THE ROLE OF EXTRACELLULAR SIGNAL-REGULATED KINASE IN β-ADRENOCEPTOR-MEDIATED VASODILATATION

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July 2012

Abstract

β-Adrenoceptors (β-ARs) mediate vasodilatation by activating various mechanisms that collectively contribute to vascular smooth muscle (VSM) relaxation. It has been shown that β_2-AR stimulation in cultured cells results in activation of extracellular signal-regulated kinase (ERK). As the functional relevance of this was not known, the aim of the current investigation was determine the role of ERK in β-AR-mediated vasodilatation.

Isoprenaline-induced relaxation of porcine coronary artery (PCA) segments pre-contracted with the thromboxane mimetic U46619 was significantly enhanced by inhibition of ERK activation. Relaxations to the β_2-AR agonist salbutamol, but not those to the β_1-AR agonist xamoterol or the adenylyl cyclase activator forskolin, were also enhanced. The intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channel blocker TRAM-34 prevented the enhancement of β_2-AR-mediated responses.

Taken together, the data indicate that ERK inhibits β_2-AR-mediated vasodilatation by interacting with a cyclic 3', 5'-adenosine monophosphate-independent relaxation pathway involving K^+ channels. This may occur through a direct regulatory action on the IK_{Ca} channel via phosphorylation.

Furthermore, the finding that increased ERK activation in a rat model of Type II diabetes was associated with significantly impaired β-AR-mediated vasodilatation raises the possibility that ERK may represent a promising therapeutic target in the treatment of disease states characterised by abnormal vascular function.

Publications

Papers:

Uhiara CO, Alexander SPH, Roberts RE (2009). Effect of inhibition of extracellular signal-regulated kinase on relaxations to β-adrenoceptor agonists in porcine isolated blood vessels. *British Journal of Pharmacology* **158**: 7: 1713-1719.

Abstracts:

Uhiara CO, Alexander SPH, Roberts RE (2008). The role of extracellular signal-regulated protein kinase (ERK) in β-adrenoceptor-mediated vasodilatation in porcine coronary artery. *pA_2 online* **6**: 4.

Uhiara CO, Alexander SPH, Roberts RE (2008). β-Adrenoceptor-mediated vasodilatation is impaired in superior mesenteric arteries from Zucker diabetic rats: Effect of inhibition of ERK-MAP kinase activation. *pA_2 online* **6**: 4.

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Acknowledgements

First and foremost, I would like to thank Dr. Richard Roberts and Dr. Stephen Alexander, my supervisors, for giving me this opportunity. Their guidance and encouragement since day one have been invaluable.

Thanks must also go to Dr. Michael Garle for his continuous support; to Dr. William Dunn, Dr. Vincent Wilson, Dr. Michael Randall and Dr. Vera Ralevic for their advice; to Dr. Paul Smith and Liaque Latif for showing me the ropes; to Jagdish Heer, Andrea Burchell and Anne Keeling for their support; and to countless others in E34 for their friendship over the years.

None of this would have been possible without the funding provided by the BBSRC, so to them I owe a debt of gratitude.

I would like to express my gratitude to Ellen Lam and family for their support. Finally, to my parents and brothers, your unwavering love and support have sustained me through my studies and life in general. Thank you.

ABBREVIATIONS

β-AR	β-adrenoceptor
AA	Arachidonic acid
AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ANOVA	Analysis of variance
AP-1	Activator protein-1
BK_{Ca}	Large-conductance calcium-activated potassium channel
cAMP	Cyclic 3',5'-adenosine monophosphate
cGMP	Cyclic 3',5'-guanosine monophosphate
CHO	Chinese hamster ovary
COPD	Chronic obstructive pulmonary disorder
COX	Cyclooxygenase
CPI-17	Protein kinase C-potentiated inhibitory protein
CREB	Cyclic 3',5'-adenosine monophosphate response element-binding protein
DAG	Diacyl glycerol
DHET	Dihydroxyeicosatrienoic acid
EC_{50}	Half-maximal effective concentration
ECE	Endothelin-converting enzyme
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
EET	Epoxyeicosatrienoic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
FFA	Free fatty acid
FPP	Farnesyl pyrophosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GGPP	Geranylgeranyl pyrophosphate
GK	Goto-Kakizaki
GPCR	G-protein-coupled receptor
GTP	Guanosine triphosphate
HB-EGF	Heparin-binding epidermal growth factor

HDL	High density lipoprotein
HEK	Human embryonic kidney
HETE	Monohydroxyeicosatetraenoic acid
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HUVEC	Human umbilical vein endothelial cells
IK_{Ca}	Intermediate-conductance calcium-activated potassium channel
IP_3	Inositol-1,4,5-triphosphate
JIP	c-Jun NH2-terminal kinase-interacting protein
JNK	c-Jun NH2-terminal kinase
K_{ATP}	ATP-sensitive potassium channel
K_B	Antagonist affinity
K_{Ca}	Calcium-activated potassium channel
kDa	Kilodalton
KH	Krebs-Henseleit
K_{IR}	Inward-rectifier potassium channel
K_V	Voltage-gated potassium channel
LDL	Low density lipoprotein
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinase
MAPK-APK	Mitogen-activated protein kinase-activated protein kinase
MAPKK/MAP2K	Mitogen-activated protein kinase kinase
MAPKKK/MAP3K	Mitogen-activated protein kinase kinase kinase
MCP-1	Monocyte chemotactic protein-1
MLC_{20}	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
M_W	Molecular weight
NO	Nitric oxide
OLETF	Otsuka Long-Evans Tokushima Fatty
PC12	Phaeochromocytoma 12
PCA	Porcine coronary artery
PDE	Phosphodiesterase
PGI_2	Prostacyclin
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A_2

PLCβ	Phospholipase Cβ
PON	Paraoxonase
RAS	Renin-angiotensin system
RhoK	Rho-associated kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
SEM	Standard error of the mean
SERCA	Sarco(endo)plasmic Ca^{2+}
sGC	Soluble guanylyl cyclase
SHR	Spontaneously hypertensive rats
SK_{Ca}	Small-conductance calcium-activated potassium channel
SMA	Superior mesenteric artery
SR	Sarcoplasmic reticulum
SUR	Sulphonylurea receptor
TP	Thromboxane-prostanoid
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle
WHO	World Health Organisation
WOKW	Wistar Ottawa Karlsburg W
ZDF	Zucker Diabetic Fatty

CHAPTER 1: INTRODUCTION

1.1. VASODILATATION IN HEALTH AND DISEASE

The regulation of vascular tone is a complex process involving the opposing actions of vasodilators and vasoconstrictors. Referring to the widening of blood vessels, vasodilatation is associated with increased blood flow and reduced vascular resistance, and occurs as a result of vascular smooth muscle (VSM) relaxation. VSM contraction causes vasoconstriction, which, in contrast, is associated with increased resistance and reduced blood flow.

Factors known to regulate blood flow are diverse and include endogenous vasoactive substances, ischaemia and metabolic rate. Endogenous regulators of vascular tone include substances that activate cell-surface receptors and ultimately either increase or decrease VSM tone. For instance, the hormone adrenaline, when acting at β-adrenoceptors (β-ARs), relaxes VSM cells, causing vasodilatation. Similarly, thromboxane A_2 (TXA_2), a product of the vascular endothelium, causes vasoconstriction by activating on thromboxane-prostanoid (TP) receptors on adjacent VSM cells.

Impairment of vasodilatation is a characteristic feature of cardiovascular diseases such as hypertension and coronary artery disease. In fact, the actions of nicorandil and the nitrate drugs, indicated in the treatment of ischaemic heart disease, involve vasodilatation (Joint Formularly Committee, 2011). Effective treatment is critical in preventing the loss of structural integrity that accompanies vascular growth in cardiovascular disease states. A mediator of this complex process is the mitogen-activated protein kinase ERK (extracellular signal-regulated kinase), which is also known to promote VSM contraction.

This investigation will attempt to reconcile the finding that β-AR agonists, which are known to induce vasodilatation, have been shown to activate ERK in cultured cells expressing β-ARs (Baker *et al.*, 2003; Friedman *et al.*, 2002).

1.2. THE ROLE OF ERK IN THE REGULATION OF VASCULAR TONE

1.2.1. Structure of blood vessels

The vascular system is a collection of blood vessels that is responsible for transporting materials throughout the body and maintaining cellular homeostasis. Supported by a complex network of arterioles, capillaries and venules, arteries and veins are key components of the vascular system. As shown in Figure 1.1, they are broadly similar anatomically, possessing a characteristic three-layer structure composed of the intima, media and adventitia.

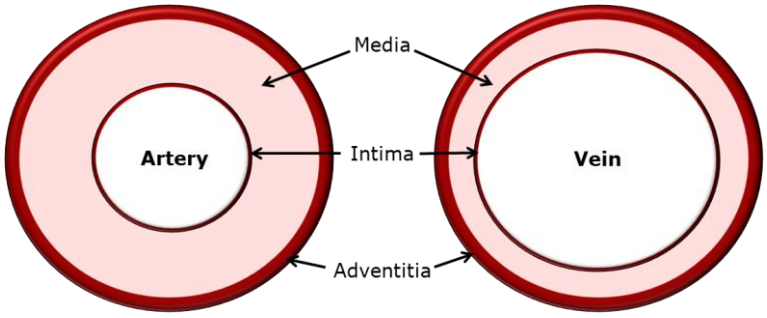

Figure 1.1. *Cross-sectional structures of an artery and a vein. The intima (innermost layer) is composed of a monolayer of endothelial cells and a subendothelial layer; smooth muscle cells and elastin fibres predominate the media; and the adventitia contains collagenous and elastic fibres.*

1.2.2. Vascular smooth muscle (VSM) contraction

1.2.2.1. Intracellular Ca^{2+}

A schematic representation of VSM contraction is shown in Figure 1.2. VSM contraction is dependent on intracellular calcium concentration ($[Ca^{2+}]_i$). The Ca^{2+}-ATPase pump and the Na^+/Ca^{2+} exchanger mediate the efflux of Ca^{2+} from the cytosol and are key in maintaining basal $[Ca^{2+}]_i$, which, at ~100 nM, is roughly 10,000-fold lower than $[Ca^{2+}]_e$ (Marin *et al.*, 1999).

Increases in $[Ca^{2+}]_i$ represent the primary means by which vasoconstrictors evoke contraction, though they may also act by increasing the sensitivity of contractile elements to Ca^{2+}.

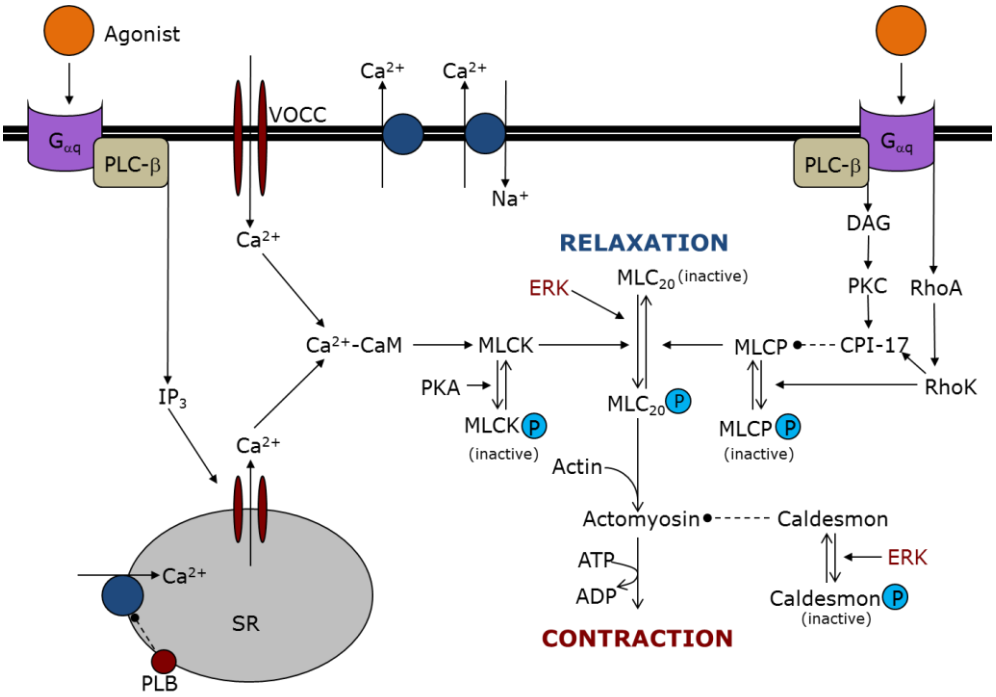

Figure 1.2. *A schematic representation of the main pathways involved in VSM contraction. Contraction depends on the phosphorylation state of myosin light chain (MLC_{20}). Ca^{2+} is sourced from the extracellular space and sarcoplasmic reticulum (SR) following activation of, for example, $G\alpha_{q/11}$-coupled receptors (left). The Ca^{2+}-binding protein calmodulin (CaM) binds Ca^{2+}, forming a complex capable of activating myosin light chain kinase (MLCK). MLCK phosphorylates MLC_{20}, allowing it to interact with actin. Subsequent actomyosin ATPase activity results in contraction. Myosin phosphatase (MLCP) causes relaxation by dephosphorylating MLC_{20} and is itself regulated by PKC-potentiated inhibitory protein (CPI-17). Rho-associated kinase (RhoK) promotes contraction by activating CPI-17 and inactivating MLCP. ERK disinhibits actomyosin ATPase activity by phosphorylating and inactivating caldesmon. ERK may also directly phosphorylate MLC_{20}. $[Ca^{2+}]_i$ is reduced by the plasma membrane Ca^{2+} pump and Na^+-Ca^{2+} exchanger. The sarco(endo)plasmic Ca^{2+} (SERCA) pump maintains Ca^{2+} in the SR and its activity is inhibited by phospholamban (PLB). PLC-β (phospholipase Cβ), IP_3 (inositol-1,4,5-triphosphate), VOCC (voltage-operated Ca^{2+} channel), PKA (protein kinase A) and DAG (diacyl glycerol) are also shown. Dashed lines with rounded tips indicate inhibition.*

The main source of Ca^{2+} is the extracellular solution, from which L-type Ca^{2+} channels conduct Ca^{2+} to the intracellular space. Opening of these

channels is voltage-sensitive and occurs under depolarised conditions, which arise in the presence of high $[K^+]_e$ or following agonist-induced receptor activation (Ganitkevich *et al.*, 1990; Matsuda *et al.*, 1990; Shimoda *et al.*, 2000).

Under resting conditions, the vast majority of intracellular Ca^{2+} is sequestered in intracellular stores such as the sarcoplasmic reticulum (SR; Figure 1.2). Much of this is due to the action of the sarco(endo)plasmic Ca^{2+} (SERCA) pump, which accumulates Ca^{2+} in the SR (Orallo, 1996). However, stored Ca^{2+} can also be utilised. For instance, activation of $G\alpha_{q/11}$-coupled receptors causes a phospholipase Cβ (PLCβ)-dependent increase in the levels of the second messenger inositol-1, 4, 5-triphosphate (IP_3), which in turn activates IP_3 receptors on the SR, causing Ca^{2+} release. Stimulation of this signalling pathway also results in the activation of protein kinase C (PKC) via an additional second messenger known as diacylglycerol (DAG). PKC is involved in Ca^{2+}-independent routes to contraction, discussed below.

As shown in Figure 1.2, elevations of $[Ca^{2+}]_i$ are detected by the Ca^{2+}-binding protein calmodulin, which forms a complex with Ca^{2+}. Ca^{2+}-bound calmodulin interacts with the regulatory domain of myosin light chain kinase (MLCK), causing its activation. Phosphorylation at Ser19 of the regulatory, 20 kDa chain of myosin (MLC_{20}) by activated MLCK triggers the binding of MLC_{20} to actin, accelerating actomyosin ATPase activity and cross bridge cycling (Somlyo *et al.*, 1994).

1.2.2.2. Ca^{2+} sensitisation

VSM contraction is enhanced by mechanisms which increase the Ca^{2+} sensitivity of the contractile elements. The phosphorylation state of MLC_{20} is also regulated by myosin phosphatase (MLCP), which promotes relaxation by dephophosphorylating MLC_{20}. However, the activity of MLCP itself is subject to regulation by Rho-associated kinase (RhoK), which promotes contraction by phosphorylating and inactivating MLCP. MLCP activity is also reduced via direct inhibition of its catalytic subunit by PKC-potentiated inhibitory protein (CPI-17), which itself is activated by PKC and RhoK. These mechanisms are illustrated in Figure 1.2.

1.2.2.3. The endothelium in VSM contraction

1.2.2.3.1. Endothelin-1

The peptide endothelin-1 (ET-1) is recognised as one of the most powerful endogenous vasoconstrictors. Formed in endothelial cells by endothelin-converting enzyme (ECE), ET-1 induces Ca^{2+}- and PKC-dependent vasoconstriction by activating the $G\alpha_{q/11}$-coupled ET_A receptors on adjacent VSM cells (Griendling *et al.*, 1989; Ohnaka *et al.*, 1990; Takuwa *et al.*, 1990). ET_B receptors are expressed on ECs, where they mediate relaxation responses via a nitric oxide (NO)-dependent mechanism (Hosoda *et al.*, 1991; Takayanagi *et al.*, 1991).

1.2.2.3.2. Thromboxane-A_2 (TXA_2)

TXA_2 is a cyclooxygenase (COX)-derived arachidonic acid (AA) metabolite known to contribute to abnormalities in various cardiovascular diseases (Sellers *et al.*, 2008). Its effects are mediated by $G\alpha_{q/11}$-coupled TP receptors, which, when activated on VSM, produce powerful vasoconstriction. The synthetic thromboxane mimetic U46619 has been shown to activate ERK in VSM (Miggin *et al.*, 2002), suggesting that the reported effects of TXA_2, such vasoconstriction and cell growth, may involve ERK.

1.2.2.4. RAS

The renin-angiotensin system (RAS) plays an important role in the regulation of cardiovascular and fluid homeostasis. Interactions between key the components of the RAS system are illustrated in Figure 1.3. Renin, secreted from juxtaglomerular kidney cells, converts liver-derived angiotensinogen to angiotensin I, which in turn is metabolised by angiotensin-converting enzyme (ACE) to form angiotensin II, the main effector peptide of the RAS. Angiotensin II produces its effects by activating AT_1 and AT_2 receptors and stimulates aldosterone release from the adrenal cortex.

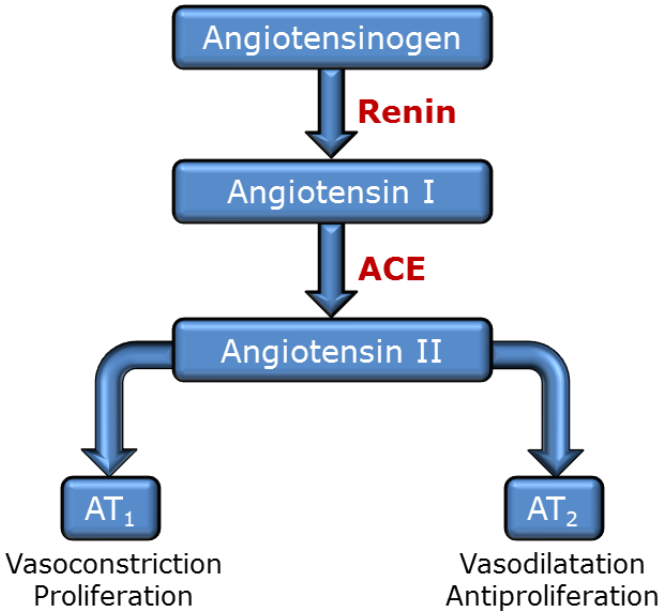

Figure 1.3. *Schematic diagram of the RAS. Angiotensin II, formed via the sequential actions of renin and ACE, acts at AT_1 or AT_2 receptors to constrict or dilate blood vessels. ACE also promotes the breakdown of bradykinin, a vasodilator, to inactive peptides.*

Although the systemic effects of circulating RAS have been well described, it has been shown that the components of the RAS are expressed in a wide range of tissues where they may perform important functions (Nguyen Dinh Cat *et al.*, 2011). In blood vessels, for example, angiotensin II synthesised and released by endothelial cells may contribute to blood vessel tone by releasing vasoactive substances such as prostaglandins (Nguyen Dinh Cat *et al.*, 2011).

1.2.2.5. Mitogen-activated protein kinase (MAPK)

The MAPK superfamily consists of distinct signalling enzymes that function to regulate a wide range of cellular processes in response to extracellular stimuli. Their physiological significance is underscored by their evolutionary conservation in eukaryotic organisms and expression in virtually all cell types. Six distinct subgroups of MAPKs have been described in mammalian

cells: ERKs 1 and 2, p38 MAPK, c-Jun NH_2-terminal kinase (JNK), ERKs 3 and 4, ERK 5 and ERKs 7 and 8 (Krishna *et al.*, 2008). A wide range of stimuli are capable of activating MAPKs. Examples include cell-surface receptor activation by hormones, growth factors and cytokines; and physical and chemical stresses such as osmolarity, cold/heat shock, radiation and hypoxia/ischaemia.

The activation of individual MAPK enzymes is mediated by a signal transduction cascade involving two upstream protein kinases. Thus MAPK signalling cascades are composed of a prototypical three-kinase module (Figure 1.4). Initially, an upstream MAP3K, or MAPK kinase kinase (MAPKKK), is activated by either small GTP-binding proteins or protein kinases. Thereafter, the activated MAP3K dual-phosphorylates its downstream signalling partner, a MAP2K (or MAPK kinase, MAPKK), allowing the MAP2K to activate the terminal MAPK by dual phosphorylation at threonine/tyrosine residues. Finally, the activated MAPK is able to regulate nuclear and cytosolic processes by phosphorylating a range of enzymes including transcription factors, protein kinases, phospholipases and phosphodiesterases.

Figure 1.4. *Schematic diagram of the MAPK signalling pathway. The cascade is composed of a three-kinase module in which each kinase is phosphorylated and activated in sequence. The ERK signalling cascade is shown as an example (in parentheses).*

1.2.2.5.1. p38 MAPK

The p38 MAPKs are so named as a result of the independent discovery of a 38 kDa protein, later termed p38α, in four laboratories (Freshney *et al.*, 1994; Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). Three further members of the p38 MAPK family have since been identified and characterised: p38β, p38δ and p38γ (Coulthard *et al.*, 2009).

The p38s regulate cellular processes in response to diverse external stimuli ranging from environmental stresses to inflammation. These stimuli are able to engage various mechanisms that stimulate the many MAP3Ks involved in p38 signalling. In turn, three dual-specificity MAP2Ks, namely MKK3, MKK4, MKK6, are activated and proceed to dual-phosphorylate p38 MAPK at Thr180 and Tyr182 (Johnson *et al.*, 2002). Downstream effectors of p38 MAPK include protein kinases such as MAPK-activated protein kinase-2 (MAPK-APK-2) (Manke *et al.*, 2005) and MAPK-APK-5 (New *et al.*, 2003) and transcription factors such as p53 (Bulavin *et al.*, 1999).

The pyridinylimidazole SB 203580, a p38 inhibitor, has been instrumental in studies of p38 function, despite demonstrating non-specific effects on distinct signalling pathways (Clerk *et al.*, 1998; Kalmes *et al.*, 1999). More specific p38 inhibitors have demonstrated therapeutic potential and have reached clinical development in disease states characterised by chronic inflammation (Behr *et al.*, 2003; Miwatashi *et al.*, 2005).

1.2.2.5.2. c-Jun NH_2-terminal kinase (JNK)

The JNK family of MAPKs includes three gene products, termed JNK1–3, which were initially characterised by their ability to phosphorylate the NH_2-

terminus of the transcription factor c-Jun (Davis, 2000). Activation of JNKs occurs primarily in response to cytokines and environmental stresses, the latter explaining why they are often called stress-activated protein kinases, or SAPKs. The components of the JNK signalling pathway, including various MAP3Ks, MAP2Ks and JNKs, are arranged into multi-protein complexes by the scaffold protein JNK-interacting protein (JIP) (Morrison *et al.*, 2003).

JNK regulates gene transcription predominantly by activating the transcription factor activator protein-1 (AP-1) (Davis, 2000). Studies of JNK function have employed the small molecule JNK inhibitor SP 600125 (Han *et al.*, 2001), which, however, has frequently been reported to produce effects independently of JNK inhibition (Ito *et al.*, 2011; Kim *et al.*, 2010; Martial *et al.*, 2008; Tanemura *et al.*, 2009). Nonetheless, inhibitors of JNK activation represent promising tools in the treatment of degenerative disorders, as dysregulation of JNK signalling has been implicated in accelerated cell death (Cui *et al.*, 2007).

1.2.2.5.3. Extracellular signal-regulated kinase (ERK)

This subgroup of the MAPK family consists of the 44 and 42 kDa proteins ERK1 and ERK2, alternatively known as p44- and p42-MAPK, respectively (Seger *et al.*, 1995). Alternatively spliced variants of both ERK1 and ERK2, namely ERK1b (Yung *et al.*, 2000), ERK1c (Aebersold *et al.*, 2004) and ERK2b (Gonzalez *et al.*, 1992), have also been described. The ERK signalling cascade involves the sequential activation of Raf (MAP3K), MEK (MAP2K) and, finally, ERK (MAPK) (Figure 1.4). Raf is typically activated by the small G-protein Ras and exists in three isoforms, namely A-Raf, B-Raf and C-Raf (or Raf-1), of which the latter two are more important activators of MEK (Shaul *et al.*, 2009; Wellbrock *et al.*, 2004). The MEK isoforms

MEK1 and MEK2 activate ERK1 and ERK2, respectively, by phosphorylation of Tyr-183 and Thr-185 residues (Robbins *et al.*, 1993).

As mentioned earlier, cell-surface receptors represent a means through which external stimuli activate ERK. Of these, two main receptor superfamilies have been particularly well studied with regard to ERK activation: receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs). Much of the current understanding of RTK structure and function stems from study of the epidermal growth factor receptor (EGFR), the first RTK to be discovered (Downward *et al.*, 1984). RTKs, which include receptors for hormones, growth factors and cytokines, are characterised by an intracellular tyrosine kinase domain and autophosphorylation of activated receptor dimers. They activate ERK by triggering a recruitment of protein complexes to the cell membrane, resulting in the switching of membrane-bound Ras from its inactive GDP-bound state to its active, GTP-bound state. In brief, activation of RTKs permits the docking to receptors of adapter proteins possessing Src Homology 2 (SH2) or phospho-tyrosine-binding (PTB) domains. Adaptor proteins also associate with the guanine nucleotide exchange factor son of sevenless (Sos), which is required for the activation of Ras. Finally, as mentioned above, Ras stimulates ERK by interacting with its upstream MAP3K Raf.

Agonist occupancy of GPCRs triggers a series of events resulting in the regulation of the activity of associated effector proteins such as adenylyl cyclase (AC), which itself acts to increase intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP). Such effector systems have been implicated in ERK phosphorylation following the activation of a wide variety of GPCRs. For instance, in human embryonic kidney (HEK) 293 cells

expressing β_2-ARs, the β-AR agonist isoprenaline activated ERK via cAMP-dependent protein kinase (PKA), the small G-protein Rap1 and B-Raf (Schmitt *et al.*, 2000). An additional means by which GPCRs have been described to activate ERK involves transactivation of RTKs, a process in which intermediates of GPCR signalling release soluble ligands that are able to bind to RTKs (Liebmann, 2011). For example, GPCR-coupled stimulation of EGFR occurs via the release of heparin-binding EGF (HB-EGF) (Prenzel *et al.*, 1999). ERK activation subsequently occurs via Ras stimulation, as described above.

The role of β-arrestin proteins in GPCR desensitisation and internalisation are well described (DeWire *et al.*, 2007). β-arrestins are also known to facilitate ERK activation by acting as scaffolds for the various elements of ERK signalling. Furthermore, when acting in this capacity, β-arrestins may prevent the nuclear translocation of ERK, thereby directing ERK to phosphorylate cytosolic targets (Cervantes *et al.*, 2010; Tohgo *et al.*, 2002). Regulation of the subcellular compartmentalisation of activated ERK is an important determinant of the cellular response produced by ERK activation.

When activated, ERK acts by phosphorylating target proteins. The vast range of targets for ERK underscores its ability to influence numerous cellular processes. In the nucleus, for example, ERK has been shown to interact with transcription factors including elk-1 (Babu *et al.*, 2000), c-fos (Murphy *et al.*, 2002) and p53 (Milne *et al.*, 1994). Cytosolic targets of ERK include phosphodiesterase (PDE) isoforms, which are phosphorylated to either reduce (Hoffmann *et al.*, 1999) or increase (Liu *et al.*, 1999) their activities. The phosphorylation of phospholipase enzymes by ERK has also been described (Lin *et al.*, 1993). Further common cytosolic targets include

protein kinases such as p90 ribosomal S6 kinase (RSK), a MAPK-APK capable of activating transcription factors and exerting feedback inhibition on ERK signalling (Frodin *et al.*, 1999). Such feedback regulation may explain the commonly observed transient nature of ERK activation (Santos *et al.*, 2007). Equally, mechanisms of sustained ERK activation have also been proposed and include direct phosphorylation and activation of Raf-1 by ERK (Balan *et al.*, 2006). The temporal characteristics of ERK activation are thought to direct the cellular response evoked by activated ERK. For instance, sustained ERK activation in phaeochromocytoma 12 (PC12) cells is required for their differentiation into the post-mitotic phenotype, whereas transient activation promotes cell proliferation (Marshall, 1995; Yaka *et al.*, 1998) .

The development of inhibitors of ERK activation have not only provided useful research tools but have also given rise to clinical trials of agents with therapeutic potential in the treatment of cancers (Friday *et al.*, 2008). The structurally dissimilar compounds PD98059 and U0126 (Figure 1.5) are the most commonly used MEK inhibitors, producing potent inhibition at low micromolar concentrations, whilst PD184952 (Figure 1.5), also structurally unrelated, inhibits MEK activity at high nanomolar concentrations (Mattingly *et al.*, 2006). They inhibit ERK activation by preventing the activation of MEK, and their specificity stems from their low affinities for the ATP-binding site common to all protein kinases (Alessi *et al.*, 1995; Davies *et al.*, 2000; Favata *et al.*, 1998).

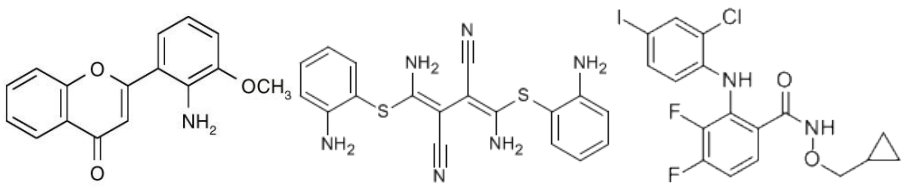

Figure 1.5. *Chemical structures of MEK inhibitors. PD98059 (left), U0126 (centre) and PD184352 are structurally dissimilar, non-competitive MEK inhibitors.*

The high molecular weight (M_W; 93 kDa) splice variant of the protein caldesmon is expressed solely in smooth muscle, whereas the low M_W, 60 kDa variant is ubiquitously expressed (Marston *et al.*, 1991; Sobue *et al.*, 1991). By binding actin, caldesmon exerts an inhibitory influence on actomyosin ATPase activity and thus attenuates contraction (Wang, 2001). Phosphorylation of caldesmon by ERK has been proposed to disinhibit actomyosin ATPase activity, suggesting a role for ERK in smooth muscle contraction (D'Angelo *et al.*, 2002; Gerthoffer *et al.*, 1996; Gorenne *et al.*, 2004). Consistent with this proposal is the finding that ERK contributed to α_2-AR-mediated vasoconstriction of porcine palmar lateral vein via a myosin phosphatase-independent pathway (Roberts, 2004). Furthermore, it has been suggested that ERK causes contraction by directly phosphorylating MLC_{20} at the same site as MLCK (D'Angelo *et al.*, 2002; Roberts, 2004). These actions of ERK, along with other regulatory mechanisms of VSM contraction, are illustrated in Figure 1.2.

1.3. THE ROLE OF β-ADRENOCEPTORS IN THE REGULATION OF VASCULAR TONE

The importance of β-ARs in the regulation of vascular tone has long been appreciated. Studies using subtype-selective compounds and knock-out animals have pointed to the β_2-ARs as the more relevant subtypes involved in this process, though variations in the involvement of each subtype exist between blood vessels (Chruscinski *et al.*, 1999; Osswald *et al.*, 1983; Rohrer *et al.*, 1999). In blood vessels, β-ARs are expressed on endothelial cells and smooth muscle cells (Guimaraes *et al.*, 2001). Activation of vascular β-ARs relaxes smooth muscle cells via diverse mechanisms, thereby causing vasodilatation. The associated reduction in peripheral vascular resistance is particularly relevant during periods of physical activity, allowing increased blood flow to skeletal muscles.

1.3.1. β-adrenoceptors

β-ARs are $G\alpha_s$-coupled cell surface receptors which mediate the effects of the endogenous catecholamines of the sympathetic nervous system, adrenaline and noradrenaline. Ubiquitously expressed in peripheral tissues, they play central roles in the regulation of diverse physiological processes in numerous organ systems, most notably the cardiovascular and respiratory systems. For decades, this receptor group has been the subject of intense study, the fruits of which have led to a more comprehensive understanding of human physiology as well as to the development of effective drug treatments in many disease states (Emilien *et al.*, 1998).

That the biological effects of adrenaline-like amines were mediated by two distinct receptor systems (α- and β-ARs) was a concept familiar to researchers as far back as the 1940s (Ahlquist, 1948). By the 1960s, the existence of multiple β-AR subtypes became evident and the β_1- and β_2-ARs were identified (Lands *et al.*, 1967). Evidence suggesting the existence of an atypical β-AR emerged in the 70s and 80s (Bojanic *et al.*, 1985; Harms *et al.*, 1977), and led to the characterisation of the β_3-AR (Bylund *et al.*, 1994). Recent reports of a putative β_4-AR (Kaumann, 1997) are likely related to multiple conformational states of existing β-ARs as opposed to a novel receptor subtype *per se* (Kaumann *et al.*, 2001). The endogenous catecholamine ligands adrenaline and noradrenaline differ in their relative potencies for the various β-AR subtypes. Specifically, they possess equal potency at the β_1AR, whilst adrenaline is more potent at β_2 and less potent at β_3 (Bylund *et al.*, 1994). Adrenaline has similar potency for α- and β-ARs, whilst noradrenaline is considered α-adrenoceptor-selective. The development of β-AR agonist ligands has been based on the molecular structures of these endogenous catecholamines, and initially led to the synthesis of isoprenaline in the 1940s (Ahlquist, 1948).

Unlike its endogenous counterparts, isoprenaline exhibits marked selectivity for β-ARs over α-ARs due to presence of a relatively large isopropyl moiety at the terminal amine group (Ariens, 1967). Furthermore, isoprenaline possess greater potency at β_1- and β_2-ARs and is equipotent with noradrenaline at β_3 (Bylund *et al.*, 1994). Although isoprenaline was once the drug of choice in the treatment of asthma (Scott *et al.*, 1961), it has since been replaced by more β_2-selective compounds and remains relevant as a tool in the study of β-AR function. Other notable β-AR-altering drugs include the bronchodilators salbutamol and salmeterol, short- and long-acting β_2-AR agonists, respectively, which offer greater

selectivity than isoprenaline for the β_2 subtype, the receptor implicated in asthma and chronic obstructive pulmonary disorder (COPD) (Waldeck, 2002). Myocardial β_1-ARs regulate heart rate and contractility and are targeted by antagonist compounds, often called β-blockers, in cardiovascular disease states including heart failure and hypertension (Joint Formularly Committee, 2011). Finally, the therapeutic potential of β_3-AR agonists as anti-obesity and anti-diabetic drugs was suggested by the expression of this receptor in adipocytes and its role in lipolysis (de Souza *et al.*, 2001).

1.3.1.1. The β_2-adrenoceptor

GPCRs consist of seven transmembrane-spanning α-helical domains with three extracellular and three intracellular loops. The human β_2-AR is a 47 kDa polypeptide composed of 413 amino acid residues (Kobilka *et al.*, 1987; Rybin *et al.*, 2000). Of these, several have been identified as important sites in various aspects of receptor function, including ligand binding, G-protein coupling and receptor desensitisation (Liggett, 2002).

The signal transduction events following ligand binding to GPCRs is well described (Rang *et al.*, 2003). Briefly, ligand binding induces a conformational change in the receptor, allowing it to bind with the GTP-bound α subunit of the G-protein, which also consists of β and γ subunits. This in turn causes GTP to displace GDP from the α subunit. As a result, GTP-bound Gα dissociates from both the receptor and $\beta\gamma$ subunits in order to activate effector enzymes. β-ARs couple to AC via Gα_s which results in the accumulation of cAMP. In turn, cAMP is able to bind to, and therefore activate, a number of downstream proteins which possess cyclic nucleotide binding (CNB) domains. The most notable example is cAMP-dependent

protein kinase (PKA), though cAMP is also able to activate the related cyclic 3',5'-guanosine monophosphate (cGMP)-dependent protein kinase (or protein kinase G, PKG), which shares significant sequence homology with PKA (Jiang *et al.*, 1992). Also of note are Epac1 and Epac2 (exchange protein directly activated by cAMP) which act as guanine nucleotide exchange factors for the small GTPases Rap1 and Rap2, respectively (Bos, 2006).

Even at the time of its discovery, PKA was known to phosphorylate multiple intracellular proteins (Walsh *et al.*, 1968). It is capable of catalysing numerous cellular reactions by phosphorylating targets at serine/threonine residues. Its role in the regulation of ion channels is well described and relevant in various cell types (Ismailov *et al.*, 1995; Levitan, 1994). For example, in VSM cells, β-AR activation has been shown to increase ATP-sensitive K^+ channel (K_{ATP}) activity in a PKA-dependent manner (Shi *et al.*, 2007). PKA is also involved in the desensitisation of the β_2-AR, a means by which responses mediated by the receptor are regulated. The phosphorylation of the receptor at serine/threonine residues within its third intracellular loop and cytoplasmic tail results in the binding of β-arrestin, which results in the uncoupling of the activated receptor from $G\alpha_s$. In addition, β-arrestin may recruit cAMP-specific PDE4, which catalyses the hydrolysis of cAMP. Furthermore, the genomic effects of the cAMP-PKA pathway are triggered by PKA-stimulated phosphorylation of the transcription factor cAMP response element-binding protein (CREB).

1.3.2. The AC-cAMP-PKA pathway in β-AR-mediated vasodilatation

The mechanisms by which the β-AR relaxes VSM cells in various tissues have been under investigation for several years, and the AC-cAMP-PKA pathway has been frequently implicated. It is thought that phosphorylation and inactivation of MLCK by PKA underlies the relaxation induced by β-AR agonists (Payne *et al.*, 1987). PKA, known to target ion channels, may also increase Ca^{2+} efflux by stimulating the Ca^{2+}-ATPase pump and the Na^{+}/Ca^{2+} exchanger, resulting in reduced $[Ca^{2+}]_i$ (Raymond *et al.*, 1996). A further possible target for PKA in β-AR-mediated vasodilatation is the transmembrane protein phospholamban, which modulates the activity of the SERCA pump. By phosphorylating phospholamban, PKA prevents its interaction with the SERCA pump, thereby increasing Ca^{2+} extrusion from the cytosolic compartment to the SR (Colyer, 1998).

1.3.3. The role of the endothelium in β-AR-mediated vasodilatation

That endothelial cells express β-ARs is now widely accepted (Guimaraes *et al.*, 2001). Nonetheless, studies probing their roles in β-AR agonist-induced VSM relaxation have produced contrasting results. For example, depending on the pre-contractile agent used, endothelium denudation either had no effect or enhanced isoprenaline-induced relaxations of canine coronary artery, suggesting endothelial β-ARs may not be involved in the relaxation response (Macdonald *et al.*, 1987). Yet an earlier study of the same vessel demonstrated that the effect of isoprenaline was inhibited by endothelium removal (Rubanyi *et al.*, 1985). Furthermore, β-AR-mediated vasodilatation

of rat aorta was shown by different groups to be completely endothelium-dependent (Gray *et al.*, 1992), completely endothelium-independent (Eckly *et al.*, 1994) or dependent on both endothelial and non-endothelial factors (Ferro *et al.*, 2004). However, in a more recent study of the same vessel, it was proposed, firstly, that the intact endothelium mediated relaxation and may have even inhibited the effect of smooth muscle cAMP, and secondly, that endothelium removal prevented the inhibitory effect, allowing the smooth muscle layer to mediate vasodilatation (Kang *et al.*, 2007).

1.3.3.1. Nitric oxide (NO)

The gaseous free radical nitric oxide (NO), also called endothelium-derived relaxing factor (EDRF), is synthesised in endothelial cells via a reaction requiring L-arginine and catalysed by endothelial NO synthase (eNOS). Central to its paracrine effect on VSM, NO is able to diffuse to adjacent smooth muscle cells where it activates soluble guanylyl cyclase (sGC), which in turn catalyses the conversion of GTP to the second messenger cyclic GMP. Subsequently, PKG is activated by cyclic GMP and regulates a number of proteins via serine/threonine phosphorylation (Figure 1.6). β_2-AR stimulation has been shown to increase eNOS activity in human umbilical vein endothelial cells (HUVEC) in a cAMP-dependent manner (Ferro *et al.*, 1999). In the same study, associated relaxations were characterised as endothelium dependent. Furthermore, other studies have shown that N-nitro-L-arginine methyl ester (L-NAME) and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), inhibitors of eNOS and sGC, respectively, inhibit or abolish the vascular effects of β-AR agonists both *in vitro* and *in vivo* (Akimoto *et al.*, 2002; Ritter *et al.*, 2006).

