishes agonist-induced receptor phosphorylation and desensitisation. However, an A_{2A}AR mutant in which two residues in the membrane proximal region of the C-terminal tail (Thr298 and Ser305) were replaced with alanine residues failed to exhibit any significant desensitisation response (Palmer and Stiles, 1997, figure 1.8). Generation of receptors with single mutations at these residues revealed that short-term desensitisation of the A_{2A}AR relies on the presence of the threonine residue at position 298. The mutant receptor lacking Thr298 also failed to undergo agonist-induced phosphorylation suggesting that phosphorylation of this single residue may be required for short-term desensitisation of the A_{2A}AR (Palmer *et al.*, 1997).

Because elevation of cAMP through stimulation of AC with forskolin does not induce desensitisation of the A_{2A}AR (Palmer *et al.*, 1994), it is unlikely that PKA is responsible for agonist-induced phosphorylation and desensitisation of the receptor. This indicates the involvement of a GRK, a theory which has been investigated further using NG108-15 cells which express endogenous A_{2A}ARs and GRK2 (Mundell *et al.*, 1997). Following short-term agonist treatment, the A_{2A}AR desensitised in these cells in a similar manner to that observed in CHO cells. However, on introduction of a mutant GRK2 (Lys220Arg) which lacks kinase activity, this response was significantly reduced. In a subsequent study, suppression of GRK2 expression using anti-sense cDNA revealed that this effect was due specifically to loss of GRK2 activity rather than effects on other GRKs present in these cells, indicating that GRK2 is at least partly responsible for mediating short-term desensitisation of natively expressed A_{2A}ARs (Mundell *et al.*, 1999).

1.6.1.3.2 Role of the C-terminal tail

In comparison to other adenosine receptors and to GPCRs in general, the A_{2A}AR has an unusually long C-terminal tail (Zezula and Freissmuth, 2008). However, the functional significance of this is not clear. As discussed above, truncation of the tail has no effect on agonist-induced phosphorylation and desensitisation of the receptor. Similarly, it has been

found that the agonist-binding properties of a mutant canine A_{2A}AR in which the last 102 residues have been replaced with a hexahistidine sequence are comparable to those of the wild-type receptor (Piersen *et al.*, 1994). The C-terminal tail also appears to be dispensable for G protein coupling and stimulation of AC (Palmer and Stiles, 1997). However, it does appear to be important for constitutive receptor signalling as truncated forms exhibit a reduced ability to activate AC in the absence of agonist (Klinger *et al.*, 2002b). The difference in AC activation between wild-type and truncated receptors only occurred in intact cells and so it was proposed that the higher levels of constitutive activity observed for the wild-type receptor were dependent on an additional as yet unidentified factor which bound to the receptor tail (Klinger *et al.*, 2002b).

The presence of 12 serine and threonine residues within the C-terminal tail of the A_{2A}AR suggests that phosphorylation in this region may be important for regulation of receptor activity (figure 1.8). Agonist-induced phosphorylation of the A_{2A}AR is associated with its desensitisation (Palmer *et al.*, 1994). However, the canine A_{2A}AR has also been shown to undergo PKC-mediated phosphorylation in response to PMA treatment or following stimulation of endogenous receptors which activate PKC (Palmer and Stiles, 1999). The canine A_{2A}AR contains three consensus PKC phosphorylation sequences within the C-terminal tail (Thr298, Ser320 and Ser335, figure 1.8). However, a mutant receptor in which these sites were disrupted displayed levels of basal and PMA-induced phosphorylation comparable with the wild-type receptor, indicating that although PKC regulates phosphorylation of the A_{2A}AR, it does not occur at these PKC consensus sites. Furthermore, PKC-mediated phosphorylation did not induce desensitisation as no significant changes in the signalling capacity or cell surface expression of the receptor were observed following PMA treatment (Palmer and Stiles, 1999). Thus, the role of PKC-mediated phosphorylation of the C-terminal tail of the A_{2A}AR remains undetermined. One possibility is that phosphorylation regulates the ability of the receptor to bind C-terminal interacting proteins. Several proteins have been reported to bind to the C-terminal tail of the A_{2A}AR as listed in Table 1. These interactions are discussed further in Chapter 4.

Interacting protein	**Interaction site on A_{2A}AR**	**Reference**
ARNO	291-312	Gsandtner *et al.*, 2005
α-actinin	293-321	Burgueño *et al.*, 2003
Ubiquitin-specific protease 4 (USP4)	385-412	Milojević *et al.*, 2006
TRAX	Not determined	Sun *et al.*, 2006
14-3-3τ	Not determined	M. Freissmuth, personal communication
D_2-dopamine receptor	Not determined	Fuxe *et al.*, 2005

Table 1: C-terminal interaction partners of the A_{2A}AR

ARNO, α-actinin, USP4 and TRAX have been identified as C-terminal interaction partners of the A_{2A}AR based on evidence from yeast two-hybrid screens and from co-immunoprecipitation and GST pull-down assays. The A_{2A}AR has been found to interact with the D_2-dopamine receptor through the use of co-immunoprecipitation and FRET analysis, confirming an association that has long been predicted to exist on the basis of physiological evidence.

Aims

The finding that levels of inflammatory cytokines and components of the NFκB signalling pathway are upregulated in A_{2A}AR-deficient mice suggest that one way in which the A_{2A}AR may limit inflammation is through suppression of pro-inflammatory signalling pathways. One of the aims of this study was to determine whether modulation of the NFκB and JAK/STAT pro-inflammatory signalling pathways by the A_{2A}AR plays a significant role in suppressing endothelial inflammation *in vivo* by examining the levels and activation status of components of these pathways in the aortae of A_{2A}AR-deficient mice.

A second aim of this study was to determine whether the human A_{2A}AR is regulated by phosphorylation as has been shown previously for the canine receptor. This was to be achieved by identifying stimuli which induce phosphorylation of the A_{2A}AR and the kinase responsible. In addition, it was of interest to determine whether stimuli-induced phosphorylation of the A_{2A}AR could have consequences for regulating interactions between the A_{2A}AR and 14-3-3τ and TRAX, two proteins which have been identified as interaction partners of the C-terminal tail of the receptor.

2 Materials and methods

2.1 Materials

American Radiolabeled Chemicals, St Louis, MO, USA

^{3}H-ZM241385 (20 Ci/mmol)

Bio-rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK

Precision Plus Protein Kaleidoscope Standards

Brandel Inc, Gaithersberg, MD, USA

Glass fibre filters

Cambridge Bioscience Ltd, Cambridge, UK

Cell Biolabs Inc QuickTitre Addenovirus Immunoassay Kit

GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK

Glutathione-Sepharose, protein G-Sepharose, [^{32}P] orthophosphate (8500-9120 Ci/mmol)

Inverclyde Biologicals, Bellshill, Lanarkshire, UK

Whatman Protran nitrocellulose membrane

Invitrogen, Paisley, UK

BioSource Mouse Inflammatory Four-Plex kit, Opti-mem, Lipofectamine, Oligofectamine, Gibco low-phosphate Dulbecco's modified Eagle's medium (DMEM) with GlutaMax

Lonza, Cambridge, UK

Endothelial basal medium (EBM) plus supplements, DMEM, Ham's F-12 medium

May and Baker Ltd, London, UK

Sagatal

Merck Chemicals Ltd, Nottingham, UK

Novagen GeneJuice

Perkin-Elmer Life Sciences, Waltham, MA, USA

Western Lightning Plus Enhanced chemiluminescence (ECL) substrate

Promega UK Ltd, Southampton, UK

*Xba*I and *Hind*III restriction enzymes, 1 kb DNA markers

Promocell, Heidelberg, Germany

Human umbilical vein endothelial cells (HUVECs)

Qiagen, Crawley, West Sussex, UK

Proteinase K, Taq DNA polymerase, dNTPs, PCR buffer, Q-solution, Plasmid Maxi Kit

Santa Cruz Biotechnology Inc, Santa cruz, CA, USA

Horseradish peroxidise (HRP)-conjugated bovine anti-goat IgG, short interfering RNA (siRNA)

Sigma-Aldrich, Poole, Dorset, UK

Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, endotoxin-free phosphate-buffered saline (PBS), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin solution, trypsin-EDTA solution, trypsin-EDTA for endothelial cells, IgG-free bovine serum albumin (BSA), 30% acrylamide/bis-acrylamide solution, HRP-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG

2.2 Methods

2.2.1 Characterisation of A_{2A}AR-deficient mice

2.2.1.1 Breeding of A_{2A}AR-deficient mice

All animal experiments, genotyping and cytokine assays were carried out by Dr Claire Rutherford (IBLS, University of Glasgow, UK) under the Home Office project license PPL 60/3119.

CD-1 mice heterozygous for an inactive allele of the A_{2A}AR were produced as described by Ledent *et al.* (1997) and were supplied alongside wild-type CD-1 mice by Charles River Laboratories, Margate, Kent, UK. Briefly, the wild-type murine A_{2A}AR was cloned and the coding sequence interrupted by insertion of the neomycin gene under control of the phosphoglycerine kinase promoter in the first exon, thereby replacing the first 102 codons of the A_{2A}AR gene which encode transmembrane segments 1 to 3. The resulting construct was introduced into R1 embryonic stem (ES) cells to allow homologous recombination. Clones carrying the recombinant A_{2A}AR were selected for by resistance to G418 and screened by Southern blotting after digestion with *Dra*I using a 2 800-bp *Eco*RI fragment complementary to a region spanning the site of insertion. Clumps of recombinant ES cells were allowed to aggregate with single CD-1 eight-cell stage embryos from which the *zona pellucida* had been removed and the resulting embryos were transferred into the uteri of pseudopregnant recipients to generate chimaeric mice. Chimaeras, when mated with wild-type CD-1 mice, produced animals heterozygous for the inactive A_{2A}AR gene ($A_{2A}AR^{-/+}$). On arrival, $A_{2A}AR^{-/+}$ mice were bred for several generations to generate homozygous animals which were then selected for further breeding to create a colony of A_{2A}AR-deficient ($A_{2A}AR^{-/-}$) mice.

2.2.1.2 Genotyping of A_{2A}AR-deficient mice

2.2.1.2.1 Extraction of DNA from tail-snips

Mice were confirmed as carriers of the mutant allele of the A_{2A}AR using polymerase chain reaction (PCR) analysis of DNA extracted from tail-snips. A 0.3 cm tail-snip was removed from each animal at the time of death and stored at – 80 °C for future processing. Each tail-snip was digested by incubation with 300 μl TNES buffer (100 mM Tris, pH 7.5, 200

mM NaCl, 5mM EDTA, 0.2 % (w/v) sodium dodecyl sulphate (SDS)) and 40 µg proteinase K at 55 °C overnight. Once all tissue was digested, 100 µl saturated (approximately 6 M) NaCl was added and the mixture was vortexed for 15 seconds. Insoluble debris was pelleted by centrifugation (16 000 g, 5 mins, room temperature (RT)) and the supernatant removed to a fresh microfuge tube. DNA was precipitated by addition of 300 µl room temperature isopropanol. The mixture was centrifuged again (16 000 g, 5 mins, RT) and the isopropanol supernatant removed. The DNA pellet was washed with 300 µl room temperature 70 % (v/v) ethanol and centrifuged once more. The ethanol was decanted and the pellet allowed to dry at room temperature before resuspension in 60 µl TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA).

2.2.1.2.2 Polymerase chain reaction (PCR)

DNA from tail-snips was screened for the presence of the wild-type and mutant alleles of the A_{2A}AR in a single PCR assay using the following primers:

A2R3: 5' – CTC CAC CAT GAT GTA CAC CG – 3'

Neo R3: 5' – AGG GAA GGG TGA GAA CAG AG – 3'

A2D3: 5' – CAT GGT TTC GGG AGA TGC AG – 3'

Primers were designed by Catherine Ledent (Institut de Recherche Interdisciplinaire, Universite de Bruxelles, Brussels, Belgium) and synthesised by Thermo Electron Corporation, Ulm, Germany. A2R3 and A2D3 amplified a 229 bp sequence from the wild-type A_{2A}AR while Neo R3 and A2D3 amplified a 570 bp sequence from the mutant allele. Reactions were carried out using the Qiagen Taq DNA polymerase system in a total volume of 26 µl containing 1 µg of DNA extracted from tail-snips, 5 pmol of each primer, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 mM $MgCl_2$, 1.5 U Taq DNA polymerase plus Qiagen PCR buffer and Q-solution as recommended by the manufacturer. PCR was performed using a Progene Techne thermal cycler programmed to give an initial denaturation step of 94 °C for 2 minutes followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 1 minute and elongation at 72°C for 30 seconds and finishing with a final elongation at 72 °C for 10 minutes.

PCR products were viewed using agarose gel electrophoresis (1.2 % (w/v) agarose) as described in Section 2.2.6.7.

2.2.1.3 Bacterial endotoxin-induced septic shock

Endotoxic shock was induced in equal numbers of male and female wild-type and $A_{2A}AR^{-/-}$ mice by intraperitoneal injection of 400 μl (200 μg) lipopolysaccharide (LPS) in order to produce an acute vascular inflammatory response. A similar group of mice were injected with an equal volume (400 μl) of phosphate-buffered saline (PBS) to give four experimental groups in total: (i) Wild-type mice + PBS, (ii) Wild-type mice + LPS, (iii) $A_{2A}AR^{-/-}$ mice + PBS, (iv) $A_{2A}AR^{-/-}$ mice + LPS. Animals were then returned to their cages and observed closely. After 4 hours, mice were euthanised by anaesthetic overdose via intraperitoneal injection of 0.3 ml Sagatal. Following confirmation of death, a small blood sample (approximately 0.5 ml) was taken for measurement of cytokine levels and animals were then perfused with PBS through a needle inserted into the left ventricle. Aortas were dissected out, snap-frozen by immersion in liquid nitrogen and stored at – 80 °C until required for preparation of protein samples.

2.2.1.4 Measurement of proinflammatory cytokines in serum

Blood collected at time of death was allowed to clot around a cocktail stick by incubating at room temperature for 1 hour and then at 4° C overnight. The following day, the clot was discarded and the remaining serum was transferred to a microfuge tube. Samples were centrifuged (16 000 g, 13 mins, 4°C) and the clear supernatant was collected for measurement of cytokine levels.

Levels of the proinflammatory cytokines TNFα, IL-6, IL-1β and GMCSF in serum samples were measured using the BioSource Mouse Inflammatory Four-Plex fluorescent immunoassay kit according to the manufacturer's instructions. This assay relies on specific interactions between antibodies conjugated to polystyrene beads and a particular cytokine in the serum sample. Four different cytokines can be measured simultaneously by mixing four populations of beads, each displaying different antibodies. Each population of beads is internally dyed with fluorophores to give individual spectral properties allowing them to be distinguished from one another during the detection process.

Serum samples and standards of known concentration were incubated with beads in a filter-bottom microplate for 2 hours at room temperature to allow TNFα, IL-6, IL-1β and GMCSF to bind to the antibodies on the beads. The beads are light sensitive and so all incubations were carried out in the dark. After washing, biotinylated detector antibodies

which recognise epitopes on the bound cytokines were added and incubated for a further hour at room temperature. Excess biotinylated antibody was washed away and streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE) was added to the beads and incubated at room temperature for 30 minutes. Streptavidin-RPE binds to the biotinylated antibody which is associated with the cytokine-antibody complex on the bead. After washing, the beads were loaded into a BioSource Luminex 100 instrument for analysis. The Luminex 100 monitors both the spectral properties of the beads and the level of fluorescence associated with RPE to generate a standard curve allowing calculation of the concentration of each cytokine in the sample.

2.2.2 Cell culture and transfections

2.2.2.1 Cell maintenance

Human umbilical vein endothelial cells (HUVECs) were cultured in 150 cm^2 flasks in endothelial basal media (EBM) supplemented with 2 % (w/v) fetal bovine serum, 0.04 % (v/v) hydrocortisone, 0.1 % (v/v) ascorbate and recombinant growth factors as recommended by the supplier. Cells were passaged on reaching 70 to 80 % confluence (approximately once a week). Because HUVECs are particularly sensitive to trypsin, a non-standard method of subculturing was employed. Cell monolayers were washed twice with 5 ml warm PBS before addition of endothelial grade trypsin-EDTA solution (5 units/ml porcine trypsin, 1.8 % (w/v) EDTA). Cells were then incubated at room temperature for 5 minutes and adherent cells dislodged from flasks by gentle tapping. Trypsin was neutralised by addition of fresh media and cells were pelleted by centrifugation (200 g, 5 mins, RT). Cell pellets were resuspended in a volume of media determined to give a suitable cell density for counting using a cytometer (typically 5 ml per 150 cm^2 flask).

HUVECs were used for experiments between passages 2 and 5. Beyond passage 5, HUVECs have been found to express altered levels of cell adhesion molecules in response to proinflammatory stimuli such as TNFα and LPS (Muller *et al.*, 2002). This suggests that in later passages HUVECs may begin to lose their suitability as a model for the endothelium *in vivo*. Cells were therefore discarded at this stage.

Human embryonic kidney 293 (HEK 293) cells were maintained in Dulbecco's modified Eagles's medium (DMEM) and Chinese hamster ovary (CHO) and C6 rat glioma cells

were maintained in Ham's F-12 medium, each supplemented with 10 % (v/v) FBS, 1 % (v/v) L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were passaged when approximately 80 % confluent using buffered trypsin (0.5% (w/v) porcine trypsin, 0.2 % (w/v) EDTA).

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

2.2.2.2 Transient transfection of HEK 293 and CHO cells

HEK 293 or CHO cells were plated in 10 cm dishes at a density of 8×10^5 cells dish and cultured overnight in complete DMEM or Ham's F-12, respectively. The following day, cells were transfected with plasmids encoding either wild-type or truncated forms of the human A_{2A}AR. For each dish, 12 μg DNA and 400 μl Opti-mem serum-free medium were mixed in a sterile microfuge tube. In a separate tube, 18 μl of the transfection reagent Lipofectamine were added directly to 400 μl Opti-mem and mixed thoroughly. The Lipofectamine/Opti-mem mix was then transferred to the tube containing the DNA, mixed thoroughly and incubated at room temperature in the dark for 20 minutes. During the incubation, cells were washed once with 5 ml Opti-mem which was then replaced with 5.2 ml fresh Opti-mem. The Lipofectamine/DNA mix was then added dropwise over the surface of the cells and the plate was rocked gently to ensure even distribution. Cells were incubated for 3 hours at 37 °C. The transfection medium was then replaced with normal complete medium. Transfection efficiency was assessed by examining cells transfected in tandem with a plasmid encoding GFP. First, the total number of cells per field was estimated by counting 5 fields in bright-field using a 10 × objective lens and calculating the mean. Fluorescent GFP-expressing cells were then counted using fluorescent microscopy and the numbers compared to estimate the percentage of transfected cells. Transfection was deemed to be successful if the percentage of fluorescing cells exceeded 50 %.

2.2.2.3 Transient transfection of C6 cells

C6 cells were plated in 6-well dishes at a density of 3×10^5 cells/well and cultured overnight in complete Ham's F-12 medium. The following day, cells were fed with fresh medium 1 hour prior to transfection using the transfection reagent, GeneJuice. 200 μl/well of Opti-mem and 1 μg DNA was placed in a sterile microfuge tube and mixed gently. GeneJuice (7.5 μl/well) was added directly to the medium in the tube, mixed thoroughly

and incubated at room temperature for 20 minutes. The appropriate volume of GeneJuice/DNA mixture was then added dropwise over the surface of cells in complete medium and plates were rocked gently to ensure even distribution. Cells were incubated for 24 hours at 37°C. The transfection mixture was then removed and replaced with fresh complete medium.

2.2.2.4 Transfection of HUVECs with short interfering RNA

Target-specific short interfering RNAs (siRNAs) designed to knock down PKCα and PKCε and non-targeting control siRNA were introduced into HUVECs using the transfection reagent Oligofectamine. HUVECs were plated in 6cm dishes and grown until 70 % confluent. For each dish, 50 pmol siRNA was mixed with 240 µl Optimem in a sterile microfuge tube. In a separate tube, 4.5 µl Oligofectamine was mixed with 18 µl Opti-mem. Following a 5 minute incubation, the Oligofectamine mixture was added to the tube containing the siRNA and incubated for 20 minutes at room temperature. Meanwhile, cells were washed twice with 2 ml Optimem which was then replaced with 1.5 ml fresh Optimem. The siRNA/Oligofectamine mix was added dropwise over the surface of the cells and the dishes were agitated gently to ensure even distribution. Cells were incubated for 5 hours at 37 °C and then the serum-free transfection medium was supplemented with an equal volume of fresh complete medium. The transfection was repeated the following day and cells were used in experiments one day later. Efficiency of knock-down by siRNA was assessed by immunoblotting as described in Section 2.2.5.

2.2.3 Generation and maintenance of recombinant adenovirus

2.2.3.1 Generation of myc-tagged human A_{2A}AR-expressing adenovirus

Recombinant adenovirus encoding the myc-tagged human A_{2A}AR (myc-hA_{2A}AR) was generated by Dr William Sands (University of Glasgow, Glasgow, UK) using the "AdEasy" system (He *et al.*, 1997) and has been described previously (Sands *et al.*, 2004). In brief, the myc-humA_{2A}AR was first cloned into the shuttle vector, pAdTrackCMV. The resultant construct was then linearised by digestion with *Pme*I and co-transformed into *E. coli* BJ5183 cells with the adenoviral backbone plasmid, pAdEasy1. Successful recombination of pAdEasy1/myc-hA_{2A}AR was confirmed by *PmeI* digestion and PCR using myc-hA_{2A}AR-specific primers. Recombinants were expanded in *E. coli* XL1 Blue

cells and linearised plasmids were transfected into HEK 293 cells which acted as a packaging cell line to allow viral production.

The pAdEasy1 plasmid contains an open reading frame encoding GFP which is maintained in the recombinant adenovirus and so viral infection of HEK 293 cells can be detected by fluorescence microscopy. Six days post-infection, HEK 293 cells were harvested and disrupted by freeze-thawing to release adenovirus particles. Cleared lysate was used to infect two 150 cm^2 tissue culture flasks of 70 % confluent HEK 293 cells. Following successful infection, cells were harvested and viral particles collected as before in order to infect twenty 150 cm^2 flasks for a large scale preparation.

Recombinant adenovirus encoding GFP alone was kindly donated by Professor Robert White (Beatson Institute for Cancer Research, Glasgow, UK).

2.2.3.2 Large scale preparation of recombinant adenoviruses

Pure high titre stocks of recombinant adenovirus were obtained by amplification and purification with reference to the method described by Nicklin and Baker (1999). Confluent 150 cm^2 flasks of low-passage HEK 293 cells were infected with either crude viral extract from previously infected HEK 293 cells or with plaque-purified recombinant adenovirus at an MOI of 0.1-10 per flask and incubated for 2-6 days at 37 °C, 5 % (v/v) CO_2. Once the cytopathic effect of the virus had caused the cells to detach from the flasks, cells were harvested and pelleted by centrifugation (250 g, 10 mins, RT). Pellets were stored at - 80° C, ready for viral harvesting and purification.

Cell pellets from twenty 150 cm^2 flasks were defrosted at room temperature and pooled by resuspension in a total volume of 10 ml room temperature PBS followed by centrifugation (250 g, 10 mins, RT). The resultant single pellet was resuspended in 5 ml PBS and cells were lysed by 5 cycles of freeze/thawing in a dry ice/methanol bath followed by incubation with agitation in a 37 °C water bath. The cell suspension was vortexed vigorously for 30 seconds between cycles to encourage cell breakage. The lysate was cleared by centrifugation (7000 g, 10 mins, 4 °C) and the supernatant containing the adenovirus was collected for further purification.

Adenovirus obtained by the freeze/thawing method is contaminated with cellular protein and viral debris which may be cytotoxic when used *in vitro*. To obtain a pure preparation,

the supernatant from the previous step was separated on a discontinuous CsCl density gradient. The CsCl gradient was created by underlying 3 ml of 1.2 g/ml CsCl solution with 1.5 ml of 1.4 g/ml CsCl solution in a 14 × 95 mm Ultra-Clear centrifuge tube (Beckman). The crude adenovirus extract was applied to the top of the gradient and centrifuged (90 000 g, 1.5 h, 8 °C) with zero deceleration to produce a translucent white band between the two layers of CsCl, representing pure adenovirus. Zero deceleration was selected during the centrifugation step to prevent disruption of the delicate band by turbulence during braking. The adenovirus band was extracted using a syringe and a 21-gauge needle to puncture the side of the centrifuge tube and then transferred to a 3 ml Slide-A-Lyser dialysis cassette (Pierce). The extract was dialysed overnight at 4 °C in 600 ml TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0) with three changes. The following day, the purified adenovirus was diluted in an equal volume of sterile storage buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 0.1 % (w/v) BSA, 50 % (v/v) glycerol) and stored at – 80 °C in 10 µl aliquots.

2.2.3.3 Titration of adenoviruses

Purified adenovirus was titred using a Cell Biolabs Inc QuickTitre Adenovirus Immunoassay Kit according to the manufacturer's instructions. HEK 293 cells were seeded in poly-D-lysine-coated 24-well tissue culture plates and incubated for 1 hour at 37 °C, 5 % (v/v) CO_2. A series of 10-fold dilutions of the CsCl-purified adenovirus preparation was prepared and used to infect the HEK 293 cells in duplicate. Forty-eight hours later, cells were fixed using ice-cold methanol and then immunostained using a primary antibody directed against the adenoviral capsid protein, hexon (supplied) and a secondary horseradish peroxidise (HRP)-conjugated antibody which recognises the anti-hexon antibody (supplied). Binding of the HRP-conjugated antibody was detected by incubation with a solution of the HRP substrate, diaminobenzidine (DAB; supplied). DAB undergoes oxidative polymerisation in the presence of HRP to produce a dark brown precipitate. Adenovirus-infected cells stained rapidly and were clearly visible under light microscopy as discrete brown patches in the cell monolayer. Positively stained cells were counted in ten fields at a virus dilution that gave 5-50 positive cells/field when viewed using a 10 × objective. The mean result was determined and used to calculate the number of infected cells per ml of the original adenovirus preparation to give a titre value in infectious units/ml (ifu/ml).

2.2.3.4 Infection of HUVECs with recombinant adenoviruses

HUVECs were seeded in tissue culture dishes at a density that would produce 70 % confluence on the following day. This equated to 3×10^5 cells/well in 6-well plates, 6×10^5 cells in 6 cm dishes or 8×10^5 cells in 10 cm dishes. The next day, cells were infected with adenovirus encoding either the human $A_{2A}AR$ ($adA_{2A}AR$) or GFP alone (adGFP) at a level of 30 ifu/cell or as described in figure legends. Cells were incubated for 24 hours and then infective media was replaced with fresh media. Experiments were performed 24 hours later.

2.2.4 Preparation of protein samples for immunoblotting

2.2.4.1 Preparation of aortic extracts

For each snap-frozen aorta to be crushed, 250 μl 2 % SDS sample buffer (2 % (w/v) SDS, 50 mM Tris, pH 7.5, 10 % (v/v) glycerol, 0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, 5 mg/ml complete EDTA-free protease inhibitor cocktail tablet) was crushed to a fine powder using a liquid nitrogen-cooled mortar and pestle resting on dry ice. The aorta was added to the mortar and crushed together with the 2 % SDS sample buffer to produce a fine frozen powder. The crushed tissue extract was then transferred to a chilled microfuge tube, allowed to thaw and then sonicated to disperse aggregated material. Extracts were centrifuged (16 000 g, 15 mins, 4 °C) to separate soluble protein from cellular lipids and insoluble debris. The soluble supernatant was transferred to a fresh tube and the centrifugation process was repeated twice more to obtain a homogenous protein sample which was then frozen at – 80 °C for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4.2 Preparation of extracts from cultured cells

Protein extracts for SDS-PAGE and immunoblotting were prepared from confluent cells grown in 6-well plates. Cells were first treated as described in figure legends. Reactions were terminated by placing dishes on ice and washing cells three times in 1 ml ice-cold PBS prior to solubilisation in 100 μl RIPA buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 10 mM sodium fluoride, 5 mM EDTA, 10 mM sodium phosphate, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, 5 mg/ml complete

EDTA-free protease inhibitor cocktail tablet). For HUVEC samples, the volume of RIPA buffer was reduced to 50 µl per well to compensate for the low protein content of these cells. Samples were incubated on ice for 30 minutes to aid solubilisation before centrifugation (10 000 g, 15 mins, 4 °C) to pellet insoluble debris. Protein concentration of supernatants was measured using a bicinchoninic acid (BCA)-based method as described below.

2.2.4.3 Determination of protein concentration using the bicinchoninic acid (BCA) assay

For each experiment, 10 µl aliquots of bovine serum albumin (BSA) of known concentration (ranging from 0 to 2 mg/ml) dissolved in the appropriate lysis buffer, and 10 µl of each protein sample were added in duplicate to a 96-well plate. 100 µl of BCA reagent (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 hydrogen carbonate, 0.08 % (w/v) copper (II) sulphate) was added to each well and the plate was incubated at room temperature for 10 minutes before measuring absorbance at 490 nm using a Dynex MRX-TC Revelation microplate reader. Upon mixing with protein, Cu2+ ions in the BCA reagent are reduced to Cu^{+} which then reacts with BCA to produce a colour change from blue to purple which is detectable at 490 nm. The extent of the colour change is directly proportional to the amount of protein in a sample. The absorbance measurements obtained for the BSA standards were used to derive a straight line graph from which the concentrations of the protein samples were calculated using Dynex Revelation software.

2.2.5 SDS-PAGE and immunoblotting

Equal quantities of protein (30 - 60 µg per sample, diluted to a final volume of 15 µl) were denatured in an equal volume of 12 % (w/v) SDS sample buffer (12 % (w/v) SDS, 50 mM Tris, pH 6.8, 10 % (v/v) glycerol, 10 mM dithiothreitol (DTT), bromophenol blue). Of this, 25 µl samples were fractionated by SDS-PAGE on 10 % (w/v) acrylamide resolving gels (10 % (w/v) acrylamide, 375 mM Tris, pH 8.8, 0.1 % (w/v) SDS) with 4 % (w/v) acrylamide stacking gels (4 % (w/v) acrylamide, 125 mM Tris, pH 6.8, 0.1 % (w/v) SDS). To allow size estimation of immunoreactive protein bands, Biorad Rainbow molecular weight markers were fractionated alongside protein samples. Electrophoresis was performed in 1 % (w/v) SDS running buffer (0.1 % (w/v) SDS, 192 mM glycine, 25 mM

Tris, pH 8.3) at a constant voltage of 180 V for approximately 1.5 hours until the dye front reached the bottom of the gel.

