ROLE OF ALTERED SIGNALING PATHWAYS IN ABNORMAL VASOCONSTRICTOR RESPONSES IN MESENTERIC ARTERIES FROM STZ-DIABETIC RATS

by

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ABSTRACT

Cardiovascular complications are recognized to be the major cause of morbidity and mortality associated with diabetes mellitus. One of the most common features of vascular dysfunction in well-established diabetes is the enhanced reactivity of blood vessels to vasoconstrictors. We and others have consistently found that contractile responses of arteries from rats with well-established diabetes to stimulation of G-protein coupled receptors (GPCRs), such as α_1-adrenoceptor (α_1-AR) and endothelin-1 (ET-1) receptors, are enhanced. Previous studies from this lab have demonstrated that the increased contractile responses of arteries from chronic (12-14 weeks) streptozotocin- (STZ-) diabetic rats to α_1-AR stimulation result from a change in the signal transduction process downstream from the receptor.

Protein kinase C (PKC) has been suggested to contribute to enhanced contractile responses of arteries from STZ-diabetic rats to stimulation of GPCRs. This was investigated in the present study by comparing the effects of the PKC inhibitors, Ro-318220 and calphostin C on contractile responses of mesenteric arteries from diabetic and age-matched control rats to the α_1-AR agonist, norepinephrine (NE) and to ET-1. Since translocation of PKC to the membrane is considered a hallmark of its activation, the effects of NE and ET-1 on particulate (membrane) levels of three isoforms of PKC (PKCα, δ and ε) that have been implicated in contraction were determined. The effect of NE on phosphorylation of CPI-17, a substrate for PKC, was also investigated. Contractile responses of endothelium-denuded arteries from diabetic rats to NE were enhanced, but were normalized by the PKC inhibitors. In contrast, no change in contractile responses of diabetic arteries to ET-1 could be detected, and PKC inhibition attenuated ET-1 responses to a similar extent in both control and diabetic tissues. NE produced a small translocation of PKCε in control arteries, but a significant translocation of PKCα and a much larger translocation of PKCε in diabetic arteries. ET-1 increased translocation of PKCα, δ and ε to the same extent in both control and diabetic arteries. NE significantly enhanced CPI-17 phosphorylation in diabetic, but not control, arteries and this was blocked by calphostin C.

In addition to PKC, the RhoA/Rho kinase (RhoK) signaling pathway is also believed to play an important role in vasoconstriction. In the next part of this study, the role of RhoK in enhanced contractile responses of diabetic mesenteric arteries to stimulation of α_1-ARs was

investigated. The selective RhoK inhibitors, Y-27632 and H-1152, produced greater inhibition of contractile responses to phenylephrine (PE) in diabetic than control mesenteric arteries and normalized the difference between them. Contractile responses to ET-1 were not different between control and diabetic arteries, and were not affected by RhoK inhibition. Since translocation of RhoK from the cytosolic to the membrane fraction is considered a marker of enzyme activity, the effects of PE on particulate levels of the two RhoK isoforms (ROCK I and II) were determined. A maximum concentration of PE produced significant translocation of ROCK I and II that was inhibited by Y-27632 in both control and diabetic arteries. The PE-induced translocation of ROCK II was significantly greater in diabetic tissues. PE also produced significant translocation of PKC α, δ and ε in diabetic but not control arteries. Y-27632 blocked the translocation of these isoforms in diabetic arteries but had no effect on ET-1 induced translocation of PKC isoforms in control arteries.

These data suggest that increased activation of the PKC/CPI-17 as well as the RhoK pathway contribute to the enhanced contractile responses of diabetic mesenteric arteries to α_1-AR stimulation. They further suggest that there is an interaction between the RhoK and PKC pathways on stimulation of α_1-ARs in diabetic mesenteric arteries, and that RhoK may be upstream of PKC. On the other hand, RhoK does not appear to contribute to contractile responses to ET-1 in either control or diabetic mesenteric arteries; this may be the reason why vasoconstrictor responses to this agonist are not altered in diabetic arteries.

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$[Ca^{2+}]_i$	intracellular calcium concentration
5-HT	5-hydroxytryptamine
7TM	seven transmembrane
AA	Arachidonic acid
ACh	acetylcholine
ADA	American Diabetes Association
AG II	angiotensin II
AGE	advanced glycation end products
aPKC	atypical protein kinase C
Ca^{2+}	calcium ion
CaD	caldesmon
CaP	calponin
CICR	Ca^{2+}-induced Ca^{2+} release
CPI-17	protein kinase C potentiated inhibitor protein of 17 kDa
cPKC	conventional protein kinase C
$cPLA_2$	Cytosolic Ca^{2+} dependent phospholipase A_2
CV	cardiovascular
CVD	cardiovascular disease
DAG	diacylglycerol
DM	diabetes mellitus
EDCF	endothelium derived constricting factors
EDRF	endothelium derived relaxing factors
ET-1	endothelin-1
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine 5`-diphosphate
GDPβS	guanosine 5`-O- (2-thiodiphosphate), a G-protein inhibitor
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
G-protein	heterotrimeric guanine nucleotide binding protein
GTP	guanosine 5`-triphosphate
GTPγS	guanosine 5`-O- (3-thiotriphosphate), a non-hydrolysable GTP analogue
IDDM	insulin dependent diabetes mellitus
IP_3	inositol 1, 4, 5 trisphosphate
$iPLA_2$	Cytosolic Ca^{2+} independent phospholipase A_2
KCl	potassium chloride
LPA	lysophosphatidic acid
MAB	mesenteric arterial bed
MAP	mitogen activated protein
MLC_{20}	regulatory light chains of myosin
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MYPT	110-130kDa regulatory subunit of MLCP

NE	norepinephrine
NEFA	non-esterified fatty acid
NIDDM	non-insulin dependent diabetes mellitus
NO	nitric oxide
NOS	nitric oxide synthase
nPKC	novel protein kinase C
PA	phosphatidic acid
PC	phosphatidylcholine
pD_2	- log EC_{50}
PdBu	phorbol 12, 13 dibutyrate
PDK-I	phosphoinositide dependent kinase I
PE	phenylephrine
PIP_2	phosphatidylinositol 4, 5 bisphosphate
PIP5K	phosphatidylinositol 4-phosphate 5-kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PP1c	37-38kDa catalytic subunit of MLCP
PS	phosphatidylserine
PTX	pertussus toxin
RACKS	receptors for activated C-kinase
RGS	regulators of G-protein signaling
RhoK	Rho kinase
ROS	reactive oxygen species
Ser	serine
SNP	sodium nitroprusside
$sPLA_2$	secretory phospholipase A_2
SR	sarcoplasmic reticulum
STZ	streptozotocin
Thr	threonine
TK	tyrosine kinase
TXA_2	thromboxane A_2
UT	untreated
VGCC	voltage gated calcium channel
α_1-AR	alpha$_1$-adrenoceptor
β-AR	beta-adrenoceptor

1. INTRODUCTION

1.1 DIABETES

Diabetes mellitus (DM) is a heterogeneous group of disorders that are characterized by abnormally high levels of glucose in the blood. Hyperglycemia is usually due to defects in insulin secretion or resistance to the actions of insulin or a combination of these. DM affects approximately 100 million people worldwide, and by the year 2010 this number is expected to increase to 221 million (Amos *et al.*, 1997). Intensive research showed that there were different types of DM with varying etiologies and there was a need for appropriate classification and systematic categorization for proper management of the disease. In 1980, the World Health Organization (WHO) endorsed the classification developed by an international group referred to as the National Diabetes Data Group (NDDG). According to this, two major forms of DM were identified: insulin dependent DM (IDDM, Type 1 DM) and non-insulin dependent DM (NIDDM, Type 2 DM) (World Health Organization, 1980). However in 1996 – 1997, the American Diabetes Association (ADA) proposed some changes to this classification scheme (American Diabetes Association Expert Committee, 1997). According to the new system, the terms insulin dependent DM and non-insulin dependent DM as well as their acronyms, IDDM and NIDDM, were eliminated and instead the two major kinds of DM were simply referred to as Type 1 and Type 2 DM.

Type 1 DM involves destruction of pancreatic β-cells, either immune mediated or due to unknown etiologies, and comprises about 5 - 10% of all cases of diabetes (Harris, 2004). It usually begins early in life, affecting children and adolescents, and is often called juvenile diabetes. However it has also been shown to affect adults (> 30 years of age) (Melton *et al.*, 1983; Laakso & Pyorala, 1985). The primary cause of immunologic β-cell destruction is the development of antibodies to the pancreatic islet cells and to insulin. The initiation of β- cell autoimmunity may be induced in any person at any time (Knip, 1997). Amongst the etiologic agents that initiate and subsequently accelerate β-cell destruction are environmental factors (e.g. viral infections).

Type 2 DM involves impaired insulin-mediated glucose disposal (insulin resistance) and defects in insulin secretion. It comprises about 90% of all cases of diabetes (Harris, 2004). It mostly affects adults, starting off with symptoms of insulin resistance and eventually leading

to a decline in insulin secretion. Although there is a strong genetic basis for this disease, lifestyle choices play a very important role in the development of Type 2 DM. There is a strong correlation between a sedentary lifestyle, physical inactivity, high calorie intake and obesity (especially central obesity) and increased incidence of Type 2 DM (Chisholm *et al.*, 1997; Gerich, 1998).

1.2 DIABETES AND CARDIOVASCULAR DISEASE

Both Type 1 and Type 2 DM are independent risk factors for the development of cardiovascular (CV) complications such as coronary heart disease, stroke, peripheral arterial disease and cardiomyopathy (Grundy *et al.*, 1999; Creager *et al.*, 2003). Cardiovascular disease (CVD) occurs at a significantly higher rate in diabetic patients as compared to the general population (Kowalska *et al.*, 2001; Hu *et al.*, 2002; Norhammar *et al.*, 2002). Not only is there an increased incidence of CV pathologies but also lower survival outcomes, significantly increasing the mortality and morbidity associated with this disease (Nesto, 2004). CVD is the major cause of death in more 65% of the diabetic population.

Most of the CV complications of diabetes are associated with tissue damage in the macro- and microvasculature. Macrovascular complications occur in the large blood vessels such as peripheral arteries of the lower limbs (e.g. femoral, popliteal), cerebral and coronary arteries and lead to the development of atherosclerosis, peripheral vascular occlusive disease and myocardial infarction. Microvascular disease affects the small arteries such as those supplying the kidneys, retina and nerves. Many of the long-term complications of diabetes stem from microvascular complications such as retinopathy, nephropathy and neurovascular defects associated with autonomic neuropathy. Clinical studies have shown that tight glycemic control with the administration of insulin results in significant retardation of the development and progression of microvascular complications (Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998).

Although multiple biochemical mechanisms have been proposed to explain how hyperglycemia leads to development of diabetic vascular complications (as reviewed by King & Brownlee, 1996; Cooper *et al.*, 2001; Setter *et al.*, 2003), the four major proposed mechanisms are:

1) Increased glucose metabolism through the polyol pathway. Through this pathway glucose is reduced to sorbitol by aldose reductase, coupled with oxidation of NADPH to NADP. The sorbitol is then oxidized to fructose by sorbitol dehydrogenase coupled with reduction of NAD to NADH. Persistent hyperglycemia can lead to accumulation of sorbitol (which can increase osmotic stress and cell permeability), fructose, vasodilatory prostaglandins and unbound NADH. However in vivo studies using polyol pathway inhibitors have been disappointing overall, showing little improvement in diabetes-induced retinopathy or nephropathy (Sorbinil Retinopathy Trial Research Group, 1990; Engerman *et al.*, 1994)

2) Increased production of advanced glycation end products (AGEs) by non-enzymatic and spontaneous reactions between reducing sugars and amino groups on proteins, lipids and nucleic acids. AGEs can contribute to basement membrane thickening of various tissues as well as alteration in the functional properties of several matrix molecules (e.g. collagen). This can result in altered structure and function of intact vessels. A number of clinical studies have shown that AGE inhibitors like aminoguanidine can prevent chronic manifestations of diabetes in the microvasculature (Eldenstein & Brownlee, 1992; Brownlee, 1995; Brownlee, 2001).

3) Generation of reactive oxygen species (ROS) by a number of mechanisms such as auto-oxidation of glucose and AGEs, increased activity of oxidative enzymes by enhanced NADH from the polyol pathway as well as enhanced activation of PKC. In fact overproduction of ROS such as superoxide is the common consequence of polyol pathway activation, formation of glycoxidation products and accumulation of AGEs. ROS can increase microvascular permeability and damage protein and mitochondrial DNA (Baynes, 1991; Taylor, 2001). It has also been shown that antioxidant levels are decreased in diabetes (Jennings *et al.*, 1987). Antioxidant supplementation in animal models and humans has been shown to ameliorate some of diabetes-induced microvascular abnormalities (Nagamatsu *et al.*, 1995; Bursell *et al.*, 1999).

4) Increased activation of the DAG/PKC pathway (discussed in section 1.10)

1.3 EXPERIMENTAL MODELS OF DIABETES

Intensive research conducted over the last few decades has enhanced our knowledge about the pathophysiology of DM. However despite significant progress, this disease at best can be

treated but is usually refractory to prevention, particularly with respect to Type 1 DM. Animal models of DM have allowed investigators to acquire more information about all aspects of this disease, as well as test new and innovative therapies for treating it. Type 1 DM can be induced in animals with the help of toxins and infectious agents (e.g. streptozotocin (STZ), synthesized from *streptomycetes acromogenes*) that act as selective pancreatic β cell cytotoxins. This model is of research interest because environmental factors can often trigger the onset of Type I DM in humans (Akerblom *et al.*, 2002; Hawa *et al.*, 2002). STZ is taken up by pancreatic β cells via the glucose transporter GLUT 2 (Schnedl *et al.*, 1994) and induces β–cell death primarily due to alkylation of DNA (as reviewed by Szkudelski, 2001). In addition to this, STZ generates reactive oxygen species such as the superoxide anion and is also a nitric oxide (NO) donor, and these effects can contribute to DNA fragmentation and damage (as reviewed by Szkudelski, 2001).

A number of studies have shown that the diabetogenic effects of STZ are related to its dose (Junod *et al.*, 1969). An intermediate dose of STZ, 50 – 70 mg/kg injected intravenously can induce Type 1 DM that is stable over a long time (Ar'Rajab & Ahren, 1993). Lower doses of STZ (30 – 40 mg/Kg) induce a transient diabetic state with complete recovery after a few days (Ar'Rajab & Ahren, 1993). Junod et al (1969) showed that when STZ was administered at a dose of 55 – 65 mg/kg (a dose commonly used in a lot of cardiovascular studies including the present investigation), rats developed hyperglycemia but were able to survive without insulin supplementation. At a higher dose of 100 mg/kg, rats were able to survive only with insulin treatment (Junod *et al.*, 1969). Within 7 – 10 hours after STZ administration, there is massive β-cell necrosis associated with increased serum insulin levels and hypoglycemia. Within 1 – 28 days pancreatic insulin levels are reduced to less than 5% of normal values and a state of prolonged hyperglycemia develops (Junod *et al.*, 1967).

Rats treated with STZ display many features seen in human patients with uncontrolled diabetes mellitus, such as hyperglycemia, hypoinsulinemia, development of cataracts, increased urinary glucose levels and consequently polyuria, polydipsia and weight loss. Studies focused on vascular dysfunction in the STZ diabetic rat are usually carried out within a few months of STZ treatment and therefore reflect the effects of relatively acute insulin deficiency in newly diagnosed Type I patients (as reviewed by Tomlinson *et al.*, 1992). The continued use of this model is evidence of the fact that it can provide valuable information

about the underlying pathophysiological conditions contributing to this debilitating disease and can be used for the development of treatments besides insulin therapy, which can arrest or inhibit the development of these conditions.

1.4 ABNORMALITIES IN THE DIABETIC VASCULATURE

The cardiovascular complications of diabetes mellitus have been shown to arise in part from altered reactivity of vascular smooth muscle to neurotransmitters and circulating hormones like norepinephrine (NE) and angiotensin II (AG II). While it has been well established that diabetes leads to abnormalities in vascular function, studies conducted on vascular reactivity in Type I diabetic animal models have not all been in agreement with each other and there have been conflicting reports on whether responsiveness is increased or decreased. We and others have consistently found that contractile responses of arteries from rats with well-established diabetes to stimulation of G-protein coupled receptors (GPCRs), such as alpha adrenoceptors (α-ARs) and endothelin (ET) receptors, are enhanced (MacLeod, 1985; Abebe & MacLeod, 1991a; Abebe & MacLeod, 1992; Inoguchi *et al.*, 1992; Ishikawa *et al.*, 2004; Yousif *et al.*, 2004). Significantly elevated responses have been observed in the aorta (Hattori *et al.*, 1995), mesenteric resistance (Taylor *et al.*, 1994), renal (Inazu *et al.*, 1991), carotid (Llorens *et al.*, 2004) and coronary (Tickerhoof *et al.*, 2003) arteries from diabetic animals on stimulation by agonists when compared to corresponding age-matched controls. However, in contrast, some studies have shown that diabetic arteries exhibit attenuated responses to agonist-induced stimulation (James & Hodgson, 1995; Makino & Kamata, 2000a; Misurski *et al.*, 2001). The precise reason for this discrepancy is not clear. One possibility could be differences in the severity and duration of diabetes, which has been shown to significantly affect the development of vascular dysfunction. Enhanced reactivity to agonists has usually been observed after a relatively long-term diabetic state (> 12 weeks) (Jackson & Carrier, 1981; MacLeod & McNeill, 1985; Van Buren *et al.*, 1998). Similarly most studies conducted on Type I diabetic patients have shown vasoconstrictor responses to be increased (Christlieb *et al.*, 1976; Drury *et al.*, 1984; Eichler *et al.*, 1992; McIntyre *et al.*, 2001), although decreased reactivity has also been reported (McNally *et al.*, 1994).

Another commonly reported vascular abnormality in Type I diabetic patients as well as experimental animal models is the development of endothelial dysfunction (as reviewed by De Vriese *et al.*, 2000). The integrity of the endothelium is usually assessed by measuring

vasodilatation in response to agents like acetylcholine (ACh) or bradykinin. Arteries like the aorta (Tesfamariam *et al.*, 1993; Pieper & Siebeneich, 1998) and the superior mesenteric artery (Heygate *et al.*, 1995; Palmer *et al.*, 1998) isolated from STZ-diabetic animals exhibit impaired relaxation to ACh with no change in vasodilator responses to sodium nitroprusside (SNP, endothelium-independent vasodilator). However, impaired endothelium-dependent vasodilatation is not consistently observed in diabetic vessels. Our lab reported that ACh-induced vasodilatation in aorta and mesenteric arteries from STZ-diabetic rats was not significantly different from the corresponding controls (Harris & MacLeod, 1988). Similarly it has been shown that endothelium-dependent relaxation is preserved in aorta (Head *et al.*, 1987; Wakabayashi *et al.*, 1987) and renal arteries (Yousif *et al.*, 2002) from diabetic animals. The reason for this discrepancy is not known but may be because of differences in the species used, the strain of rats, or the duration of the diabetes.

Endothelial dysfunction in diabetes is believed to arise predominantly from an imbalance in availability and production of endothelium derived relaxing factors (EDRFs) and endothelium derived constricting factors (EDCFs) (as reviewed by De Vriese *et al.*, 2000). A hyperglycemia-induced increase in steady-state levels of reactive oxygen species results in inactivation of NO (an EDRF) (Pieper *et al.*, 1992; Giugliano *et al.*, 1996). At the same time there can also be enhanced production of EDCFs such as prostanoids, which can oppose the action of EDRFs (Mayhan *et al.*, 1991; Shimizu *et al.*, 1993). Another factor that can contribute to endothelial dysfunction in diabetes is impaired release of endothelium-derived hyperpolarizing factor (EDHF), which has been suggested to be a major determinant of vascular tone in small arteries (Garland *et al.*, 1995). For example, attenuation of endothelium-dependent relaxation in mesenteric arteries from STZ-diabetic rats was suggested to be due to a diminished contribution of EDHF (Fukao *et al.*, 1997; Makino *et al.*, 2000b; Wigg *et al.*, 2001).

In most cases the abnormal vasoreactivity can be restored to normal by administration of insulin (MacLeod, 1985; Takiguchi *et al.*, 1989; Tanz *et al.*, 1989; Hodgson & King, 1992), suggesting that hyperglycemia and associated metabolic abnormalities predominantly mediate vascular dysfunction in diabetes .

1.5 PREVIOUS FINDINGS FROM OUR LAB

Our lab has consistently reported that contractile responses to α_1-AR stimulation in diabetic arteries are significantly elevated as compared to age and gender matched controls. The enhanced responses are due to the diabetic state as insulin treatment can prevent or reverse these effects (MacLeod, 1985). Intensive investigation of the factors that could be contributing to this revealed that the enhanced responses of diabetic arteries were not due to:

1) A generalized increase in the reactivity of diabetic tissues, because contractile responses to potassium depolarization were not significantly elevated (MacLeod, 1985).
2) Release of endothelium-derived vasoactive factors, since enhanced responses were also observed in the absence of a functional endothelium (Harris & MacLeod, 1988).
3) The development of autonomic neuropathy or changes in β receptor function or extraneuronal uptake mechanisms (Weber & MacLeod, 1994).
4) Differences in total α_1-AR number or affinity or change in G-protein ($G_{i2/3}$ and $G_{q/11}$) levels (Weber & MacLeod, 1997).

These results suggest that significantly enhanced contractile responses in diabetic arteries are due to an intrinsic alteration at the level of the smooth muscle, such as increased efficiency of coupling of the α_1-AR to its signal transduction pathway.

1.6 G-PROTEIN COUPLED RECEPTORS

G proteins are heterotrimeric guanine nucleotide binding proteins that serve as transducers for GPCRs. These receptors constitute the largest, most ubiquitous and most versatile family of membrane receptors. They are characterized by a signature seven transmembrane (7TM) configuration with three extracellular loops and three intracellular cytoplasmic loops (hence GPCRs are also referred to as heptahelical or serpentine receptors). G-proteins consist of three subunits, α, β and γ, of which Gα is responsible for GDP and GTP binding. Stimulation of GPCRs with their cognate agonists results in the formation of a transient high affinity complex of agonist-activated receptor and G-protein. GDP is released from the G-protein and is replaced by GTP, promoting its dissociation into the GTP bound Gα-subunit and a G$\beta\gamma$ dimer, both of which have the capacity to activate a number of different effector systems and second messenger cascades (as reviewed by Pierce *et al.*, 2002). Regulators of G-protein

signaling (RGS) mediate hydrolysis of GTP to GDP, leading to the reassociation of the subunits and termination of the activation cycle. To date 16 α, 5 β and 12 γ proteins have been identified.

G proteins are generally referred to by their α subunits. So for example the Gq complex contains Gαq. Four different α subunit families have been recognized (as reviewed by Landry & Gies, 2002) -

1) Gs: including α_s and α_{olf}, which couple to stimulation of adenylyl cyclase.

2) G_i: including α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, α_{t1}, α_{t2}, α_z and α_{gust}, which couple to inhibition of adenylyl cyclase and activation of G-protein coupled inwardly rectifying potassium channels (GIRK).

3) Gq: including α_q, α_{11}, α_{14}, α_{15} and α_{16}, which couple to stimulation of phospholipase Cβ (PLCβ).

4) G_{12}: including α_{12} and α_{13}, which couple to stimulation of Rho guanine nucleotide exchange factors (GEFs).

The two GPCRs that are most pertinent to this project are the α_1-AR and the ET receptor. The α_1-ARs play an important role in regulating the vascular and cardiac effects of the endogenous sympathomimetic catecholamines, epinephrine and NE and mediate the vasoconstrictor actions of exogenously administered α-AR agonists (as reviewed by Guimaraes & Moura, 2001). They constitute three pharmacologically defined subtypes: α_{1A}-AR, α_{1B}-AR and α_{1D}-AR. All three α_1 ARs have been shown to be expressed in smooth muscles of different rat arteries (Faure *et al.*, 1994; Scofield *et al.*, 1995). While these subtypes share structural homology, they exhibit considerable functional heterogeneity as well as differences in tissue distribution and affinities for agonists and antagonists. The α_{1B}-AR has been shown to be predominantly expressed on the cell surface (Hrometz *et al.*, 1999) whereas the α_{1D}-AR was detected mainly intracellularly (McCune *et al.*, 2000). α_{1A}-AR was detected both on the cell surface as well as intracellularly (Hirasawa *et al.*, 1997; McCune *et al.*, 2000).