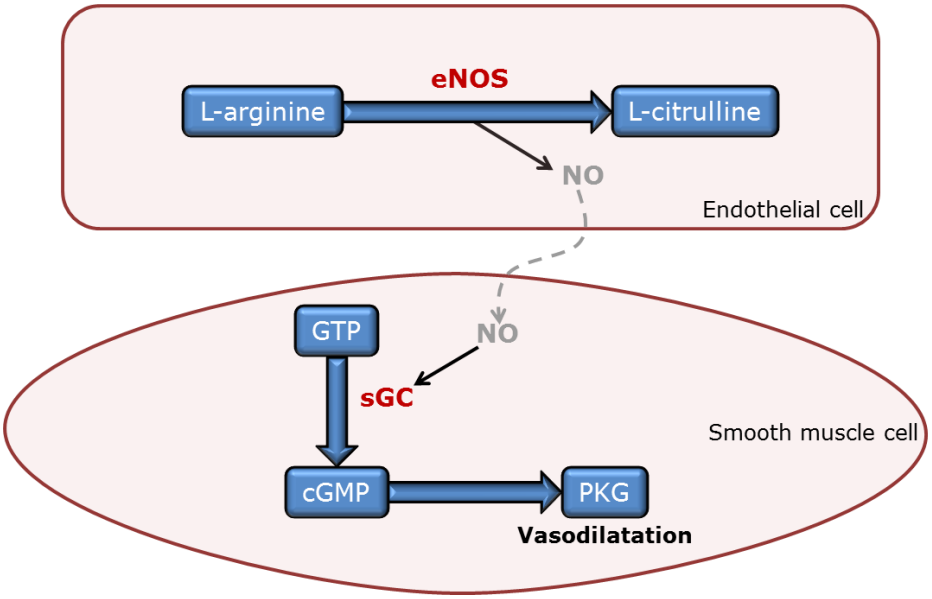

Figure 1.6. *A schematic representation of the biosynthesis and signalling of nitric oxide (NO) in blood vessels. NO is synthesised in the endothelium by a reaction catalysed by endothelial NO synthase (eNOS). NO diffuses to adjacent vascular smooth muscle (VSM) cells where it activates soluble guanylyl cyclase (sGC), which causes the accumulation of cyclic-3',5'-guanosine monophosphate (cGMP). Finally, cGMP activates cyclic-GMP-dependent protein kinase (PKG), which causes relaxation by phosphorylating various effector molecules (Bryan et al., 2009).*

1.3.3.2. Arachidonic acid (AA) metabolites

AA metabolites play key roles in the regulation of diverse processes in both physiological and pathological states. Their vasoactive properties are evident from the finding that AA itself, when applied to blood vessels, produces relaxation (Rosolowsky *et al.*, 1993).

1.3.3.2.1. Cyclooxygenase (COX)-derived metabolites of AA

Prostacyclin (PGI_2) has long been recognised as the chief AA metabolite in blood vessels, where it acts to mediate vasodilatation (Dusting *et al.*, 1979). However, there is scant evidence suggesting a link between PGI_2 and β-AR-mediated vasodilatation. In fact, it was shown that whereas α-AR agonists were able to stimulate PGI_2 synthesis in rat aorta, isoprenaline

and salbutamol had no effect (Jeremy *et al.*, 1985). Furthermore, in a later study the COX inhibitor indomethacin did not alter isoprenaline-induced relaxations in the same vessel (Satake *et al.*, 1997). It has been proposed that β-AR-mediated vasodilatation in rat coronary circulation may be regulated by a COX-dependent endothelium-derived contracting factor (EDCF), which regulates the vasodilatation response by opposing the relaxing actions of endothelium-derived hyperpolarising factor (EDHF) and NO (Vazquez-Perez *et al.*, 2001). However, studies have failed to consistently demonstrate an effect of indomethacin on β-AR-agonist-induced relaxations, suggesting COX metabolites may not be important regulators of this process in many vascular beds.

1.3.3.2.2. Cytochrome P450-derived metabolites of AA

The products of AA metabolism by cytochrome P450 system are epoxyeicosatrienoic acids (EETs), monohydroxyeicosatetraenoic acids (HETEs) and dihydroxyeicosatrienoic acids (DHETs). Of these, EETs are most relevant in blood vessels and have been shown to mediate vasodilatation in a paracrine manner by activating large-conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels on adjacent VSM cells (Huang *et al.*, 2005). The importance of the vasodilator and anti-inflammatory properties of EETs in blood vessels is highlighted by accumulating evidence that their dysregulation is linked with an elevated risk of cardiovascular disease (Zordoky *et al.*, 2010). The cytochrome P450 inhibitor metyrapone (Hildebrandt, 1972), has been used to probe the role of cytochrome P450 metabolites in blood vessels. Isoprenaline-induced relaxation of rat aorta has been shown to be sensitive to metyrapone, suggesting a role for the cytochrome P450 system in β-AR-mediated vasodilatation (Honda *et al.*, 1998; Satake *et al.*, 1997).

1.3.3.3. Endothelium-derived hyperpolarising factor (EDHF)

The term EDHF was coined to describe an endothelium-derived entity, distinct from NO and PGI_2, capable of causing relaxation by hyperpolarising smooth muscle through K^+ channel activation. However, the current consensus is that the opening of EC K_{Ca} channels initiates the spread of hyperpolarisation to the VSM through a number of mechanisms (Busse *et al.*, 2002). Connexin proteins form gap junctions, intercellular channels which allow electrical communication between adjacent cells of the vasculature. Experiments in which electrical communication between ECs and VSM cells was disrupted suggest that gap junctions may be important in EDHF responses (Chaytor *et al.*, 1998). Other possible mediators of the EDHF response include the activation of smooth muscle K_{Ca} by EETs (Quilley *et al.*, 2000) and the activation of VSM inward-rectifier K^+ (K_{IR}) channels and the Na^+/K^+-ATPase pump by K^+ released from ECs (Edwards *et al.*, 1998).

1.3.4. The role of K^+ channels in β-AR-mediated vasodilatation

Although K^+ channels are known to participate in the remodelling of the vasculature, for instance by controlling smooth muscle cell proliferation and migration, greater interest lies in their ability to influence vascular tone and thus regulate blood flow.

A marked K^+ concentration gradient is maintained across the cell by the Na^+/K^+-ATPase pump, which actively extrudes K^+ in exchange for Na^+. By mediating the efflux of K^+ ions along this gradient, K^+ channels hyperpolarise the cell membrane and prevent Ca^{2+} influx via L-type Ca^{2+} channels, causing smooth muscle relaxation. This property of K^+ channels

has led to their targeting in the treatment of angina, for which the selective ATP-sensitive K^+ (K_{ATP}) channel opener nicorandil is indicated (Joint Formularly Committee, 2011). Various K^+ channels have been linked to β-AR-mediated vasodilatation, including K_{ATP} channels, voltage-gated K^+ (K_V) channels and the Ca^{2+}-activated K^+ (K_{Ca}) channels. Furthermore, K^+ channel activation by β-ARs has variously been reported to either require cAMP (Chang, 1997) or to proceed via cAMP-independent pathways (Husken *et al.*, 1997; Randall *et al.*, 1995).

1.3.4.1. K_{ATP} channels

Since their first identification in 1983 (Noma, 1983), K_{ATP} channels, named after their characteristic inhibition by intracellular ATP, have been the subject of considerable interest and study. Although K_{ATP} channels are important regulators of numerous cellular processes, reflecting their ubiquitous expression, they are perhaps best known for their role in insulin secretion in the pancreatic β-cell. The blocking of K_{ATP} channels with sulphonylurea drugs such as glibenclamide is routinely indicated in the treatment of Type II diabetes (Joint Formularly Committee, 2011).

The K_{ATP} channel is composed of eight subunits, four of which form the channel pore and are inwardly rectifying K^+ channel (K_{IR}) subunits. Each K_{IR} subunit associates with a single sulphonylurea receptor (SUR), which are required for channel regulation. Two K_{IR} ($K_{IR}6.1$ and $K_{IR}6.2$) genes and three SUR genes (SUR1, SUR2A and SUR2B) have thus far been identified, giving rise to several receptor-subunit combinations. In the pancreatic β-cell, K_{ATP} channel composition is $K_{IR}6.2$/SUR1 (Sakura *et al.*, 1995), whilst in VSM $K_{IR}6.2$/SUR2B predominates (Isomoto *et al.*, 1996). In addition to their expression on the cell surface, K_{ATP} channels are also located on

mitochondrial (Inoue *et al.*, 1991) and nuclear (Quesada *et al.*, 2002) membranes.

K_{ATP} channels have been shown to influence vascular tone under resting conditions and in response to endogenous and synthetic vasodilators. For instance, application of the K_{ATP} channel blocker glibenclamide to dog and rabbit coronary circulation significantly reduced resting blood flow (Samaha *et al.*, 1992). However, glibenclamide did not cause contraction of porcine isolated coronary artery rings (O'Rourke, 1996), suggesting species differences in the ability of K_{ATP} channels to influence resting tone. Endogenous activators of K_{ATP} channels rely on phosphorylation of channels by protein kinases, such as PKA and PKG, to cause vasodilatation; conversely, endogenous vasoconstrictors, such as ET-1, may depolarise VSM by closing K_{ATP} channels, again via phosphorylation-dependent mechanisms (Brayden, 2002). Synthetic K_{ATP} channel openers cause vasodilatation and are of particular interest in cardiovascular disease, hence the use of nicorandil in the treatment of angina pectoris (Joint Formularly Committee, 2011). Finally, K_{ATP} channels are activated in pathophysiological states such as hypoxia and ischaemia/reperfusion, possibly as a result of altered ATP, ADP or adenosine regulation (Brayden, 2002).

It has been frequently reported in the literature that K_{ATP} channels contribute to β-AR-mediated vasodilatation in a range of vascular beds both *in vivo* and *in vitro* (Chang, 1997; Dumas *et al.*, 1999; Ming *et al.*, 1997; Randall *et al.*, 1995; Sheridan *et al.*, 1997). However, other studies have failed to demonstrate an involvement under physiological conditions (Husken *et al.*, 1997; Satake *et al.*, 1996; White *et al.*, 2001). It is possible that the mechanism of K_{ATP} activation following β-AR stimulation

may vary from vascular bed to vascular bed, as cAMP-dependent (Chang, 1997) and –independent (Randall *et al.*, 1995) routes have both been observed.

1.3.4.2. Voltage-gated K^+ (K_V) channels

K_V channels are members of the voltage-gated superfamily of ion channels, the members of which are characterised by their voltage sensitivity and include channels for Na^+ and Ca^{2+}. Structurally, the K_V channel is composed of four α subunits, all of which contain a voltage sensor and form the central pore (Yellen, 2002). Thus far, as many as 40 K_V channel α subunits have been identified (Gutman *et al.*, 2011). The α subunits associate with four accessory β subunits, which alter channel expression and properties (Pongs *et al.*, 1999).

K_V channels are believed to influence resting vascular tone, as K_V channel blockers have been shown to cause phasic contractions of canine and porcine isolated coronary artery rings (Nakazawa *et al.*, 1988; O'Rourke, 1996). Investigations of K_V activity during β-AR-mediated vasodilatation are uncommon. One study showed that isoprenaline enhanced K_V currents in rabbit portal vein smooth muscle cells via PKA-dependent phosphorylation of the channel (Aiello *et al.*, 1995). Furthermore, in rat aortic rings, isoprenaline was proposed to activate K_V channels via the activation of β_1-ARs and subsequent stimulation of a cAMP-dependent pathway (Satake *et al.*, 1996). Conflicting evidence presented in a later study of the same vessel demonstrated that β_3- but not β_1- or β_2-AR-mediated vasodilatation was inhibited by 4-aminopyridine (4-AP), a K_V channel blocker (Matsushita *et al.*, 2006).

1.3.4.3. Ca^{2+}-activated potassium (K_{Ca}) channels

K_{Ca} channels form a large family of K^+ channels which are activated by elevations in $[Ca^{2+}]_i$. Three subtypes exist, differing in both their voltage sensitivity and single-channel conductance: large-conductance K_{Ca} (BK_{Ca}), intermediate-conductance K_{Ca} (IK_{Ca}) and small-conductance K_{Ca} (SK_{Ca}).

1.3.4.3.1. BK_{Ca} channels

BK_{Ca} channels, also called Maxi-K, have large single-channel conductance (100 pS - 250 pS). Like the other K_{Ca} channels, they display Ca^{2+}-sensitivity but differ in that they are capable of sensing voltage – specifically, their activity increases with depolarisation. Thus, in VSM, where they are densely expressed, BK_{Ca} channels may act to oppose, or modulate, the effects of L-type Ca^{2+} channels (Nelson *et al.*, 1995a).
Structurally, BK_{Ca} channels are composed of four pore-forming α subunits which associate with four modulatory β subunits (Vergara *et al.*, 1998). Investigations of BK_{Ca} function have relied on the scorpion venom-derived toxins iberiotoxin and charybdotoxin; the former produces specific blockade of BK_{Ca} channels, whilst the latter is a mixed BK_{Ca}/IK_{Ca} channel blocker (Doughty *et al.*, 1999; Galvez *et al.*, 1990; Hanner *et al.*, 1998). Furthermore, NS1619, a benzimidazolone compound, selectively opens BK_{Ca} channels and has been routinely used alongside the toxins (Khan *et al.*, 1998).

As described earlier, endothelial stimulation may result in the activation of BK_{Ca} channels via a number of mechanisms. For example, cytochrome P450-derived EETs may hyperpolarise VSM via BK_{Ca} activation (Huang *et al.*, 2005). NO has also been shown to induce relaxations through the

activation of BK_{Ca} channels (Bychkov *et al.*, 1998), and this may occur via PKG-dependent phosphorylation of the channel (Taniguchi *et al.*, 1993). Although BK_{Ca} channels are capable of opposing the effects of raised $[Ca^{2+}]_i$ during depolarisation, some vasoconstrictors are able to prevent this feedback action by regulating BK_{Ca} activity. For instance, the TXA_2 mimetic U46619 and the endogenous peptides ET-1 and angiotensin II have been shown to inhibit BK_{Ca} activity in porcine coronary artery smooth muscle cells (Minami *et al.*, 1995; Scornik *et al.*, 1992).

Collectively, the relevant studies indicate that BK_{Ca} channels are implicated in β-AR-mediated vasodilatation. It was proposed by Scornik *et al.* (1993) that β-AR-mediated stimulation of BK_{Ca} channels in coronary smooth muscle cells may occur through either PKA-dependent and -independent phosphorylation. A later experiment using coronary smooth muscle cells attributed isoprenaline-induced BK_{Ca} channel activation to a cAMP-dependent cross-activation of PKG (White *et al.*, 2000). Furthermore, isoprenaline-induced vasodilatation of intact rat mesenteric artery occurred via a PKA-independent stimulation of BK_{Ca}, and that inhibition of this pathway unmasked a β_1-AR-induced, PKA-dependent relaxation mechanism (White *et al.*, 2001).

1.3.4.3.2. IK_{Ca} and SK_{Ca} channels

First described in 1958 (Gardos, 1958), the IK_{Ca} channel is characterised by "intermediate" single-channel conductance (20 pS – 80 pS), a marked Ca^{2+}-dependence and sensitivity to charybdotoxin (Latorre *et al.*, 1989). In addition, the antifungal clotrimazole and similar compounds, most notably TRAM-34 (Wulff *et al.*, 2000), produce block of this channel and have been employed in studies of its function. With single channel conductance

ranging from 2 pS – 20 pS, the SK_{Ca} channel is also Ca^{2+}-sensitive and is blocked by apamin or UCL 1684 (Dunn, 1999; van der Staay *et al.*, 1999). Both SK_{Ca} and IK_{Ca} channel structures are also based on pore-forming, four α subunit motifs. Ca^{2+} sensitivity is thought to be conferred by binding of the Ca^{2+}-binding protein calmodulin to the carboxy terminus of each α subunit (Fanger *et al.*, 1999; Schumacher *et al.*, 2001).

In blood vessels, IK_{Ca} channels are thought to be predominantly expressed on the endothelium, with VSM expression infrequently reported and related to non-contractile, proliferative phenotypes (Neylon *et al.*, 1999; Tharp *et al.*, 2009). However, IK_{Ca} channels have been detected on contractile smooth muscle cells (McNeish *et al.*, 2006). In any case, the ability of this channel to mediate relaxations following stimulation of the endothelium with various vasodilators is undisputed. Furthermore, it is now generally accepted that K^+ efflux via endothelial IK_{Ca} and SK_{Ca} channels initiates the events associated with the EDHF phenomenon (Busse *et al.*, 2002).

β_3-ARs were shown to be expressed on ECs of human coronary resistance arteries and to mediate relaxations to the β_3-AR agonist BRL 37344 (Dessy *et al.*, 2004). eNOS inhibition reduced the degree of relaxation, leaving a residual NO-insensitive component that was subsequently blocked by the combination of apamin and charybdtoxin, a well-known susceptibility of EDHF-mediated responses (Busse *et al.*, 2002). These findings suggest that the β_3-AR-mediated vasodilatation, in this vessel at least, may involve EDHF. Beyond the findings of this study, the relevance of EDHF to β-AR-mediated vasodilatation is unclear.

1.4. ERK AND β-ADRENOCEPTORS IN DISEASE

According to the WHO, cardiovascular disease was the leading cause of death worldwide in 2008, accounting for an estimated 30% of total deaths, equivalent to 17.3 million people (World Health Organisation, 2011a). This figure is predicted to rise to 23.6 million people by 2030, with the majority of deaths likely to occur in low- and middle-income countries (World Health Organisation, 2011a).

Cardiovascular disease refers to disorders of the heart and vasculature, and includes ischaemic heart disease, congenital heart disease, cerebrovascular disease and hypertension. Risk factors include obesity, diabetes, smoking, excessive alcohol consumption, high-fat diets, sedentary lifestyles, stress and age. In addition, transgenic animal models and human genome-wide association studies have provided insights into the influence of genes on cardiovascular risk (Delles et al., 2008; WTCCC, 2007). The prevention and treatment of cardiovascular disease often involves lifestyle changes. However, drug treatments are integral to lowering the cardiovascular disease burden. Several drug classes collectively acting on a wide range of molecular targets are employed in treatment. Among them are the statins (Section 1.4.3), whose effectiveness, tolerability and apparent versatility have led to their widespread use in both treatment and prophylaxis.

1.4.1. ERK and β-ARs in cardiovascular disease

ERK has emerged as an important regulator of VSM function. Its involvement in the proliferation and migration of VSM cells has long been

appreciated (Graf *et al.*, 1997). More recently, non-genomic functions have been reported, including the mediation of contractile responses (Roberts, 2004). A previous study reported that the angiotensin II receptor antagonist losartan and the Ca^{2+} channel blocker nifedipine produced parallel reductions of aortic ERK activation and blood pressure in a rat model of hypertension (Hamaguchi *et al.*, 1999).

The development of atherosclerosis involves, amongst other things, the recruitment and infiltration of monocytes into the arterial subendothelium (Gu *et al.*, 1998). Known to be involved in this process is the CC chemokine monocyte chemotactic protein-1 (MCP-1), which has also been shown to trigger endothelial cell migration (Weber *et al.*, 1999). More recently, MCP-1 was reported to stimulate matrix metalloproteinase (MMP) release from endothelial cells, also a feature of atherogenesis, via the activation of ERK (Werle *et al.*, 2002). In another study linking ERK to the progression of atherosclerosis, ERK expression and activity were greater in aortae of cholesterol-fed rabbits than in control aortae by factors of 2-3-fold and 3-5-fold, respectively (Hu *et al.*, 2000). This was accompanied by increased migration and proliferation of VSM cells of the atherosclerotic aorta. Increased ERK activation in cardiovascular disease has also been observed in non-vascular tissues. For instance, the increased activation of ERK observed in the myocardia of CHF patients was proposed to contribute to the pathology (Dong *et al.*, 2006). Furthermore, elevated myocardial ERK activation was associated with susceptibility to viral myocarditis following infection with Group B coxsackievirus in mice (Opavsky *et al.*, 2002).

β-AR function is impaired in various cardiovascular diseases. In a canine model of heart failure, for example, abnormalities in myocardial β-ARs

were accompanied by altered β-AR-mediated vasodilatation of coronary artery (Larosa *et al.*, 1996). In a human study, β_2-AR-mediated vasodilatation was shown to be impaired in coronary artery disease and was accompanied by reduced endothelium-dependent relaxations (Barbato *et al.*, 2005). Furthermore, studies using rat models of hypertension have consistently reported deficits of β-AR-mediated vasodilatation; for a review, see Feldman *et al.* (1998). Studies providing evidence of impaired β-AR-mediated vasodilatation in Type II diabetes and obesity are presented in Section 1.4.2.4.

1.4.2. Type II diabetes and obesity

As significant causes of morbidity and mortality, particularly in the developed world, Type II diabetes and obesity are among the leading public health issues of the 21st century. Due to considerable overlap between the clinical features of Type II diabetes and obesity, leading international health agencies have recently moved to group the two conditions under the same umbrella, known as the metabolic syndrome (Alberti *et al.*, 1998; Alberti *et al.*, 2006; Grundy *et al.*, 2006; NCEP, 2001). Although prevention and lifestyle changes are emphasised, pharmacological treatments are key in reducing the burden of metabolic syndrome and intensive research efforts continue to fuel progress in the development of successful drug therapies.

1.4.2.1. Type II diabetes

Formerly known as adult-onset diabetes or non-insulin-dependent diabetes mellitus (NIDDM), Type II diabetes is characterised by insulin resistance, chronic hyperglycaemia and dyslipidaemia. It is estimated that Type II

diabetes accounts for approximately 90–95% of the total diabetes burden. Signs and symptoms of diabetes include polyuria, polydipsia, persistent or recurrent infections, tiredness and lethargy and tingling/pain/numbness in the hands/feet/legs.

In the UK, antidiabetic drugs are indicated in Type II diabetes if non-pharmacological measures, such as increased physical activity and reduced caloric intake, do not provide adequate glycaemic control (Joint Formularly Committee, 2011). Currently used antidiabetics include the sulphonylurea drugs, including glibenclamide and gliclazide, the biguanide metformin and recently approved agents such as the long-acting glucagon-like peptide liraglutide (Joint Formularly Committee, 2011).

In addition to acute complications such as hypoglycaemia and diabetic ketoacidosis, uncontrolled diabetes is associated with a raft of long-term microvascular and macrovascular complications. Microvascular complications include diabetic retinopathy, nephropathy and neuropathy, affecting the eyes, kidneys and peripheral nerves, respectively. Macrovascular complications include stroke, coronary artery disease and peripheral arterial disease. Thus Type II diabetes is a significant risk factor for cardiovascular disease.

1.4.2.2. Obesity

In 2008, it was estimated that over 200 million men and 300 million women were obese (World Health Organisation, 2011c). Obesity is classified as a body mass index (BMI) is of greater than or equal to 30. Approximately 44% of the diabetes burden is attributable to overweight and obesity (World Health Organisation, 2011c).

The associations between obesity and other related disease states, or co-morbidities, have long been appreciated, with Type II diabetes, dyslipidaemia, hypertension and cancer being particularly worthy of note (Basen-Engquist *et al.*, 2011; Travers *et al.*, 2011). As in Type II diabetes, the strategies for managing obesity involve dietary and lifestyle changes. This aims to offset the prevailing trends of increasingly sedentary lifestyles and greater consumption of high calorie foods.

In the UK, pharmacological treatment of obesity is only indicated in clinically obese individuals (i.e., BMI $\geq$ 30) in whom at least 3 months of managed care failed to produce adequate weight reduction (Joint Formularly Committee, 2011). Currently, only orlistat, a lipase inhibitor which reduces the absorption of fat, is indicated in the pharmacological management of obesity in the UK (Joint Formularly Committee, 2011).

1.4.2.3. Animal models of Type II diabetes and obesity

Analogous to the Spontaneously hypertensive rat (SHR) model of hypertension (Okamoto *et al.*, 1964), the Zucker rat and Zucker diabetic fatty (ZDF) rats have long been used to model obesity and diabetes, respectively. The characteristic obese phenotype of the Zucker rat is derived from an autosomal recessive mutation of the leptin receptor (Phillips *et al.*, 1996). As a result, Zucker rats display classic signs and symptoms associated with metabolic syndrome: they are hyperinsulinaemic, hyperlipidaemic, hyperglycaemic and borderline hypertensive (Banday *et al.*, 2004).

The ZDF rat emerged during attempts to inbreed Zucker fatty rats of diabetic lineage (Peterson *et al.*, 1990). ZDF rats are characterised by

altered metabolic characteristics, including hyperglycaemia, obesity, hyperinsulinaemia and hyperlipidaemia, in addition to altered vascular reactivity among other cardiovascular changes (Chirieac *et al.*, 2004; Oltman *et al.*, 2008; Zhou *et al.*, 1999). Other genetic animal models include the Goto-Kakizaki (GK) rat and Akita mouse, both of which are non-obese models of Type II diabetes (Srinivasan *et al.*, 2007).

1.4.2.4. ERK and β-AR-mediated vasodilatation in Type II diabetes and obesity

Whilst several studies have investigated the effects of Type II diabetes and obesity on β-AR-mediated vasodilatation, less is known about the interaction between ERK and these disease states. That ERK activation may be increased in hyperglycaemic states is suggested by the observation that high glucose conditions cause marked increases in ERK activation *in vitro* (Bandyopadhyay *et al.*, 2000; Natarajan *et al.*, 1999). A study using the GK rat model demonstrated that ET-1-induced contractions of mesenteric artery were increased in comparison to Wistar control rats (Matsumoto *et al.*, 2009). The enhancement of the contractile response was accompanied by increased ERK activation and abolished by MEK inhibitors.

It has been suggested that attenuated ERK signalling may also contribute to pathological changes in these disease states. Compensation for chronic myocardial ischaemia in coronary artery disease occurs via the development of coronary collateral vessels, which increase myocardial delivery and confer a survival benefit (Williams *et al.*, 1976). However, collateral vessel development is compromised in diabetes and obesity

(Abaci *et al.*, 1999; Yilmaz *et al.*, 2003). It has been proposed that a contributory factor may be the reduced ability of insulin to phosphorylate ERK and synthesize vascular endothelial growth factor (VEGF) in VSM cells (Doronzo *et al.*, 2004).

In a human study of the effects of Type II diabetes on vascular reactivity in males, dorsal hand vein diameter was measured following the infusion of various vasoactive agents (Harada *et al.*, 1999). Isoprenaline-induced venodilatation was significantly impaired in diabetic patients compared to controls. The finding that responses to noradrenaline and nitroglycerin did not differ between the two groups suggests that the diabetic state altered vascular properties unique to β-AR-mediated vasodilatation. Despite the small sample size (n=8 in each group) and the fact that all subjects were male, the findings were not confounded by differences in age, blood pressure or lipid status.

β-AR-mediated vasodilatation is also impaired in animal models of diabetes and obesity. Firstly, isoprenaline-induced increases in mesenteric blood flow were reduced in Zucker rats compared to control rats (D'Angelo *et al.*, 2006). Second, maximal isoprenaline-induced relaxations of arterioles from the hindlimb vascular bed were significantly reduced in pre-diabetic, short-term diabetic and long-term diabetic ZDF rats as compared to their respective age-matched controls (Lesniewski *et al.*, 2008). Thirdly, in the Wistar Ottawa Karlsburg W (WOKW) rat model of metabolic syndrome and the ZDF rat model of type II diabetes, Grisk *et al.* (2007) demonstrated impaired β-AR-mediated vasodilatation of coronary arteries at 16 months and 3 months of age, respectively.

Similar impairments of other vasodilator-stimulated pathways are observed in these disease states. For instance, ACh-induced, endothelium-dependent relaxations of penile artery and forearm circulation were impaired in Zucker rats and humans, respectively (Gazis *et al.*, 1999; Sanchez *et al.*, 2010). Likewise, reduced K^+ channel function has been reported in blood vessels of human diabetics (Miura *et al.*, 2003) and in mesenteric artery of the ZDF rat (Burnham *et al.*, 2006). Furthermore, dysregulation of endogenous contractile factors in both Zucker rats (Ouchi *et al.*, 1996) and ZDF rats (Lesniewski *et al.*, 2008) has been shown to accompany impaired vasodilatations.

In summary, although it is known that alterations in the expression and activation of ERK occur in cardiovascular disease, the relevance of this to the functional impairments of β-AR-mediated vasodilatation has not been studied.

1.4.3. Statins in cardiovascular disease

Statins are lipid regulators and exert this effect by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis. Thus, statins are employed primarily to reduce low-density lipoprotein (LDL) cholesterol. Moreover, statins have been reported to have additional, pleiotropic effects, independent of their main lipid-lowering activity and likely related to anti-inflammatory actions. As a result statins reduce the risk of cardiovascular disease and are first-line treatments in the primary and secondary prevention of cardiovascular disease and in the treatment of hypercholesterolaemia and hypertriglyceridaemia (Joint Formularly Committee, 2011). Statins have also been proposed as potential therapeutic options in disease states as varied as colorectal cancer (Bardou *et al.*, 2010) and Alzheimer's disease (McGuinness *et al.*, 2010)

In the UK, five statins are licensed for use, namely atorvastatin, fluvastatin, pravastatin sodium, rosuvastatin and simvastatin (Joint Formularly Committee, 2011). Their chemical structures are shown in Figure 1.7. Although statins are generally well tolerated, their use in hepatic impairment is cautioned as they are metabolised by the liver, which, incidentally, is their principal site of action. Furthermore, statins may produce muscle pains and in severe cases rhabdomyolysis, though these side-effects are rare.

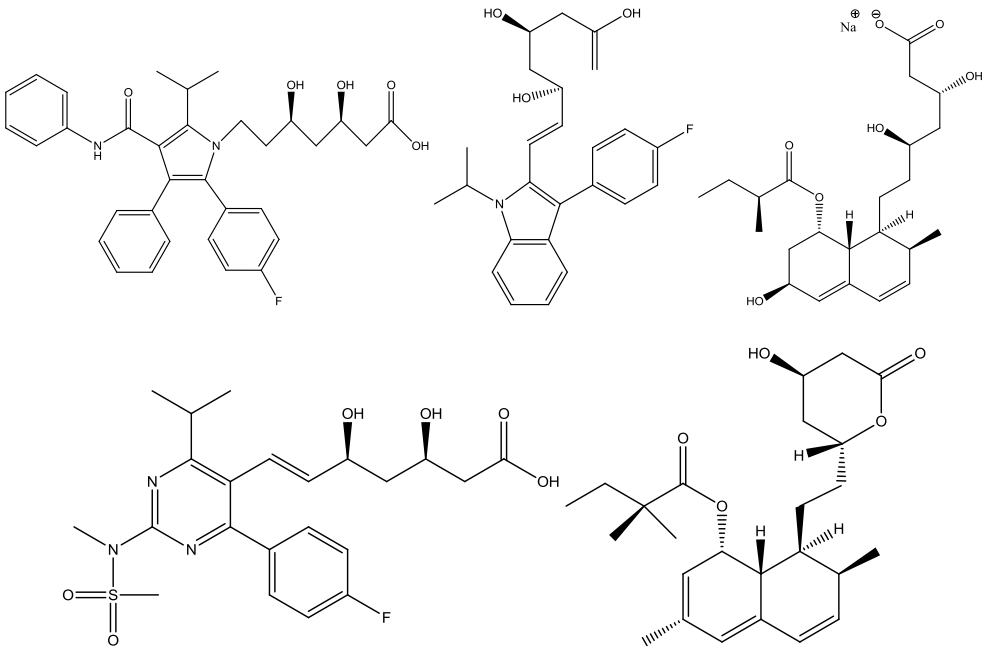

Figure 1.7. *Chemical structures of common statins. Clockwise from top-left: atorvastatin, fluvastatin, pravastatin sodium, simvastatin and rosuvastatin. Structures, adapated from Weitz-Schmidt (2002), were drawn using ChemDraw software (CambridgeSoft, Cambridge, MA).*

1.4.3.1. The mevalonic acid pathway

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, targeted by statins, is involved in the rate-limiting step in cholesterol biosynthesis (Figure 1.8). Firstly, acetyl-CoA is converted to HMG-CoA. HMG CoA reductase, as its name implies, reduces HMG-CoA to mevalonate, which in turn is converted to cholesterol via a number of isoprenoid intermediates.

The isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) play key roles in the activation of Ras and Rho GTPases. Specifically, FPP and GGPP are involved in the farnesylation and geranylgeranylation Ras and Rho, respectively. These post-translational modifications allow the small G-proteins to associate with the plasma membrane, where they are activated. Therefore, inhibition of the upstream reductase disrupts Ras and Rho activation. The consequences of this effect are varied and depend on cell type. As discussed in Section 1.2.2.2, RhoK

is involved in VSM contraction, meaning that inhibition of Rho, through the inhibition of HMG-CoA reductase, may inhibit contractions. Indeed, simvastatin and fluvastatin have been shown to inhibit contractile responses to various agents in a GGPP-dependent manner (Shiga *et al.*, 2005).

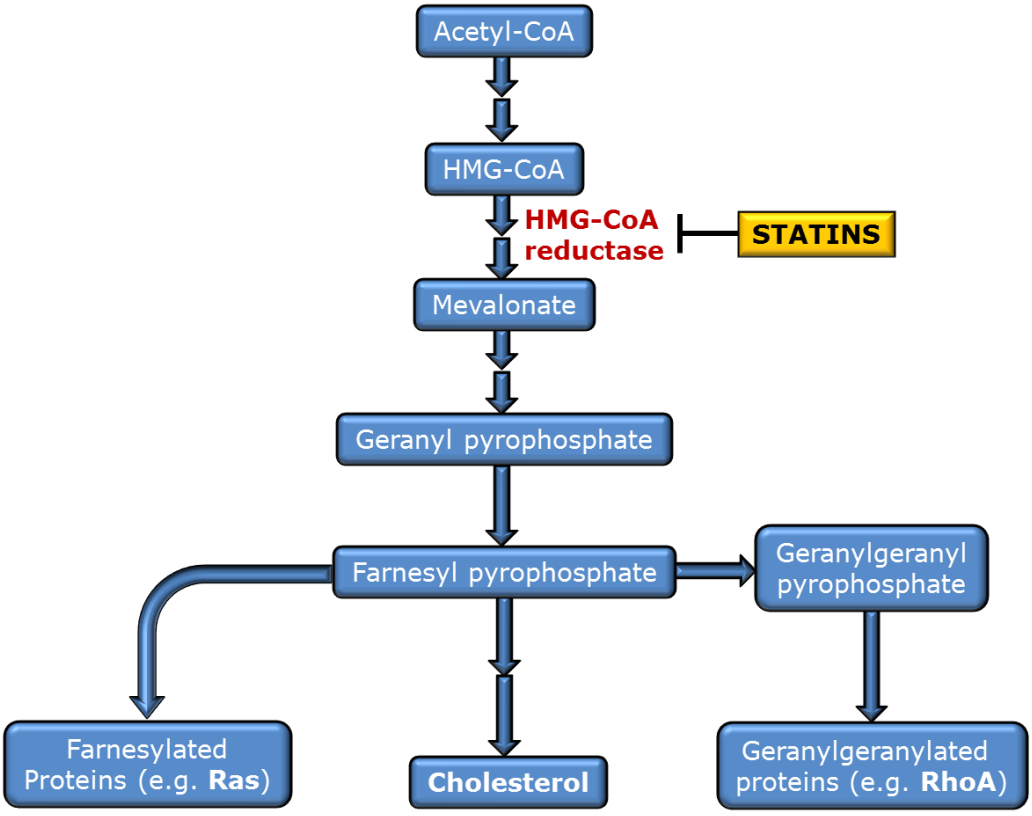

Figure 1.8. *A schematic representation of the mevalonate pathway. Statins inhibit HMG-CoA reductase and therefore prevent the formation of mevalonate.*

1.4.3.2. The effect of statins on ERK activation

As described in Section 1.2.2.5.3, Ras can trigger the ERK signalling cascade by activating Raf. Therefore, inhibition of HMG-CoA reductase by statins may prevent ERK signalling via the inhibition of upstream Ras. In fact, it was shown that simvastatin inhibited ERK actvation in isolated vascular smooth muscle cells (Tristano *et al.*, 2007). The consequences of inhibition of ERK activity would be associated with reduced cell

differentiation, cell growth and vasoconstriction, as ERK is involved in these processes. As vascular remodelling and excessive contractility are generally considered injurious to cardiovascular health, these theorized effects of statins would be welcomed, particularly in cardiovascular disease states characterised by increased ERK activation. Therefore, inhibition of ERK activation using statins represents a promising therapeutic strategy in the treatment of cardiovascular disease.

1.4.3.3. The effects of statins in vitro

Due to the ubiquitous expression of the mevalonate pathway enzymes in eukaryotes, HMG-CoA inhibition by statins has the potential to influence numerous processes in a range of tissues. However, the ability of statins to penetrate the plasma membrane, a property defined by their intrinsic lipophilicity, may represent a barrier to the actions of weakly lipophilic, strongly hydrophobic statins. Variations between the lipophilicities of individual statins are appreciated, with simvastatin and pravastatin, for example, differing in relative lipophilicity by a factor of > 1000:1 (Serajuddin *et al.*, 1991). Furthermore, the ionic charge of a statin is likely to influence its lipophilicity, this being relevant because various statins can be acquired in salt form (typically Na^+). This property has been used to explain discrepancies in the effects of statins in a number of tissues. For instance, simvastatin and lovastatin, also liphophilic, were shown to alter rat basilar arterial tone, whereas pravastatin was without effect (Bergdahl *et al.*, 2003). However, the roles of specific membrane transporter molecules should not be ignored, as they represent a means by which weakly lipophilic statins may enter the cell. This is proposed to underlie the ability of such statins to inhibit cholesterol synthesis in hepatocytes (Yamazaki *et al.*, 1993). On the other hand, unopposed access to virtually

any cell, a freedom shared by lipophilic statins, would inevitably be related to a greater chance of undesirable effects.

A further issue relating to the effect of statins is the requirement of HMG-CoA reductase, also called mevalonate dependence. Assessments of the mevalonate dependence of statins are made by supplementing the system under investigation with mevalonate. As statins inhibit HMG-CoA reductase competitively, mevalonate supplementation would be expected to reverse the effects of statins that occur as a result of HMG-CoA reductase inhibition. Conversely, mevalonate supplementation would not be expected to alter any effects of statins that are independent of HMG-CoA reductase inhibition.

1.5. SUMMARY

Although β-AR-mediated vasorelaxation has been extensively studied for several decades, the exact mechanisms involved are not fully understood and may vary from blood vessel to blood vessel. Indeed, the finding that isoprenaline and other β-AR ligands were able to activate ERK (Daaka *et al.*, 1997; Baker *et al*., 2003), suggests ERK may be involved in this process. However, it will be noted that these observations were made in cultured cells and may not be physiologically relevant. Increased ERK activation has been observed in hypertension (Kim *et al.*, 1997) and heart failure (Dong *et al.*, 2006), suggesting that the influence of ERK on β-AR-mediated vasodilatation may be greater in such disease states. Furthermore, the fact that statins have been shown to inhibit ERK activation in vascular smooth muscle cells (Tristano *et al.*, 2007) suggests they may alter any influence of ERK on β-AR-mediated vasodilatation.

The primary aims of this investigation are to determine whether ERK is involved in β-AR-mediated vasodilatation and, if so, to describe its role in this process. The porcine coronary artery (PCA) will be used as the primary preparation in this investigation owing to the favourable price and availability of porcine tissue and the anatomical and physiological similarities between pigs and humans. However, animal models of named disease states will also be employed where possible. Finally, the therapeutic potential of statins within the context of the findings will be explored. The outline of investigation is shown in Figure 1.9.

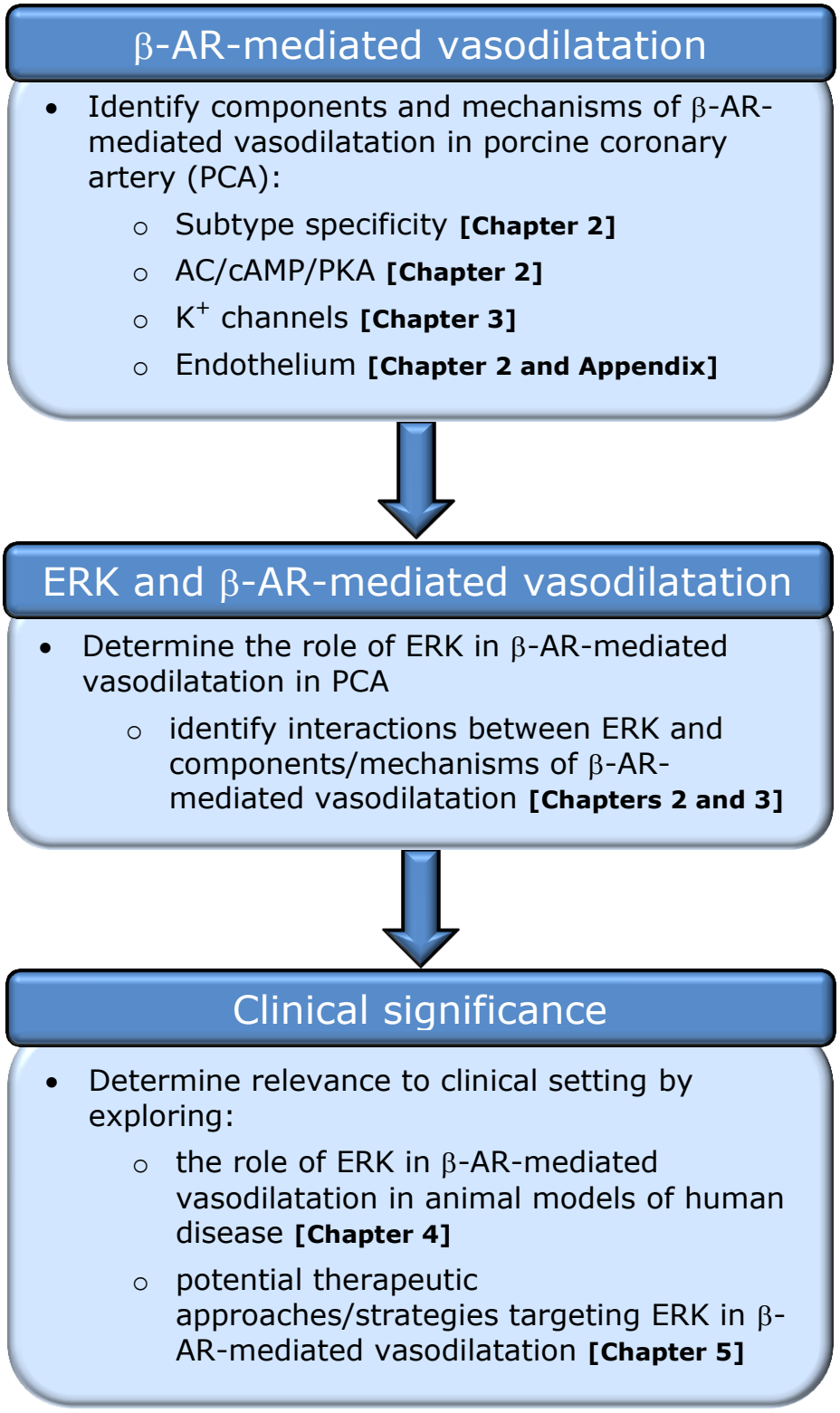

Figure 1.9. *Plan of investigation. The effect of ERK on β-AR-mediated vasodilatation in the primary preparation (PCA) and in animal models of human disease will be investigated.*

CHAPTER 2: THE ROLE OF ERK IN β-ADRENOCEPTOR-MEDIATED VASODILATATION

2.1. INTRODUCTION

β-Adrenoceptors (β-ARs) have been extensively studied owing to their widespread expression in mammalian tissues, and the fruits of this research have led to successful treatments for a number of disease states, including those affecting the cardiovascular system. Although β-AR agonists have not been used in the treatment of cardiovascular disease, their potential use is suggested by their ability to induce vasodilatation when applied to blood vessels. Various mechanisms have been proposed to underlie this effect, including the increased production of the second messenger cAMP (cAMP) (Murray, 1990) and the opening of K^+ channels (White *et al.*, 2001).