Proteins were electrotransferred from the gel on to a Protran nitrocellulose membrane (0.2 μm pore size) for 45 min at a constant current of 400 mA in a transfer buffer containing 192 mM glycine, 25 mM Tris, pH 8.3 and 20 % (v/v) methanol. Membranes were then blocked for at least 1 hour at room temperature in immunoblotting buffer (20 mM Tris, pH 7.4, 140 mM NaCl, 0.1 % (v/v) Tween 20, 5 % (w/v) milk proteins) prior to incubation with rotation with primary antibody for either 1 hour at room temperature or overnight at 4°C. Antibodies used during this study are listed in Table 1. Antibodies were diluted in either immunoblotting buffer or 5 % (w/v) BSA in Tris-buffered saline/1 % (v/v) Tween 20 (TBST; 20 mM Tris, pH 7.4, 140 mM NaCl, 1 % (v/v) Tween 20). After 3×5 minute washes in TBST, membranes were exposed to the appropriate horseradish peroxidise-conjugated secondary antibody diluted 1/1000 in immunoblotting buffer for 1 hour at room temperature. Membranes were then washed 5×5 minutes in TBST. Immunoreactive proteins were visualised using Perkin-Elmer enhanced chemiluminescence (ECL) detection reagents, according to the manufacturer's instructions.

Antibody reactivity	**Supplier**	**Catalogue Number**	**Dilution**
Phospho-IκBα (Ser $^{32/36}$) (5A5)	Cell Signalling Technology	9246	1:1000
IκBα (C-21)	Santa Cruz Biotechnology, Inc.	Sc -371	1:1000
NFκB p65 (A)	Santa Cruz Biotechnology, Inc.	Sc -109	1:400
VCAM-1	R & D Systems	AF643	1:1000
ICAM-1	R & D Systems	AF796	1:500
Phospho-STAT1 (Tyr^{701})	Cell Signalling Technology	9171	1:1000
STAT1	Cell Signalling Technology	9172	1:1000
Phospho-STAT3 (Tyr^{705}) (D3A7)	Cell Signalling Technology	9145	1:1000
STAT3	Cell Signalling Technology	9132	1:1000
TAP-1 (M-18)	Santa Cruz Biotechnology, Inc.	Sc-11465	1:500
Glyceraldehyde-3-phosphate dehydrogenase	abcam	ab9484	1:20 000
Phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) (E10)	Cell Signalling Technology	9106	1:1000
c-myc (9E10)	Eurogentec	Ascites fluid	1:1000
A_{2A} Adenosine Receptor	Cambridge Bioscience	PA1-042	1:1000
PKC (A-9)	Santa Cruz Biotechnology, Inc.	Sc-17804	1:500
PKCε (22B10)	Cell Signalling Technology	2683	1:250
PKCα	Cell Signalling Technology	2056	1:250

Table 2: Antibodies used for immunoblotting

2.2.6 Molecular Biology

2.2.6.1 Plasmid DNA constructs

Plasmids encoding the wild-type human A_{2A}AR (pcDNA3.1/mycHis-hum A_{2A}AR) and the carboxyl terminus-truncated mutant receptors (pcDNA3.1/mycHis-hum A_{2A}AR 1-311 and pcDNA3.1/mycHis-hum A_{2A}AR 1-360) were constructed by Dr Tim Palmer (Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK). Briefly, a myc epitope (EQKLISEEDL) and His_6 sequences were added to the carboxyl terminus of the human A_{2A}AR by PCR using pCMV5/human A_{2A}AR as a template. Primers were designed to amplify the A_{2A} region while removing the stop codon and introducing an *Xba*I site. The resultant PCR product was digested with *Hind*III and *Xba*I and ligated into similarly digested pcDNA3.1/mycHisA in-frame and upstream of the myc and His sequences. Plasmids encoding truncated human A_{2A}AR mutants were created in the same way but using different antisense primers designed to remove either 101 or 52 amino acids from the carboxyl terminus to generate pcDNA3.1/mycHis-hum A_{2A}AR 1-311 and pcDNA3.1/mycHis-hum A_{2A}AR 1-360 respectively.

pGEX-TRAX (Sun *et al.*, 2006) was kindly donated by Dr Yijuang Chern (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan).

pGEX-14-3-3τ (Ward and Milligan, 2005) was a gift from Professor Graeme Milligan (Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK).

2.2.6.2 Bacterial Strains and Media

E. coli XL1 Blue cells were used for the propagation of plasmid vectors while recombinant proteins were expressed in *E. coli* BL21 cells. *E. coli* were grown in sterile Luria-Bertani broth (LB; 10 g/l bacto-tryptone, 10 g/l NaCl, 5 g/l, pH 7.5) supplemented with ampicillin (50 μg/ml) where necessary (LB^{Amp}). LB^{Amp} plates were made by inclusion of 1.5 % (w/v) agar and stored at 4 °C until required.

2.2.6.3 Transformation of competent *E. coli*

Aliquots of *E. coli* XL1 Blue or BL21 competent bacteria were thawed on ice and 40 μl per transformation were immediately transferred to chilled microfuge tubes containing 30-

50 ng DNA. Cells were incubated on ice for 15 minutes before heat shocking at 42 °C for 45 seconds. The tubes were immediately returned to ice for 2 minutes. LB (1 ml) was then added and the cells were incubated with shaking at 37 °C for 1 hour. 200 µl of this mix was plated out on LB^{Amp} plates and grown overnight at 37 °C to allow growth of bacterial colonies.

2.2.6.4 Preparation of plasmid DNA

DNA was purified from bacterial cultures using the Qiagen Plasmid Maxi kit as recommended by the manufacturer. An isolated colony from an agar plate was used to inoculate 5 ml LB^{Amp} and grown for eight hours at 37 °C with shaking at 200 rpm. This culture was used to inoculate 250 ml LB^{Amp} which was grown for a further 16 hours in the same conditions. Typically, 5 ml of culture was reserved for preparation of glycerol stocks. Bacteria were harvested from the remaining culture by centrifugation (6 000g, 15 mins, 4°C) and then resuspended in 10 ml Buffer P1 (50 mM Tris, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Cells were lysed by addition of 10 ml Buffer P2 (200 mM NaOH, 1 % (w/v) SDS) and incubation at room temperature for 5 minutes. The lysates were neutralised by addition of Buffer P3 (3 M potassium acetate, pH 5.5), mixed by inversion and incubated on ice to facilitate precipitation of potassium dodecyl sulphate, SDS-denatured proteins, genomic DNA and cellular debris. The lysate was then cleared by centrifugation (20 000 g, 10 mins, 4 °C). The supernatant containing soluble plasmid DNA was applied to a Qiagen-tip 500, pre-equilibrated with 10 ml Buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15 % (v/v) isopropanol, 0.15 % (v/v) Triton X-100) and allowed to enter the resin within the tip by gravity flow. The tip was then washed twice with 30 ml Buffer QC (1 M NaCl, 50 mM MOPS, pH 7.0, 15 % (v/v) isopropanol) and DNA was eluted using 15 ml Buffer QF (1.25 M NaCl, 50 mM Tris, pH 8.5, 15 % (v/v) isopropanol). DNA was precipitated by addition of 10.5 ml room temperature isopropanol and incubation at room temperature for 30 minutes before being pelleted by centrifugation (15 000 g, 30 mins, 4 °C). The DNA pellet was washed with 5 ml room temperature ethanol, centrifuged once more and allowed to air-dry for 10 minutes before resuspension in 500 µl sterile TE buffer. DNA concentration was determined by diluting the preparation 1 in 500 in distilled water and measuring absorbance at 260 nm (A_{260}), assuming that a 50 µg/ml solution of double-stranded DNA has an A_{260} of 1 unit. Absorbance at 280 nm (A_{280}) was also measured and used to determine DNA purity assuming that pure DNA has an A_{260}/A_{280} ratio of 1.8.

2.2.6.5 Preparation of glycerol stocks

Glycerol stocks were prepared for long-term storage of plasmid DNA. For each glycerol stock, 0.7 ml overnight culture was added to 0.3 ml sterile 50 % (v/v) glycerol in a sterile cryovial. Vials were vortexed vigorously to ensure even dispersal of glycerol, frozen rapidly on dry ice and stored at – 80 °C.

2.2.6.6 Restriction digestion of plasmid DNA

Plasmids encoding wild-type and truncated forms of myc-hA_{2A}AR were digested using *Hind*III and *Xba*I. DNA (1μg) was digested in a reaction mixture containing 5 U each of *Hind*III and *Xba*I, 3 μl of Promega Buffer E (6mM Tris, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.5) and nuclease-free water to a total volume of 10 μl. Reactions were allowed to proceed for 1.5-3 hours and fragments were analysed by agarose gel electrophoresis using 1 % (w/v) agarose gels as described below.

2.2.6.7 Agarose gel electrophoresis

Plasmid DNA and PCR products were analysed by agarose gel electrophoresis using 1 % (w/v) and 1.2 % (w/v) gels respectively. Gels were prepared by dissolving 0.4 or 0.48 g of agarose in 40 ml TAE buffer (40 mM Tris, 1 mM EDTA, 40 mM glacial acetic acid) with heating. Gels were cooled to hand-warm and 4 μl of 10 mg/ml ethidium bromide solution was added in order to stain DNA bands for visualisation under UV light. Before loading, 2 μl DNA loading Buffer (0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose in TAE buffer) was added to samples to be analysed. Samples were run alongside 1 kb step ladder markers at 100 V/ 250 mA in TAE buffer for approximately 1 hour until the dye front reached the end of the gel. Bands were detected by ethidium bromide staining and viewed using a UV transilluminator.

2.2.6.8 Preparation of GST fusion proteins

A scraping from a glycerol stock of *E. coli* BL21 cells transformed with pGEX plasmid DNA encoding either GST-14-3-3τ, GST-TRAX or GST alone was used to inoculate 10 ml LB^{Amp}. Cells were cultured for 8 hours (GST-TRAX) or overnight (GST and GST-14-3-3τ), at 37 °C with shaking at 200 rpm. This culture was used to inoculate 300 ml LB^{Amp} and cells were then grown for approximately 2 hours until an OD_{600} of 0.3 was reached,

indicating that bacteria were growing exponentially. Isopropyl β-thiogalalactopyranoside (IPTG) (0.2 - 1 mM) was then added to induce expression of recombinant protein and bacteria were grown for a further 4 hours at 37°C (GST and GST-14-3-3τ) or overnight at 25 ° C (GST-TRAX). Bacteria were harvested by centrifugation (6700 g, 15 mins, 4 °C) and pellets were stored at – 80 °C for protein purification the following day.

Pellets containing GST-tagged proteins were defrosted at room temperature and resuspended in 20 ml lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 % (v/v) Triton X-100). Samples were probe sonicated on ice for 6 × 20 seconds with 20 second intervals to prevent build-up of heat and centrifuged (27 000 g, 30 mins, 4 °C) to pellet insoluble material. The cleared lysate was mixed with 0.3 ml of a 50 % (v/v) glutathione Sepharose bead suspension in lysis buffer and incubated for 1 hour at 4 °C with rotation in order to immobilise GST-tagged proteins on the beads. The beads were pelleted by gentle centrifugation (335 g, 1 min, 4 °C), washed twice in 10 ml PBS and then transferred to a microfuge tube for a final wash in 1 ml PBS. PBS was aspirated from tubes and beads were resuspended in 0.3 ml 50 % (v/v) glycerol in PBS supplemented with protease inhibitors (0.1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine) and stored at – 20 °C.

Concentration of GST-tagged proteins was determined by SDS-PAGE on 10 % (w/v) polyacrylamide resolving gels as described in Section 2.2.5. Proteins were eluted from beads following a brief spin to allow removal of the glycerol/PBS storage solution. Beads were resuspended in 20 μl 12 % (w/v) SDS sample buffer and incubated at 65 °C for 1 hour with occasional vortexing. Eluted samples were transferred to fresh microfuge tubes using a Hamilton syringe and then run on gels in parallel with known quantities of BSA ranging between 0.2 and 2 μg. To view bands, gels were stained in 0.25 % (w/v) Coomassie brilliant blue, 10 % (v/v) acetic acid, 45 % (v/v) methanol for 1 hour and destained in 10 % (v/v) acetic acid, 10 % (v/v) methanol. Gels were scanned and the densities of bands produced by the BSA standards and the GST-tagged proteins were quantitated by densitometry using Non-linear Dynamics TotalLab software. Results from the BSA standards were then used to generate a straight line graph from which the concentration of the eluted GST-tagged proteins could be calculated.

To track expression and recovery of fusion proteins, 100 μl samples were reserved at different stages of the procedure as described in figure legends and mixed with an equal

volume of 12 % (w/v) SDS sample buffer for analysis by SDS-PAGE and Coomassie staining as described above.

2.2.7 GST pull-down assay

HUVECs were seeded in 10 cm dishes and infected with adA_{2A}AR at 30 ifu/cell as described in Section 2.2.3.4. Cells were treated as described in figure legends and reactions were terminated by placing dishes on ice and washing twice with 5 ml ice-cold PBS. Cells were harvested by scraping into 750 µl pull-down lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 % (v/v) Triton X-100) supplemented with protease inhibitors (0.1 mM PMSF, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine, 5 mg/ml complete EDTA-free protease inhibitor cocktail tablet, 100 µM sodium orthovanadate) and incubated for 1 hour with rotation at 4 °C to aid solubilisation. Samples were then centrifuged (20 000 g, 15 mins, 4 °C) to pellet insoluble material and assessed for protein concentration using a BCA assay as described in Section 2.2.4.3. Extracts containing equivalent amounts of protein in a volume of 680 µl were added to microfuge tubes containing the appropriate volume of GST-fusion protein glutathione Sepharose beads to give 20 µg of either GST, GST-14-3-3τ or GST-TRAX. Samples were then incubated overnight at 4 °C with rotation to allow protein complexes to form. The following day, beads and any associated protein complexes were pelleted by gentle centrifugation (600 g, 1 min, 4 °C) and then washed 3 times in 1 ml ice-cold pull-down lysis buffer. Bound proteins were eluted by adding 40 µl 12 % (w/v) SDS sample buffer and incubating at 65 °C for 30 minutes, vortexing every 10 minutes. Samples were transferred to fresh microfuge tubes using a Hamilton syringe and analysed by SDS-PAGE and immunoblotting as described in Section 2.2.5.

2.2.8 Intact cell receptor phosphorylation assay

For characterisation of wild-type A_{2A}AR phosphorylation, HUVECs were grown in 6-well dishes and infected with 30 ifu/cell adA_{2A}AR or adGFP as described in Section 2.2.3.4. Forty-eight hours post-infection, cells were washed twice with 2 ml/well low-phosphate DMEM and incubated for 90 min at 37° C, 5 % CO_2 in 0.75 ml of the same media supplemented with 0.8 mCi/ml [^{32}P] orthophosphate in order to label the intracellular pool of ATP. Cells were then treated as described in figure legends. Reactions were terminated by placing dishes on ice and washing twice in ice-cold PBS. Cells were solubilised by

scraping into 250 µl RIPA buffer supplemented with 100 µM sodium orthovanadate followed by rotation at 4 ° C for 1 hour. Samples were cleared of cellular debris by centrifugation (10 000 g, 15 mins, 4 °C) and 10 µl portions of undiluted supernatants were assayed for protein content as described in Section 2.2.4.3. For immunoprecipitation of phosphorylated receptors, extracts containing equivalent amounts of protein in a volume of 180 µl were added to microfuge tubes containing 30 µl of a 50 % suspension of protein G-sepharose beads, 5 µl 9E10 ascites fluid and 100 µl 0.2 % (w/v) IgG-free BSA. Extracts were incubated with beads on a rotating wheel for 90 minutes at 4° C. Immune complexes conjugated to beads were then recovered by brief centrifugation (10 000 g, 15 secs, 4 °C) and washed 3 times in 1 ml ice-cold RIPA buffer. Pelleted beads were resuspended in 12% (w/v) SDS sample buffer and complexes were eluted by vortexing and incubation at room temperature overnight. The following day, beads were pelleted by brief centrifugation (10 000 g, 15 secs, RT) and supernatants were transferred to fresh microfuge tubes using a Hamilton syringe. Samples were boiled at 95° C for 5 minutes to denature antibody heavy and light chains prior to loading on to 10 % (w/v) polyacrylamide gels for analysis by SDS-PAGE and autoradiography. Gels were dried under vacuum with heat for 2 hours and exposed to film between 2 intensifying screens for 2-7 days at -80 ° C.

For analysis of phosphorylation of truncated A_{2A} adenosine receptors, HEK 293, C6 glioma or CHO cells were plated in 10 cm dishes. On reaching 70 % confluence, cells were transfected with constructs encoding wild-type or truncated forms of the A_{2A}AR as described in Sections 2.2.2.2 and 2.2.2.3. The following day, each 10 cm dish was split into 6-well plates in order to minimise variation in transfection efficiency between wells. Intact cell phosphorylation assays were carried out as described above with minor alterations depending on the efficiency of recovery of protein from different cell types. HEK 293, CHO and C6 glioma cells were labelled with 0.4 mCi/ml [^{32}P] orthophosphate rather than 0.8 mCi/ml and were solubilised in 500 µl RIPA buffer rather than 250 µl allowing 430 µl of each sample to be used in the immunoprecipitation step.

2.2.9 Saturation binding assay

2.2.9.1 HUVEC membrane preparation

HUVECs were seeded in 10 cm dishes and infected with adA_{2A}AR or adGFP as described in Section 2.2.3.4. Forty-eight hours post infection, dishes were placed on ice and washed

3 times with 5 ml ice-cold PBS. Cells were scraped into 5 ml/dish lysis buffer (10 mM Hepes, pH 7.5 at RT, 5 mM EDTA) and lysed on ice by 20 up-and-down strokes of a 7 ml Dounce homogeniser. The homogenate was transferred to a chilled centrifuge tube and membranes were pelleted by centrifugation (14 000g, 15 mins, 4 °C). The membrane pellet was resuspended in 400 µl binding buffer (50 mM Hepes, pH 6.8 at RT, 10 mM $MgCl_2$) and subjected to further homogenisation by 20 up-and-down strokes in a 1 ml Dounce homogeniser. A 50 µl sample was removed and stored at – 20 °C for determination of protein concentration at a later date. The membrane preparation was then diluted to a final volume of 4 ml with binding buffer supplemented with 1 U/ml adenosine deaminase to degrade endogenous adenosine. The extract was homogenised once more before immediate use in binding assays.

2.2.9.2 ^{3}H-ZM241385 saturation binding assay

Binding assays were performed in duplicate in a total volume 250 µl containing 150 µl membrane preparation and 50 µl ^{3}H-ZM241385 at final concentrations ranging from approximately 0.25 nM to 8 nM. Non-specific binding was defined in parallel by inclusion of NECA at a final concentration of 50 µM. Samples were incubated for 1 hour with shaking in a 37 °C water bath to allow binding to reach equilibrium. Bound radioligand was isolated by rapid vacuum filtration over 0.3 % (v/v) polyethyleneimine solution-soaked GF/C glass fibre filters using a Brandel cell harvester. Filters were then washed three times with 3 ml ice-cold wash buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4 at 4 °C) supplemented with 0.03 % (w/v) CHAPS detergent to minimise non-specific binding. Filter discs for each sample were added to scintillation vials containing 5 ml scintillation fluid and incubated at 4 °C overnight to reduce chemiluminescence before scintillation counting. Non-specific counts were subtracted from total counts to give values for specific binding in dpm which were then plotted against ^{3}H-ZM241385 concentration (nM) using GraphPad Prism software. The data was fitted to a hyperbola using a non-linear regression equation in order to determine the total number of receptors expressed (B_{max}) and the equilibrium dissociation constant (K_d). Samples reserved during membrane preparation were assayed for protein content using a BCA protein assay and the results used to calculate B_{max} in pmol/mg.

2.2.10 Statistical analysis

All statistical analyses were carried out using a one-way ANOVA with a Bonferroni comparisons post test using GraphPad Prism software.

3 The role of the A_{2A} adenosine receptor in suppressing vascular inflammation

3.1 Introduction

Inflammation of the vascular endothelium is central to the development of major diseases including atherosclerosis. In the earliest stages of inflammation, endothelial cells are activated by various stimuli to express adhesion molecules such as VCAM-1, ICAM-1 and E-selectin which initiate the recruitment of inflammatory cells (Muller, 2002). This is a critical step in the development of atherosclerosis, as macrophage infiltration of the vessel wall and subsequent foam-cell formation leads to the development of atherosclerotic plaques (Glass and Witztum, 2001). Activated endothelial cells also elaborate numerous inflammatory mediators such as IL-6 and IL-1 which contribute to disease progression in many ways including by promoting macrophage differentiation, smooth muscle cell proliferation, and further cytokine expression (von der Thüsen *et al.*, 2003). It is important to study how these events are regulated as this could reveal new targets for therapeutic intervention and allow development of better strategies for the treatment of inflammatory disease.

One way that the body naturally deals with the risk of excessive inflammation and tissue damage is through accumulation of extracellular adenosine. Adenosine exerts its effects through stimulation of A_1, A_{2A}, A_{2B} and A_3 receptors, of which the A_{2A}AR appears to be most important in mediating anti-inflammatory responses (Linden, 2001). Endothelial cells are key targets for adenosine as they express both A_{2A} and A_{2B}ARs. The A_{2A}AR has been reported to be the predominant form in large blood vessels which is significant in terms of disease as this is the site of atherosclerotic lesion formation (Feoktistov *et al.*, 2002). Endothlelial cells are also significant producers of adenosine both directly and via the sequential dephosphorylation of adenine nucleotides released from damaged cells to adenosine by the ecto-apyrase CD39 and the ecto-nucleotidase CD73, which they express on their cell surface (Lennon *et al.*, 1998). Gene-targeting studies have shown that a lack of functional CD39 (Eltzschig *et al.*, 2003) or CD73 (Thompson *et al.*, 2004) results in excessive vascular leakage in response to hypoxia which points to the crucial nature of this pathway and suggests that its defective regulation may predispose toward disease. A_{2A}AR activation therefore potentially represents an important endogenous mechanism for limiting vascular inflammation.

Several *in vitro* studies have demonstrated the potential for adenosine and signalling through the A_{2A}AR to modulate inflammatory responses in endothelial cells. For example, activation of A_{2A}ARs with the A_{2A}AR-selective agonist CGS21680 has been shown to inhibit phorbol ester-stimulated adhesion of neutrophils to porcine aortic endothelial cells (Felsch *et al.*, 1995). In HUVECs stimulated with LPS, TNFα or IL-1β, adenosine inhibited release of the pro-inflammatory cytokines IL-6 and IL-8 and also reduced expression of the adhesion molecules VCAM-1 and E-selectin which mediate monocyte adhesion (Bouma *et al.*, 1996). A specific role for the A_{2A}AR in regulating leukocyte recruitment has been demonstrated in HUVECs through adenoviral-mediated gene transfer. In these cells, increased presence of the A_{2A}AR was sufficient to inhibit TNFα-induced E-selectin expression and monocyte adhesion to the endothelium (Sands *et al.*, 2004).

The beneficial effects of signalling through the A_{2A}AR *in vivo* have been shown using animal models of inflammation and disease. For example, in the murine carotid artery ligation model of arterial inflammation, mice treated with the A_{2A}AR agonist ATL-146e displayed significantly reduced neutrophil and macrophage recruitment and expression of VCAM-1, ICAM-1 and P-selectin and this was associated with markedly reduced lesion formation (McPherson *et al.*, 2001). Furthermore, a physiological role for the A_{2A} receptor in *in vivo* regulation of inflammation has been confirmed through creation of an A_{2A}-deficient mouse strain. When these mice were subjected to experimentally induced models of hepatitis and septic shock, it was found that even low doses of inflammatory stimuli which had little effect on wild-type mice caused extensive inflammation and tissue damage (Ohta and Sitkovsky, 2001). This was accompanied by elevated levels and a prolonged presence of pro-inflammatory cytokines such as TNFα and IFNγ and IL-6.

Despite the wealth of evidence regarding the anti-inflammatory properties of the A_{2A}AR, the molecular mechanisms behind these effects have not been well studied. Signalling through the A_{2A}AR produces diverse but consistently anti-inflammatory effects in a wide range of cell types and disease models suggesting that there could be a common mechanism. Despite the complexity of responses to tissue damage and inflammation, the majority are mediated through activation of only a few major signalling pathways including the NFκB and JAK/STAT pathways. If the A_{2A}AR could influence signalling through these pathways, this would provide an explanation for its wide range of anti-inflammatory actions. This case is strengthened by the fact that all of the adhesion molecules and inflammatory mediators that have been shown to be affected by adenosine or A_{2A}AR stimulation have their expression regulated by NFκB and STAT proteins. In

addition, *in vitro* studies have shown that signalling through the A_{2A}AR suppresses NFκB (Sands *et al.*, 2004) and JAK/STAT (Sands *et al.*, 2006) pathway activation in cultured endothelial cells. Whether this is a relevant physiological mechanism for limiting inflammation *in vivo* is not known.

A_{2A}AR-deficient mice have been used to demonstrate the protective role of the A_{2A}AR in several models of inflammatory disease (Naganuma *et al.*, 2006; Day *et al.*, 2004; Ohta and Sitkovsky, 2001). If the A_{2A}AR mediates this effect through suppression of NFκB and JAK/STAT signalling, then it can be hypothesised that these signalling pathways will be hyperactivated in A_{2A}AR-deficient mice. To determine whether this is the case, in this study, A_{2A}AR-deficient mice were subjected to LPS-induced septic shock to induce vascular inflammation and then activation of NFκB and JAK/STAT pathways was compared with wild-type mice by detecting levels of activated signalling proteins in the aorta.

3.2 Results

The lack of a functional A_{2A}AR has been shown to exacerbate inflammation and tissue damage in several animal models of inflammatory disease (Naganuma *et al.*, 2006; Day *et al.*, 2004; Ohta and Sitovsky, 2001). However, there is little information available regarding the molecular mechanisms which produce these effects *in vivo*. Two major pathways involved in the inflammatory response are the NFκB and JAK/STAT pathways. Hyperactivation of the NFκB and JAK/STAT signalling pathways is found in several inflammatory diseases and so they present important potential targets for the anti-inflammatory actions of the A_{2A}AR (Miagkov *et al.*, 1998; Hajra *et al.*, 2000; Gharavi *et al.*, 2007; Schreiber *et al.*, 2002). Specifically, the importance of NFκB in the development of atherosclerosis is indicated by studies using $LDLR^{-/-}$ mice in which the NFκB pathway was found to be primed for activation in endothelial cells in atherosclerotic lesion-prone regions of the aorta (Hajra *et al.*, 2000). Stimulation of A_{2A}ARs has been shown to reduce arterial inflammation and lesion formation in the murine carotid artery ligation model (McPherson *et al.*, 2001) but whether this involves suppression of pro-inflammatory signalling has not been addressed. In this study, the physiological role of the A_{2A}AR in regulating pro-inflammatory signalling *in vivo* was assessed by measuring activation of NFκB and JAK/STAT signalling in the aortae of $A_{2A}AR^{-/-}$ mice.

To examine whether the A_{2A}AR has an effect on pro-inflammatory signalling *in vivo*, a colony of A_{2A}AR-deficient mice was created as described previously (Ledent *et al.*, 1997). Carriers of the wild-type and the non-functional mutant A_{2A}AR were identified by PCR analysis of DNA extracted from tail-snips using specific primers. As shown in figure 3.1, samples from wild-type mice generated a single 229 bp PCR product while samples from mice homozygous for the mutant allele generated a single 570 bp fragment. These mice were selected for use in subsequent studies.

In order to study inflammatory responses in the presence and absence of a functional A_{2A}AR, mice were subjected to an LPS-induced model of sepsis which has been used previously to induce acute systemic activation of the aortic endothelium (Hajra *et al.*, 2000). Mice were injected intraperitoneally with either 200 μg LPS or an equal volume of PBS and then euthanized 4 hours later. Serum levels of the pro-inflammatory cytokines TNFα, IL-6, IL-1 and GM-CSF were measured (figure 3.2). Levels of TNFα, IL-6 and GM-CSF were significantly elevated in wild-type mice treated with LPS compared to PBS-

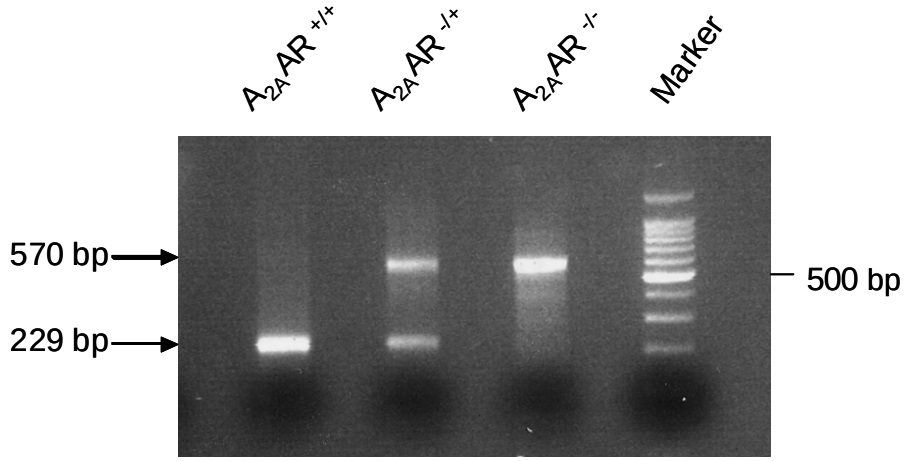


Figure 3.1 Genotyping of wild-type and A_{2A}AR-deficient mice

CD-1 mice heterozygous for an inactive allele of the A_{2A}AR gene were bred to produce a colony of A_{2A}AR-deficient ($A_{2A}AR^{-/-}$) mice. Genomic DNA extracted from tail-snips was screened for the presence of wild-type and mutant alleles of the A_{2A}AR using PCR analysis with specific primers. PCR products were separated on a 1.5 % (w/v) agarose gel. Fragments representing the wild-type (229 bp) and the mutant A_{2A}AR (570 bp) are indicated.