In vascular smooth muscle, α_1-ARs mediate two important effects: vasoconstriction and vascular growth (as reviewed by Varma & Deng, 2000). Arterial vasoconstriction is mediated

primarily by postjuctional α_1-ARs, although depending on the artery, different subtypes participate in this response (Hrometz *et al.*, 1999). For example it has been shown that the α_{1D}-AR is predominantly activated in the aorta, femoral and iliac artery (Piascik *et al.*, 1995; Buckner *et al.*, 1996; Gisbert *et al.*, 2003), α_{1A}-AR in the renal and caudal artery (Hrometz *et al.*, 1999; Gisbert *et al.*, 2003) and α_{1B}-AR and α_{1D}-AR in the mesenteric artery (Hussain & Marshall, 2000). α_1-AR stimulation by catecholamines has been shown to trigger not only immediate responses but also long term actions such as enhanced growth and proliferation in vascular smooth muscle cells (Hu *et al.*, 1996; Xin *et al.*, 1997). Abnormally increased sympathomimetic activity due to elevated plasma catecholamine levels has been shown to produce vascular hypertrophic effects via increased α-actin mRNA and total cell protein (Chen *et al.*, 1995) and increased DNA synthesis (van Kleef *et al.*, 1992). Mitogenesis in vascular smooth muscle due to α_1-AR stimulation is primarily due to tyrosine phosphorylation of several proteins (Hu *et al.*, 1999). The hypertrophic effects of α_1-ARs in arterial smooth muscle cells have been shown to be predominantly mediated by the α_{1B} and α_{1D} subtype (Chen *et al.*, 1995; Xin *et al.*, 1997).

There are two kinds of ET receptors, ET_A and ET_B in mammalian cells and they are stimulated by three endogenous isoforms of ET, ET-1, ET-2 and ET-3 (as reviewed by Sokolovsky, 1995; Sugden, 2003). The ET_A receptor has high affinity for ET-1 and ET-2 but a low affinity for ET-3 whereas the ET_B receptor has equal affinity for all three ET isoforms. ET_A receptors are located primarily on vascular smooth muscle cells and regulate vasoconstriction and cellular proliferation and mitogenesis (Schiffrin, 1995). These effects are opposed by stimulation of ET_B receptors that are predominantly expressed by vascular endothelial cells. Their activation leads to generation of prostacyclin and NO by stimulation of nitric oxide synthase (NOS) and helps to prevent apoptosis (Niwa *et al.*, 2000). Therefore the vasoregulatory action of ETs can be controlled by a number of different factors such as number and type of receptors activated, receptor affinity and the kind of tissue being stimulated. To a lesser extent, ET_B receptors are also expressed in vascular smooth cells and can mediate vasoconstriction like the ET_A receptors. In pathological conditions there could be upregulation of ET_B receptors on vascular smooth muscle and together with ET_A

receptors, they could amplify the vasoconstrictive and mitogenic effects of ET (Haynes *et al.*, 1995).

1.7 GPCRs AND ASSOCIATED SIGNAL TRANSDUCTION PATHWAYS

Both α_1-ARs (α_{1A}, α_{1B} and α_{1D}) (as reviewed by Zhong & Minneman, 1999; Varma & Deng, 2000) and ET receptors (ET_A and ET_B) (as reviewed by Sokolovsky, 1995; Sugden, 2003) have been shown to couple to the pertussus toxin (PTX)-insensitive $G_{q/11}$ G protein. Activation of this G protein leads to the stimulation of the phosphoinositide-specific phospholipase Cβ (PLCβ) that hydrolyzes the membrane phospholipid, phosphatidylinositol 4, 5 bisphosphate (PIP_2). This leads to the production of two second messengers: diacylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP_3). DAG remains in the cell membrane and mediates the activation of protein kinase C (PKC). IP_3 diffuses into the cytoplasm and binds to receptors present on a number of intracellular organelles, primarily the sarcoplasmic reticulum (SR), and mediates Ca^{2+} release.

As far as α_1-ARs are concerned, studies have suggested that there are differences in the coupling of the different receptor subtypes to their second messenger systems. α_{1A}-ARs have been shown to be the most effective activators of phosphoinositide hydrolysis and have been reported to be much more efficiently coupled to the activation of PKC than α_{1B} or α_{1D}-ARs (Schwinn *et al.*, 1995; Theroux *et al.*, 1996; Taguchi *et al.*, 1998).

The α_1-ARs (as reviewed by Seasholtz *et al.*, 1999; Fukata *et al.*, 2001) and ET receptors (Gohla *et al.*, 2000) have also been shown to couple to the PTX-insensitive $G_{12/13}$ G-protein. These G-proteins regulate the activity of the small monomeric GTPase Rho via direct activation and regulation of RhoGEFs (guanine nucleotide exchange factors) (as reviewed by Sah *et al.*, 2000). In the inactive state, Rho is bound to GDP and GDI (guanine nucleotide dissociation inhibitor). Activation of RhoGEFs by $G_{12/13}$ catalyzes nucleotide exchange on Rho, replacing GDP with GTP, thereby activating Rho. Since RhoGEFs facilitate RhoA activation, they can regulate the signaling duration and intensity of GPCRs. The two GEFs identified in vascular smooth muscle that have been shown to be activated by $G_{12/13}$ are p115RhoGEF and PDZ-RhoGEF (Hart *et al.*, 1998; Fukuhara *et al.*, 1999). Inactivation of

small G-proteins is regulated by GAPs (GTPase activating proteins) that accelerate their intrinsic GTPase activity.

Stimulation of α_1-ARs and ET receptors has also been shown to increase the activity of phospholipase D (PLD) and phospholipase A_2 (PLA_2) (as reviewed by Sokolovsky, 1995; Varma & Deng, 2000). PLD hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA), which is then converted into DAG. This signaling pathway provides an additional source of DAG in addition to the rapid but transient increase in DAG following agonist induced PI hydrolysis. The activated PKC resulting from PLD-induced DAG formation further enhances PLD activation. This produces a sustained increase in DAG formation and hence prolonged maintenance of PKC activation, a pathway that could be useful for long-term cellular responses such as cell growth (Nishizuka, 1992; Eskildsen-Helmond *et al.*, 1996).

PLA_2 represents a superfamily of enzymes broadly classified into (as reviewed by Balsinde *et al.*, 2002):

1) Secretory ($sPLA_2$)
2) Cytosolic Ca^{2+} dependent ($cPLA_2$)
3) Cytosolic Ca^{2+} independent ($iPLA_2$)

On activation, PLA_2 catalyzes the hydrolysis of phospholipids into a free fatty acid and a lysophospholipid. Arachidonic acid (AA) production from phospholipids is primarily mediated via the catalytic activity of PLA_2. $sPLA_2$ isoforms are low molecular weight extracellular enzymes (~ 14kDa), which require millimolar concentrations of Ca^{2+} for activation (Balsinde *et al.*, 1999). They do not exhibit fatty acid selectivity *in vitro* and so are not specific for phospholipids containing AA. Both $iPLA_2$ and $cPLA_2$ are intracellular enzymes and have been shown to have similar molecular weights (~ 85 kDa) and catalytic mechanisms (Balsinde & Dennis, 1997). $iPLA_2$ isoforms are Ca^{2+} independent whereas $cPLA_2$ isoforms require micromolar concentrations of Ca^{2+} for activation and are selective for AA-containing phospholipids. Free AA is the precursor of eicosanoids, which include the prostaglandins (produced via the cyclooxygenase reaction) and the leukotrienes (produced via the lipoxygenase reaction) (Smith, 1992).

Stimulation of α_1-ARs (Toma *et al.*, 1995; Ward *et al.*, 1995; Jin *et al.*, 1996) and ET receptors (Ohanian *et al.*, 1997) has also been associated with increased phosphorylation of proteins on tyrosine residues. Traditionally, activation of tyrosine kinases (TKs) has been

associated with long-term effects such as oncogenesis, cell growth and proliferation. However recent evidence has shown that TKs can be phosphorylated within seconds in response to vasoconstrictor hormones, and can influence vascular tone (Hughes & Wijetunge, 1998). These are usually non-receptor TKs, e.g. Src kinases that are present in the cytosol and, unlike receptor TKs whose activity is regulated by extracellular ligands; they usually do not have extracellular recognition domains for ligands. However, their activity can be regulated at an intracellular level by GPCRs that themselves lack intrinsic TK activity. One of the mechanisms used by GPCRs to signal through non-receptor TKs is via adaptor/scaffolding proteins that facilitate interaction between receptor and their downstream signaling effectors (Luttrell *et al.*, 1999; Imamura *et al.*, 2001).

1.8 VASCULAR SMOOTH MUSCLE CONTRACTION

The principle trigger initiating contraction in smooth as well as striated (cardiac and skeletal) muscle is an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$). In the resting state, vascular smooth muscle has a negative membrane potential, with $[Ca^{2+}]_i$ ranging from 120-270 nmol. Membrane depolarization (positive potentials) can result in the opening of voltage-gated calcium channels (VGCC) and the influx of Ca^{2+} from the extracellular space. This in turn also leads to Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR). An increase in $[Ca^{2+}]_i$ to 500-700 nmol triggers the binding of Ca^{2+} to calmodulin, a multi-functional Ca^{2+} binding protein. This in turn induces a conformational change in the calmodulin molecule as a result of which it binds to the catalytic sub-unit of myosin light chain kinase (MLCK) and activates it. MLCK then phosphorylates serine 19 on the regulatory light chains of myosin (MLC_{20}, 20kDa) and allows the actomyosin ATPase to be activated by actin. The myosin cross bridges slide over the actin filaments, as a result of which there is development of contractile force. A fall in the $[Ca^{2+}]_i$ inactivates MLCK and allows myosin light chain phosphatase (MLCP) to dephosphorylate MLC_{20}, producing relaxation (as reviewed by Somlyo & Somlyo, 1994; Horowitz *et al.*, 1996c).

The development of contraction that is dependent on changes in membrane potential and $[Ca^{2+}]_i$ is referred to as electromechanical coupling. Another physiologically important component of the contractile process is pharmacomechanical coupling (Somlyo & Somlyo, 1968) that operates via the ability of intracellular signaling pathways to affect force without changes in membrane potential. An important mechanism of pharmacomechanical coupling

is the regulation of Ca^{2+} sensitivity. The development of force is not always proportional to the $[Ca^{2+}]_i$ and neurohumoral stimulation of smooth muscle can increase the MLCK/ MLCP activity ratio, hence enhancing the sensitivity of the contractile proteins to Ca^{2+}. This is referred to as Ca^{2+} sensitization, that is the development of tension without change in $[Ca^{2+}]_i$. It has been shown that agonist-induced stimulation of GPCRs, including α_1-AR, ET, thromboxane A_2 (TXA_2) and AT receptors, can induce Ca^{2+} sensitization, and this can be mimicked by GTPγS (a non-hydrolysable GTP analogue) and is inhibited by GDPβS (a G-protein inhibitor) (Kitazawa *et al.*, 1989; Himpens *et al.*, 1990; Iizuka *et al.*, 1997), suggesting that G-proteins are a vital factor in this process.

The principal mechanisms for GPCR-mediated Ca^{2+} sensitization have been suggested to be via inhibition of MLCP and inhibition of actomyosin ATPase by thin filament associated proteins.

Inhibition of MLCP

MLCP is a heterotrimeric complex composed of a 37 - 38kDa catalytic subunit (PP1c), a 110-130 kDa regulatory subunit (MYPT) and a 20kDa subunit of unknown function. While the catalytic subunit is common to all type I phosphatases, it is MYPT that confers the high selectivity of MLCP for phosphorylated myosin. It enhances the catalytic activity of PP1c and targets the entire MLCP complex towards phosphorylated myosin. Initially characterized as an unregulated 'house-keeping enzyme', it was soon established that MLCP is a G-protein regulated enzyme (Somlyo *et al.*, 1989) and inhibition of MLCP is the major mechanism for GPCR mediated Ca^{2+} sensitization (Somlyo & Somlyo, 2003; Ito *et al.*, 2004). The inhibition of MLCP results in increased MLC_{20} phosphorylation and enhanced contractile force at a given $[Ca^{2+}]_i$. MLCP is predominantly inhibited via phosphorylation of MYPT and the site most commonly phosphorylated by a number of different kinases (such as ZIP-like kinase (Borman *et al.*, 2002), integrin-linked kinase (Deng *et al.*, 2001) and Rho kinase (RhoK) (Feng *et al.*, 1999a) is Thr 696. However only RhoK-induced inhibition of MLCP has been shown to play a significant role in GPCR-mediated Ca^{2+} sensitization and contraction in vascular smooth muscle (discussed in section 1.16).

MLCP can also be inhibited by other mechanisms of action. It was shown that AA dissociated the MLCP holoenzyme and since the isolated PP1c is much less active compared to the MYPT-PP1c complex towards phosphorylated myosin, its activity was significantly

attenuated (Gong *et al.*, 1992). MLCP can also be inhibited via phosphorylated CPI-17, a predominant PKC substrate (discussed in detail in section 1.23) (Eto *et al.*, 1995; Eto *et al.*, 1997).

Inhibition of actomyosin ATPase by thin filament associated proteins

Smooth muscle thin filaments are regulated by a number of proteins and the two most well characterized are caldesmon (CaD) and calponin (CaP). Both CaD and CaP have been shown to bind to actin and inhibit actomyosin ATPase under physiological conditions *in vitro*. They can compete with myosin for binding sites on actin and inhibit actomyosin ATPase by a number of mechanisms. CaD has been shown to decrease the affinity between actin and myosin as a result of which the ATPase cannot be activated (Hemric & Chalovich, 1988). It has also been shown to decrease the rate of reaction of the ATPase cycle (Hemric *et al.*, 1994). CaP on the other hand does not affect the affinity between actin and myosin. Instead it can produce a conformational change in the actin molecule as a result of which there is a significant decrease in the activation rate of the ATPase (Horiuchi & Chacko, 1991; Noda *et al.*, 1992). Phosphorylation of CaD and CaP by kinases such as PKC and mitogen activated protein (MAP) kinases results in 'disinhibition' of actomyosin ATPase activity, leading to enhanced contractile force without change in $[Ca^{2+}]_i$ (as reviewed by Horowitz *et al.*, 1996c). However while the role of CaD and CaP in GPCR-mediated Ca^{2+} sensitization has been demonstrated *in vitro*, their *in vivo* significance and functions have yet to be established.

The agonist-G-protein complex is physically separated from the myosin-bound MLCP and the thin filament associated CaD and CaP (as reviewed by Somlyo & Somlyo, 1994). PKC, RhoK, AA and TKs can serve as messengers that can communicate the Ca^{2+} sensitization signals to MLCP and/or CaD and CaP.

1.9 THE PROTEIN KINASE C FAMILY

PKC was identified in bovine cerebellum by Nishizuka and colleagues in 1977 (Inoue *et al.*, 1977; Takai *et al.*, 1977). Two years later it was found that PKC was activated by phosphatidylserine (PS) and DAG in a Ca^{2+} dependent manner (Takai *et al.*, 1979). More than 25 years later, intensive research has revealed PKC to be a large extended superfamily and not a single protein. At present the PKC family comprises of 12 known isoforms, which differ in structure, function and co-factor requirements. In fact the PKC family is the largest serine/threonine kinase family known, which could explain the many signal transduction

functions that have been attributed to this family (as reviewed by Webb *et al.*, 2000). The 12 isoforms are divided into three families on the basis of co-factor requirements:

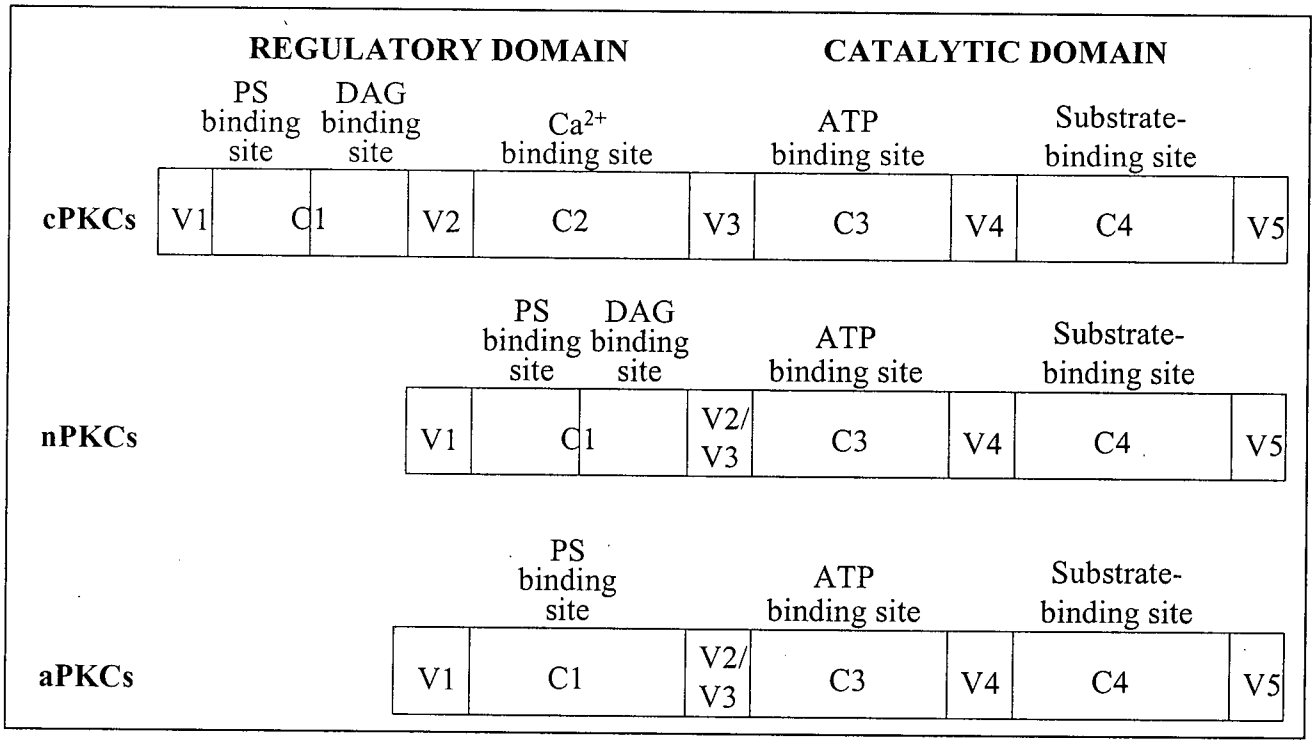
1) The conventional PKCs (cPKC: α, β_I, β_{II}, γ) that require PS, DAG and Ca^{2+} for activation

2) The novel PKCs (nPKC: δ, ε, η, θ, μ) that are Ca^{2+}-independent and require PS and DAG for activation

3) The atypical PKCs (aPKC: ζ, τ, λ) that only require PS for activation

The PKC isoforms share a highly conserved catalytic domain (containing the ATP (C3) and substrate (C4) binding site) and so have similar substrate specificities (Fig. 1.1). However they tend to differ in their membrane targeting regulatory domains, which contain the binding sites for PS (C1), DAG (C1) and Ca^{2+} (C2).

Figure 1.1



Diagrammatic representation of the structure of PKC isoforms
(Modified from Webb *et al.*, 2000)

In the unstimulated state, PKC isoforms are present mainly in the cytosol, whereas their activators are hydrophobic and reside in the particulate fraction. When the PKC molecule is inactive, the regulatory domain covers the catalytic site preventing access to the protein

substrate. The critical events in the regulation of PKC activity are phosphorylation by phosphoinositide dependent kinase I (PDK-I) (Dutil *et al.*, 1998) and at least one subsequent autophosphorylation in the catalytic domain (Le Good *et al.*, 1998). These phosphorylations are essential for PKC to be activated by second messengers (Dutil *et al.*, 1998; Dempsey *et al.*, 2000). Upon receptor stimulation and subsequent Ca^{2+} mobilization (critical for activation of cPKCs), PKC translocates from the cytosolic to the membrane fraction (Zidovetzki & Lester, 1992). The availability of DAG and PS is essential for activation of cPKCs and nPKCs and facilitate their penetration through the cell membrane (Lester *et al.*, 1990). This results in a conformational change in the PKC molecule, such that the regulatory domain is unmasked from the catalytic site, allowing substrate entry and catalysis to occur, hence activating PKC for downstream signaling. Translocation of PKC isoforms from the cytosolic to membrane fraction is often used as an index of their activation (Taggart *et al.*, 1999).

With the help of immunofluorescence techniques, it was shown that agonist-induced stimulation of PKC is associated with isozyme-selective localization in the cell (Mochly-Rosen *et al.*, 1990; Disatnik *et al.*, 1994) and this is achieved by binding of PKC to specific anchoring proteins (Mochly-Rosen & Gordon, 1998). These are referred to as RACKS (receptors for activated C-kinase). RACKS bind to PKC in the presence of activators and help to regulate its intracellular compartmentalization and localization to its sites of action. To date two RACKS have been characterized:

1) RACK1 – found to be selective for PKC β_2 (Ron *et al.*, 1994)
2) β`-COP - found to be selective for PKC ε (Csukai *et al.*, 1997)

1.10 HYPERGLYCEMIA-INDUCED ACTIVATION OF PKC

A number of mechanisms have been put forward to explain vascular dysfunction in diabetes (discussed in section 1.2). One of the major pathways implicated in the pathogenesis of hyperglycemia-induced vascular injury is DAG-mediated PKC activation (as reviewed by King & Brownlee, 1996; Idris *et al.*, 2001). DAG is generated from 4 major sources (Fig. 1.2):

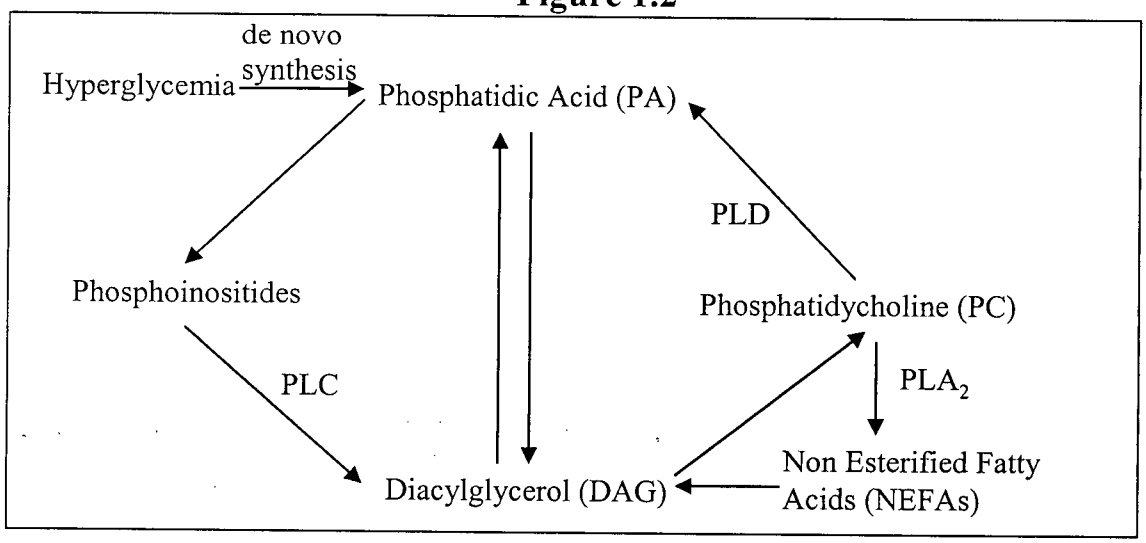
1) Receptor-mediated, PLC-catalyzed hydrolysis of PIP_2, resulting in the production of IP_3 and DAG

2) PLD-mediated hydrolysis of PC to PA, which is then converted to DAG by phosphatidic acid phosphohydrolase

3) PLA_2 mediated release of non-esterified fatty acids (NEFAs) from precursor lipids (e.g. PC) which are then converted into DAG

4) de novo synthesis or direct metabolism of glucose to DAG.

Figure 1.2



Principal Sources of DAG Production
(Modified from Idris *et al.*, 2001)

A number of studies have shown that hyperglycemia stimulates the DAG-PKC pathway in a variety of microvascular and macrovascular tissues (Craven & DeRubertis, 1989; Inoguchi *et al.*, 1992; Inoguchi *et al.*, 1994). This was primarily shown to be due to enhanced de novo synthesis of DAG (Inoguchi *et al.*, 1994; Xia *et al.*, 1994). Under conditions of hyperglycemia, the amount of glucose metabolized through glycolysis is increased and glycolytic intermediates such as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are converted into PA. This is then converted to DAG through the action of phosphatidic acid phosphohydrolase. In addition to this, there has been evidence to indicate that PC turnover is also significantly elevated in diabetes (Okurama *et al.*, 1991; Li *et al.*, 1994). The increased activation of PKC resulting from elevation of DAG levels has been shown to be one of the major factors contributing to functional and structural abnormalities leading to diabetic micro- and macroangiopathy (as reviewed by Meier & King, 2000; Idris *et al.*, 2001; Way *et al.*, 2001).

One of the ways found to ameliorate hyperglycemia induced DAG-PKC activation in the vasculature was administration of d-α-tocopherol (vitamin E). Vitamin E has been suggested to indirectly modulate the PKC signal transduction pathway in diabetes in a number of ways. It does not affect already-activated PKC (Kunisaki *et al.*, 1995). However it can enhance DAG kinase activity, which reduces DAG levels by increasing its metabolism to PA (Lee *et al.*, 1999a). Vitamin E has also been shown to inhibit PKC by preventing its translocation to the cell membrane (Boscoboinik *et al.*, 1991) and by activating phosphatase type 2A activity, which can dephosphorylate and inactivate PKC (Azzi *et al.*, 2000). Some studies have shown that vitamin E supplementation can produce a significant reduction in the development and progression of vascular complications by a combination of both its anti-oxidant properties and its inhibitory effect on the DAG-PKC pathway (Bursell *et al.*, 1999; Way *et al.*, 2001; Wigg *et al.*, 2004). However at the same time a number of investigations have reported that vitamin E supplementation had no effect on cardiovascular complications in diabetic patients (Gazis *et al.*, 1999; Yusuf *et al.*, 2000) and in some cases also had a worsening effect (Economides *et al.*, 2005).