β-AR signalling has also been linked to extracellular signal-regulated kinase (ERK) activity. For instance, the β-AR agonist isoprenaline increased ERK activation in Chinese Hamster Ovary (CHO) cells stably expressing human β_2-ARs (Baker *et al.*, 2003). ERK has previously been studied in the context of blood vessels, where its activation has been associated with contractile responses (Roberts, 2001). In this chapter, experiments will aim to reconcile the seemingly divergent findings that β-AR agonists elicit smooth muscle relaxation and also activate ERK, which itself mediates contraction. The interaction between ERK and various components of β-AR signalling will be investigated.

2.2. MATERIALS AND METHODS

2.2.1. Isometric Tension Recordings

2.2.1.1. Tissue preparation

Hearts were obtained from freshly-slaughtered pigs courtesy of a local abattoir. The hearts were transferred from the abattoir at ice-cold temperature in Krebs-Henseleit (KH) solution, which was pre-gassed with O_2-CO_2 (95:5) and contained, in mM, the following: NaCl, 128; KCl, 4.8; $MgSO_4$, 1.1; $NaHCO_3$, 25; KH_2PO_4, 1.2; d-glucose, 12; $CaCl_2$, 1.25. The anterior, descending coronary artery was dissected from each heart, stripped of its adipose and connective tissue and finally stored overnight at 4°C in pre-gassed KH solution containing 2% (w/v) Ficoll.

The following day 2 mm rings segments (1 mm diameter) were dissected from the distal portion of each coronary artery under a light microscope, and were fitted to a Mulvany, four-channel wire myograph which itself was attached to a MacLab data acquisition system (ADInstruments Ltd, Charlsgrove, UK). Each ring segment was bathed in 5 ml KH solution gassed with O_2/CO_2 mixture (95:5) at 37°C. After a 20 minute equilibration period, a resting tension of 2.5 g (determined in pilot studies to yield reproducible contractions to 60 mM KCl) was applied to each ring segment.

2.2.1.2. Experimental procedure

KCl was applied to each well to achieve a final concentration of 60 mM in order to confirm tissue viability and to determine maximal tissue

contractile capacities. The arteries were then thoroughly rinsed with KH solution and allowed to re-equilibrate for 15 to 20 mins. The process was repeated twice.

2.2.1.2.1. The effect of MEK inhibition on β-AR-mediated relaxation

In order to determine the role of ERK in β-AR-mediated vasodilatation, arteries were incubated for 45 mins with KH solution containing the MEK inhibitor PD98059 (10 μM or 50 μM) (Alessi *et al.*, 1995). PD98059, insoluble in water, was reconstituted in DMSO whilst control arteries were incubated for the same length of time in KH solution containing DMSO only (0.26% (v/v) DMSO for 50 μM PD98059 and 0.05% (v/v) DMSO for 10 μM PD98059). At the end of the incubation period, arteries were immediately exposed to the thromboxane mimetic U46619 (10 nM – 20 nM) in order to evoke a contractile tone equivalent to 65–80% of the maximal KCl response and against which subsequent relaxation responses would be standardised. Once a stable tone was achieved, cumulative concentrations of the non-selective β-AR agonist isoprenaline (1 nM – 10 μM) were applied to relax the tissues.

The general experimental protocol described above was repeated for experiments in which arteries were incubated with the structurally dissimilar MEK inhibitors U0126 (Davies *et al.*, 2000) or its inactive congener U0124 (10 μM) (Favata *et al.*, 1998), rather than PD98059.

Finally, to examine the importance of the contraction phase of the experiment on the effect of PD98059, arteries pre-incubated with PD98059 were exposed to endothelin-1 (1 nM – 10 nM), rather than U46619, prior to relaxation with cumulative concentrations of salbutamol.

2.2.1.2.2. The effect of MEK inhibition on β_1- and β_2-AR-mediated relaxation

In experiments investigating the roles of specific β-AR subtypes, β_1- and β_2-AR-selective agonists and antagonists were employed. Following incubation with PD98059 and contraction using U46619, arteries were relaxed with cumulative concentrations of the β_2-AR agonist salbutamol (10 nM – 30 μM) or the β_1-AR agonist xamoterol (1 nM – 30 μM). The MEK inhibitor PD184352 (5 μM) (Allen *et al.*, 2003) was also assessed against relaxations to salbutamol.

Arteries were exposed to the β_1-AR antagonist CGP20712A (Gustafsson *et al.*, 2000) before being pre-contracted and then relaxed with xamoterol. In addition, arteries were incubated with the combination of PD98059 and CGP20712A prior to assessing salbutamol relaxations.

2.2.1.2.3. The effect of MEK inhibition on cAMP-mediated relaxation

The adenylyl cyclase (AC) activator forskolin (1 nM – 3 μM) (de Souza *et al.*, 1983) was used to relax pre-contracted arteries incubated with PD98059 in order to assess the role of the cAMP-PKA signalling pathway. In later experiments, arteries were incubated with the AC inhibitor SQ22536 (100 μM) (Hourani *et al.*, 2001) prior to relaxation with forskolin. Furthermore, separate experiments assessed the combined effect of SQ22536 with 2',5'-dideoxyadenosine (DDA, 50 μM) (Sabouni *et al.*, 1991) and adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMPS, 100 μM) (Hirshman *et al.*, 2005), inhibitors of AC and PKA, respectively.

2.2.1.2.4. The role of the endothelium in β-AR-mediated relaxation

In order to determine the role of endothelium-derived NO, isoprenaline relaxation curves were performed following 45 mins incubation with the NO synthase inhibitor L-NAME (300 μM).

2.2.1.2.5. The effect of MEK inhibition on TP receptor-mediated contraction

The effect of MEK inhibition on TP receptor-mediated contraction was determined by performing cumulative contraction curves to U46619 in the absence and presence of PD98059 (60 mins incubation period).

2.2.2. Measurement of ERK activation

As previously described in our laboratory (Roberts, 2001), the phosphorylation state of ERK at Thr-202 and Tyr-204 was measured using Western immunoblotting.

2.2.2.1. Tissue preparation

Porcine coronary artery ring segments were set up in 5 ml organ baths attached to a computer via a MacLab data acquisition system. The baths contained KH solution and were maintained at 37°C. After three consecutive KCl (60 mM) challenges, arteries received one of the following treatments: U46619 (10 – 20 nM), in order to achieve 60–85% of the maximal KCl contraction; a submaximal concentration of isoprenaline (1 μM); or U46619 until a steady contractile tone was achieved, followed by isoprenaline. Control arteries received no treatment.

Efforts were made to be consistent with the timing of drug addition and the removal of arteries from their baths prior to freezing. Thus, arteries exposed to U46619 alone were removed from their baths at the point at which a steady contractile plateau was reached; control arteries were removed at the same time. Isoprenaline was added to the two remaining arteries once the second artery receiving U46619 reached its plateau. Once the relaxation to isoprenaline had reached its maximum, both arteries were removed from their baths and frozen by rapidly placing the arteries on dry ice immediately. Finally, the arteries were stored overnight at -80°C.

After defrosting, arteries were chopped into smaller pieces and homogenised for approximately 30 seconds (FisherBrand 0.1ml glass-glass homogeniser) in ice-cold buffer containing a mixture of components which collectively acted to (1) release constituent proteins within the tissue sample, allowing them to migrate individually through electrophoresis gels, and (2) reduce the rate of protein degradation by inhibiting protease and phosphatase enzymes. Specifically, the agents used were: 80 mM sodium β-glycerophosphate, 20 mM imidazole [pH 7.6], 1 mM dithiothreitol (DTT), 1 mM sodium fluoride (NaF), 500 μM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 1 μM trans-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64), 10 μg ml^{-1} aprotonin, 1 μM leupeptin and 500 μM EDTA (Roberts, 2001).

2.2.2.2. Bradford Protein Assay

A fraction of each homogenate was used to estimate protein concentration using the Bradford protein assay. Firstly, a standard concentration curve of bovine serum albumin (BSA) was prepared using the following concentrations: 2 mg ml^{-1}, 1 mg ml^{-1}, 0.5 mg ml^{-1}, 0.25 mg ml^{-1} and 0.125

mg ml^{-1}. The diluent used contained homogenisation buffer at concentrations listed above. 10 μl of each sample was loaded in pairs onto a 96-well plate, along with each concentration of BSA and a pair of blanks (diluent only). Each well was topped up with 40 μl BioRad protein assay dye reagent concentrate and 150 μl distilled water. Absorbance readings, measured at 595 nm, were generated using a microplate reader and used to estimate the concentration of protein in each sample. These estimates in turn were used to determine the volume of each homogenate sample to be used in the next stage of the experiment.

2.2.2.3. Western immunoblotting

The remainder of the homogenised samples were diluted (1:1) in Laemmli sample buffer (4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) bromophenol blue and 0.125 M Tris HCl at pH 6.8) and heated at 95°C for 5 min. Equal amounts of each sample (5 μg protein) were carefully loaded into wells of pre-cast 10% (w/v) SDS-PAGE gels. Protein loading was not corrected for using a "housekeeping" protein such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Loaded gels were slotted into an electrode tank containing electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS in distilled H_2O at pH 8.3). A potential difference of 150 V was placed across the gel for 65 mins, causing protein separation. Protein was transferred from gel to nitrocellulose membrane (GE Healthcare Life Sciences, Amersham, UK) using a mini Transblot (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) immersed in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol in distilled H_20 at pH 8.3). A voltage of 100 V was applied for 60 mins.

To prevent non-specific attachment of antibodies to the nitrocellulose membrane, the membrane was 'blocked' by incubating for 60 min in 5% (w/v) milk solution (Marvel skimmed milk powder dissolved in Tris-buffered saline [25 mM Tris, 125 mM NaCl to pH 7.6 in distilled H_20] containing 0.1% Tween-20). Membranes were incubated overnight at 4°C in 5% (w/v) milk solution containing a 1 in 1000 dilution of "primary" antibodies specific to (i) the dual-phosphorylated forms of ERK1/2 and (ii) total ERK1/2 (New England Biolabs, Hitchin, UK).

The following morning, membrane blots were incubated at room temperature for 60 mins in 5% (w/v) milk solution containing fluorescently-tagged "secondary" antibodies (1 in 10,000 dilution) raised in goat against mouse (LI-COR IRDye 680 nm [phosphorylated ERK]) or rabbit (LI-COR IRDye 800 nm [total ERK]) proteins (LI-COR Biotechnology Ltd., Cambridge, UK). The blots were then scanned at 700 nm and 800 nm wavelengths using an Odyssey Infrared Imaging System (LI-COR Biotechnology Ltd., Cambridge, UK). Odyssey software (LI-COR Biotechnology Ltd., Cambridge, UK) was used to analyse the optical densities and molecular weights of the visualised bands.

2.2.3. Statistical and data analyses

Data were analysed using GraphPad Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA). Relaxation response measurements are expressed as means ± SEM. Statistical comparisons between groups were made using the two-tailed, unpaired Student's t-test except where stated. As there were insufficient repeat experiments to determine distribution, the data were assumed to be normally distributed and parametric tests were employed. A P value < 0.05 was considered statistically significant. In all

experiments, "n" numbers represent the number of animals from which tissues were obtained.

The following equation, based on Gaddum analysis (Gaddum, 1957; Lazareno *et al.*, 1993), was used to estimate the binding affinity of competitive antagonists, K_D:

$$K_D = \frac{[\text{antagonist}]}{(\text{dose ratio} - 1)}$$

[Antagonist] represents a fixed concentration of antagonist and the dose ratio is the ratio of EC_{50} values of functional responses measured in the presence and absence of the antagonist.

2.2.4. Materials

2-Amino-3-methoxyflavone (PD98059) was obtained from Calbiochem, Beeston, Nottingham. Forskolin, salbutamol (sulphate salt) and (Z)-7-[(1S,3S,4S)-3-[(E,3S)-3-hydroxyoct-1-enyl]-5-oxabicyclo[2.2.1]heptan-2-yl]hept-5-enoic acid (U46619) were obtained from Axxora (Bingham, Nottinghamshire, UK).

Xamoterol, salbutamol (neutral and hemisulphate salt), (2Z,3Z)-2,3-bis[amino-(2-aminophenyl)sulfanylmethylidene]butanedinitrile (U0126), (Z,2Z)-4-amino-2-[amino(methylsulfanyl)methylidene]-3-isocyano-4-methylsulfanylbut-3-enenitrile (U0124), 2-(2-chloro-4-iodoanilino)-N-(cyclopropylmethoxy)-3,4-difluorobenzamide (PD184352), 2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)imidazol-2-yl]phenoxy]propyl]amino]ethoxy]benzamide (CGP20712A), 9-(oxolan-2-yl)purin-6-amine (SQ22536), (*R*)-adenosine cyclic-3,5-hydrogenphosphorothioate triethylammonium (Rp-cAMPS, 6-(6-aminopurin-9-yl)-2-oxido-2-sulfanylidene-4a,6,7,7a-tetrahydro-4H-

furo[3,2-d][1,3,2]dioxaphosphinin-7-ol) and 4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine (SB 203580) were obtained from Tocris Bioscience (Bristol, UK). N'-Nitro-L-arginine-methyl ester hydrochloride (L-NAME, methyl (2S)-2-amino-5-[[amino(nitramido)methylidene]amino]pentanoate), 2',5'-dideoxyadenosine (DDA, (2R,3S,5R)-5-(6-aminopurin-9-yl)-2-methyloxolan-3-ol)), endothelin 1, isoprenaline and the remaining chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK).

2.3. RESULTS

2.3.1. The effect of MEK inhibition on β-AR-mediated relaxation

Application of β-AR agonists produced a concentration-dependent relaxation of pre-contracted coronary artery segments. A representative trace is shown in Figure 2.1. Relaxations to isoprenaline (1 nM – 3 μM) were enhanced by pre-incubation with the MEK inhibitor PD98059 (10 μM), with pEC_{50} values increasing from 7.9 ± 0.1 in controls (0.05% (v/v) DMSO) to 8.1 ± 0.1 (P<0.05; Figure 2.2).

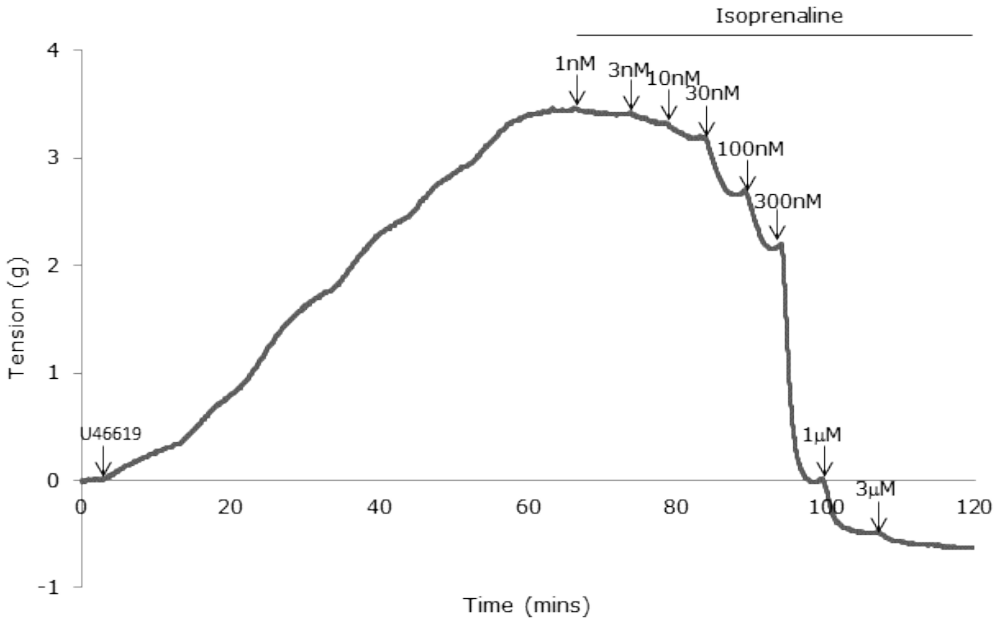

Figure 2.1. *A representative trace of U46619- and isoprenaline-evoked responses in PCA. U46619 (10–20 nM) was used to contract PCA before relaxations were induced using cumulative concentrations of isoprenaline.*

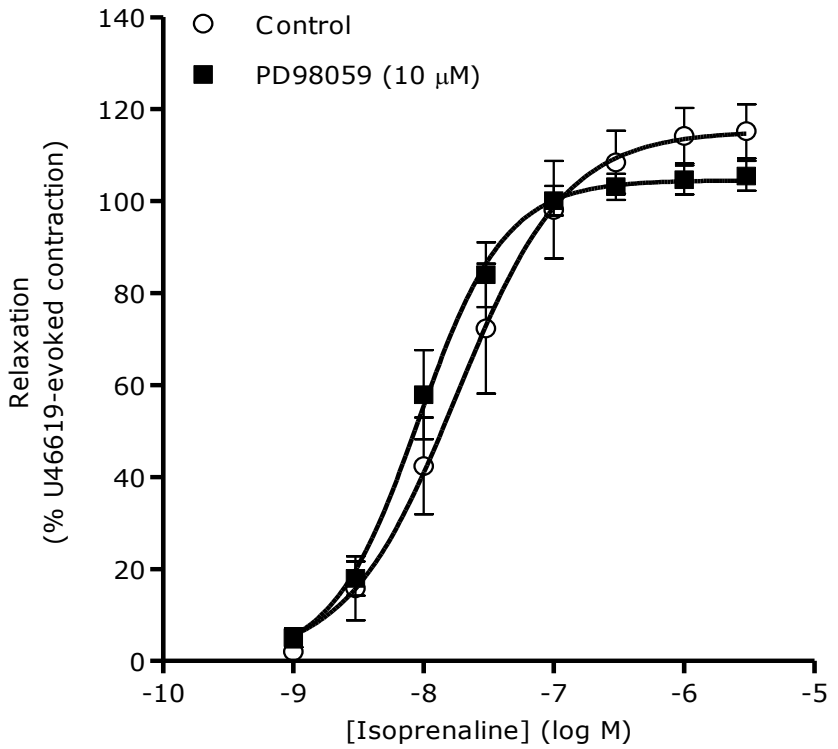

Figure 2.2. *The effect of MEK inhibition on isoprenaline relaxations in PCA (n=7). PD98059 (10 μM) increased isoprenaline pEC_{50} values from 7.9 ± 0.1 in controls (0.05% (v/v) DMSO) to 8.1 ± 0.1 (P<0.05).*

50 μM PD98059 also caused an enhancement of isoprenaline responses, increasing pEC_{50} values from 7.5 ± 0.1 in controls (0.26% (v/v) DMSO) to 8.1 ± 0.1 (P<0.05; Figure 2.3). Neither concentration of PD98059 significantly affected the maximal response to isoprenaline.

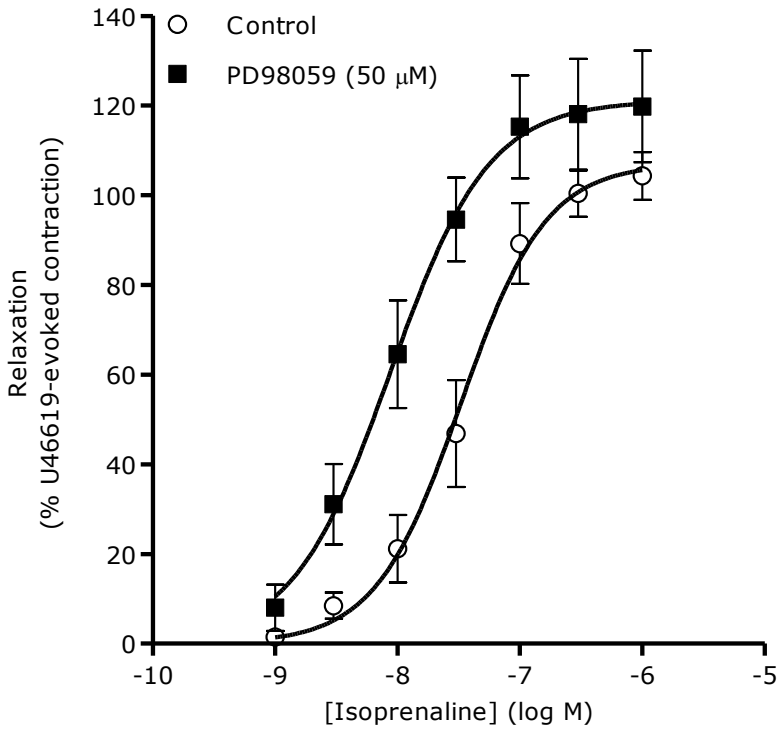

Figure 2.3. *The effect of MEK inhibition on isoprenaline relaxations in PCA (n=6). Incubation with PD98059 (50 μM) resulted in a leftward shift of the isoprenaline response curve, with pEC_{50} values increasing from 7.5 ± 0.1 in controls (0.26% (v/v) DMSO) to 8.1 ± 0.1 (P<0.05).*

The structurally dissimilar MEK inhibitor U0126 (10 μM) produced similar effects: pEC_{50} values were increased from 8.0 ± 0.1 in controls (0.05% (v/v) DMSO) to 8.4 ± 0.2 (P<0.05; Figure 2.4). However, as shown in Figure 2.5, U0124 (10 μM), a pharmacologically inactive variant of U0126, failed to alter isoprenaline relaxations (pEC_{50} value of 7.9 ± 0.1 in both control and U0124-exposed vessels; P>0.05).

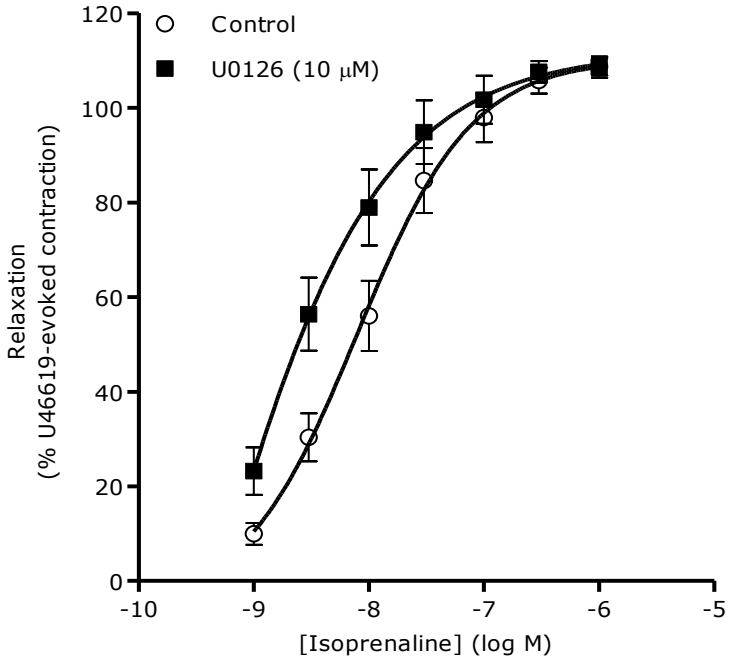

Figure 2.4. *The effect of the MEK inhibitor U0126 on isoprenaline relaxations in PCA (n=15). In comparison to control experiments (0.05% (v/v) DMSO), U0126 (10 μM) enhanced isoprenaline relaxations, increasing pEC_{50} values from 8.0 ± 0.1 in controls (0.05% (v/v) DMSO) to 8.4 ± 0.2 (P<0.05).*

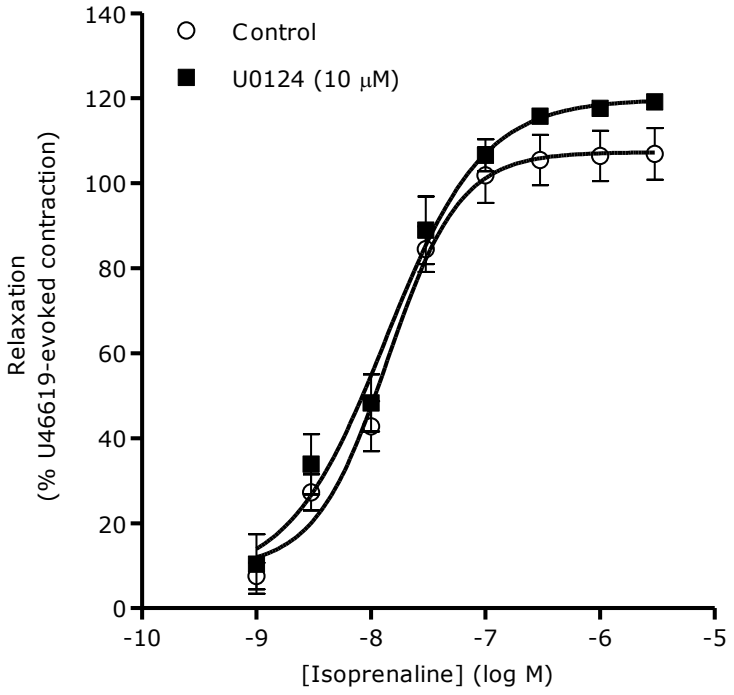

Figure 2.5. *Isoprenaline concentration-response curves in the absence (0.05% (v/v) DMSO) and presence of U0124, the inactive congener of the MEK inhibitor U0126 (n=4 or 5). In contrast to U0126, U0124 did not enhance isoprenaline relaxations (pEC50 value of 7.9 ± 0.1 in both experiments; P>0.05).*

2.3.2. The effect of MEK inhibition on β_1- and β_2-AR-mediated relaxation

As shown in Figure 2.6, relaxations induced by the β_2-AR agonist salbutamol (10 nM – 30 μM) were also enhanced by MEK inhibition. PD98059 (50 μM) significantly increased relaxation responses to salbutamol at 1 μM (12 ± 4% in controls to 35 ± 11%; $P<0.05$), 3 μM (28 ± 7 % to 59 ± 13%; $P<0.05$), 10 μM (44 ± 9% to 83 ± 13%; $P<0.05$) and 30 μM (54 ± 10% to 92 ± 13%; $P<0.05$).

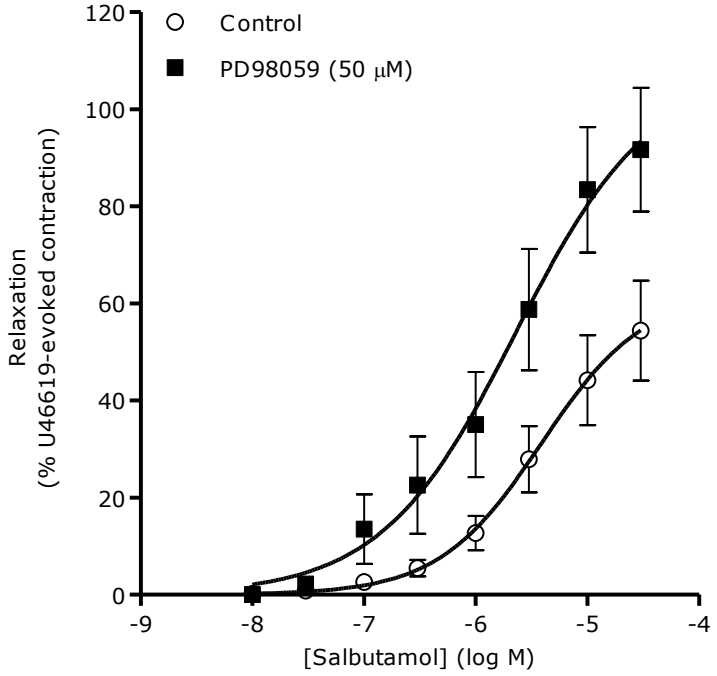

Figure 2.6. *The effect of MEK inhibition on relaxations to salbutamol in PCA rings pre-contracted with U46619 (n=9). Salbutamol produced a concentration-dependent relaxation of PCA rings pre-contracted with U46619. Compared to corresponding relaxations in control experiments (0.26% (v/v) DMSO), relaxations to 1 μM, 3 μM, 10 μM and 30 μM salbutamol in PD98059-exposed ring segments were significantly greater (P<0.05).*

The MEK inhibitor PD184352 (5 μM) also produced an enhancement of salbutamol responses, increasing the log concentration required to produce 50% of the response to 30 μM salbutamol from -7.1 ± 0.2 in controls to -7.7 ± 0.3 ($P<0.05$; Figure 2.7). A similar enhancement was observed when

artery segments pre-exposed to PD98059 were pre-contracted with endothelin-1, rather than U46619, and subsequently relaxed with salbutamol (relaxation response to 30 μM salbutamol 40 ± 7% in controls compared to 66 ± 7% in PD98059-incubated arteries; *P*<0.05; Figure 2.8).

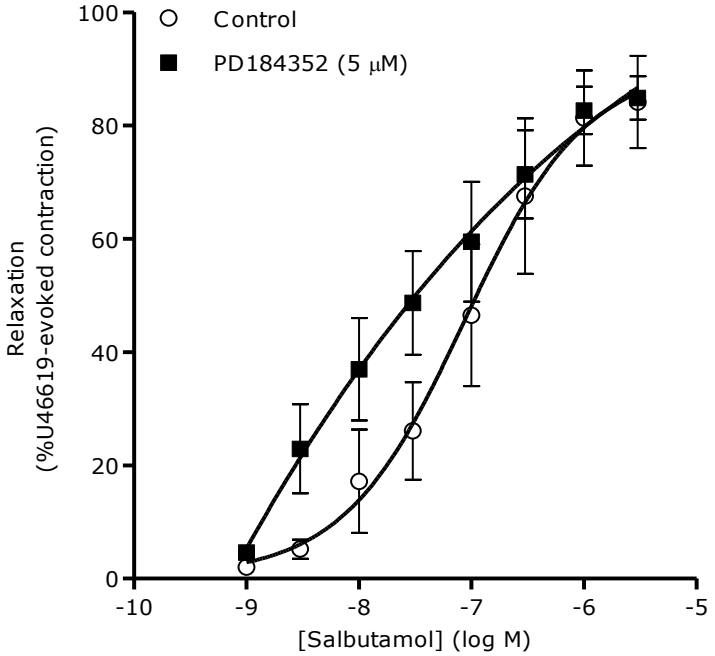

Figure 2.7. *The effect of the MEK inhibitor PD184352 (5 μM) on salbutamol-induced relaxations in PCA (n=4 or 5). PCA rings were pre-contacted using U46619. PD184352 increased the log concentration required to produce 50% of the response to 30 μM salbutamol from -7.1 ± 0.2 in controls (0.1% (v/v) DMSO) to -7.7 ± 0.3 (P<0.05).*

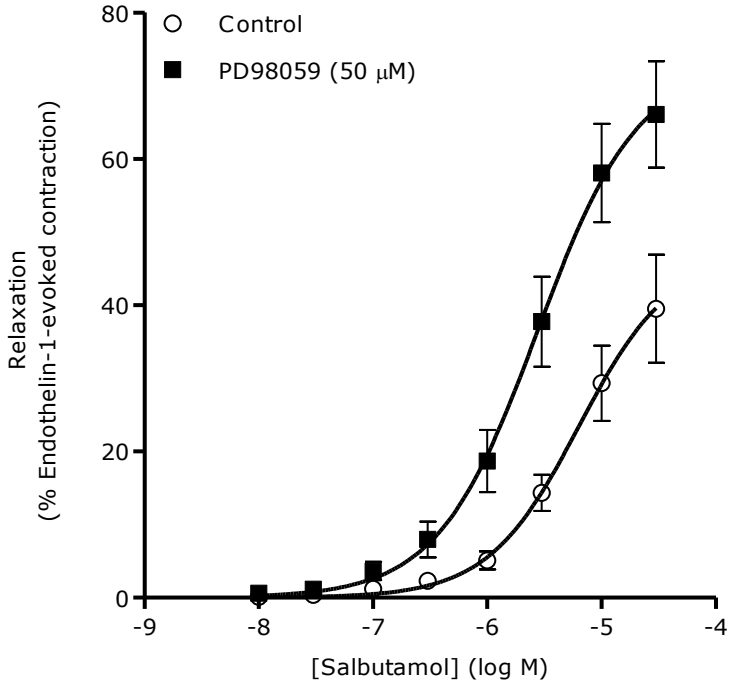

Figure 2.8. *The effect of MEK inhibition on salbutamol relaxations in PCA rings pre-contracted with endothelin-1 (n=16 or 17). The relaxation response to 30 μM salbutamol was greater in PCA rings incubated with 50 μM PD98059 (66 ± 7%) than in controls (0.26% (v/v) DMSO; 40 ± 7% relaxation; P<0.05).*

PD98059 (50 μM) did not change the relaxation characteristics of the β_1-AR agonist xamoterol (1 nM – 30 μM; R_{MAX} in controls = 56 ± 15% relaxation compared to 58 ± 5% in PD98059-incubated vessels, *P*>0.05; pEC_{50} in controls = 6.2 ± 0.2 compared to 6.2 ± 0.1 in PD98059-incubated vessels, *P*>0.05; Figure 2.9).

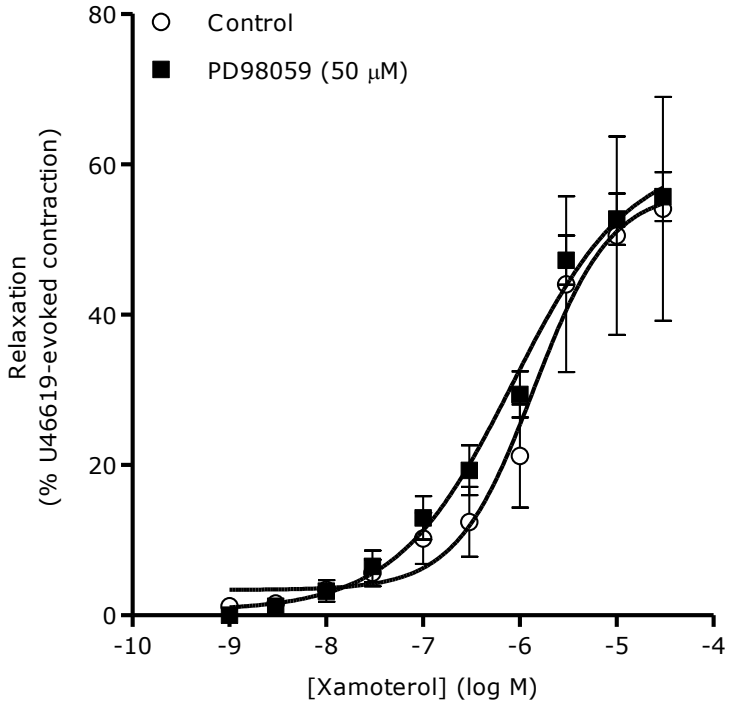

Figure 2.9. *The effect of the MEK inhibitor PD98059 on β_1-AR-mediated vasodilatation induced by xamoterol (n=7). PD98059 (50 μM) did not alter relaxations to the β_1-AR agonist xamoterol (control R_{MAX} = 56 ± 15% relaxation versus 58 ± 5% in PD98059-treated vessels, P>0.05; control pEC_{50} = 6.2 ± 0.2 compared to 6.2 ± 0.1 in PD98059-treated vessels, P>0.05). Control experiments were performed in 0.26% (v/v) DMSO.*

The β_1–AR antagonist CGP20712A (10 nM) significantly inhibited relaxations to xamoterol, reducing the control pEC_{50} value from 7.2 ± 0.3 to 5.6 ± 0.3 (P<0.01; Figure 2.10; K_B = 0.26 nM). CGP20712A did not prevent the enhancement of salbutamol-induced relaxations by PD98059 (response to 10 μM salbutamol of 74 ± 6% in controls, versus 97 ± 4% in the presence of PD98059 and CGP20712A; P<0.01; Figure 2.11).

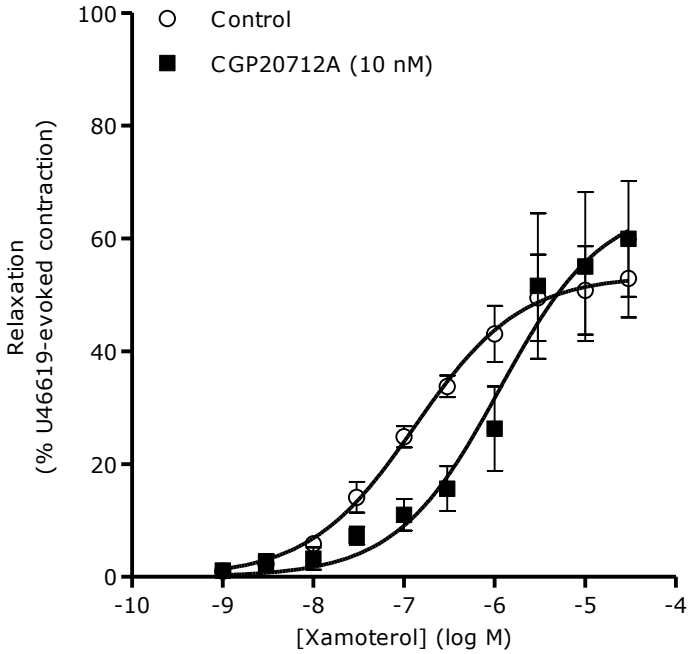

Figure 2.10. *The effect of β_1-AR blockade on relaxations to xamoterol in PCA (n=4 or 5). The β_1-AR antagonist CGP20712A (10 μM) shifted the xamoterol relaxation curve to the right, corresponding to a reduction in pEC_{50} from 7.2 $\pm$ 0.3 in control rings (0.26% (v/v) DMSO) to 5.6 $\pm$ 0.3 in CGP20712A-treated rings (P<0.01; K_B = 0.26 nM).*

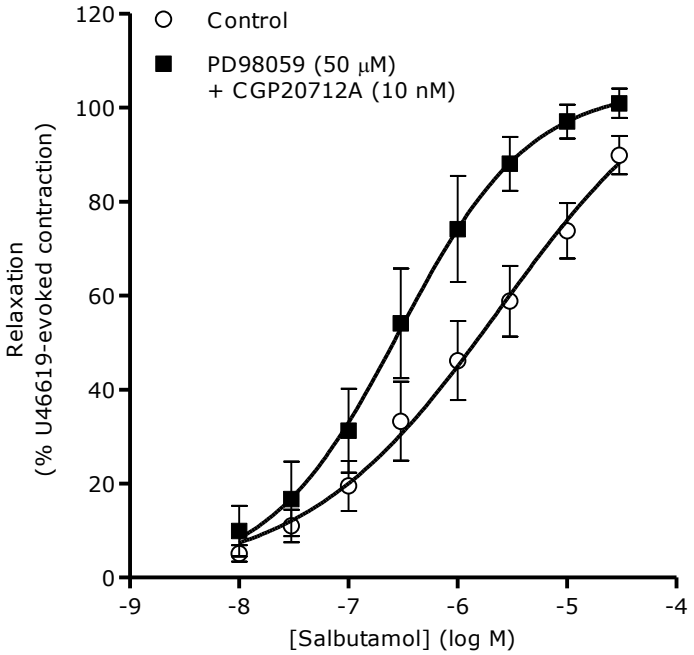

Figure 2.11. *The effect of MEK inhibition on salbutamol-induced relaxations in PCA rings treated with the β_1-AR antagonist CGP20712A (n=8 or 11). Pre-incubation with CGP20712A (10 μM) did not prevent enhancement of salbutamol relaxations by PD98059. Relaxations to 10 μM salbutamol were significantly greater in PD98059+CP20712A-treated rings (pEC_{50} value 7.2 $\pm$ 0.3) than in controls (0.26% (v/v) DMSO; pEC_{50} value 5.6 $\pm$ 0.3; P<0.01).*

2.3.3. The effect of MEK inhibition on cAMP-mediated relaxation

Pre-contracted porcine coronary artery segments were relaxed by the adenylyl cyclase activator forskolin (1 nM – 3 μM) (de Souza *et al.*, 1983) in a concentration-dependent manner. Pre-incubation with PD98059 had no effect on the relaxation characteristics (Figure 2.12).