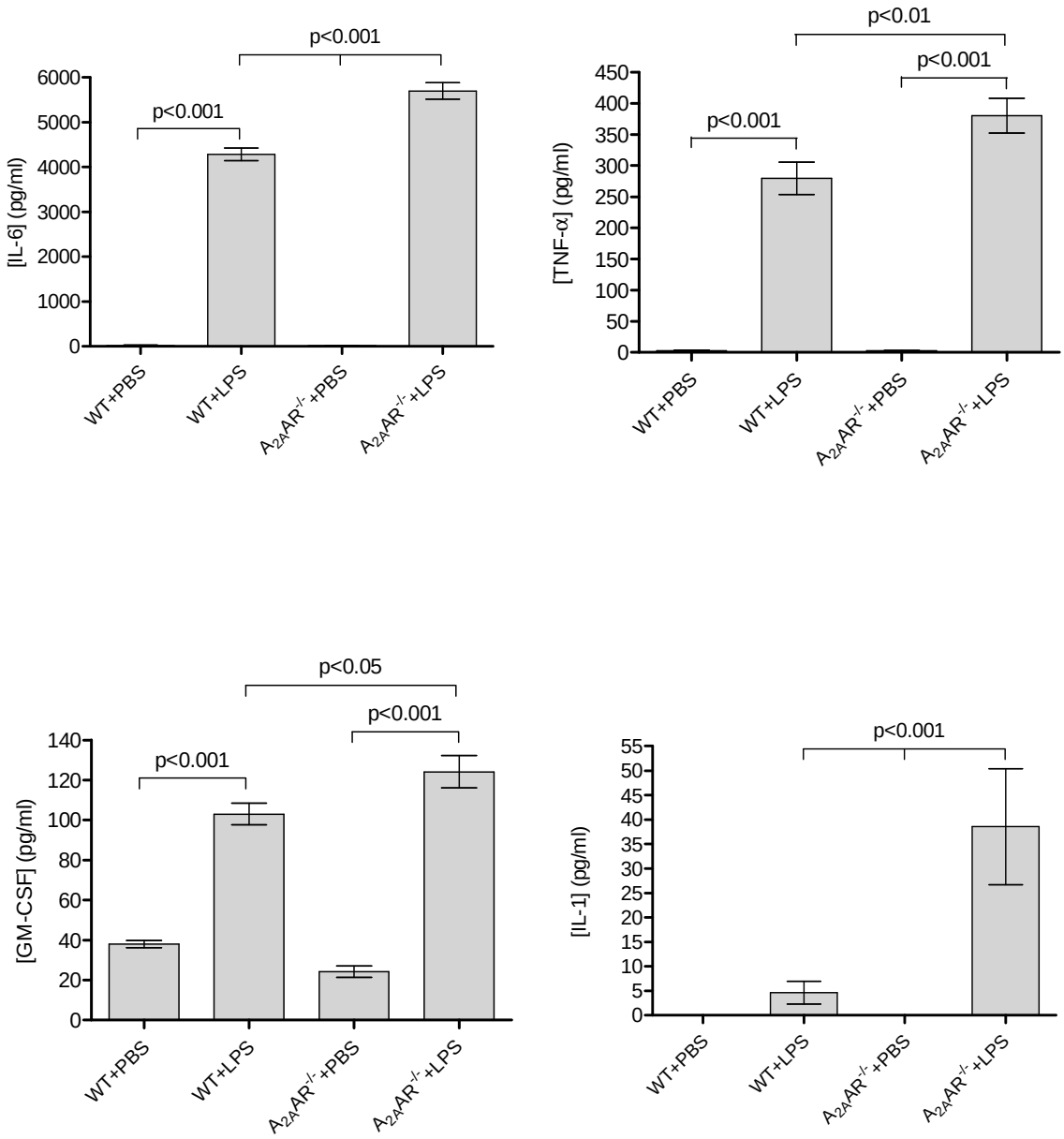


Figure 3.2 Serum levels of proinflammatory cytokines are enhanced in $A_{2A}AR$-deficient mice treated with LPS

Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle. Animals were euthanised after 4 hours and blood was collected for preparation of serum samples. Levels of specific proinflammatory cytokines in serum were measured using a BioSource four-plex fluorescent immunoassay kit and detected using a Luminex instrument as described in Section 2.2.1.4. Values were obtained for 40 mice and are represented in the graphs as the mean ± S.E.

treated controls which confirmed the effectiveness of the LPS administration. This response was potentiated in A_{2A}AR-deficient mice which also displayed a large increase in IL-1 following LPS treatment which was not seen in wild-type mice. There were no significant differences in cytokine levels between wild-type and A_{2A}AR-deficient mice treated with PBS. Thus, LPS-induced pro-inflammatory cytokine production is greatly enhanced in the absence of the A_{2A}AR.

In vitro studies have shown that one mechanism by which the A_{2A}AR may exert its anti-inflammatory effects is through suppression of pro-inflammatory signalling pathway activation. For example, increasing expression of the A_{2A}AR in C6 glioma cells and HUVECs has been shown to reduce NFκB binding to target DNA in response to LPS and TNFα via cell type-specific mechanisms (Sands *et al.*, 2004). In C6 cells, this was associated with almost complete inhibition of IκBα phosphorylation and degradation while in HUVECs, IκBα degradation was unaffected but NFκB translocation to the nucleus is severely impaired. A role for the A_{2A}AR in regulating NFκB has also been demonstrated using $A_{2A}AR^{-/-}$ mice (Lukashev *et al.*, 2004). Following injection with CpG DNA, NFκB DNA-binding activity was found to be enhanced in macrophages derived from $A_{2A}AR^{-/-}$ compared to those from wild-type mice. This appeared to be due to a greater availability of active NFκB as levels of IκB phosphorylation and degradation were also increased.

To see whether the absence of a functional A_{2A}AR potentiates NFκB signalling in the aorta, protein samples were analysed by SDS-PAGE and immunoblotting using phospho-specific and total IκBα antibodies (figure 3.3). In its inactive state, NFκB is found in the cytoplasm in complex with an inhibitory protein such as IκBα. In response to stimuli such as LPS, IκBα is phosphorylated by IKK on Ser32 and Ser36 which marks it for ubiquitination and degradation by the proteasome. Active NFκB is thereby released from the inhibitory complex and translocates to the nucleus to modulate transcription (Mercurio and Manning, 1999). Because phosphorylation of IκB and its subsequent degradation are critical regulatory steps in the NFκB pathway (Karin and Ben-Neriah, 2000), phosphorylated and total levels of IκB can be used to measure NFκB activation. Low levels of phosphorylated IκBα were detected in both LPS and PBS treated wild-type mice while samples from $A_{2A}AR^{-/-}$ mice had greatly elevated levels of IκBα regardless of LPS treatment. Intriguingly, although phosphorylation of IκBα usually leads to its degradation, total levels of IκBα remained constant between different groups of mice.

Figure 3.3 IκBα phosphorylation is increased in $A_{2A}AR^{-/-}$ mice

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and normalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using phospho-specific IκBα (pIκBα) and total IκBα antibodies. **B.** Immunoreactive bands were quantitated by densitometry. Levels of phosphorylated and total IκBα are shown on the graph as a mean percentage of the maximal response ± S.E. (n=12).

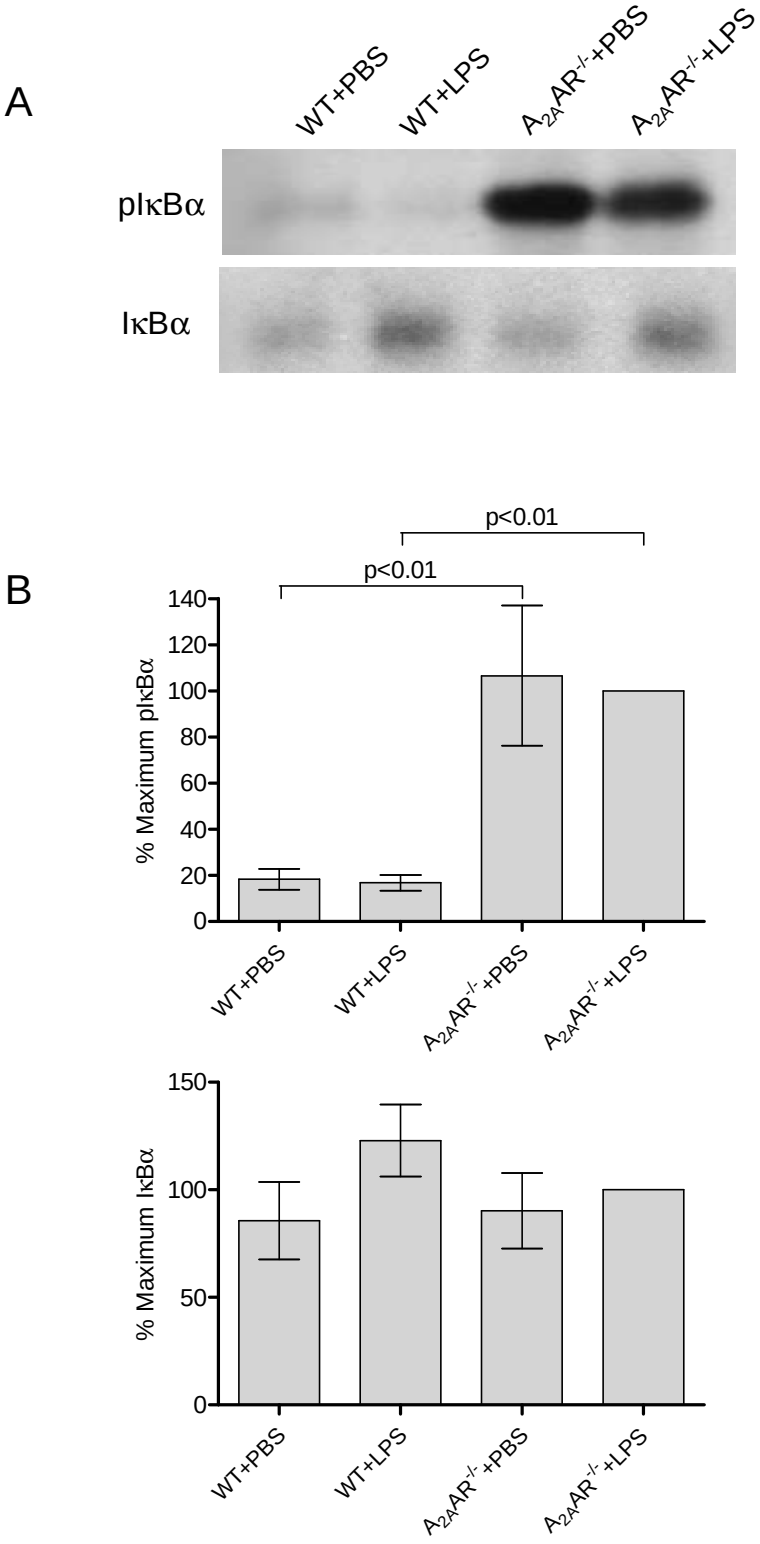


Figure 3.3 IκBα phosphorylation is increased in $A_{2A}AR^{-/-}$ mice

In addition to activation status, the relative abundance of NFκB subunits could potentially have an effect on NFκB signalling. NFκB constitutes a family of structurally related proteins which form homo- or heterodimers. The p50/p65 dimer is the predominant form found in endothelial cells (Read *et al.*, 1994) and is of particular interest due to its role in regulating expression of VCAM-1 (Shu *et al.*, 1993) and E-selectin (Read *et al.*, 1994) which are involved in key steps of leukocyte recruitment during inflammatory responses in the vasculature (Muller, 2002). Levels of p65 in aortic extracts were measured by immunoblotting. However, no significant changes were observed in p65 levels in the aortae of wild-type or $A_{2A}AR^{-/-}$ mice in response to LPS treatment (figure 3.4).

$A_{2A}AR^{-/-}$ mice displayed dramatically increased levels of phosphorylated IκBα compared wild-type mice even in the absence of LPS stimulation. However, this did not appear to translate into increased degradation of IκBα. To determine whether the increases in IκBα phosphorylation had an effect on downstream targets of NFκB, immunoblots were probed for VCAM-1 and ICAM-1, the expression of which is dependent upon NFκB (Schu *et al.*, 1993; Roebuck and Finnegan, 1999). VCAM-1 was barely detectable in samples from PBS treated wild-type or $A_{2A}AR^{-/-}$ mice but was strongly induced in response to LPS treatment (figure 3.5). There was no notable difference in the LPS-induced response between wild-type and $A_{2A}AR^{-/-}$ mice. Similarly, low levels of ICAM-1 were detected in PBS treated wild-type and $A_{2A}AR^{-/-}$ mice while it was strongly upregulated to a similar extent in both wild-type and $A_{2A}AR^{-/-}$ mice treated with LPS (figure 3.6).

In addition to its effects on activation of the NFκB pathway, the $A_{2A}AR$ has also been found to influence pro-inflammatory signalling through suppression of the JAK/STAT pathway. In studies using HUVECs, stimulation of endogenous $A_{2A}ARs$ resulted in reduced STAT3 phosphorylation in response to IL-6 treatment which was associated with induction of SOCS3 (Sands *et al.*, 2006). In addition, increasing expression of the $A_{2A}AR$ has been shown to inhibit IL-6 and IFNγ-induced JAK/STAT signalling via ubiquitination and proteasomal degradation of STAT proteins (Safhi *et al.*, submitted for publication). To determine whether inhibition of JAK/STAT signalling by the $A_{2A}AR$ is a significant mechanism *in vivo*, aortae dissected from wild-type and $A_{2A}AR^{-/-}$ mice were used to make protein samples which were then analysed for the presence of active JAK-phosphorylated STAT proteins using SDS-PAGE and immunoblotting. Levels of phosphorylated STATs were measured as STAT phosphorylation by JAKs represents the key hormone-regulated step in activation of the JAK/STAT pathway and because tyrosine phosphorylation of

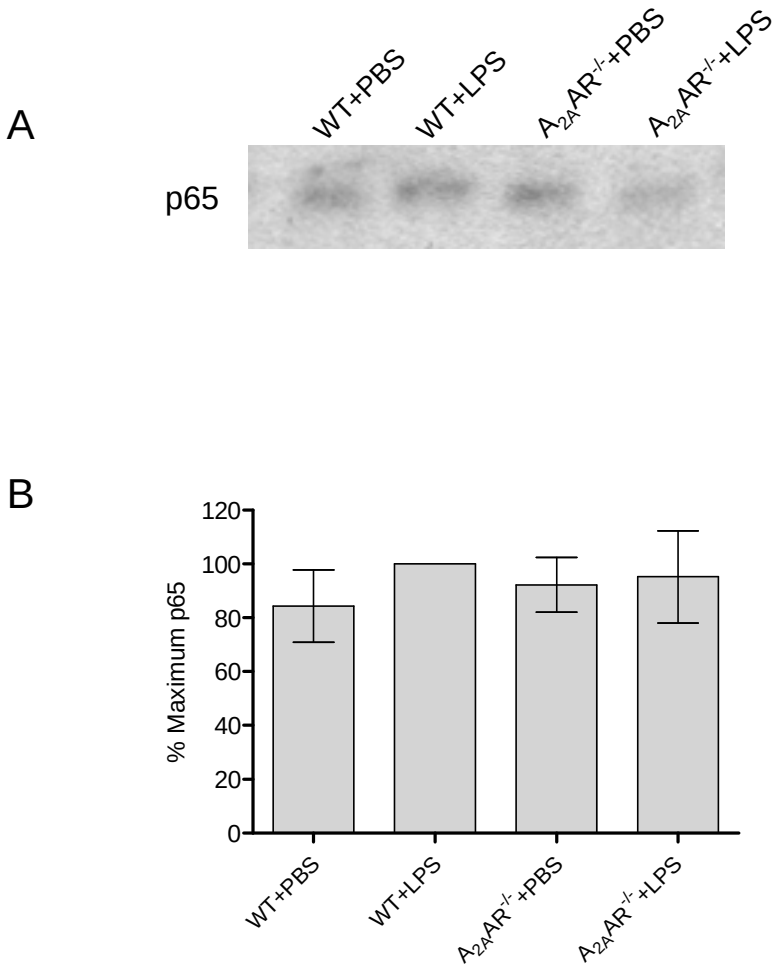


Figure 3.4 Total levels of the NFκB subunit p65 remain unchanged in $A_{2A}AR^{-/-}$ mice subjected to LPS-induced septic shock

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and normalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using an antibody directed against p65. **B.** Immunoreactive bands were quantitated by densitometry. Values are represented on the graph as a mean percentage of the maximal response ± S.E. (n=12).

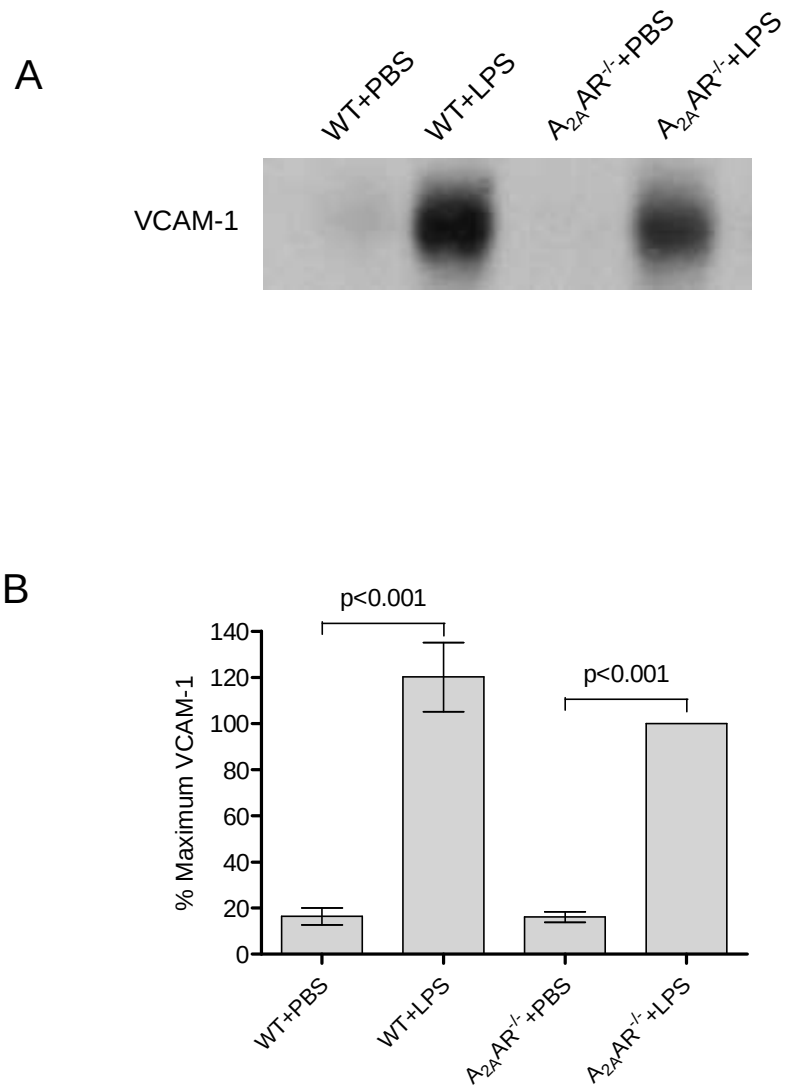


Figure 3.5 Induction of VCAM-1 is unaffected by $A_{2A}AR$ gene deletion

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and bnormalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using an antibody directed against VCAM-1. **B.** Immunoreactive bands were quantitated by densitometry. Values are represented on the graph as a mean percentage of the maximal response ± S.E. (n=12).

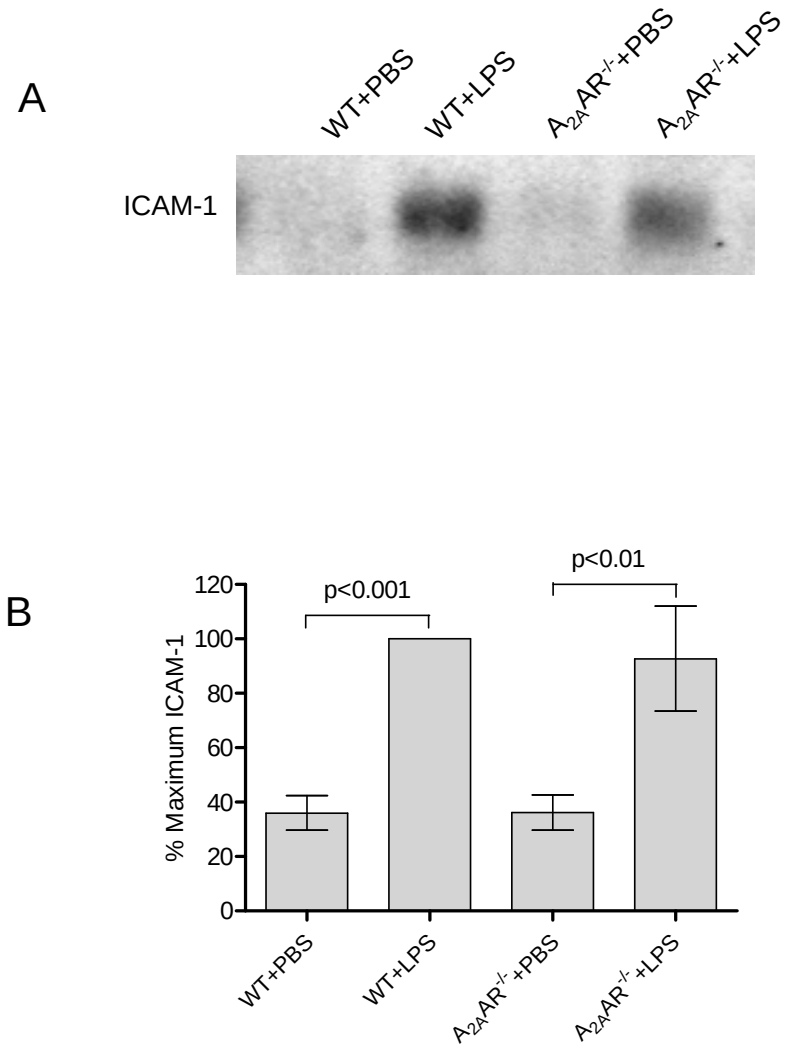


Figure 3.6 Induction of ICAM-1 is unaffected by $A_{2A}AR$ gene deletion

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and normalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using an antibody directed against ICAM-1. **B.** Immunoreactive bands were quantitated by densitometry. Values are represented on the graph as a mean percentage of the maximal response ± S.E. (n=12).

STATs is required for STAT dimerisation and translocation to the nucleus to allow transcriptional activity (Shuai *et al.*, 1993, Kaptein *et al.*, 1996).

Tyr^{701}-phosphorylated STAT1 was barely detectable in PBS-treated wild-type or $A_{2A}AR^{-/-}$ mice but was increased in LPS-treated mice indicating activation of the JAK/STAT pathway (figure 3.7 A, top panel). Interestingly, the observed increase in STAT1 phosphorylation was significantly greater in $A_{2A}AR^{-/-}$ mice compared to wild-type mice ($p<0.001$; figure 3.7 B). This was not due to changes in the abundance of STAT1 protein in $A_{2A}AR^{-/-}$ samples as total STAT1 levels did not vary significantly between groups of mice.

Levels of Tyr^{705}-phosphorylated STAT3 were also very low in PBS-treated mice but were elevated in mice injected with LPS (figure 3.8 A, top panel). While the increase in STAT3 phosphorylation did appear to be greater in $A_{2A}AR^{-/-}$ mice, the difference was not judged to be statistically significant ($p>0.05$; figure 3.8 B). Total levels of STAT3 remained unchanged between wild-type and $A_{2A}AR^{-/-}$ samples.

Since $A_{2A}AR^{-/-}$ mice had increased levels of phosphorylated STAT1 compared to wild-type mice when treated with LPS, it was possible that these mice would express elevated levels of STAT1-dependent gene products such as transporter of antigenic peptides 1 (TAP-1). TAP-1 is part of the TAP complex which transports antigenic peptides generated by the proteasome into the endoplasmic reticulum and facilitates their loading on to class I MHC molecules (Owen and Pease, 1999; Neefjes *et al.*, 1993). TAP-1 expression is induced in response to LPS (Marqués *et al.*, 2004) and pro-inflammatory cytokines such as interferons and TNFα (Epperson *et al.*, 1992) and has been shown to be dependent on STAT1 activation in several cell types including endothelial cells (Mahboubi and Pober, 2002; Cramer *et al.*, 2000; Min *et al.*, 1996). To determine whether increased STAT1 phosphorylation in $A_{2A}AR^{-/-}$ mice had effect on TAP-1 expression, aortic protein samples were subjected to SDS-PAGE and immunoblotting using an anti-TAP-1 antibody. TAP-1 was detected in PBS-treated wild-type and $A_{2A}AR^{-/-}$ mice representing basal expression. However, no significant increases were observed in response to LPS treatment (figure 3.9).

Figure 3.7 Effect of LPS treatment on STAT1 activation in the aortae of A_{2A}AR-deficient mice

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and normalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using antibodies specific for phosphorylated STAT1 (pSTAT1), total STAT1 and GAPDH as indicated. **B.** Immunoreactive bands were quantitated by densitometry. Levels of phosphorylated and total STAT1 were normalised to GAPDH to account for variations in protein loading and are shown on the graph as a mean percentage of the maximal response ± S.E. (n=12).

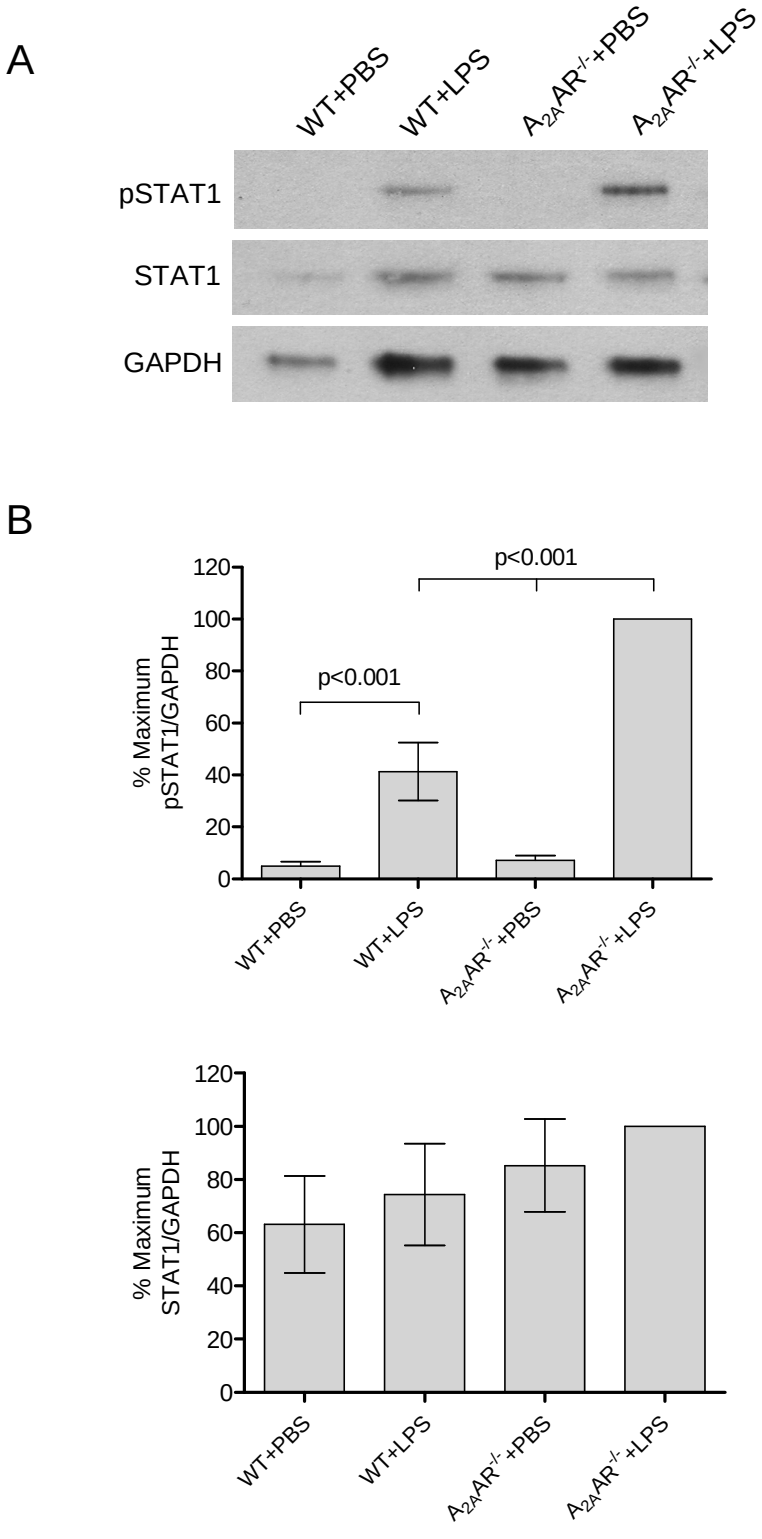


Figure 3.7 Effect of LPS treatment on STAT1 activation in the aortae of A_{2A}AR-deficient mice

Figure 3.8 Effect of LPS treatment on STAT3 activation in the aortae of A_{2A}AR-deficient mice

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and normalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using antibodies specific for phosphorylated STAT3 (pSTAT3), total STAT3 and GAPDH as indicated. **B.** Immunoreactive bands were quantitated by densitometry. Levels of phosphorylated and total STAT3 were normalised to GAPDH to account for variations in protein loading and are shown on the graph as a mean percentage of the maximal response ± S.E. (n=12).