Hyperglycemia-induced PKC activation is isoform and tissue specific, and out of the 12 known isoforms of PKC, it has been shown that there is preferential activation of PKCβ_2 as well as increased expression of this isoform in a number of different cardiovascular tissues (Inoguchi *et al.*, 1992; Guo *et al.*, 2003; Tickerhoof *et al.*, 2003; Wigg *et al.*, 2004). PKC isoforms play a very important role in cell signaling, and nonspecific inhibition of PKC *in vivo* is liable to have major detrimental effects. Therefore attempts have been made to develop isoform selective inhibitors and one of the most promising compounds is LY333531, a bisindolymaleimide that can be administered orally and has been shown to selectively inhibit PKCβ. Treatment of diabetic animals with LY333531 produced significant amelioration of diabetes-induced cardiomyopathy and nephropathy (as reviewed by Idris *et al.*, 2001; Way *et al.*, 2001). This suggests that PKCβ plays a critical role in the development of cardiovascular complications in diabetes.

In addition to PKCβ, enhanced activity and/or expression of other PKC isoforms have also been reported in other diabetic tissues. For example, in the diabetic rat retina PKC α, β and ε were increased in the membrane and cytosolic fractions (Shiba *et al.*, 1993). In the diabetic rat glomeruli, the expression of PKCα and β_1 in the membrane fraction was enhanced and

this was associated with an increased phosphorylation of these isoforms, suggesting a state of activation (Koya *et al.*, 1997).

A number of reasons could explain the differential activation of PKC isoforms in diabetes (Ishii *et al.*, 1998; Way *et al.*, 2001). For example, there could be differences in the sensitivity of the PKC isoforms to DAG, there could be increased DAG production at specific subcellular sites accessible to only certain PKC isoforms pools or the rates of protein synthesis and degradation of the isoforms could be different.

1.11 VASOREACTIVITY AND PKC ACTIVATION IN DIABETES

Enhanced contractility of vascular smooth muscle in Type I diabetic animal models has been reported frequently, and a number of studies have implicated increased activation of PKC as the underlying reason for this observation. Significantly increased contractions to ET-1, 5-hydroxytryptamine (5-HT) and the thromboxane A_2 mimetic, U-46619, in aortas from 8-12 week diabetic rats were suggested to be due to a greater influx of Ca^{2+} through transmembrane Ca^{2+} channels resulting from increased PKC-activated processes (Hattori *et al.*, 1995; Hattori *et al.*, 1999). However these conclusions were based on pharmacological inhibition of PKC and Ca^{2+} channels and did not involve direct measurement of $[Ca^{2+}]_i$ or PKC activation. Resistance arteries from 4 week diabetic hamsters exhibited significantly enhanced contractions to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and an enhancement of the PKC pathway was implicated in the augmented contractility (Georgescu & Popov, 2003). Increased aortic reactivity to NE in 6-7 week diabetic Wistar rats was attributed to a PKC-mediated increase in DAG kinase activity (Nobe *et al.*, 2002). Coronary arteries from 6-week diabetic rats exhibited significantly greater contractile responses to ET-1, which were normalized following treatment with a selective PKC inhibitor (Tickerhoof *et al.*, 2003). Significantly enhanced sensitivity to PE-induced contractions in a 4-week diabetic mouse model was reversed by treatment with LY333531 (10 mg/kg/day), suggesting that this was due to enhanced PKCβ activation (Nangle *et al.*, 2003). Consistent with these reports, our lab also has preliminary data to implicate PKC in significantly enhanced contractile responses to NE in arteries from 12-14 week diabetic rats. It was shown that NE mediated stimulation of phosphoinositide metabolism was significantly elevated in diabetic arteries, resulting in an increased production of IP_3, total inositol phosphates and phosphatidic acid (Abebe & MacLeod, 1991a; Abebe & MacLeod, 1991b). Moreover the enhanced responses were

normalized following treatment with the relatively non-specific PKC inhibitor staurosporine (Abebe & MacLeod, 1990).

1.12 PKC AND CALCIUM SENSITIZATION

A role for PKC in Ca^{2+} sensitization was suggested when phorbol esters such as phorbol 12, 13 dibutyrate (PdBu), which are structural analogs of DAG and cause direct activation of PKC, increased contractile force without a significant increase in $[Ca^{2+}]_i$ (Jiang & Morgan, 1987; Jiang & Morgan, 1989; Gailly *et al.*, 1997; Miura *et al.*, 1997). Agonists that stimulate the phosphoinositide pathway leading to activation of PKC also have the ability to sensitize the contractile proteins to Ca^{2+} (Jiang & Morgan, 1989; Horowitz *et al.*, 1996c; Kitazawa *et al.*, 1999; Kanashiro & Khalil, 2001; Wickman *et al.*, 2003). Of the twelve known PKC isoforms, the three that have been most frequently implicated in Ca^{2+} sensitization and contraction in response to stimulation of GPCRs in vascular smooth muscle are PKC α, δ and ε (Lee *et al.*, 1999b; Eto *et al.*, 2001).

Proposed mechanisms by which PKC may induce Ca^{2+} sensitization are:

1) Direct phosphorylation of MYPT (within residues 40-296) which diminishes its affinity for PP1c (Toth *et al.*, 2000), hence decreasing the activation of MLCP. However this has only been demonstrated *in vitro* and its physiological relevance has yet to be established.

2) Activation of PLA_2 and production of AA (Gong *et al.*, 1995; Parsons *et al.*, 1996; Miura *et al.*, 1997), which has the ability to enhance MLC phosphorylation via a number of mechanisms (discussed in section 1.18).

3) Inhibitory phosphorylation of CaP and CaD (Horowitz *et al.*, 1996a; Horowitz *et al.*, 1996b; Pohl *et al.*, 1997; Sohn *et al.*, 2001). PKC-mediated reversal of the inhibitory action of CaP and CaD on actomyosin ATPase has been demonstrated *in vitro* and there is conflicting evidence regarding the importance of this mechanism under physiological conditions. In one study, phosphopeptide maps of caldesmon phosphorylated in intact porcine carotid arteries suggested that PKC was not the phosphorylating kinase (Adam *et al.*, 1989). Moreover increasing evidence suggests that MAP kinase is the most likely candidate to induce CaD phosphorylation under physiological conditions (Adam *et al.*, 1993). In some studies calponin phosphorylation was undetectable in response to agonist-induced contraction in a

number of different smooth muscles (Barany & Barany, 1993; Adam *et al.*, 1995). The importance of PKC-catalyzed phosphorylation of CaP and CaD in agonist-induced Ca^{2+} sensitization requires further investigation.

4) Phosphorylation of CPI-17, the most widely accepted mechanism of Ca^{2+} sensitization by PKC (see below).

In 1995, Eto and colleagues reported that a heat stable protein, referred to as CPI-17 (short for: PKC potentiated inhibitor protein of 17kDa) was able to inhibit both the catalytic and regulatory subunit of MLCP when it was phosphorylated by PKC at Thr 38 (Eto *et al.*, 1995). Phosphorylation at Thr 38 increased the inhibitory potency of CPI-17 >1000 fold. This investigation unveiled the signaling pathway between PKC and inhibition of MLCP. Cloning and sequencing analysis of CPI-17 revealed that it was a novel protein with a unique amino acid sequence distinct from all other known proteins. It was also unique in its mechanism of action in that it did not phosphorylate MLCP but rather inhibited both the catalytic and regulatory subunits of the phosphatase (Eto *et al.*, 1995). In fact recent studies have shown that phosphorylated CPI-17 forms a stable inactive complex with MYPT and PP1c, which allows MLC phosphorylation and contraction to increase (Eto *et al.*, 2004).

CPI-17 was shown to be exclusively expressed in smooth muscle but Northern and Western blotting experiments showed that expression of CPI-17 tended to vary depending on whether smooth muscle was vascular or visceral (Woodsome *et al.*, 2001). It was shown that CPI-17 content was greater in vascular (e.g. aorta) than in visceral (e.g. vas deferens) smooth muscle and within these tissues, that the tonic muscle phenotype contained more CPI-17 than the phasic muscles. Contractile force generated by PdBu was found to be directly proportional to the total amount of CPI-17 in a muscle and was associated with increases in CPI-17 and MLC phosphorylation, suggesting that PKC physiologically mediated Ca^{2+} sensitization by phosphorylating CPI-17 (Woodsome *et al.*, 2001). It was initially suggested that PKCα and δ were the principle isoforms involved in mediating CPI-17 phosphorylation in response to agonist-induced stimulation of smooth muscle, with the latter being a more efficient kinase (Eto *et al.*, 2001). However it has been shown that all PKC isoforms can bind to CPI-17 *in vitro*, and that PKCα and ε are equally efficient in phosphorylating it (Zemlickova *et al.*, 2004).

Initially believed to be a PKC substrate, a number of kinases, including RhoK, ZIP-like kinase, integrin-linked kinase and PKN have now been shown to phosphorylate CPI-17 in vitro (as reviewed by Somlyo & Somlyo, 2003). However whether the CPI-17 phosphorylation mediated by these kinases plays a physiological role in Ca^{2+} sensitization and contraction in vascular smooth muscle has yet to be shown.

1.13 RhoA AND Rho KINASE

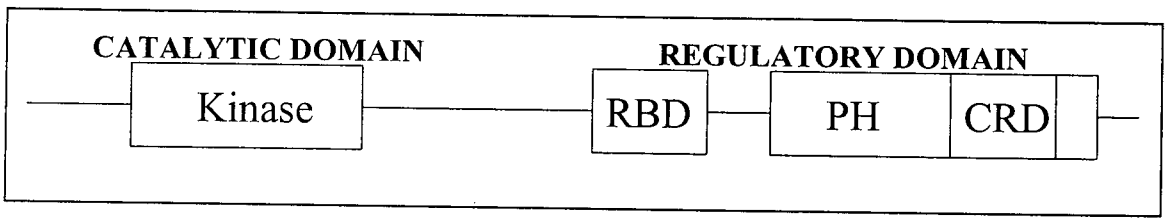
The Rho (Ras homologous member) GTPases form a subgroup of the Ras superfamily of low molecular weight (20-30 kDa) G-proteins. In mammalian cells at least 10 distinct proteins of this sub-group have been identified: RhoA, RhoB, RhoC, RhoD, RhoE, RhoG, Rac (Ras related C3 botulinum toxin substrate) 1 and 2, Cdc42 (cell division cycle 42) and TC10 (Van Aelst & D'Souza-Schorey, 1997; as reviewed by Seasholtz *et al.*, 1999). These proteins have a conserved primary structure and share 50-55% structural homology. Most of the functions of the Rho family are based on studies conducted on RhoA. These proteins have been implicated in membrane trafficking, regulation of cytoskeletal reorganization, cell growth control and development.

In the inactive GDP bound state, RhoA is predominantly cytosolic. On activation, when GDP is replaced with GTP, there is an increase in membrane associated RhoA with a corresponding decrease in cytosolic RhoA levels (as reviewed by Seasholtz *et al.*, 1999). A key step in the activation of RhoA is the attachment of geranylgeraniol, which is an isoprenoid intermediate of cholesterol biosynthesis. This posttranslational lipid modification, referred to as geranylgeranylation, is essential for membrane binding. Translocation to the particulate (membrane) fraction is often used as an index of RhoA activation (Taggart *et al.*, 1999). The downstream effectors of RhoA include protein kinase N (PKN), RhoK, citron, citron kinase, rhophilin, rhotekin and p140mDia (as reviewed by Fukata *et al.*, 2001). Of these the first and most well characterized RhoA effector is RhoK, particularly with respect to the effects of RhoA on vascular smooth muscle contractility.

RhoK is a serine/threonine kinase that exists in two isoforms: ROCK I (also known as ROKβ, 160 kDa) and ROCK II (ROKα, 150 kDa). RhoK contains an amino-terminal catalytic domain, and a C-terminal regulatory domain which contains the Rho binding site (RBD) and a pleckstrin homology (PH) domain (Fig. 1.3) (as reviewed by Riento & Ridley, 2003). Both ROCK I and II share a 92% similarity in their kinase domains, and there is very

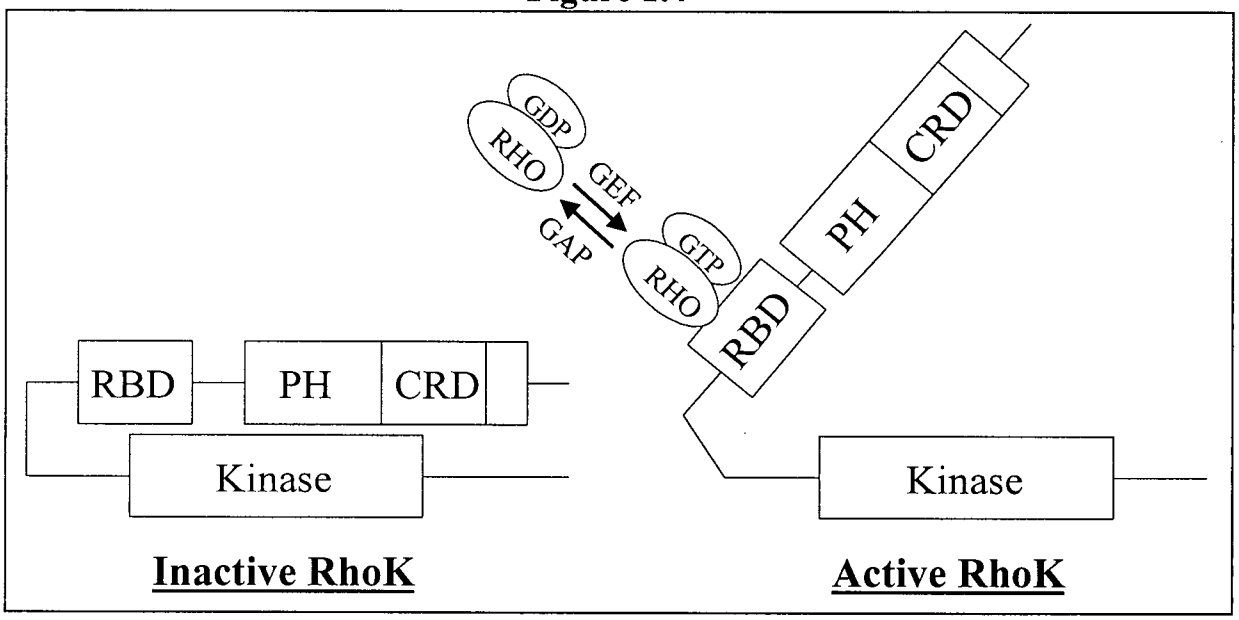
little evidence to show that they phosphorylate different substrates. Some substrates that have been shown to be phosphorylated by RhoK include MYPT, MLC, adducin, the ERM family (ezrin, radixin, moesin), LIM kinase and vimentin. In the inactive state, the regulatory site binds to the catalytic domain, forming an autoinhibitory loop (Fig. 1.4). Binding of RhoA induces a conformational change in the kinase which results in the disruption of the negative regulatory interaction between the catalytic and regulatory domain (Fig. 1.4) (as reviewed by Riento & Ridley, 2003). RhoA binds to RhoK only in the activated GTP bound form and on binding enhances its kinase activity (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). Like RhoA, RhoK translocates to the membrane on activation and this is often considered as a marker of enzyme activity (Obara *et al.*, 1999; Miao *et al.*, 2002b; Kandabashi *et al.*, 2003).

Figure 1.3



Diagrammatic Representation of the Structure of RhoK. The amino-terminal kinase region constitutes the catalytic domain whereas the Rho binding domain (RHD) and the pleckstrin homology domain (PH) containing an internal cysteine rich domain (CRD) constitute the regulatory subunit of RhoK. (Modified from Riento & Ridley, 2003)

Figure 1.4



Regulation of Activation of RhoK. In the inactive state, the regulatory domain negatively regulates the kinase activity. However binding of GTP bound RhoA disrupts this interaction resulting in activation of the enzyme. (Modified from Riento & Ridley, 2003)

1.14 RhoA/RhoK AND CARDIOVASCULAR DISEASE

Under normal physiological conditions, RhoA/RhoK serve a number of homeostatic functions that are essential for normal cell growth and development. However their sustained activation in pathological conditions can have detrimental consequences. So much so that they have come to be regarded as a formidable force in the field of cardiovascular disease (as reviewed by Shimokawa, 2002; Wettschureck & Offermanns, 2002).

Increased activation of the RhoA/RhoK pathway has been found in various conditions of abnormal vascular smooth muscle contractility. A link between RhoA/RhoK and hypertension was established by the ground-breaking work of Uehata et al. (Uehata *et al.*, 1997). They found that administration of Y-27632, a selective RhoK inhibitor, was able to correct hypertension in several experimental hypertensive rat models without a significant effect on blood pressure of normotensive rats. RhoK inhibition by fasudil also improved forearm circulation in humans, suggesting that enhanced activation of this pathway was a significant contributor to increased peripheral vascular resistance in human hypertension (Masumoto *et al.*, 2001).

RhoA/RhoK activation has also been implicated in vasospasm. RhoK inhibitors significantly improved cerebral vasospasm after subarachnoid hemorrhage in humans as well as experimental animal models (Shibuya *et al.*, 1992; Sato *et al.*, 2000b). Coronary artery spasm is a common feature of ischemic heart diseases such as angina pectoris, myocardial infarction and ventricular arrhythmias. Inhibition of RhoK significantly ameliorated symptoms of angina in humans and experimental animal models (Utsunomiya *et al.*, 2001; Masumoto *et al.*, 2002). For example, in a dog model of effort angina, hydroxyfasudil protected the myocardium subjected to pacing-induced ischemia by increasing regional blood flow in the absence of any inotropic or chronotropic effects (Utsunomiya *et al.*, 2001). Similarly in patients with vasospastic angina, fasudil effectively prevented myocardial ischemia resulting from ACh-induced coronary artery spasm (Masumoto *et al.*, 2002).

Lung diseases such as bronchial asthma are associated with airway inflammation and hyper responsiveness. Elevated bronchial levels of RhoA/RhoK and enhanced activation of this pathway were reported in animals suffering from airway hyper-reactivity (Chiba *et al.*, 1999; Chiba *et al.*, 2001; Hunter *et al.*, 2003; Sylvester, 2004). The main treatment for asthmatic patients is administration of β_2-AR antagonists, whose beneficial effects can be limited after chronic use due to desensitization (Johnson, 1998). Inhibition of RhoA/RhoK could serve as a therapeutic alternative for these patients.

Increased activation/expression of RhoA/RhoK has also been implicated in pre-term labor (increased uterine contractions) (Tahara *et al.*, 2002), glaucoma (increased intraocular pressure) (Honjo *et al.*, 2001; Rao *et al.*, 2001) and erectile dysfunction (decreased intracavernosal pressure) (Chitaley *et al.*, 2001; Mills *et al.*, 2001).

Most vascular pathologies are characterized by vascular smooth muscle proliferation and migration as well as vascular remodeling which involves the rearrangement of cellular and extracellular components. In addition to mediating enhanced vascular smooth muscle contractility, RhoA/RhoK have also been shown to participate in vascular remodeling processes. RhoA/RhoK influence cell cycle progression and cell proliferation by a number of mechanisms, for example, by reducing the expression of cell cycle inhibitors, which leads to increased DNA synthesis (Olson *et al.*, 1998; Laufs *et al.*, 1999) or by increased phosphorylation of extracellular-regulated kinase (ERK) (Welsh *et al.*, 2001; Roovers *et al.*, 2003). This increased vascular smooth muscle cell migration and proliferation contributes to

the pathophysiological progression of vascular diseases including hypertension, and could participate in the pathogenesis of arteriosclerosis/atherosclerosis.

1.15 RhoA/RhoK AND DIABETES

The role of the RhoA/RhoK pathway has been investigated in various diabetic complications. It has been shown that diabetes mellitus adversely affects endothelial and nitrergic relaxation in the corpus cavernosum of humans and animals leading to erectile dysfunction. Increased expression of RhoA and ROCK II was detected in the STZ diabetic rat penis (Bivalacqua *et al.*, 2004) and RhoK antagonism was shown to have a beneficial therapeutic effect in diabetic mice suffering from erectile dysfunction (Buyukafsar & Un, 2003). Over expression of ROCK I mediating increased contractility to ET-1 was proposed to be causing the increased tone of the corpus cavernosum smooth muscle from chronic diabetic rabbits, which leads to erectile dysfunction in this model (Chang *et al.*, 2003).

Increased activation of RhoA has been implicated in diabetic renal injury. It was shown that the renal cortex of STZ diabetic rats had significantly increased levels of membrane bound RhoA without any change in total RhoA expression (Massey *et al.*, 2003). This was accompanied by renal pathologies such as hypertrophy, proteinuria and glomerulopathy. This was supported by another report where inhibition of RhoA by statins prevented high glucose induced proliferation of mesangial cells, an early development in diabetic nephropathy (Danesh *et al.*, 2002).

Upregulation of basal RhoA expression and activity was found in basilar arteries from STZ-diabetic rats, and it was suggested to be involved in cerebral vascular pathogenesis during diabetes mellitus, such as stroke and cerebral edema (Miao *et al.*, 2002a). However unlike the DAG/PKC pathway that has been intensively investigated with respect to peripheral arterial dysfunction in diabetes, there is very little information regarding the role of the RhoA/RhoK pathway in abnormal vasoreactivity in diabetes. Increased activation of this pathway has been implicated in the enhanced contractile responsiveness to thrombin in aortic smooth muscle cells from a type II diabetic model, the Goto-Kakizaki rat, due to loss of insulin-mediated inhibition of the pathway (Begum *et al.*, 2000). However whether the RhoA/RhoK pathway also contributes to abnormal vasoconstrictor responses in hypoinsulinemic STZ-diabetic rats has not yet been investigated.

1.16 RhoA/RhoK AND CALCIUM SENSITIZATION

GPCRs have been shown to be highly effective activators of the RhoA/RhoK pathway in vascular smooth muscle. Agonists like ET, phenylephrine (PE), thrombin, TXA_2, and lysophosphatidic acid (LPA) have been shown to stimulate the activity of this pathway leading to enhanced contractile force via increased Ca^{2+} sensitization of the contractile apparatus (as reviewed by Somlyo *et al.*, 1999; Somlyo & Somlyo, 2000). Inhibition of RhoA by bacterial exoenzymes such as EDIN (epidermal differentiation inhibitor from staphylococcus aureus) or exoenzyme C3 (clostridium botulinum) by ADP-ribosylation (which blocks the biological activity of RhoA and inhibits its translocation to the plasma membrane) results in significant attenuation of agonist induced Ca^{2+} sensitization and force development in smooth muscle (Gong *et al.*, 1996; Otto *et al.*, 1996; Fujihara *et al.*, 1997; Lucius *et al.*, 1998). Similarly inhibition of RhoK by selective inhibitors like Y-27632 significantly reduces contraction produced by excitatory agonists (Uehata *et al.*, 1997; Fu *et al.*, 1998; Yoshii *et al.*, 1999). These observations have established RhoA/RhoK as being major effectors in the regulation of pharmacomechanical coupling in smooth muscle.

The predominant molecular mechanism by which RhoA regulates Ca^{2+} sensitization induced by agonist stimulation is the activation of its downstream effector, RhoK. Although GTP bound RhoA can interact with MYPT, this association was found to have no significant effect on MLCP activity (Kimura *et al.*, 1996). However RhoK has been suggested to produce Ca^{2+} sensitizing effects via a number of different mechanisms:

1) Phosphorylation of MYPT resulting in inhibition of MLCP, an increase in the MLCK/MLCP activity ratio and enhanced MLC phosphorylation. RhoK has been shown to phosphorylate MYPT at Thr 696 *in vivo* in response to a number of different agonists, suggesting that MLCP is predominantly a RhoK substrate (Kimura *et al.*, 1996). In one study, point mutation at Thr 696 on the MYPT subunit resulted in complete loss of RhoK mediated inhibition of MLCP, suggesting that it was the major inhibitory site (Feng *et al.*, 1999a). RhoK has also been shown to phosphorylate two other sites on MYPT *in vitro*, Thr 853 and Ser 852. Thr 853 lies within a region that has been identified as a myosin-binding domain (Johnson *et al.*, 1997) and can affect targeting function of MLCP (Velasco *et al.*, 2002). Phosphorylation of this site by RhoK decreases binding of MLCP to MLC and could be one of the mechanisms

responsible for RhoK mediated Ca^{2+} sensitizing effects. Data regarding the relevance of Ser 852 phosphorylation by RhoK has been conflicting. In one study RhoK did not produce any detectable phosphorylation of this site (Velasco *et al.*, 2002) while in another, Ser 852 phosphorylation was only partially blocked by RhoK inhibitors (Kawano *et al.*, 1999).