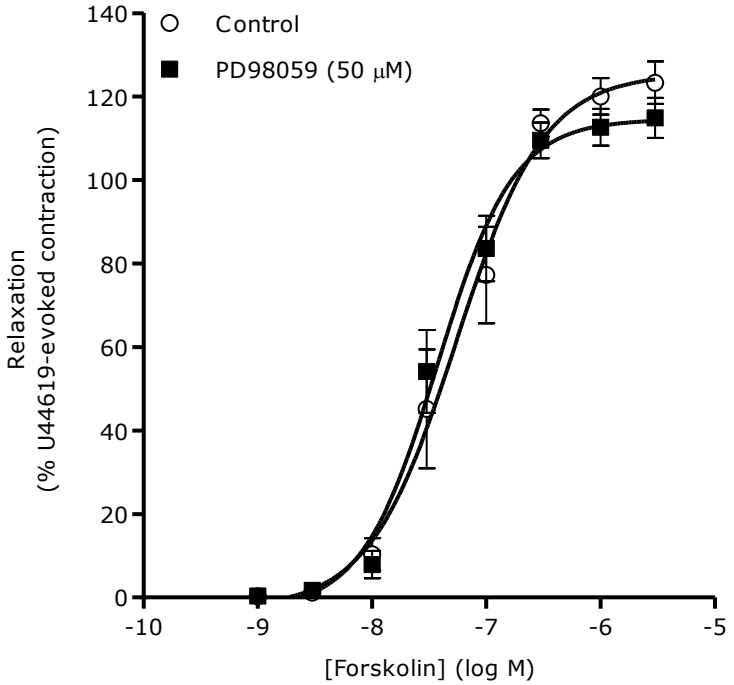

Figure 2.12. *The effect of PD98059 on relaxations to the AC activator forskolin in PCA (n=6). Pre-incubation with PD98059 (50 μM) did not alter relaxations to forskolin in comparison to control experiments, which were performed in 0.26% (v/v) DMSO. Forskolin produced R_{MAX} values of 126 ± 6% and 115 ± 4% in control and PD98059 experiments, respectively (P>0.05); corresponding pEC_{50} values were 7.3 ± 0.1 and 7.4 ± 0.1, respectively (P<0.05).*

SQ 22536, an adenylyl cyclase inhibitor (Hourani *et al.*, 2001), had no effect on forskolin relaxations, either alone (100 μM; Figure 2.13) or in combination with other inhibitors of cAMP signalling, namely the adenylyl cyclase inhibitor DDA (50 μM) and PKA inhibitor Rp-cAMPS (100 μM; Figure 2.14). As these agents were unable to inhibit forskolin relaxations, they were not assessed for effects against β-AR agonist-induced relaxations.

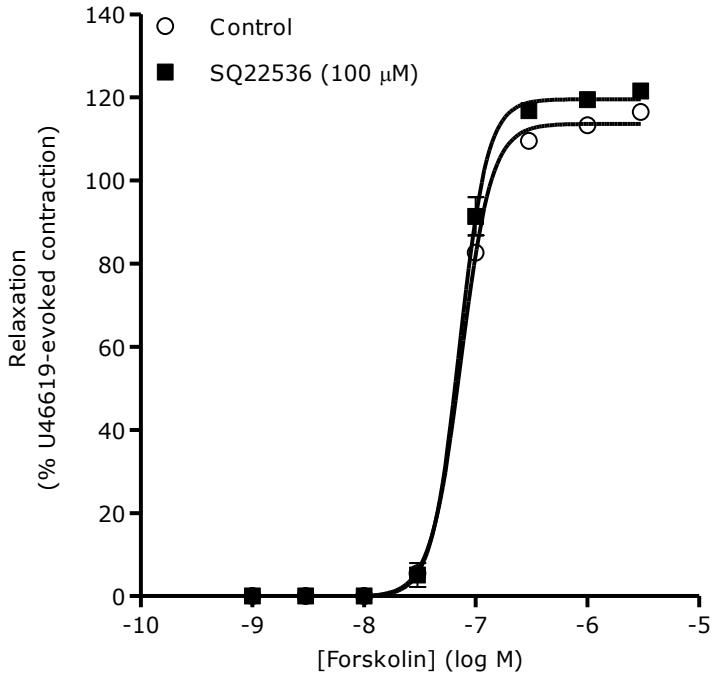

Figure 2.13. *Forskolin-induced relaxations in PCA rings pre-incubated with an inhibitor of adenylyl cyclase (n=2). The relaxation characteristics of forskolin in PCA were very similar in the absence and presence of SQ22536 (100 μM).*

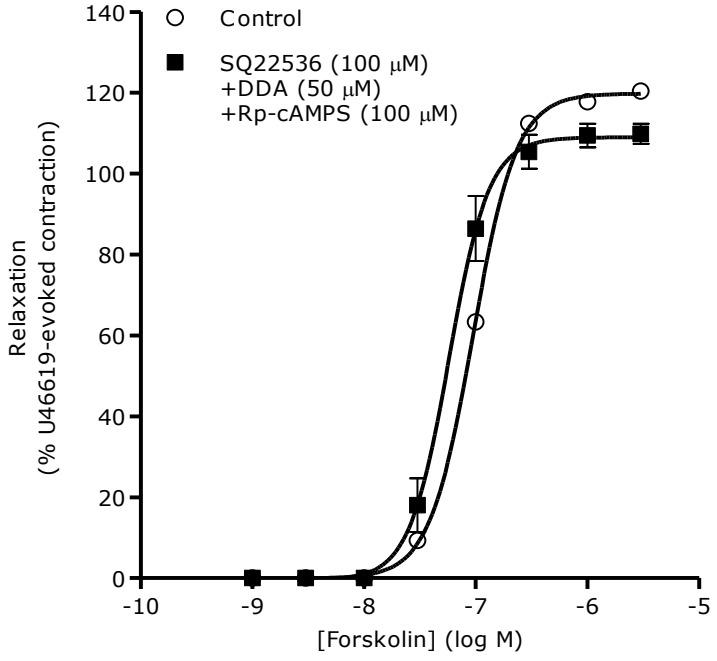

Figure 2.14. *The effect of inhibition of the AC/cAMP/PKA pathway on forskolin-induced relaxations (n=2). The combination of the AC inhibitors SQ22536 (100 μM) and DDA (50 μM) and the PKA inhibitor Rp-cAMPS (100 μM) failed to alter relaxations to forskolin.*

2.3.4. The role of the endothelium in β-AR-mediated relaxation

In experiments assessing the effect of NO synthase inhibition on β-AR-mediated relaxations, the potency of isoprenaline was reduced by L-NAME (300 μM), with pEC_{50} values falling from 7.7 ± 0.1 in controls to 7.5 ± 0.1 in treated artery segments (P<0.05, two-tailed, unpaired Student's t-test; Figure 2.15). In contrast, R_{MAX} values were increased by L-NAME, rising from 112 ± 3% in controls to 122 ± 4% (P<0.05).

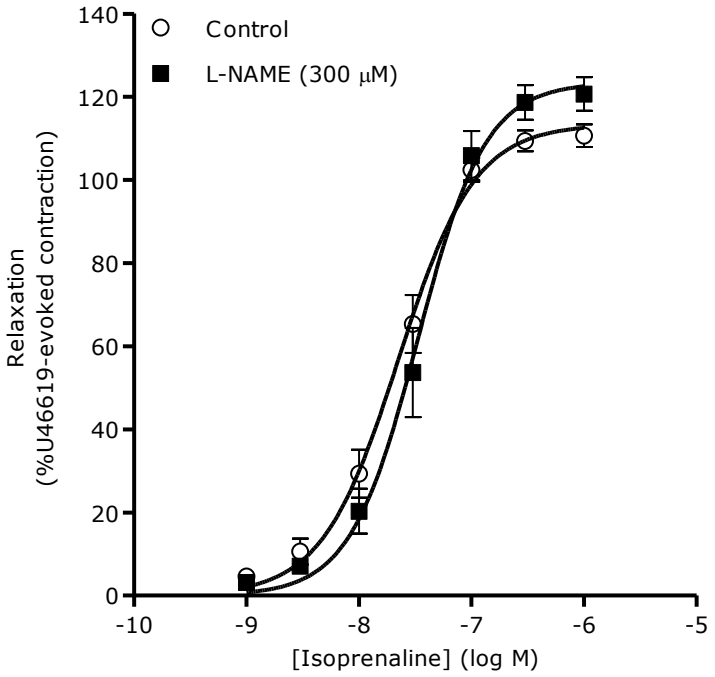

Figure 2.15. *The effect of eNOS inhibition on relaxation curves to isoprenaline in PCA (n=7). L-NAME (300 μM) reduced the potency of isoprenaline relaxations (7.5 ± 0.1 versus 7.7 ± 0.1 in controls; P<0.05) but increased R_{MAX} (122 ± 4% versus 112 ± 3% in controls; P<0.05).*

2.3.5. The effect of MEK inhibition on TP receptor-mediated contraction

U46619 evoked a concentration-dependent contraction of PCA rings (Figure 2.16). Pre-incubation with PD98059 caused a rightward shift of the

concentration response curve, with the mean pEC_{50} value falling from 7.6 ± 0.1 in control arteries to 7.2 ± 0.1 in PD98059-treated arteries (P<0.05).

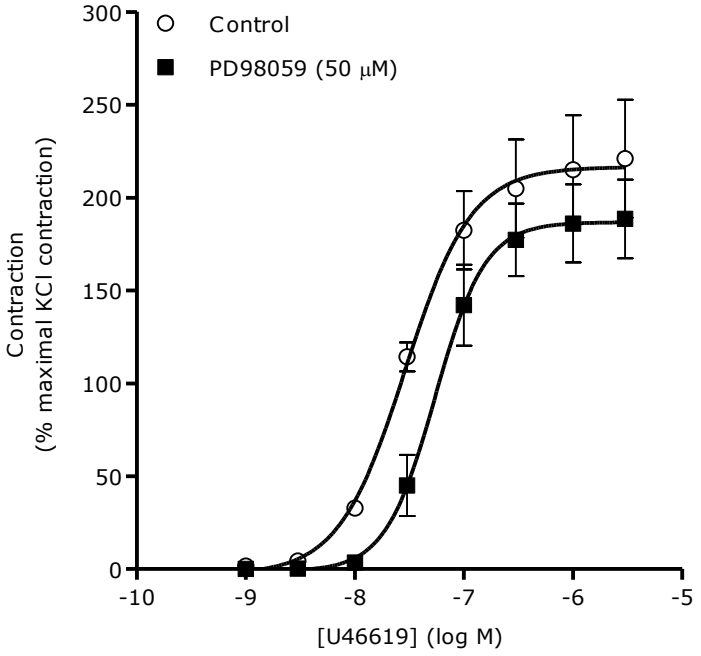

Figure 2.16. *The effect of MEK inhibition on TP receptor-mediated contractions in PCA (n=5). Contractions to U46619 were inhibited by 50 μM PD98059 (mean pEC_{50} value fell from 7.6 ± 0.1 in controls (0.26% (v/v) DMOS) to 7.2 ± 0.1; P<0.05).*

2.3.6. The effect of TP receptor and β-AR activation on ERK activation

The effects of various treatments on levels of phosphorylated ERK (pERK), total ERK (tERK) and the ratio of pERK to tERK in PCA, including a representative blot, are shown in Figure 2.17. Values in Figures 2.17B (ERK2) and 2.17C (ERK1) are normalised to basal levels (control arteries received no treatment).

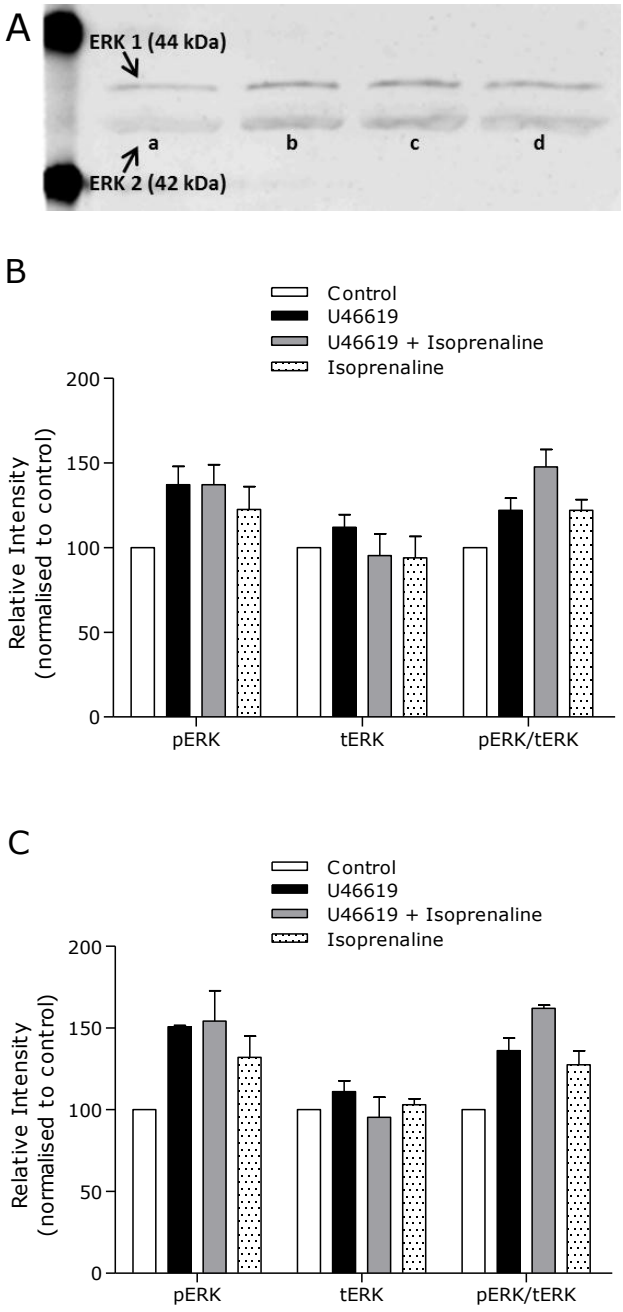

Figure 2.17. *The effect of TP receptor and β-AR activation on ERK activation in PCA.* ***(A)*** *Representative immunoblot of ERK activation. Prior to freezing and homogenisation, PCA segments were pre-treated as follows: (a) no treatment (control), (b) U46619, (c) U46619 + isoprenaline and (d) isoprenaline.* ***(B)*** *and* ***(C)*** *The effect of U46619, isoprenaline and both agents in combination on levels of pERK, tERK and pERK expressed as a fraction of tERK (ERK2 (B) and ERK1 (C)). All data, shown as means ± SEM (n=3), are normalised to the corresponding control value (arteries not exposed to any compound).*

The intensity of pERK2 in arteries treated with U46619, isoprenaline and both agents in combination were raised compared to controls but did not

differ significantly from each other (P>0.05, one-way ANOVA followed by Dunnett's multiple comparisons test). Total ERK2 levels were unchanged in all treatment groups. In arteries treated with the combination of U46619 and isoprenaline, the ratio of pERK2 to tERK2 increased by a factor of 1.48 ± 0.10 (P<0.05, one-way ANOVA followed by Dunnett's multiple comparisons test).

U46619 and the combination of U46619 and isoprenaline increased levels of pERK1 by factors of 1.51 ± 0.09 and 1.54 ± 0.10, respectively (P<0.05, one-way ANOVA followed by Dunnett's multiple comparisons test). Isoprenaline slightly increased pERK1 levels but the change was statistically non-significant. As observed in the tERK2 dataset, none of the treatments altered tERK1 levels. The ratio of pERK1 to tERK1 was increased from control in each treatment group. U46619 alone, U46619 + isoprenaline and isoprenaline alone produced a 1.36 ± 0.08 –fold (P<0.01, one-way ANOVA followed by Dunnett's multiple comparisons test), 1.62 ± 0.02 –fold increase (P<0.005, one-way ANOVA followed by Dunnett's multiple comparisons test) and 1.28 ± 0.08 –fold (P<0.05, one-way ANOVA followed by Dunnett's multiple comparisons test) increase in the ratio (one-way ANOVA followed by Dunnett's multiple comparisons test).

2.4. DISCUSSION

Porcine coronary artery (PCA) rings were pre-contracted with the thromboxane mimetic U46619, and the cumulative application of isoprenaline (1 nM – 3 μM) completely reversed the contractile tone. β-ARs are known to mediate relaxation responses in blood vessels and indeed the vasorelaxant effect of isoprenaline observed in the current investigation has frequently been reported in the literature (Begonha, 1995; Graves *et al.*, 1993; Ushio-Fukai *et al.*, 1993).

Variations in isoprenaline-induced relaxations were observed between sets of experiments. For instance, isoprenaline relaxed control PCA rings with greater potency in the experiment shown in Figure 2.2 (pEC_{50} = 7.9 ± 0.1) than in the experiment shown in Figure 2.3 (pEC_{50} = 7.5 ± 0.1). Similar observations were made with salbutamol-induced relaxations. These variations may be explained by the heterogeneity of the animals from which tissues were obtained (pigs used were of either sex and, unlike the rat models described in Chapter 4 of this report, were reared in largely uncontrolled environments). A possible approach to controlling for inter-animal variability involves performing both "arms" of the experiment on the same tissue segment. Initially, the experiment would be performed in the absence of investigational drug(s), i.e. control conditions. After thorough washout, the procedure would be repeated in the presence of investigational drug(s).

PCA rings that were pre-incubated with the selective MEK inhibitor PD98059 (10 μM) (Alessi *et al.*, 1995) were more responsive to the vasorelaxant effects of isoprenaline, as indicated by a significant leftward shift in the concentration-response curve. Pre-incubation with a higher

concentration of PD98059, namely 50 μM, produced a more noticeable leftward-shift of the concentration-response curve to isoprenaline, suggesting a concentration-dependent effect of the MEK inhibitor.

The MEK inhibitors U0126 (10 μM) (Davies *et al.*, 2000) and PD184352 (5 μM) (Allen *et al.*, 2003) caused a similar enhancement of the β-AR-mediated relaxation responses. Although the three MEK inhibitors produced the same effect in these experiments, their chemical structures are unrelated (Figure 1.5). This lends credence to the proposal that their common effect is related to MEK inhibition, rather than a result of interactions with non-specific targets. In further support of this is the observation that U0124 (10 μM) (Favata *et al.*, 1998), the inactive analogue of U0126, did not alter relaxation responses to isoprenaline. Taken together, these data suggest that ERK acts to limit the degree of vasodilatation elicited by β-AR stimulation and that this inhibitory influence is removed in the presence of MEK inhibitors.

Using radioligand binding studies, Schwartz *et al.* (1983) detected the expression of β_1- and β_2-ARs in PCA and calculated that the receptor subtypes existed in a ratio of approximately 65:35. A similar ratio (70:30) was later described in the proximal region of the same vessel (Brehm *et al.*, 1999). Activation of each of β-AR subtype with selective agonists in bovine coronary artery resulted in artery dilatation, which itself was abolished by corresponding subtype-selective receptor antagonists (Vatner *et al.*, 1986). Xamoterol (Nuttall *et al.*, 1982) and salbutamol (Cullum *et al.*, 1969) have previously been shown to be selective agonists at the β_1- and β_2-AR, respectively.

In the current investigation, activation of β-ARs using these agents caused vasodilatation of pre-contracted PCA, suggesting that both β_1- and β_2-AR may regulate PCA blood flow. The selectivity of xamoterol was assessed using CGP20712A (10 nM), a β_1-AR-selective antagonist (Gustafsson *et al.*, 2000). Relaxations to xamoterol were competitively inhibited by CGP20712A, lending support to earlier characterisations of its status as a β_1-AR agonist. Furthermore, the antagonist affinity of CGP20712A for the β_1AR, estimated to be 0.26 nM, is consistent with figures reported elsewhere in the literature (Baker, 2005) and suggests the effects of xamoterol were mediated via the β_1-AR.

As discussed earlier, MEK inhibition resulted in an enhanced relaxation of PCA treated with isoprenaline. However, since isoprenaline activates β-ARs in a non-selective manner, it is not possible to use this agonist to determine whether the MEK inhibitors mediate their effects via specific receptor subtypes. Relaxations of PCA elicited by salbutamol, but not xamoterol, were enhanced by PD98059, suggesting that the effect of MEK inhibitors on β-AR-mediated relaxations is specific to the β_2-AR subtype. In support of this is the observation that enhancement of salbutamol-induced responses by PD98059 persisted in the presence of the β_1-AR antagonist CGP20712A (10 nM).

The classical signalling pathway associated with β-ARs involves the stimulation of AC, followed by increases in intracellular cAMP levels and the subsequent activation of protein kinase A (PKA). In smooth muscle, PKA is thought to mediate relaxation by phosphorylating, and thus inactivating, myosin light chain kinase (MLCK) and RhoA (Conti *et al.*, 1980; Murthy *et al.*, 2003). In tissues expressing β-ARs, direct activation of the AC/cAMP/PKA axis has been shown to produce effects mimicking receptor

activation. For instance, isoprenaline, forskolin and dibutyryl cAMP concentration-dependently relaxed phenylephrine-pre-contracted rat aortae (El-Hajj *et al.*, 2006).

In the current investigation, application of cumulative concentrations of forskolin to U46619-pre-contracted PCA resulted in a concentration-dependent relaxation response, matching the observations made with β-AR agonists. A combination of AC and PKA inhibitors failed to alter relaxations to forskolin, suggesting that higher concentrations of the inhibitors may have been required to produce an inhibitory effect. Alternatively, the inhibitors may not have been able to enter tissues in order to produce an effect. The AC inhibitor 9-cyclopentyladenine, which has been shown to inhibit cAMP-mediated relaxations in guinea pig taenia coli (King *et al.*, 2008), may have proven a more successful means of disrupting the AC/cAMP/PKA pathway. Likewise, measurements of cAMP accumulation in parallel with functional studies would have given a clearer indication of the activity (or inactivity) of the inhibitors. Unlike relaxations to salbutamol and isoprenaline, forskolin-induced relaxations were unaffected by MEK inhibition. This finding rules out an interaction between ERK and the AC/cAMP/PKA pathway and suggests that ERK may regulate β-AR-mediated relaxations via effects on other signalling systems.

MEK inhibition was associated with an enhancement of β-AR-mediated vasodilatation but also produced effects on the pre-contraction evoked by U46619. Indeed, PD98059 inhibited U46619-induced contractions of PCA. This raises the possibility that relaxations were enhanced simply as a result of an impairment of the preceding contraction phase of the experiment. However, this explanation is unlikely for four reasons.

Firstly, when conducting the experiment, care was taken to pre-contract arteries to the same extent; vessels treated with MEK inhibitors received greater concentrations of U46619 in order to achieve this. Secondly, MEK inhibition failed to alter the relaxation characteristics of forskolin and xamoterol despite inhibiting U46619-induced pre-contractions in the same experiments; therefore, if the enhancement was due to inhibition of the pre-contraction, all subsequent relaxation responses would have been enhanced. Thirdly, in porcine palmar lateral vein, PD98059 enhanced β-AR-mediated relaxations despite having no effect on U46619-evoked contractions (unpublished data). Finally, salbutamol-induced relaxations of PCA following pre-contraction using endothelin-1 (ET-1) were enhanced by MEK inhibition in a manner similar to those observed following pre-contraction using U46619. In comparison to U46619-induced pre-contractions, ET-1 responses appeared more resistant to MEK inhibition. Furthermore, data previously collected in this laboratory in PCA showed that contractile responses evoked by cumulative concentrations of ET-1 were not affected by PD98059 (50 μM).

As discussed earlier, PKA is widely regarded as a key mediator of relaxations induced by β-AR agonists. Evidence for the involvement of K^+ channels also exists in abundance and will be discussed in Chapter 3 along with the relevant data collected in this study. NO is thought to be yet another key regulator of β-AR-mediated vasodilatation. However, studies in this area have often produced conflicting findings (Eckly *et al.*, 1994; Ferro *et al.*, 2004; Gray *et al.*, 1992). In the current experiment, NO signalling was disrupted by inhibition of NOS using L-NAME. Treatment with L-NAME inhibited the potency of isoprenaline-induced relaxations but not the maximal response. An explanation for this is that the β-AR may recruit NO

in the presence of relatively low concentrations of β-AR agonists, whilst relying on other mediators at higher concentrations. However, the magnitude of the inhibition (pEC_{50} value reduced from 7.7 to 7.5) suggests that NO is unlikely to be a major mediator of β-AR-mediated relaxation. The finding that the maximal response to isoprenaline was significantly enhanced by L-NAME treatment is counter-intuitive, but similar findings have previously been presented. Liang *et al.*, (2010) showed that NO inhibited natriuretic peptide-induced relaxations of PCA by desensitising K^+ channels. It is possible that inhibition of NO signalling in PCA augments β-AR-mediated relaxation via enhanced K^+ channel function; however, further experimentation would be required to test this hypothesis. In addition, future experiments would aim to assess the effect of endothelium removal on β-AR-mediated vasodilatation (the results of preliminary experiments are shown in Figures A3 and A4, Appendix).

In conclusion, inhibition of ERK activation is associated with an enhancement of β-AR-mediated vasodilatation of PCA, suggesting ERK may somehow regulate this process. That MEK inhibitors produced some effect is consistent with findings from immunoblotting experiments that suggest ERK may not only be activated under basal conditions but its activation may also be stimulated by TP receptor and β-AR activation. The observed enhancement of relaxations is specific to the β_2-AR subtype and is unlikely to be related to non-specific effects on contractile responses or to desensitisation, which was not detectable in this tissue (Figures A1 and A2, Appendix). Rather, the enhancement may be the result of interactions between ERK and cAMP-independent relaxation mechanisms. Activation of K^+ channels represents one such mechanism and will be the subject of the following chapter.

CHAPTER 3: THE ROLE OF POTASSIUM CHANNELS IN ENHANCED β-ADRENOCEPTOR-MEDIATED VASODILATATION

3.1. INTRODUCTION

K^+ channels are ubiquitously expressed and contribute to the regulation of numerous cellular processes. In vascular smooth muscle, activation of K^+ channels permits the efflux of K^+ ions, resulting in cell hyperpolarisation and relaxation. Thus, by altering blood vessel diameter, K^+ channels are able to regulate blood flow, and agents promoting opening of K^+ channels are used in the prevention and treatment of angina pectoris (Joint Formularly Committee, 2011).

Activation of vascular β-adrenoceptors (β-ARs) results in vasodilatation. Several intracellular factors, including cAMP-dependent protein kinase (PKA) (Murray, 1990) and nitric oxide (NO) (Graves *et al.*, 1993), have been implicated in this process, though their relative roles are disputed. K^+ channels are also thought to play a role β-AR-mediated vasodilatation (White *et al.*, 2001). In this chapter, the contributions of distinct K^+ channel subtypes to β-AR-mediated vasodilatation will be investigated. Furthermore, as data presented in Chapter 2 linked ERK activation to reduced β–AR-mediated vasodilatation, experiments in this chapter will aim to determine whether this association may involve K^+ channels.

3.2. MATERIALS AND METHODS

3.2.1. Isometric Tension Recordings

3.2.1.1. Tissue preparation

Porcine coronary artery (PCA) rings were prepared and set up in a Mulvany wire myograph as described in Section 2.2.1.1.

3.2.1.2. Experimental procedure

PCA rings were challenged with KCl (60 mM) to confirm tissue viability and to determine their maximal contractile capacities. The arteries were then thoroughly rinsed with Krebs-Henseleit (KH) solution and allowed to re-equilibrate for 15 to 20 mins. The process was repeated twice.

3.2.1.2.1. The role of K^+ channels in enhanced β-AR-mediated vasodilatation

PCA rings were incubated for 45 mins in KH solution containing PD98059 (50 μM) before performing the pre-contraction phase of the experiment using KCl (25 mM – 40 mM) rather than U46619. Subsequent relaxation curves were performed using isoprenaline (1 nM – 3 μM). Similar experiments assessed the role of K^+ channels by exposing arteries to a combination of PD98059 and tetraethylammonium (TEA, 10 mM), a non-selective K^+ channel blocker (Iwaki *et al.*, 1988), before pre-contracting and relaxing using U46619 and isoprenaline, respectively.

3.2.1.2.2. The effect of K_{ATP} channel blockade on enhanced β-AR-mediated vasodilatation

PCA ring segments were incubated for 45 mins in Krebs-Henseleit (KH) solution containing glibenclamide (10 μM), a blocker of ATP-sensitive potassium (K_{ATP}) channels (Zhang *et al.*, 1995). U46619 (10 nM – 20 nM) was used to pre-contract the arteries, which were subsequently relaxed by applying cumulative concentrations of isoprenaline. Higher concentrations of U46619 (20 nM – 40 nM) were required to pre-contract glibenclamide-incubated arteries. Control arteries were incubated in 0.1% (v/v) DMSO.

In a related experiment, arteries were incubated for 45 mins in KH solution containing either PD98059 alone (50 μM), PD98059 with glibenclamide (1 μM) or PD98059 with glibenclamide (10 μM). Again, isoprenaline was used to relax the arteries following U46619-evoked contraction. Respective vehicle controls were as follows: 0.36% (v/v) DMSO (control), 0.1% (v/v) DMSO and 0.09% (v/v) DMSO.

The effect of glibenclamide on relaxation responses to the β_2-AR agonist salbutamol was also assessed. In this experiment, arteries were pre-contracted with U46619 after a 45 min exposure to either PD98059 (50 μM), glibenclamide (10 μM) or both agents in combination. The respective vehicle controls were as follows: 0.36% (v/v) DMSO (control), 0.1% (v/v) DMSO and 0.26% (v/v) DMSO.

3.2.1.2.3. The effect of BK_{Ca} channel blockade on enhanced β-AR-mediated vasodilatation

Similar experiments were carried out using the large-conductance, calcium activated K^+ (BK_{Ca}) channel blocker iberiotoxin (Au *et al.*, 2004). Arteries were treated for 45 mins with iberiotoxin (100 nM), PD98059 (50 μM) or a combination of the two agents before they were contracted with U46619 and subsequently relaxed with either isoprenaline or salbutamol. In a related experiment, U46619-pre-contracted arteries were relaxed using 100 nM – 10 μM NS1619, a BK_{Ca} channel activator (Khan *et al.*, 1998), in the absence and presence of PD98059 (50 μM).

3.2.1.2.4. The effect of IK_{Ca} channel blockade on enhanced β-AR-mediated vasodilatation

The effect of TRAM-34, a blocker of intermediate-conductance calcium-activated K^+ (IK_{Ca}) channels (Gluais *et al.*, 2005), on salbutamol-induced relaxations was investigated. Arteries were exposed for 45 mins to a combination of TRAM-34 (10 μM) and PD98059 (50 μM) as well as to each compound alone. Vehicle effects were controlled for as follows: 0.36% (v/v) DMSO (control), 0.1% (v/v) DMSO in PD98059-incubated arteries and 0.26% (v/v) in TRAM-34-incubated arteries.

3.2.2. Electrophysiology experiments

3.2.2.1. Tissue preparation

Porcine coronary artery (PCA) rings were prepared and stored overnight at 4°C in KH solution as described in Section 2.2.1.1.

3.2.2.2. Development of a method of smooth muscle cell isolation from PCA for patch clamp recording

Initial attempts to isolate PCA cells were based on a procedure previously developed in the laboratory to isolate rat superior mesenteric artery (SMA) cells. Briefly, PCA were finely dissected into flat strips of roughly 5 mm^2 and incubated at 37°C in KH solution containing dithiothreitol (DTT; 1mg ml^{-1}) and papain (1.5mg ml $^{-1}$) for 30 mins. The artery segments were then incubated in KH solution containing collagenase type H (1.5 mg ml^{-1}) for 15 mins. Afterwards, the segments were extracted from solution following gentle centrifugation and washed with KH solution and triturated by gently agitating the solution using a shortened plastic pipette tip. Unfortunately, this protocol yielded isolated PCA smooth muscle cells infrequently, and any cells produced appeared damaged when observed under a light microscope.

A number of changes to the procedure were therefore implemented. Firstly, the duration of incubation in collagenase was varied, and it was determined that an incubation period of at least 20 mins was required to produce cells. However, isolated cells still appeared damaged. Suspicions that overnight storage of the arteries might have reduced their viability led

to the third modification of the above procedure. Although functional experiments of PCAs stored overnight had not suggested reduced tissue viability, it was possible that cell-based investigations were more sensitive to such means of storage. Thus PCA rings were prepared and digested on the same day they were transported to the laboratory rather than on the morning after. This meant that the cell isolation procedure was completed within 5 hours of animal sacrifice rather than after roughly 20 hours as was previously the case. However, the modification did not appear to improve cell quality. The decision was finally taken to abandon the isolation procedure, which appeared more suitable for isolating rat SMA cells than PCA cells.

The enzymatic dissociation of PCA smooth muscle has previously been described (Au *et al.*, 2004). Following the disappointing attempts described above to isolate PCA smooth muscle cells, methods used in this earlier study were adapted. Initially, arteries were stored overnight at 4°C in KH solution. A low [Ca^{2+}] solution was prepared in distilled water and contained, in mM, the following: NaCl, 110; KCl, 5; NaH_2PO_4, 0.5; KH_2PO_4, 0.5; $NaHCO_3$, 10; HEPES (H^+), 10; pyruvic acid, 5; glucose, 11; $CaCl_2$, 0.16; $MgCl_2$, 2; EDTA, 0.5; and L-ascorbic acid, 0.3. After gassing with O_2:CO_2 (95:5), pH was corrected to 7.0 using NaOH.

PCAs were dissected into flat, 2 mm^2 strips and incubated at 37°C in the above solution containing DTT (6.0 mg ml^{-1}), papain (2.0 mg ml^{-1}) collagense type II (7.5mg ml^{-1}) and bovine serum albumin (fatty-acid free; 2.0 mg ml^{-1}) for 45 – 60 mins. Subsequent yields were much greater than in earlier attempts and smooth muscle cells, apparently healthier, appeared characteristically long and spindle-shaped. However, the outlines of most cells appeared rough under a light microscope, suggesting a

degree of membrane damage. This was accompanied by an inability of a large proportion of cells to stick to the petri dish upon plating, even when using glass-bottomed petri dishes. Attempts to generate high-resistance seals using glass capillary electrodes (see later) were predominantly unsuccessful.

It was therefore likely that tissues were being overly digested. Several variables were modified in attempts to determine optimal digestion conditions. The concentration of collagenase was reduced to 5.0 mg ml^{-1} and digested arteries were washed three times in enzyme-free low [Ca^{2+}] solution. Also, centrifugation prior to rinsing was omitted as this may have contributed to tissue damage; instead, forceps were used to gently transfer digested PCA segments from enzyme solution to enzyme-free solution. As attempted before, the digestion process was performed on the same day tissues were received in the laboratory. Alas, these changes failed to produce sticky, patchable cells. Despite further adjustments which appeared to yield healthier cells, including reductions in enzyme incubation durations (20 – 30 mins), the fraction of cells amenable to sealing remained low in subsequent yields.

3.2.2.3. Patch clamp recording

Patch micropipettes were made from borosilicate glass capillaries (GC150T-15; Clark Electromedical Instruments, Pangbourne, Reading, UK). Sylgard was carefully cured onto the upper shaft area immediately below the pipette tip in order to reduce electrical noise during recording. The pipettes were briefly fire-polished to produce a tip resistance of 2 – 5 MΩ. High [K^+] solution (see Section 3.2.2.4) was then back-filled into the pipettes using a microsyringe, with care taken to remove bubbles from the tip.

Following cell isolation, PCA smooth muscle cells were plated on glass-bottomed petri dishes and allowed to settle for up to 10 mins. Prior to recording, plated cells were superfused with high [K^+] solution to, first, wash out debris and, second, determine whether cells had stuck to the glass base. Only long, spindle-shaped cells that had stuck were chosen for recordings. Gigaohm seals were achieved by bringing the patch pipette into contact with the cell of interest and then gently applying negative pressure to the pipette using an attached syringe. Recordings were made in this (cell-attached) or inside-out configuration using an Axopatch patch clamp amplifier (Molecular Devices Inc., Sunnyvale, CA, USA). Single-channel currents were filtered at 2 kHz prior to digitisation at 10 kHz using Clampfit 10.3 (Molecular Devices Inc., Sunnyvale, CA, USA).

As discussed earlier, it proved difficult to isolate healthy, compliant smooth muscle cells. Only a few cells stuck to the glass bottom, and of those, fewer still were not dislodged upon contact with the pipette. Furthermore, attempts to generate GΩ seals using suction were frequently unsuccessful, presumably due to a loss of cell membrane integrity during enzymatic digestion. Finally, on occasions when such seals were formed, the tendency was for the seals to prematurely break, particularly during superfusion, resulting in incomplete experiments.

Solutions containing salbutamol (1 μM or 10 μM) or quinine (300 μM) were superfused onto plated cells 1 min prior to data recording. In some experiments, cells were plated in high [K^+] solution containing PD98059 (50 μM) for 25 – 35 min prior to current recordings. Current-voltage relationships were assessed by recording currents for 15 seconds at voltage intervals ranging from either -60 mV to +60 mV or -100 mV to +100 mV. All experiments were performed at 22–23°C.

3.2.2.4. Solutions

In order to clamp membrane potential to 0 mV, all recordings were made in symmetrical, high [K^+] conditions, with both pipette and extracellular solutions containing, in mM, the following: KCl, 145; NaCl, 5; $MgCl_2$, 1.15; $CaCl_2$, 5.5; HEPES (H^+), 10; glucose, 5.5; EGTA, 5; with pH adjusted to 7.2 using KOH (Srivastava *et al.*, 2006). The concentration of free Ca^{2+} in this solution at pH 7.2 was 1 μM.

3.2.3. Statistical analyses

Data recorded in wire myography experiments were analysed using GraphPad Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA). Relaxation response measurements are expressed as means ± SEM. Statistical comparisons between groups were made using the two-tailed, unpaired Student's t-test except where stated. As there were insufficient repeat experiments to determine distribution, the data were assumed to be normally distributed and parametric tests were employed. A *P* value < 0.05 was considered statistically significant. In all experiments, "n" numbers represent the number of animals from which tissues were obtained.

Patch-clamp data were analysed using Clampfit 10.3 (Molecular Devices Inc., Sunnyvale, CA, USA) and GraphPad Prism 5.0. Single channel current amplitudes were calculated by fitting all-points histograms to a Gaussian function. Open probabilities (NP_O) were calculated by expressing the area under the curve for the open components of the histogram as a fraction of the total area under the curve. Conductance values were estimated using the gradients of straight-line components of current-voltage plots fitted with either linear regression or third-order polynomials.

3.2.4. Materials

Tetraethylammonium acetate, (R)-[(2S,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]octan-2-yl]-(6-methoxyquinolin-4-yl)methanol (quinine), 5-chloro-N-[2-[4-(cyclohexylcarbamoylsulfamoyl)phenyl]ethyl]-2-methoxybenzamide (glibenclamide), iberiotoxin, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619), 1-[(2-chlorophenyl)-diphenylmethyl]pyrazole (TRAM-34),

4-aminopyridine, Papain from papaya latex and collagenase (Types II and H) from *Clostridium histolyticum* were acquired from Sigma (Poole, Dorset, UK). The remaining chemicals were obtained as stated in Section 2.2.4.

3.3. RESULTS

3.3.1. The role of K^+ channels in enhanced β-AR-mediated vasodilatation

Figure 3.1 shows that PD98059 does not alter relaxations to isoprenaline in PCA segments pre-contracted with KCl (pEC_{50} and R_{MAX} values: 7.6 ± 0.1 and 115 ± 5%, respectively, in controls versus 7.8 ± 0.1 and 109 ± 4%, respectively, in 50 μM PD98059-treated arteries; *P*>0.05).

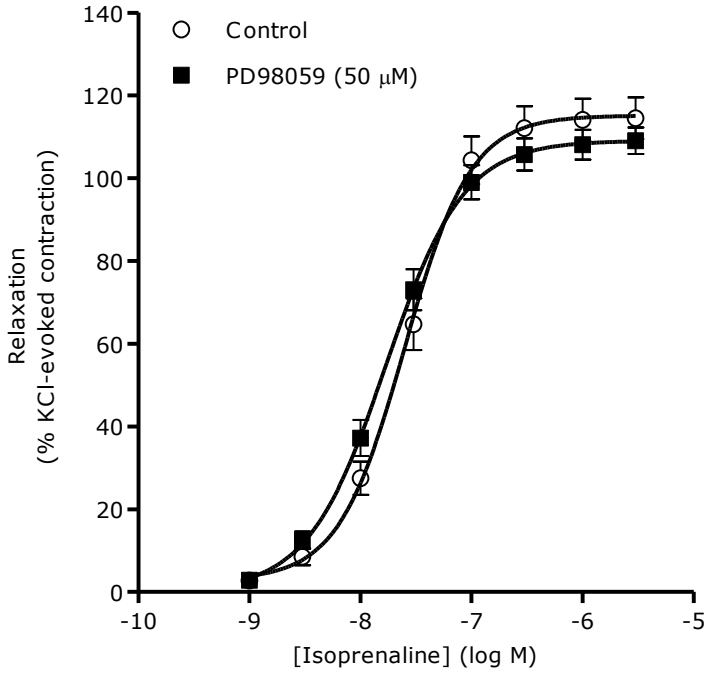

Figure 3.1. *The effect of MEK inhibition on β-AR-mediated vasodilatation in PCA rings pre-contracted with KCl (n=5). PD98059 (50 μM) failed to alter the relaxation characteristics of isoprenaline. There were no differences between rings treated with PD98059 (50 μM) and corresponding controls (0.26% (v/v) DMSO) with respect to pEC_{50} (7.6 ± 0.1 versus 7.8 ± 0.1; P>0.05) or R_{MAX} (115 ± 5% versus 109 ± 4%; P>0.05).*

In arteries pre-contracted with U46619 following pre-incubation with the combination of PD98059 and the K^+ channel blocker TEA (10 mM), isoprenaline-induced relaxations were not significantly different from control arteries (pEC_{50} and R_{MAX} values: 7.1 ± 0.2 and 123 ± 8%,

respectively, in controls versus 7.5 ± 0.2 and 113 ± 4%, respectively, in PD98059-treated arteries; *P*>0.05; Figure 3.2).