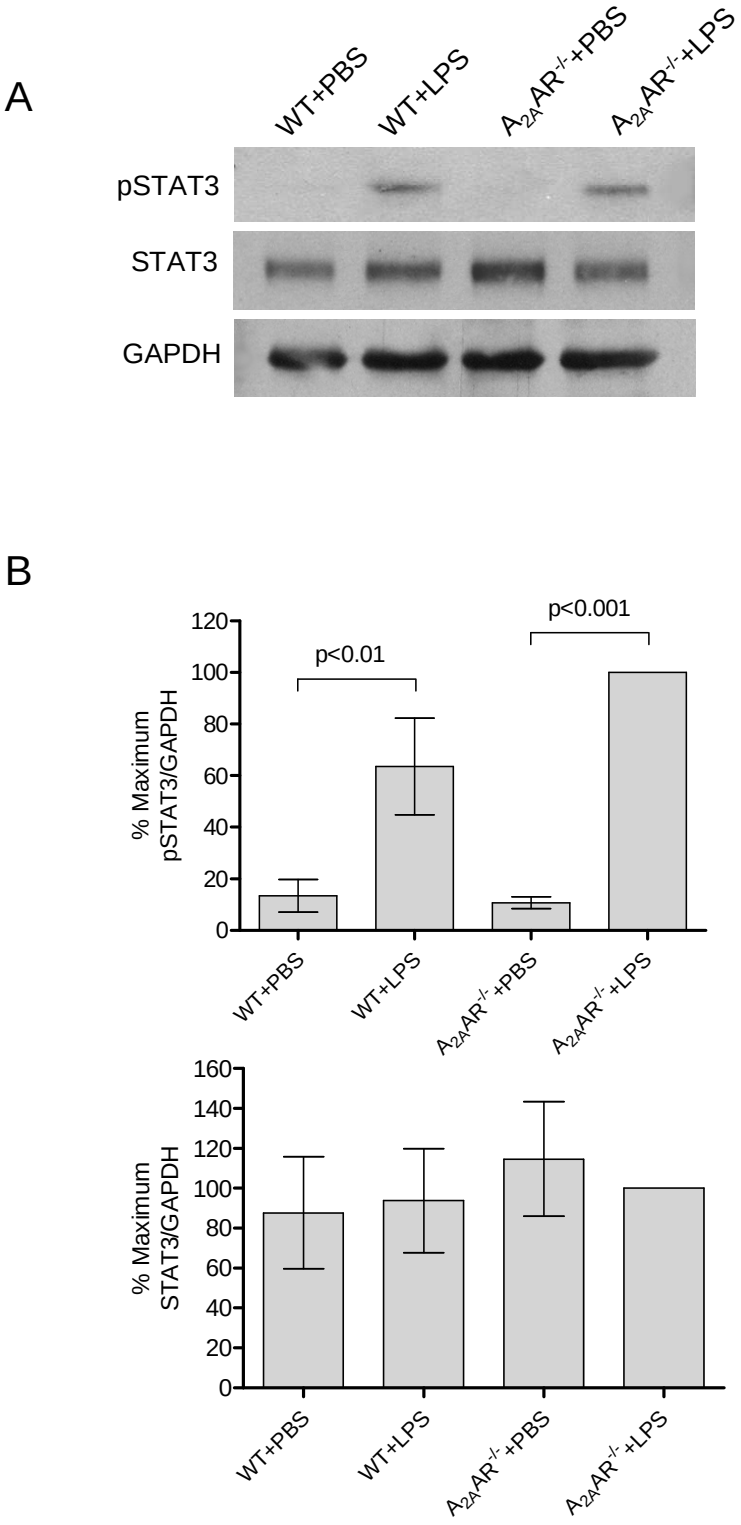


Figure 3.8 Effect of LPS treatment on STAT3 activation in the aortae of A_{2A}AR-deficient mice

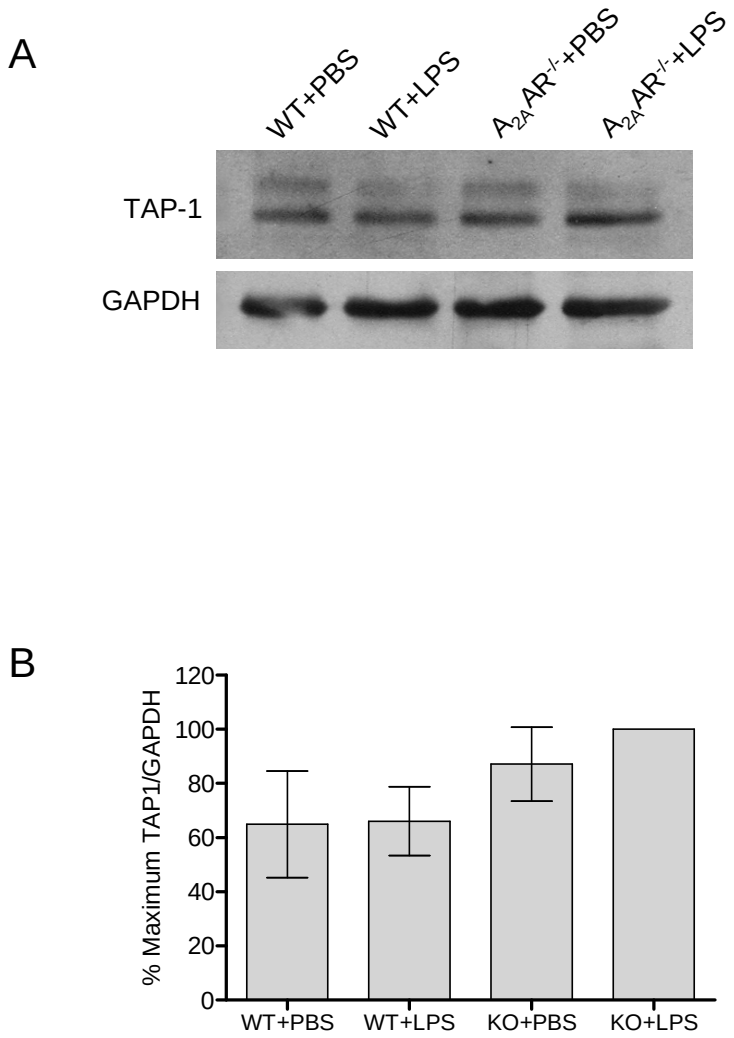


Figure 3.9 Levels of the STAT1-dependent protein TAP-1 are unchanged in LPS-challenged $A_{2A}AR^{-/-}$ mice

A. Wild-type (WT) and $A_{2A}AR^{-/-}$ mice were subjected to intraperitoneal injection of 200 μg LPS or PBS vehicle and then euthanised 4 hours later. Protein extracts were prepared from aortic tissue and normalised for protein content before fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using an antibody directed against TAP-1 and GAPDH as indicated. **B.** Immunoreactive bands were quantitated by densitometry. Levels of TAP-1 were normalised to GAPDH to account for variations in protein loading and are shown on the graph as a mean percentage of the maximal response ± S.E. (n=12).

3.3 Discussion

It is important to understand mechanisms by which the body regulates inflammatory responses as excessive and inappropriate inflammation contribute to the pathology of major diseases including atherosclerosis (Hansson, 2005; Libby, 2002). Studies using A_{2A}AR-deficient mice have shown that signalling through the A_{2A}AR represents an endogenous means of limiting inflammatory responses and tissue damage (Ohta and Sitkovsky, 2001). However, the mechanisms behind this effect are not well understood. Findings from *in vitro* studies indicate that the A_{2A}AR could mediate some of its effects through suppression of the NFκB (Sands *et al.*, 2004) and JAK/STAT (Sands *et al.*, 2006) pro-inflammatory signalling pathways but whether this is a significant mechanism *in vivo* is not known. The aim of this study was to investigate the molecular mechanisms behind the anti-inflammatory actions of the A_{2A}AR *in vivo* using A_{2A}AR-deficient mice. This was achieved through examination of NFκB and JAK/STAT pathway activation in the aortae of A_{2A}AR-deficient mice subjected to LPS-induced septic shock.

Data presented here show that in mice lacking the A_{2A}AR, LPS-induced pro-inflammatory cytokine production is markedly enhanced compared to wild-type mice. Consistent with this observation, activation of the JAK/STAT pathway in response to LPS was potentiated in the aortae of these animals as shown by elevated levels of phosphorylated STAT1. However, STAT1-dependent gene expression as assessed by detecting levels of TAP-1 was unaffected. NFκB signalling was also altered in A_{2A}AR-deficient mice as shown by elevated levels of IκB phosphorylation even in the absence of LPS stimulation. However, total levels of IκBα and RelA/P65 were unaffected as was expression of the NFκB-dependent gene products VCAM-1 and ICAM-1.

Interruption of the A_{2A}AR gene in A_{2A}AR-deficient mice was confirmed by PCR as shown by the increased size of the PCR product representing the gene with the neomycin cassette inserted compared to the wild-type gene. Although expression of receptor protein was not measured in the current study, the lack of a functional A_{2A}AR in A_{2A}AR-deficient mice created in the same way has been confirmed previously (Ledent *et al.*, 1997). In the original study characterising A_{2A}AR-deficient mice, binding of [^{3}H]CGS21680 was detected in brain slices and membrane preparations from the striatum of wild-type mice but not those in which the A_{2A}AR gene had been interrupted, demonstrating a lack of binding sites in A_{2A}AR-deficient mice (Ledent *et al.*, 1997).

Although it was found in this study that the presence of a functional A_{2A}AR was important for regulation of the NFκB and JAK/STAT signalling pathways, it is possible that other adenosine receptor subtypes may have been upregulated in A_{2A}AR-deficient mice and masked some of the effects of the loss of the A_{2A}AR. For example, the A_{2B}AR is also coupled to G_s and so may be able to compensate for the loss of some of the A_{2A}AR's cAMP-mediated effects. Levels of the different receptor subtypes were not examined in this study. However, in previous studies comparing mRNA expression of A_1, A_{2B} or A_3 receptors in the lympoid organs of A_{2A}AR-deficient mice and wild-type mice, no significant changes were observed (Lukashev *et al.*, 2003). This indicates that loss of the A_{2A}AR does not affect expression of other adenosine receptor subtypes in normal conditions. However, there is evidence that adenosine receptors expression is subject to regulation by cytokines produced during inflammatory conditions. For example IL-1β and TNFα have been found to increase expression of the A_{2A}AR in lung epithelial A549 cells, (Morello *et al.*, 2006), human monocytic THP-1 cells (Khoa *et al.*, 2001) and in rat PC12 cells (Trincavelli *et al.*, 2002). Meanwhile, in studies using human microvascular endothelial cells, IL-1 and TNFα have been found to increase expression of the A_{2A}AR and the A_{2B}AR while IFNγ also increased expression of the A_{2B}AR but decreased expression of the A_{2A}AR (Khoa *et al.*, 2003). Expression of the A_{2B}AR is also upregulated in macrophages in response to IFNγ treatment (Xaus *et al.*, 1999). These studies indicate that proinflammatory cytokines can alter the adenosine receptor profile of various different tissues, potentially with different effects in wild-type and adenosine receptor-deficient mice. This is likely to have significance in the present study where levels of these cytokines were increased following LPS treatment.

In this study, LPS-induced septic shock was used as a model of vascular inflammation. LPS activates toll-like receptor 4 (TLR4) on macrophages and neutrophils to induce an acute inflammatory response (Bosshart and Heinzelmann, 2007; Han and Ulevitch, 2005). Activation of pro-inflammatory signalling pathways in macrophages results in expression of cytokines and other inflammatory mediators including TNFα, IL-1, IL-6 and GM-CSF (Murphy *et al.*, 2008). In this study, administration of LPS effectively induced an inflammatory response as shown by the increased levels of these cytokines detected in serum from LPS-treated mice compared to PBS-treated mice. In $A_{2A}AR^{-/-}$ mice, the increase was significantly greater than in wild-type mice indicating that the A_{2A}AR had a suppressive role in regulating pro-inflammatory cytokine production. This is in agreement with data from previous studies which showed increased serum levels of IL-6 and TNFα in

LPS-treated $A_{2A}AR^{-/-}$ mice compared to wild-type mice (Ohta and Sitkovsky, 2001). In further *in vivo* studies, the increases in cytokine production observed in A_{2A}AR-deficient mice were found to be due to positive transcriptional regulation as mRNA levels of pro-inflammatory cytokines were increased in LPS-treated $A_{2A}AR^{-/-}$ mice compared to wild-type mice (Lukashev *et al.*, 2004). This effect could be simulated by pharmacological inactivation of the A_{2A}AR in wild-type mice using the A_{2A}AR-selective antagonist ZM241385. In contrast, activation of the A_{2A}AR by injection of the A_{2A}AR-selective agonist CGS21680 resulted in reduced expression of pro-inflammatory cytokine mRNA, providing further evidence that signalling through the A_{2A}AR regulates inflammation *in vivo* through suppression of pro-inflammatory cytokine expression (Lukashev *et al.*, 2004).

To begin to investigate mechanisms potentially responsible for the increased inflammatory responses seen in A_{2A}AR-deficient mice, protein samples produced from the aortae of LPS and PBS-treated wild-type and A_{2A}AR-deficient mice were examined for the presence of components of the NFκB and JAK/STAT signalling pathways.

In this study, activation of the NFκB pathway as determined by IκB phosphorylation was strikingly upregulated in both PBS and LPS-treated $A_{2A}AR^{-/-}$ mice compared to wild-type mice suggesting that the A_{2A}AR plays a role in regulating activation of the pathway even in the absence of stimuli. A negative regulatory effect of the A_{2A}AR on NFκB signalling has been observed previously in macrophages from $A_{2A}AR^{-/-}$ mice (Lukashev *et al.*, 2004) and has also been demonstrated *in vitro* in C6 cells where increasing expression of the A_{2A}AR resulted in a severe reduction in IκB phosphorylation in response to TNFα or LPS (Sands *et al.*, 2004). The reason for the lack of effect of LPS treatment on IκBα phosphorylation in $A_{2A}AR^{-/-}$ mice is not clear. It could be that the NFκB pathway is maximally activated even in basal conditions. However, this cannot be concluded from data presented here as although levels of phosphorylated IκBα are clearly elevated in A_{2A}AR mice, it is not possible to tell what percentage of total IκB levels this represents. This question could be addressed through the use of two-dimensional gel electrophoresis to fractionate protein samples on the basis of charge as well as size. This would allow separation of phosphorylated IκBα from the unphosphorylated form, thereby allowing quantitation of their relative abundances.

IKK-mediated phosphorylation of IκBα on Ser32 and Ser36 is generally thought to lead to its Lys48 polyubiquitination and degradation by the proteasome (Karin and Ben-Neriah,

2000). Surprisingly, despite the dramatic increases in IκBα phosphorylation observed here in $A_{2A}A^{-/-}$ mice, there were no differences in total levels of IκBα between different groups of mice. A possible explanation for this is that in $A_{2A}AR^{-/-}$ mice, negative feedback mechanisms are induced or upregulated to compensate for the increased activation of the NFκB pathway. IκBα is itself a target for NFκB transcriptional activity (de Martin *et al.*, 1993) and its resynthesis and binding to activated NFκB proteins in the nucleus is a crucial step in the regulation of NFκB signalling (Karin and Ben-Neriah, 2000). In an auto-regulatory loop, newly synthesised IκBα enters the nucleus where it binds to NFκB and removes it from DNA, thereby terminating its transcriptional activity (Arenzana-Seisdedos *et al.*, 1997). The NFκB/IκB complex is then transported back into the cytoplasm by the nuclear protein CRM1 which recognises nuclear export sequences on IκB (Huang *et al.*, 2000; Johnson *et al.*, 1999). It is possible that in $A_{2A}AR^{-/-}$ mice, the efficiency of this pathway is upregulated and that increased degradation of phosphorylated IκB does occur but is not detected because it is rapidly resynthesised resulting in no net change in abundance.

Another possible explanation for the apparent lack of IκBα degradation is that there is a defect in the proteasome of $A_{2A}AR^{-/-}$ mice. Alternatively, there could be alterations in the activity of the enzymes which mediate ubiquitination of phosphorylated IκBα and target it for degradation by the proteasome. The E3 ubiquitin ligase complex which recognises phosphorylated IκBα and promotes its polyubiquitination is regulated by post-translational modification by the ubiquitin-like protein Nedd8 (Read *et al.*, 2000). Neddylation of the cullin-1 subunit of the IκB-specific Skp1-cullin-F-Box (SCF) ubiquitin ligase is required for its activity while deneddylation represents a means of regulating NFκB activity by preventing degradation of IκB (Read *et al.*, 2000). This mechanism has been found to be activated by adenosine acting through A_{2B}ARs and to contribute to the protective effects of hypoxic preconditioning in mice (Khoury *et al.*, 2007). It is possible that in $A_{2A}AR^{-/-}$ mice, this protective mechanism is upregulated and neddylation of cullin-1 is reduced. This would result in IκBα levels remaining constant despite increased phosphorylation.

Other NFκB-induced feedback inhibitors which could be at work to prevent inappropriate activation in A_{2A}AR-competent mice include the deubiquitinating proteins CYLD and A20. CYLD inhibits NFκB by reversing the K63-linked ubiquitination of upstream signalling molecules such as TRAF2 and TRAF6 which is required for LPS-induced activation of the IKK complex (Courtois, 2008). CYLD expression has been found to be

upregulated in atherosclerotic lesions and its overexpression in cultured endothelial cells resulted in inhibition of TNFα-induced NFκB activation through deubiquitination of TRAF2 (Takami *et al.*, 2008). These findings suggest it could play an important role in suppressing inflammation in the aorta. A20 was originally identified as regulator of TNFα-induced NFκB activation with the ability to change the ubiquitination profile of RIP thereby targeting it for proteasomal degradation but it could also affect activation by LPS as it has effects on signalling from TLRs through deubiquitination of TRAF2 and IKKγ (Heyninck and Beyaert, 2005).

Despite the observed dysregulation of IκBα phosphorylation in $A_{2A}AR^{-/-}$ mice, no changes were seen in the induction of VCAM-1 and ICAM-1 expression in response to LPS, perhaps reflecting the efficiency of feedback mechanisms induced. However, it is also possible that increases in VCAM-1 and ICAM-1 expression were not detected due to increased shedding of the extracellular domains of these proteins into the circulation of $A_{2A}AR^{-/-}$ mice. Increased levels of the soluble forms of VCAM-1 and ICAM-1 are found in a number of inflammatory diseases (Gearing and Newman, 1993). In this study, VCAM-1 and ICAM-1 were detected in the aorta using an antibody directed against their N-terminal extracellular domains which would not take account of molecules which have shed their extracellular domains. Total levels of VCAM-1 and ICAM-1 could be measured using an antibody directed against their intracellular domains or by detecting levels of shed molecules in serum.

In addition to the NFκB pathway, the JAK/STAT pathway is also activated in response to LPS. LPS signalling through TLR4 has not been reported to directly activate the JAK/STAT pathway but rather to rapidly induce expression of interferons in order to achieve optimal expression of LPS-inducible genes (Ohmori and Hamilton, 2001). In this study, STAT1 phosphorylation in response to LPS treatment was elevated in $A_{2A}AR^{-/-}$ mice compared to wild-type mice. It is tempting to suggest that this might be due to suppression of STAT1 phosphorylation in wild-type mice by members of the SOCS family as stimulation of the $A_{2A}AR$ has been shown to reduce IL-6-induced STAT3 phosphorylation in cultured endothelial cells through induction of SOCS3 (Sands *et al.*, 2006). However, SOCS3 mediates this effect on STAT3 activation by binding to tyrosine-phosphorylated IL-6 receptors and inhibiting the activity of associated JAKs (Sasaki *et al.*, 1999). Since regulation by SOCS3 is at the level of JAK activity, it would be expected that if SOCS3 is involved then both STAT1 and STAT3 phosphorylation would be affected in $A_{2A}AR^{-/-}$ mice. SOCS1 also inhibits JAK activity (Yasukawa *et al.*, 1999) and

so can influence phosphorylation of both STAT1 and STAT3. Since only STAT1 phosphorylation is significantly elevated in $A_{2A}AR^{-/-}$ mice, it seems unlikely that decreased activity of SOCS1 or SOCS3 is responsible.

Despite increased STAT1 phosphorylation in response to LPS, STAT1-regulated expression of TAP-1 was not altered in $A_{2A}AR^{-/-}$ mice. However, the significance of this is not clear as TAP-1 expression was not increased in LPS-treated mice. High basal expression of this protein may have masked changes occurring in response to LPS or it is possible that although the dose of LPS used was sufficient to elicit STAT1 phosphorylation, it may not have activated the JAK/STAT pathway strongly enough to produce detectable effects downstream. Alternatively, it may be that $A_{2A}AR$-mediated regulation of STAT1-dependent gene expression is target-specific and examining expression of other STAT1-regulated genes may still reveal functional effects of the absence of the $A_{2A}AR$. In order to investigate this possibility, attempts were made during this study to determine the effects of $A_{2A}AR$ gene deletion on expression of the inducible form of nitric oxide synthase (iNOS). iNOS expression can be induced in endothelial cells in response to combinations of cytokines such as IFNγ and TNFα (Wagner *et al.*, 2002) or by IFNs and LPS (Koide *et al.*, 2007) through activation of both STAT1 and NFκB which act synergistically at the iNOS promoter (Ganster *et al.*, 2001). However, in this study, it was not possible to assess iNOS expression as immunoblotting using an anti-iNOS antibody produced multiple bands of approximately the correct size. Attempts were made to obtain clearer results using a second iNOS antibody directed against a separate epitope and through use of different positive controls. However, it was still not possible to confidently identify a single band as iNOS.

Mice lacking the $A_{2A}AR$ have been shown previously to express elevated levels of pro-inflammatory cytokines and to suffer enhanced tissue damage in response to inflammatory stimuli (Ohta and Sitkovsky, 2001). The model of vascular inflammation used in this study revealed significant perturbations in both NFκB and JAK/STAT signalling in the aortae of $A_{2A}AR^{-/-}$ mice which could perhaps account for these effects. It was of particular interest to study pro-inflammatory signalling pathway activation in the aorta because in a previous study, the NFκB pathway was found to be primed for activation in atherosclerotic lesion-prone areas of the aorta in LPS-treated wild-type mice (Hajra *et al.*, 2000). If $A_{2A}AR$ expression in the aorta can regulate NFκB activation then it may play a role in preventing development of atherosclerosis. However, further analysis of NFκB and

STAT-dependent expression of specific target genes including VCAM-1 and ICAM-1 which mediate leukocyte recruitment during atherogenesis did not reveal any changes in protein levels in the absence of the A_{2A}AR. This does not however rule out a role for the A_{2A}AR in regulating inflammatory events involved in atherosclerosis. Several aspects of the model used in this study mean that it was not possible to fully evaluate the effects of the loss of the A_{2A}AR on specific events occurring in the endothelium during atherogenesis. For example, LPS induces an acute form of inflammation while atherosclerosis is a chronic inflammatory condition. Although LPS does induce expression of inflammatory mediators and adhesion molecules believed to be important in development of atherosclerosis, the actual similarity to the disease is not known. In addition, results presented here are based on samples produced from whole aortas which included endothelial cells but also the surrounding smooth muscle layer. The A_{2A}AR may have specific effects in the endothelium which are masked by responses occurring in smooth muscle cells. Similarly, by examining the whole aorta, it was not possible to detect differences in inflammatory pathway activation and target protein expression specifically in lesion-prone areas of the aorta where regulation could be most crucial. This problem could be addressed by using immunohistochemistry to examine NFκB and JAK/STAT pathway activation in particular lesion-prone areas of the intact endothelium.

An additional concern in this study was that since mice were euthanized and organs harvested 4 hours following LPS-treatment it was only possible to examine protein expression in aortic tissue samples at a single time point. The NFκB and JAK/STAT pathways are subject to regulation at many stages through feedback mechanisms and through interaction with other signalling pathways. It is likely that many changes occurred before the 4 hour time point or may have occurred later if the study had been continued for longer. However, in order to produce samples at different time points, it would have been necessary to use much larger numbers of mice which would have been prohibitively expensive.

Regardless of the limitations mentioned above, data presented here clearly mark out a role for the A_{2A}AR in modulating pro-inflammatory signalling through the NFκB and JAK/STAT pathways in the aorta. This not only adds to the collective evidence for a physiological role for the A_{2A}AR for limiting inflammation *in vivo* but more importantly provides new evidence regarding the mechanisms behind this effect. Future studies *in vitro* should be aimed at further analysis of the role of the A_{2A}AR in regulating NFκB and

JAK/STAT signalling and will hopefully allow identification of specific mechanisms involved.

4 Regulation of the A_{2A} adenosine receptor by phosphorylation

4.1 Introduction

The anti-inflammatory and tissue protective effects of signalling through the A_{2A}AR are well documented (Palmer and Trevethick, 2008; Sitkovsky *et al.*, 2004; Sitkovsky, 2003). Elucidation of the mechanisms behind the beneficial effects of the A_{2A}AR is a current topic of investigation and progress is being made in identifying means by which the A_{2A}AR may modulate pro-inflammatory signalling pathways as exemplified by findings described in chapter 3. In contrast, a subject that has not received much attention is how the A_{2A}AR is regulated at a molecular level. This is of particular interest since it has become apparent that in addition to heterotrimeric G proteins and proteins involved in desensitisation, numerous other proteins can interact with the intracellular portions of GPCRs. These proteins have the potential to regulate the activity of GPCRs either by directly activating alternative signalling pathways or by acting as adaptors or scaffolds to recruit proteins with the potential to modulate G protein-dependent signalling or initiate G protein-independent signalling events (Kristiansen, 2004; Hall and Lefkowitz, 2002; Heuss and Gerber, 2000).

The A_{2A}AR has an unusually long C-terminal tail in comparison to many other GPCRs and particularly in contrast to other adenosine receptors (122 amino acids in man compared to only 34 in the C-terminal tail of the A_1AR; Zezula and Freissmuth, 2008). The presence of numerous serine and threonine residues in this region suggests potential for regulation of the A_{2A}AR by phosphorylation. Indeed, agonist-induced desensitisation of the canine A_{2A}AR is associated with rapid phosphorylation of the receptor (Palmer *et al.*, 1994). Furthermore, studies using the canine A_{2A}AR expressed in C6 glioma cells have shown that stimulation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) or endothelin-1 (ET-1) which activates PKC through endogenous ET_A receptors results in dramatically increased phosphorylation of the A_{2A}AR over basal levels (Palmer and Stiles, 1999). Through the use of inhibitors, the involvement of PKC in mediating this effect has been confirmed and the PKCδ isoform in particular identified as being partly responsible.

The role of the extended C-terminal tail of the A_{2A}AR and the significance of phosphorylation events in this region has not been determined. In fact, it has been demonstrated that deletion of 95 amino acids from the C-terminus of the canine A_{2A}AR

which removes ten of the twelve potential phosphorylation sites has no effect on agonist-induced phosphorylation or desensitisation of the receptor (Palmer and Stiles, 1997). Similarly, using truncated forms of the human A_{2A}AR it has been shown that the last 95 amino acids of the C-terminal tail are not required for agonist-stimulated G protein-coupling and stimulation of adenylyl cyclase (Palmer and Stiles, 1997). In addition, it has been found that the agonist-binding properties of a mutant canine A_{2A}AR lacking the last 102 residues are comparable to those of the wild-type receptor (Piersen *et al.*, 1994). Since truncation of the A_{2A}AR appears to have no effect on the classical signalling or regulatory mechanisms associated with GPCRs, other potential roles for the C-terminal tail must be considered. A number of proteins have been reported to interact with this region of the A_{2A}AR indicating that it may act as a scaffold for recruitment of proteins involved in G protein-independent signalling pathways (Zezula and Freissmuth, 2008). This theory is supported by the finding that G-protein independent activation of ERK by the A_{2A}AR is dependent on an interaction between the C-terminal tail of the A_{2A}AR and ARF nucleotide site opener (ARNO) which is a guanine nucleotide exchange factor for the ADP-ribosylation factor (ARF) family of monomeric G proteins (Gsandtner *et al.*, 2005). The cytoskeletal protein α-actinin has also been identified as a C-terminal binding partner of the A_{2A}AR. This interaction has been found to be important for agonist-mediated clustering and internalisation of the receptor (Burgueño *et al.*, 2003). However, α-actinin can also interact with components of the ERK cascade, suggesting an additional role as a scaffold protein involved in organisation of ERK signalling (Christerson *et al.*, 1999; Leinweber *et al.*, 1999). Translin-associated protein X (TRAX) is another protein which binds to the C-terminus of the A_{2A}AR with effects on A_{2A}AR-mediated signalling that appear to be both G_s and ERK-independent (Sun *et al.*, 2006). TRAX has been shown to be involved in the ability of the A_{2A}AR to suppress proliferation of PC12 cells and restore nerve growth factor (NGF)-induced neuronal differentiation that is impaired by p53 inactivation. However, the mechanism by which this occurs is unclear (Sun *et al.*, 2006). Yet another recently identified interaction partner of the A_{2A}AR is the deubiquitinating enzyme ubiquitin-specific protease 4 (USP4). Binding of USP4 to the C-terminus of the A_{2A}AR results in deubiquitination of the receptor which is required for its export from the ER during synthesis (Milojević *et al.*, 2006). Ubiquitination status is, however, also an important determinant of the fate of endocytosed GPCRs (Wojcikiewicz, 2004) and so it is possible that binding of USP4 could have effects on the rate of receptor recycling following desensitisation.

The list of proteins reported to interact with the C-terminal tail of the A_{2A}AR is growing rapidly. Despite the exaggerated length of this region compared to other adenosine receptors, it is unlikely that it is long enough to accommodate binding of numerous interaction partners simultaneously. Moreover, ARNO (Gsandtner *et al.*, 2005) and α-actinin (Burgueño *et al.*, 2003) have been reported to bind in the same juxtamembrane portion of the receptor tail. This highlights the need for regulation of binding events and indeed it has been suggested that phosphorylation of thr 298 which is present in the binding sites of both ARNO and α-actinin may be important for determining which interactions occur (Zezula and Freissmuth, 2006). As mentioned above, there are twelve potential phosphorylation sites present within the C-terminal tail of the receptor and so it seems highly likely that phosphorylation events could have consequences for binding of particular accessory proteins. This could provide a mechanism by which different pathways can interact with the A_{2A}AR to regulate its activity.

The aim of the present study was to identify stimuli that induce increased phosphorylation of the human A_{2A}AR and to determine how this affects recruitment of C-terminal-interacting molecules involved in regulating A_{2A}AR activity and downstream signalling events. Information currently available in this area is derived from work using the canine A_{2A}AR expressed in C6 glioma, CHO and COS cells (Palmer and Stiles, 1999). Although A_{2A}ARs have fairly high species homology (92% between human and canine), most differences occur within the C-terminal tail. For this reason, findings from studies using canine receptors may not reflect mechanisms that regulate human A_{2A}ARs. In addition, since C6, CHO and COS cells do not express A_{2A}ARs, they may not have the regulatory mechanisms in place that would be found in cells expressing endogenous receptors. In order to properly understand how the human A_{2A}AR is regulated, it is necessary to study human receptors in human cells that express endogenous receptors. Therefore, in the following experiments receptor phosphorylation has been studied using a myc-tagged human A_{2A}AR expressed in human umbilical vein endothelial cells (HUVECs).