2) Direct phosphorylation of MLC_{20} at serine 19 (the site that is also phosphorylated by MLCK) leading to enhanced activation of actomyosin ATPase by actin (Amano *et al.*, 1996; Kureishi *et al.*, 1997; Feng *et al.*, 1999b). Whether RhoK induces MLC_{20} phosphorylation under physiological conditions has yet to be established.

3) Phosphorylation of CPI-17 at Thr 38 (demonstrated *in vitro*) which converts it into a potent MLCP inhibitor (discussed in section 1.12) (Koyama *et al.*, 2000).

4) Inhibitory phosphorylation of CaP (demonstrated *in vitro* and physiological importance not established). RhoK induced phosphorylation of calponin at a number of sites and inhibited its binding to actin (Kaneko *et al.*, 2000).

1.17 CALCIUM SENSITIZATION BY AA

It has been shown in a wide variety of tissues that agonist-induced AA release is primarily via stimulation of $cPLA_2$, which translocates to the particulate fraction on activation (Anderson *et al.*, 1997; Muthalif *et al.*, 1998; Trevisi *et al.*, 2002). AA release induced by NE (LaBelle & Polyak, 1998) and ET-1 (Trevisi *et al.*, 2002) in rat tail artery was inhibited by treatment with $AACOCF_3$, an inhibiter selective for $cPLA_2$ (Street *et al.*, 1993). However in these studies the effect of $AACOCF_3$ on NE and ET-1-induced contraction was not investigated. In a recent study PE-induced AA release in vascular smooth muscle was found to be via stimulation of $iPLA_2$ (Guo *et al.*, 2003). A number of studies have implicated AA in agonist-induced Ca^{2+} sensitization. It was shown that ONO-RS-082 (a non-isoform specific PLA_2 inhibitor) and BEL (a selective $iPLA_2$ inhibitor) significantly attenuated PE-induced Ca^{2+} sensitization and AA production in permeabilized rabbit femoral artery (Gailly *et al.*, 1997) and rabbit portal veins (Guo *et al.*, 2003), respectively. Similarly quinacrine (a non-isoform specific PLA_2 inhibitor) inhibited 5-hydroxytryptamine- (5-HT-), but not PE-, induced Ca^{2+} sensitization in permeabilized rabbit mesenteric artery (Parsons *et al.*, 1996). The different mechanisms by which AA has been suggested to mediate GPCR-induced Ca^{2+} sensitization are discussed below.

1) AA was shown to directly inhibit MLCP (discussed in section 1.8) in permeabilized smooth muscle resulting in increased MLC phosphorylation (Gong *et al.*, 1992; Gong *et al.*, 1995; Gailly *et al.*, 1996). However this has only been demonstrated in permeabilized rabbit femoral arteries.

2) AA can stimulate RhoK activation independent of RhoA (Fu *et al.*, 1998; Feng *et al.*, 1999b; Araki *et al.*, 2001). This was demonstrated in permeabilized smooth muscle when treatment with Y-27632 resulted in the loss of AA induced Ca^{2+} sensitizing effects. AA activated RhoK by binding to its PH domain and releasing the autoinhibition mediated by the interaction between the C-terminal regulatory domain and the catalytic site.

3) AA can stimulate the activation of aPKCs (Nakanishi & Exton, 1992; Gailly *et al.*, 1997). In bovine kidney, PKCζ was found be activated by AA and other unsaturated fatty acids (Nakanishi & Exton, 1992). In vascular smooth muscle there is one study showing that inhibition of aPKCs by a pseudopeptide inhibitor resulted in significant attenuation of AA-induced Ca^{2+} sensitization of force and MLC_{20} phosphorylation in rabbit femoral artery (Gailly *et al.*, 1997).

1.18 CALCIUM SENSITIZATION BY TKs

TKs have been shown to participate in GPCR-mediated contraction via modulation of ion channels leading to an increase in $[Ca^{2+}]_i$. TK inhibitors have prevented agonist induced Ca^{2+} influx via VGCC (Gould *et al.*, 1995; Toma *et al.*, 1995) as well as Ca^{2+} release from intracellular stores (Liu & Sturek, 1996). However at the same time, activation of TKs following agonist-induced stimulation of GPCRs have also increased myofilament force sensitivity to Ca^{2+}. It was shown that Ca^{2+} sensitization mediated by PE and histamine (Sasaki *et al.*, 1998) and ET-1 (Ohanian *et al.*, 1997) was significantly blocked by TK inhibitors in permeabilized mesenteric arteries. In other studies TKs were shown to play a role in both Ca^{2+} dependent and independent contractile processes in response to classical vasoconstrictors in mesenteric resistance (Toma *et al.*, 1995) and human omental (Martinez *et al.*, 2000) arteries. The degree of physiological importance of TKs in modulating GPCR-mediated Ca^{2+} sensitivity is uncertain but some potential mechanisms linking tyrosine phosphorylation to this process have been suggested:

1) Increased activation of PLD, leading to enhanced production of DAG and a resultant increase in PKC activation (Ward *et al.*, 1995; Jinsi *et al.*, 1996). However these studies are based on the effects of TK inhibition on PLD activation in response to agonist-induced stimulation in vascular smooth muscle, and do not show how this affects agonist-induced Ca^{2+} sensitization.

2) Increased activation of MAP kinases, which require phosphorylation of tyrosine and threonine residues for complete activation (Ward *et al.*, 2002). MAP kinases have the ability to phosphorylate and inhibit CaD (Adam *et al.*, 1995; Dessy *et al.*, 1998).

3) There is no direct evidence suggesting activation of RhoK by TKs but one study has suggested that there is an interaction between them. Sphingosylphosphorylcholine (SPC)-induced Ca^{2+} sensitization and contraction was suggested to be mediated by RhoK as well as Src family protein tyrosine kinases (SrcPTKs) (Nakao *et al.*, 2002). PP1 (a SrcPTKs kinase-inhibitor) and eicosapentaenoic acid (EPA, a SrcPTKs translocation-inhibitor) but not Y-27632 abolished SPC-induced activation of SrcPTKs. PP1 and EPA also inhibited activation and translocation of RhoK in response to SPC, suggesting that SrcPTKs maybe acting upstream of RhoK.

1.19 HYPOTHESIS AND OBJECTIVES

CVD is the major burden of DM and vascular complications significantly enhance the morbidity and mortality associated with this disease. In spite of many advances and breakthroughs in our understanding of this debilitating disease, there is a paucity of data related to the pathogenesis of vascular disease in diabetes and this has hindered the development of new preventative and therapeutic strategies for the treatment of diabetic vascular disorders.

Abnormal vasoreactivity in the diabetic vasculature has been the subject of much investigation. Our lab has consistently reported that vasoconstrictor responses to stimulation of α_1-ARs are significantly elevated in mesenteric arteries isolated from STZ-induced diabetic rats as compared to corresponding controls. In order to investigate if this was due to increases in mean cytosolic $[Ca^{2+}]_i$, changes in $[Ca^{2+}]_i$ and contractile tension in response to stimulation with NE were measured in mesenteric arteries from control and diabetic rats (Chow *et al.*, 2001). The results demonstrated that the enhanced sustained contractile responses of mesenteric arteries from diabetic rats to a maximum concentration of NE were not associated with a greater increase in Ca^{2+} influx or mean cytosolic Ca^{2+}. This suggests that enhanced vasoreactivity of diabetic arteries is due an increase in the Ca^{2+} sensitivity of the contractile proteins.

Diabetes-induced increases in the expression and/or activity of some PKC isoforms have been demonstrated in various cardiovascular tissues and based on functional studies, PKC has been suggested to be involved in abnormal vasoconstrictor responses in arteries from STZ-diabetic rats. However, the effect of diabetes on agonist-induced changes in PKC isoforms involved in Ca^{2+} sensitization has not been reported. Moreover, the contribution of RhoK, TKs and $cPLA_2$ to abnormal vasoconstrictor responses in tissues from Type I diabetic rats has not been investigated. Since PKC, RhoK, TKs and $cPLA_2$ have been shown to participate in GPCR-mediated Ca^{2+} sensitization and contraction, we hypothesized that one or more of these pathways play a role in the altered contractile responses to α_1-AR and ET-1 receptor stimulation in mesenteric arteries from diabetic rats. It is also possible that these pathways are not activated independent of each other but in fact interact at various points to mediate abnormal vasoconstrictor responses in diabetic arteries.

In order to investigate the role of PKC in α_1-AR and ET-1 receptor-mediated contractile responses in mesenteric arteries from diabetic rats, functional and biochemical studies were conducted. In the functional studies, pharmacological experiments were carried out to investigate the effects of calphostin C and Ro-318220 (non-isoform selective PKC inhibitors) and rottlerin (an inhibitor selective for PKCδ) on contractile responses to NE and ET-1 in mesenteric arteries from control and diabetic rats. Since activation of PKC has been shown to involve its translocation from the cytosol to isoform-specific binding sites at cell membranes, changes in particulate (membrane) levels of the isoforms are often used as an index of their activation. Therefore in the biochemical studies, western blotting experiments were conducted to measure the change in the particulate levels of the PKC isoforms, α, δ and ε, in response to a maximum concentration of NE and ET-1 in mesenteric arteries from control and diabetic rats. PKC-mediated CPI-17 phosphorylation has been proposed to be the primary mechanism by which PKC increases Ca^{2+} sensitization in vascular smooth muscle. To investigate if activation of PKC was associated with increased phosphorylation of this downstream target, the effects of NE on the phosphorylation of CPI-17 in the absence and presence of the PKC inhibitor, calphostin C, in control and diabetic mesenteric arteries were measured by western blotting.

In order to investigate the role of RhoK in α_1-AR and ET-1 receptor-mediated contractile responses in mesenteric arteries from diabetic rats, functional and biochemical studies were conducted. Functional studies consisted of pharmacological experiments investigating the effects of specific RhoK inhibitors, Y-27632 and H-1152, on contractile responses to PE and ET-1 in mesenteric arteries from control and diabetic rats. Like PKC, translocation of RhoK isoforms from the cytosol to the membrane fraction is considered a marker of the enzyme activity. Therefore in the biochemical studies, western blotting experiments were conducted to measure the degree of translocation of the RhoK isoforms, ROCK I and ROCK II in response to a maximum concentration of PE and ET-1 in mesenteric arteries from control and diabetic rats. To investigate the possibility of an interaction between the PKC/RhoK pathways, the effect of RhoK inhibition by Y-27632 on agonist-induced changes in levels of PKC isoforms in the particulate fraction was also determined.

In order to investigate the role of TKs and $cPLA_2$ in PE and ET-1-induced contractile responses in mesenteric arteries from control and diabetic rats, pharmacological experiments

were conducted. In these studies the effects of structurally different TK inhibitors, tyrphostin A23 and genistein, and cPLA$_2$ inhibitor, AACOCF$_3$, on contractile responses to PE and ET-1 in mesenteric arteries from control and diabetic rats were investigated.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Materials used to make the Krebs buffer:

Sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$) and dextrose were purchased from Sigma (St. Louis, MO).

2.1.2 Materials used in the tissue bath contractility studies:

Heparin (Hepalean from porcine intestinal mucosa) was obtained from Organon Teknika, Toronto, Canada. NE, PE, ET-1, acetylcholine (Ach), phorbol 12,13 dibutyrate (PdBu), phentolamine, Ro-318220 (3-[1-[3-(amidinothio)propyl-1H-indol-3yl]-3-(1-methyl-1H-indol-3-Bisindolylmaleimide IX methanesulphonate), rottlerin and calphostin C, $AACOCF_3$, genistein and tyrphostin A23 were purchased from Sigma (St. Louis, MO). Y-27632 ((+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate) and H-1152 ((S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulphonyl]-homopiperazine) were purchased from Calbiochem (La Jolla, CA).

2.1.3 Materials used in Western Blotting:

Tris (hydroxymethyl) aminomethane (Tris-HCl), 2-mercaptoethanol, sodium dodecyl sulphate (SDS), glycine, protein assay dye reagent, acrylamide, bis N,N` methylene-bis-acrylamide and temed were purchased from Bio-Rad (Hercules, CA).

Polyoxyethylenesorbitan monolaurate (Tween 20), ethylene glycol-bis(β-aminoethyl ether)-N,N,N`,N`-tetraacetic acid (EGTA), EDTA, sodium fluoride (NaF), leupeptin, aprotinin, deoxycholic acid, NP-40 were purchased from Sigma (St. Louis, MO).

4-(2-aminoethyl)benzenesulfonylflouride (AEBSF) was purchased from Calbiochem (La Jolla, CA).

Hyperfilms and enhanced chemiluminescence (ECL) detection kits were purchased from Amersham Pharmacia, Piscataway, N.J.

The primary antibodies for PKC and RhoK isoforms (rabbit polyclonal) and actin (goat polyclonal) and secondary antibodies (goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The primary

antibodies for CPI-17 and phospho-CPI-17 (Thr38) (rabbit polyclonal) were purchased from Upstate (Charlottesville, VA).

2.2 METHODS

2.2.1 Control and Diabetic Rats

Male Wistar rats weighing between 150-175g were either obtained from the University of British Columbia Animal Care Unit or from Charles River Laboratories Inc., Montreal, Quebec. They were housed and treated in accordance with the guidelines of the Canadian Council on Animal Care. Rats were randomly divided into two groups. In one group, diabetes was induced by injection of 60mg/kg STZ (Sigma, St. Louis, MO) into the lateral tail vein of rats lightly anesthetized with halothane. The other group received the citrate buffer vehicle and served as the age and weight matched control group. STZ-treated rats with blood glucose levels of 13mmol/L or greater, measured with an Ames glucometer 1 week after injection, were considered diabetic and were kept for experiments. The blood for this preliminary glucose measurement was taken by nicking the end of the rat's tail with a scapel and bleeding just enough to cover a glucometer strip. Control and diabetic rats were housed separately and given free access to food (rat chow, LabDiet 5001, PMI Nutrition International, Inc., Brentwood MO) and water. Twelve to fourteen weeks later, animals were weighed and given an overdose of sodium pentobarbital. Blood was collected by cardiac puncture with a syringe containing heparin (0.1ml of 1000USP units/ml) and then centrifuged in an eppendorf microcentrifuge for 15 minutes at 4°C. The plasma was removed with a Pasteur pipette, divided into aliquots and stored at –20°C for later assay of plasma insulin and glucose levels.

2.2.2 Tissue Bath Studies

The superior mesenteric artery was excised, placed in Krebs solution (composition in mM: NaCl 124, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 and dextrose 11.5) at room temperature and cleaned of fat and connective tissue. The artery was then cut into 4mm segments. The endothelium was removed from each segment by rubbing the internal lumen of the vessel gently against a thin wire. The rings were then suspended in isolated tissue baths using triangular hooks, one of which was attached to a fixed tissue support, while the other was connected to a Grass FT.03 force displacement transducer which in turn was attached to a Grass polygraph (Model 7E, Grass Instruments Co., Quincy, Mass.). The rings

were equilibrated for 90 minutes under 1g resting tension in Krebs solution continuously aerated with 95% O_2/5% CO_2 and maintained at a temperature of 37°C.

Tissues were allowed to equilibrate for 60 minutes with the bathing solution being changed every 10-15 minutes. The loss of the endothelium in the tissues was first confirmed by demonstrating their inability, when pre-contracted with 3μM PE, to relax in response to 10μM Ach. The tissues were then washed for 1 hour and cumulative concentration response curves to NE, PE and ET-1 were obtained in the presence or absence of:

1) Ro-318220, 3μM for 30 minutes
2) Calphostin C, 3μM for 30 minutes
3) Rottlerin, 10 μM for 30 minutes
4) Y-27632, 1μM for 15 minutes
5) H-1152, 0.1μM for 15 minutes
6) $AACOCF_3$, 10 μM for 30 minutes
7) Genistein, 10 μM for 30 minutes
8) Tyrphostin A23, 10 μM for 30 minutes

Only one agonist and one antagonist were tested in each tissue. The maximum response to KCl (0.1M) in the presence of 1μM phentolamine was also measured. In some cases the effect of the inhibitors on KCl and PdBu induced contractions was also determined.

2.2.3 Bio-Rad Protein Assay

Protein content of freshly prepared samples was determined using the Bio-Rad® assay system, which is based upon the protein dye binding method of Bradford. Bovine serum albumin (BSA) was used as a protein standard. Standard curves were performed in triplicate, using at least 5 different protein concentrations ranging from 3 – 15μg. Triplicates of 10μl of each sample solution/tube were used to measure total protein levels. The EGTA buffer was used for the blank and 2.5ml of the diluted and filtered reagent was added to each tube and vortexed. The mixture was allowed to react for 5 minutes at room temperature after which 200μl of each standard and sample tube was pipetted into separate microtiter plate wells. The absorbance was measured at 595nm and the protein content of the unknown sample solutions was calculated from the linear standard curve.

2.2.4 Western Blotting

While the specific tissue treatments and extraction procedures for identification of the various proteins of interest are discussed in Section 2.2.4, 2.2.5 and 2.2.6, this section will deal with the general aspects of the procedure.

Preparation of the polyacrylamide gels

Separating gel (11%): (mixture for two gels)

30 / 0.8% acrylamide / bisacrylamide = 29.4 ml

Separating gel buffer (1.5M Tris-HCl, pH 8.8, 0.4% SDS) = 26.4 ml

Distilled water = 24.2 ml

20% ammonium persulphate = 160µl

Temed = 28 µl

Stacking gel: (mixture for two gels)

30 / 0.8% acrylamide / bisacrylamide = 4 ml

Stacking gel buffer (0.5M Tris-HCl, pH 6.8, 0.4% SDS) = 7.5 ml

Distilled water = 18.5 ml

20% ammonium persulphate = 90µl

Temed = 30 µl

The separating gel was poured in the glass plates and sealed with 70% ethanol. It was allowed to set for at least an hour, after which the ethanol was poured off and the gel was repeatedly rinsed with distilled water. The combs (15 wells) were inserted into the glass plates and the stacking gel was poured in. It was allowed to set for at least an hour after which the combs were removed. The wells were repeatedly flushed with running buffer (1.4% glycine, 0.3% Tris and 0.1% SDS). After the protein samples were poured into the wells, the gels were run at constant current (16 - 18 m Amp overnight).

Transfer of Separated Proteins from Gel to Membrane

The sponges, filter papers, membrane (nitrocellulose or PVDF) and gel were wetted in transfer buffer (0.3% Tris, 0.01% SDS and 20% methanol). The gel was assembled in a `sandwich` - sponge – filter paper – membrane – gel – filter paper – sponge. Membrane transfer was conducted at 4°C in transfer buffer for three hours at 4 Amp. The transferred proteins were visualized by reversibly staining the membranes with Ponceau (0.2g Ponceau

in 5% glacial acetic acid), a protein stain for serum proteins separated by agarose gel and cellulose acetate electrophoresis. Excess stain was rinsed off with water.

Detection by Enhanced Chemiluminescence (ECL)

Each membrane was treated with the respective primary and secondary antibodies and then allowed to soak in ECL western blotting detection reagent (equal volumes of solution 1 and 2 mixed together) for one minute at room temperature. The membrane was wrapped in saran wrap. It was placed in a film cassette with a sheet of film on top of it and the cassette was closed. Exposure time varied from 5 – 15 seconds. The film was then processed using the GBX developer/fixer pack (Sigma). It was thoroughly washed with distilled water and allowed to dry.

Densitometric Analysis of Immunoblots

The immunoblots were quantified with a pdi 420 oe scanning densitometer (pdi Inc., Huntington Station, NY) connected to a personal computer using pdi Quantity One® Ver. 3.0 software. The background was subtracted from each film. A contour was drawn around each band and the contour quantity (contour area x optical density) was obtained for each band.

2.2.5 Western Blotting: Total levels of PKC and RhoK isoforms

Mesenteric arteries from control and diabetic animals were cleaned, denuded and frozen in liquid nitrogen. The arteries were powdered (with the help of pestle and mortar) and placed in 240µl ice-cold EGTA buffer (composition: Tris HCl 20mM, mercaptoethanol 50mM, EGTA 5mM, EDTA 2mM, NaF 10mM, AEBSF 1mM, leupeptin 25µg/ml, aprotinin 2µg/ml, NP40 1%, SDS 0.1% and deoxycholic acid 0.5%). The mixture was sonicated on ice and then centrifuged at 100,000g for 1 hour at 4°C. The supernatant was collected and total protein content was determined using the Bradford assay. Sample dilution buffer was added (5 X) to equal amounts of sample protein (20µg) and the mixture was vortexed and boiled for 4 minutes. The boiled protein was stored at –70°C to be later used for separation by gel electrophoresis.

Samples of total protein (20µg) were subjected to SDS-PAGE on 11% polyacrylamide gels and the resolved proteins were electrophoretically transferred to nitrocellulose membranes, as described in section 2.2.4. The membranes were then blocked with 5% non-fat milk/0.05% Tween-TBS for 2 hours at room temperature. They were then incubated with the appropriate PKC isoform-specific (rabbit polyclonal, 1:500) or RhoK isoform specific (rabbit polyclonal,

1:180) primary antibody overnight at 4°C. Actin was used as an internal control and membranes were incubated with actin primary antibody (goat polyclonal, 1:200) in the manner described above.

In preliminary experiments, each of the antibodies for PKCα, β_2, δ, and ε were tested against the purified standard for each isoform, and no cross-reactivity was detected. The same dilution of each PKC and RhoK isoform antibody and the same amount of protein were used for all the control and diabetic samples. These were found in preliminary experiments to give signals in the linear range of the optical density vs. protein concentration curve (Fig. 2.1). Immune complexes were detected following incubation of membranes with horseradish peroxidase conjugated anti-rabbit (for PKC and RhoK) or anti-goat (for actin) secondary antibody for 2 hours at room temperature (1:20,000 in 3% non-fat milk) using an enhanced chemiluminescence detection kit. Band intensity was analyzed by densitometry and normalized for actin on the same membrane to correct for any inaccuracies that might have occurred during gel loading and transfer. Total actin levels were not significantly different in control and diabetic mesenteric arteries (Fig. 2.2A).

As an alternative to actin, some of the membranes were analyzed by the method described by Ping (Ping *et al.* 1997). Following Ponceau staining, the most predominant band on the membrane was quantified with the help of an image scanning densitometer. Each PKC or RhoK signal was normalized to the corresponding Ponceau stain signal. The results corrected by this method and by actin were found to be similar. Total levels of PKC isoforms in diabetic arteries were expressed as a percent of levels of the same isoform in control arteries.

2.2.6 Western Blotting: Particulate levels of PKC and RhoK isoforms

Following the equilibration period, artery rings from control and diabetic animals were divided into 7 groups –

1) Untreated
2) NE, 30 μM for 5 minutes
3) ET-1, 0.03 μM for 7 minutes
4) Y-27632, 1 μM for 15 minutes
5) PE, 30 μM for 5 minutes in the absence of Y-27632
6) PE, 30 μM in the presence of Y-27632 (1 μM for 15 minutes)

7) ET-1, 0.03 μM in the presence of Y-27632 (1 μM for 15 minutes)

Once the peak sustained contractile response was reached, the rings were quickly rinsed in ice-cold Krebs solution, blotted, frozen in liquid nitrogen and stored at –70°C. The frozen rings were powdered then sonicated in EGTA buffer (without detergents), and then centrifuged at 100,000g for 1 hour. The supernatant was retained as the soluble fraction, and the pellets were re-suspended in the EGTA buffer containing NP-40 1%, SDS 0.1% and deoxycholic acid 0.5%. Following centrifugation at 100,000g for 1 hour, the supernatant was collected and used as the particulate fraction. The protein content of the particulate fraction was determined using the Bradford assay. Equal amounts of protein (20μg) were subjected to SDS-PAGE on 11% polyacrylamide gels and the resolved proteins were electrophoretically transferred to a nitrocellulose membrane. Membranes were treated with PKC or RhoK isoform-specific antibodies and results were normalized for actin as described above. Neither diabetes nor treatment with agonist had any effect on particulate levels of actin (Fig. 2.2B). Particulate levels of PKC and RhoK isoforms following NE, PE and ET-1 treatment were calculated relative to particulate levels in untreated control tissues.

2.2.7 Western Blotting: Total and phosphorylated levels of CPI-17

In preliminary experiments, artery rings from control animals were either left untreated or treated with PdBu (3 μM for 10 minutes). To investigate the effect of NE on CPI-17 phosphorylation, artery rings from control and diabetic animals were divided into 3 groups:

1) Untreated
2) NE, 30 μM for 5 min
3) Calphostin C, 3 μM for 35 min, with 30 μM NE being added for the last 5 min.

The rings were then frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10mM dithiothreitol (DTT) cooled with dry ice, and stored at –70°C. Frozen tissues were gradually warmed to –20°C, then 4°C and finally washed several times with ice-cold acetone to remove the TCA. The tissues were ground in a motor-driven glass homogenizer (Kontes) in EGTA buffer containing 20mM DTT and 0.5% SDS. They were sonicated on ice and then centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was collected and equal amounts of protein were subjected to SDS-PAGE on 16% polyacrylamide gels.