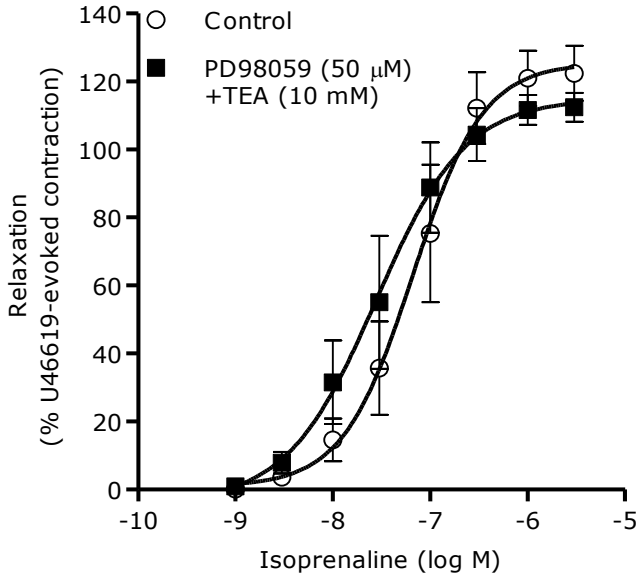

Figure 3.2. *The effect of MEK inhibition on β-AR-mediated relaxations in PCA rings treated with the non-selective K^+ channel blocker TEA. U46619 was used to pre-contract rings prior to inducing relaxations with isoprenaline. The combination of 10 mM TEA and 50 μM PD98059 did not alter relaxations to isoprenaline, with pEC_{50} and R_{MAX} values (7.1 ± 0.2 and 123 ± 8%, respectively) unchanged compared to corresponding controls (7.5 ± 0.2 and 113 ± 4%, respectively; P>0.05 in both cases). Control experiments were performed in KH solution containing 0.26% (v/v) DMSO.*

3.3.2. The role of K_{ATP} channels in enhanced β-AR-mediated vasodilatation

The K_{ATP} channel blocker glibenclamide (10 μM) had no effect on the relaxation characteristics of isoprenaline in porcine coronary artery segments pre-contracted with U46619 (Figure 3.3). Consistent with earlier findings, pre-incubation of PD98059 enhanced isoprenaline-induced relaxations versus control (Figure 3.4). Pre-incubation with the combination of PD98059 and glibenclamide caused a comparable enhancement of the relaxation response (pEC_{50} values: control = 7.6 ± 0.1, PD98059 = 8.0 ± 0.1, PD98059 + 1 μM glibenclamide = 8.1 ± 0.1,

PD98059 + 10 μM glibenclamide = 8.2 ± 0.1; $P<0.05$ for PD98059 (± glibenclamide) experiments versus control experiment, one-way ANOVA followed by Tukey's multiple comparisons test).

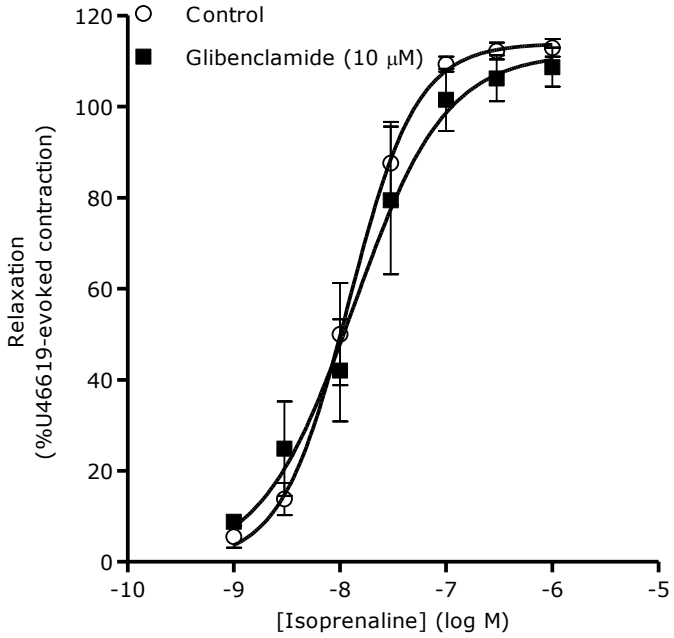

Figure 3.3. *The effect of K_{ATP} channel blockade on β-AR-mediated relaxations in PCA rings (n=3 or 4). Under control conditions (0.1% (v/v) DMSO), isoprenaline produced pEC_{50} and R_{MAX} values (7.9 ± 0.1 and 114 ± 4%, respectively) that did not significantly differ from corresponding values in glibenclamide (10 μM) experiments (7.9 ± 0.1 and 112 ± 8%, respectively; P>0.05 in both cases).*

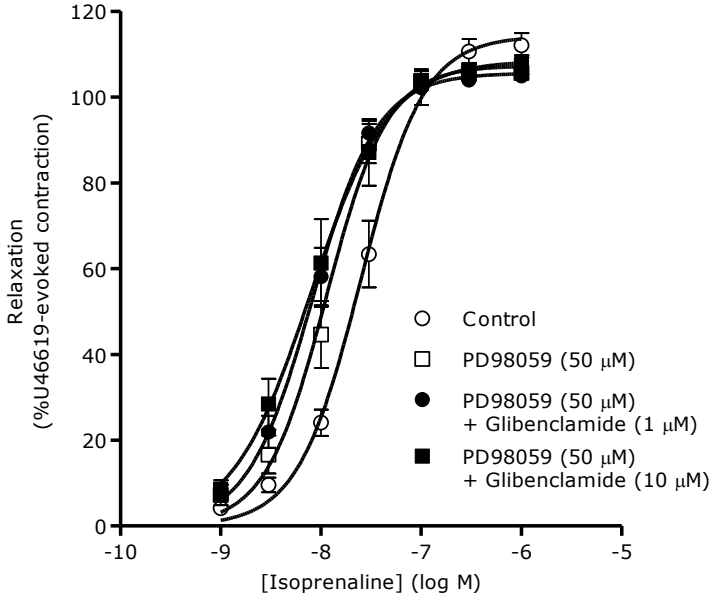

Figure 3.4. *The effect of MEK inhibition on β-AR-mediated relaxations in PCA (n=5–10). Recordings were made in KH solution containing the following: 0.36% (v/v) DMSO (control), PD98059 (50 μM) + 0.1% (v/v) DMSO, PD98059 (50 μM) + glibenclamide (1 μM) + 0.09% (v/v) DMSO, or*

PD98059 (50 μM) + glibenclamide (10 μM). PD98059 enhanced isoprenaline relaxations in each experiment (pEC_{50} values versus control pEC_{50} value of 7.6 ± 0.1: PD98059 = 8.0 ± 0.1, PD98059 + 1 μM glibenclamide = 8.1 ± 0.1, PD98059 + 10 μM glibenclamide = 8.2 ± 0.1; P<0.05 versus control in each experiment).

As shown in Figure 3.5, similar phenomena were observed when relaxations were evoked using salbutamol, with glibenclamide failing to reverse the PD98059-induced enhancement (pEC_{50} values: control = 5.6 ± 0.2, PD98059 = 6.3 ± 0.2, 10 μM glibenclamide = 5.7 ± 0.2, PD98059 + 10 μM glibenclamide = 6.4 ± 0.1; *P*<0.05, one-way ANOVA followed by Tukey's multiple comparisons test).

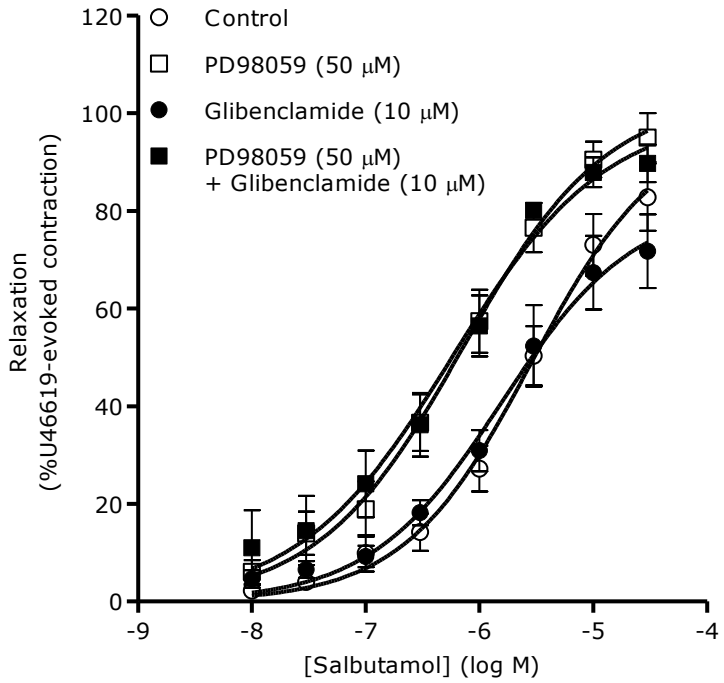

Figure 3.5. *The effect of the MEK inhibitor PD98059 with and without the K_{ATP} channel blocker glibenclamide on β_2-AR-mediated relaxations in PCA (n=4 or 5). Recordings were made in KH solution containing the following: 0.36% (v/v) DMSO (control), PD98059 (50 μM) + 0.1% (v/v) DMSO, glibenclamide (10 μM) + 0.26% (v/v) DMSO, or PD98059 (50 μM) + glibenclamide (10 μM). Relaxations to salbutamol were enhanced by PD98059 alone and in combination with glibenclamide (pEC_{50} values versus control pEC_{50} value (5.6 ± 0.2): PD98059 = 6.3 ± 0.2, PD98059 + 10 μM glibenclamide = 6.4 ± 0.1; P<0.05 versus control in each experiment). Glibenclamide alone failed to alter relaxations (pEC_{50} value 5.7 ± 0.2; P>0.05 versus control pEC_{50}).*

3.3.3. The role of BK_{ca} channels in enhanced β-AR-mediated vasodilatation

Iberiotoxin (100 nM), like glibenclamide, had no effect on relaxations to either isoprenaline or salbutamol (Figures 3.6 and 3.7, respectively). However, PD98059, alone or in combination with iberiotoxin, also failed to enhance isoprenaline or salbutamol responses in these experiments, though statistically non-significant leftward shifts of the relaxation curves were observed (isoprenaline pEC_{50} values: control = 7.7 ± 0.1, PD98059 = 8.0 ± 0.2, iberiotoxin = 7.7 ± 0.1, PD98059 + iberiotoxin = 7.9 ± 0.1, P>0.05, one-way ANOVA followed by Tukey's multiple comparisons test; salbutamol pEC_{50} values: control = 7.1 ± 0.2, PD98059 = 7.5 ± 0.2, iberiotoxin = 7.0 ± 0.1, PD98059 + iberiotoxin = 7.3 ± 0.2, P>0.05, one-way ANOVA followed by Tukey's multiple comparisons test).

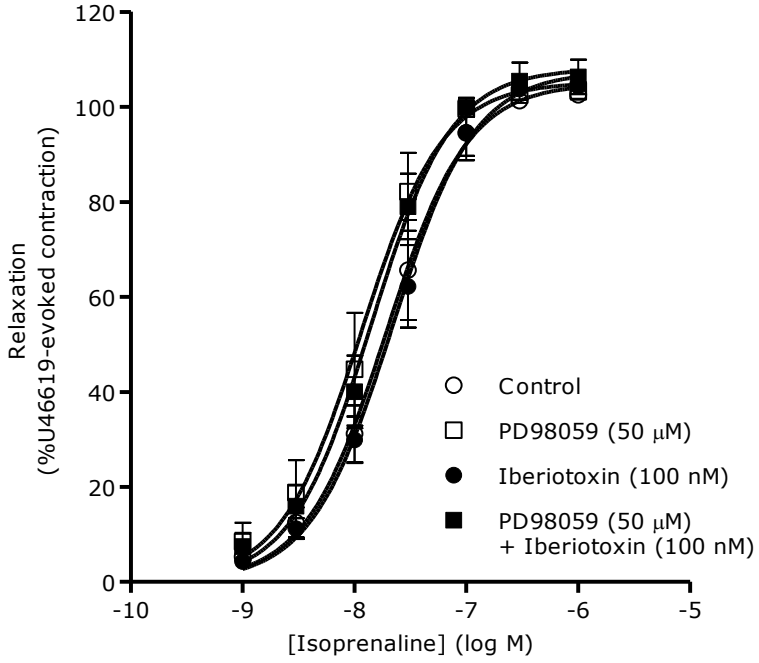

Figure 3.6. *The effect of MEK inhibition and BK_{Ca} channel blockade on β-AR-mediated relaxations (n=4). Recordings were made in KH solution containing the following: 0.26% (v/v) DMSO (control), PD98059 (50 μM), iberiotoxin (100 nM) + 0.26% (v/v) DMSO, or PD98059 (50 μM) + iberiotoxin (100 nM). None of the treatment experiments were associated with altered relaxation characteristics of isoprenaline in comparison with control experiments.*

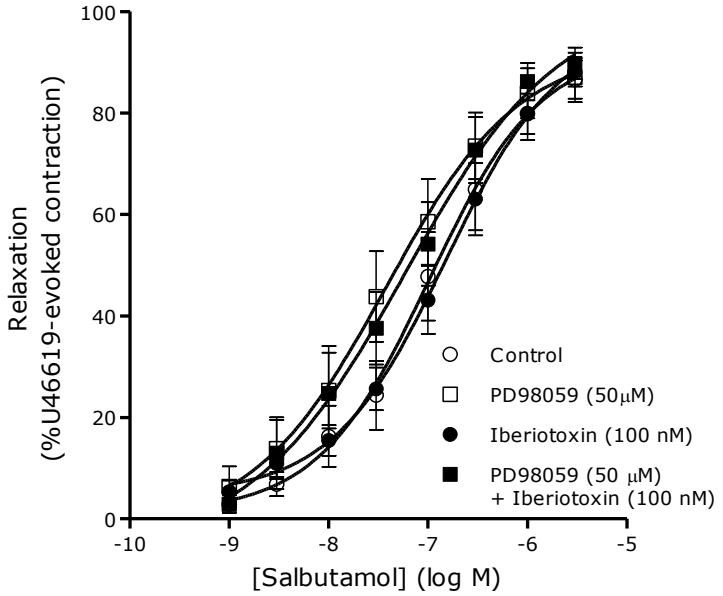

Figure 3.7. *The effect of MEK inhibition and BK_{Ca} channel blockade on β_2-AR-mediated relaxations in PCA (n=6–9). Recordings were made in KH solution containing the following: 0.26% (v/v) DMSO (control), PD98059 (50 μM), iberiotoxin (100 nM) + 0.26% (v/v) DMSO, or PD98059 (50 μM) + iberiotoxin (100 nM). Salbutamol relaxations were unaffected by PD98059, iberiotoxin or a combination of both agents.*

In a related experiment, NS1619-induced relaxations were assessed against PD98059 (Figure 3.8). Unfortunately, the data analysis software was unable to estimate R_{MAX} and pEC_{50} values from this dataset. Therefore, the following were calculated and used as substitutes for R_{MAX} and pEC_{50} values, respectively: (1) the relaxation response produced by the highest concentration of NS1619 (*i.e.,* 10 μM) and (2) the negative log concentration of NS1619 producing 50% of this response. Incubation with PD98059 did not change these values (control = 106 ± 13% and 5.5 ± 0.04, PD98059 = 110 ± 8% and 5.6 ± 0.03; $P>0.05$).

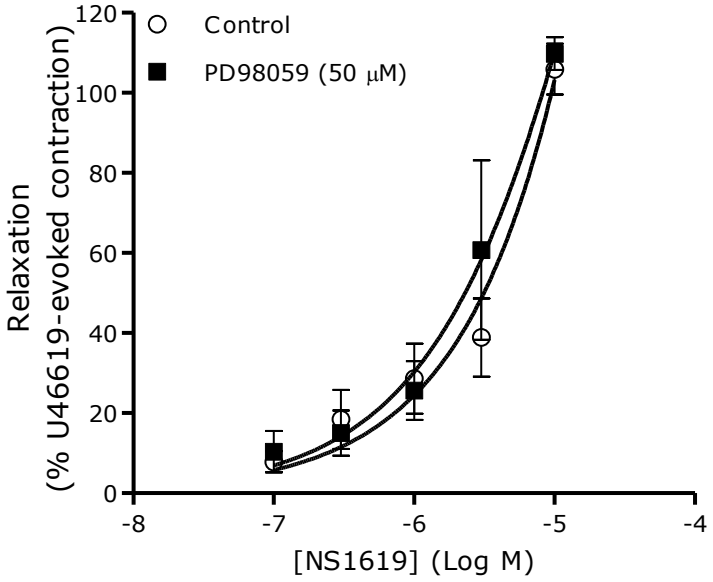

Figure 3.8. *The effect of MEK inhibition on relaxations to the BK_{Ca} channel opener NS1619 in PCA (n=7). NS1619 evoked a concentration-dependent relaxation of U46619-pre-contracted PCA rings that was unaffected by PD98059 (50 μM). Control experiments were performed in 0.26% (v/v) DMSO. The relaxation responses produced by 10 μM NS1619 under control conditions and in the presence of 50 μM PD98059 were not significantly different (106 ± 13% and 110 ± 8%, respectively; P>0.05). Equally, the potency (as assessed by the negative log of the concentration of NS1619 producing 50% of the highest measured response) did not differ between controls and PD98059-incubated rings (5.5 ± 0.04 versus 5.6 ± 0.03, respectively; P>0.05).*

3.3.4. The role of IK_{Ca} channels in enhanced β-AR-mediated vasodilatation

Figure 3.9 shows the effects of PD98059 and the IK_{Ca} channel blocker TRAM-34 (10 μM) on relaxation responses to salbutamol. TRAM-34 alone did not significantly alter the relaxation, though there was a slight trend towards inhibition at the lower concentrations of salbutamol. PD98059 produced a significant leftwards shift of the response curve. However, this shift was abolished when arteries were pre-incubated with the combination of PD98059 and TRAM-34 (pEC_{50} values: control = 6.1 ± 0.1, PD98059 = 6.6 ± 0.1, TRAM-34 = 5.9 ± 0.1, PD98059 + TRAM-34 = 6.2 ± 0.1; $P<0.05$, one-way ANOVA followed by Tukey's multiple comparisons test).

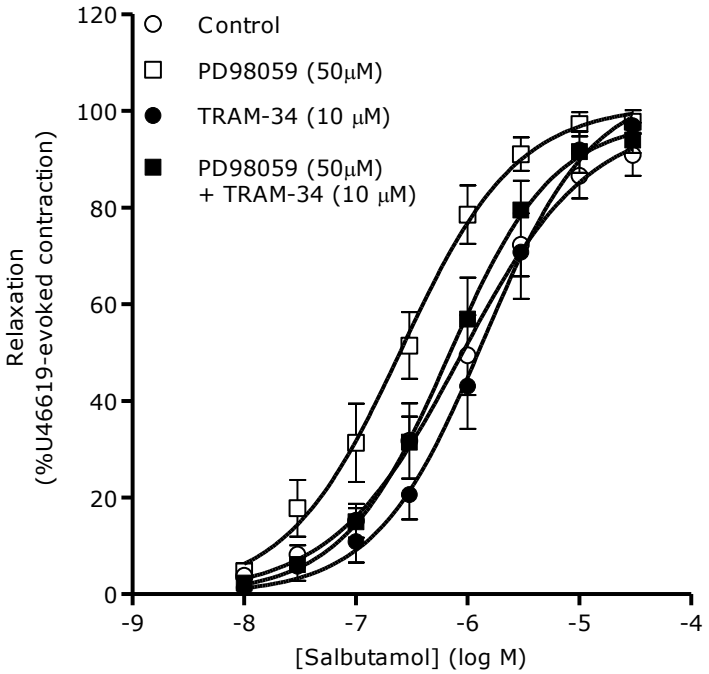

Figure 3.9. *The effect of MEK inhibition and IK_{Ca} channel blockade on salbutamol-induced relaxations in PCA rings (n=5 or 6). Recordings were made in KH solution containing the following: 0.36% (v/v) DMSO (control), PD98059 (50 μM) + 0.1% (v/v) DMSO, TRAM-34 (10 μM) + 0.26% (v/v) DMSO, or PD98059 (50 μM) + TRAM-34 (10 μM). Salbutamol relaxations (control pEC_{50} value = 6.1 ± 0.1) were enhanced by PD98059 (pEC_{50} value = 6.6 ± 0.1; P<0.05) but not by the combination of PD98059 and TRAM-34 (pEC_{50} value = 6.2 ± 0.1; P>0.05) or by TRAM-34 alone (pEC_{50} value = 5.9 ± 0.1; P<0.05).*

3.3.5. Electrophysiological studies of K^+ channels in porcine coronary artery

Using cell-attached and inside-out patch-clamp experiments, single-channel currents from at least two distinct channel types were observed. Figure 3.10A shows representative 10 s traces of one channel from a single cell-attached patch of an isolated PCA smooth muscle cell. In this experiment, the pipette and extracellular solutions contained the same $[K^+]$, i.e. 145 mM. Channel activity was only observed at positive membrane potentials in the cell-attached configuration. Thus, all traces represent recordings performed at +60 mV.

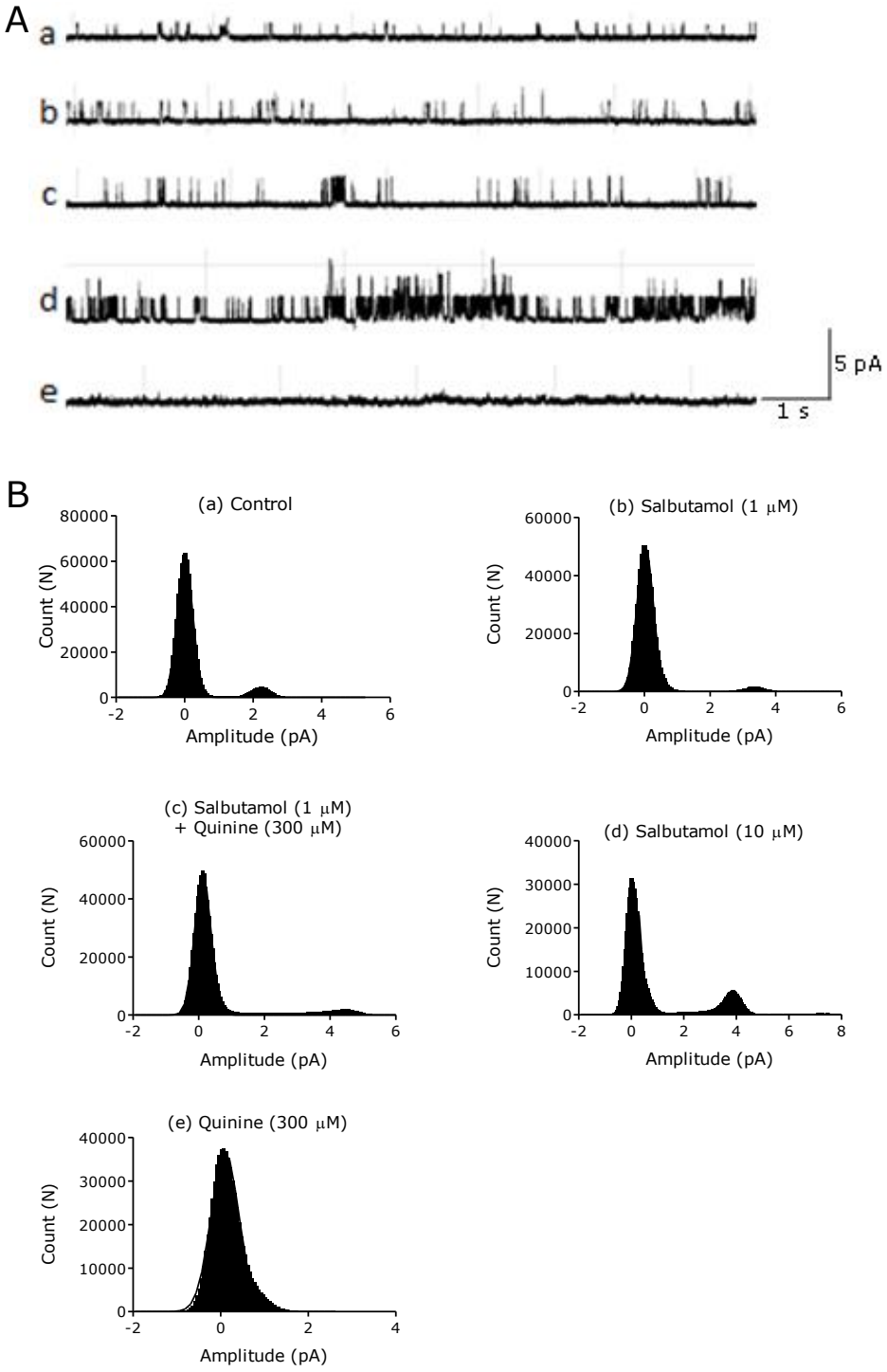

Figure 3.10. *The effects of salbutamol and quinine on channel currents in a single cell-attached patch.* ***(A)*** *Five representative ~10 s traces of channel activity. Recordings were made at +60 mV following voltage steps from a holding potential of 0 mV under the following conditions: (a) control, (b) salbutamol (1 μM), (c) salbutamol (1 μM) + quinine (300 μM), (d) salbutamol (10 μM) and (e) quinine (300 μM).* ***(B)*** *All-points histograms of current amplitude in a single cell-attached patch corresponding to trace recordings shown in (A). Recordings were made at +60 mV following voltage steps from a holding potential of 0 mV under the following conditions: (a) control, (b) salbutamol (1 μM), (c) salbutamol (1 μM) + quinine (300 μM), (d) salbutamol (10 μM) and (e) quinine (300 μM).*

Figure 3.10B shows all-points current amplitude histograms derived from corresponding 60 sec recordings of the same cell-attached patch shown in Figure 3.10A. In each case, the larger peak represents the closed state of the channel whilst a smaller peak, if present, indicates a conducting level. NP_O values derived from amplitude histograms were as follows: 0.08 under control conditions, 0.03 in the presence of salbutamol (1 μM), 0.06 in both salbutamol (1 μM) and quinine (300 μM) and 0.19 in salbutamol (10 μM). Analyses of current-voltage relationships estimated corresponding unitary conductances of 37 pS, 55 pS, 87 pS and 65 pS, respectively. In the presence of quinine (300 μM) alone, conductance and NP_O values were negligible. Quinine has previously been shown to block voltage-dependent (K_V) and ATP-sensitive (K_{ATP}) K^+ channels (Fatherazi *et al.*, 1991); however, attempts to further characterise this channel using the inside-out configuration were unsuccessful as the seal was lost.

Representative trace recordings and corresponding all-points current amplitude histograms of a second patched channel are shown in Figures 3.11A and 3.11B. Cells were exposed to 50 μM PD98059 for 25 – 35 min prior to data collection. Recordings were made under symmetrical high [K^+] conditions in the cell attached (a) and inside-out configurations (b and c). Channel activity was not detected at negative membrane potentials in the cell-attached configuration.

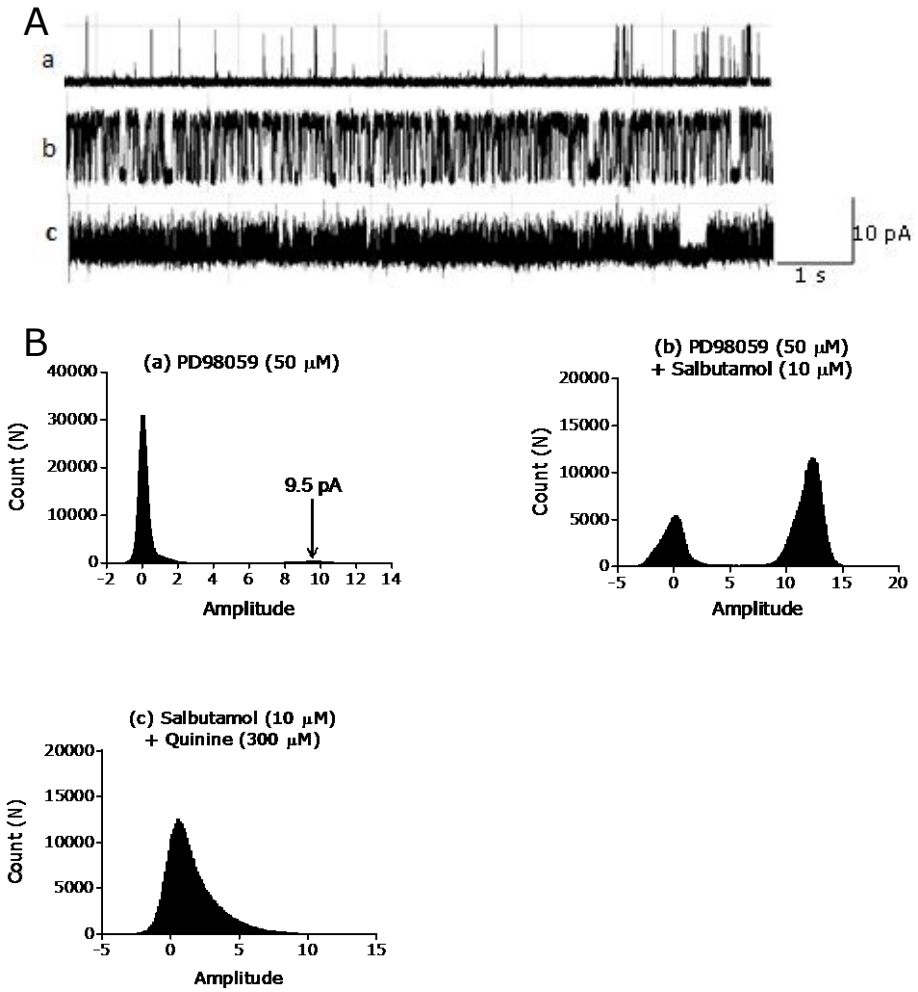

Figure 3.11. *Channel currents in a single patch-clamp experiment in which cells were pre-incubated in PD98059 (50 μM) for 35 mins.* ***(A)*** *Representative ~10 s traces of ion channel activity. Recordings were made at +60 mV following voltage steps from a holding potential of 0 mV under the following conditions: (a) PD98059 (50 μM), (b) PD98059 (50 μM) + salbutamol (10 μM) and (c) salbutamol (10 μM) + quinine (300 μM).* ***(B)*** *All-points histograms of current amplitude in a single cell-attached patch corresponding to trace recordings shown in (A). Recordings were made at +60 mV following voltage steps from a holding potential of 0 mV under the following conditions: (a) PD98059 (50 μM), (b) PD98059 (50 μM) + salbutamol (10 μM) and (c) salbutamol (10 μM) + quinine (300 μM).*

In the presence of PD98059 alone, NP_O and conductance were 0.03 and 137 pS, respectively, at +60 mV. Superfusion of high $[K^+]$ solution containing PD98059 (50 μM) and salbutamol (10 μM) resulted in the excision of the patch to the inside-out configuration. Channel activity was detected at negative membrane potentials in this configuration. The conductance derived from the associated current-voltage relationship

(Figure 3.12) was 210 pS. Channel activity also increased, with NP_O rising to 0.70 at +60 mV. The voltage sensitivity of the channel is described by the P_O-voltage relationship shown in Figure 3.13.

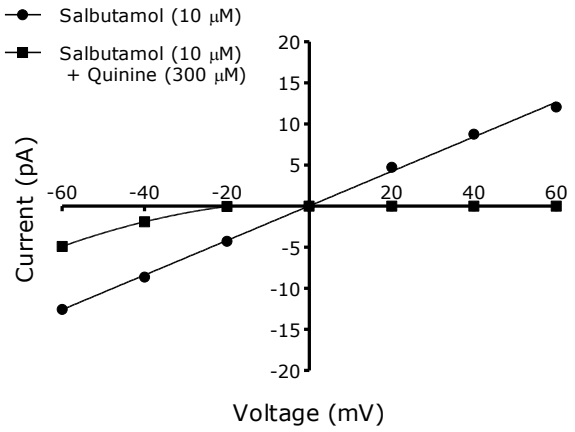

Figure 3.12. *Current-voltage relationships in a single cell-attached patch of a large conductance channel. Recordings were made at 20 mV increments for 15 secs in the presence of either salbutamol (10 μM) or the combination of salbutamol (10 μM) and quinine (300 μM).*

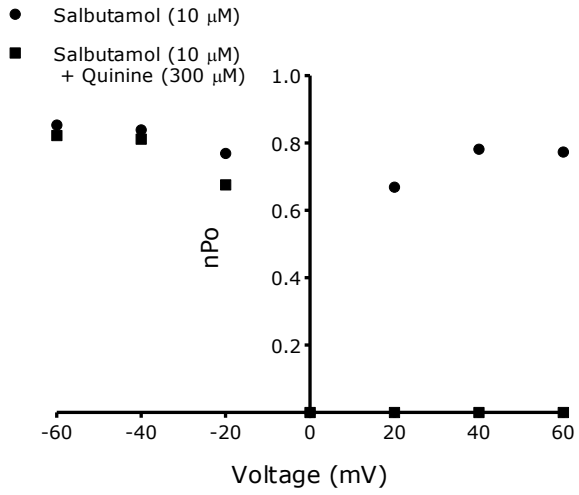

Figure 3.13. *P_O-voltage relationships in a single cell-attached patch corresponding of a large conductance channel. Recordings were made at 20 mV increments for 15 secs in the presence of either salbutamol (10 μM) or the combination of salbutamol (10 μM) and quinine (300 μM).*

Application of salbutamol (10 μM) and quinine (300 μM) prevented channel activation across a range of voltages (0 mV to +60 mV) and reduced current amplitude between -20 mV and -60 mV (Figure 3.12), producing a conductance of 30 pS. In addition to reducing current amplitude, quinine caused a rapid flickering of the current trace, representing increased

frequency of channel closures. Changes in voltage-sensitivity caused by quinine are shown in Figure 3.13. Channel opening was prevented at positive voltages but persisted at negative voltages, a discrepancy likely related to the experimental protocol. Specifically, after the application of quinine, currents were recorded at each voltage for 15 secs, with recordings made sequentially every 20 mV from -60 mV to +60 mV. The channel had therefore been exposed to quinine for a longer period of time during data recording at positive voltages.

Based on the large single-channel conductance, voltage-sensitivity and blockade by quinine (Bokvist *et al.*, 1990; Mancilla *et al.*, 1990; Nelson *et al.*, 1995b), these recordings represent the large-conductance calcium-activated K^+ (BK_{Ca}) channel.

3.4. DISCUSSION

As important regulators of vascular tone, K^+ channels in blood vessels have long been subjected to intensive research, the fruits of which have contributed to the treatment of ischaemic heart disease. In virtually all cells K^+ is the predominant intracellular cation, existing at much higher concentrations in the cytoplasm than outside the cell. This chemical gradient is maintained by the Na^+/K^+-ATPase pump and provides the driving force for the outward movement of K^+ through open channels. K^+ efflux is accompanied by a reduction in cell membrane potential, termed hyperpolarisation, and, as a result, reduces the influx of Ca^{2+} through L-type Ca^{2+} channels. The effects of reduced intracellular Ca^{2+} levels are dependent on cell type, with relaxation occurring in vascular smooth muscle cells.

K^+ channels are expressed on both endothelial cells and smooth muscle cells, and both receptor populations are known to participate in regulating vascular tone. For example, activation of small- and intermediate-conductance Ca^{2+}-activated K^+ channels (SK_{Ca} and IK_{Ca}, respectively) on endothelial cells results in smooth muscle relaxation; it is believed that this phenomenon may be mediated by an as yet unidentified endothelium-derived hyperpolarising factor (EDHF) (Coleman *et al.*, 2004). Equally, activation of smooth muscle K^+ channels, such as the large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), leads to vasorelaxation (Balwierczak *et al.*, 1995).

Vascular K^+ channels are regulated by numerous factors, including G-protein-coupled receptors (GPCRs). For example, ATP-sensitive K^+ channels (K_{ATP}) have been proposed as mediators of β-AR agonist-induced

relaxations (Chang, 1997; Ming *et al.*, 1997; Randall *et al.*, 1995; Sheridan *et al.*, 1997). In Chapter 2 of the present study, it was shown that inhibition of extracellular signal-regulated kinase (ERK) activation in porcine coronary artery (PCA) enhances subsequent β_2-AR-mediated vasodilatation. Furthermore, ERK activation has been associated with reduced dilator responses to K^+ channel openers in pig pial artery (Ross *et al.*, 2003). In the current chapter, experiments aimed to determine whether K^+ channels are implicated in the enhancement phenomenon.

In initial experiments, attempts were made to prevent K^+ channel activation during β-AR-mediated vasodilatation. This was done in the first instance by performing pre-contractions with KCl rather than the thromboxane mimetic U46619. By pre-contracting with KCl, extracellular $[K^+]$ is increased to an extent that abolishes the concentration gradient responsible for driving the efflux of K^+ ions through open K^+ channels. Subsequent relaxations to isoprenaline were not enhanced by incubation with PD98059, in contrast to relaxations made following U46619-induced pre-contraction. This finding suggests that K^+ channels are involved in the enhancement of β-AR-mediated caused by MEK inhibition.

In support of this is the finding that isoprenaline-induced relaxations of arteries incubated with the non-selective K^+ channel blocker TEA were not enhanced by PD98059. However, there appeared to be a slight, albeit statistically insignificant, leftward shift of the response curve, indicating a degree of enhancement by PD98059. Incomplete K^+ channel blockade by TEA may explain this discrepancy. An alternative interpretation is that PD98059 enhances relaxations via a K^+ channel-independent signalling pathway, and that K^+ channel blockade, which itself acts to inhibit relaxations, indirectly masks the enhancement. The net result is no overall

change in the relaxation response. This explanation could be confirmed or ruled out by further experiments designed to assess the effect of TEA alone on isoprenaline-induced relaxations.

The data above suggest that inhibition of ERK activation enhances β-AR-mediated relaxations through an increase in K^+ channel activation. Therefore further experiments were carried out to determine which K^+ channels are involved. Previous studies have suggested that β-AR relaxations are mediated through activation of K_{ATP} channels (Randall *et al.*, 1995). Therefore it was determined whether blocking K_{ATP} channels with glibenclamide prevented the enhancement by PD98059.

Since its development as a potent hypoglycaemic agent (Loubatieres *et al.*, 1968), the sulphonylurea glibenclamide has been successfully used in the treatment of Type II diabetes owing to its ability to block K_{ATP} channels in insulin-secreting cells (Schmid-Antomarchi *et al.*, 1987). Glibenclamide has also been frequently employed in cardiovascular research as a blocker of K_{ATP} channels in blood vessels. As mentioned earlier, several authors have reported an involvement of K_{ATP} channels in β-AR-mediated vasodilatation (Chang, 1997; Ming *et al.*, 1997; Randall *et al.*, 1995; Sheridan *et al.*, 1997).

In the current study, glibenclamide was found to have no effect on relaxations induced by either isoprenaline or salbutamol. This is in contrast to the previously quoted studies, which collectively demonstrated an inhibitory effect of glibenclamide on both β_{1-} and β_2-AR-mediated vasorelaxation. The concentrations of glibenclamide used in these studies ranged from 1 μM to 10 μM, suggesting that the lack of effect of glibenclamide in the current experiment was not related to its

concentration (10 μM). This finding has been observed in other tissues, such as rat isolated thoracic aorta (Husken *et al.*, 1997) and mesenteric artery (White *et al.*, 2001), suggesting it is not specific to PCA. The possibility that the negative result in this study was related to the absence of glibenclamide-sensitive channels in PCA is ruled out by (1) the detection of functional K_{ATP} channels in cultured PCA smooth muscle cells (Wakatsuki *et al.*, 1992) and (2) the observation that vasorelaxation of PCA was induced by the K_{ATP} channel openers pinacidil (Figure 5.7) and levcromakalim (McPherson *et al.*, 1997). Nonetheless, further experimentation in PCA to assess relaxation responses to pinacidil in the presence of glibenclamide is warranted. It is possible that blockade of K_{ATP} channels in PCA is compensated for by enhanced signalling via other pathways. As discussed earlier, MEK inhibition in the presence of raised extracellular K^+ or the non-selective blocker TEA did not result in enhancement of β-AR-mediated vasodilatation. When selectively blocking K_{ATP} channels using glibenclamide, MEK inhibition produced enhancements of isoprenaline- and salbutamol-induced vasodilatation, which suggests that K_{ATP} channels are not involved in the enhancement mechanism.

Patch-clamp experiments of PCA smooth muscle cells in the presence of PD98059 (50 μM) revealed a voltage-dependent, quinine-sensitive channel with large conductance (137 pS). The BK_{Ca} channel is characterised by its voltage sensitivity, large unit conductance (> 100 pS), and is blocked by 200 - 300 μM quinine (Bokvist *et al.*, 1990; Mancilla *et al.*, 1990; Nelson *et al.*, 1995b). The open probability of this channel in unstimulated conditions was low (NP_O = 0.03 at +60 mV), suggesting that the channel may not be important in setting resting arterial tone. Due to difficulties in isolating smooth muscle cells, experiments controlling for the effects of PD98059 or vehicle (DMSO) were not performed, so it is also possible that the low

channel activity was due to effects of these compounds. However, this explanation is unlikely as low basal activity of the BK_{Ca} channel has previously been observed in these cells (Deenadayalu *et al.*, 2001; Hu *et al.*, 1997).