4.2 Results

The myc-tagged human A_{2A}AR was introduced to HUVECs via adenoviral mediated gene transfer using an moi of 30 ifu/cell. Successful expression of the A_{2A}AR was confirmed by detection of the myc tag of the receptor by immunoblotting using the 9E10 antibody (figure 4.1, *lower panel*). Immunoblotting produced two bands at approximately 50 and 40 kDa in samples from adA_{2A}AR-infected cells as has been observed previously using the 9E10 antibody to detect the myc-tagged A_{2A}AR expressed in HUVECs using adenovirus (Sands *et al.*, 2004). A third band was detected below the 37 kDa marker in both adA_{2A}AR and adGFP-infected cells and so was determined to be non-specific. The 50 kDa band corresponds to the size of the native A_{2A}AR which has been detected as a 45-46 kDa band using photoaffinity labelling of the A_{2A}AR in human striatal membranes (Ji *et al.*, 1992) and cardiac tissues (Marala *et al.*, 1998). This is likely to represent the fully glycosylated, full-length form of the receptor. This has been investigated by immunoprecipitating the A_{2A}AR from cells treated with biotin hydrazide to label cell-surface carbohydrate groups (T.M. Palmer, unpublished observation). Blotting with HRP-streptavidin produced a single band at approximately 50 kDa representing the glycosylated receptor. The 40 kDa band is likely to represent a partially processed form of the A_{2A}AR rather than a degradation product as degradation of the A_{2A}AR typically involves loss of the C-terminal tail (Nanoff *et al.*, 1990). The myc tag is at the C-terminus of the receptor and thus the tail must be intact to be recognised by 9E10. Furthermore, the 40 kDa band is consistent with the predicted molecular weight of the deglycosylated receptor and is of a similar size to the 38 kDa bovine striatal A_{2A}AR detected by photoaffinity labelling following deglycosylation (Barrington *et al.*, 1990).

Expression of the myc-tagged A_{2A}AR was specific to adA_{2A}AR-infected cells as recombinant receptor was not detected in adGFP-infected cells. Stimulation of the receptor using the selective agonist CGS21680 produced a time-dependent increase in ERK phosphorylation indicating that the receptor was functional (figure 4.1, *upper panel*). ERK phosphorylation in response to CGS216080 was also detected in adGFP-infected cells but not to the extent seen in adA_{2A}AR-infected cells indicating activation of endogenous receptors. Saturation binding analysis using ^{3}H-ZM241385 revealed that in adA_{2A}AR-infected cells, the myc-tagged A_{2A}AR had a K_d value of 1.4 ± 0.4 nM and was expressed at a level of 80 ± 7 pmol/mg protein (figure 4.2). Despite detection of functional endogenous receptors via CGS21680 stimulation of ERK phosphorylation, no ^{3}H-

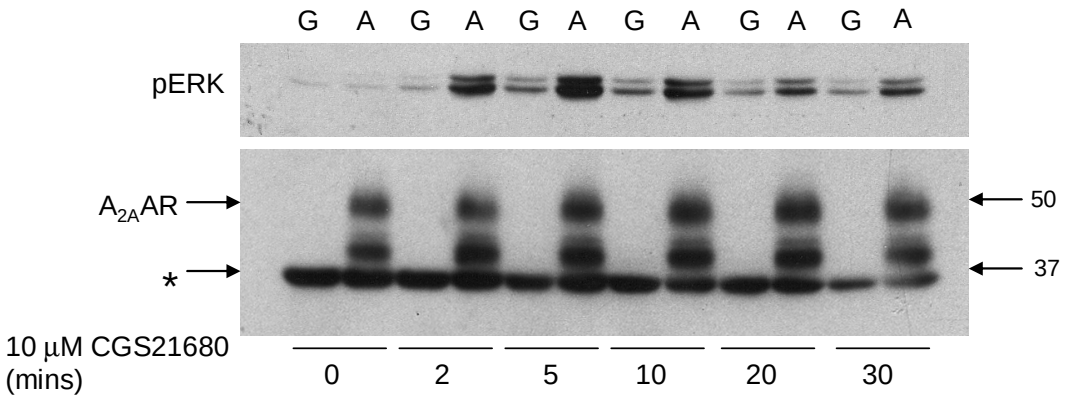


Figure 4.1 Adenovirus-mediated expression of the human A_{2A}AR in HUVECs

HUVECs were infected with A_{2A}AR-expressing adenovirus (A) or control adenovirus expressing GFP alone (G). Cells were incubated with 10 μM CGS21680 for the times indicated before preparation of protein extracts. Extracts were normalised for protein content prior to fractionation by SDS-PAGE on 10 % (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose for immunoblotting using a phospho-specific ERK antibody (pERK) and the 9E10 antibody which recognises the myc tag of the A_{2A}AR. This is an example of two such experiments. The asterisk denotes a non-specific band.

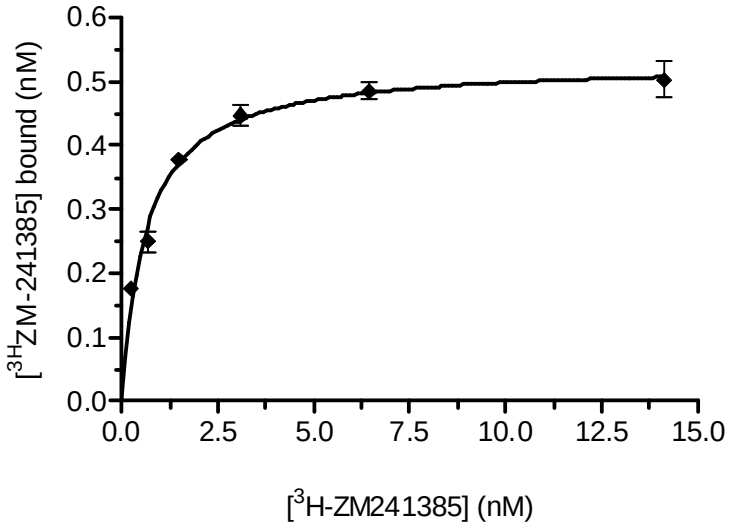


Figure 4.2 Saturation analysis of ^{3}H-ZM241385 binding to the myc-tagged human A_{2A}AR

HUVECs were infected with A_{2A}AR-expressing adenovirus before preparation of membrane extracts for use in saturation binding assays using a range of concentrations of the radiolabelled A_{2A}AR antagonist ^{3}H-ZM241385. This is a representative example of three such experiments.

ZM241385 binding was observed in adGFP-infected cells. However, this lack of detectable binding has been found in previous studies in HUVECs (Sands *et al.*, 2004) and T-cells (Armstrong *et al.*, 2001) using ^{3}I-ZM241385.

To allow analysis of receptor phosphorylation in whole cells, it was necessary to be able to isolate the A_{2A}AR from other proteins present in whole cell extracts. This was achieved by immunoprecipitation using the 9E10 antibody which recognises the myc tag of the A_{2A}AR. Immunoblotting using an A_{2A}AR-specific antibody revealed that the A_{2A}AR was successfully immunoprecipitated from adA_{2A}AR-infected cells but not from control adGFP-infected cells and only in the presence of the 9E10 antibody showing that the procedure was specific for the myc-tagged A_{2A}AR (figure 4.3). In addition, recovery of the receptor was good as immunoprecipitated receptors were detected at levels proportionally comparable to total input levels of the A_{2A}AR in whole cell lysates.

For initial analysis of receptor phosphorylation, HUVECs infected with adA_{2A}AR or adGFP were subjected to an intact cell phosphorylation assay in the presence or absence of 1μM PMA which has been shown to induce phosphorylation of the canine A_{2A}AR in C6 cells (Palmer and Stiles, 1999). The phosphorylated A_{2A}AR was detected as two bands at approximately 45-50 kDa and 40 kDa corresponding to the bands detected in immunoblots. A third band above 50 kDa was also detected in some cases but appears to be non-specific as it is present in lanes when the other bands are not detected and is also faintly detectable in cells that have not been infected with adA_{2A}AR.

Stimulation with PMA resulted in an increase in receptor phosphorylation over basal levels in adA_{2A}AR-infected cells but not adGFP-infected cells showing that it was the phosphorylated A_{2A}AR that was being detected specifically (figure 4.4 A). PMA activation of novel and conventional isoforms of PKC results in Raf- mediated activation of the ERK pathway (Schönwasser *et al.*, 1998) in manner that can be either Ras-dependent (Chiloeches *et al.*, 1999) or -independent (Ueda *et al.*, 1996). Induction of ERK phosphorylation by PMA was therefore a useful measure to confirm PMA activity as shown in figure 4.4 B (*upper panel*). The presence of the A_{2A}AR in adA_{2A}AR-infected cells but not adGFP-infected cells was confirmed by immunoblotting (figure 4.4B, *lower panel*).

To begin to characterise phosphorylation of the A_{2A}AR, HUVECs infected with adA_{2A}AR were subjected to an intact cell phosphorylation assay in the presence of 10 μM

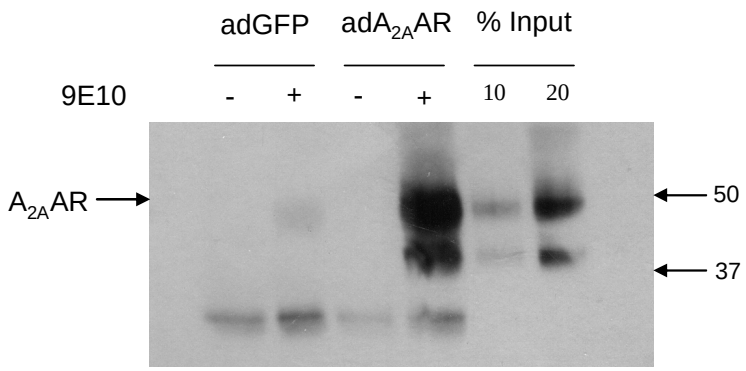


Figure 4.3 Immunoprecipitation of the A_{2A}AR

HUVECs were infected with adenovirus expressing the myc-tagged A_{2A}AR (adA_{2A}AR) or GFP alone (adGFP). Cell extracts were prepared and equalised for protein content before subjection to an immunoprecipitation protocol in the presence or absence of the anti-myc 9E10 antibody as indicated. Immunoprecipitated receptors and total levels of receptors in cell lysates (Input) were detected using SDS-PAGE followed by immunoblotting using an A_{2A}AR-specific antibody.

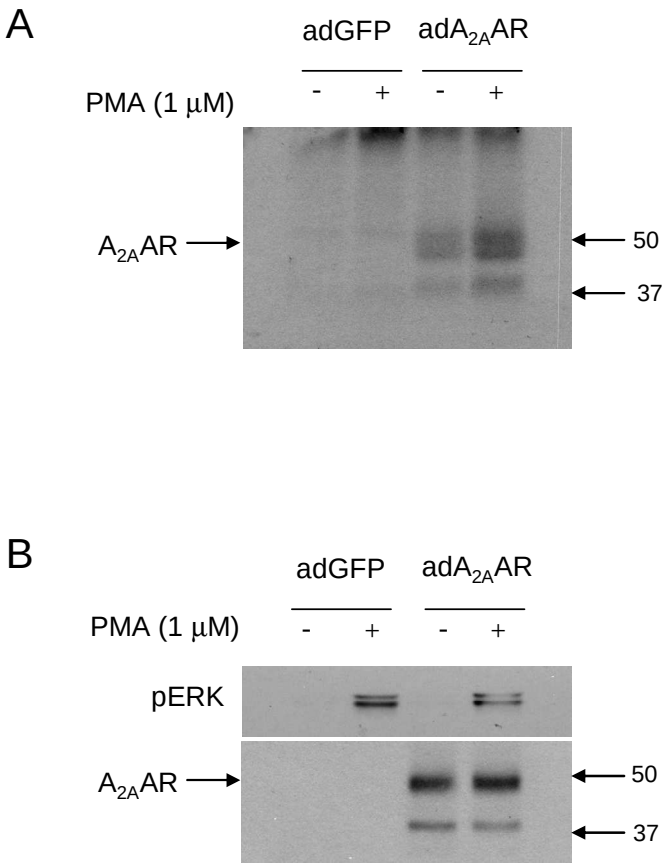


Figure 4.4 Specific immunoprecipitation and phosphorylation of the A_{2A}AR

HUVECs infected with adenovirus expressing the myc-tagged A_{2A}AR ($adA_{2A}AR$) or GFP alone (adGFP) were labelled with ^{32}P orthophosphate for 90 minutes. Cells were incubated in the presence or absence of 1 μM PMA for 30 minutes before preparation of protein extracts. Extracts were equalised for protein content and used to immunoprecipitate the A_{2A}AR using the anti-myc 9E10 antibody. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Levels of phosphorylated ERK (pERK) and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells. The 9E10 antibody was used to detect the A_{2A}AR. Shown are examples of results from two replicated experiments.

CGS21680, 1 μM PMA, 50 μM forskolin, 10 ng/ml TNFα, 1μg/ml LPS or 5 ng/ml IL-6. CGS21680 was included as GPCRs in general are phosphorylated in response to agonist stimulation as discussed in Section 1.5.4.1. In addition, CGS21680 has previously been shown to induce phosphorylation of the canine A_{2A}AR expressed in CHO cells (Palmer *et al.*, 1994). Forskolin stimulates AC resulting in elevation of cAMP, thereby mimicking one of the effects of A_{2A}AR stimulation and so may have had the potential to activate feedback mechanisms to induce receptor phosphorylation. The A_{2A}AR has also been reported to regulate activation of the NFκB pathway by TNFα and LPS (Sands *et al.*, 2004) and the JAK/STAT pathway by IL-6 (Sands *et al.*, 2006; Sahfi *et al.*, submitted for publication). It was therefore of interest to include these stimuli as an effect on receptor phosphorylation could indicate a means of reciprocal regulation by these pathways. As shown in figure 4.5, only PMA had a significant effect on A_{2A}AR phosphorylation, inducing a 2.5 to 5-fold increase over basal levels ($p < 0.001$, $n = 3$). All stimuli were active as shown by their ability to induce ERK phosphorylation (figure 4.5 C, *upper panel*). Moreover, differences in phosphorylation levels were not due to alterations in receptor expression as levels of the A_{2A}AR detected by immunoblotting were not affected by any of the chosen stimuli (figure 4.5 C, *lower panel*). Thus, the A_{2A}AR is strongly phosphorylated in response to PMA treatment but not following stimulation with agonist or any other stimuli tested.

The time-dependence of PMA-induced A_{2A}AR phosphorylation was assessed by performing an intact cell phosphorylation assay with adA_{2A}AR-infected HUVECs treated with 1μM PMA for different times. As shown in figure 4.6, phosphorylation was rapid, being detectable at the first time point tested (15 seconds). It reached a maximum at 10 minutes and was sustained at high levels for at least 30 minutes. These results closely match the values obtained in previous studies using the canine A_{2A}AR (Palmer and Stiles, 1999). However, in contrast to the canine receptor, phosphorylation of the human A_{2A}AR appeared to occur in two stages, reaching a first peak at 1 minute and levelling off slightly until the 5 minute time point before rising to a maximum after 10 minutes. The response was concentration-dependent reaching a maximum of (3.6 ± 0.4)-fold above basal at 10 nM (figure 4.7 B) which is lower than the value previously observed for the canine A_{2A}AR ((11.2 ± 2.5)-fold above basal levels at 1 μM PMA; Palmer and Stiles, 1999). Curve-fitting of data pooled from three experiments produced an EC_{50} value for PMA of 1.7 nM (figure 4.7 B) which is consistent with published values for the affinity of phorbol esters for PKC (Dimitrijević *et al.*, 1995).

Figure 4.5 The human A_{2A}AR is phosphorylated in response to PMA

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to stimulation with 10 μM CGS21680 (CGS), 1 μM PMA, 50 μM forskolin (Fsk), 10 ng TNFα, 1 μg LPS or 5 ng IL-6/25 ng IL-6 sRα (IL-6) for 30 minutes. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean fold increase ± SE as shown in the graph. **C.** Levels of phosphorylated ERK and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells (n = 2). The 9E10 antibody was used to detect the A_{2A}AR.

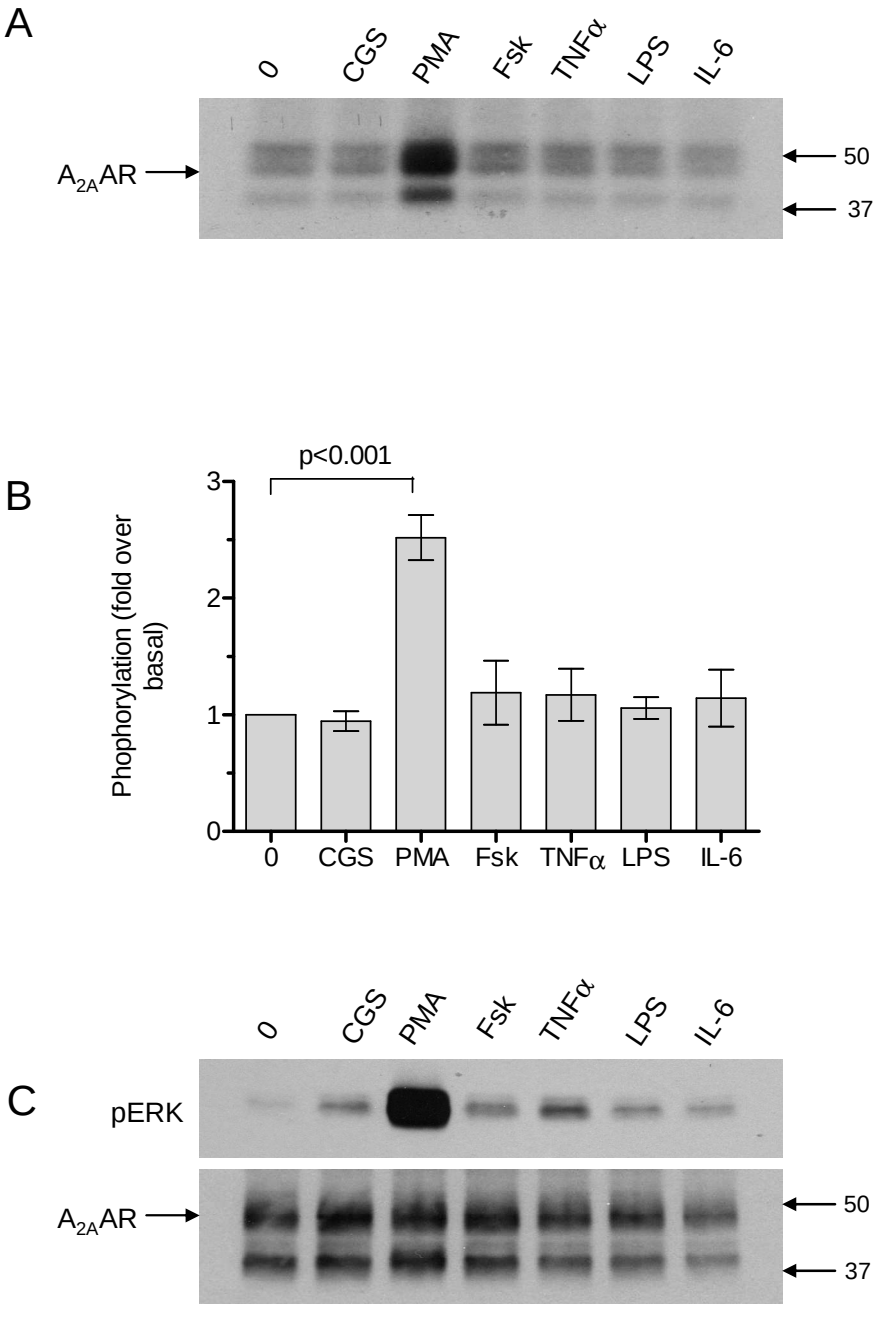


Figure 4.5 The human A_{2A}AR is phosphorylated in response to PMA

Figure 4.6 PMA induces rapid phosphorylation of the human A_{2A}AR

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to stimulation with 1 μM PMA for the times indicated. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean percentage of the maximal response ± SE as shown in the graph. **C.** Identical experiments were carried out in parallel using unlabelled cells (*n = 1*). Protein extracts were prepared for analysis by SDS-PAGE and immunoblotting using the 9E10 antibody to detect total levels of the A_{2A}AR.

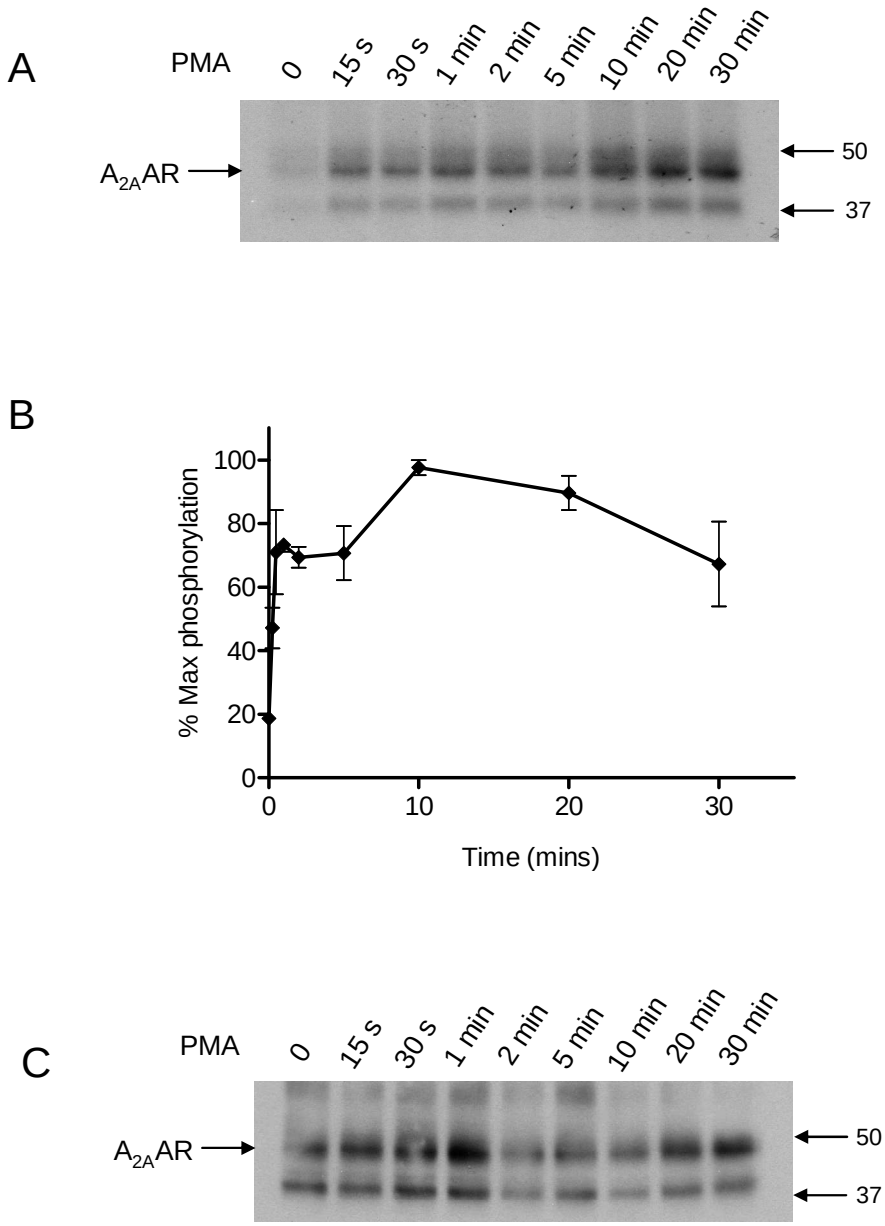


Figure 4.6 PMA induces rapid phosphorylation of the human A_{2A}AR

Figure 4.7 PMA induces phosphorylation of the human A_{2A}AR at low concentrations

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to stimulation with PMA at the concentrations indicated for 20 minutes. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean percentage of the maximal response ± SE as shown in the graph. **C.** Identical experiments were carried out in parallel using unlabelled cells. Protein extracts were prepared for analysis by SDS-PAGE and immunoblotting using the 9E10 antibody to detect total levels of the A_{2A}AR (*n = 1*).

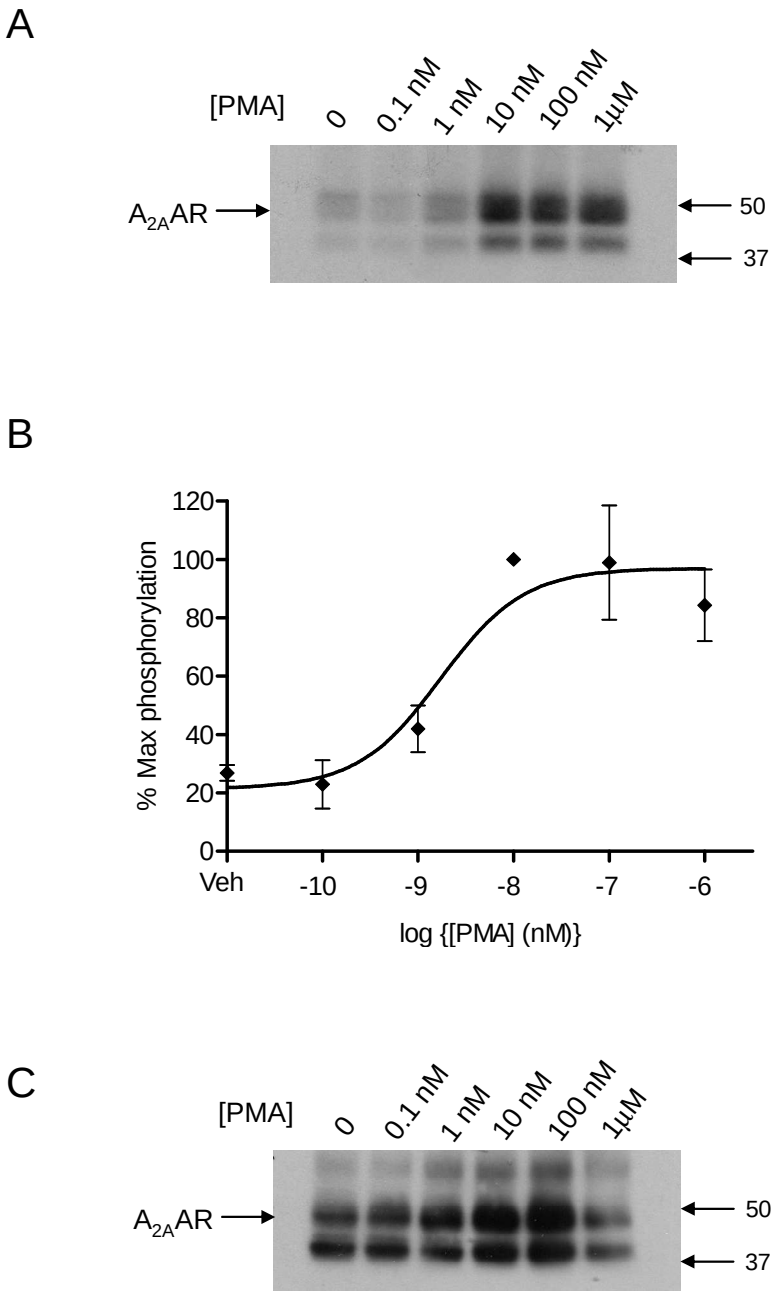


Figure 4.7 PMA induces phosphorylation of the human A_{2A}AR at low concentrations

PMA is an activator of conventional and novel isoforms of PKC (Bell and Burns, 1991). To determine whether A_{2A}AR phosphorylation could be induced by stimulation of endogenous receptors which activate PKC, adA_{2A}AR-infected HUVECs were stimulated with histamine. In HUVECs, histamine activates histamine H1 receptors (Li *et al*., 2003), which leads to stimulation of PLCβ and activation of PKC (Hill *et al.*, 1997). Treatment of HUVECs with 1 μM histamine stimulated a time-dependent increase in A_{2A}AR phosphorylation (figure 4.8) which paralleled the agonist's ability to induce ERK phosphorylation (figure 4.8 C, *upper panel*). Histamine induced maximal phosphorylation at 2 minutes (70 ± 17 % above basal levels, $p = 0.05$, $n = 4$) but was not as effective as parallel PMA treatment which induced a 245 ± 38 % increase in stimulation ($p < 0.01$, $n = 4$; figure 4.5). Thus, phosphorylation of the A_{2A}AR can be induced both through treatment with PMA and by stimulation of endogenous histamine H1 receptors which activate PKC in HUVECs.

To begin to investigate the role of PKC in PMA-induced phosphorylation of the A_{2A}AR, an intact cell phosphorylation assay was carried out using adA_{2A}AR-infected HUVECs which had been treated with increasing concentrations of GF109203X, an inhibitor of classical and novel forms of PKC (Martiny-Baron *et al.*, 1993; Toullec *et al.*, 1991), before stimulation with 10 nM PMA. As before, PMA induced receptor phosphorylation in the absence of GF109203X. However, pre-treatment with GF109203X resulted in a dose-dependent inhibition of receptor phosphorylation with an IC_{50} of 200 nM (figure 4.9 A,B), a value which is consistent with concentrations of GF10923X required to inhibit phosphorylation of other known substrates of novel and conventional isoforms of PKC (Überall *et al.*, 1997). PMA-stimulated ERK phosphorylation was similarly inhibited in GF109203X-treated cells (figure 4.9 C, *upper panel*). The reduction in receptor phosphorylation could not be explained by changes in receptor expression as shown in figure 4.9 C, *lower panel*, indicating that PMA induced phosphorylation of the A_{2A}AR is dependent on the involvement of classical or novel isoforms of PKC.