The resolved proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat milk/0.05% Tween-TBS overnight at 4°C and then incubated with the appropriate anti-CPI-17 (0.2μg/ml) or anti-phospho-CPI-17 (Thr38, 2μg/ml) (rabbit polyclonal) primary antibody for 3 h at room temperature. Actin was used as an internal control and membranes were incubated with actin primary antibody as described above. Membranes were incubated with the horseradish peroxidase conjugated anti-rabbit (1:12500 for total CPI-17 and 1:7575 for anti-phospho-CPI-17 in 3% non-fat milk) or anti-goat (for actin, 1:20,000 in 3% non-fat milk) secondary antibody for two hours at room temperature. Levels of phosphorylated CPI-17 were expressed as a percent of total CPI-17 levels in each group.

2.2.8 Statistical Analyses

All data are presented as the mean ± S.E.M. `n` indicates the number of animals in each group. The concentration response curves were analyzed by non-linear regression using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA) for calculation of pD_2 (-log EC_{50}) values and maximum responses (Rmax). Statistical significance was evaluated by one-way or two-way ANOVA followed by Newman-Keuls post hoc test for multiple comparisons, using Number Cruncher Statistical System (NCSS, Kaysville, Utah). A $p<0.05$ was considered statistically significant.

Figure 2.1.A

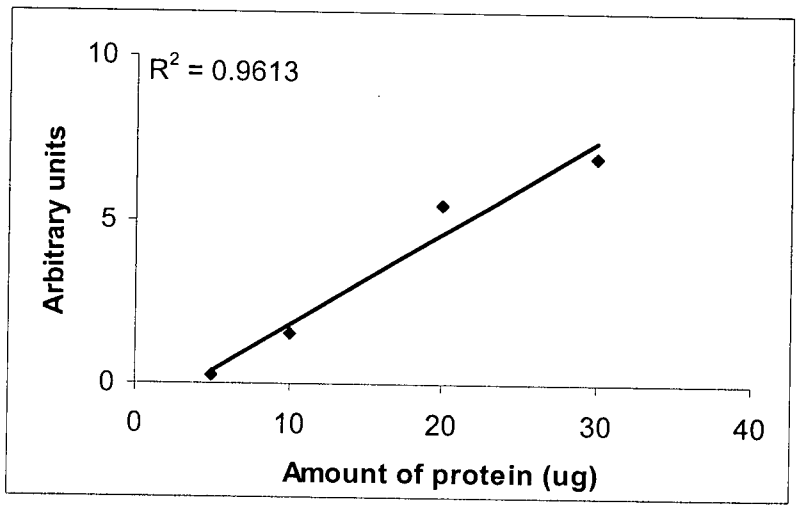


Figure 2.1.B

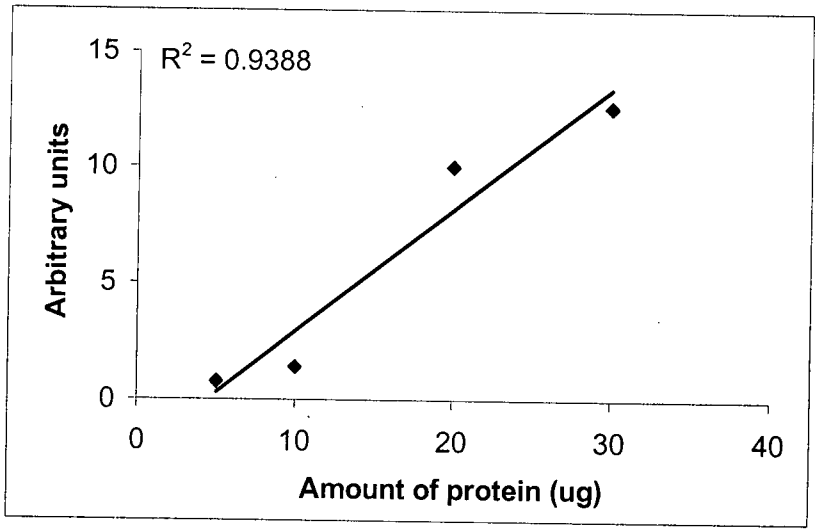


Figure 2.1 Preliminary experiments for the selection of optimal loading concentration. Optical density vs. protein concentration for particulate levels of PKCα (A) and PKCε (B).

Figure 2.2.A

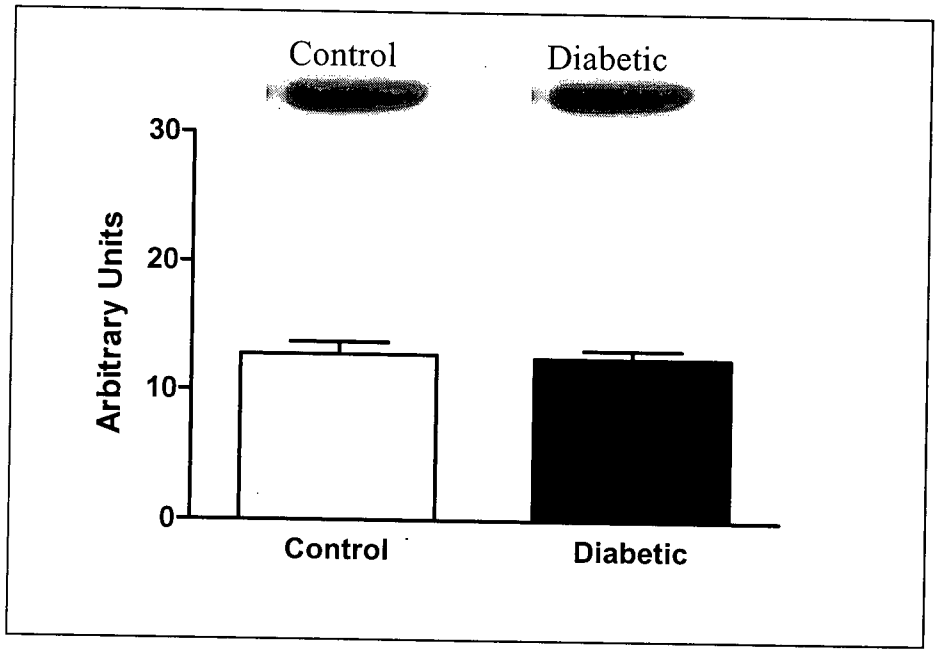


Figure 2.2.B

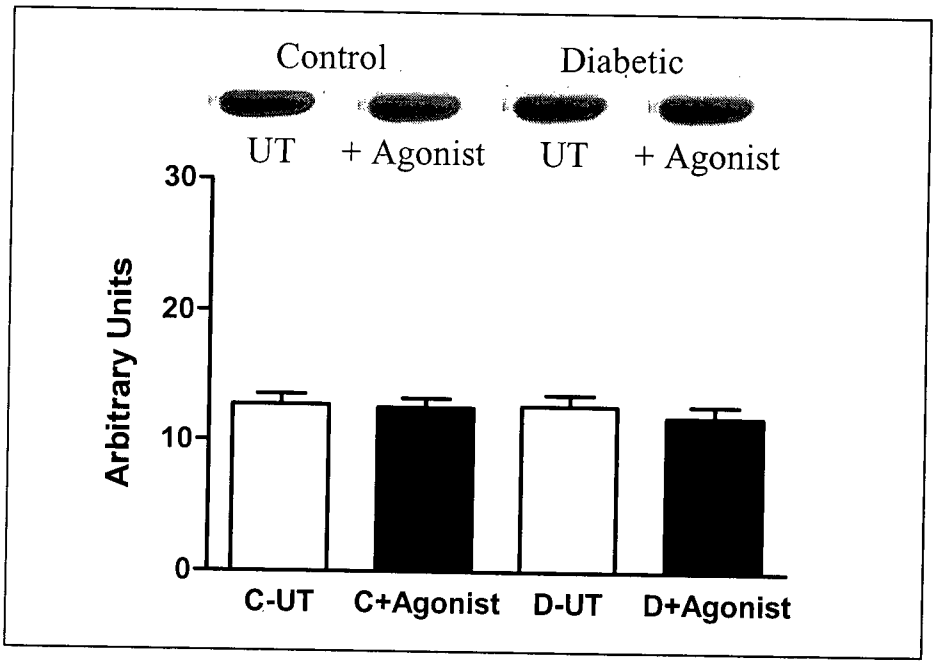


Figure 2.2 Total levels of actin in control and diabetic arteries (A). Particulate levels of actin in the absence (UT) and presence of agonist in particulate fractions of control (C) and diabetic (D) arteries (B)

3. RESULTS

3.1 CHARACTERISTICS OF CONTROL AND DIABETIC ANIMALS

Some characteristics of rats used in the present investigation are shown in Table 3.1. Diabetic rats weighed significantly less than their corresponding controls, and had much lower serum insulin levels, associated with significant hyperglycemia. STZ-treated rats also exhibited other symptoms of diabetes including polydipsia, polyuria, development of cataracts and increased food intake. However, there was no significant difference in the cross-sectional areas of arteries from the two groups of animals.

TABLE 3.1

Characteristics of Control and Diabetic Rats

	Body Weight (g)	Blood Glucose (mmol/L)	Serum Insulin (ng/ml)	Mesenteric Artery Cross-Sectional Area (mm^2)
Control (n=30)	533.9 ± 6.6	8.2 ± 0.1	5.0 ± 0.4	0.35 ± 0.02
Diabetic (n=30)	344.9 ± 9.4*	22.9 ± 0.6*	0.3 ± 0.03*	0.35 ± 0.02

Values represent mean ± S.E.M of number of animals in brackets.

*$p<0.05$ compared to control value.

(One-way ANOVA)

3.2 ROLE OF PKC/CPI-17 PATHWAY IN ENHANCED CONTACTILE RESPONSES OF MESENTERIC ARTERIES FROM DIABETIC RATS TO α_1-AR STIMULATION

3.2.1 Effect of PKC Inhibitors on Contractile Responses to NE and ET-1

Consistent with our previous report, mesenteric arteries from diabetic rats exhibited enhanced contractile responses to NE (Fig. 3.1.B, 3.2.B, 3.3B, Table 3.2) without a corresponding increase in contractile responses to depolarization with 0.1M KCl in the presence of 1μM phentolamine. The response to KCl was 0.6 ± 0.1g in the control and 0.7 ± 0.1g in the diabetic mesenteric arteries.

Incubation of mesenteric arteries with 3μM Ro-318220 for 30 min had no significant effect on the contractile response to 0.1M KCl plus 1μM phentolamine (Fig. 3.1.A), but completely abolished the contractile response to direct activation of PKC with phorbol 12,13 dibutyrate (PdBu) (data not shown). In mesenteric arteries from control rats, Ro-318220 had no significant effect on the NE Rmax, but produced a significant shift in the NE concentration–response curve, resulting in a decrease in the NE pD_2 value (Fig. 3.1.B, Table 3.2). Ro-318220 had a greater inhibitory effect on contractile responses of mesenteric arteries from diabetic rats to NE, producing a significant decrease in both the pD_2 value and Rmax to NE. In the presence of this antagonist, there was no significant difference between control and diabetic arteries in NE pD_2 values or Rmax (Table 3.2).

Figure 3.1.A

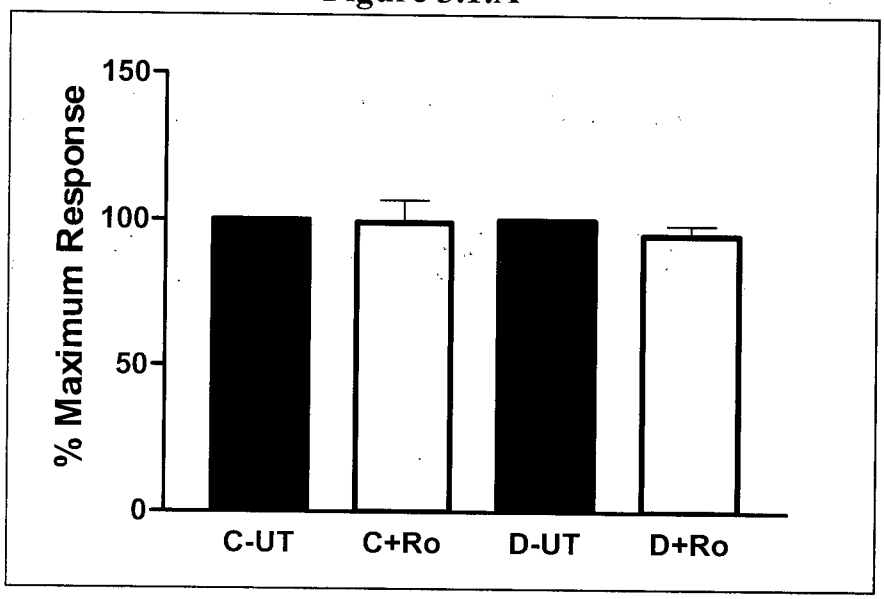


Figure 3.1.B

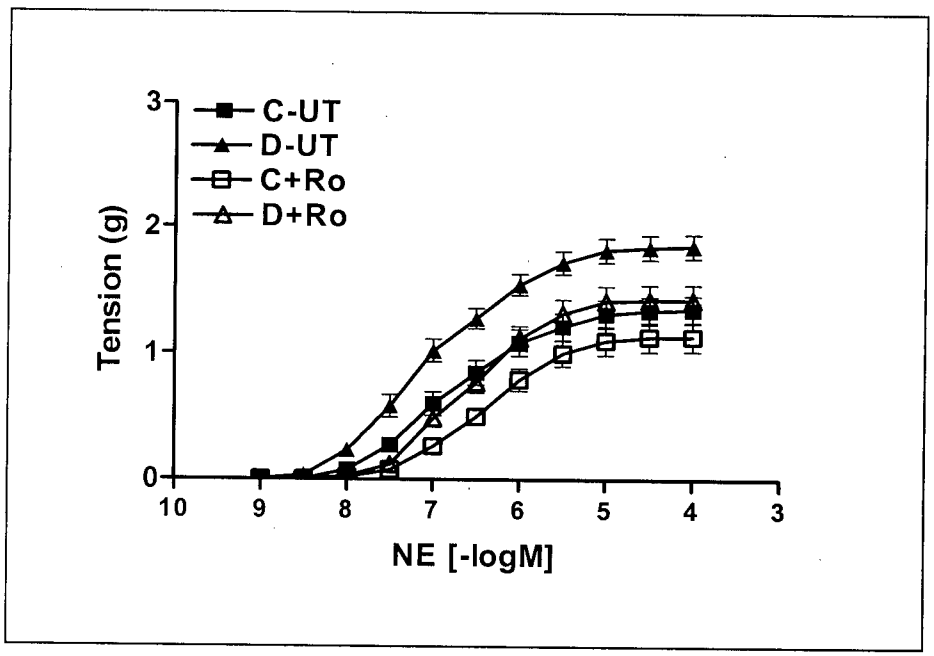


Figure 3.1 Effect of the non-isoform selective PKC inhibitor Ro-318220 (Ro, 3μM for 30min) on maximum KCl contractions (A, n=3) and NE concentration response curves (Fig. B, n=13) in control and diabetic mesenteric arteries. The response to KCl was 1.7 ± 0.1g in the control mesenteric arteries.

Similar results were obtained with calphostin C, a second non-isoform selective PKC inhibitor. In preliminary experiments, incubation of mesenteric arteries with 3μM calphostin C for 30 min had little effect on the contractile response to 0.1M KCl in the presence of phentolamine (Fig. 3.2.A). As was found with Ro-318220, calphostin C was without effect on the NE Rmax, but produced a small but significant decrease in the NE pD_2 value in mesenteric arteries from control rats (Fig. 3.2.B, Table 3.2). Calphostin C produced only a small shift in the NE response in mesenteric arteries from diabetic rats, and in the presence of this antagonist, the NE pD_2 value remained greater than that in control arteries (Table 3.2). However, this antagonist depressed the NE Rmax in mesenteric arteries from diabetic rats, and in the presence of calphostin C there was no significant in the NE Rmax between arteries from the two groups of animals.

The effect of rottlerin, an inhibitor reported to be specific for PKCδ (Gschwendt *et al.*, 1994), on contractile responses to NE in control and diabetic mesenteric arteries was very different from that of Ro-318220 and calphostin C. While 10μM rottlerin for 30 min did not affect the contractile response to 0.1M KCl in the presence of phentolamine in diabetic arteries, it significantly depressed KCl-induced contractions in control tissues (Fig. 3.3.A). For this reason the concentration was not increased any further. Rottlerin had no significant effect on the NE Rmax or pD_2 in control mesenteric arteries (Fig. 3.3.B). It produced a significant shift in the NE response in mesenteric arteries from diabetic rats. However this antagonist did not significantly affect the NE Rmax in diabetic arteries and in the presence of rottlerin, the Rmax continued to be significantly higher when compared to the control arteries (Table 3.2).

In contrast to NE, neither the maximum contractile response nor the sensitivity of mesenteric arteries from diabetic rats to ET-1 were significantly different from control (Fig. 3.4 and 3.5, Table 3.2). Incubation with Ro-318220 resulted in marked attenuation of maximum responses of both control and diabetic arteries to ET-1, without affecting the ET-1 pD_2 value (Fig. 3.4, Table 3.2). Similar results were obtained with calphostin C (Fig. 3.5), which significantly decreased the ET-1 Rmax in both control and diabetic arteries (Table 3.2) without altering the sensitivity to this agonist. There was no significant difference between control and diabetic arteries in response to ET-1 in the presence of either antagonist.

The effect of rottlerin on the ET-1 response in control arteries was also investigated. While it had no affect on the contractile responses to NE, rottlerin significantly attenuate the ET-1 Rmax (Fig. 3.6, Table 3.2).

Figure 3.2.A

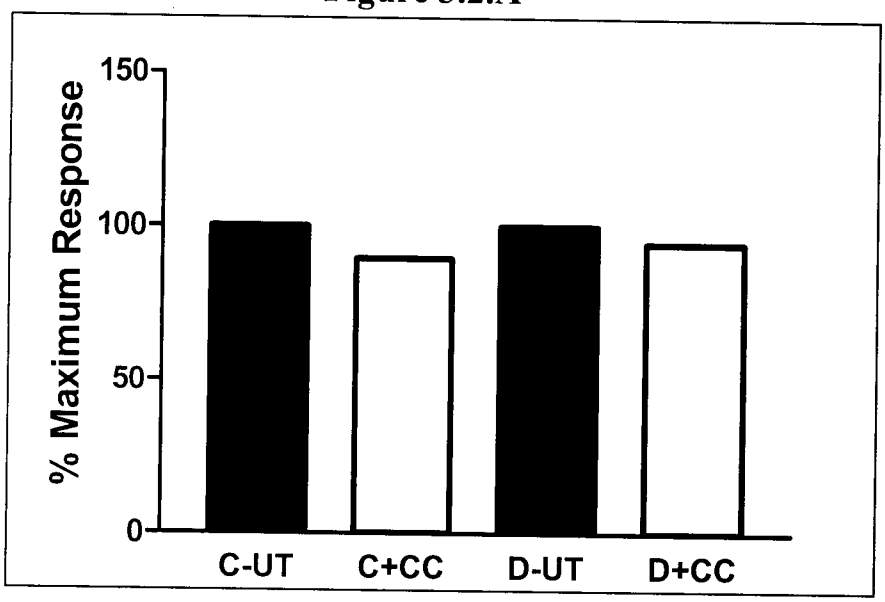


Figure 3.2.B

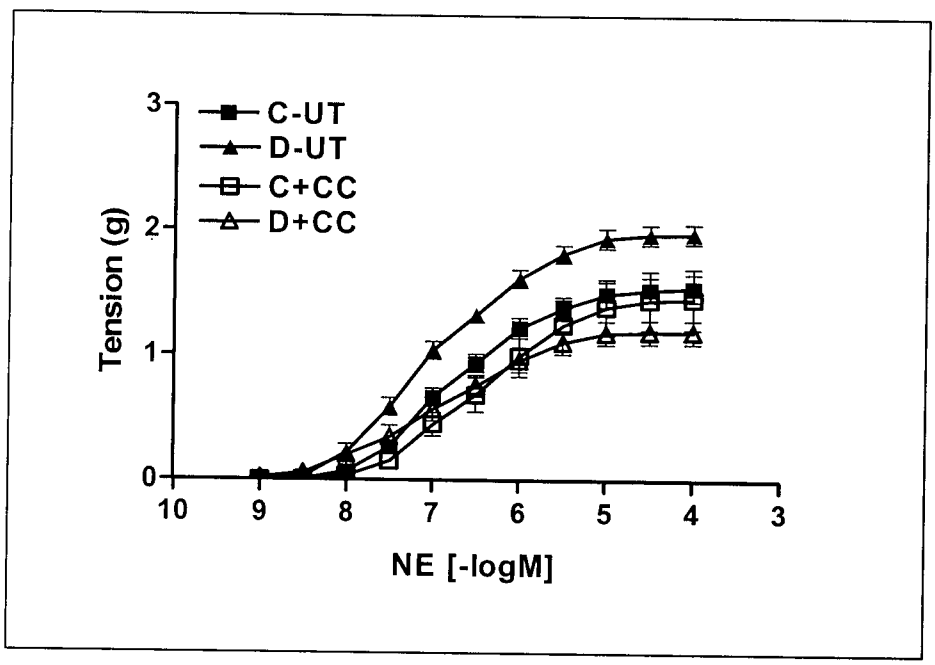


Figure 3.2 Effect of the non-isoform selective PKC inhibitor calphostin C (CC, 3μM for 30min) on maximum KCl contractions (A, n=2) and NE concentration response curves (B, n=8) in control (C) and diabetic (D) mesenteric arteries.

Figure 3.3.A

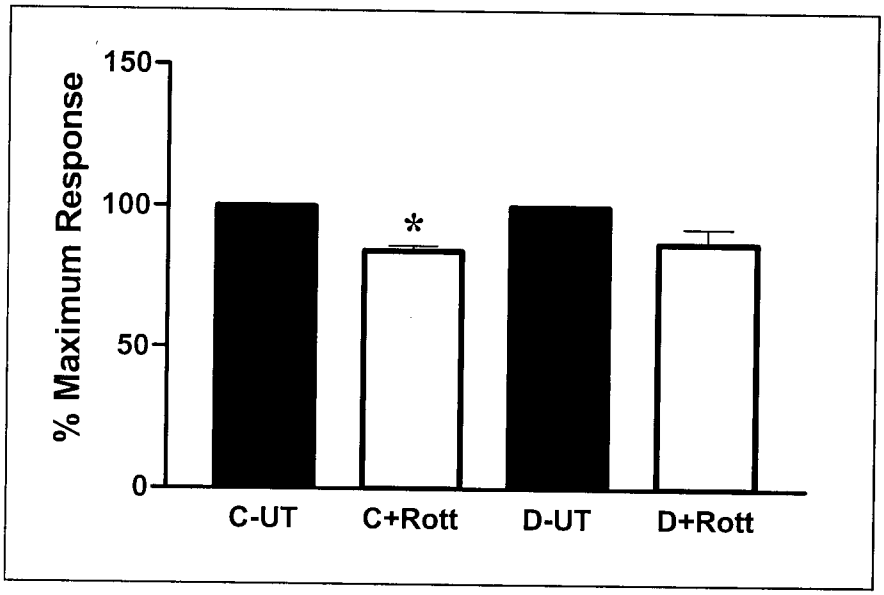


Figure 3.3.B

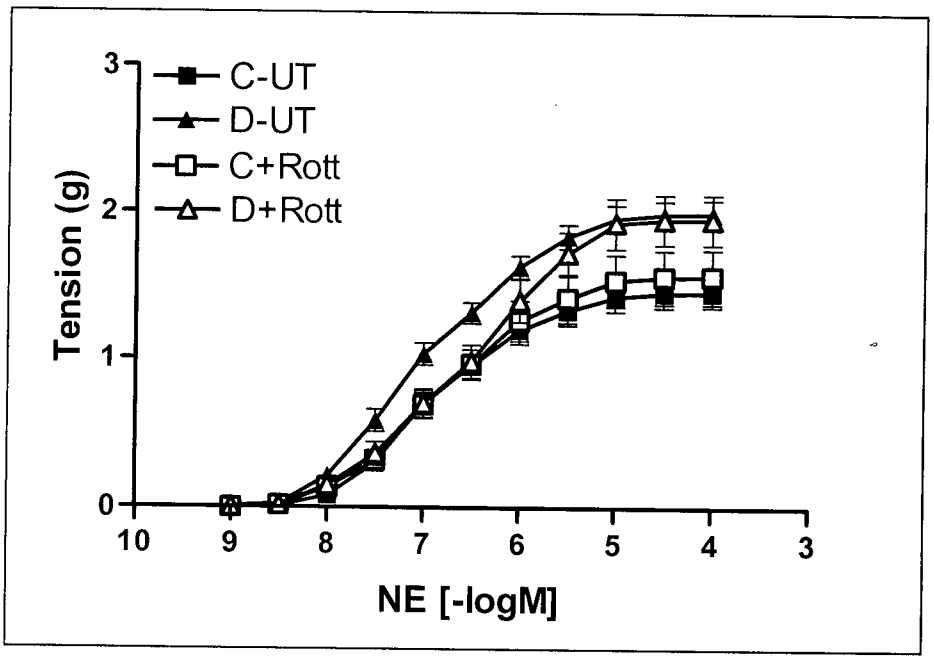


Figure 3.3 Effect of rottlerin, the PKC inhibitor specific for PKCδ, (Rott, 10μM for 30min) on maximum KCl contractions (A, n=4) and NE concentration response curves (B, n=8) in control (C) and diabetic (D) mesenteric arteries. * $p<0.05$ compared to the corresponding untreated. The response to KCl was 1.1 ± 0.1g in the control mesenteric arteries.