Application of salbutamol increased channel activity (NP_O 0.70 at +60 mV), suggesting an interaction between the β_2-AR and BK_{Ca} channels. However, excision of the patch occurred during superfusion of salbutamol. As a result, the intracellular face of the channel was exposed to 1 μM Ca^{2+}, which was present in the bathing solution and may have contributed to the increased channel activity. On the other hand, prior to excision, the intact cell was bathed in depolarising concentrations of K^+ which normally cause Ca^{2+} influx via L-type Ca^{2+} channels. In fact, cytosolic Ca^{2+} concentrations of up to 1 μM have been measured in arterial smooth muscle under such conditions (Abe *et al.*, 1990; Hirano *et al.*, 1991). It is therefore likely that the increased BK_{Ca} activity occurred at least in part as a result of β_2-AR stimulation. Furthermore, the activation of BK_{Ca} channels by isoprenaline in PCA smooth muscle cells has previously been reported, with the authors proposing cross-activation of PKG by cAMP as the underlying mechanism (White *et al.*, 2000).

Related isometric tension experiments used iberiotoxin which, at 100 nM, the concentration used in this study, has been shown to reduce BK_{Ca} channel activity and associated relaxations in PCA (Leung *et al.*, 2007; Yang *et al.*, 2008). Like glibenclamide, iberiotoxin failed to alter relaxations to either isoprenaline or salbutamol. PD98059 appeared to shift agonist curves to leftwards, though this effect was statistically non-significant. Co-incubation with PD98059 and iberiotoxin also appeared to enhance agonist curves though, again, these effects were not statistically

significant. These data suggest that PD98059 does not enhance β-AR-mediated relaxations by altering BK_{Ca} channel activity and are consistent with the subsequent finding that vasorelaxations induced by the BK_{Ca} channel opener NS1619 were insensitive to PD98059. Further supporting evidence is provided by the facts that isoprenaline-induced activation of BK_{Ca} channels in PCA smooth muscle cells occurred via a cAMP-dependent pathway (White *et al.*, 2000); however, as indicated in Chapter 2, the enhancement of β-AR-mediated relaxations by PD98059 appears to occur through a cAMP-independent pathway.

IK_{Ca} channels are characterised by their Ca^{2+} dependence, voltage-independence and sensitivity to the scorpion venom charybdotoxin and the triarylmethane clotrimazole. A related triarylmethane derivative, namely TRAM-34, was recently developed (Wulff *et al.*, 2000) and has since been used (1 μM – 10 μM) to study IK_{Ca} function in blood vessels (Crane *et al.*, 2003; Eichler *et al.*, 2003; McSherry *et al.*, 2005). In the current study, TRAM-34 was used in conjunction with PD98059 to determine whether the enhancement of β_2-AR agonist-mediated vasodilatation involves IK_{Ca} channels. By itself, TRAM-34 (10 μM) failed to alter relaxation responses to salbutamol, though there appeared to be a slight, statistically non-significant inhibitory effect at lower concentrations. In the same experiments, PD98059 alone, but not in combination with TRAM-34, enhanced relaxations. The fact that TRAM-34 blocked the enhancement of the salbutamol-induced relaxation by PD98059 indicates that the IK_{Ca} channel may be involved in the enhancement of β_2-AR-mediated vasodilatation. In other words, β_2-AR-mediated vasodilatation may be inhibited by an interaction between ERK and the IK_{Ca} channel, with inhibition of ERK activation resulting in the disinhibition of IK_{Ca} channel activity and, therefore, the β_2-AR-mediated response. This finding also

suggests that reduced IK_{Ca} channel-mediated dilator activity may contribute to the pathology of cardiovascular disease states characterised by increased ERK activation (see Chapter 4).

The EDHF phenomenon has been the subject of considerable interest and study (Busse *et al.*, 2002). It is thought that elevations of cytosolic $[Ca^{2+}]$ in endothelial cells results in the activation of IK_{Ca} and SK_{Ca} channels causing hyperpolarisation which is subsequently conducted to smooth muscle cells. IK_{Ca} channel expression in blood vessels has frequently been observed in endothelial cells and less commonly in smooth muscle cells (McNeish *et al.*, 2006). In the current experiment, attempts to probe for the existence of IK_{Ca} currents using electrophysiology in PCA smooth muscle cells were unsuccessful due to difficulties in isolating healthy, patchable cells. Immunohistochemistry techniques using IK_{Ca}–specific antibodies may be warranted to confirm or rule out the expression of the channel in PCA smooth muscle cells. However, the up-regulation of vascular smooth muscle IK_{Ca} channel expression has been observed in human coronary artery disease and both mouse and pig models of atherosclerosis, suggesting that, in smooth muscle, IK_{Ca} channels may be relevant in proliferative but not contractile phenotypes (Tharp *et al.*, 2006; Toyama *et al.*, 2008). On the other hand, the upregulation of endothelial IK_{Ca} channels observed in a rat models of hypertension (Giachini *et al.*, 2009) and obesity (Chadha *et al.*, 2010) served to restore impaired endothelium-dependent vasodilation. To further investigate these discrepancies, application of the IK_{Ca} channel opener 1-EBIO (Adeagbo, 1999) to endothelium-denuded PCAs would be warranted, as the observation of vasodilatation under these conditions would indicate the presence of functional IK_{Ca} channels on smooth muscle.

A possible role for the SK_{Ca} channel which, along with IK_{Ca} channels, has been implicated in EDHF-mediated responses was not investigated in this study. However, the suggestion from previous investigations that smooth muscle IK_{Ca} channels may be unimportant within the contractile phenotype opens the possibilities that, firstly, the interaction between ERK and the IK_{Ca} may exist at the level of the endothelium and, secondly, that the interaction may also involve SK_{Ca} channels and EDHF.

It is not known whether voltage-dependent K^+ (K_V) channels are involved in the enhancement of β-AR-mediated vasodilatation. Attempts to block their activity using 4-aminopyridine (Doggrell *et al.*, 1999) produced strong phasic contractions, making the measurement of relaxation responses very difficult, and suggesting that these channels may play an important role in regulating resting arterial tone, which is consistent with the literature (Yuan, 1995). Two quinine-sensitive channels were observed in patch-clamp experiments, both of which were activated by salbutamol, but it was possible to fully characterise only one of these, namely the BK_{Ca} channel. Unfortunately, attempts to investigate the enhancement phenomenon using patch-clamp experiments were unsuccessful.

In conclusion, the β-AR has frequently been reported to dilate blood vessels via pathways involving K^+ channels. The lack of effect of K^+ channel blockers on β–AR-agonist-induced vasodilatation may have been related to the compensatory recruitment of alternative signalling pathways. Inactivation of K^+ channels provided evidence that the enhancement of β_2-AR-mediated vasodilatation by MEK inhibitors involves K^+ channels. The use of selective K^+ channel blockers ruled out roles for the K_{ATP} and BK_{Ca} channels in this phenomenon and revealed that at least one K^+ channel subtype, namely the IK_{Ca} channel, may be involved.

The interaction between ERK and the IK_{Ca} channel may involve direct phosphorylation of the channel by ERK or an ERK-dependent kinase, resulting in reduced channel activity. Further experiments probing the nature of this interaction are warranted, particularly in light of the facts that (i) ERK has been reported to regulate K^+ channel activity (Li *et al.*, 2006; Ross *et al.*, 2003) and (ii) the IK_{Ca} channel is known to be regulated by phosphorylation (Gerlach *et al.*, 2000).

CHAPTER 4: THE ROLE OF ERK IN β-ADRENOCEPTOR-MEDIATED VASODILATATION IN ANIMAL MODELS OF TYPE II DIABETES AND OBESITY

4.1. INTRODUCTION

Also known as non-insulin-dependent diabetes mellitus, Type II diabetes is a complex metabolic condition characterised by insulin resistance and hyperglycaemia. It is estimated that 346 million people worldwide suffer from this condition, with many cases undiagnosed (World Health Organisation, 2011b). Type II diabetes is associated with considerable lifestyle disruption and serious long-term consequences, including macrovascular complications such as coronary artery disease and stroke. Defined by the WHO as "abnormal or excessive fat accumulation that may impair health", obesity has more than doubled since 2008, with an estimated 500 million obese adults worldwide in 2008 (World Health Organisation, 2011c). Obesity is associated with elevated insulin resistance and hyperglycaemia, and represents a significant risk factor for Type II diabetes.

As both Type II diabetes and obesity are responsible for significant morbidity and mortality, considerable efforts have been undertaken to develop effective treatment options. In biomedical research, animal models of these conditions have been developed and provide valuable insights into the pathological changes that occur in these disease states. The obese phenotype of the Zucker obese rat is derived from an autosomal recessive mutation of the leptin receptor (Chua *et al.*, 1996; Phillips *et al.*, 1996). Efforts to inbreed selected Zucker obese rats of diabetic lineage produced the Zucker diabetic fatty (ZDF) rat (Peterson *et al.*, 1990).

Neither the Zucker rat nor the ZDF rat is a perfect model of human disease. First, leptin abnormalities, which underlie the pathological changes in these animals, rarely occur in humans and account for a small fraction of

the disease burden in obesity and Type II diabetes (Farooqi *et al.*, 2007). In addition, differences in pathophysiology exist between human and rodent, an example being the observation that islet amyloid deposition is a key pathological feature of Type II diabetes in humans but not in ZDF rats (Matveyenko *et al.*, 2006). Despite these differences, the Zucker rat and ZDF rat exhibit characteristics that also occur in humans with obesity and Type II diabetes, respectively, and justify their use as models of these disease states. The Zucker rat is hyperphagic and develops obesity by 3–5 weeks of age (Zucker *et al.*, 1972); hypertriglyceridaemia and hyperinsulinaemia (but not gross hyperglycaemia) also occur in early life (Ionescu *et al.*, 1985). Similarly, the ZDF rat develops hyperinsulinaemia, obesity and hyperglycaemia by 6–8 weeks of age and exhibits micro- and macrovascular pathologies that also complicate human disease (Belin de Chantemele *et al.*, 2009; Danis *et al.*, 1993; Hempe *et al.*, 2012). The timeframe of disease progression in these animals (weeks, compared to several years in humans) facilitates the study of the underlying pathophysiology and the development of treatment interventions.

As shown in Chapter 2, β-AR-mediated vasodilatation is modulated by ERK. A recent study using the ZDF rat model of Type 2 diabetes reported reduced β-AR function in coronary artery of this animal (Grisk *et al.*, 2007). The current Chapter will explore a possible role for ERK in β-AR-mediated vasodilatation in animal models of Type II diabetes and obesity. Additionally, *ex vivo* experiments exposing animal vessels to high glucose concentrations in order to mimic the hyperglycaemic state will be performed.

4.2. MATERIALS AND METHODS

4.2.1. Isometric Tension Recordings

4.2.1.1. Tissue preparation

Thoracic aortae and superior mesenteric arteries (SMA) were dissected from freshly-sacrificed, 12-week old male Zucker diabetic fatty rats (ZDF), Zucker obese rats and Wistar rats (Charles River Laboratories International, Inc.). Wistar rats were used as controls where Zucker lean rats were not available. Weight and glucose levels of the rats were not available. The vessels were stored in KH solution and prepared as described in Section 2.2.1.1. After mounting the rat SMA and aorta to a Mulvany wire myograph, a resting tension of 0.5 g and 1.0 g, respectively, was applied. In experiments using porcine coronary arteries (PCA), the protocol described in Section 2.2.1.1 was used.

4.2.1.2. Experimental procedure

Mounted arteries were challenged with KCl (60 mM) in order to confirm tissue viability and to determine their maximal contractile capacities. The arteries were then thoroughly rinsed with KH solution and allowed to re-equilibrate for 15 to 20 min. The process was repeated twice.

4.2.1.2.1. The effect of MEK inhibition on β-AR-mediated relaxation

To assess the role of ERK activation in β-AR-mediated relaxation, arteries were incubated with either PD98059 (50 μM) or 0.26% (v/v) DMSO. This

was followed by contraction with the thromboxane mimetic U46619 (10 – 20 nM) to 60–70% of the maximum response to KCl. Finally, cumulative concentrations of isoprenaline (1 nM – 3 μM) were used to relax the pre-contracted arteries.

4.2.1.2.2. The effect of high glucose concentration on β-AR-mediated relaxation

In a separate series of experiments, the effect of overnight incubation of PCA in high glucose conditions was investigated. The aim was to determine whether the effects of diabetes on β-AR-mediated relaxation could be mimicked *in vitro*. In the following experiments, arteries were prepared in KH solution modified to contain 5.5 mM glucose.

Arteries were stored in pre-gassed KH solution containing 25 mM glucose at 4°C for roughly 16 hours. Control arteries were stored in KH solution containing 5.5 mM glucose. To account for the osmotic effects of high glucose concentrations, a second control group was stored overnight in mannitol (19.5 mM). Immediately after the incubation period, arteries were set up on a Mulvany wire myograph in order to carry out isometric tension recordings. Following KCl challenges and pre-contraction with U46619, cumulative concentrations of salbutamol (10 nM – 30 μM) were applied to generate concentration-response curves. The KH solution used during the course of the experiment was continuously gassed and contained 5.5 mM glucose.

In a similar set of experiments, arteries were stored overnight as described above. However, during isometric tension recordings arteries were exposed only to the same solutions in which they were incubated. A related

experiment was performed in which the arteries were incubated for 16 hours in their respective solutions at 37°C. To prevent microbial growth, solutions were supplemented with penicillin-streptomycin (500 units/0.5 mg ml^{-1}) and all manipulations prior to isometric tension recordings were carried out under aseptic conditions. Again, during isometric tension recordings, arteries were exposed only to the same solutions in which they were incubated.

4.2.2. Measurement of ERK activation

As previously described in our laboratory (Roberts, 2001), the phosphorylation state of ERK at Thr-202 and Tyr-204 was measured using immunoblotting.

4.2.2.1. Tissue preparation

SMA dissected from ZDF rats and their Zucker lean controls were set up on a Mulvany wire myograph as described earlier. Following KCl challenges and re-equilibration periods, arteries were either contracted with U46619 to 60–70% of the maximal KCl response or left to incubate in KH solution (controls). As soon as the contractile response to U46619 had reached a plateau, the arteries were dismounted from the myograph and immediately frozen on dry ice. They were stored at -80°C. After defrosting, arteries were prepared for immunoblotting experiments as described in Section 2.2.2.1.

4.2.2.2. Immunoblotting

The arteries were assessed for changes in the dual phosphorylation of ERK using immunoblotting as described in Section 2.2.2.3.

4.2.3. Statistical analyses

Data were analysed using GraphPad Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA). Relaxation response measurements are expressed as means ± SEM. Statistical comparisons between groups were made using the two-tailed, unpaired Student's t-test except where stated. As there were insufficient repeat experiments to determine distribution, the data were assumed to be normally distributed and parametric tests were employed. A *P* value < 0.05 was considered statistically significant. In all experiments, "n" numbers represent the number of animals from which tissues were obtained.

4.2.4. Materials

Penicillin-streptomycin (10,000 units/10 mg ml^{-1}) was ordered from Sigma (Poole, Dorset, UK). The remaining chemicals were acquired as stated in Section 2.2.4.

4.3. RESULTS

4.3.1. The effect of MEK inhibition on β-AR-mediated vasodilatation in Zucker diabetic fatty rats

In ZDF rat aortae, isoprenaline reversed the U46619-evoked pre-contraction to a maximal relaxation of 50 ± 7% (Figure 4.1). This was significantly lower than the corresponding value in aortae from Wistar control rats (93 ± 5%; $P<0.05$; Figure 4.2).

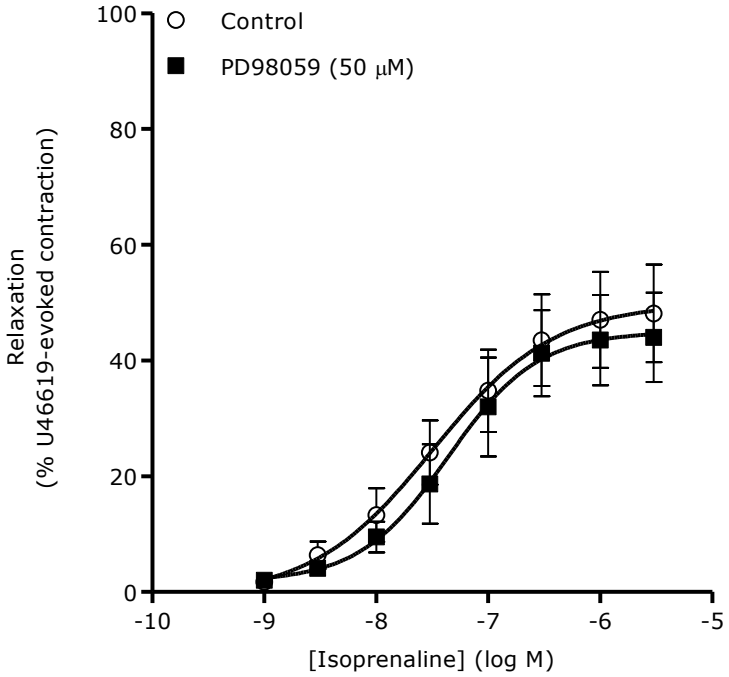

Figure 4.1. *The effect of MEK inhibition on relaxations to the β-AR agonist isoprenaline in ZDF rat aorta (n=6 or 7). Isoprenaline relaxations in control rings (treated with 0.26% (v/v) DMSO) were similar to those in PD98059-treated rings: there were no differences in either pEC_{50} (7.5 ± 0.3 versus 7.4 ± 0.2; P>0.05) or R_{MAX} values (45 ± 5% versus 50 ± 7%; P>0.05).*

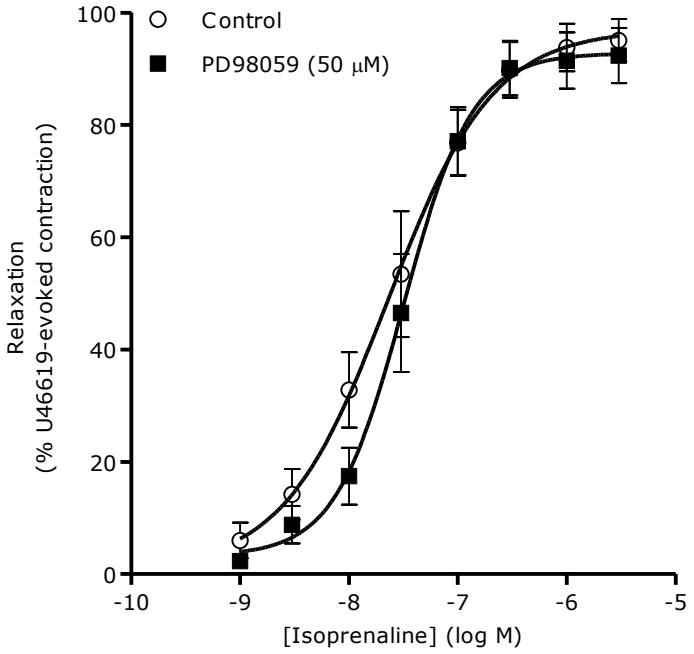

Figure 4.2. *The effect of the MEK inhibitor PD98059 on β-AR-mediated relaxations in Wistar rat aorta (n=5 or 6). PD98059 (50 μM) did not alter relaxation responses to isoprenaline with respect to either pEC_{50} (7.5 ± 0.1 versus 7.6 ± 0.1 in controls; P>0.05) or R_{MAX} (93 ± 5% from 97 ± 5% in controls; P>0.05). Control experiments were performed in (0.26% (v/v) DMSO).*

As shown in Figure 4.1, PD98059 appeared to inhibit isoprenaline-induced relaxations of pre-contracted ZDF aortae, though this effect was not statistically significant (R_{MAX}: 45 ± 5% from 50 ± 7% in controls, *P*>0.05; pEC_{50}: 7.4 ± 0.2 from 7.5 ± 0.3 in controls; *P*>0.05). Likewise, Figure 4.2 shows that PD98059 did not alter isoprenaline responses in Wistar rat aorta, though there appeared to be a trend towards inhibition at lower concentrations of isoprenaline (R_{MAX}: 93 ± 5% from 97 ± 5% in controls; *P*>0.05, two-tailed, unpaired Student's t-test; pEC_{50}: 7.5 ± 0.1 from 7.6 ± 0.1 in controls; *P*>0.05).

Figures 4.3 and 4.4 show the effect of PD98059 on isoprenaline-induced relaxations of SMA from ZDF rats and Wistar rats, respectively. Whilst isoprenaline was able to reverse the U46619-evoked pre-contraction in Wistar SMA almost completely, the responses in ZDF rats were markedly

blunted. In fact, the latter artery failed to respond to high concentrations of isoprenaline (1 μM and 3 μM).

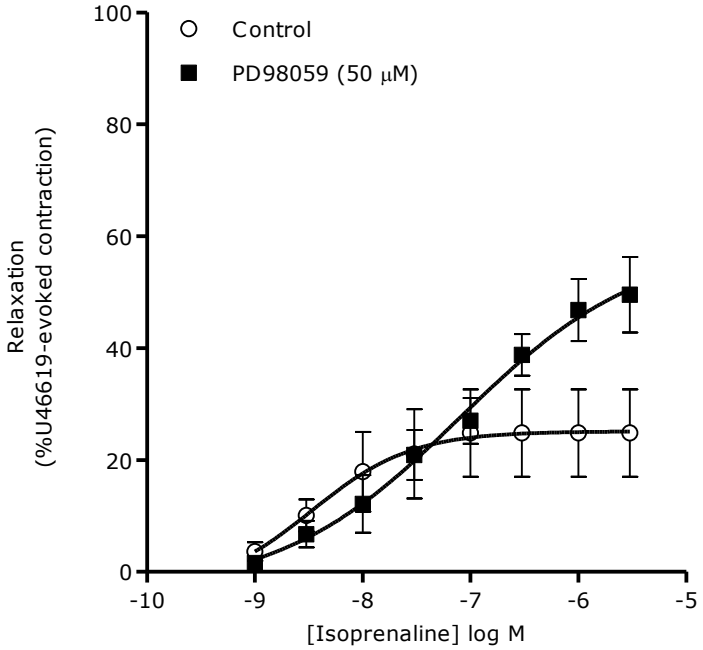

Figure 4.3. *The effect of MEK inhibition on β-AR-mediated relaxations in ZDF rat SMA (n=7). Isoprenaline reversed the U46619-induced contraction by only 25 ± 8% under control conditions (0.26% (v/v) DMSO), and PD98059 (50 μM) enhanced the isoprenaline response (50 ± 7% reversal of U46619-evoked contraction; P<0.05).*

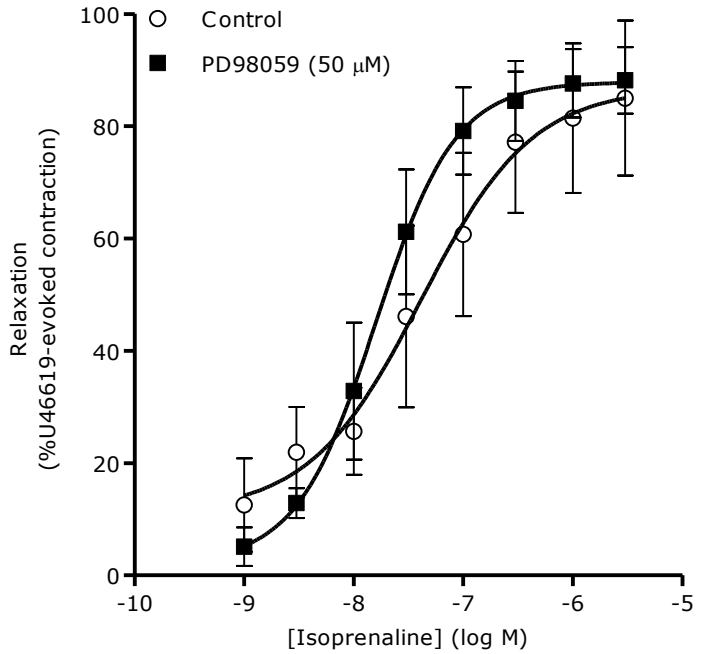

Figure 4.4. *The effect of MEK inhibition on β-AR-mediated relaxations in Wistar rat SMA (n=4). Isoprenaline response curves performed in the presence of 50 μM PD98059 were not significantly different to those performed under control conditions (0.26% (v/v) DMSO).*

As it was not possible to determine R_{MAX} values in the ZDF rat SMA dataset using the computer software, maximal responses are instead quoted as the response to the highest concentration of isoprenaline (3 μM). At 3 μM isoprenaline, ZDF rat SMA relaxed to 25 ± 8% compared to 85 ± 14% in SMA from Wistar rats (P<0.01). PD98059 increased the response in ZDF rat SMA (50 ± 7%; P<0.05) but not Wistar rat SMA (88 ± 6%; P>0.05).

4.3.2. The effect of MEK inhibition on β-AR-mediated vasodilatation in Zucker obese rats

Figure 4.5 shows that isoprenaline induced concentration-dependent relaxations of Zucker obese rat aortae. Isoprenaline reversed the U46619-evoked contractile tone by only 34 ± 8%, compared to 95 ± 4% in Wistar rat aortae (Figure 4.2; P<0.001; values represent responses to 3 μM isoprenaline).

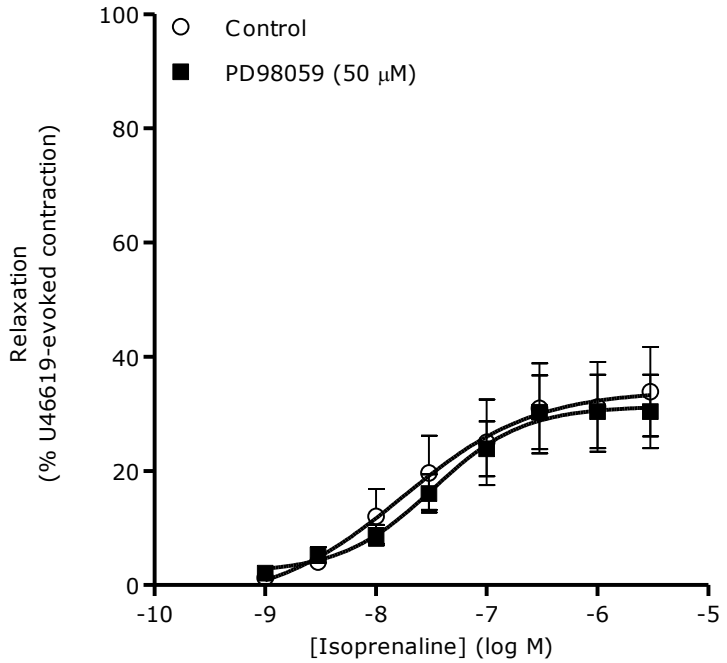

Figure 4.5. *The effect of MEK inhibition on β-AR-mediated relaxations in Zucker obese rat aorta (n=7 or 8). Under control conditions (0.26% (v/v) DMSO), the U46619-evoked contraction was reversed by only 34 ± 8% following the application of 3 μM isoprenaline. The corresponding response in rings exposed to PD98059 (50 μM) was 30 ± 6% (P>0.05).*

PD98059 did not alter the response to isoprenaline in Zucker obese rat aorta (30 ± 6% relaxation to 3 μM isoprenaline; *P*>0.05, two-tailed, unpaired Student's t-test; Figure 4.5). In addition, the concentration of isoprenaline required to produce 50% of the response to 3 μM isoprenaline also did not differ between controls and PD98059 (7.6 ± 0.2 versus 7.7 ± 0.2, respectively; *P*>0.05). In contrast to its effect on Wistar rat SMA (R_{MAX} = 85 ± 14%; Figure 4.4), isoprenaline produced only modest relaxations in Zucker obese rat SMA (R_{MAX} = 28 ± 4%; *P*<0.01; Figure 4.6). PD98059 increased the maximal relaxation response to isoprenaline in Zucker obese rat SMA from 28 ± 4% in controls to 43 ± 9% (*P*<0.05). However, there was no change in the potency (pEC_{50} values of 7.5 ± 0.3 in controls and 7.3 ± 0.4 with PD98059; *P*>0.05).

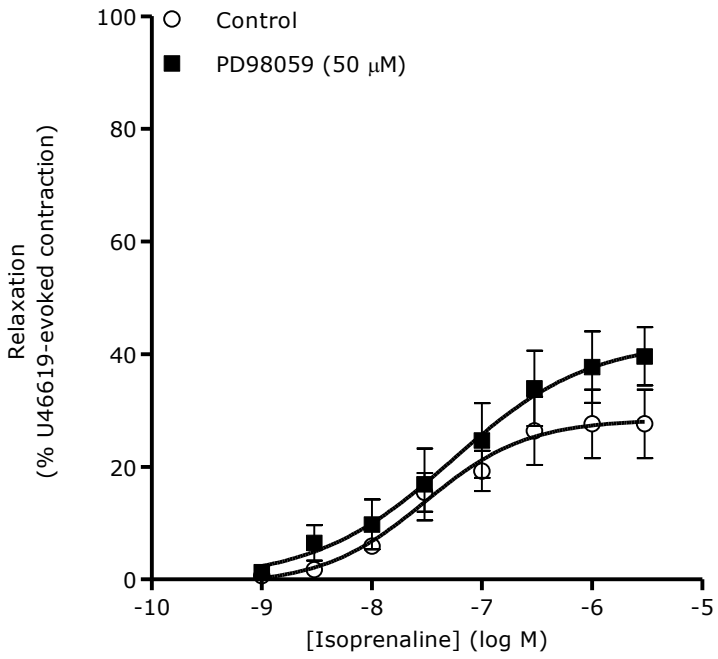

Figure 4.6. *The effect of MEK inhibitor PD98059 (50 μM) on β-AR-mediated relaxations in Zucker obese rat SMA (n=6 or 7). The maximal relaxation to isoprenaline was greater in PD98059-treated SMA rings than in controls (0.26% (v/v) DMSO): 43 ± 9% versus 28 ± 4%; P<0.05.*

4.3.3. Summary of β-AR vasodilatation in ZDF rats and Zucker obese rats

A summary of the differences in β–AR-mediated relaxations that exist between the various rat strains is shown in Table 4.1, along with effects of MEK inhibition.

	Aorta		SMA	
Strain	Relaxations compared to control rats	Effect of MEK inhibition on relaxations	Relaxations compared to control rats	Effect of MEK inhibition on relaxations
Wistar	NA	↔	NA	↔
ZDF	↓	↔	↓	↑
Obese	↓	↔	↓	↑

Table 4.1. *Summary of changes in β-AR-mediated relaxation in rat models of Type II diabetes and obesity. Impairments (↓), enhancements (↑) and no changes (↔) are indicated.*

4.3.4. The effect of TP receptor activation on ERK activation in ZDF rats

A representative blot of western immunoblotting experiments assessing the effect of TP receptor activation on ERK activation in SMA of ZDF and Zucker lean rats is shown in Figure 4.7. The associated densitometric analysis is summarised in Table 4.2. In both preparations, the levels of total ERK1 (tERK1) and tERK2 were unchanged by U46619 ($P>0.05$, two-way ANOVA followed by Bonferroni post-hoc test). In Zucker lean rats, U46619 had no effect on phosphorylated ERK1 (pERK1) or pERK2. However, in ZDF rats U46619 increased the levels of pERK1 and pERK2 by factors of 1.56 and 1.84, respectively ($P<0.01$, two-way ANOVA followed by Bonferroni post-hoc test).

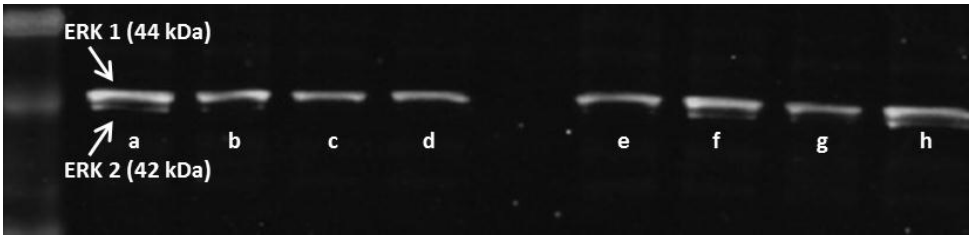

Figure 4.7. *Representative blot of western immunoblotting experiments assessing the effect of TP receptor activation on ERK activation in SMA taken from Zucker lean rats (a-d) and ZDF rats (e-h). Prior to freezing and homogenisation, SMA rings were incubated in tissue baths for 45 mins in the absence (a, c, e and g) or presence (b, d, f and h) of U46619.*

Taken together, the data indicate that treatment with U46619 stimulates ERK activation in ZDF rat SMA, whilst having no effect on ERK activation in SMA of Zucker lean controls.

	Zucker Lean (n=4)		**Zucker Diabetic (n=5)**	
	Basal	*U46619*	*Basal*	*U46619*
pERK1	4.63 (0.8)	4.25 (0.7)	4.72 (0.7)	**7.34 (1.2)****
tERK1	5.45 (0.3)	4.98 (0.2)	5.35 (0.3)	6.19 (0.3)
pERK2	1.68 (0.4)	2.09 (0.8)	2.16 (0.6)	**3.98 (0.7)****
tERK2	2.75 (0.4)	2.83 (0.2)	3.24 (0.3)	3.55 (0.3)

Table 4.2. *The effect of U46619 on the levels of pERK1/2 and tERK1/2 in SMA of ZDF rats and Zucker lean rats. Values are mean (SD) optical densities (arbitrary units) derived from analysis using Odyssey software (LI-COR Biotechnology Ltd., Cambridge, UK). U46619 caused a significant increase (versus basal) in pERK1 and pERK2 activation in ZDF rats but not in Zucker lean rats. ** represents P<0.01 (two-way ANOVA followed by Bonferroni post-hoc test).*

4.3.5. The effect of high glucose concentration on β-AR-mediated vasodilatation

To determine whether the impaired β-AR-mediated vasodilator responses observed in Zucker obese rats and ZDF rats could be replicated *in vitro*, PCA segments were exposed to high glucose conditions in order to mimic the hyperglycaemic state. Salbutamol-induced relaxations of porcine coronary artery segments pre-contracted with U46619 were unaffected by

pre-treatment with high concentrations of glucose at 4°C (Figure 4.8). Control vessels relaxed to a maximum of 83 ± 8% with a pEC_{50} value of 6.0 ± 0.1; 25 mM glucose-incubated vessels relaxed to 94 ± 5% with a pEC_{50} value of 5.8 ± 0.1; and vessels incubated with 19.5 mM mannitol (osmotic control) relaxed to 95 ± 13% with a pEC_{50} value of 5.8 ± 0.2.

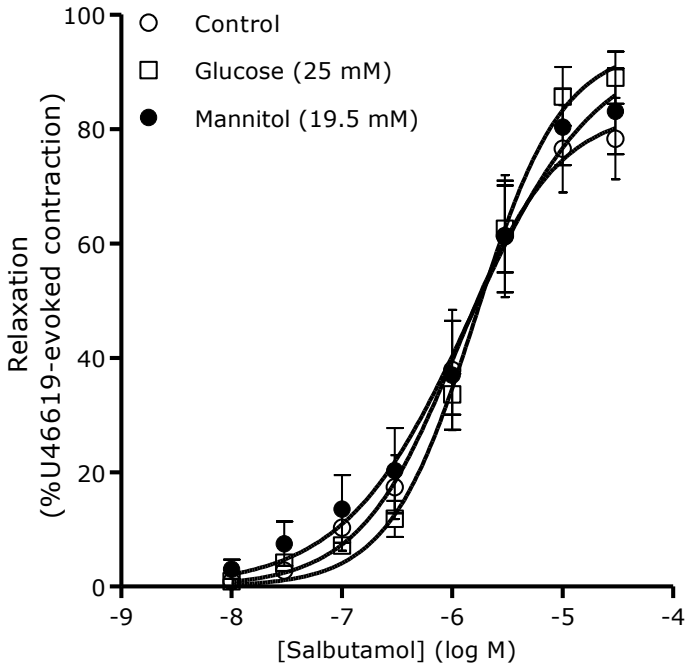

Figure 4.8. *The effect of high glucose concentrations on β-AR-mediated relaxations in PCA rings incubated overnight at 4℃ (n=2). PCA rings had previously been incubated at 4℃ in KH solution containing either 5.5 mM glucose (open circles), 25 mM glucose (open squares) or 5.5 mM glucose/19.5 mM mannitol (osmotic control; closed circles), and experiments were carried out in KH solution containing 5.5 mM glucose. Neither high glucose (25 mM) nor mannitol appeared to alter the relaxation response to salbutamol.*

Figure 4.9 shows the effect of incubating coronary arteries with 25 mM glucose for 16 hours prior to experimenting, as well as during the experiment itself. There was no significant difference between the relaxation characteristics of isoprenaline in each dataset (R_{MAX} and pEC_{50} values: control = 80 ± 7% and 5.8 ± 0.1, 25 mM glucose = 95% and 5.7, 19.5 mM mannitol = 81 ± 5% and 5.6 ± 0.1).

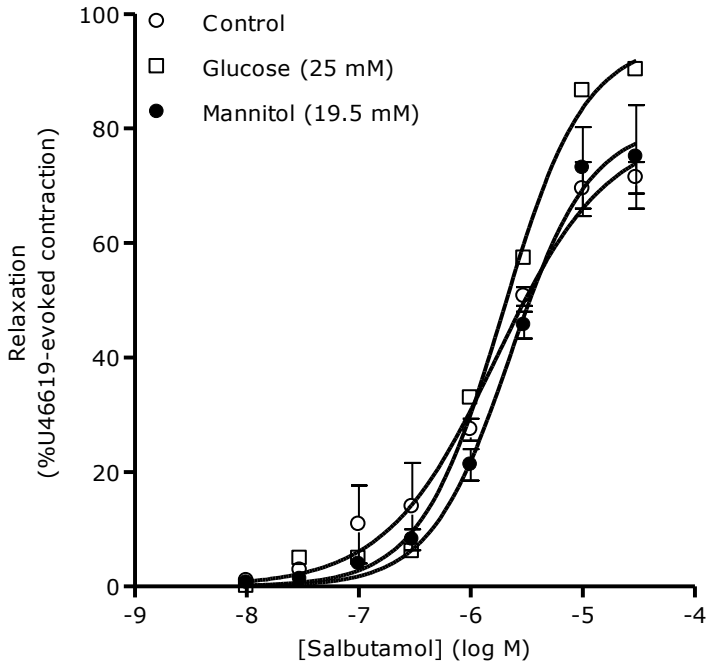

Figure 4.9. *The effect of high glucose concentrations on β-AR-mediated relaxations in PCA rings incubated overnight at 4℃ (n=2). Log concentration–response curves to salbutamol in PCA. Relaxations, shown as means ± SEM (n=1 or 2), are expressed as a percentage of the U46619-evoked contraction. PCA rings had previously been incubated at 4℃ in KH solution containing either 5.5 mM glucose (open circles), 25 mM glucose (open squares) or 5.5 mM glucose/19.5 mM mannitol (osmotic control; closed circles), and experiments were carried out in the corresponding solutions. The relaxation response to salbutamol was not altered by either high glucose (25 mM) or mannitol.*

Coronary arteries incubated in KH solution containing 5.5 mM glucose for 16 hours at 37°C relaxed to 88 ± 4%, compared to 87 ± 8% and 85% in arteries incubated in KH solution containing 25 mM glucose and 19.5 mM mannitol, respectively, in response to salbutamol (Figure 4.10). There appeared to be a trend towards inhibition of the relaxation response as a result of exposure to 25 mM glucose (pEC_{50} values: 7.0 ± 0.1 in controls, 6.3 ± 0.2 in high glucose-incubated arteries, and 6.9 in osmotic controls).

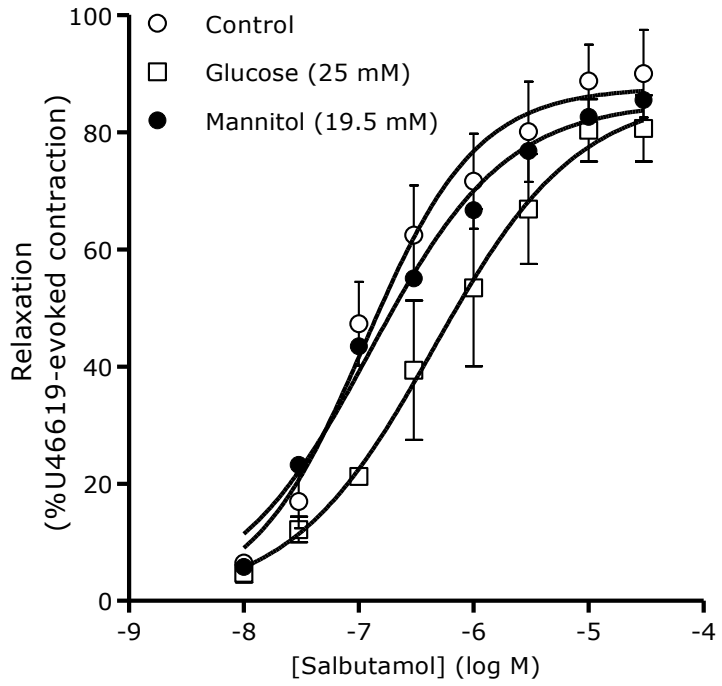

Figure 4.10. *The effect of high glucose concentration on β-AR-mediated relaxations in PCA rings incubated overnight at 37℃ (n=1 or 2). PCA rings had previously been incubated at 37℃ in KH solution containing either 5.5 mM glucose (open circles), 25 mM glucose (open squares) or 5.5 mM glucose/19.5 mM mannitol (osmotic control; closed circles), and experiments were carried out in the corresponding solutions. Incubation with glucose (25 mM) but not mannitol appeared to inhibit salbutamol relaxations.*

4.4. DISCUSSION

In this Chapter, animal models of diabetes and obesity were used in conjunction with isometric tension and immunoblotting techniques to assess the role of ERK in regulating blood vessel tone in these disease states. Two blood vessels derived from Zucker obese rats and Zucker diabetic fatty (ZDF) rats were assessed for β-AR-mediated vasodilatation, namely the thoracic aorta and superior mesenteric artery (SMA).