An alternative method for analysing the involvement of PKC is to deplete cellular levels by chronic treatment with PMA. Prolonged exposure to phorbol esters triggers the down-regulation of PMA-sensitive PKC isoforms by increasing the rate of proteolytic degradation (Liu and Heckman, 1998). The effect of PKC depletion on PMA-induced receptor phosphorylation was investigated by incubating HUVECs with 100 nM PMA for 36 hours before performing an intact cell phosphorylation assay. In cells which had not received chronic treatment with PMA, a 20 minute treatment with 10 μM PMA induced

Figure 4.8 The human A_{2A}AR is phosphorylated in response to stimulation of endogenous histamine H1 receptors which activate PKC

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to stimulation with 1 μM histamine for the times indicated. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean fold increase ± SE as shown in the graph. **C.** Levels of phosphorylated ERK and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells (n = 2). The 9E10 antibody was used to detect the A_{2A}AR.

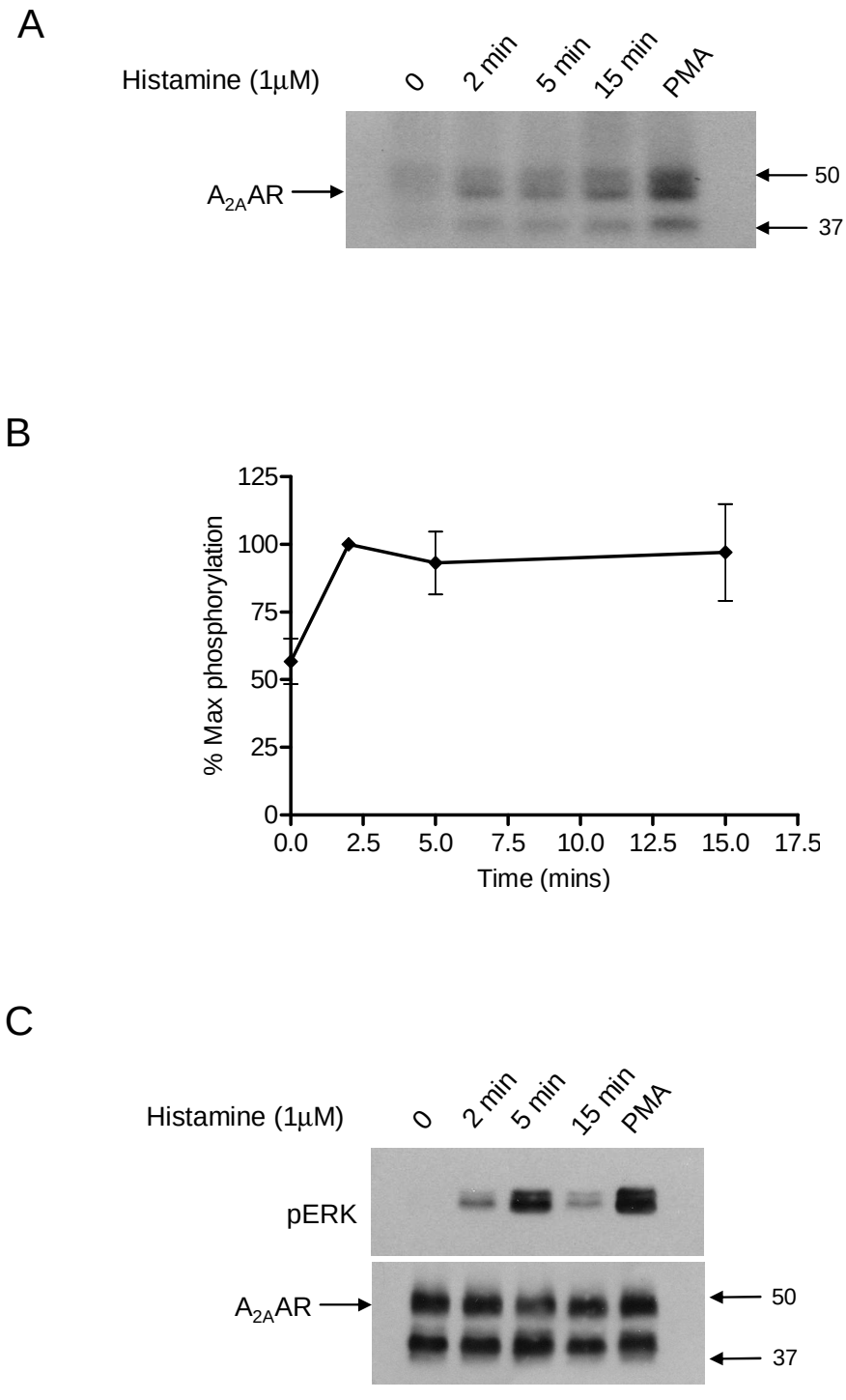


Figure 4.8 The human A_{2A}AR is phosphorylated in response to stimulation of endogenous histamine H1 receptors which activate PKC

Figure 4.9 PKC plays a role in PMA-induced phosphorylation of the human A_{2A}AR

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to addition of the PKC inhibitor GF109203X (GFX) at the concentrations indicated. After 30 minutes, cells were stimulated with 10 nM PMA (+) or DMSO vehicle (–) for 20 minutes. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean percentage of the maximal response ± SE as shown in the graph. **C.** Levels of phosphorylated ERK and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells (n = 2). The 9E10 antibody was used to detect the A_{2A}AR.

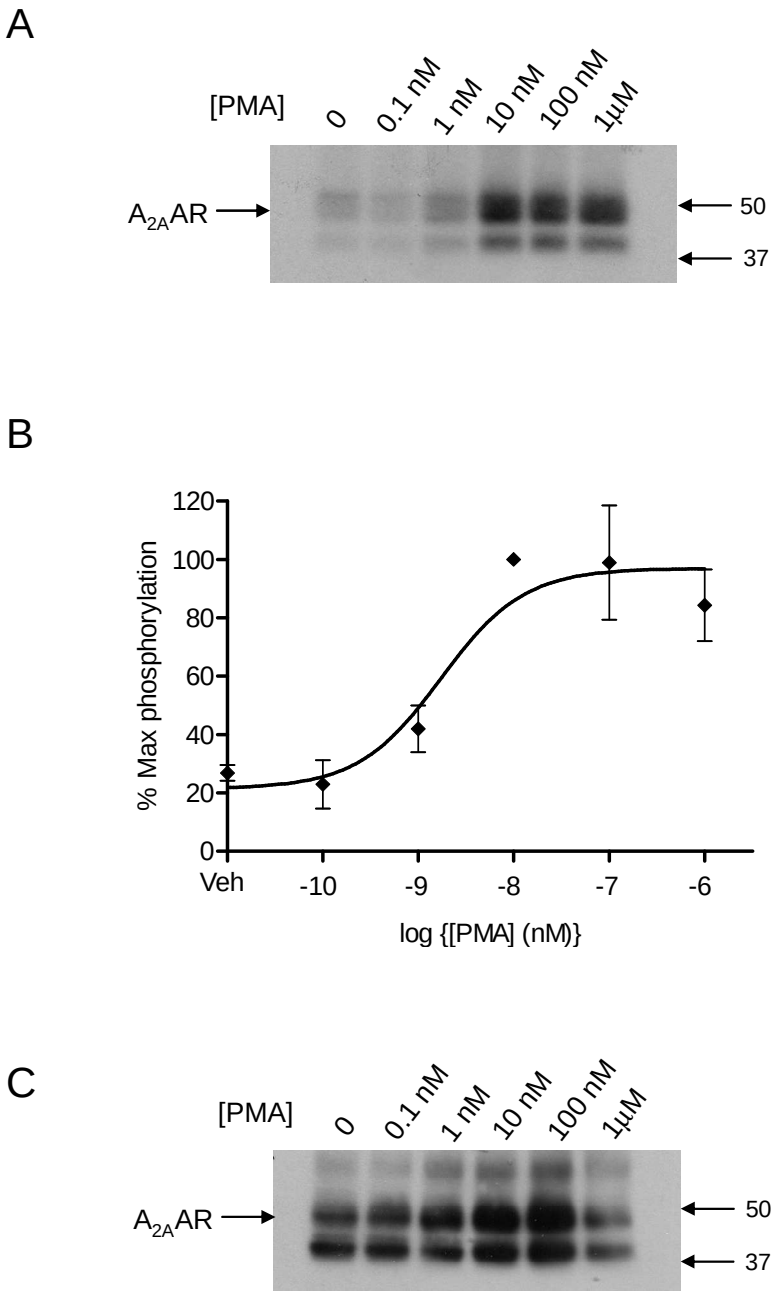


Figure 4.9 PKC plays a role in PMA-induced phosphorylation of the human A_{2A}AR

strong phosphorylation as observed in the experiments described above (figure 4.10). However, in cells which had been treated with 100 nM PMA for 36 hours, this response was reduced by an average of 59 ± 9 % (p = 0.05, n = 3). This was not due to effects of PMA other than on PKC as in cells pre-incubated for 36 hours with an equivalent concentration of the control drug, 4α phorbol which does not activate PKC, receptor phosphorylation was detected at similar levels as that seen in cells which had not undergone chronic treatment. Immnoblotting using a pan-PKC antibody showed that the PKC-depletion protocol was effective (figure 4.10 C, *upper panel*). In accordance with a reduction in PKC activity, PMA-induced ERK phosphorylation was reduced or undetectable in cells subjected to chronic PMA treatment. The changes in phosphorylation observed were not due to any effects of the drugs used on receptor expression as levels of the A_{2A}AR did not vary significantly between conditions (figure 4.10 C, *lower panel*). Thus PKC depletion severely impairs the ability of PMA to induce phosphorylation of the A_{2A}AR.

Results obtained using the PKC inhibitor GF109203X and by depleting cellular levels of PKC indicate that PKC plays a role in mediating phosphorylation of the A_{2A}AR. HUVECs express 5 isoforms of PKC (α, δ, ε, θ and ζ; Haller *et al.*, 1996) of which all apart from ζ are activated by DAG and can be down-regulated by chronic phorbol ester treatment (Liu and Heckman, 1998). To begin to investigate which isoform may be responsible for phosphorylation of the A_{2A}AR, intact cell phosphorylation assays were carried out with cells treated with rottlerin which is an inhibitor of PKCδ and has been shown previously to inhibit phosphorylation of the canine A_{2A}AR expressed in C6 cells (Palmer and Stiles, 1999). Cells were treated with different concentrations of rottlerin for 30 minutes before stimulation with 10 nM PMA. As shown in figure 4.11, phosphorylation of the A_{2A}AR was unaffected by rottlerin except at the highest concentration used (10 μM). At this concentration, rottlerin may inhibit kinases other than PKCδ (Davies *et al.*, 2000) and so it is unclear whether or not PKCδ is responsible for phosphorylating the A_{2A}AR.

PKC isoforms can be differentiated on their requirement for calcium. HUVECs express both calcium-dependent PKCα and calcium-independent PKCδ, PKCε and PKCθ (Haller *et al.*, 1996). In order to test whether a calcium-dependent isoform was involved, PMA-induced receptor phosphorylation was assessed in cells which had been pre-incubated with the cell-permeable calcium chelator BAPTA/AM (10 μM) for 30 minutes. As shown in figure 4.12, PMA induces receptor phosphorylation which is not affected by BAPTA/AM.

Figure 4.10 PKC depletion reduces PMA-stimulated phosphorylation of the human A_{2A}AR

HUVECs infected with adenovirus expressing the A_{2A}AR were incubated with 10 nM PMA or DMSO vehicle (–) or with 10 nM 4α-phorbol for 36 hours before stimulation with 10 nM PMA or vehicle for 20 minutes. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean percentage of the response observed for cells treated with 4α-phorbol ± SE as shown in the graph. **C.** Levels of phosphorylated ERK and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells (n = 2). The 9E10 antibody was used to detect the A_{2A}AR.

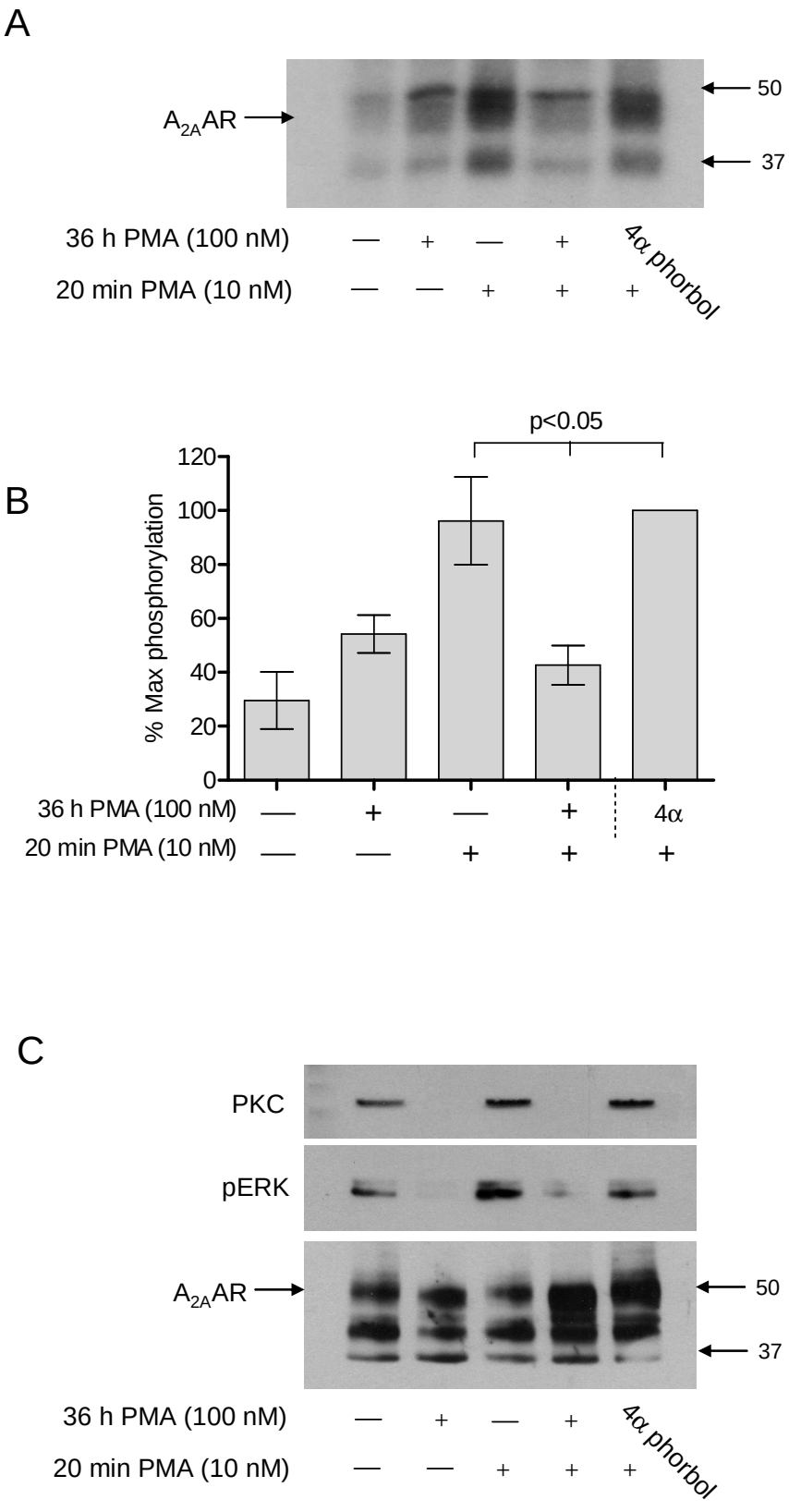


Figure 4.10 PKC depletion reduces PMA-stimulated phosphorylation of the human A_{2A}AR

Figure 4.11 PKCδ does not appear to be involved in PMA-induced phosphorylation of the human A_{2A}AR

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to addition of the PKCδ inhibitor rottlerin at the concentrations indicated. After 30 minutes, cells were stimulated with 10 nM PMA (+) or DMSO vehicle (–) for 20 minutes. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean percentage of the maximal response ± SE as shown in the graph. **C.** Levels of phosphorylated ERK and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells (n = 2). The 9E10 antibody was used to detect the A_{2A}AR.

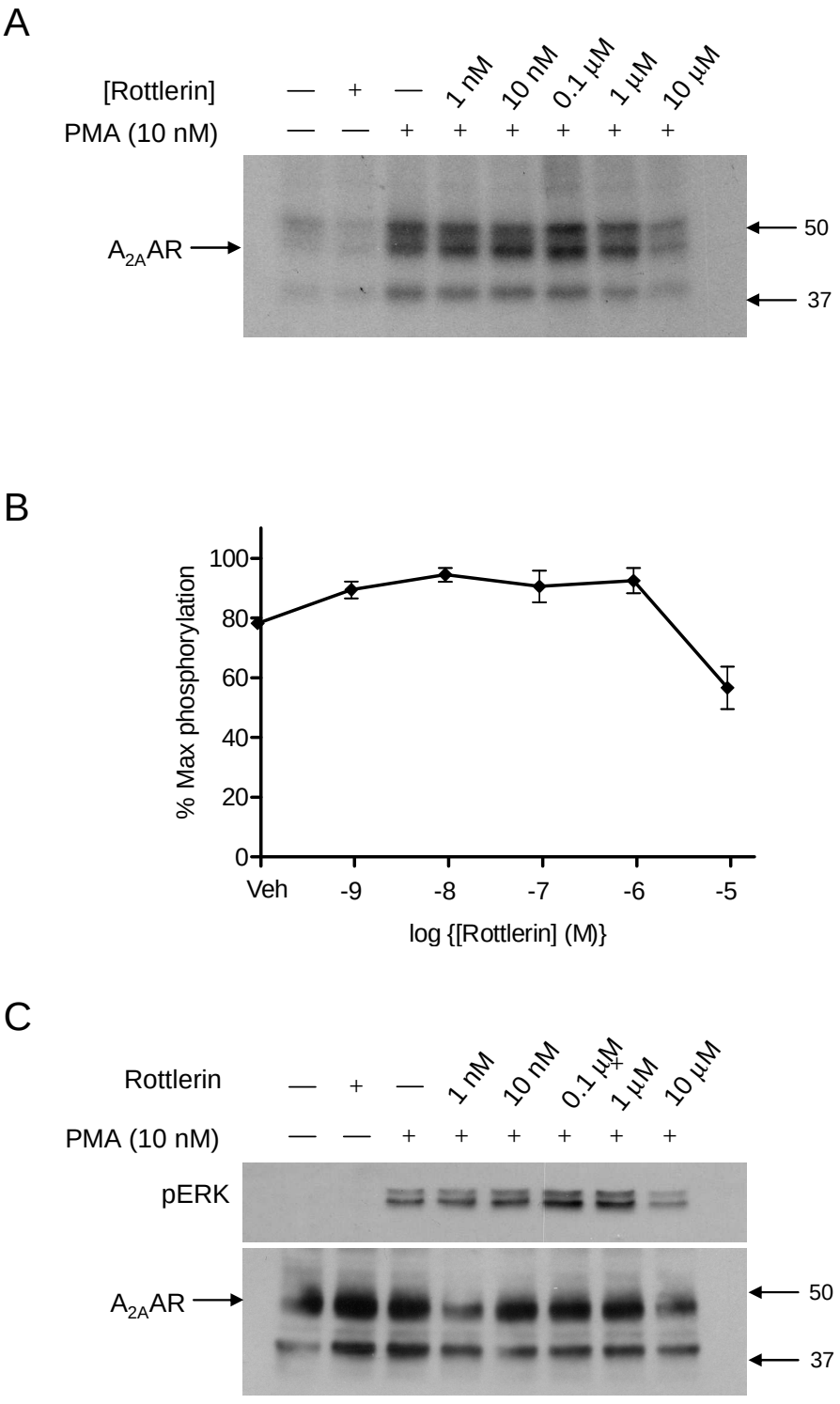


Figure 4.11 PKCδ does not appear to be involved in PMA-induced phosphorylation of the human A_{2A}AR

Figure 4.12 Effect of calcium chelation on PMA-induced phosphorylation of the human A_{2A}AR

HUVECs infected with adenovirus expressing the A_{2A}AR were labelled with ^{32}P orthophosphate for 90 minutes prior to addition of 10 μM BAPTA/AM. After 30 minutes, cells were stimulated with 10 nM PMA, 1μM ionomycin or DMSO vehicle (–) as indicated. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE. **B.** Bands were quantitated by densitometry and values expressed as a mean percentage of the maximal response ± SE as shown in the graph. **C.** Levels of phosphorylated ERK and total A_{2A}AR expression were detected by SDS-PAGE and immunoblotting of protein samples prepared from experiments carried out in parallel using unlabelled cells (n = 2). The 9E10 antibody was used to detect the A_{2A}AR.

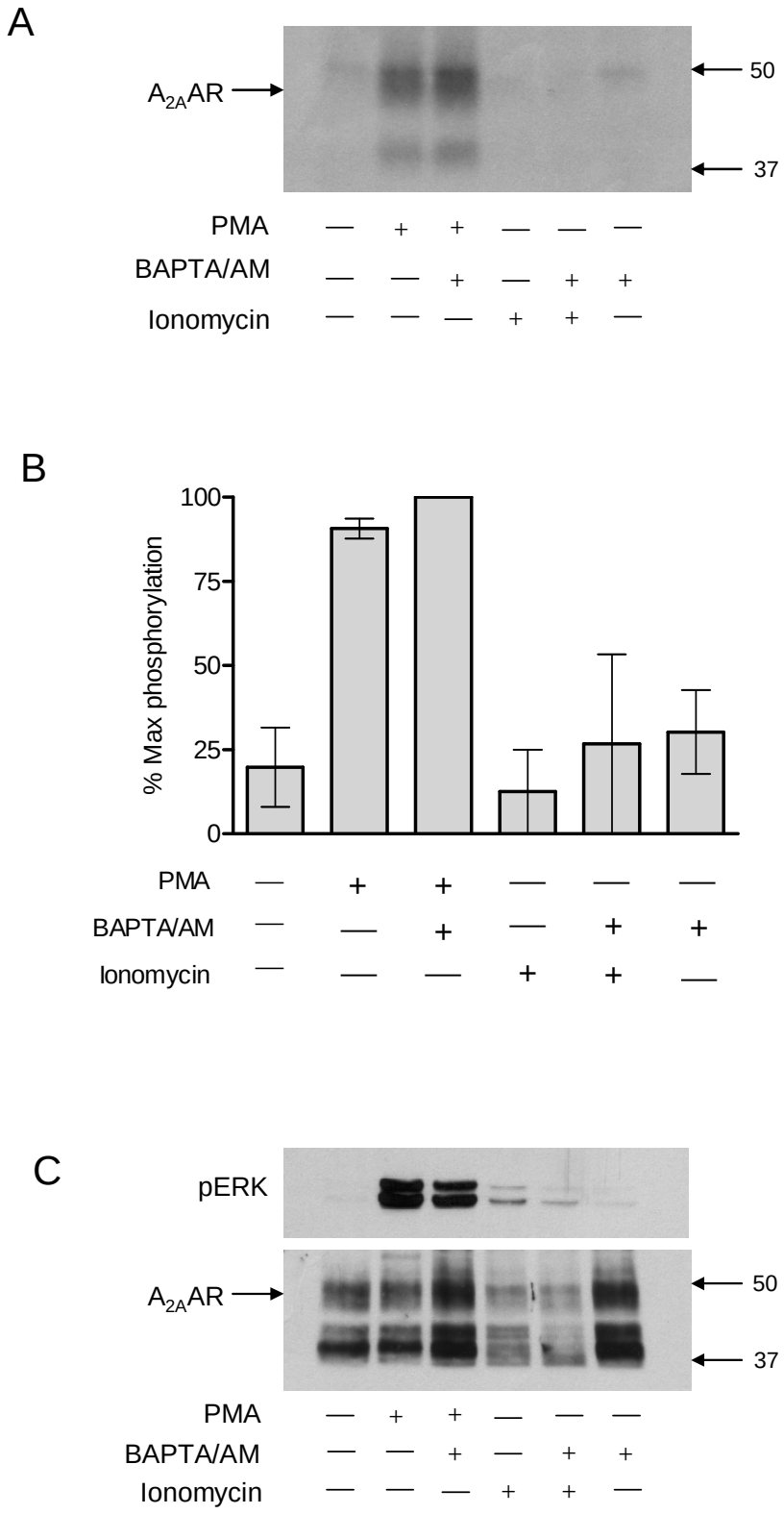


Figure 4.12 Effect of calcium chelation on PMA-induced phosphorylation of the human A_{2A}AR

To confirm that BAPTA/AM was effective, cells were treated with the ionophore ionomycin which mobilises intracellular calcium stores (Liu and Hermann, 1978). This results in calcium-dependent ERK activation via activation of calmodulin and calmodulin binding proteins which can modulate Ras activity (Agell *et al.*, 2002). Calcium chelation was judged to be successful as calcium-dependent activation of ERK induced by treating cells with 1 μM ionomycin for 20 minutes was reduced in cells pre-incubated with BAPTA/AM (figure 4.12 C, *upper panel*). It should be noted that in cells treated with ionomycin, receptor expression was reduced (figure 4.12 C, *lower panel*). However, since the extent of the reduction was similar for both BAPTA/AM-treated and untreated cells, the effect of BAPTA/AM on ERK phosphorylation was still considered relevant. Thus, A_{2A}AR phosphorylation appears to be mediated by a calcium-independent isoform of PKC which rules out the involvement of PKCα. Excluding PKCδ, the remaining calcium-independent, PMA-sensitive isoforms expressed by HUVECs are PKC ε and θ.

Although many PKC inhibitors exist, their selectivity for different isoforms is limited. A more specific approach to achieve inhibition of individual PKC isoforms is to use siRNA gene silencing. To assess the involvement of PKCε in A_{2A}AR phosphorylation, siRNAs targeted against human PKCε, non-targeted control siRNA or PKCα were introduced into HUVECs prior to infection with adA_{2A}AR. PKCα siRNA was included as a relative kinase control to show that effects observed were specific to PKCε and that PKCα was not involved. As before, PMA induced phosphorylation of the A_{2A}AR (figure 4.13 A). No decrease in this response was observed in cells transfected with siRNAs directed against PKCε, PKCα or control siRNA suggesting that PKCε is not required for PMA-mediated A_{2A}AR phosphorylation. This also corroborated findings presented above which indicated that calcium-dependent isoforms such as PKCα are not required for PMA-induced phosphorylation. As shown in figure 4.13 B (*top panel*) PKCε was effectively depleted in cells transfected with PKCε-targeted siRNA while levels were constant in untransfected cells and those transfected with PKCα, confirming the effectiveness of the technique. PKCα expression was also significantly reduced specifically in cells transfected with PKCα-targeted siRNA (figure 4.13 B, *centre panel*). Expression of the A_{2A}AR remained constant between conditions (figure 4.13 B, *bottom panel*). Thus, PKCε does not appear to be required for PMA-induced A_{2A}AR phosphorylation.

It was one of the aims of this study to determine which region of the A_{2A}AR was phosphorylated in response to PMA. To begin to address this question, cells were

Figure 4.13 Effect of PKCε and PKCα siRNA gene silencing on PMA-induced A_{2A}AR phosphorylation

HUVECs were transfected with siRNA targeted against PKCε or PKCα and infected with adenovirus expressing the A_{2A}AR. Cells were labelled with ^{32}P orthophosphate for 90 minutes prior to stimulation with 1 μM PMA for 20 minutes. Cell extracts were prepared and equalised for protein content before immunoprecipitation of the A_{2A}AR. **A.** The ^{32}P-labelled A_{2A}AR was detected by autoradiography following SDS-PAGE (*n* = 2). **B.** Identical experiments were carried out in parallel using unlabelled cells. Protein extracts were prepared for use in SDS-PAGE and immunoblotting to confirm successful siRNA-mediated knock-down of PKCε and PKCα using PKC isoform-specific antibodies and to show total levels of A_{2A}AR expression using the 9E10 antibody (*n* = 2).

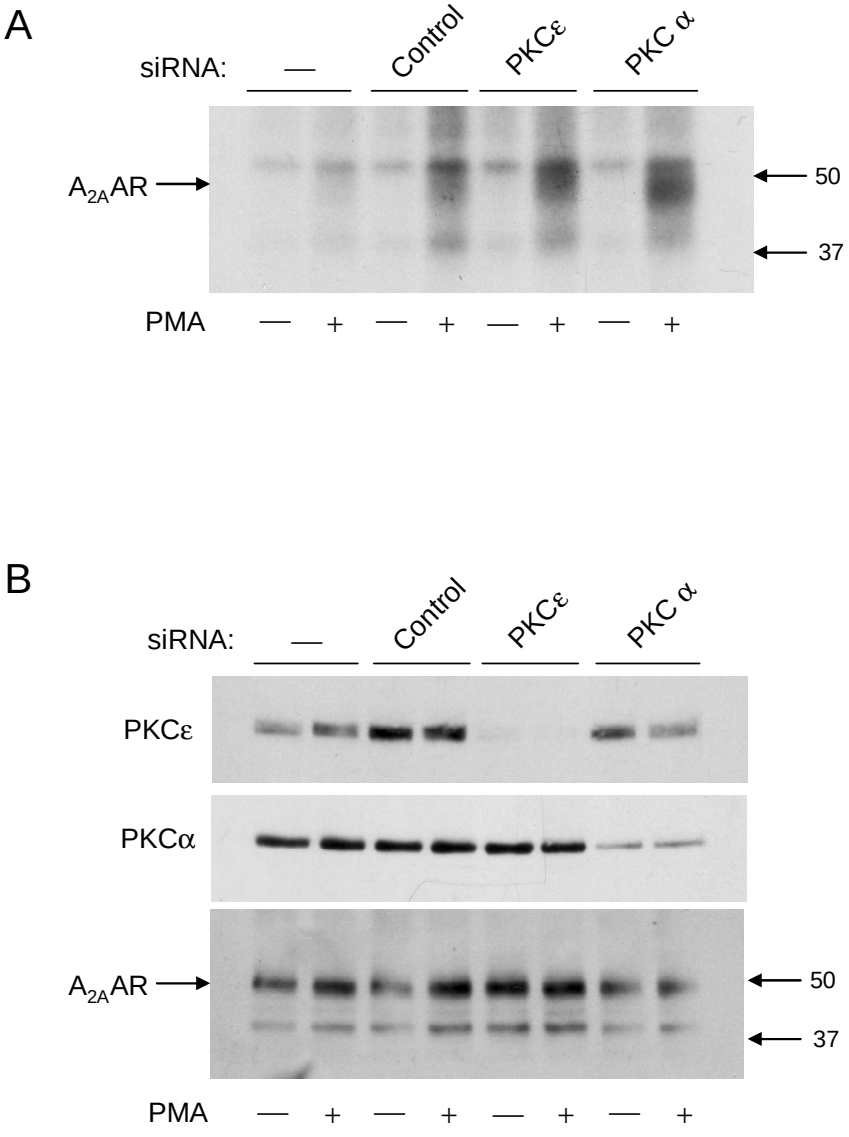


Figure 4.13 Effect of PKCε and PKCα siRNA gene silencing on PMA-induced A_{2A}AR phosphorylation

transfected with one of two A_{2A}AR truncation mutants in order to assess which regions of the C-terminal tail were required for phosphorylation. One construct (1-360) lacked five potential phosphorylation sites from the receptor tail and the other (1-311), the most severely truncated, lacked eleven sites (Klinger *et al.*, 2002b, figure 4.14). HUVECs do not respond well to transfection protocols and attempts to express the receptor constructs proved to be unsuccessful (data not shown). For this reason, experiments were initially carried out using CHO cells which are a more tractable cell line.