Figure 3.4

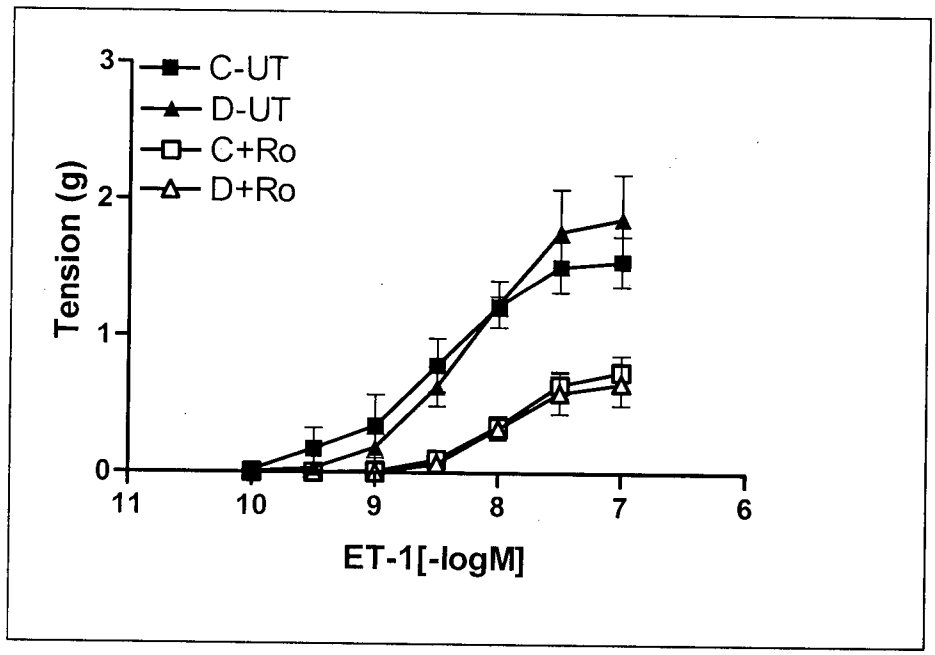


Figure 3.4 Effect of the non-isoform selective PKC inhibitor Ro-318220 (Ro, 3μM for 30min, n=4) on ET-1 concentration response curves in control (C) and diabetic (D) mesenteric arteries.

Figure 3.5

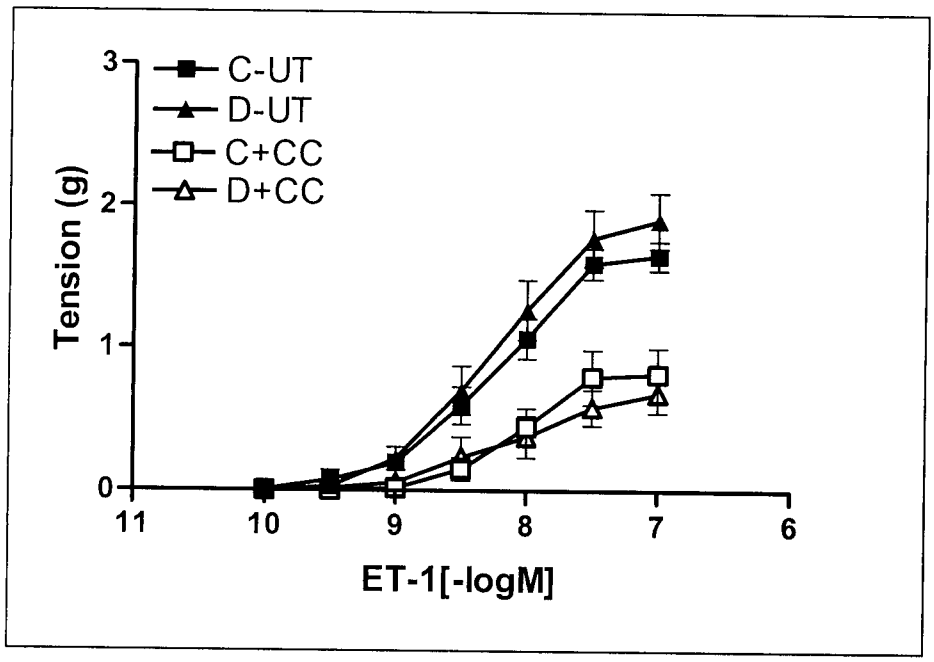


Figure 3.5 Effect of the non-isoform selective PKC inhibitor calphostin C (CC, 3μM for 30min, n=5) on ET-1 concentration response curves in control (C) and diabetic (D) mesenteric arteries.

Figure 3.6

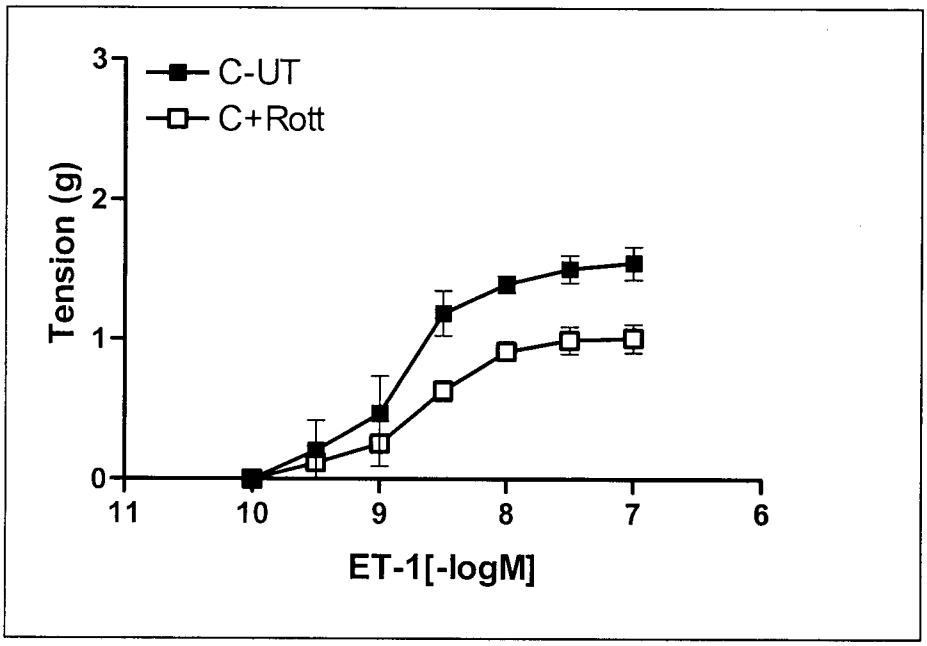


Figure 3.6 Effect of rottlerin, the PKC inhibitor specific for PKCδ, (Rott, 10μM for 30min, n=5) on ET-1 concentration response curves in control (C) mesenteric arteries.

TABLE 3.2

Effect of PKC Inhibitors on NE and ET-1-Induced Contractions

	CONTROL		DIABETIC	
	Rmax (g)	pD_2 (- log EC_{50})	Rmax (g)	pD_2 (- log EC_{50})
NORADRENALINE				
Untreated	1.4 ± 0.1	6.8 ± 0.1	1.9 ± 0.1*	7.1 ± 0.1*
Ro-318220	1.2 ± 0.1	6.4 ± 0.1$^{@}$	1.4 ± 0.1$^{@}$	6.6 ± 0.04$^{@}$
Untreated	1.5 ± 0.1	6.8 ± 0.1	1.9 ± 0.1*	7.1 ± 0.1*
Calphostin C	1.4 ± 0.2	6.5 ± 0.1$^{@}$	1.2 ± 0.1$^{@}$	6.9 ± 0.1*$^{@}$
Untreated	1.5 ± 0.1	6.9 ± 0.04	2.0 ± 0.1*	7.1 ± 0.1
Rottlerin	1.6 ± 0.2	6.9 ± 0.1	2.0 ± 0.2*	6.6 ± 0.2$^{@}$
ENDOTHELIN-1				
Untreated	1.6 ± 0.2	8.2 ± 0.1	1.9 ± 0.3	8.2 ± 0.2
Ro-318220	0.8 ± 0.1$^{@}$	8.0 ± 0.1	0.7 ± 0.2$^{@}$	8.0 ± 0.1
Untreated	1.7 ± 0.1	8.4 ± 0.2	1.9 ± 0.2	8.3 ± 0.1
Calphostin C	0.8 ± 0.2$^{@}$	8.1 ± 0.2	0.7 ± 0.1$^{@}$	8.1 ± 0.2
Untreated	1.5 ± 0.1	8.9 ± 0.3		
Rottlerin	1.0 ± 0.1$^{@}$	8.7 ± 0.3		

* $p<0.05$ compared to corresponding control value

$^{@}p<0.05$ compared to corresponding untreated value

(Two-way ANOVA followed by Newman Keuls post-hoc test)

3.2.2 Effect of Diabetes on Levels of PKC Isoforms in Mesenteric Arteries

PKCα, β_2, δ, and ε were all detected in mesenteric arteries from both control and diabetic rats (Fig. 3.7). No significant differences in total levels of PKCα or PKCδ were detected between control and diabetic arteries. However, levels of PKCβ_2 and PKCε in diabetic arteries were increased to 195 ± 26.9% and 140 ± 13.2%, respectively, of levels in control arteries.

Figure 3.7

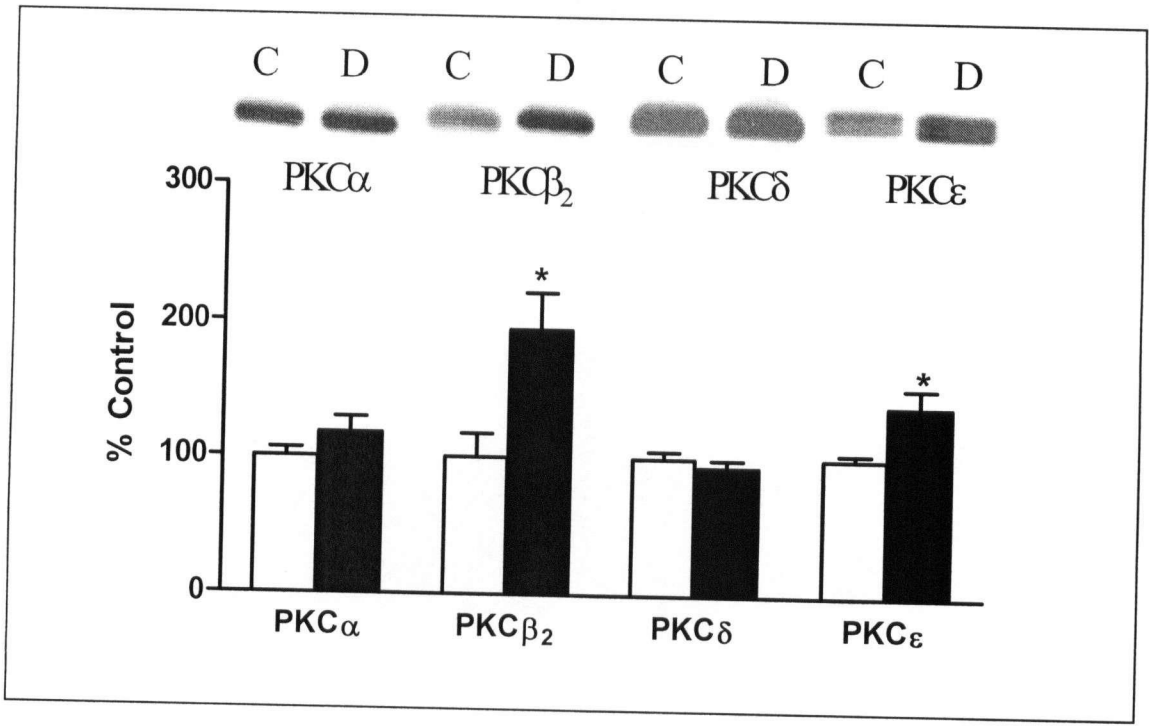


Figure 3.7 Total levels of PKC α, β_2, δ and ε in control (open bars) and diabetic (closed bars) mesenteric arteries (n=5). Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.5), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins. Results were expressed as a percent of levels of the isoform in control mesenteric arteries. * $p<0.05$ compared to the corresponding control fraction (One-way ANOVA).

3.2.3 Effect of NE and ET-1 on Particulate Levels of PKC Isoforms in Mesenteric Arteries

Treatment with 30μM NE for 5 minutes, the time required for the sustained contractile response to this agonist to reach its maximum in both control and diabetic arteries, had no significant effect on particulate levels of PKCα in control arteries, but resulted in a marked increase in levels of PKCα in the particulate fraction of diabetic mesenteric arteries, to 190 ± 22% of levels in untreated control arteries (Fig. 3.8.A). NE treatment had no detectable effect on particulate levels of $PKC\beta_2$ (data not shown) or PKCδ (Fig. 3.8.B) in mesenteric arteries from either control or diabetic rats. However, NE did result in a small but significant increase of PKCε in the particulate fraction of control mesenteric arteries, to 125 ± 8% of those in untreated arteries (Fig. 3.8.C). Particulate levels of PKCε in untreated diabetic arteries were significantly greater than those in untreated control arteries, and treatment with NE resulted in a further significant increase in PKCε particulate levels, to 230 ± 19% of control. The NE-induced increase in PKCε in the particulate fraction was significantly greater in diabetic than in control mesenteric arteries.

Treatment with 0.03μM ET-1 for 7 minutes, the time required for the sustained contractile response to this agonist to reach its maximum, resulted in similar increases in particulate levels of PKCα in mesenteric arteries from both control and diabetic rats. Particulate levels of PKCα were increased by ET-1 to 192 ± 15% in control and to 180 ± 12% in diabetic arteries of levels in untreated control arteries (Fig. 3.9.A). Like NE, ET-1 had no significant effect on particulate levels of $PKC\beta_2$ (data not shown). However, ET-1 produced significant increases in particulate levels of PKCδ in both control and diabetic arteries (Fig. 3.9.B). The ET-1-induced increase in particulate PKCδ was significantly less in diabetic (117 ± 7%) than in control (153 ± 5%) arteries. In mesenteric arteries from control rats, ET-1 treatment resulted in a significant increase in levels of PKCε in the particulate fraction, to 166 ± 7% of levels in the untreated control (Fig. 3.9.C). A similar increase in particulate levels of PKCε in response to ET-1, to 181 ± 8% of control, was also detected in diabetic mesenteric arteries.

Figure 3.8

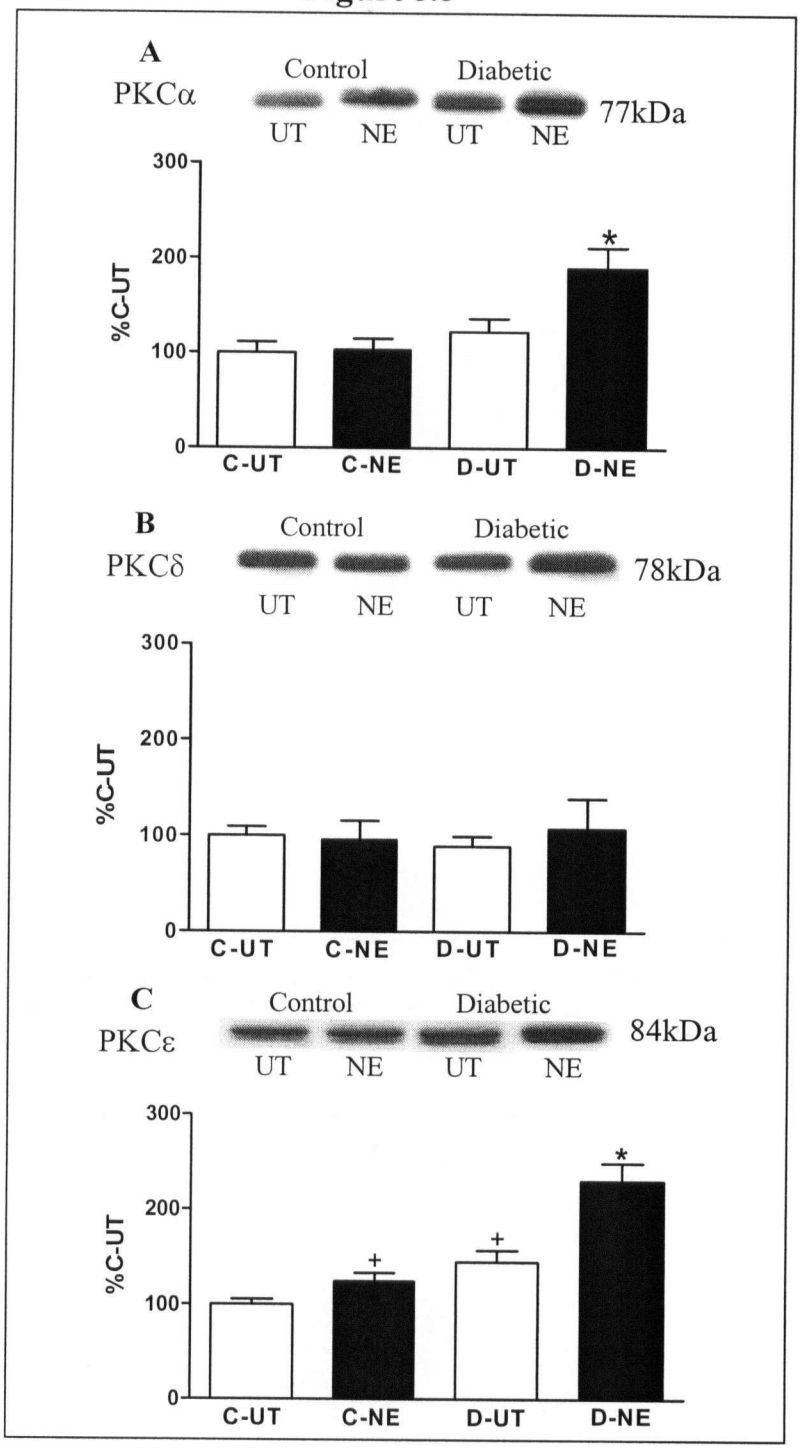


Figure 3.8 Particulate levels of PKCα (A), δ (B) and ε (C) in the absence (UT) and presence of NE (n=5) in control (C) and diabetic (D) arteries. Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.6), centrifuged and 20 μg of the particulate fraction was loaded onto 11% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to all the other corresponding groups; + $p<0.05$ compared to the corresponding control basal group. (Two-way ANOVA followed by Newman Keuls post-hoc test)

Figure 3.9

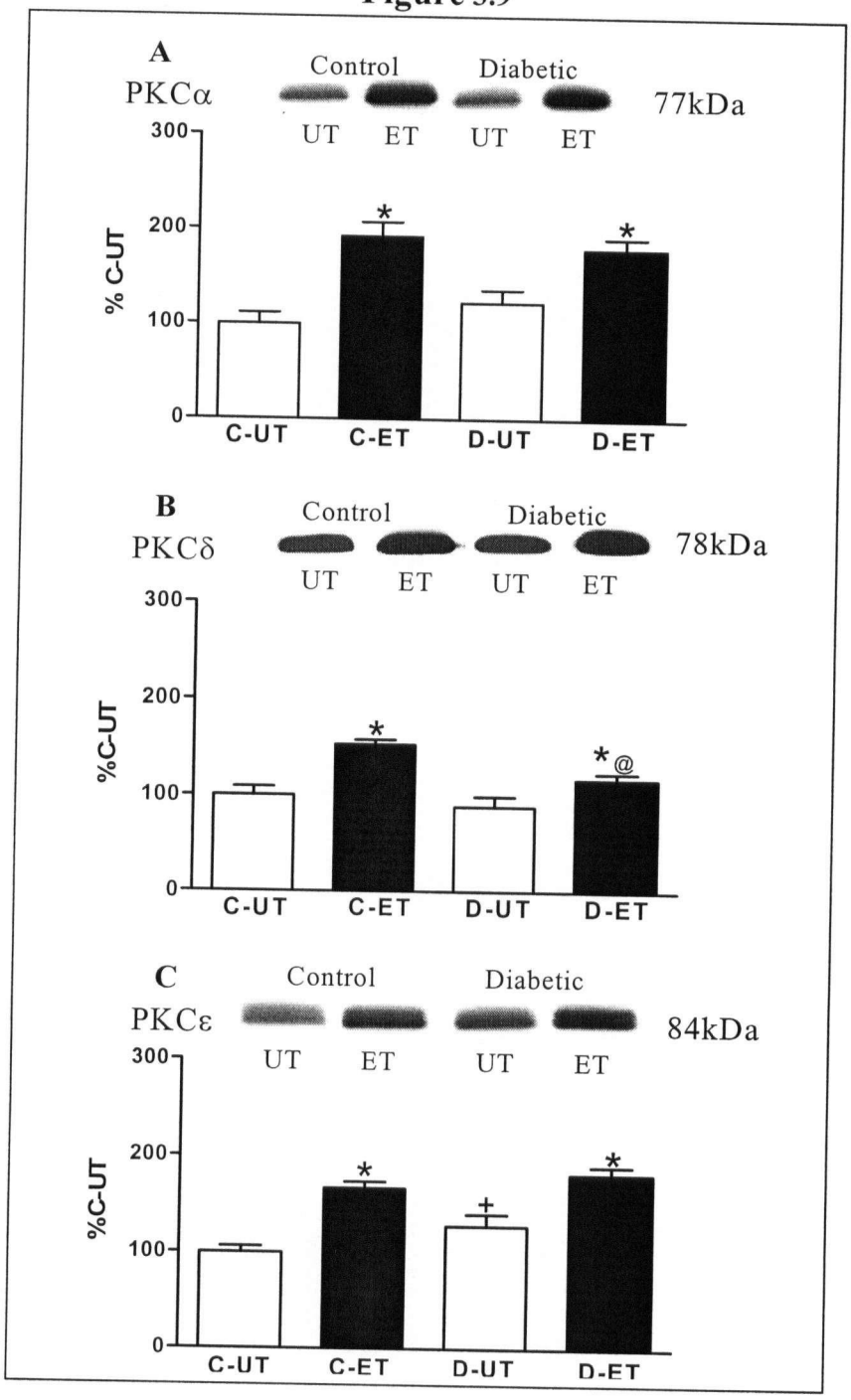


Figure 3.9 Particulate levels of PKCα (A), δ (B) and ε (C) in the absence (UT) and presence of ET-1 (n=5) in control (C) and diabetic (D) arteries. Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.6), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to the corresponding basal group; + $p<0.05$ compared to the corresponding control basal group; @ $p<0.05$ compared to the corresponding control ET-1 treated group (Two-way ANOVA followed by Newman Keuls post-hoc test).

3.2.4 Effect of PdBu and NE on Phosphorylation of CPI-17

We investigated whether stimulation of arteries with PdBu and NE was associated with increased phosphorylation of the downstream target of PKC, CPI-17. In preliminary experiments, treatment of control arteries with PdBu (3 μM for 10 minutes) produced a significant increase in CPI-17 phosphorylation levels (Fig. 3.10). No significant differences in total protein levels of CPI-17 were detected between control and diabetic mesenteric arteries (Fig. 3.11.A). NE had no significant effect on the phosphorylation of CPI-17 in control arteries (Fig. 3.11.B). Levels of phosphorylated CPI-17 were lower in untreated diabetic than in untreated control arteries, although this difference was not statistically significant. However, NE produced a significant increase in CPI-17 phosphorylation in diabetic mesenteric arteries, and in the presence of NE, levels of phosphorylated CPI-17 were significantly greater in diabetic than in control arteries. The NE-induced increase in CPI-17 phosphorylation in diabetic arteries was blocked by the same concentration of calphostin C (3 μM) that normalized the contractile response of diabetic mesenteric arteries to NE (Fig. 7B).