Isoprenaline-induced relaxations of arteries taken from Zucker obese rats and ZDF rats were significantly impaired as compared to corresponding measurements in arteries from Wistar rats. These findings are consistent with the observation in a previous study that mesenteric blood flow responses to isoprenaline were markedly blunted in Zucker obese rats (D'Angelo *et al.*, 2006). Similar impairments of vasodilator responses to isoprenaline have been observed in skeletal muscle arterioles from ZDF rats (Lesniewski *et al.*, 2008). The observation that MEK inhibition failed to enhance β-AR-mediated relaxations in healthy Wistar rats is not consistent with findings from corresponding experiments in healthy pigs (Section 2.3.1). Different levels of basal and agonist-stimulated ERK activation between the two species may explain this discrepancy.

In this study, it was difficult to determine whether reduced vasodilatation in diseased rat arteries was accompanied by exaggerated contractile responses as cumulative concentration-contraction curves to vasoconstrictors were not performed. Nonetheless, detailed assessments of vascular reactivity in these models have been performed elsewhere, with reports of augmented contractility in Zucker obese rats (Frisbee, 2004;

Ouchi *et al.*, 1996) and ZDF rats (Belin de Chantemele *et al.*, 2009; Lesniewski *et al.*, 2008).

A combination of impaired vasodilatation and enhanced contractility would be expected to compromise the ability of Zucker obese rats and ZDF rats to adequately regulate blood flow and maintain physiological arterial pressures. Although changes were detected in the function of both the thoracic aorta and SMA of diseased rats, it is more likely that alterations in the latter are more relevant. Owing to its proximity to the mesenteric vascular bed and smaller lumen diameter (roughly 0.5 mm versus 2 mm in aorta), the SMA may be a more important contributor to vascular resistance than the thoracic aorta, which mainly serves as a conduit vessel. However, investigations of higher-order mesenteric arteries may be warranted, as it is thought that arteries with lumen diameters of less than 0.4 mm are the most relevant to vascular resistance (Intengan *et al.*, 2000).

As demonstrated in Chapter 2, ERK activation is associated with reduced β-AR-mediated vasodilatation in porcine coronary arteries (PCA). To test the hypothesis that ERK contributes to the observed impairment of β-AR-mediated vasodilatation in obese and diabetic rats, experiments were carried out in the absence and presence of the MEK inhibitor PD98059. In SMA of Zucker obese rats and ZDF rats, PD98059 partially restored functional responses to isoprenaline, whereas no enhancement was observed in thoracic aorta. These findings suggest that ERK activation may be increased in SMA but not in aorta. Although biochemical assessments of ERK activation in aorta were not performed, immunoblotting experiments using SMA showed an increased U46619-induced activation of ERK in ZDF rats as compared to Zucker lean rats. An alternative interpretation is that

SMA and aorta may exhibit similar ERK activation characteristics, but ERK does not regulate β–AR-mediated vasodilatation in aorta. Additionally, given that β_2- but not β_1-AR-mediated vasodilatation was enhanced by PD98059 (Section 2.3.2), β_2-ARs may be more relevant in isoprenaline-induced relaxations of SMA, whilst the β_1 subtype may predominate in aorta. In future immunoblotting experiments, a "housekeeping" protein such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) would be employed in order to correct for protein loading and thus substantiate changes, if any, in pERK and tERK levels.

Although PD98059 partially corrected isoprenaline-induced relaxations of SMA from ZDF rats and Zucker obese rats, the degree of enhancement appeared greater in the former preparation. Again, this may be related to differences in ERK activation and/or the relative expression of β-AR subtypes between the two preparations. The partial, rather than complete, correction of isoprenaline-induced responses by PD98059 may reflect incomplete inhibition of ERK activation at the concentration of PD98059 used in the experiment (50 μM). A more likely explanation is that additional signalling pathways may contribute to the impairment of β-AR-mediated vasodilatation. A possible candidate is the MAPK p38, the activation of which in VSM cells has been linked to high glucose concentrations (Igarashi *et al.*, 1999). In fact, data presented in the current investigation suggests a role for p38 MAPK in β-AR-mediated vasodilatation (Figure A5, Appendix). However, further experimentation would be required to confirm the involvement of p38 MAPK in impaired β-AR-mediated vasodilatation in ZDF rats and Zucker obese rats.

Regulation of vascular tone by the endothelium has been the subject of considerable research in recent decades (Mas, 2009). Endothelial function

has previously been investigated in rat models of Type II diabetes. Initially, Bohlen *et al.* (1995) demonstrated that endothelial-dependent vasodilatation of intestinal arterioles were comparable between ZDF rats and Zucker lean rats. In contrast, it was later shown in a different rat model, namely the Otsuka Long-Evans Tokushima Fatty (OLETF) rat, that endothelium-dependent relaxation of thoracic aorta was reduced relative to non-diabetic controls (Sakamoto *et al.*, 1998).

In the present study, it is not known whether the blunted β-AR-mediated vasodilatation observed in arteries from ZDF rats (12-week-old) was caused by endothelial dysfunction. However, a recent study showed that impaired β-AR-mediated relaxation of coronary artery from 12-week-old ZDF rats was not accompanied by endothelial dysfunction (Grisk *et al.*, 2007). This is in agreement with an earlier study which reported that endothelial dysfunction emerged after 16 – 24 weeks in ZDF rats (Oltman *et al.*, 2006). Furthermore, β-AR-mediated relaxation of dorsal hand vein was shown to be reduced in diabetic patients as compared to controls, whilst endothelium-dependent relaxations were similar between the two groups (Harada *et al.*, 1999). Together, these data suggest that the impairment of β-AR-mediated vasodilatation in Type II diabetes may precede endothelial dysfunction.

Investigations in diabetic humans have also demonstrated impairments of endothelium-dependent vasodilatation. Gazis *et al.* (1999) showed that acetylcholine (ACh)-induced increases of forearm blood flow was blunted in diabetic subjects compared to controls. Blood flow changes to sodium nitroprusside (SNP), an endothelium-independent vasodilator, were similar between the two groups, suggesting that endothelium dysfunction was responsible for the impairment of ACh-mediated vasodilatation. However,

these findings were confounded by the fact that systolic blood pressure, triglycerides and HDL cholesterol were also significantly different between the two groups. Impaired endothelium-dependent vasodilatation has also been shown in *in vivo* studies of human coronary artery (Nitenberg *et al.*, 1993) and brachial artery (Enderle *et al.*, 1998). The mechanisms by which Type II diabetes reduce endothelium-dependent vasodilatation are not fully understood. However, it is possible that the release and/or signalling of endothelium-derived vasodilator substances, such as nitric oxide (NO) and endothelium-derived hyperpolarising factor (EDHF), are in some way disrupted.

In Zucker obese rats, impaired endothelium-dependent vasorelaxations have been demonstrated in several blood vessels. In aorta of 16-week old rats, for example, endothelium-dependent vasorelaxation was blunted and accompanied by enhanced vasoconstriction, possibly via changes in Akt- and Rho-dependent pathways, respectively (Nishimatsu *et al.*, 2005), Furthermore, it was shown in penile arteries of 17 – 18-week-old Zucker obese rats that impaired ACh-induced relaxation responses were related to an inability of prostanoids to mediate vasodilatation (Sanchez *et al.*, 2010). Again, it is not known whether the impaired β-AR-mediated vasodilatation observed in Zucker obese rats in the current study was associated with altered endothelial function. Although previous studies have shown reduced endothelial function in this model, the animals used were older than those used in the current study (16 – 18-week-old versus 12-week-old). Furthermore, Oltman *et al.* (2006) demonstrated that endothelium dysfunction occurs after 28 weeks in Zucker obese rats. Human studies have also reported abnormal endothelial function in obese individuals compared to lean controls (Oflaz *et al.*, 2003; Weil *et al.*, 2011; Worthley *et al.*, 2009).

It has been frequently reported that K^+ channel activity is reduced in blood vessels of human diabetics (Irat *et al.*, 2006; Miura *et al.*, 2003). As discussed in Chapter 3, the inhibitory actions exerted by ERK on β-AR-mediated vasodilatation may involve an interaction with K^+ channels. Activation of ERK in U46619-stimulated SMA from ZDF rats was significantly higher than in lean rats. This may have produced impaired β-AR-mediated vasodilatation via a greater disruption of K^+ channels. To test this hypothesis further, the effects of K^+ channel blockers and openers, in conjunction with MEK inhibitors, would need to be studied in arteries from ZDF rats.

Similar experiments in other laboratories have demonstrated that K^+ channel function in rat models of diabetes may be altered. For instance, ATP-sensitive K^+ channels (K_{ATP}) and calcium-activated K^+ channels (K_{Ca}) in arteries from fructose-fed, insulin-resistant rats have been shown to function abnormally (Erdos *et al.*, 2002). A later study showed, using voltage clamp recordings, that NS1619, a selective large-conductance, calcium activated K^+ (BK_{Ca}) channel opener, produced significantly smaller currents in mesenteric artery smooth muscle cells derived from ZDF rats compared to those from their lean littermates (Burnham *et al.*, 2006).

The BK_{Ca} channel is activated by both membrane depolarisation and increases in intracellular Ca^{2+}, and, in addition to mediating responses to vasodilator agents, may also play a role in modulating vasoconstriction (Nelson *et al.*, 1995a). Thus, an expected consequence of reduced BK_{Ca} channel function would be enhanced contractile responses due to a loss of this regulatory mechanism. It is not possible to endorse this hypothesis based on the data presented in the current study; as mentioned earlier, cumulative contraction-response curves to vasoconstrictors were not

performed. Nonetheless, the clinical significance of these findings is highlighted by a previous study in mice, where disruption of BK_{Ca} channel function via genetic deletion of the β1 subunit resulted in hypertension (Pluger *et al.*, 2000). Moreover, based on investigations of a rat model of hypertension, Amberg *et al.* (2003) proposed that loss of BK_{Ca} channel function, via reduced expression of the β1 subunit, may contribute to the development of hypertension.

As discussed earlier, the effects of long-term hyperglycaemia on vascular function have been intensely studied, both in humans and animal models of Type II diabetes. In attempts to replicate hyperglycaemic conditions, porcine coronary artery (PCA) rings were incubated in Krebs-Henseleit (KH) solution containing 25 mM glucose at 4°C and 37°C. Glucose concentrations as high as 44 mM have previously been used to mimic hyperglycaemia (Tesfamariam *et al.*, 1990; Weisbrod *et al.*, 1993). In the present investigation, potential osmotic effects due to high glucose concentration (25 mM) were controlled for through the use of an osmotically equivalent concentration of mannitol (19.5 mM). Cumulative concentration-relaxation curves to salbutamol were subsequently performed and the results suggest that osmotic effects were not important, as mannitol failed to alter salbutamol-induced relaxations in all experiments. Overnight incubation with 25 mM glucose at 4°C had no effect on the relaxation characteristics of salbutamol, even when the arteries were exposed to 25 mM glucose during the course of the experiment. Overnight incubation of arteries at 37°C appeared to shift the concentration-relaxation curves leftwards when compared to values measured following overnight incubation at 4°C (pEC_{50} values for control arteries = 7.0 ± 0.1 at 37°C and 5.8 ± 0.1 at 4°C). Temperature has been shown to influence vasorelaxation (Garcia-Villalon *et al.*, 1995; Saito *et al.*,

1998), possibly by altering ion channel activity. However, in the present experiment, all relaxation responses were recorded at the same temperature, 37°C, suggesting the enhanced relaxation was due to overnight incubation at a higher temperature.

Whereas salbutamol-induced relaxations were unaffected by high glucose treatment at 4°C, relaxations following incubation with high glucose at 37°C appeared to be inhibited, as shown by a modest right-shift of the relaxation curve. Maximal relaxation responses, however, were unaffected by high glucose treatment, in contrast to the dramatic reductions of maximal relaxations observed in arteries from diabetic rats. This difference suggests that chronic exposure to high glucose produce greater deficits of β-AR-mediated vasodilatation than acute exposure. In any case, further experimentation relating to the acute effects of high glucose concentrations is warranted as the small number of repeats in each experiment may have obscured findings. It would also be worth investigating the effect of this treatment on ERK activation, and whether inhibition of ERK activation restores relaxation capacity.

The finding that U46619-stimulated ERK activation was greater in ZDF rats than in lean controls. Increased ERK activation in ZDF rats may occur via a number of mechanisms. One possibility is the hyperglycaemic state *per se*, as high glucose treatment has been associated with marked increases in ERK phosphorylation in range of tissues (Bandyopadhyay *et al.*, 2000; Cohen *et al.*, 2003; Farrokhnia *et al.*, 2005; Natarajan *et al.*, 1999). Secondly, by inducing the non-enzymatic glycation of various plasma proteins and lipids, chronic hyperglycaemia accelerates the production of advanced glycation end-products (AGEs), which are implicated in diabetes complications and have been shown to activate ERK (Berrou *et al.*, 2009;

Huang *et al.*, 2008). Furthermore, hyperglycaemia-related activation of ERK may also occur via intermediate glycated products. For instance, glycated albumin, which, incidentally, is recognised as a reliable marker of short-term glycaemic control in diabetic patients, has been shown to stimulate ERK activity in VSM and other tissues (Cohen *et al.*, 2003; Hattori *et al.*, 2001; Treins *et al.*, 2001).

Both the ZDF rat and Zucker obese rat strains are characterised by hyperlipidaemia, with overtly raised triglyceride and cholesterol levels compared to healthy, lean rats (Hoshida *et al.*, 2000; Liszka *et al.*, 1998). In humans, it has long been known that derangements of lipid levels present an increased risk of cardiovascular disease (Keys *et al.*, 1984; Stamler *et al.*, 1986). Free fatty acids (FFAs), as well as elevated glucose levels, were reported to activate PKC and reactive oxygen species (ROS) (Inoguchi *et al.*, 2000), both of which are implicated in cardiovascular complications of diabetes (Idris *et al.*, 2001; Jay *et al.*, 2006). Moreover, low-density lipoprotein (LDL), known to contribute to the development of atherosclerosis and endothelial dysfunction, was shown to activate ERK in human endothelial cells and rat aortic smooth muscle cells (Pintus *et al.*, 2003; Velarde *et al.*, 2001).

The fatty acid arachidonic acid (AA) and its numerous eicosanoid metabolites are recognised as key regulators of diverse functions. In blood vessels, for instance, accumulations of prostacyclin and thromboxane, both cyclooxygenase (COX) products of AA metabolism, are respectively associated with contraction and relaxation of VSM, reflecting a contractile phenotype (Shibamoto *et al.*, 1995; Shimokawa *et al.*, 1988). Previous studies have linked AA/eicosanoids with ERK activation in blood vessels. For example, a lipoxygenase (LOX) metabolite of AA, namely leukotriene

B_4 (LTB_4), was recently shown to mediate VSM cell migration via activation of ERK2 (Moraes *et al.*, 2010). These signalling mechanisms may be more relevant in disease states characterised by hyperlipidaemia and/or increased ERK activation. Thus it is possible that such disease states may exhibit altered regulation of processes such as cell proliferation and migration. The resulting changes in blood vessel structure, reflecting a shift from a contractile to a proliferative phenotype, would likely produce functional deficits similar to those observed in the current study.

In summary, β-AR-mediated vasodilatation was impaired in rat models of Type II diabetes and obesity. This impairment was observed both in SMA and thoracic aorta of Zucker obese rats and ZDF rats. Inhibition of ERK activation partially restored β-AR-mediated relaxations in SMA but not in aorta. Furthermore, TP receptor-stimulated ERK activation was elevated in SMA of ZDF rats compared to those of Zucker lean rats. In experiments designed to mimic the hyperglycaemic state, β-AR-mediated vasodilatation appeared to be blunted by high glucose concentrations, though a greater number of repeat experiments are required. Taken together, these data suggest that impairments of β-AR-mediated vasodilatation caused by Type II diabetes and obesity may occur as a result of increased ERK activation. Further experimentation would be required to determine the precise mechanism(s) involved, including a possible role for ERK in the regulation of K^+ channels.

CHAPTER 5: THE EFFECT OF STATINS ON β-ADRENOCEPTOR-MEDIATED VASODILATATION

5.1. INTRODUCTION

Statins are widely used in the prevention and treatment of cardiovascular disease. Their cholesterol-lowering effects are caused by the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which prevents the formation of mevalonate, a key precursor in the biosynthesis of cholesterol (Figure 1.8).

HMG-CoA reductase is also involved in the formation of the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which in turn play key roles in the post-translational modification of GTP-binding proteins (also known as GTPases) such as Ras and Rho. These modifications occur via prenylation, the covalent attachment of an isoprenoid group to the GTPase, allowing it associate with the cell membrane, where it is activated. By inhibiting HMG-CoA reductase, statins may decrease levels of FPP and GGPP and therefore prevent the activation of Ras and Rho. This mechanism might underlie the beneficial effects of statins unrelated to cholesterol lowering.

As shown in Chapter 2, inhibition of extracellular signal-regulated kinase (ERK) activation results in the enhancement of relaxation responses to β-adrenoceptor (β-AR) agonists. In the aforementioned experiments, specific inhibitor compounds, namely PD98059, PD184352 and U0126, were used to prevent activation of ERK. However, it is possible that inhibition of ERK activation may be achieved using statins due to their ability to inhibit the activation of Ras, which lies upstream of ERK. This raises the possibility of extending the clinical use of statins to include inhibition of ERK activation in order to enhance β-AR-mediated vasodilatation, and is supported by the following observations. Firstly, in a mouse osteosarcoma cell line, statins

were shown to disrupt ERK signalling via inhibitory effects on GGPP biosynthesis (Tsubaki *et al.*, 2011); secondly, lipopolysaccharide-stimulated activation of ERK in mononuclear cells was inhibited by simvastatin, and this inhibition was reversed by GGPP (Sundararaj *et al.*, 2008).

The aim of these experiments was to determine the effect of statins on relaxations induced by β-AR agonists in order to assess their potential utility as enhancers of β-AR-mediated vasodilatation in the clinical setting.

5.2. MATERIALS AND METHODS

5.2.1. Tissue preparation

Porcine coronary artery (PCA) rings were prepared and set up in a Mulvany wire myograph as described in Section 2.2.1.1.

5.2.2. Experimental procedure

5.2.2.1. The effect of statins on β-AR-mediated relaxation

The effects of various statins on the relaxation characteristics of salbutamol were assessed. Artery rings were contracted with U46619 to 60–85% of their maximal response to KCl (60 mM) after a 45 min incubation period with one of the following statins, as shown in Table 5.1: simvastatin (5 μM and 10 μM), lovastatin (10 μM), pravastatin (10 μM) and simvastatin Na^+ (5 μM). Control tissues received vehicle only (0.09% (v/v) DMSO for 10 μM simvastatin/lovastatin and 0.05% (v/v) DMSO for 5 μM simvastatin/lovastatin; the water-soluble pravastatin and Na^+ salt of simvastatin did not require vehicle control).

The concentration of U46619 required to pre-contract arteries to 60–85% was 10 nM – 20 nM with the exception of arteries treated with simvastatin (5 μM) and lovastatin, which required 20 nM – 50 nM, and simvastatin (10 μM) which required in excess of 100 nM. In the latter case, subsequent attempts to induce relaxations with salbutamol were unsuccessful.

Statin	Concentration used	Reference
Simvastatin	5 μM, 10 μM	Nagaoka *et al.* (2007)
Lovastatin	10 μM	Fatehi-Hassanabad *et al.* (2006)
Pravastatin	10 μM	Sonmez Uydes-Dogan *et al.* (2005)
Simvastatin Na^+	5 μM	Seto *et al.* (2007)

Table 5.1. *Concentrations of statins used in the current experiments. Arteries were incubated with a statin for 45 min prior to contraction with U46619 and relaxation with salbutamol, forskolin, pinacidil or NS1619.*

5.2.2.2. The effect of statins on cAMP-mediated relaxation

In related experiments, arteries incubated for 45 min with simvastatin (5 μM) and contracted with U46619 were relaxed using cumulative concentrations of the adenylyl cyclase (AC) activator forskolin (1 nM – 3 μM), the ATP-sensitive K^+ (K_{ATP}) channel opener pinacidil (1 nM – 30 μM(Gojkovic-Bukarica *et al.*, 1999) or the large-conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channel opener NS1619 (10 nM – 30 μM; (Edwards *et al.*, 1994).

5.2.2.3. The effect of mevalonate supplementation on statin-induced inhibition of β-AR-mediated relaxation

In order to determine the involvement of HMG-CoA reductase in the effect of statins on salbutamol-induced relaxations, PCA were incubated with simvastatin (5 μM), mevalonolactone (250 μM) or both agents in combination for 45 min. Control tissues received vehicle only (0.09% (v/v) ethanol for 250 μM mevalonolactone). U46619 was subsequently applied to pre-contract the vessels before relaxations were evoked using salbutamol.

5.2.2.4. The effect of Rho kinase inhibition on β-AR-mediated relaxation

In a separate set of experiments, arteries were incubated for 45 mins in Krebs-Henseleit (KH) solution containing the Rho kinase inhibitor Y27632 (5 μM) before being contracted with U46619. In vitro experiments investigating the effect of Y27632 typically use a concentration of 10 μM (Maekawa *et al.*, 1999). However, in the current experiment Y27632 caused a powerful impairment of the U46619-evoked pre-contraction at that concentration, preventing the establishment of a stable contractile tone. The impairment was much less significant at 5 μM, and the concentration of U46619 required to pre-contract Y27632-incubated arteries was 50 nM – 100 nM, compared to 10 nM – 20 nM in control arteries. Finally, cumulative concentrations of salbutamol were applied to pre-contracted arteries in order to induce relaxation.

5.2.3. Statistical analyses

Data were analysed using GraphPad Prism 5.0 (Graphpad Software Inc., La Jolla, CA, USA). Relaxation response measurements are expressed as means ± SEM. Unless otherwise stated, statistical comparisons between groups were made using two-way ANOVA followed by a Bonferroni post-test. As there were insufficient repeat experiments to determine distribution, the data were assumed to be normally distributed and parametric tests were employed. A P value < 0.05 was considered statistically significant. In all experiments, "n" numbers represent the number of animals from which tissues were obtained.

5.2.4. Materials

(2S)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-Hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthalenyl-2-methyl butanoate (lovastatin), (βR,δR,1S,2S,6S,8S,8aR)-1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-[(2S)-2-methyl-1-oxobutoxy]-1-naphthaleneheptanoic acid monosodium salt (pravastatin sodium salt) and (1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthalenyly-2,2-dimethyl butanoate (simvastatin) were acquired from Tocris Bioscience (Bristol, UK). Simvastatin sodium salt was acquired from Calbiochem (Nottingham, Nottinghamshire, UK). (±)-β-Hydroxy-β-methyl-δ-valerolactone (mevalonolactone), 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS 1619) and (±)-N-cyano-N-4-pyridinyl-N”-(1,2,2-trimethylpropyl)guanidine monohydrate (pinacidil) were acquired from Sigma (Poole, Dorset, UK).
The remaining chemicals were purchased as stated in Section 2.2.4.

5.3. RESULTS

5.3.1. The effect of statins on β-AR-mediated vasodilatation

Salbutamol-induced relaxations of porcine coronary artery ring segments pre-contracted with U46619 were significantly inhibited by simvastatin and lovastatin (Figures 5.1 and 5.2, respectively). Simvastatin (5 μM) reduced the response to 30 μM salbutamol from 90 ± 13% in controls to 26 ± 12% (P<0.001; n=4), whilst also significantly inhibiting salbutamol responses at 10 μM, 3 μM (P<0.001) and 1 μM (P<0.05).

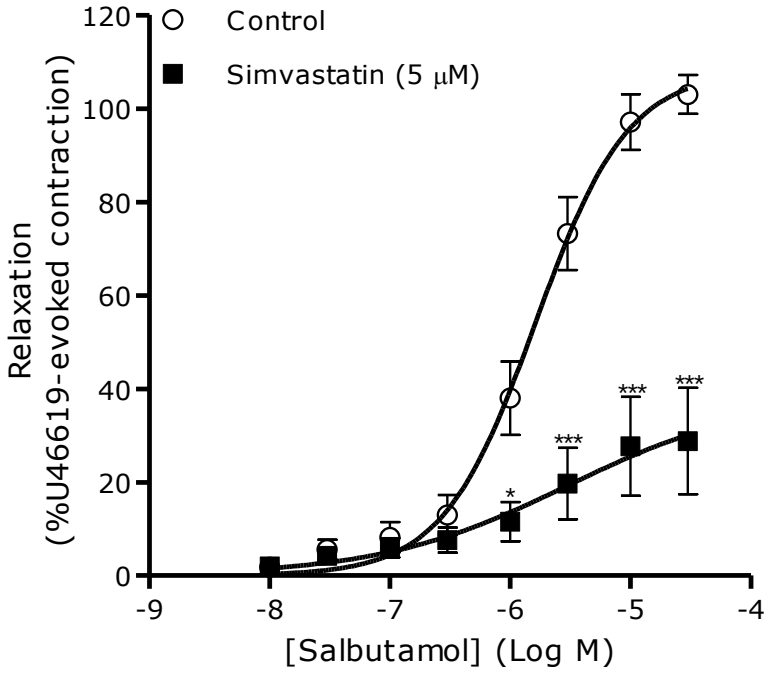

Figure 5.1. *The effect of simvastatin on β-AR-mediated relaxations in PCA (n=4).*
*Simvastatin (5 μM) inhibited relaxations induced by salbutamol. Control experiments were pre-treated with 0.05% (v/v) DMSO. * indicates P<0.05 and *** indicates P<0.001.*

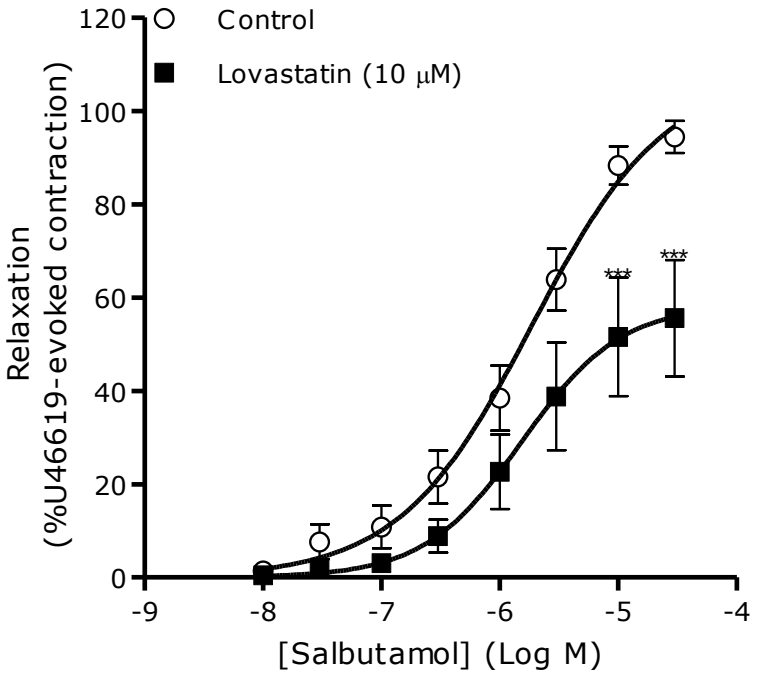

Figure 5.2. *The effect of lovastatin on β-AR-mediated relaxations in PCA (n=7–9). Relaxations to salbutamol were blunted in the presence of lovastatin (10 μM). Control experiments were performed with 0.05% (v/v) DMSO. *** indicates P<0.001.*

Salbutamol was unable to relax pre-contracted arteries following incubation with simvastatin (10 μM). Lovastatin inhibited relaxation responses to salbutamol at 30 μM (94 ± 3% in controls to 56 ± 12% in lovastatin-incubated arteries; $P<0.001$; n=7–9) and 10 μM ($P>0.001$). Figures 5.3 and 5.4 show that neither pravastatin ($P>0.05$; n=4) nor simvastatin Na^+ ($P>0.05$; n=5 or 6) altered relaxation responses to salbutamol.

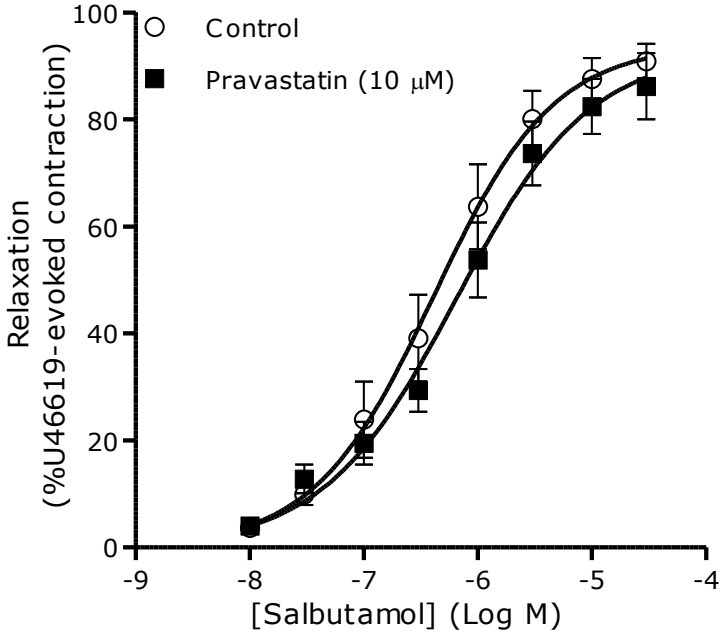

Figure 5.3. *The effect of the hydrophilic statin pravastatin on β-AR-mediated relaxations in PCA (n=4). Salbutamol-induced relaxations were unaltered in the presence of pravastatin.*

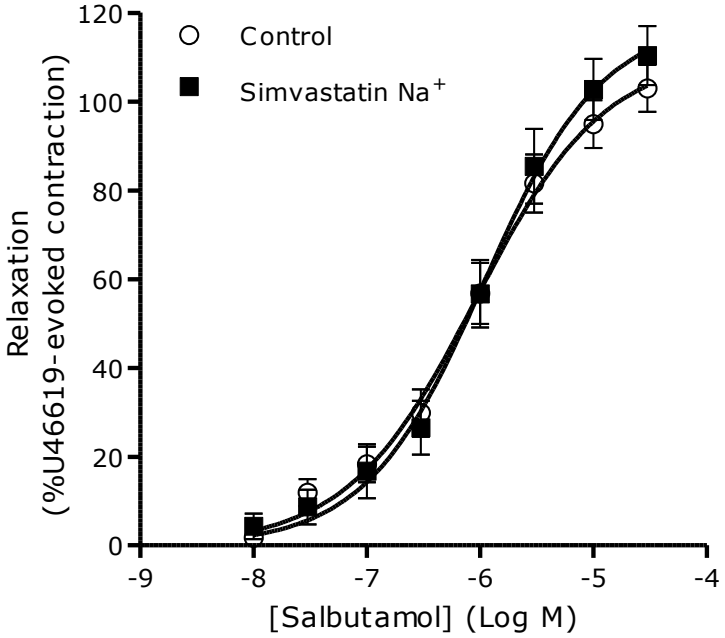

Figure 5.4. *The effect of the Na^+ salt of simvastatin on β-AR-mediated vasodilatation in PCA (n=5 or 6). The hydrophilic simvastatin Na^+ (5 μM) did not affect relaxation responses to salbutamol.*

5.3.2. The effect of statins on cAMP-mediated vasodilatation

Simvastatin (5 μM) had no effect on the relaxation characteristics of forskolin in porcine coronary arteries pre-contracted with U46619 (n=4 or 5, Figure 5.5). Maximum response values were 124 ± 4% in controls and 118 ± 4% in simvastatin-incubated arteries (P>0.05), whilst pEC_{50} values were 7.4 ± 0.1 and 7.3 ± 0.1, respectively (P>0.05).

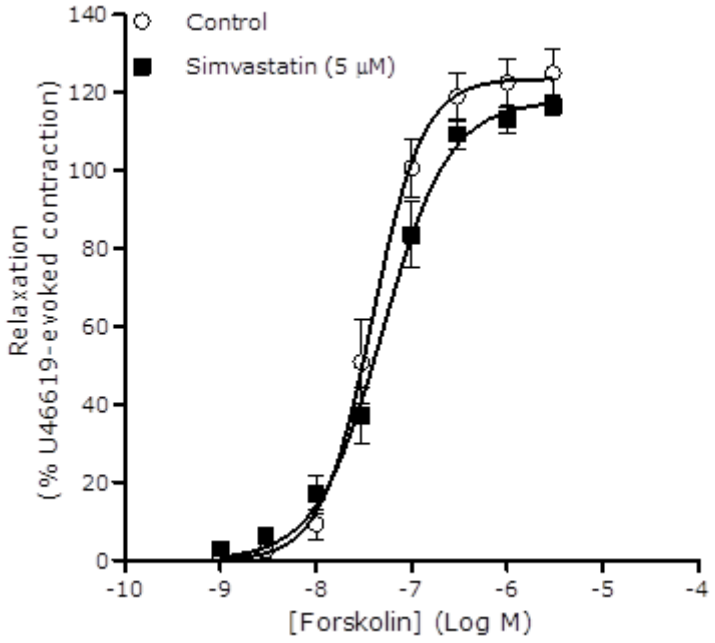

Figure 5.5. *The effect of simvastatin on the AC activator forskolin in PCA (n=4 or 5). Relaxation responses to forskolin were not altered by simvastatin (5 μM). Control experiments were conducted in (0.05% (v/v) DMSO).*

5.3.3. The effect of statins on K^+ channel-mediated vasodilatation

Cumulative concentrations of the BK_{Ca} channel opener NS1619 (10 nM – 30 μM) relaxed pre-contracted artery segments in a concentration-dependent manner (Figure 5.6). Simvastatin (5 μM) inhibited responses to NS1619 at 1 μM, 3 μM, 10 μM (P<0.001; n=5 or 6) and 30 μM (P<0.05).

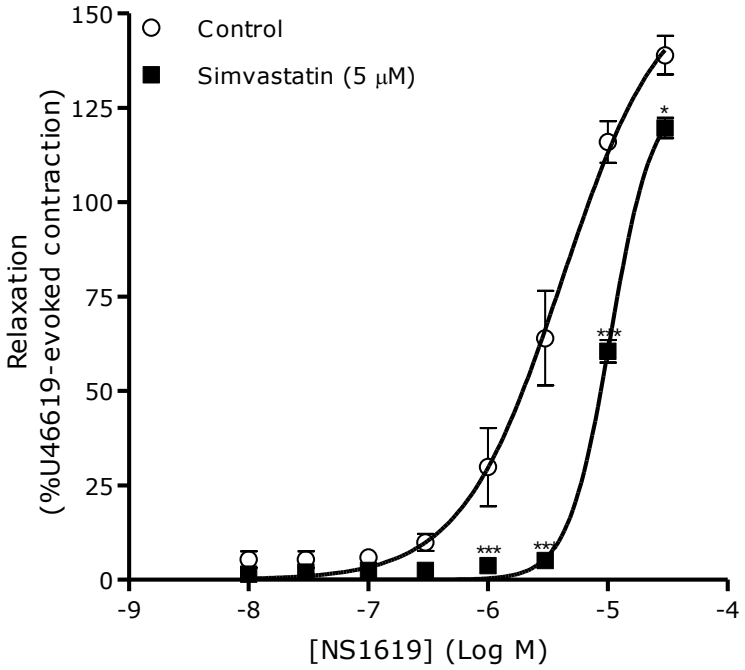

Figure 5.6. *The effect of simvastatin on relaxations to the BK_{Ca} channel opener NS1619 in PCA (n=5 or 6). Simvastatin (5 μM) inhibited NS1619-induced relaxations. Control recordings were made in 0.05% (v/v) DMSO. * indicates P<0.05 and *** indicates P<0.001.*

The K_{ATP} channel opener pinacidil (1 nM – 30 μM) also evoked a concentration-dependent relaxation of pre-contracted coronary artery ring segments (Figure 5.7) and was inhibited by 5 μM simvastatin (responses to pinacidil at 1 μM, 3 μM, 10 μM and 30 μM were significantly inhibited; *P*<0.001; n=3 or 4).

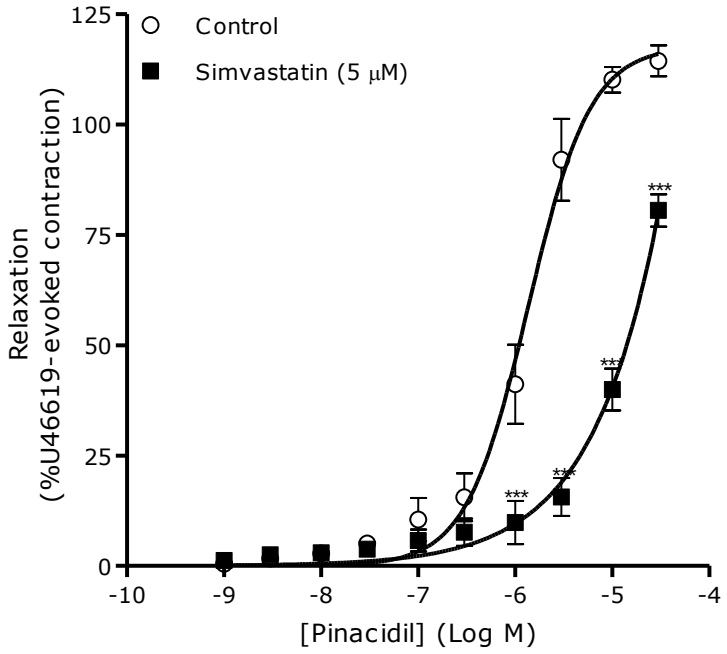

Figure 5.7. *The effect of simvastatin on relaxations to the K_{ATP} channel opener NS1619 in PCA (n=3 or 4). Pinacidil produced concentration-dependent relaxations that were inhibited by simvastatin (5 μM). Control recordings were made in 0.05% (v/v) DMSO. * indicates P<0.05 and *** indicates P<0.001.*

5.3.4. The effect of mevalonate supplementation on the inhibitory effect of statins

Simvastatin (5 μM) inhibited salbutamol-induced relaxations at 1 μM (P<0.05), 3 μM, 10 μM and 30 μM (P<0.001; n=4–6; Figure 5.8). Mevalonolactone (250 μM) alone did not alter the relaxation characteristics of PCA to salbutamol. In addition, relaxations carried out in the presence of both mevalonolactone and simvastatin were inhibited at 3 μM, 10 μM and 30 μM salbutamol (P<0.001) but did not differ from those carried out in the presence of simvastatin alone.

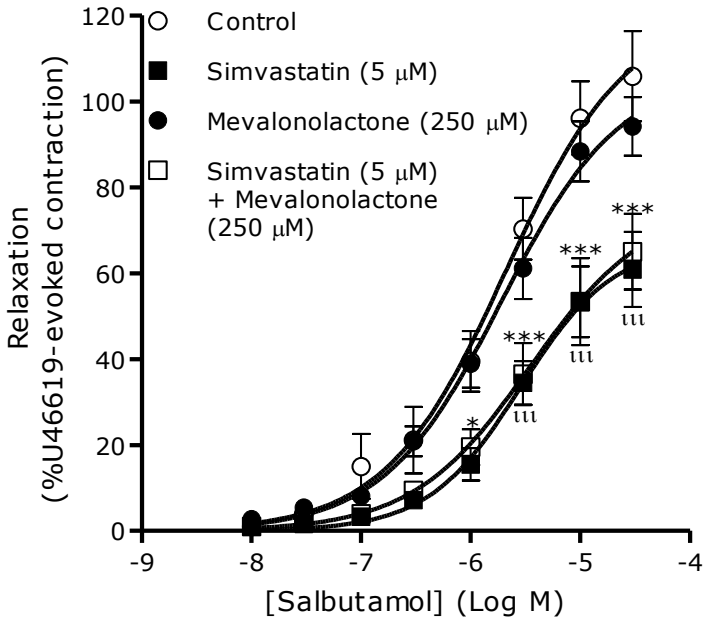

Figure 5.8. *The effect of simvastatin on β-AR-mediated relaxations in PCA rings supplemented with mevalonolactone (n=4–6). Recordings were made in the absence or presence of simvastatin (0.05% (v/v) DMSO or 5 μM, respectively), mevalonolactone (0.09% (v/v) ethanol or 250 μM, respectively) or both agents in combination. Simvastatin inhibited relaxations to salbutamol, even in the presence of mevalonolactone, while mevalonolactone alone was without effect. * indicates P<0.05 and *** indicates P<0.001 for simvastatin versus control; and ιιι indicates P<0.001 for simvastatin + mevalonate versus control.*

5.3.5. The effect of Rho kinase inhibition on β-AR-mediated vasodilatation

Figure 5.9 shows the effect of the Rho kinase inhibitor Y27632 (5 μM) on salbutamol-induced relaxations. Y27632 inhibited relaxations to 10 μM salbutamol (from 80 ± 6% in controls to 62 ± 6%; *P*<0.05) and 30 μM salbutamol (from 85 ± 6% in controls to 68 ± 7%; *P*<0.05).