Transfection of CHO cells was successful as shown in figure 4.15 A, *upper panel*. Wild-type A_{2A}AR was detected as two bands between 37 and 75 kDa, while the 1-360 and 1-311 truncated forms are represented as bands of lower molecular mass owing to the absence of 50 or 100 amino acids respectively. However, in intact cell phosphorylation assays using transfected cells stimulated with 10 μM CGS21680 or 1 μM PMA, no phosphorylation of the wild-type or truncated receptors was detected (figure 4.15 B). This was despite confirmation that the CGS21680 and PMA were active as shown by their ability to induce ERK phosphorylation (figure 4.15 A, *lower panel*). The lack of detectable A_{2A}AR phosphorylation was not due to a failure in the immunoprecipitation procedure as the A_{2A}AR was detected by immunoblotting immunoprecipitated samples with an A_{2A}aR-specific antibody (figure 4.15 C). Thus, it appears that the human A_{2A}AR is not subject to PMA-induced phosphorylation in CHO cells, raising the possibility that this is a cell type-specific phenomenon. To further investigate this possibility, phosphorylation was assessed in HEK 293 and C6 cells transfected with the wild-type or truncated forms of the A_{2A}AR. Immunoblotting revealed that the wild-type A_{2A}AR was expressed in HEK 293 cells at easily detectable levels while the 1-311 and 1-360 truncated forms were present at lower levels (figure 4.16 A (i), *upper panel*). Stimulation with CGS21680 and PMA induced ERK phosphorylation (figure 4.16 A (i), *lower panel*) but did not result in receptor phosphorylation (figure 4.16 A (ii)). The wild-type and truncated A_{2A}ARs were also successfully expressed in C6 cells but detection by immunoblotting was hindered by a strong band at approximately 60 kDa possibly representing endogenous myc (figure 4.16 B (i), *upper panel*). Again, PMA induced ERK phosphorylation (figure 4.16 B (i)) but did not result in detectable receptor phosphorylation (figure 4.16 B (ii)). Therefore, it appears that phosphorylation of the human A_{2A}AR in response to PMA only occurs in certain cell types including HUVECs but not CHO, HEK 293 or C6 cells.

Previous studies have shown that a large part of the A_{2A}AR tail is dispensable for agonist binding (Piersen *et al.*, 1994), desensitisation (Palmer and Stiles, 1997) and G-protein

```
HUMAN A2A WT     QTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSDGEQVSLRLNGHPPGVW 347
HUMAN A2A 1-360  QTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSDGEQVSLRLNGHPPGVW 347
HUMAN A2A 1-311  QTFRKIIRSHVLRQ---------------------------------- 311
                 **************************************************

HUMAN A2A WT     ANGSAPHPERRPNGYALGLVSGGSAQESQGNTGLPDVELLSHELKGVCPE 396
HUMAN A2A 1-360  ANGSAPHPERRPN---------------------------------- 360
HUMAN A2A 1-311  ------------------------------------------------
                 ************

HUMAN A2A WT     PPGLDDPLAQDGAGVS 412
HUMAN A2A 1-360  ----------------
HUMAN A2A 1-311  ----------------
```

Figure 4.14 Sequences of the C-terminal tail of DNA constructs representing the wild-type and truncated forms of the human A_{2A}AR

Figure 4.15 Expression of wild-type and truncated forms of the A_{2A}AR in CHO cells

CHO cells were transfected with plasmids encoding a myc-tagged WT human A_{2A}AR (WT) or one of two myc-tagged carboxyl-terminus truncation mutants (1-311 and 1-360). **A.** Cells were stimulated with 10 μM CGS21680, 1 μM PMA or DMSO vehicle for 20 minutes before preparation of protein extracts. Extracts were normalised for protein content prior to fractionation by SDS-PAGE and immunoblotting using a phospho-ERK (pERK)-specific antibody or the 9E10 antibody which recognises both WT and truncated forms of the A_{2A}AR (*n* = 2). **B.** Transfected cells were labelled with ^{32}P for 90 minutes prior to stimulation with 10 μM CGS21680, 1 μM PMA or DMSO vehicle for 20 minutes. Cell extracts were prepared and equalised for protein content before subjection to an immunoprecipitation protocol using the 9E10 antibody. Samples were fractionated by SDS-PAGE and dried gels were exposed to film to allow detection of any ^{32}P-labelled receptor (*n* = 3). **C.** CHO cells were left untransfected (control) or were transfected with the WT A_{2A}AR. Cell extracts were prepared and equalised for protein content before subjection to an immunoprecipitation protocol in the presence or absence of the 9E10 antibody as indicated. Immunoprecipitated receptors and total levels of receptors in cell lysates (Input) were detected using SDS-PAGE followed by immunoblotting using an A_{2A}AR-specific antibody (*n* = 2).

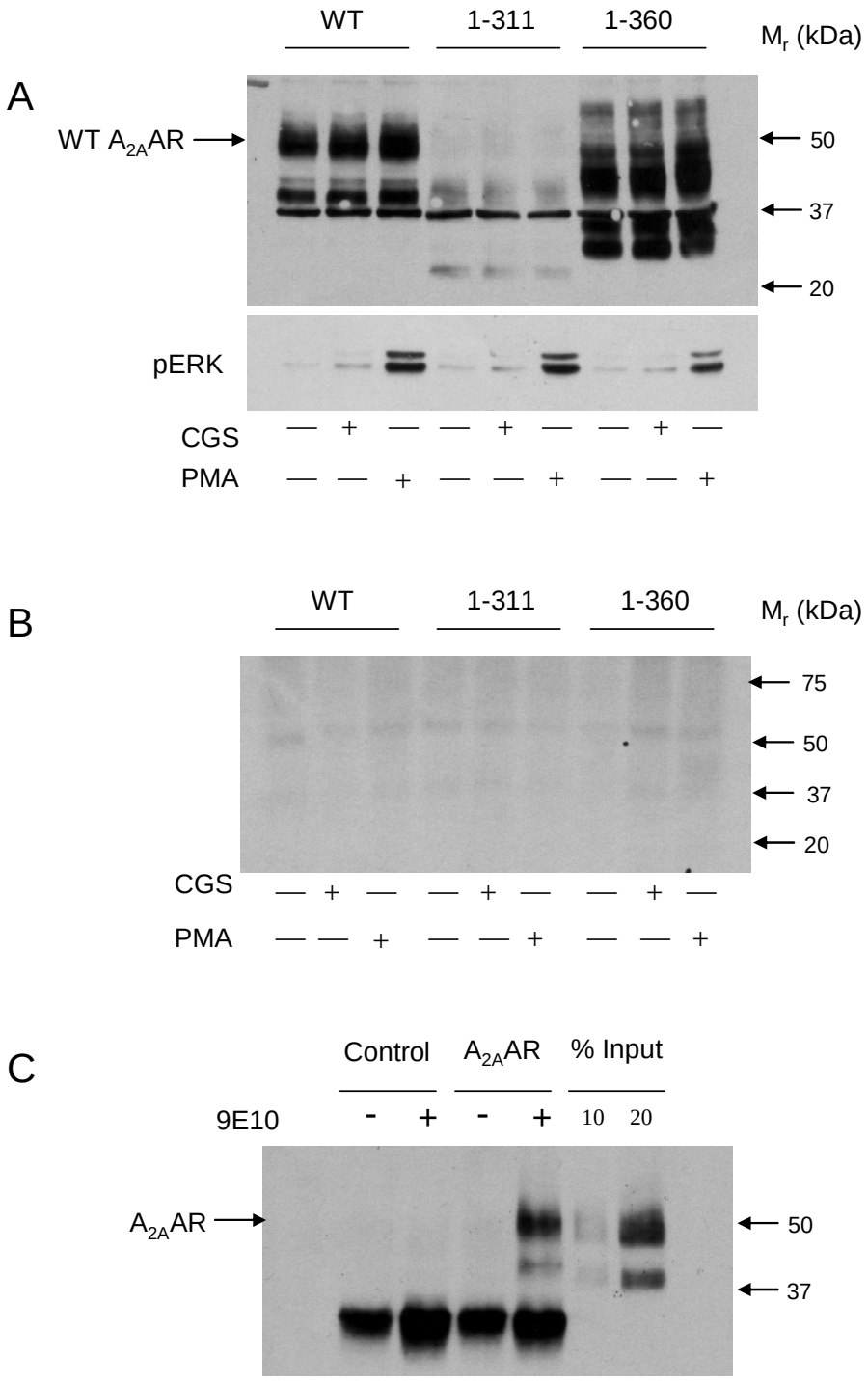


Figure 4.15 Expression of wild-type and truncated forms of the A_{2A}AR in CHO cells

Figure 4.16 Expression of wild-type and truncated forms of the A_{2A}AR in HEK 293 and C6 cells

HEK 293 (**A**) or C6 cells (**B**) were transfected with plasmids encoding a myc-tagged WT human A_{2A}AR (WT) or one of two myc-tagged carboxyl-terminus truncation mutants (1-311 and 1-360). (i) Cells were stimulated with 10 μM CGS21680, 1 μM PMA or DMSO vehicle for 20 minutes before preparation of protein extracts. Extracts were normalised for protein content prior to fractionation by SDS-PAGE and immunoblotting using a phospho-ERK (pERK)-specific antibody or the 9E10 antibody which recognises both WT and truncated forms of the A_{2A}AR (*n* = 2). (ii) Transfected cells were labelled with ^{32}P for 90 minutes prior to stimulation with 10 μM CGS21680, 1 μM PMA or DMSO vehicle for 20 minutes. Cell extracts were prepared and equalised for protein content before subjection to an immunoprecipitation protocol using the 9E10 antibody. Samples were fractionated by SDS-PAGE and dried gels were exposed to film to allow detection of any ^{32}P-labelled receptor (*n* = 3).

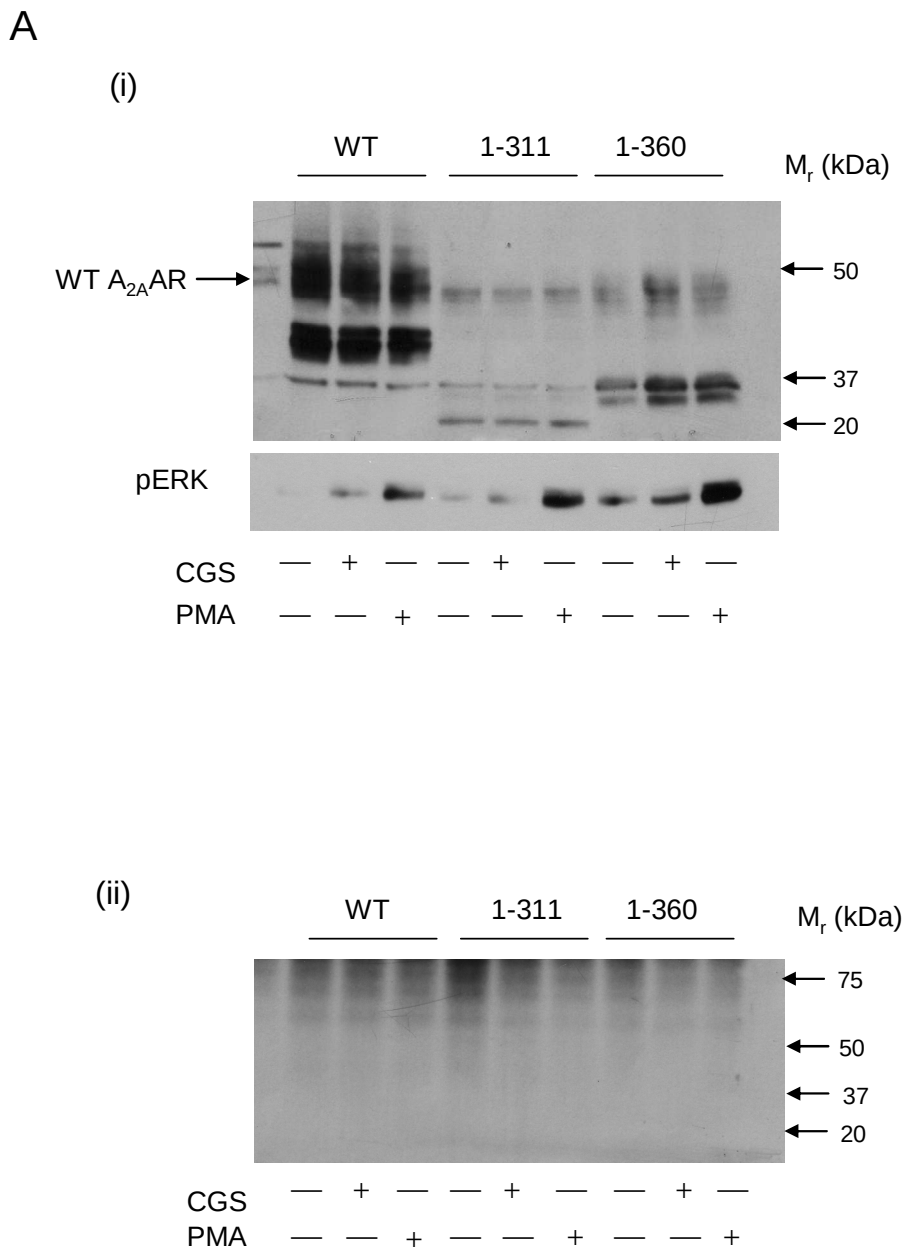
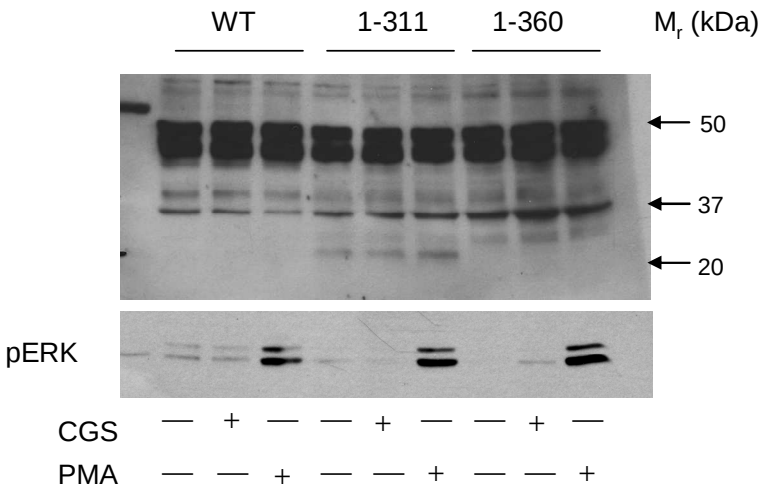


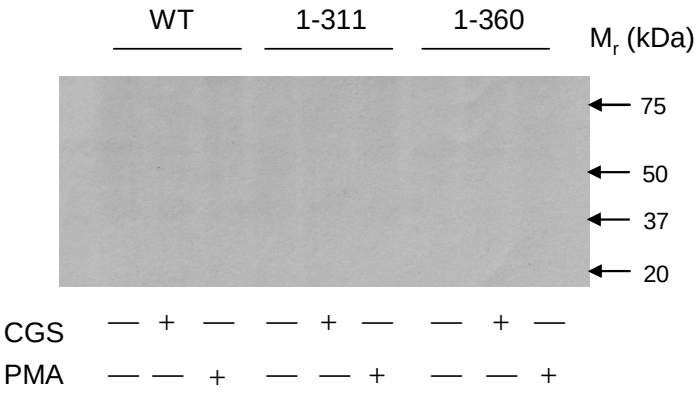
Figure 4.16 Expression of wild-type and truncated forms of the A_{2A}AR in HEK 293 and C6 cells

B

(i)


WT
1-311
1-360
M_r (kDa)
50
37
20
pERK
CGS — + — — + — — + —
PMA — — + — — + — — +

(ii)


WT
1-311
1-360
M_r (kDa)
75
50
37
20
CGS — + — — + — — + —
PMA — — + — — + — — +

coupling (Klinger *et al.*, 2002b). Therefore, the significance of phosphorylation events in this region is unclear. However, the A_{2A}AR has been reported to interact with a number of accessory proteins, including 14-3-3 proteins and TRAX (Gsandtner and Freissmuth, 2006), prompting the question of whether phosphorylation of the A_{2A}AR tail could be important for determining which proteins bind. It was of interest to determine whether PMA-induced phosphorylation observed in this study had an effect on such interactions. This was investigated using pull-down assays to detect interactions *in vitro* between the A_{2A}AR and 14-3-3τ or TRAX under conditions where the A_{2A}AR would or would not be phosphorylated. To perform these assays, it was necessary to obtain purified GST fusion proteins. GST-14-3-3τ and GST-TRAX were expressed in *E. coli* and purified on glutathione Sepharose beads. GST-14-3-3τ was successfully expressed and was detected by Coomassie staining as shown in figure 4.17 A by a clean band at approximately 55 kDa. TRAX was expressed at reasonable levels as shown by the strong band at approximately 60 kDa present 4 hours after induction (figure 4.17 B) although the weaker bands detected at later points show that significant amounts of TRAX protein were lost during the purification process.

Purified GST-tagged 14-3-3τ and TRAX were used in pull-down assays with cell lysates produced from adA_{2A}AR-infected cells which had been incubated in the presence or absence of 1μM PMA for 20 minutes to induce optimal receptor phosphorylation (figure 4.18). As shown in figure 4.18 A, the A_{2A}AR was detected in samples from pull-down assays using GST-14-3-3τ regardless of PMA treatment confirming that 14-3-3τ does interact with the A_{2A}AR in this cell system. However, since there was no change in levels detected when cells had been stimulated with PMA, it does not appear to be dependent on PMA-induced A_{2A}AR phosphorylation. No receptor was detected when parallel pull-downs were performed with GST alone indicating that observations were not a result of non-specific interactions. The A_{2A}AR was also detected when pull-downs were performed with unstimulated cells in the presence of GST-TRAX. Strikingly, levels of the A_{2A}AR were drastically reduced in samples from PMA-stimulated cells (71 ± 17 % reduction compared to unstimulated, $p < 0.0001$, $n = 3$) indicating that the interaction of TRAX with the A_{2A}AR may be dependent on phosphorylation status of the A_{2A}AR tail.

In summary, data presented here shows that phosphorylation of the human A2AAR can be induced in HUVECs by treatment with PMA or by stimulation of endogenous histamine H1 receptors. A role for PKC was indicated by the ability of both a PKC inhibitor and

Figure 4.17 Expression and purification of GST-tagged 14-3-3τ and TRAX.

E. coli were transformed with plasmids encoding either GST-14-3-3τ or GST-TRAX. **A.** Expression of GST-14-3-3τ was induced by incubation of bacterial cultures with 1 mM IPTG for 4 hours at 37 °C and recombinant protein was purified by immobilisation on glutathione Sepharose beads. Bound proteins were eluted from beads and protein analysed by SDS-PAGE and Coomassie staining. **B.** GST-TRAX protein expression was induced by addition of 0.5 mM IPTG for 16 hours at 25 °C and recombinant protein was purified by immobilisation on glutathione Sepharose beads. Eluted protein and samples taken from cultures at each hour following induction and from whole cell lysates (a), sonicated lysates (b) and cleared lysates (c) were analysed by SDS-PAGE and Coomassie staining.

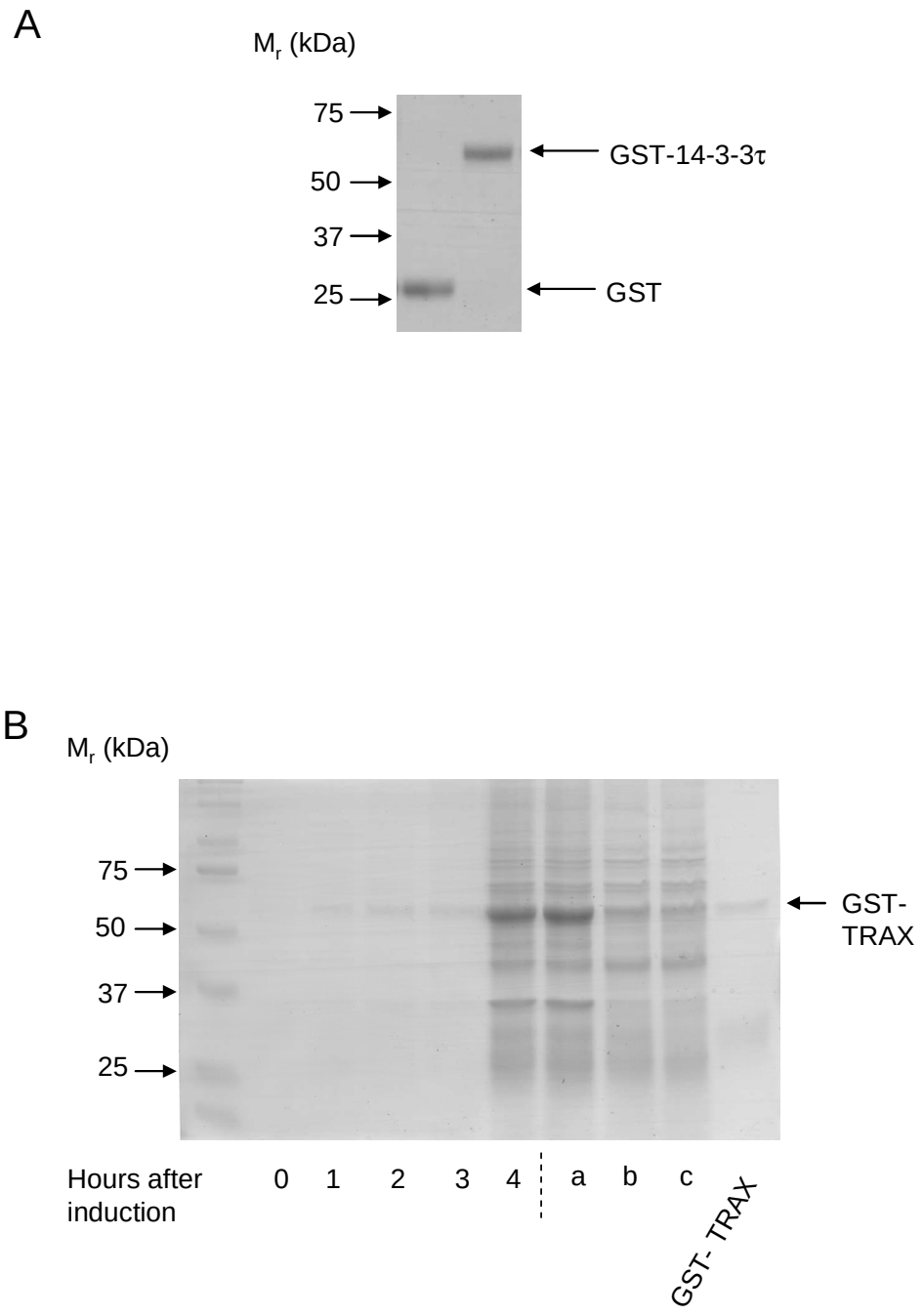


Figure 4.17 Expression and purification of GST-tagged 14-3-3τ and TRAX

Figure 4.18 14-3-3τ and TRAX interact with the human A_{2A}AR

HUVECs infected with adenovirus expressing the A_{2A}AR were incubated with or without 1 μM PMA for 20 minutes. Cell extracts were prepared and used in a pull-down assay using GST-fusion protein glutathione Sepharose beads representing 20 μg of either GST, GST-14-3-3τ or GST-TRAX. **A.** Protein complexes eluted from beads and samples of untreated and PMA-treated cell lysates (input) were subjected to SDS-PAGE and immunoblotting using the 9E10 antibody to detect the A_{2A}AR. **B.** Interactions of the receptor with **(i)** GST-14-3-3τ and **(ii)** GST-TRAX were quantitated by densitometry of immunoreactive bands and values were expressed as a mean percentage of the maximum A_{2A}AR detected ± SE.

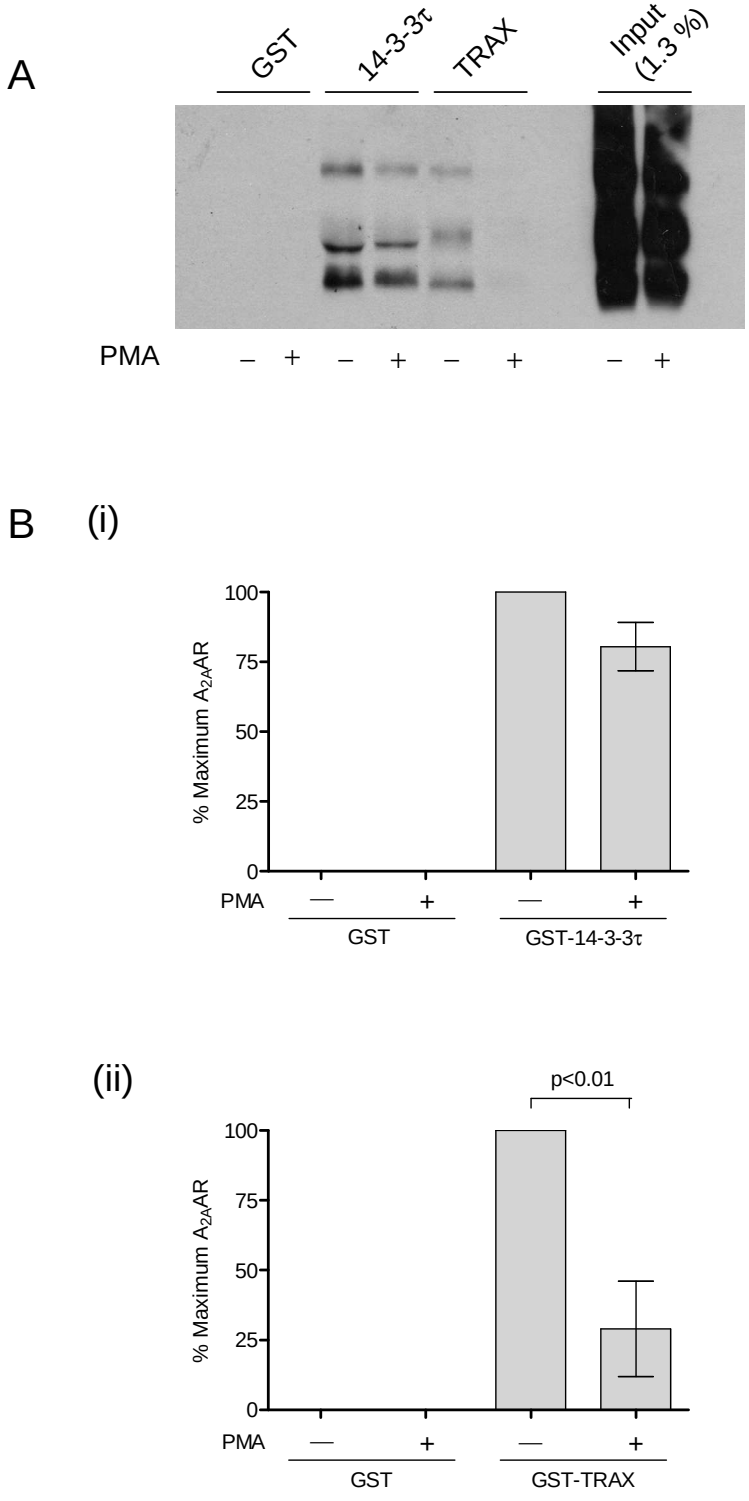


Figure 4.18 14-3-3τ and TRAX interact with the human A_{2A}AR

chronic PMA-mediated PKC depletion to inhibit receptor phosphorylation. Initial investigations showed that this effect is mediated by a calcium-insensitive isoform of PKC as chelation of intracellular calcium had no effect on levels of PMA-induced phosphorylation. An siRNA gene silencing approach confirmed that PKCα is not required and also indicated that PKCε is not required for PMA-induced phosphorylation but it was not possible to identify the specific isoform responsible. Notably, through the use of pull-down assays, it was possible to detect *in vitro* interactions between 14-3-3τ and TRAX and the A_{2A}AR. Most interestingly, while the interaction between 14-3-3τ and the A_{2A}AR was detected in both basal conditions and following PMA treatment when the receptor would be phosphorylated, the interaction of TRAX was drastically reduced when cells had been treated with PMA, indicating that the phosphorylation status of the receptor may negatively regulate TRAX binding.

4.3 Discussion

Several proteins have been reported to interact with the long C-terminal tail of the A_{2A}AR prompting the suggestion that it may act as a scaffold upon which signalling complexes can be assembled (Zezula and Freissmuth, 2008). However, the question of how these interactions might be regulated has not been addressed. A number of serine and threonine residues are present within this region of the A_{2A}AR suggesting potential for regulation by phosphorylation. The canine A_{2A}AR has been shown to be phosphorylated in response to activation of PKC but unlike other GPCRs, this is not associated with heterologous desensitisation (Palmer and Stiles, 1999). It was therefore of interest in this study to investigate whether phosphorylation of the C-terminal tail of the A_{2A}AR could play a role in regulating the binding of interacting proteins. Importantly, this question was addressed using human receptors expressed in HUVECs which express low levels of endogenous receptors as phosphorylation of the A_{2A}AR has only been examined previously using the canine receptor heterologously expressed in rat C6 glioma cells.