Figure 3.10

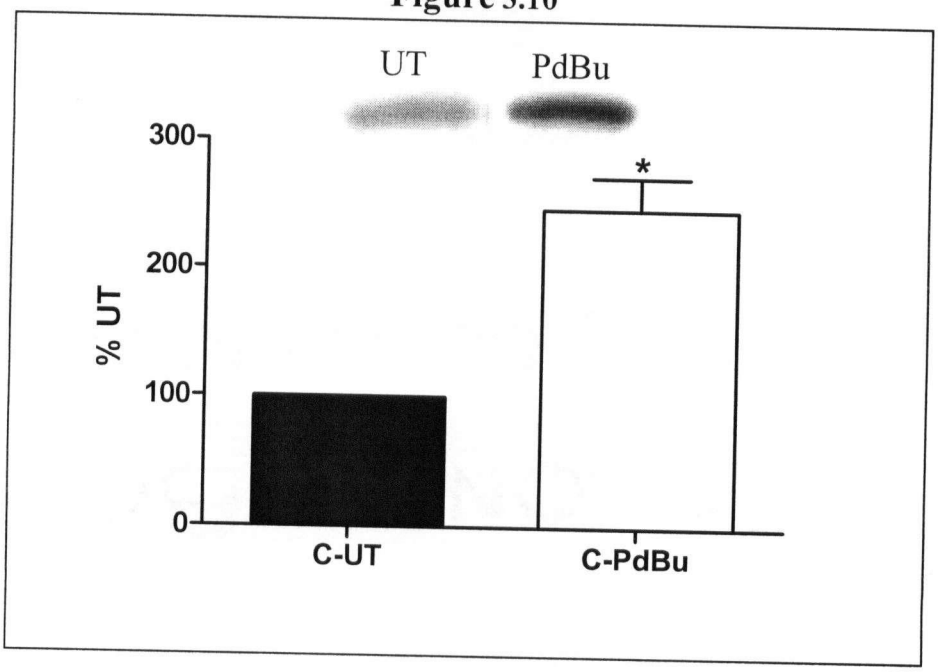


Figure 3.10 Levels of phosphorylated CPI-17 in mesenteric arteries from control (C) rats in the untreated state (UT) and in the presence of PdBu (3μM for 10 min). Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.7), centrifuged and 20 μg of total protein was loaded onto 16% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to C-UT (One-way ANOVA).

Figure 3.11.A

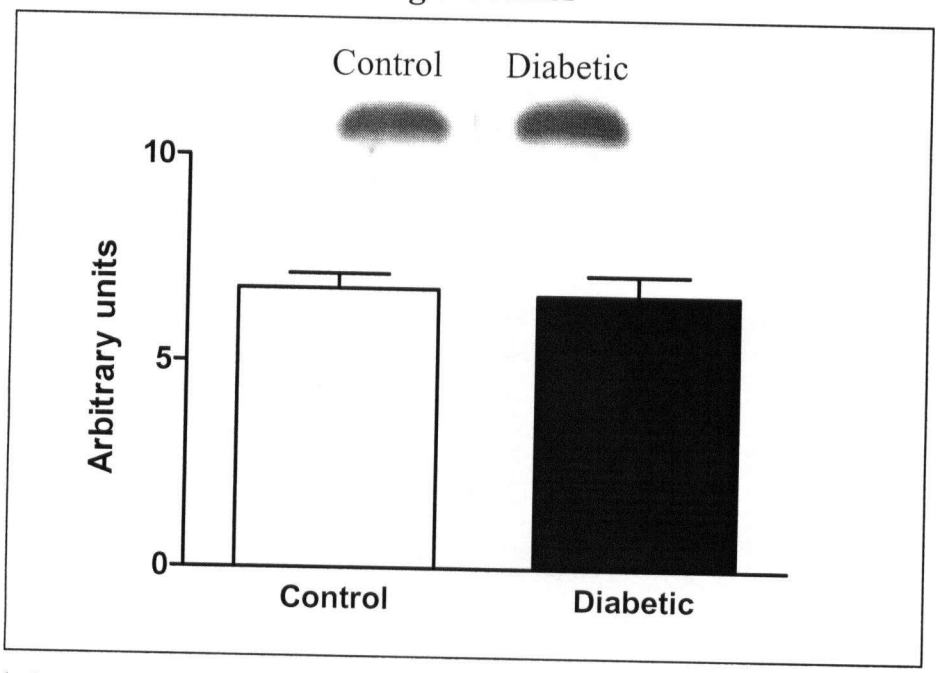


Figure 3.11.A Total levels of CPI-17 in control and diabetic arteries (n=3). Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.7), centrifuged and 20 μg of total protein was loaded onto 16% polyacrylamide gels for separation of proteins.

Figure 3.11.B

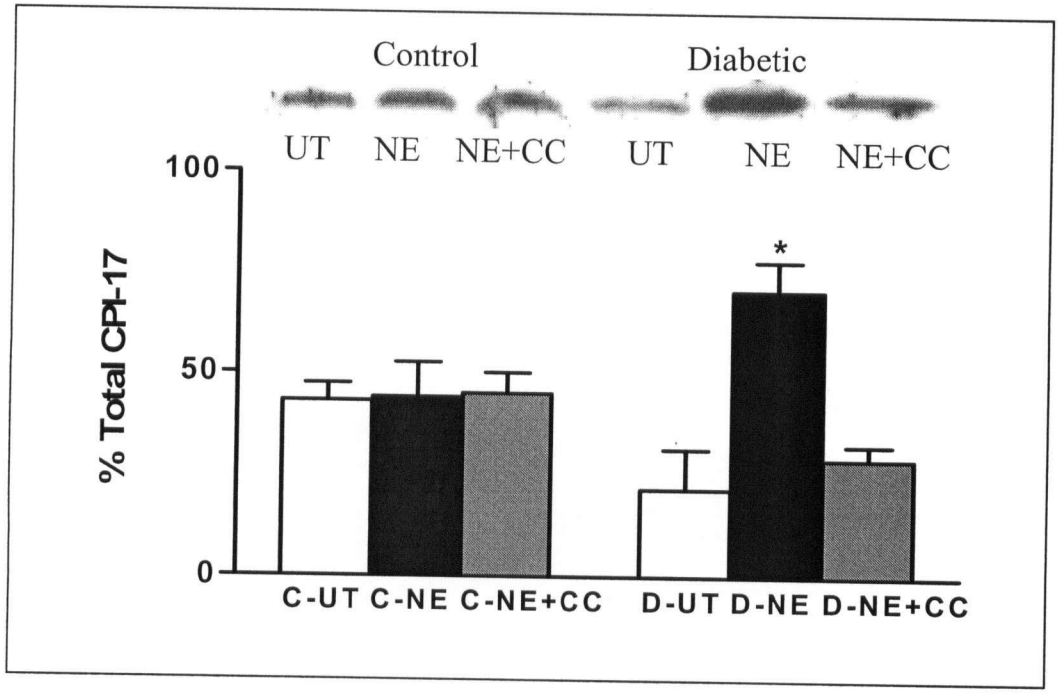


Figure 3.11.B Levels of phosphorylated CPI-17 in mesenteric arteries from control (C) and diabetic (D) rats in the untreated state (UT), in the presence of NE (30μM for 5 min) and in the presence of NE plus the non-isoform specific PKC inhibitor, calphostin C (NE + CC, 3μM calphostin C for 35 min with 30 μM NE being added for the last 5 min) (B, n=3). Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.7), centrifuged and 20 μg of total protein was loaded onto 16% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to C-NE, C-NE + CC, D-UT and D-NE + CC (Two-way ANOVA followed by Newman Keuls post-hoc test).

3.3 CONTRIBUTION OF RhoK TO THE ENHANCED CONTRACTILE RESPONSES OF DIABETIC MESENTERIC ARTERIES TO α_1-AR STIMULATION: INTERACTION WITH PKC

3.3.1 Effect of RhoK & PKC Inhibitors on Contractile Responses to PE and ET-1

As previously reported for NE, contractile responses of diabetic arteries to PE were significantly greater than control (Fig 3.12.C, 3.13.B, 3.14, 3.15, 3.16 and Table 3.3). This enhanced response was observed in the absence of any significant difference in the maximum response to KCl (0.1M in the presence of 10μM phentolamine) between the control and diabetic tissues (1.18±0.07 vs. 1.18±0.08g, control vs. diabetic).

Treatment with the RhoK inhibitor Y-27632, 1μM for 15 minutes, had no significant effect on maximum KCl (Fig. 3.12.A) or PdBu (3.12.B) induced contractions. Used at the same concentration for the same time period, it significantly decreased the sensitivity of control mesenteric arteries to PE, but had no significant effect on the Rmax (Fig. 3.12.C, Table 3.3). In arteries from diabetic rats, Y-27632 significantly attenuated the PE Rmax and also shifted the concentration response curve resulting in a significant decrease in the PE pD_2. In the presence of Y-27632, the difference in the PE response between the control and diabetic arteries was abolished.

Similar results were obtained with a second RhoK inhibitor, H-1152 (Fig. 3.13.B, Table 3.3). Used at 0.1μM for 15 minutes, H-1152 had no significant effect on the maximum KCl response (Fig. 3.13.A). However like Y-27632, in control mesenteric arteries it significantly reduced the PE pD_2 without affecting the PE Rmax. It had a greater inhibitory effect on diabetic arteries, as it significantly attenuated both the sensitivity and maximum contractile responses to PE. After treatment with the inhibitor, the significant difference in PE response between control and diabetic arteries was abolished.

In order to confirm that inhibition of PKC normalized enhanced contractile responses of diabetic arteries to PE (as previously reported for NE), we investigated the effect of the PKC inhibitors, calphostin C and Ro-318220 on contractile responses of arteries from control and

diabetic rats to PE. Ro-318220, used at 3μM for 30minutes, produced a small but significant shift in the control PE concentration response curve without affecting the Rmax (Fig. 3.14, Table 3.3). However it had a greater inhibitory effect in diabetic tissues, significantly attenuating the PE Rmax and pD_2, and normalizing responses of diabetic arteries to PE.

Similar results were obtained with the other PKC inhibitor calphostin C. At a concentration of 3μM, calphostin C produced a small but significant decrease in the PE pD_2 in control mesenteric arteries without affecting the PE Rmax (Fig. 3.15, Table 3.3). In diabetic tissues, calphostin C produced significant attenuation of both the PE Rmax and pD_2 and abolished the difference in PE response between control and diabetic arteries.

When we investigated the effect of rottlerin on PE induced contractions in control and diabetic arteries, the results were different from those observed with NE. Rottlerin produced a significant shift in the PE pD_2 in control mesenteric arteries without affecting the PE Rmax. However in diabetic arteries, it significantly attenuated both the PE Rmax and pD_2 and normalized the enhanced response to PE.

Unlike the α_1-AR responses, RhoK inhibition did not significantly affect contractile responses to ET-1 in both control and diabetic mesenteric arteries. The concentration of Y-27632 that significantly attenuated NE-induced contractile responses in control mesenteric arteries, had no significant effect on ET-1 Rmax (222.5±18.2 vs. 190.3±14.7, absence vs. presence of Y-27632) and pD_2 (8.4±0.1 vs. 8.2±0.1, absence vs. presence of Y-27632) (Fig. 3.17). Similarly in diabetic mesenteric arteries, Y-27632 little inhibitory effect on ET-1 Rmax (227.2±12.9 vs. 197.6±19.2, absence vs. presence of Y-27632) and pD_2 (8.2±0.1 vs. 8.3±0.2, absence vs. presence of Y-27632) (Fig. 3.17). The effect of H-1152 (Fig. 3.18) was investigated only in control mesenteric arteries. Like Y-27632, it had almost no inhibitory effect on ET-1-induced vasoconstriction.

Figure 3.12.A

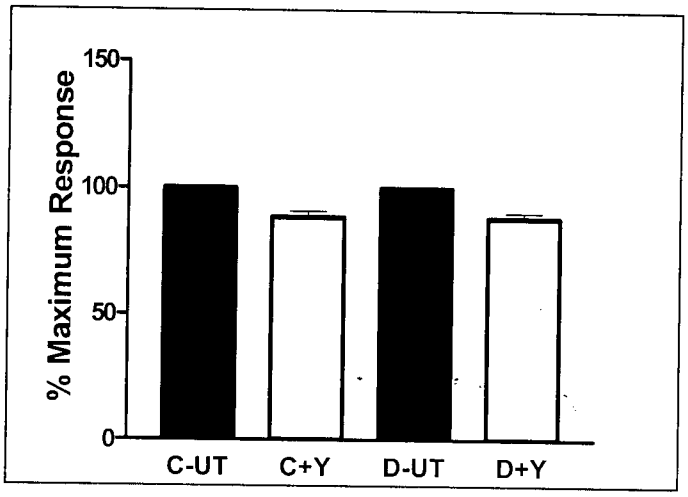


Figure 3.12.B

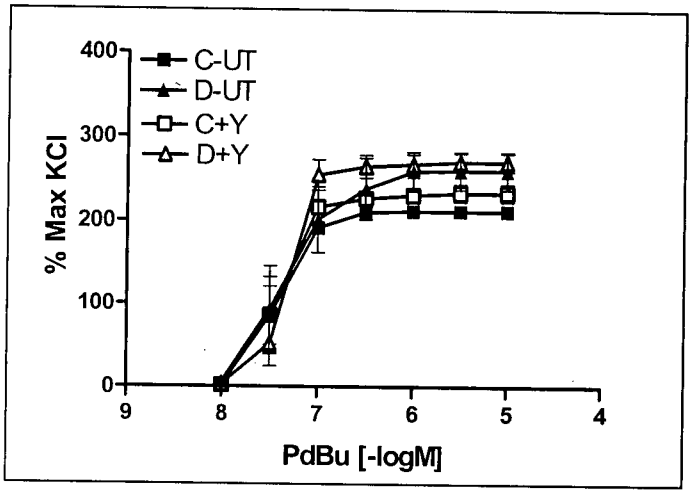


Figure 3.12 Effect of the RhoK inhibitor Y-27632 (Y, 1μM for 15min) on KCl (A, n=6) and PdBu (B, n=4) contractions in control and diabetic mesenteric arteries. The response to KCl was 1.3 ± 0.1g in the control mesenteric arteries.

Figure 3.12.C

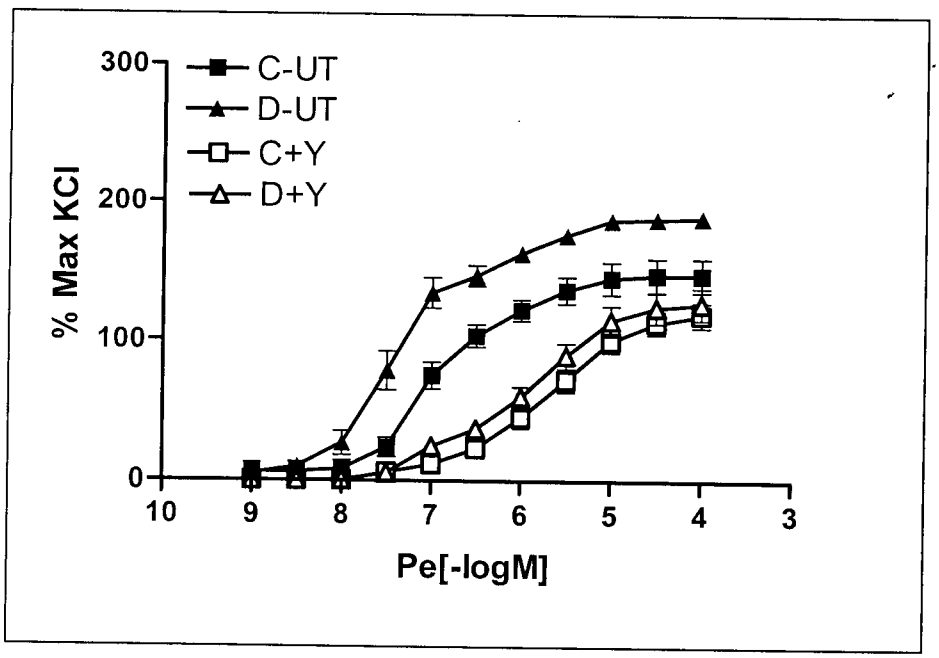


Figure 3.12.C Effect of the RhoK inhibitor Y-27632 (Y, 1μM for 15min, n=6) on PE concentration response curves in control (C) and diabetic (D) mesenteric arteries.

Figure 3.13.A

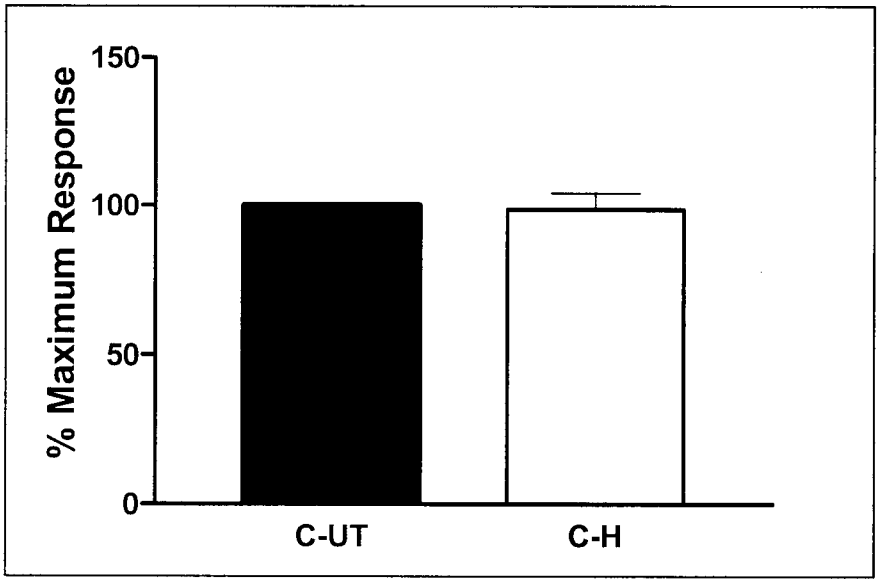


Figure 3.13.B

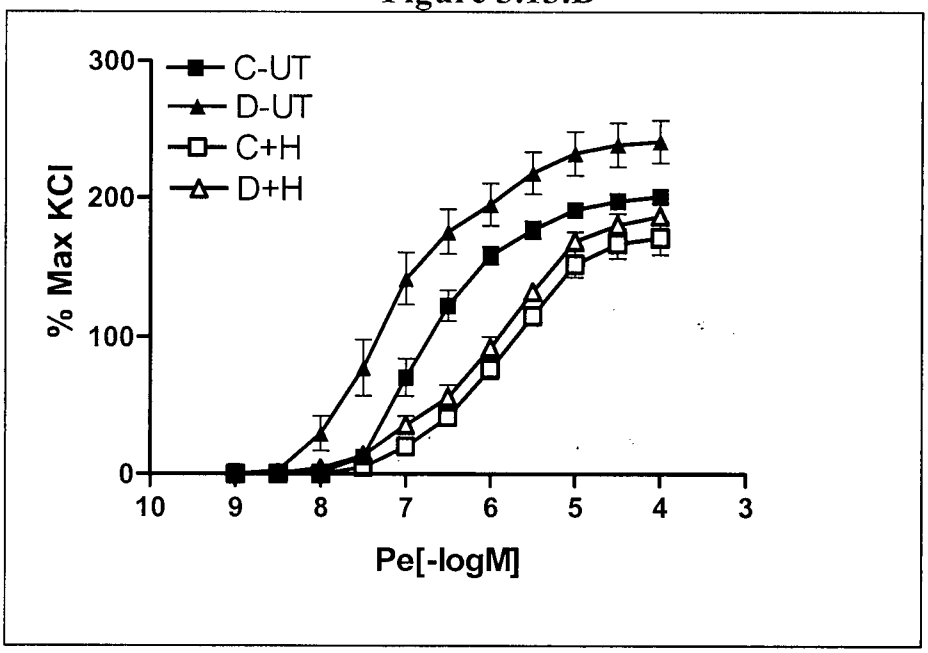


Figure 3.13 Effect of the RhoK inhibitor H-1152 (H, 0.1μM for 15min) on maximum KCl contractions (A, n=3) and PE concentration response curves (B, n=8) in control (C) and diabetic (D) mesenteric arteries. The response to KCl was 0.9 ± 0.1g in the control mesenteric arteries.

Figure 3.14

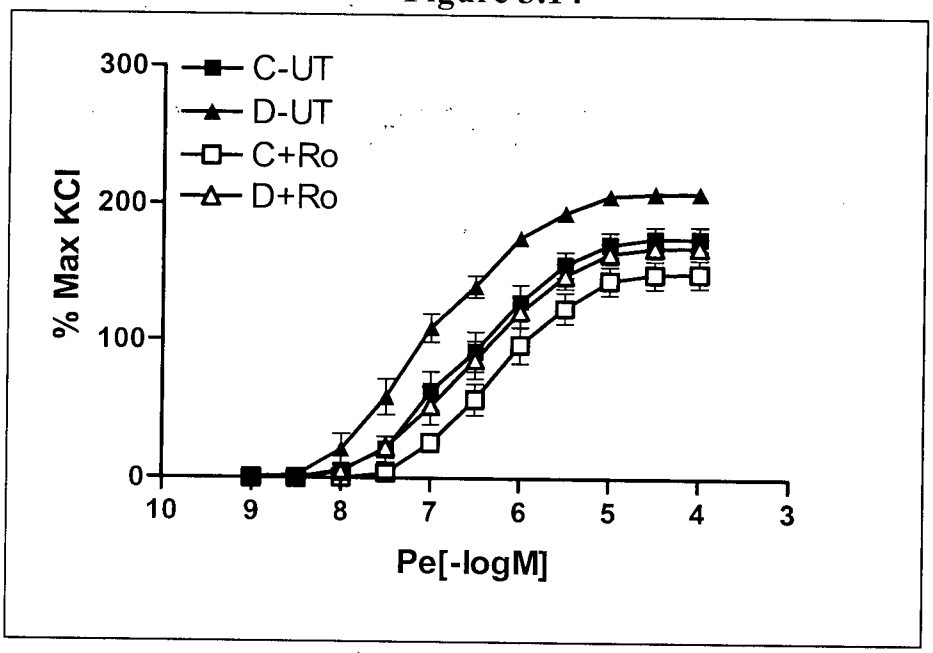


Figure 3.14 Effect of the non-isoform selective PKC inhibitor Ro-318220 (Ro, 3μM for 30min, n=7) on PE concentration response curves in control (C) and diabetic (D) mesenteric arteries.

Figure 3.15

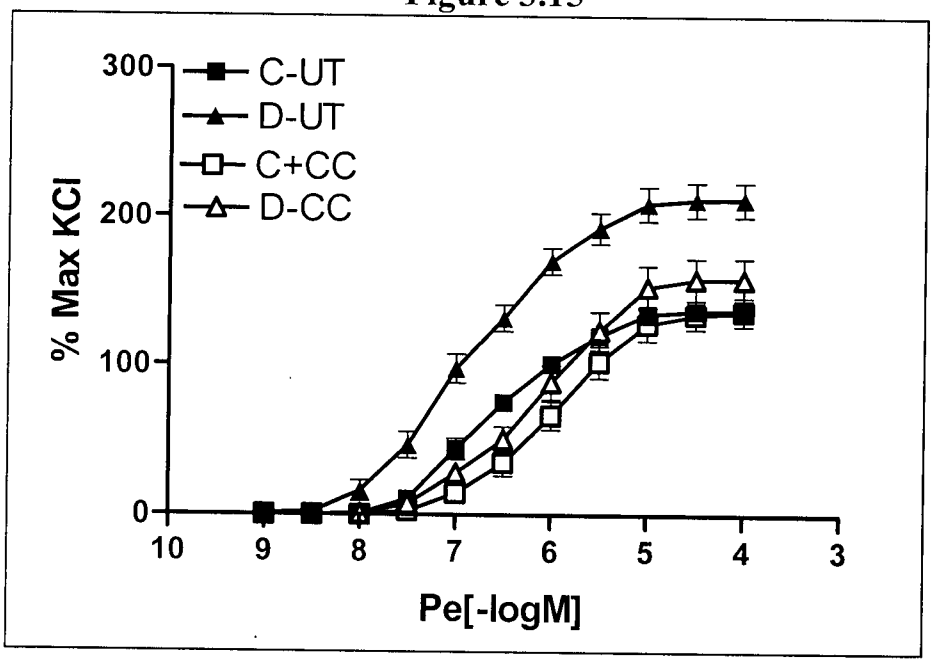


Figure 3.15 Effect of the non-isoform selective PKC inhibitor calphostin C (CC, 3μM for 30min, n=6) on PE concentration response curves in control (C) and diabetic (D) mesenteric arteries.