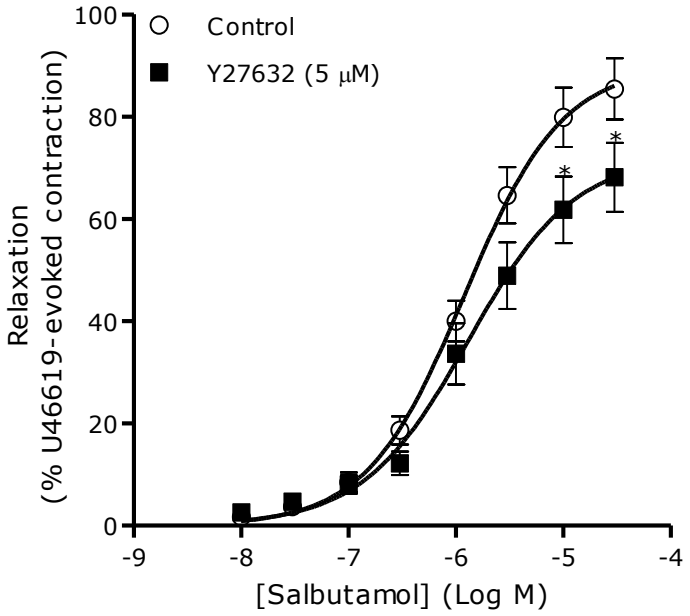

Figure 5.9. *The effect of the Rho kinase inhibitor Y27632 on β-AR-mediated relaxations in PCA (n=8–10). Relaxations were inhibited in the presence of presence of Y27632 (5 μM). * indicates P<0.05.*

5.4. DISCUSSION

The isoprenoid FPP is required for the farnesylation of Ras, allowing its association to the cell membrane where it is activated. Activated Ras can switch on the signalling cascade involving Raf and MEK and ultimately activating ERK. By inhibiting HMG-CoA reductase, statins prevent the formation of intermediates of the mevalonate pathway, such as FPP. Therefore, statins would be expected to inhibit ERK activation. Indeed, simvastatin has been reported to inhibit ERK activation in isolated vascular smooth muscle cells (Tristano *et al.*, 2007).

As described in Chapter 2, inhibition of ERK activation in PCA using specific inhibitors of MEK results in an enhancement of β-AR-mediated vasodilatation. Data presented in the current chapter, however, demonstrate that statins produced the opposite effect. Incubation with simvastatin (5 μM) and lovastatin (10 μM) significantly inhibited relaxation responses to the β_2-AR agonist salbutamol. Moreover, when a higher concentration of simvastatin (10 μM) was used, salbutamol was unable to relax pre-contracted arteries.

The inhibition of relaxations was not a universally observed phenomenon amongst the statins used. Pravastatin, unlike simvastatin and lovastatin, did not alter the relaxation characteristics of salbutamol. Likewise, the sodium salt of simvastatin was also without effect. Similar findings have been reported in rat basilar artery, in which simvastatin and lovastatin, but not pravastatin, have been shown to induce relaxation (Bergdahl *et al.*, 2003).

It is likely that the selectivity of inhibition is related to the lipophilicity of the statin. Simvastatin and lovastatin are described as lipophilic (or hydrophobic), in contrast to pravastatin and simvastatin Na^+, which are hydrophilic. In fact, based on octanol-water partition coefficients it was estimated that lovastatin and simvastatin may be approximately 75- and 200-fold, respectively, more lipophilic than pravastatin (Serajuddin *et al.*, 1991). Furthermore, according to XLogP3 values (Cheng *et al.*, 2007), shown in Table 5.2, the differences are even greater, at approximately 500- and 1250-fold, respectively. These differences, coupled with the fact that lipophilicity is an important characteristic in the ability of a molecule to penetrate the lipid-rich cell membrane, explain the disparity between the observed effects of the statins used in this experiment.

Statin	XLogP3	Relative lipophilicity
Pravastatin	1.6	1
Lovastatin	4.3	501
Simvastatin	4.7	1259

Table 5.2. *XLogP3 values of pravastatin, lovastatin and simvastatin. Relative lipophilicities are compared to the lowest value (pravastatin). Data acquired from U.S. National Library of Medicine, 2011.*

Furthermore, based on the lack of effect of pravastatin, it would be reasonable to assume that PCA smooth muscle cells do not express membrane proteins capable of transporting hydrophilic statins. Such transport mechanisms have been detected in hepatocytes and are proposed to be responsible for the uptake of hydrophilic statins into this cell type, allowing the statins to mediate their cholesterol-lowering effects (Yamazaki *et al.*, 1993).

It is not known whether the effects of statins observed in the current experiment are caused by the lactone prodrug forms in which they were

administered or their corresponding β-hydroxy acids. The latter are formed by chemical or enzymatic hydrolysis of the lactones, and have been shown to be more potent inhibitors of HMG-CoA reductase (Vickers *et al.*, 1990). Candidates for the conversion of statins are the paraoxonase (PON) enzymes (Draganov *et al.*, 2005). PONs 1 and 3 have been implicated in the hydrolysis of statin lactones in human serum (Billecke *et al.*, 2000; Suchocka *et al.*, 2006). PON-2, a predominantly intracellular protein expressed in a wide range of cell types including smooth muscle (Ng *et al.*, 2001), may hydrolyse statin lactones inside cells, though this has not yet been investigated.

Experimental assessments of statins typically evaluate reversibility by supplementation with intermediates of the mevalonate pathway. For example, mevalonate prevented the reduction in pulmonary artery pressure caused by simvastatin in a rat model of chronic pulmonary hypertension (Girgis *et al.*, 2007). In human macrophages, lipopolysaccharide-induced stimulation of phospholipase A_2 (PLA_2) expression was inhibited by simvastatin, an effect blocked by treatment with mevalonate or GGPP (Song *et al.*, 2011). In this experiment, mevalonolactone, which is hydrolysed to mevalonate in aqueous solution, had no effect on relaxation responses to salbutamol. Importantly, treatment with the combination of mevalonolactone and simvastatin failed to reverse the inhibitory effect of simvastatin on salbutamol-induced relaxations, suggesting that this effect of statins is independent of HMG-CoA inhibition.

An alternative explanation for the lack of effect of mevalonolactone is that the treatment conditions – specifically the concentration of mevalonolactone and duration of incubation – were insufficient to deliver a

pharmacologically significant amount of mevalonate to target cells. Arteries were exposed for 45 mins to 250 μM mevalonolactone, a concentration that has previously been shown to be sufficient to rescue isoprenoid function in the presence of statins (Cole *et al.*, 2005). Indeed, 250 μM mevalonolactone abolished the cytoprotective effects of lovastatin on rat cortical glial cells (Paintlia *et al.*, 2008). These findings suggest that the mechanism of action of statins in the current experiment is independent of HMG-CoA reductase inhibition.

The AC/cyclic 3',5'-adenosine monophosphate/protein kinase A (cAMP/PKA) signal transduction pathway has been closely associated with β-AR signalling. In this experiment, direct activation of AC using forskolin mimicked the response to the β-AR agonist salbutamol, namely relaxation. However, while simvastatin inhibited relaxations to salbutamol, it failed to affect forskolin-induced relaxations. This finding suggests that simvastatin does not affect signalling via the AC/cAMP/PKA pathway but may instead inhibit a separate pathway employed by the β-AR during relaxation. One such mechanism may involve K^+ channels, which have been shown to mediate β-AR-induced relaxations via a cAMP-independent mechanism (White *et al.*, 2001).

In this experiment, relaxations caused by the application of openers of K_{ATP} and BK_{Ca} channels were inhibited by simvastatin. Consistent with this is the finding in smooth muscle cells of the PCA that simvastatin inhibited BK_{Ca} channel currents (Seto *et al.*, 2007). Interestingly, in the same study neither pravastatin nor simvastatin Na^+ had any observable effect, a finding consistent with those discussed earlier in this section. These data support the idea that simvastatin inhibits βAR-mediated relaxations through inhibition of K^+ channel activity. The mechanism of K^+ channel

inhibition may involve disruption of signalling between the activated β-AR and downstream K^+ channels; alternatively, the effect may be a result of direct channel blockade by the statin. The latter hypothesis could be tested using specific configurations of the patch-clamp technique. A lack of effect of statins on K^+ channel activity under inside-out or outside-out configurations would suggest that statins may inhibit K^+ channels by targeting elements of the intracellular signalling machinery. Conversely, direct blockade of K^+ channels by statins would be detectable under these configurations.

By impairing the activity of HMG-CoA reductase, statins prevent the formation of the isoprenoid GGPP and subsequent activation of the GTP-binding protein Rho. Previous studies have linked the effects of statins to the inhibition of Rho. For instance, the Rho inhibitor C3 exoenzyme mimicked the inhibitory effect of simvastatin on LPS-induced elevation of PLA_2 expression in human macrophages (Song *et al.*, 2011). Similarly, simvastatin was shown to impair the self-renewal of mouse embryonic stem cells via inhibitory effects on Rho (Lee *et al.*, 2007). In the current investigation, the Rho kinase inhibitor Y27632 inhibited relaxation responses to salbutamol, reflecting findings made with simvastatin. Therefore, it is possible that the inhibitory effect of statins on β-AR-mediated vasodilatation may involve inhibition of Rho. However, it is worth noting that inhibition of Rho by statins would unlikely be related to any potential upstream effects on HMG-CoA reductase as mevalonate supplementation did not reverse the effect of statins.

A further possible mechanism by which statins inhibit relaxation responses to salbutamol may involve indirect effects on the U46619-evoked pre-contraction. Arteries pre-incubated with lovastatin or simvastatin (5 μM)

required approximately 20 nM – 50 nM of U46619, whilst those incubated with simvastatin (10 μM) required > 100 nM of U46619. In contrast, vehicle controls were pre-contracted using only 10 nM – 20 nM. This difference suggests that the statins inhibited contractions to U46619 meaning that higher concentrations of U46619 were required to achieve a given level of pre-contractile tone.

A previous investigation showed that lovastatin inhibited L-type Ca^{2+} currents in rat basilar artery smooth muscle cells and impaired associated contractile responses to serotonin and K^+ (Bergdahl *et al.*, 2003). Additionally, this property of statins may be associated with their ability to inhibit Rho, which itself is required for the activation of Rho kinase and subsequent signalling associated with smooth muscle contraction (Fukata *et al.*, 2001). Although the level of tone induced by U46619 was the same in the presence and absence of statins, by inhibiting one mechanism of contraction, U46619 would need to recruit an alternative mechanism to induce contraction. Increased activation of this alternative signalling pathway may inhibit the β-AR-mediated relaxation. This would be consistent with the Rho kinase inhibitor data. In any case, it is possible that greater concentrations of salbutamol were needed to overcome the U46619-evoked contraction, representing inhibition of the salbutamol responses. However, this fails to explain why forskolin-induced relaxations were not inhibited by simvastatin in these experiments. Furthermore, data presented in Chapter 2 show that U46619-evoked contraction of PCA was inhibited by PD98059 but subsequent relaxation responses to salbutamol were not impaired.

In these experiments, arteries were exposed to statins (or vehicle control) for 45 mins. Repeating the experiments with longer statin incubation

periods (for example, overnight incubation at 37°C under aseptic conditions) may yield findings more relevant to the clinical setting. Furthermore, the concentrations of statins used in this study (5 μM – 10 μM) considerably exceeded those measured in the clinical setting. It has been shown, for example, that in healthy adults a single 40 mg oral dose of simvastatin produces peak plasma lactone and acid concentrations of 7.3 ng ml^{-1} (17 nM) and 2.6 ng ml^{-1} (6.0 nM), respectively (Lilja *et al.*, 2004). An equivalent dose of pravastatin appears to produce higher serum concentrations (45.3 ng ml^{-1} or 110 nM) of its active metabolite (Lilja *et al.*, 1999), likely due to less extensive first-pass metabolism and plasma protein binding than simvastatin (Schachter, 2005).

In conclusion, statins inhibit β-AR-mediated vasodilatation of PCA. This effect, specific to lipophilic statins, appears to be cAMP-independent and may be related to inhibitory effects on K^+ channels. It is also possible that the phenomenon occurs indirectly as a consequence of impairment of pre-contraction due to inhibition of Ca^{2+} channels and/or Rho-associated signalling. The inhibition of relaxation is inconsistent with the findings that (1) inhibitors of ERK activation enhance β-AR-mediated relaxations, as shown in Chapter 2, and (2) statins inhibit ERK activation (Tristano *et al.*, 2007). Future biochemical experiments assessing the effects of statins on ERK activation in intact PCA are warranted. Although the observed inhibition of contractions by statins are consistent with their cardioprotective characteristics, the inhibition of relaxation responses is unexpected in this context and would be an undesirable effect in patients with established cardiovascular disease.

CHAPTER 6: GENERAL DISCUSSION

Vascular β–adrenoceptors (β-ARs) mediate vasodilatation by recruiting a range of effector systems capable of relaxing vascular smooth muscle (VSM) cells. The precise mechanisms involved are often complex and vary between species and vessels. The main preparation studied in this investigation was the left, anterior descending porcine coronary artery (PCA). The non-selective β-AR agonist isoprenaline concentration-dependently relaxed U46619-pre-contracted PCA rings as measured using wire myography. The subtype-selective agonists xamoterol and salbutamol also produced concentration-dependent relaxation responses, indicating the functional expression of β_1-AR and β_2-ARs, respectively, in this tissue, consistent with the literature (Brehm *et al.*, 1999; Schwartz *et al.*, 1983).

Triggered by $G_{\alpha s}$-dependent activation of adenylyl cyclase (AC), the cyclic 3',5'-adenosine monophosphate-protein kinase A (cAMP-PKA) signalling pathway is the most commonly reported route of β-AR-mediated relaxation of VSM. Consistent with this was the observation that the AC activator forskolin elicited relaxations of PCA. However, attempts to disrupt this pathway using pharmacological inhibitors of AC and protein kinase A (PKA) did not reduce β-AR-mediated relaxation. The recruitment of alternative signalling pathways is proposed as an explanation for this finding. The gaseous free radical NO is derived from the endothelium and exerts a paracrine effect on adjacent VSM by engaging the soluble guanylyl cyclase-cyclic 3', 5'-guanosine monophosphate-protein kinase G (sGC-cGMP-PKG) pathway. Studies of its potential role in β-AR-mediated vasodilatation have produced conflicting findings. In the current investigation, inhibition of endothelial nitric oxide synthase (eNOS) was associated with a modest reduction of relaxations to low concentrations of isoprenaline, suggesting an involvement, albeit minor, of nitric oxide (NO) in this process. Interestingly, eNOS inhibition was also accompanied by an enhancement of

relaxations at higher concentrations of isoprenaline, which may have been related to disinhibition of a more potent effector system, as proposed by Liang *et al.* (2010). Various K^+ channels have been reported to induce hyperpolarisation of VSM as a mechanism of β-AR-mediated vasodilatation. However, selective blockers of ATP-sensitive K^+ channels (K_{ATP}), large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) and intermediate-conductance Ca^{2+}-activated K^+ channels (IK_{Ca}) channels failed to alter β-AR-mediated vasodilatation. Again, these negative results may have been associated with increased signalling via other pathways to compensate for K^+ channel blockade. Attempts to study the effect of β-AR activation on K^+ channels in smooth muscle cells using the patch-clamp technique were unsuccessful, due to difficulties in isolating healthy cells.

β-ARs have been reported to signal via extracellular signal-regulated kinase (ERK), albeit in cultured cells (Baker *et al.*, 2003; Friedman *et al.*, 2002), and ERK has been shown to contribute to contraction of VSM (Roberts, 2001; Xiao *et al.*, 2004). However, the functional effects of β-AR-mediated activation of ERK in blood vessels has not been studied. Therefore, the primary aim of this investigation was to reconcile the implication that β-AR activation, which causes VSM relaxation, may increase the activation of ERK, which promotes VSM contraction. It was subsequently shown that inhibition of ERK activation using specific MEK inhibitors significantly enhanced vasodilatation induced by isoprenaline, suggesting an inhibitory effect of ERK on this response. The use of subtype-selective β-AR agonists demonstrated that the phenomenon was specific to the β_2-AR subtype. This finding may represent a physiological, negative-feedback regulatory mechanism of β_2-AR-mediated vasodilatation and further adds to the complexity of this process.

In experiments designed to determine the mechanism by which ERK disrupts β-AR-mediated vasodilatation, the various elements of β-AR signalling were isolated prior to assessing the effects of MEK inhibition on the relaxation response. It was proposed that ERK may interact with a cAMP-independent pathway because forskolin-induced relaxations were unaffected by MEK inhibition. Inactivation of K^+ channels, by using either the non-selective blocker tetraethylammonium (TEA) or high extracellular K^+ concentrations, prevented the enhancement of β_2-AR-mediated vasodilatation. β-ARs have been shown to activate K^+ channels via cAMP-dependent (White *et al.*, 2000) and –independent (Randall *et al.*, 1995) routes. However, the finding in the current study that ERK interacts with K^+ channels but not a cAMP-dependent pathway suggests that the K^+ channel species involved in the enhancement phenomenon is/are activated via cAMP-independent routes. The nature of this activation may involve direct stimulation of the K^+ channel by $G\alpha_s$, as has previously been demonstrated in PCA smooth muscle cells (Scornik *et al.*, 1993). β_2-AR-mediated activation of ERK in HEK293 cells also required $G_{\alpha s}$ but involved the AC-cAMP-PKA pathway (Friedman *et al.*, 2002; Schmitt *et al.*, 2000). Therefore, $G\alpha_s$ may represent a commonality in β_2-AR-mediated activation of K^+ channels and ERK in PCA, with the latter but not the former also requiring the AC-cAMP-PKA pathway.

Using an array of selective K^+ channel blockers, it was found that the IK_{Ca} channel, but not the K_{ATP} or BK_{Ca} channel, was implicated in the MEK inhibitor-induced augmentation of β-AR-mediated vasodilatation. The expression of IK_{Ca} channels on endothelial cells has frequently been reported, underscoring its central role in the endothelium-derived hyperpolarising factor (EDHF) phenomenon. In VSM cells, however, IK_{Ca} channels may be relevant in proliferative phenotypes rather than

contractile phenotypes (McNeish *et al.*, 2006; Tharp *et al.*, 2009). This raises doubts over the possibility that the interaction between ERK and IK_{Ca} channels in PCA occurs at the level of the smooth muscle. Moreover, a previous study demonstrated that MEK inhibitors enhanced bradykinin-induced, EDHF-mediated vasodilatation of PCA (Brandes *et al.*, 2002). The authors proposed that enhancement occurred via reversal of the inhibitory influence of ERK on gap junction communication, caused by phosphorylation of connexin 43. The activation of IK_{Ca} and SK_{Ca} channels appears to be a pre-requisite for EDHF-mediated relaxation, though IK_{Ca} channels alone may be sufficient in some arteries (McNeish *et al.*, 2006). In any case, IK_{Ca}/SK_{Ca} activation precedes the conduction of hyperpolarisation from the endothelium to the smooth muscle layer, which may involve myo-endothelial gap junctions (Busse *et al.*, 2002). Therefore, it is possible that the enhancement caused by MEK inhibitors in the current study was related to the effects of ERK on gap junction communication rather than on IK_{Ca} channels *per se*; indeed, TRAM-34 would still have been expected to reverse the enhancement in this scenario. Nonetheless, the IK_{Ca} channel may still represent a phosphorylation target for ERK. Previous studies have shown that ERK is able to regulate various K^+ channels (Li *et al.*, 2006; Ross *et al.*, 2003), including the IK_{Ca} channel (Lhuillier *et al.*, 2000). Future experimentation using ^{32}P labelling and immunoprecipitation would allow examination of the effect of MEK inhibition on IK_{Ca} channel phosphorylation status.

As proposed earlier, ERK may act as part of a physiological, negative-feedback mechanism limiting the degree of β-AR-mediated vasodilatation. However, increases in ERK activation, which have been reported in various cardiovascular diseases, may result in dysregulation and impairment of this process. Markedly reduced β-AR-mediated vasodilatation was observed in

arteries of Zucker Diabetic Fatty (ZDF) rats and Zucker obese rats, models of Type II diabetes and obesity, respectively. U46619-stimulated ERK activation was significantly increased in superior mesenteric artery (SMA) of ZDF rats, suggesting that the impairment of relaxations was related to greater ERK activity in these animals. Furthermore, MEK inhibition partially reversed the impairment of relaxations in SMA. Similar deficits in relaxations to β-AR agonists and other vasodilators in ZDF rats and Zucker rats have frequently been reported and often involve endothelial dysfunction. However, endothelial function is preserved in these rats at the age used in the current study (12 weeks) (Oltman *et al.*, 2006), suggesting that the impairment of β-AR-mediated relaxations by ERK in this artery may occur at the level of the smooth muscle. Additional experiments would be required to confirm this suggestion and to examine the effect of increased ERK activation on K^+ channel function. Moreover, further investigations would also aim to determine whether ERK nonetheless contributes to the development and progression of endothelial dysfunction in Zucker rats.

Several studies have reported impairments of β-AR-mediated vasodilatation in various rat models of hypertension (Arvola *et al.*, 1993; Fujimoto *et al.*, 1988; Soltis *et al.*, 1991). Furthermore, similar findings have been noted in human hypertension (Naslund *et al.*, 1990; van Brummelen *et al.*, 1981). Increased vascular ERK activation has also been reported in rat models of hypertension, and appears to occur without parallel increases in non-cardiovascular tissues (Kim *et al.*, 2000a; Kim *et al.*, 1997; Kim *et al.*, 2000b). However, the relationship between ERK activation and β-AR-mediated vasodilatation in hypertensive states has not been studied. Nonetheless, given the finding in the current investigation that inhibition of ERK activation somewhat restored β-AR function in rat

models of diabetes and obesity, which are also characterised by increased ERK activation, it is possible that similar observations would be made in corresponding studies of hypertension. Interestingly, isoprenaline-stimulated activation of ERK was shown to be significantly attenuated in aortic smooth muscle of spontaneously hypertensive rats (SHR) in comparison with normotensive controls (Gros *et al.*, 2006). In contrast, forskolin-stimulated ERK activation did not differ between the two groups, whilst the vasoconstrictor hormones vasopressin and angiotensin II, which signal via $G\alpha_q$, activated ERK to a greater degree in SHR. The authors propose that uncoupling of β-AR-$G\alpha_s$ interactions occurs in the hypertensive state, whilst G-protein-coupled receptor (GPCR)-$G\alpha_q$ coupling may be increased. Collectively, the consequences of these changes would be detrimental to vascular function for several reasons. The uncoupling of β-AR-$G\alpha_s$ would impair, firstly, the recruitment of effector systems required to elicit vasodilatation and, secondly, the long-term modulation of vascular growth, as β-AR activation is known to inhibit proliferation of VSM cells (Nakaki *et al.*, 1990). Furthermore, increased GPCR-$G\alpha_q$ coupling would have equivalent functional consequences, given the role of these receptors in VSM contraction and proliferation. In the current investigation, ERK phosphorylation in PCA was increased by β-AR and thromboxane-prostanoid (TP) receptor stimulation. It is not known whether β-AR-stimulated ERK activation was reduced in ZDF rats versus lean controls; however, TP receptor-stimulated, $G_{\alpha q}$-dependent ERK activation was increased, consistent with findings presented by Gros *et al.* (2006). It is possible that this profile of altered ERK and β-AR function in blood vessels may be a common feature of cardiovascular disease and may contribute to the alterations in vascular reactivity and growth.

In summary, a novel role for ERK in the regulation of vascular tone is proposed. Specifically, as part of a physiological control mechanism, ERK inhibits β-AR-mediated vasodilatation by reducing IK_{Ca} channel activity. This proposal represents an additional means by which ERK participates in the short-term regulation of vascular reactivity; its role in mediating vasoconstriction is well described. Furthermore, the long-term regulation of vascular function by ERK, also well described, involves VSM cell proliferation. Increased ERK activation in these contexts would be injurious to cardiovascular health via three distinct routes. First, impaired vasodilatation would render the vasculature unable to adequately respond to the circulatory demands of organs and tissues. Second, augmented contractility would increase vascular resistance and, therefore, mean arterial pressure. Indeed, this in turn may further increase ERK levels, as has been demonstrated in rat models of hypertension (Kim *et al.*, 1997). Finally, vascular growth associated with endothelial/VSM cell migration and proliferation would reduce lumen diameter, increase vessel stiffness and promote endothelial dysfunction. Thus, targeting ERK in cardiovascular disease represents a promising treatment strategy.

A possible role for statins in this context was explored in this study as statins have been shown to inhibit ERK activation (Tristano *et al.*, 2007). However, the potent inhibitory effect of statins on β-AR-mediated relaxations was likely related to the concentrations used (low micromolar range), which are significantly higher than those achieved in the clinical setting (low-to-mid nanomolar range). Nonetheless, the rationale for targeting ERK in this way is supported by studies attributing the cardiovascular benefits of statins to inhibition of ERK activation (Huang *et al.*, 2010; Takayama *et al.*, 2011).

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APPENDIX: SUPPLEMENTARY EXPERIMENTS

1. Methods

1.1. The effect of MEK inhibition on β-AR desensitisation

To determine whether ERK alters β-AR-mediated relaxations via effects on receptor desensitisation, arteries were exposed to isoprenaline (1 μM) for 60 mins and washed out (every 5 mins for 45 mins) prior to contraction with U46619 and relaxation with salbutamol. In similar experiments, arteries were exposed to isoprenaline (1 μM) for 10, 20 or 30 mins prior to washout, contraction using U46619 and relaxation with isoprenaline.

1.2. The role of the endothelium in β-AR-mediated relaxation

In experiments assessing the role of endothelial cells in isoprenaline-induced vasorelaxation, the endothelium was removed from PCA ring segments before they were fitted to a Mulvany wire myograph. A preliminary experiment was carried out in order to determine the minimum amount of tissue manipulation required to abolish endothelium-dependent relaxation. Endothelium removal was achieved by very gently "rolling" the neck of a pair of forceps inside the lumen of artery rings. To prevent the tissue segments from drying, this procedure was carried out on a paper towel thoroughly moistened with KH solution. The lumina of three adjacent ring segments from the same artery were "rolled" to varying degrees (10, 20 or 30 times). The endothelium of a fourth, control segment was left intact.

The arteries were gently washed in KH solution before being fitted to a Mulvany wire myograph as described earlier. Pre-contraction using U46619

preceded the application of the endothelium-dependent vasorelaxant substance P (100 nM). It was observed that 20 revolutions of the forcep neck were sufficient to prevent relaxations to substance P, and this protocol was used in subsequent investigations. At the end of the experiment, isoprenaline (1 μM) was applied to the arteries without washout of substance P.

In a separate experiment, the combined effects of endothelium denudation and MEK inhibition on β-AR-mediated relaxation were investigated. Prior to the experiment, four ring segments from the same PCA were dissected. Where appropriate, endothelia were removed as described above. The rings were subsequently relaxed with cumulative concentrations of isoprenaline (1 nM – 3 μM) following pre-contraction with U46619 in either the absence (0.05% (v/v) DMSO) or presence of PD98059 (50 μM; 45 min incubation). Thus, the four experimental conditions were as follows: 1. endothelium intact + DMSO; 2. endothelium intact + PD98059; endothelium denuded + DMSO; endothelium denuded + PD98059.

1.3. The effect of p38 MAPK inhibition on β-AR-mediated vasodilatation

In these experiments, the effects of p38 on β-AR-mediated vasodilatation were assessed. Arteries were incubated for 45 min in KH solution containing either the p38 inhibitor SB 203580 (10 μM) (Saklatvala *et al.*, 1996) or 0.1% (v/v) DMSO (control). After incubation, U46619 was used to pre-contract arteries, which were subsequently relaxed with isoprenaline.

2. Results

2.1. The effect of MEK inhibition on β-AR desensitisation

Salbutamol-induced relaxations were unchanged following 60 min pre-exposure to isoprenaline (1 μM; Figure A1). Likewise, relaxations to cumulative concentrations of isoprenaline were unaffected by pre-exposure to isoprenaline (10 – 30 mins; Figure A2). Taken together, these data suggest desensitisation of the β-AR does not occur under the current experimental conditions.

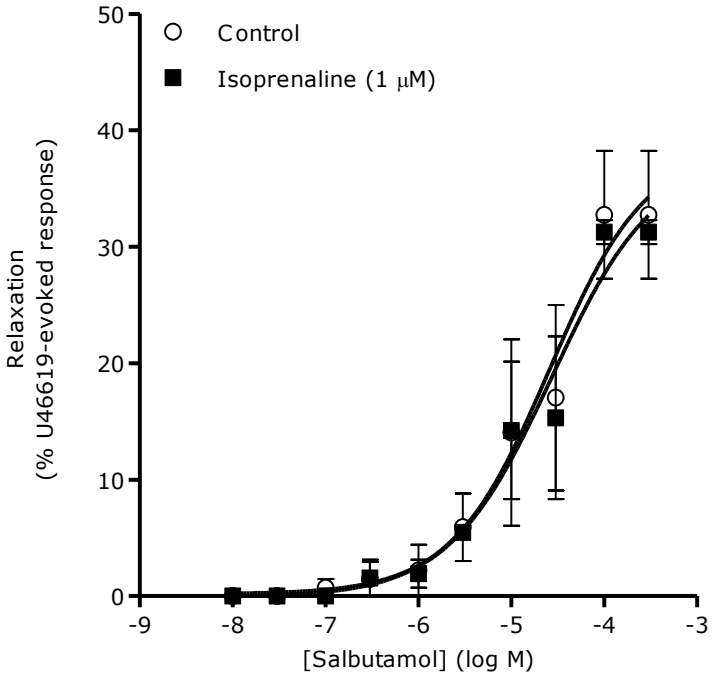

Figure A1. *The effect of pre-incubation with a β-AR agonist on β-AR-mediated vasodilatation (n=2). Relaxations to salbutamol observed following 60 min pre-exposure to isoprenaline (1 μM) and washout were similar to relaxations observed under control conditions (no isoprenaline).*

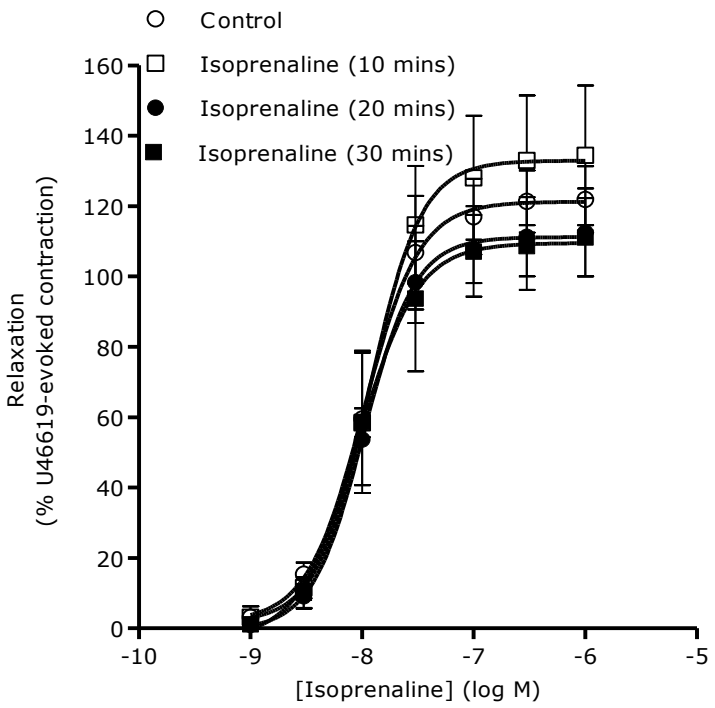

Figure A2. *The effect of pre-incubation with isoprenaline on β-AR-mediated relaxations in PCA (n=2). Pre-incubation with 1 μM isoprenaline (10–30 min) did not alter subsequent relaxations to isoprenaline.*

2.2. The role of the endothelium in β-AR-mediated relaxation

The aim of a preliminary experiment was to determine the minimum degree of luminal disruption required to abolish endothelial-dependent relaxations. The endothelium-dependent vasodilator actions of substance P have previously been described (Kuroiwa *et al.*, 1995). In the current study substance P (100 nM) elicited transient relaxation (10 min) of pre-contracted PCA ring segments in an endothelium-dependent manner (Figure A3). The control artery ring (intact endothelium) relaxed to 40% of the level of pre-contraction evoked by U46619. The artery ring "rolled" 10 times was relaxed by substance P to a similar degree, 38%. However, greater disruption of endothelial function abolished responses to substance P. Isoprenaline (1 μM) relaxed all arteries to approximately the same extent (124% - 147%).

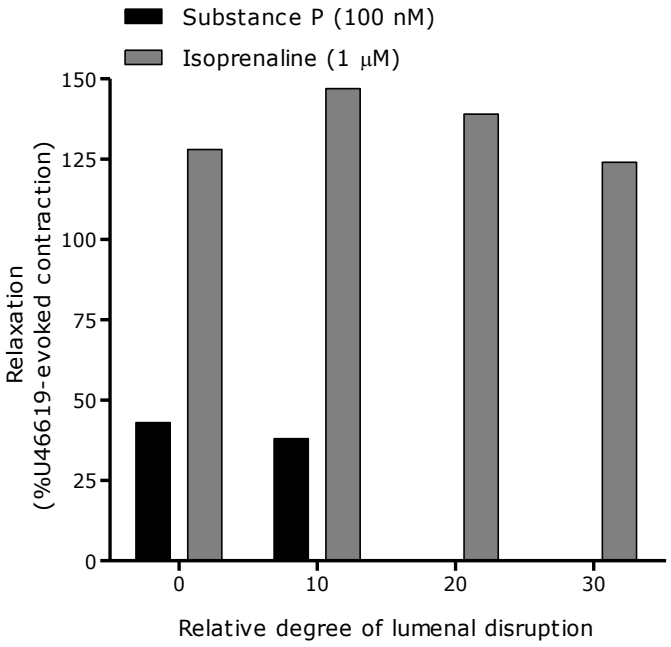

Figure A3. *The effect of endothelium disruption on relaxations to 100 nM substance P and 1 μM isoprenaline (n=1). Endothelia were agitated by gently "rolling" the neck of a pair of forceps inside the artery lumen for varying lengths of time. The endothelium of the control segment ('0') was left intact. Isoprenaline produced a similar degree of relaxation in each ring, whereas substance P either produced incomplete relaxations or failed to induce relaxations. Further repeat experiments are required.*

In an experiment investigating the combined effect of PD98059 and endothelium denudation (Figure A4), isoprenaline relaxed control arteries by 146%, with a pEC_{50} value of 7.5. In endothelium-denuded arteries, the corresponding values were similar: 138% and 7.5, respectively. PD98059 reduced the maximal response to isoprenaline in endothelium-intact arteries to 111% but increased the potency (pEC_{50} = 8.0). Similarly, in endothelium-denuded arteries exposed to PD98059, the maximal response was 107% whilst the pEC_{50} value was 7.8.

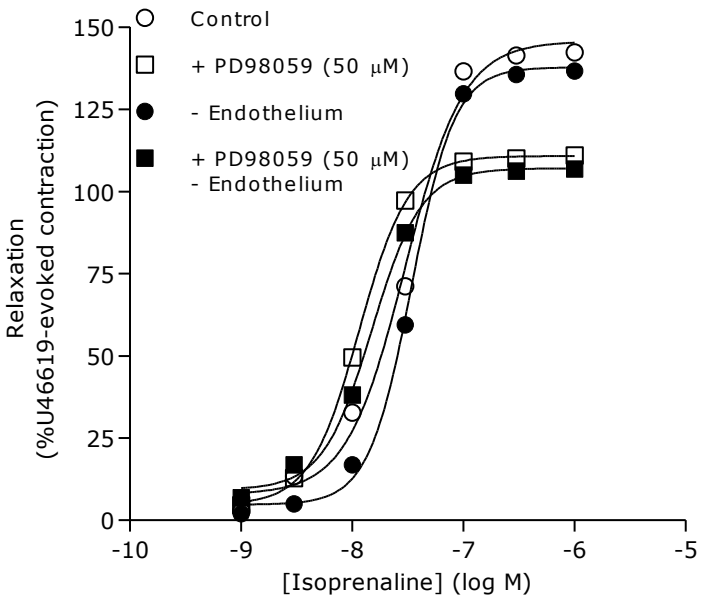

Figure A4. *The effect of MEK inhibition on β-AR-mediated vasodilatation in endothelium-denuded PCA rings (n=1). Prior to incubation with 50 μM PD98059 (controls received 0.05 % (v/v) DMSO), the endothelia of arteries were either denuded or left intact. Isoprenaline produced concentration-dependent relaxations. Further repeat experiments are warranted.*

2.3. The effect of p38 inhibition on β-AR-mediated vasodilatation

Isoprenaline-induced vasodilatation of PCA ring segments was altered by p38 inhibition (Figure A5). pEC_{50} values were raised from 7.6 ± 0.1 in controls to 8.1 ± 0.1 in SB 203580-treated arteries ($P<0.01$). On the other hand, R_{MAX} values were reduced, though the changes were not statistically significant (138 ± 10% in controls versus 118 ± 3% in SB 203580-treated arteries, $P>0.05$).

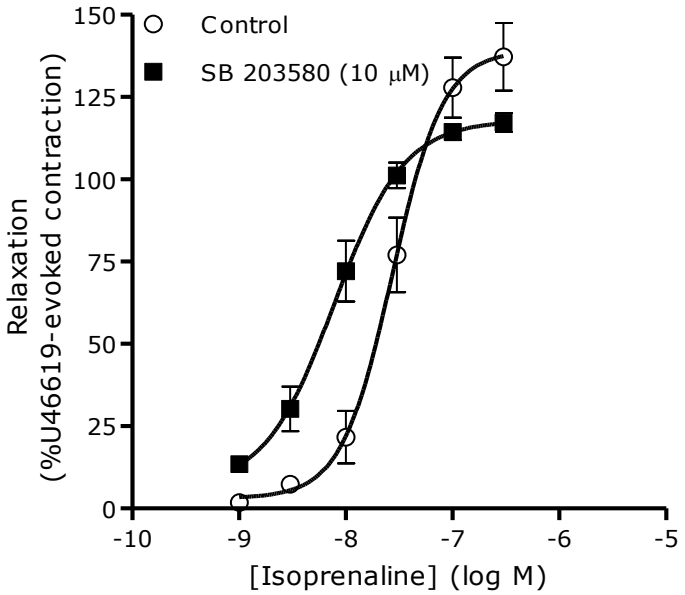

Figure A5. *Log concentration–response curves to isoprenaline in PCA. Relaxations, shown as means ± SEM (n=5), are expressed as a percentage of the U46619-evoked contraction. Recordings were made in either the absence (0.1% (v/v) DMSO) or presence of SB 203580 (10 μM).*

3. Discussion

Desensitisation of β-ARs occurs in response to repeated or prolonged exposure to β-AR agonists, and causes a reduction in subsequent responses to β-AR agonists. In brief, phosphorylation of the β-AR by PKA or β-adrenoceptor kinase (β-ARK) promotes the binding of the receptor to β-arrestin, which in turn sterically uncouples the receptor from the G-protein, allowing internalisation of the receptor. As discussed earlier, ERK appears to exert an inhibitory influence on β-AR-mediated relaxations, but the exact mechanism involved is unknown. It has been shown in HEK-293 cells that the β-AR is able to activate ERK via a β-ARK/β-arrestin-dependent pathway (Shenoy *et al.*, 2006). Thus an interaction with the mechanics of desensitisation represents a means by which ERK may regulate β-AR-mediated relaxations.

A comparable phenomenon was proposed by the authors of a previous study, in which it was shown that activation of protein kinase C (PKC) in bovine tracheal smooth muscle enhanced desensitisation of the β_2-AR (Boterman *et al.*, 2006). Inhibition of PKC in the same study enhanced isoprenaline-induced relaxations. Thus activation of ERK may promote receptor desensitisation such that when ERK is inhibited, there is less receptor desensitisation and hence a greater relaxation. It is possible that receptor desensitisation occurs over the duration of the construction of concentration-response curves to β-AR agonists (30-60 min). Therefore we investigated whether desensitisation could occur over this time period.

Attempts to observe desensitisation of β-AR-mediated relaxations of PCA were unsuccessful despite pre-exposing arteries to 1 μM isoprenaline for up to 60 mins. It is possible that incubating higher concentrations of β-AR agonists for longer time periods than those used in this experiment may have resulted in receptor desensitisation. However, the data presented suggest that it is unlikely that the effects of inhibition of ERK activation are due to prevention of receptor desensitisation.

A caveat is that resensitisation may have occurred during the washout period (arteries were rinsed with pre-gassed KH solution every 5 mins for 45 mins), meaning that β-AR density and function could have been restored prior to the repeated agonist challenge. This represents an inherent difficulty in experiments investigating desensitisation. Excessive washout may reverse and therefore mask the effects of desensitisation, whilst insufficient washout would fail to remove residual agonist from the receptor population, which may confound subsequent recordings. It is also possible that β-AR agonists produce relaxations in PCA via mechanisms which possess significant reserve capacities. A large receptor reserve, for

instance, could explain why agonist-induced receptor internalisation would not necessarily lead to reduced functional responses on agonist re-exposure. Likewise, signalling downstream of receptor activation may be amplified such that functional responses are identical despite fewer agonist-receptor interactions.

A role for p38 in β-AR-mediated vasodilatation is suggested by the finding that SB 203580, a p38 inhibitor (Saklatvala *et al.*, 1996), increased the potency of isoprenaline-induced relaxations. It is therefore possible that p38, like ERK, may somehow inhibit β-AR-mediated vasodilatation. However, the relevance of this finding should be considered in light of frequent reports of non-specific effects of SB 203580 (Clerk *et al.*, 1998; Kalmes *et al.*, 1999).

The neuropeptide substance P has been described as an endothelial-dependent vasorelaxant (Crossman *et al.*, 1989; Kuroiwa *et al.*, 1995). Consistent with this characterisation, substance P was unable to evoke relaxations in endothelium-denuded arteries in this experiment. In contrast, relaxation responses to isoprenaline did not appear to be altered by endothelium denudation, which is inconsistent with the finding that the NOS inhibitor L-NAME inhibited isoprenaline-induced relaxations. However, a greater number of experimental repeats are needed to fully ascertain the role of the endothelium in β-AR-mediated vasodilatation. The observation that the enhancement of the isoprenaline concentration curve caused by PD98059 persisted in endothelium-denuded arteries suggests that the enhancement does not involve endothelium-derived factors. Again, further repeat experiments would be required to test this proposal.