In the present study, it was found that phosphorylation of the human A_{2A}AR is rapidly elevated over basal levels following activation of PKC either by treatment with PMA or through activation of endogenous histamine H1 receptors. This effect was significantly reduced in the presence of the PKC inhibitor GF109203X or following depletion of cellular levels of PKC, thereby confirming the involvement of PKC. The specific isoform of PKC responsible for PMA-induced phosphorylation of the A_{2A}AR was determined to be calcium-insensitive and through the use of siRNA gene silencing a role for PKCε was eliminated, leaving PKCδ and PKCθ as potential candidates. Previously reported interactions between the A_{2A}AR and TRAX and 14-3-3τ were confirmed *in vitro* by GST pull-down assay. Binding of 14-3-3τ to the A_{2A}AR was detected at similar levels in samples from PMA-treated compared to untreated cells. However, A_{2A}AR complex formation with TRAX was significantly reduced in samples from PMA-stimulated cells indicating that receptor phosphorylation may regulate the interaction with TRAX.

In order to study phosphorylation of the A_{2A}AR, recombinant adenovirus was used to introduce the myc-tagged human receptor into HUVECs. Data obtained from ligand-binding studies showed that expression of the receptor was very high compared to endogenously expressed receptors (80 ± 7 pmol/mg compared to 0.95 pmol/mg previously reported for the A_{2A}AR in porcine striatum; Klinger *et al.*, 2002b). However, in this study,

it was necessary to achieve high expression in order to detect receptor phosphorylation. This allowed identification of mechanisms which may potentially regulate the activity of the A_{2A}AR. Further studies can be carried out in future to assess the functional relevance of these events in cells expressing endogenous receptors.

Phosphorylation of the human A_{2A}AR was induced by low nanomolar concentrations of PMA and in a short time frame as previously described for the canine receptor (Palmer and Stiles, 1999). However, the human receptor was not as strongly phosphorylated reaching only (3.6 ± 0.4)-fold above basal levels compared to the canine receptor which reached (11.2 ± 2.5)-fold above basal levels. In addition, in contrast to the canine receptor, phosphorylation of the human A_{2A}AR appeared to be bi-phasic, perhaps suggesting the existence of two separate phosphorylation events. Activation of histamine receptors also resulted in increased phosphorylation of the A_{2A}AR indicating that this is a physiologically relevant event. However, in contrast to studies using the canine receptor expressed in C6, CHO and COS cells (Palmer and Stiles, 1997; Palmer *et al.*, 1994), phosphorylation in response to agonist stimulation was not detected. This is perhaps surprising as agonist-induced phosphorylation by GRKs represents the major mechanism for homologous desensitisation of many GPCRs (Krupnick and Benovic, 1998). It is possible that basal levels of phosphorylation are higher in HUVECs than in CHO cells, making small agonist-induced changes difficult to detect compared to the strong response elicited by PMA. An alternative explanation is that recruitment of GRKs to the A_{2A}AR may require prior phosphorylation of the receptor by second messenger-dependent kinases. A specific consensus sequence for phosphorylation by GRKs has not been defined. However, in studies using synthetic peptides, GRK2 has been found to preferentially phosphorylate peptides with negatively charged amino acids to the N-terminal side of a serine or threonine residue (Onorato *et al.*, 1991; Benovic *et al.*, 1990). Indeed, replacement of specific acidic residues with uncharged amino acids in a peptide representing a GRK2 phosphorylation site in the α_2-adrenergic receptor completely abolished phosphorylation (Onorato *et al.*, 1991). It is possible that in the A_{2A}AR, serine and threonine residues are not present in regions sufficiently rich in negatively charged residues to allow recruitment of GRK2. However, introducing negative charges through PMA-induced phosphorylation could potentially provide this requirement and increase the affinity of the receptor for GRK2, thereby allowing subsequent agonist-induced phosphorylation.

Initial investigations into the role of PKC in mediating phosphorylation of the A_{2A}AR were made using the PKC inhibitors GF109203X and rottlerin. A potential problem with this

approach is that many PKC inhibitors are non-selective as they inhibit PKC via its ATP binding site, a domain which is highly homologous between different protein kinases. Despite this, GF109203X has been shown to be a potent and selective inhibitor of classical and novel isoforms of PKC (Toullec *et al.*, 1991) and so its ability to inhibit phosphorylation of the A_{2A}AR does indicate a role for PKC in this process. Rottlerin, which has been reported to inhibit PKC with some selectivity for PKCδ (Gschwendt *et al.*, 1994), did not inhibit phosphorylation of the A_{2A}AR except at the highest concentration tested. However, the value of this finding is questionable as in more recent studies rottlerin was found to be a very poor inhibitor of PKCδ with the ability to inhibit other unrelated kinases with much greater potency (Davies *et al.*, 2000). Further studies using siRNA to specifically knock down expression of PKCδ will be important to more accurately assess the role of PKCδ.

The ability of GF109203X to inhibit phosphorylation indicated the involvement of a novel or classical isoform of PKC. These can be differentiated based upon their requirement for calcium for activation. In this study, the calcium chelator BAPTA/AM failed to block PMA-induced phosphorylation indicating that one of the novel calcium-insensitive isoforms of PKC present in HUVECs (either PKCε, δ or θ) was responsible. However, these results are difficult to interpret as cells treated with ionomycin in control experiments to confirm the activity of BAPTA/AM appeared to suffer toxic effects. Without confirmation that BAPTA/AM was active, it is not possible to firmly conclude that the phosphorylation response is calcium-independent as similar results would have been obtained using an inactive drug. To obtain more reliable results, this experiment could be repeated using an alternative ionophore such as A23187 which may be less toxic than ionomycin.

In contrast to the use of PKC inhibitors, siRNA-mediated gene silencing provided a very specific method for examining the contribution of individual isoforms of PKC to PMA-induced phosphorylation of the A_{2A}AR. Expression of PKCε and PKCα was efficiently downregulated as determined by immunoblotting but this had no effect on levels of phosphorylation achieved providing strong evidence that these isoforms are not involved. Attempts were made to assess the role of PKCθ in a similar fashion. However, PKCθ proved extremely difficult to detect by immunoblotting which made it impossible to determine whether PKCθ expression had been successfully downregulated by RNA interference. Optimisation of immunoblotting procedures perhaps through the use of

alterative anti-PKCθ antibodies will be required to allow the use of RNA interference to determine whether PKCθ is involved in regulating PMA-induced phosphorylation of the A_{2A}AR.

Although the above findings strongly suggest a role for PKC in regulating phosphorylation of the A_{2A}AR, it is not clear whether PKC phosphorylates the receptor directly or whether an intermediate kinase is involved. This is an important consideration as PMA has been shown to activate several other targets in addition to PKC including the PKD family of serine/threonine kinases (Brose and Rosenmund, 2002). In common with PKCs, PKDs have a DAG-binding site termed the C1 site which can also bind phorbol esters (Valverde *et al.*, 1994). Following stimulation with PMA, PKD is translocated to the plasma membrane where it has the potential to interact with other signalling proteins (Matthews *et al.*, 2000). PKD is then activated through direct interaction with and phosphorylation by PKC (Waldron and Rozengurt, 2003; Zugaza *et al.*, 1996). Several isoforms of PKC have been implicated in the activation of PKD including all novel isoforms which is consistent with an effect that would be inhibited by GF109203X as observed in this study (Wang, 2006). PKD phosphorylates serine residues within the consensus motif (LXR(Q/K/E/M)(M/L/K/E/Q/A)S), the most critical residue being leucine at the -5 position (Döppler *et al.*, 2005; Nishikawa *et al.*, 1997). There are two serine residues (Ser329 and Ser370) in the C-terminal tail of the A_{2A}AR with leucine at the -5 position. However, the intervening residues do not conform to the consensus sequence and so it is not clear whether the tail of the A_{2A}AR could act as a substrate for phosphorylation by PKD. Interestingly, another potential phosphorylation site (Ser213) bearing a stronger resemblance to the PKD substrate consensus sequence is present within the third intracellular loop of the receptor. However, this is in the region implicated in G protein coupling and since phosphorylation has no effect on the ability of the receptor to stimulate AC (Palmer and Stiles, 1999), it seems unlikely that PMA-induced phosphorylation is occurring at this site. Unfortunately, there are no specific PKD inhibitors available at present which could be used to test the involvement of PKD in phosphorylation of the A_{2A}AR and so further investigations would require the use of siRNA to suppress expression of each of the four isoforms of PKD.

The failure to detect phosphorylation of the A_{2A}AR in cell types other than HUVECs presented a major problem during this study and prevented evaluation of the effects of receptor truncation on the response. Detection of phosphorylation in HUVECs required that the A_{2A}AR be overexpressed at high levels (80 ± 7 pmol/mg) which was readily

achievable through the use of recombinant adenovirus. DNA constructs encoding the wild-type and truncated forms of the A_{2A}AR were introduced to C6, CHO or HEK 293 cells by the less efficient process of transfection. It must therefore be considered that receptor phosphorylation was not detected in these cells because the receptors were not expressed at sufficiently high levels. Initial determination of receptor expression was in itself complicated by the presence of multiple non-specific bands in immunoblots. This problem could have been avoided by including samples from mock-transfected cells for comparison as these would display only non-specific bands and not those representing the different forms of the receptor. Despite the difficulties in identifying specific bands, it did appear that the 1-311 mutant in particular was expressed at lower levels than the WT receptor and the 1-360 mutant although this could perhaps be explained by the fact that the 1-311 mutant lacks the binding site for USP4. USP4 is thought to promote cell surface expression of the A_{2A}AR following synthesis by deubiquitinating it and preventing its degradation by the proteasome (Milojević *et al.*, 2006). Therefore, it seems likely that a truncated form of the receptor that does not bind USP4 would be retained in the ER and degraded. However, USP4 is reported to bind within the last 50 amino acids of the A_{2A}AR (Milojević *et al.*, 2006) which are missing from both the 1-311 and the 1-360 mutants and so it might be expected that both mutants would be expressed at similarly low levels. Thus, there may be additional reasons for the poor expression of the 1-311 mutant. For example, the additional truncated residues may be important for proper folding of the receptor, meaning that 1-311 is more prone to misfolding and therefore more likely to be degraded before reaching the cell surface. The requirement for high levels of expression of the A_{2A}AR to allow detection of phosphorylation could be tested by using adenovirus to increase expression of the receptor in C6 or CHO cells. It would also be useful to transfect cells with the canine receptor in parallel with cells transfected with the human A_{2A}AR to assess phosphorylation in response to PMA treatment. Since PMA-induced phosphorylation of the canine receptor has been observed previously in C6 and CHO cells, this would provide a positive control to highlight any failings in experimental procedures. An alternative explanation for the lack of response could be that the wild-type and mutant constructs encoded receptors which were not fully functional. However, this is unlikely as the ability of these receptors to stimulate AC activation and ERK activation has been confirmed in a previous study using the same DNA constructs (Klinger *et al.*, 2002b). It is possible that PMA-induced phosphorylation of the human A_{2A}AR was only observed in HUVECs because it requires the presence of a PKC isoform or other kinase which is not present in C6, CHO or HEK 293 cells. For example, CHO cells do not appear to express PKCθ (Megson *et al.*, 2001; Tippmer *et al.*, 1994) which was one of the isoforms of PKC

identified as a potential candidate for mediating phosphorylation of the receptor in this study.

During this study, previously identified interactions between 14-3-3τ and the A_{2A}AR and TRAX and the A_{2A}AR were observed *in vitro* using pull-down assays. The A_{2A}AR-TRAX interaction has been confirmed in intact cells by immunoprecipitating the A_{2A}AR and immunoblotting using an antibody directed against TRAX and also by colocalisation of these two proteins in the brain using double-immunohistochemical staining (Sun *et al.*, 2006). Similar studies should be carried out to confirm the interaction between A_{2A}AR and 14-3-3τ in intact cells.

The finding that 14-3-3τ interacted with the A_{2A}AR independently of PMA-induced receptor phosphorylation was surprising as a major determinant for 14-3-3 binding to many ligands is the presence of a phosphorylated serine or threonine residue (Aitken, 2006). 14-3-3 proteins exist as dimers, each monomer containing a binding pocket that can interact with proteins with either RSXpSXP and RXϕXpSXP binding motifs (Yaffe *et al.*, 1997; Muslin *et al.*, 1996). Data from crystal structures in complex with peptides indicates that a cluster of basic residues within an amphipathic groove of each 14-3-3 monomer mediates the interaction with phosphorylated residues in partner binding proteins (Aitken, 2006). It is possible that in this study, 14-3-3τ bound to serine residues of the A_{2A}AR that are phosphorylated in basal conditions and this is why no changes were detected in response to PMA treatment. However, it is also possible that the interaction occurred independently of phosphorylation as occurs in the case of some other 14-3-3-binding proteins. For example the inositol polyphosphate 5-phosphatase forms a complex with 14-3-3ζ that appears to be mediated by a non-phosphorylated RSESEE motif (Campbell *et al.*, 1997). It is thought that the presence of negatively charged Asp and Glu residues may compensate for the lack of phosphorylated serine residues, thereby allowing binding to the amphipathic groove of 14-3-3ζ in a similar way to phosphorylated ligands. In another example, 14-3-3 proteins have been found to interact with the *Pseudomonas aeroginosa* ADP-ribosyltransferase toxin exoenzyme S in a completely different fashion involving hydrophobic rather than electrostatic interactions (Ottmann *et al.*, 2007). In this case, binding requires the presence of four leucine residues. Further studies will be required to ascertain whether any of these modes of binding are employed in the interaction between the A_{2A}AR and 14-3-3τ. There are no aspartic acid or leucine-rich motifs which stand out in the sequence of the C-terminal tail of the A_{2A}AR. However, other amino acids with similar properties may allow

similar interactions to occur. Identification of the region of the A_{2A}AR involved in binding to 14-3-3τ will be important in determining whether this is the case. Firstly, to determine whether a conventional phosphorylated binding site is used, it would be useful to immunoprecipitate the A_{2A}AR and perform an immunoblot using a phospho-14-3-3 substrate antibody. If the antibody does not react with the A_{2A}AR, indicating that a conventional binding site is not being used, then to begin to investigate which part of the receptor is involved in the interaction, pull-downs or co-immunoprecipitation studies with wild-type and truncated forms of the A_{2A}AR could be used to see if the deleted parts of the truncated receptors are necessary for binding. Similarly, the effect of truncation of the receptor on colocalisation of 14-3-3τ and the A_{2A}AR could be assessed. More detailed analysis of the binding site involved could be achieved by detecting binding of purified 14-3-3τ to peptide arrays constituting the C-terminal tail region of the A_{2A}AR.

It is also not clear what functional role 14-3-3τ binding to the A_{2A}AR may play. 14-3-3 proteins have been ascribed diverse roles in many physiological processes including cell signalling, cell cycle progression, intracellular trafficking, cytoskeletal structure and transcription (Fu *et al.*, 2000). In the context of cell signalling, their ability to act as scaffolding proteins is of particular interest. Since 14-3-3 proteins exist as dimers, they are able to bind two interaction partners simultaneously (Tzivion *et al.*, 2001). It is therefore possible that 14-3-3τ could recruit signalling molecules to the tail of the A_{2A}AR and facilitate initiation of different signalling pathways. 14-3-3 proteins have been reported to bind several signalling molecules including Raf kinases, MEKKs, PKC and PI-3 kinase (Tzivion *et al.*, 2001). The interaction between 14-3-3 and Raf-1 has been particularly well studied and appears to play a critical regulatory role in Ras-mediated activation of Raf-1 during initiation of the ERK cascade (Fu *et al.*, 2000). There are conflicting reports regarding the precise role of 14-3-3 proteins in this process. However, this may be due largely to the fact that Raf-1 contains two phosphoserine-dependent binding sites (pSer259 and pSer621) and one phosphate-independent binding site for 14-3-3 proteins, each with different roles. Interactions at the pSer259 site negatively regulate Raf-1 activity while 14-3-3 proteins bound at the pSer621 site have been suggested to either act as cofactors for Raf-1 kinase activity or alternatively to stabilise Raf-1 in a confirmation that promotes its activation (Fu *et al.*, 2000). The ability of 14-3-3 proteins to regulate Raf-1 activity prompts the question of whether binding of 14-3-3τ to the A_{2A}AR could influence initiation of ERK signalling from the receptor. This has been suggested for the α_2-adrenergic receptors which bind 14-3-3ζ via their third intracellular loops (Prezeau *et al.*,

1999). Since α_2-adrenergic receptors activate ERK via Ras and Raf, it was proposed that binding of Raf to inactive receptors via 14-3-3ζ could poise it for immediate activation following agonist stimulation. It is possible that 14-3-3τ could play a similar role in ERK activation by the A_{2A}AR in endothelial cells where G protein-independent activation of ERK is believed to occur through activation of Ras (Seidel *et al.*, 1999).

A major finding in this study was that interaction of the A_{2A}AR with TRAX was severely inhibited under conditions when the A_{2A}AR would be phosphorylated. Future studies to determine the functional consequences of this effect can make use of the discovery that TRAX mediates the ability of the A_{2A}AR to induce cell cycle arrest and differentiation of PC-12 cells that is impaired in the absence of functional p53 (Sun *et al.*, 2006). First it will be important to define the binding site for TRAX in the A_{2A}AR C-terminal tail. Initially, the truncated forms of the A_{2A}AR could be used in pull-down experiments to determine which region of the receptor is involved. More detailed analysis could be achieved by constructing peptide arrays based on the tail of the A_{2A}AR and testing the ability of purified TRAX to bind to different stretches of amino acids. If the residues required for binding of TRAX to the A_{2A}AR can be identified, then peptides based on this sequence could be constructed and modified with lipids to allow introduction into PC-12 cells. If the interaction of TRAX with the A_{2A}AR is dependent on the receptor being unphosphorylated, then these peptides will displace TRAX from the A_{2A}AR and this will result in a reduced ability of the A_{2A}AR to induce neurite differentiation in the absence of functional p53. Treating cells with PMA to induce receptor phosphorylation and dissociation from TRAX would be expected to have a similar effect. Together, these experiments would show whether or not phosphorylation of the receptor is a significant factor in regulating downstream functional effects of A_{2A}AR signalling.

In summary, during this study, it was found that the human A_{2A}AR is phosphorylated in response to activation of PKC either by treatment with PMA or through stimulation of endogenous histamine H1 receptors. In HUVECs this effect appeared to be mediated by PKCδ and/or PKCθ although the involvement of an intermediate kinase could not be ruled out. Importantly, a potential functional role for these phosphorylation events was indicated by the finding that *in vitro* binding of the C-terminal-interacting protein TRAX to the A_{2A}AR was significantly reduced under conditions when the receptor would be phosphorylated while the interaction with 14-3-3τ was unaffected. This suggests that phosphorylation of the A_{2A}AR may be important in regulating binding of particular proteins to the C-terminal tail of the receptor. This is significant in the light of the

increasing number of proteins that are being identified as able to interact with the C-terminal tail of the A_{2A}AR. Regulation by phosphorylation may provide a mechanism by which different proteins can be recruited in order to initiate different signalling pathways in different cellular contexts.

5 Final discussion

The production of adenosine in response to metabolic stress represents a critical endogenous mechanism for preventing excessive inflammation and limiting tissue injury (Haskó and Cronstein, 2004). The central role of the A_{2A}AR in mediating these responses has been demonstrated in numerous studies using adenosine and A_{2A}AR selective agonists to inhibit the inflammatory responses in a variety of cell types *in vitro* as well as in *in vivo* models of inflammatory disease and in studies using A_{2A}-deficient mice (Palmer and Trevethick, 2008; Haskó and Cronstein, 2004; Sitkovsky, 2003). As an endogenous mediator of anti-inflammatory responses, the A_{2A}AR represents a particularly attractive subject for study. Increasing understanding of how the body naturally deals with excessive inflammation may reveal mechanisms of limiting inflammation and tissue damage that could be harnessed to create novel therapies for major inflammatory diseases such as atherosclerosis, sepsis and cancer.

Despite the plethora of evidence regarding the anti-inflammatory effects of signalling through the A_{2A}AR, the mechanisms behind these effects are only just beginning to be elucidated. Findings from *in vitro* studies using endothelial cells have indicated that the ability of the A_{2A}AR to exert such wide-ranging anti-inflammatory effects could be explained by its ability to regulate pro-inflammatory signalling pathways (Sands *et al.*, 2006; Sands *et al.*, 2004). The aim of the study described in Chapter 2 of this thesis was to investigate the physiological relevance of these findings by assessing the role of the A_{2A}AR in regulating activation of the NFκB and JAK/STAT pro-inflammatory signalling pathways in the aortae of A_{2A}AR-deficient mice. Data presented here show that in mice lacking the A_{2A}AR, LPS-induced pro-inflammatory cytokine production is markedly enhanced compared to wild-type mice. Consistent with this observation, activation of the JAK/STAT pathway in response to LPS was potentiated in the aortae of these animals as shown by elevated levels of phosphorylated STAT1. Similarly, heightened activation of the NFκB pathway was detected by the presence of increased levels of phosphorylated IκBα in $A_{2A}AR^{-/-}$ mice regardless of LPS-treatment. However, evaluation of the significance of this finding will require further study as no change was detected in the expression of the STAT1 and NFκB-regulated genes, VCAM-1, ICAM-1 and TAP-1.

The lack of effect of increased NFκB and JAK/STAT pathway activation on downstream signalling and gene expression was unexpected but can perhaps be explained by the

upregualtion of negative regulatory mechanisms in A_{2A}AR-deficient mice. This may have been a result of using a mouse model in which the A_{2A}AR gene had been disrupted in all tissues. Many unknown compensatory genetic changes could have occurred that might mask the effects of expressing a non-functional A_{2A}AR. A more useful method to analyse specific effects of the A_{2A}AR in the vascular endothelium would be to use tissue-specific gene deletion to target the A_{2A}AR only in endothelial cells. Another approach to assess the importance of adenosine receptor signalling in regulating pro-inflammatory signalling in the endothelium would be to examine NFκB and JAK/STAT pathway activation in mice lacking endothelial CD73 or CD39. These enzymes enable the endothelium to produce a significant amount of adenosine that is critical for maintenance of endothelial barrier function during acute inflammatory responses (Lennon *et al.*, 1998). If adenosine signalling through the A_{2A}AR is a significant mechanism for regulating pro-inflammatory signalling in the endothelium then it might be expected that CD73 or CD39-deficient mice would display similar perturbations in NFκB and JAK/STAT signalling to $A_{2A}AR^{-/-}$ mice.

In this study, pro-inflammatory signalling pathway activation was examined specifically in the aorta in an attempt to characterise mechanisms which may allow the A_{2A}AR to suppress the development of vascular inflammation and atherosclerosis. LPS-induced sepsis was used as a model of vascular inflammation. However, LPS treatment produces acute inflammation while atherosclerosis is a chronic inflammatory disease with plaques developing over several years or decades (Hansson, 2005). In order to understand changes in the endothelium during atherosclerosis, it would be useful to carry out studies using a model of chronic inflammation such as collagen-induced arthritis which develops over several weeks. This may produce quite different effects on pro-inflammatory signalling owing to changes in the cytokines present during chronic inflammation and the involvement of different cell types, particularly T cells.

A particularly striking difference between wild-type and $A_{2A}AR^{-/-}$ mice observed in this study was the increased level of IκBα phosphorylation detected in $A_{2A}AR^{-/-}$ mice even in the absence of LPS treatment. It would be interesting to investigate the mechanisms which prevent IκBα phosphorylation and therefore inappropriate activation of the NFκB pathway in A_{2A}AR-competent mice. This could occur at several steps in the pathway leading to NFκB activation. For example, through negative regulation of IKK phosphorylation or by inactivation of upstream components such as TAK1, RIP or TRAF proteins. These signalling factors rely on Lys63 polyubiquitination for activation and are subject to negative regulation by DUBs such as A20 and CYLD (Sun *et al.*, 2008). Therefore

modulation of DUB activity represents a likely mechanism for regulation of their activation status. A_{2B}AR activation has already been shown to promote deneddylation of the Cul-1 subunit of the IκBα E3 ligase complex (Khoury *et al.*, 2007). Perhaps A_{2A}AR activation in wild-type mice suppresses NFκB activation in the absence of stimuli by promoting the deubiquitination of signalling components upstream of IKK.

The effects of the A_{2A}AR on endothelial cells are of particular interest since vascular dysfunction is central to development of atherosclerosis. In this study, it was hypothesised that the presence of the A_{2A}AR might suppress pro-inflammatory pathway activation in the endothelium and therefore protect against development of vascular disease. However, these effects may not be specific to endothelial cells and it would be interesting to see if signalling in other cell types is affected by A_{2A}AR activation. T cells and NKT cells, for example, represent interesting targets as they are both involved in the development of atherosclerosis (Hansson, 2005) and have their pro-inflammatory functions suppressed by A_{2A}AR stimulation (Sevigny *et al.*, 2007; Lappas *et al.*, 2006).

Another subject for future studies might be to investigate the effect of A_{2A}AR gene-deletion on signalling pathways other than the NFκB and JAK/STAT pathways. Although these are generally considered to be the major pro-inflammatory signalling pathways, others such as the JNK and p38 pathways are also involved in activation of the endothelium and so may represent targets for regulation by the A_{2A}AR (Sumara *et al.*, 2005; Hoefen and Berk, 2002).

In summary, findings presented in Chapter 2 of this thesis indicate that signalling through the A_{2A}AR represents a significant endogenous mechanism for suppressing activation of the NFκB and JAK/STAT pro-inflammatory signalling pathways in the aorta. Further studies will be required to assess the consequences of this for downstream gene expression and development of vascular inflammation. Future studies aimed at identifying mechanisms by which the A_{2A}AR regulates pro-inflammatory signalling may reveal novel targets for therapeutic intervention. This could allow specific modulation of inflammatory events occurring in the endothelium to limit the progression of diseases such as atherosclerosis, sepsis and cancer.

While data presented in Chapter 2 of this thesis add to the large body of evidence regarding the physiological effects of signalling through the A_{2A}AR, findings described in Chapter 3 pertain to the lesser studied question of how the receptor is regulated at a molecular level.

This subject has become of particular interest recently since it has become apparent that in addition to heterotrimeric G proteins and proteins involved in desensitisation, several other proteins can interact with the long C-terminal tail of the A_{2A}AR (Zezula and Freissmuth, 2008). However, the question of how these interactions might be regulated has not been addressed. Previous studies have shown that the canine A_{2A}AR is phosphorylated in response to activation of PKC. However, unlike in the case of other GPCRs, this is not associated with heterologous desensitisation of receptor-G protein coupling (Palmer *et al.*, 1999), indicating that PKC-mediated phosphorylation may have a previously unappreciated role in regulating A_{2A}AR activity. The aim of this study was to determine whether the human A_{2A}AR, like the canine receptor, is subject to regulation by phosphorylation and whether phosphorylation events in the C-terminal tail of the A_{2A}AR could play a role in regulating the binding of interacting proteins. Data presented here indicate that the human A_{2A}AR is indeed phosphorylated in response to PKC activation. Interestingly this modification appears to have consequences for the binding of C-terminal interacting proteins as 14-3-3τ was found to bind to the A_{2A}AR in the presence or absence of PKC-activating stimuli while TRAX bound only to the unphosphorylated receptor. This suggests that PKC-mediated phosphorylation may represent a selective means of controlling binding of individual interacting proteins.

In addition to 14-3-3τ and TRAX, several other proteins have been reported to interact with the C-terminal tail of the A_{2A}AR including ARNO (Gsandtner *et al.*, 2005), α-actinin (Burgueño *et al.*, 2003) and USP4 (Milojević *et al.*, 2006). To determine whether PKC-mediated phosphorylation represents a general mechanism of regulating interactions with the A_{2A}AR, it would be interesting to repeat pull-down experiments carried out in this study with ARNO, α-actinin and USP4. It would also be interesting to find out if any of these proteins can bind simultaneously and whether the phosphorylation status of the receptor dictates which combination of proteins can bind at one time. For example, while phosphorylation of the A_{2A}AR inhibits the interaction with TRAX, dissociation from TRAX may enable another protein to bind. To properly characterise these interactions, it will be important to identify the binding sites of each of the interacting proteins. This can be achieved by assessing binding of recombinant proteins to peptide arrays representing the C-terminal tail of the A_{2A}AR. Alanine scanning mutagenesis could then be used to identify the role of individual amino acids in dictating interactions in co-immunoprecipitation and pull-down assays.

The finding that 14-3-3τ binds to the A_{2A}AR indicates that other 14-3-3 proteins may also be A_{2A}AR interactors as members of this family are highly homologous (Aitken, 2006). It would be interesting to test whether this is the case as different isoforms of 14-3-3 proteins may be present in different cell types. It would also be interesting to find out whether they share a common binding site as the A_{2A}AR C-terminal tail does not contain any classical 14-3-3 consensus binding motifs.

Future studies should be directed at determining the functional significance of interactions occurring at the C-terminal tail of the A_{2A}AR and the consequences of receptor phosphorylation. USP4 has been assigned a role in promoting A_{2A}AR cell-surface expression by regulating its ubiquitination status (Milojević *et al.*, 2006) while α-actinin appears to be important for A_{2A}AR internalisation (Burgueño *et al.*, 2003) and ARNO is involved in G-protein-independent activation of the ERK pathway by the A_{2A}AR (Gsandtner *et al.*, 2005). If receptor phosphorylation is found to regulate the interactions of any of these proteins with the tail of the A_{2A}AR, it would be interesting to see if phosphorylation results in any changes in these functions. This could be achieved by introducing cell-permeable peptides based on either the phosphorylated or unphosphorylated form of the receptor tail to displace the interacting proteins followed by assessment of downstream effects. The role of 14-3-3τ binding to the A_{2A}AR has not yet been identified but could be investigated using gene targeting to generate mice lacking 14-3-3τ and then examining the resultant phenotypes for changes that might relate to aberrant A_{2A}AR function. The likelihood that other 14-3-3 proteins could compensate for the lack of 14-3-3τ might limit the usefulness of this approach.

In summary, findings presented here show that the human A_{2A}AR is subject to PKC-mediated phosphorylation and this appears to play a role in controlling which proteins can bind to the C-terminal tail of the receptor. The functional significance of this regulation remains to be examined but it could represent a means of selectively recruiting different proteins to the receptor to allow initiation of distinct signalling pathways, thereby enabling the A_{2A}AR to induce the appropriate responses in particular cellular contexts.

6 References

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