Figure 3.16

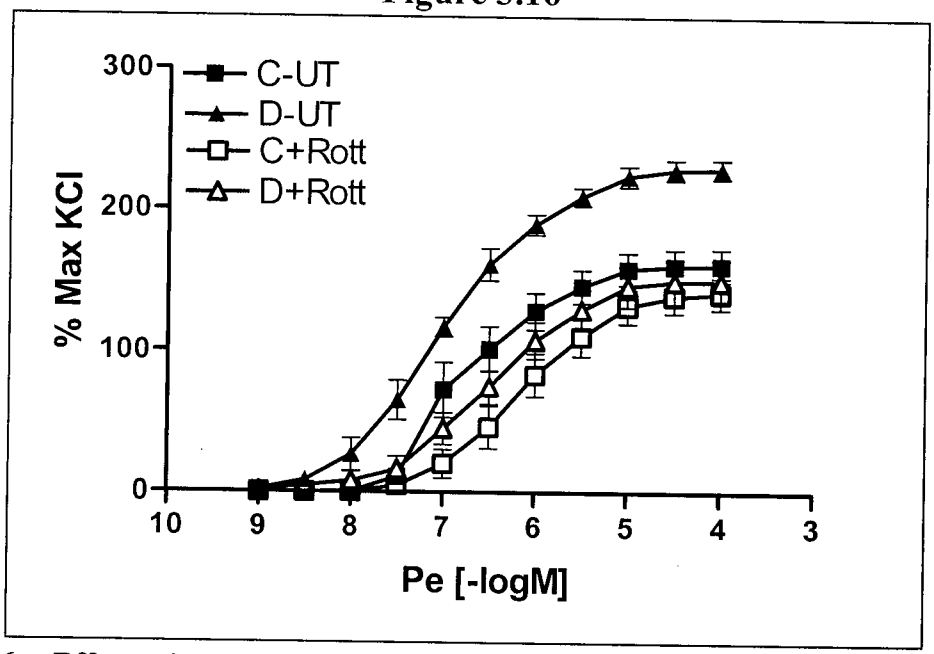


Figure 3.16 Effect of rottlerin, the PKC inhibitor specific for PKCδ, (Rott, 10μM for 30min, n=6) on PE concentration response curves in control (C) and diabetic (D) mesenteric arteries

TABLE 3.3

Effect of RhoK and PKC Inhibitors on PE-Induced Contractions

	CONTROL		DIABETIC	
	Rmax (% Max KCl)	pD_2 (- log EC_{50})	Rmax (% Max KCl)	pD_2 (- log EC_{50})
Untreated	155.9 ± 13.4	7.0 ± 0.1	182.5 ± 3.7*	7.4 ± 0.1*
Y-27632	119.8 ± 8.5	5.6 ± 0.1@	126.6 ± 11.3@	5.8 ± 0.1@
Untreated	197.3 ± 3.7	6.8 ± 0.1	232.3 ± 15.9*	7.1 ± 0.1
H-1152	172.6 ± 12.7	5.9 ± 0.1@	184.5 ± 7.8@	6.1 ± 0.1@
Untreated	174.9 ± 7.4	6.6 ± 0.2	204.6 ± 2.5*	7.0 ± 0.1*
Ro-318220	152.1 ± 9.1	6.2 ± 0.1@	169.0 ± 6.0@	6.5 ± 0.2@
Untreated	135.4 ± 6.6	6.6 ± 0.1	208.0 ± 11.3*	6.9 ± 0.1
Calphostin C	138.9 ± 10.2	6.0 ± 0.1@	160.3 ± 14.2@	6.1 ± 0.1@
Untreated	159.7 ± 11.7	6.8 ± 0.1	221.8 ± 9.1*	7.0 ± 0.1
Rottlerin	142.3 ± 11.5	6.1 ± 0.1@	148.7 ± 16.4@	6.5 ± 0.1@

* $p<0.05$ compared to corresponding control value

@ $p<0.05$ compared to corresponding untreated value

(Two-way ANOVA followed by Newman Keuls post-hoc test)

Figure 3.17

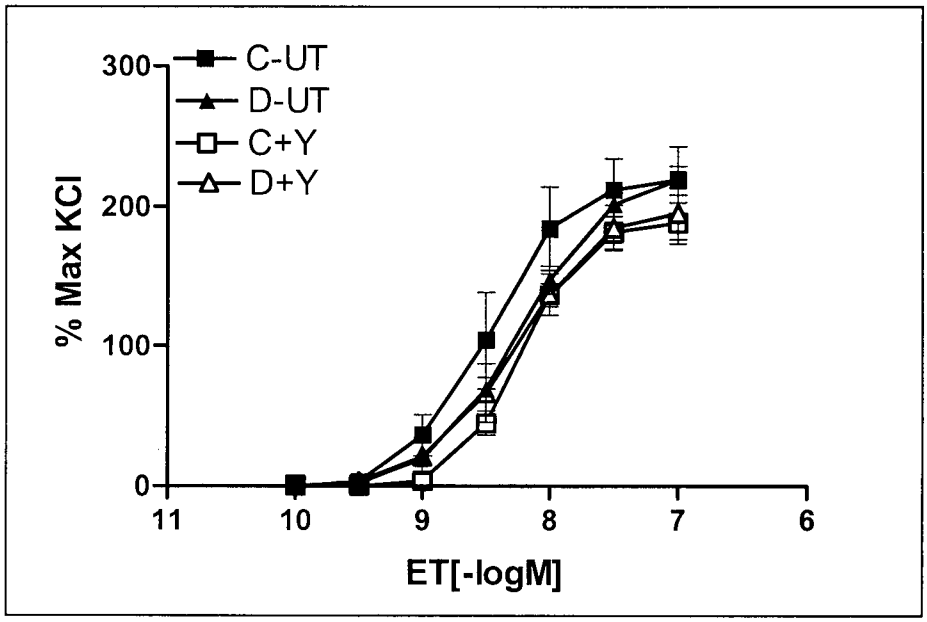


Figure 3.17 Effect of the RhoK inhibitor Y-27632 (Y, 1μM for 15min, n=6) on ET-1 concentration response curves in control (C) and diabetic (D) mesenteric arteries.

Figure 3.18

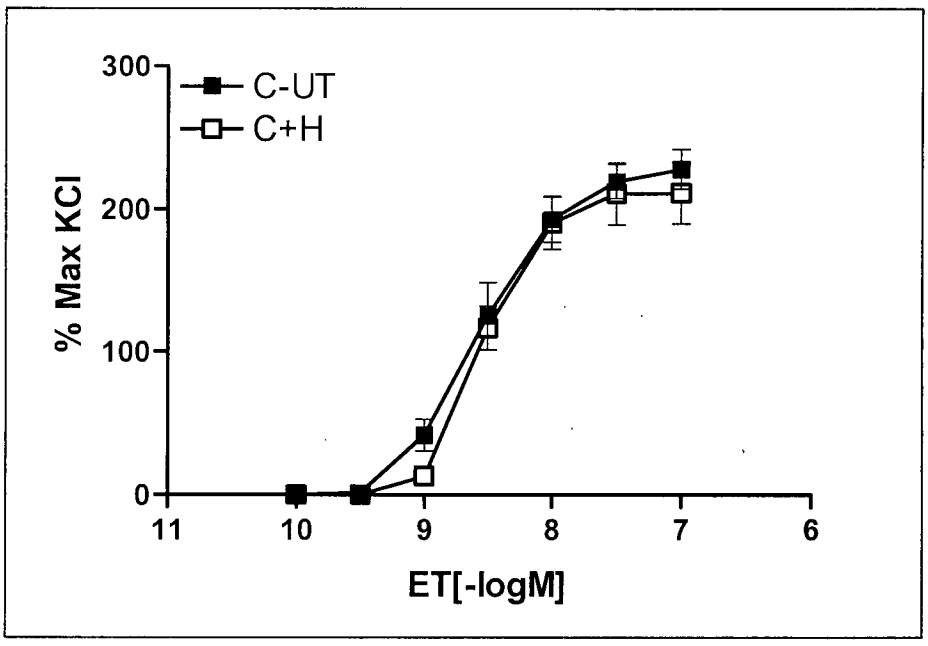


Figure 3.18 Effect of the RhoK inhibitor H-1152 (H, 0.1μM for 15min, n=5) on ET-1 concentration response curves in control (C) mesenteric arteries.

3.3.2 Effect of Diabetes on Levels of RhoK Isoforms in Mesenteric Arteries

Total levels of the RhoK isoforms in diabetic arteries were not significantly different from those in control arteries (Fig. 3.19). The total levels of ROCK I in diabetic arteries were 105.2 ± 9.0% and those of ROCK II were 106.3 ± 7.8% of levels in control tissues.

Figure 3.19

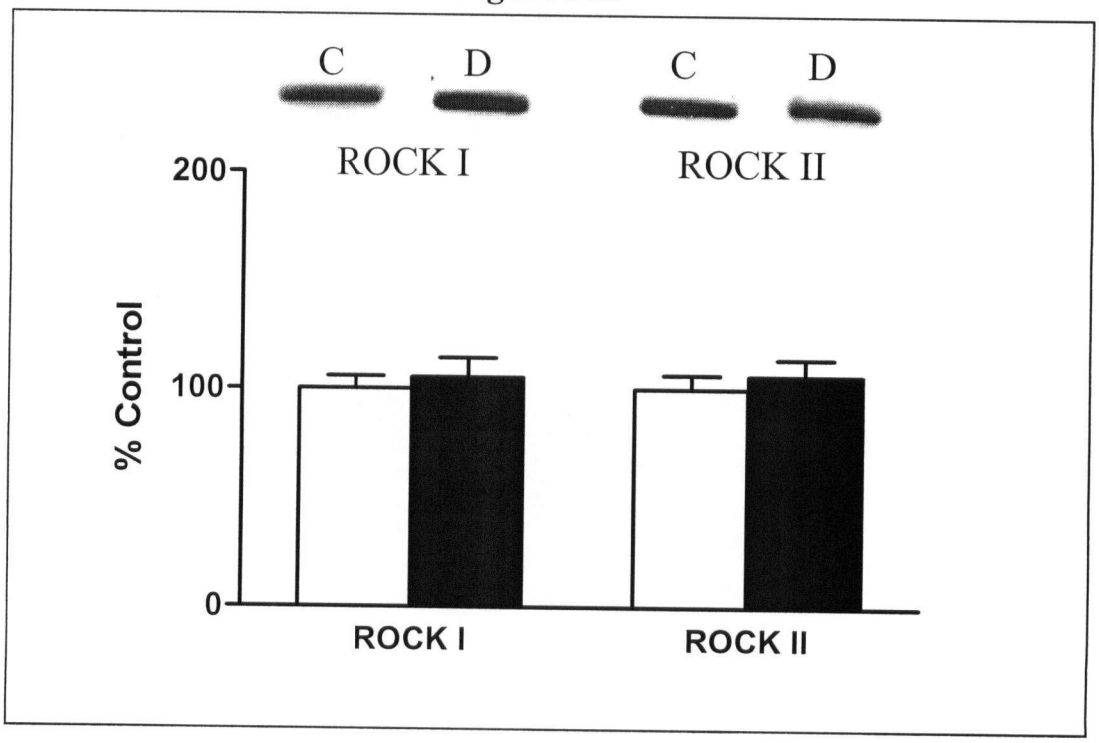


Figure 3.19 Total levels of ROCK I and II in control (open bars) and diabetic (closed bars) mesenteric arteries (n=5). Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.5), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins. Results were expressed as a percent of levels of the isoform in control mesenteric arteries.

3.3.3 Effect of PE and ET-1 on Particulate Levels of RhoK Isoforms in Mesenteric Arteries

Particulate levels of ROCK I and II in untreated arteries from control and diabetic rats were not significantly different (Fig. 3.20.A and B). Treatment with a maximum concentration of PE (30μM) for the time required to produce a sustained contraction produced a significant increase in levels of ROCK I in the particulate fraction in both control and diabetic mesenteric arteries (Fig. 3.20.A). The particulate levels of ROCK I increased to 132 ± 14.0% in control tissues, and to 152 ± 6.7% in diabetic tissues, of levels in untreated control arteries. The increment in ROCK I particulate levels was not significantly different between arteries from control and diabetic rats. PE also induced significant translocation of ROCK II in both control (137 ± 13.2%) and diabetic (187 ± 18.5%) arteries (Fig. 3.20.B). However the increase in ROCK II levels in diabetic tissues was significantly greater than control. Treatment of both control and diabetic tissues with Y-27632, 1μM for 15 minutes, did not affect basal particulate levels of ROCK I or II (data not shown). However, it did abolish the PE-induced increases in the particulate levels of these isoforms in arteries from both control and diabetic rats (Fig. 3.20.A and B). Consistent with the contractility data, treatment of tissues with 0.03 μM ET-1, had no effect on the particulate levels of ROCK I or II (Fig. 3.21.A and B) in either control or diabetic arteries.

Because both inhibition of RhoK and inhibition of PKC normalized the PE response in diabetic arteries, we investigated the effect of Y-27632 on PE–induced increases in particulate levels of PKC isoforms in control and diabetic arteries. Stimulation of control tissues with 30μM PE had no effect on the particulate levels of PKCα. However in the diabetic tissues, PE significantly increased particulate levels of PKCα by 148 ± 7.3% compared to the untreated control (Fig. 3.22.A). PE had no effect on particulate levels of PKCδ in control arteries but it also significantly increased particulate levels of this isoform to 145 ± 5.9% in diabetic arteries (Fig. 3.20.B). In diabetic arteries basal particulate levels of PKCε were significantly increased to 141 ± 8.6% of control (Fig. 3.22.C). Stimulation with PE had no effect on levels of PKCε in control tissues. However in diabetic arteries, PE induced a further significant increment in particulate levels of PKCε to 219 ± 11.9%. Treatment of both control and diabetic arteries with Y-27632, 1μM for 15 minutes, did not

affect basal particulate levels of PKCα, δ and ε (data not shown). However PE-induced increases in these PKC isoforms in diabetic arteries were abolished by Y-27632 (Fig. 3.22.A, B and C).

In order to determine whether this effect of Y-27632 was due to direct inhibition of PKC, the effect of the inhibitor on ET-1 induced translocation of PKC isoforms was investigated. In control arteries, ET-1 induced significant increases in particulate levels of PKCα, δ and ε (Fig. 3.23.A, B and C). Incubation of Y-27632 had no significant effect on these changes.

Figure 3.20

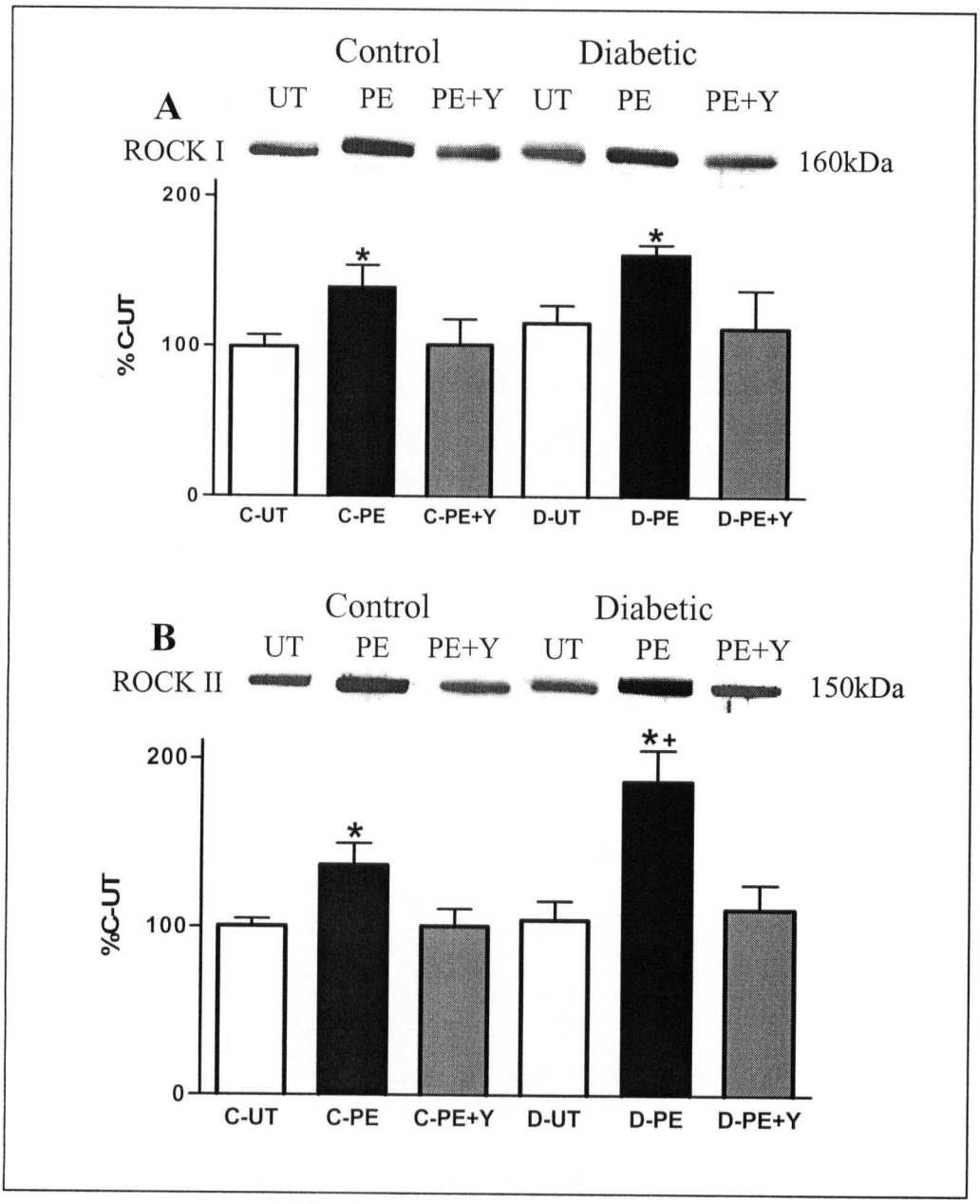


Figure 3.20 Particulate levels of ROCK I (A) and ROCK II (B) in the absence (UT) and presence of PE (n=6) in control (C) and diabetic (D) arteries. Some tissues were treated with the RhoK inhibitor, Y-27632 prior to stimulation with PE. Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.6), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to the corresponding basal group. + $p<0.05$ compared to the corresponding control group (Two-way ANOVA followed by Newman Keuls post-hoc test).

Figure 3.21

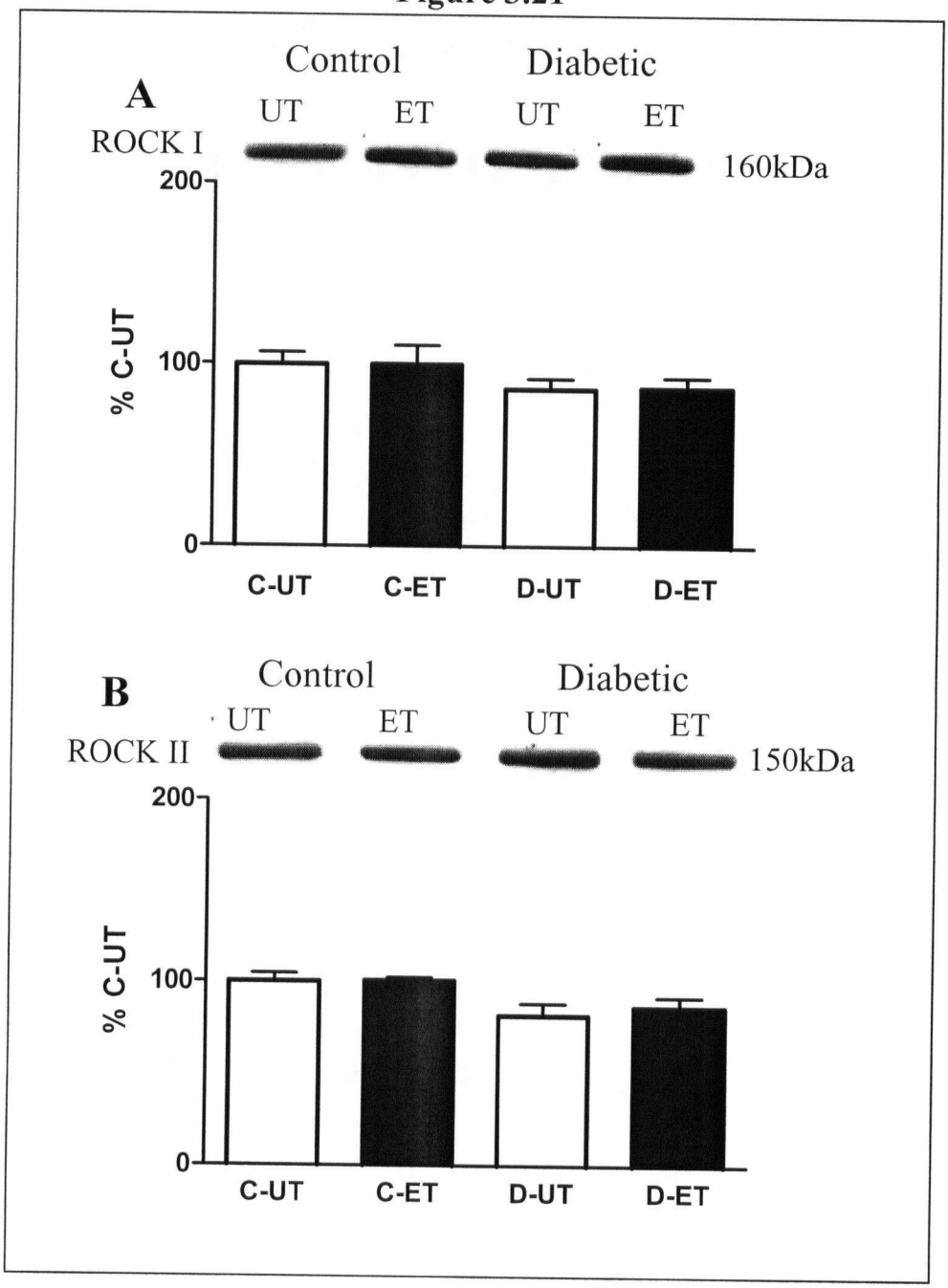


Figure 3.21 Particulate levels of ROCK I (A) and ROCK II (B) in the absence (UT) and presence of ET-1 (n=5) in control (C) and diabetic (D) arteries. Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.6), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins.

Figure 3.22

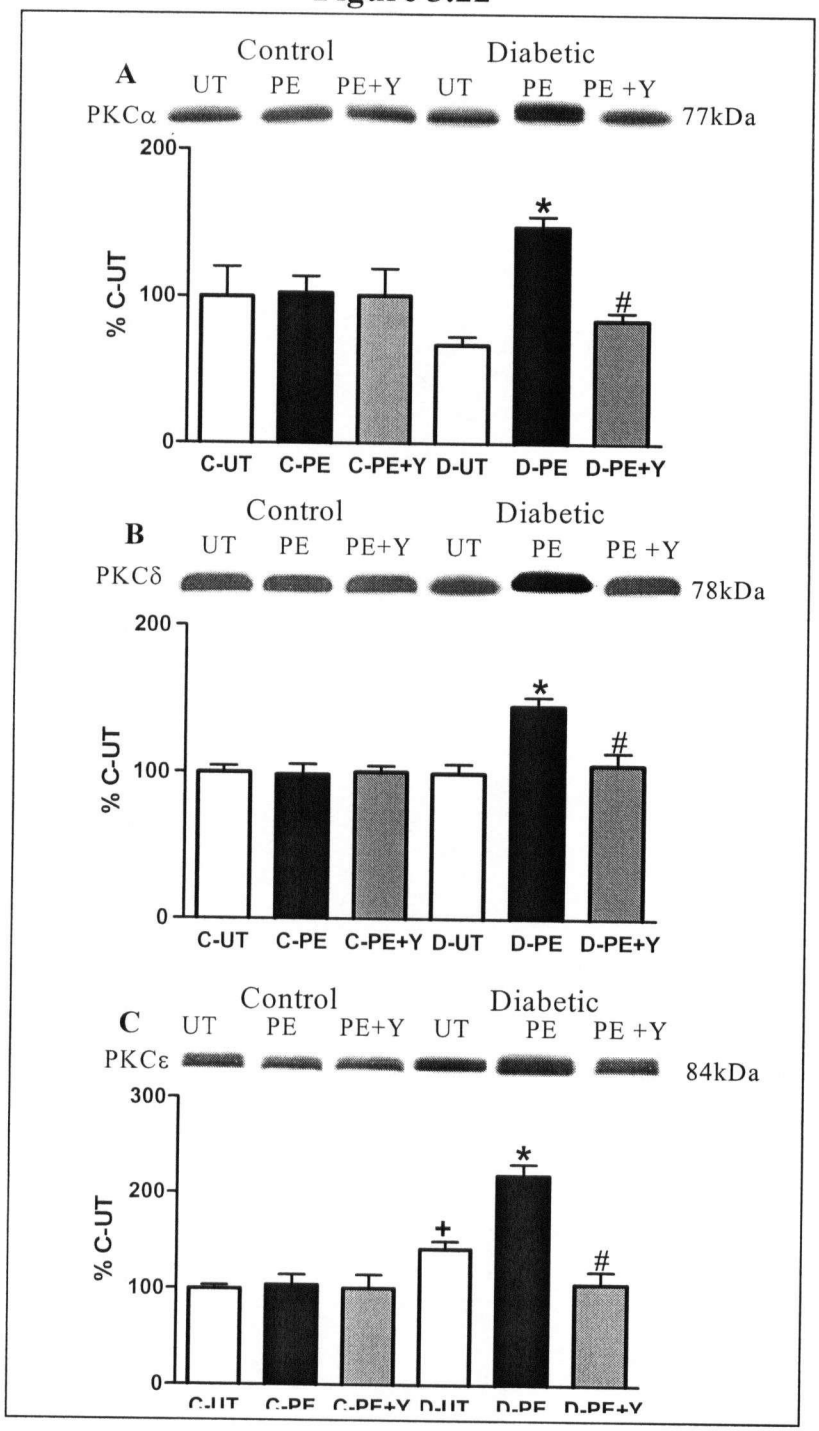


Figure 3.22 Particulate levels of PKCα (A), δ (B) and ε (C) in the absence (UT) and presence of PE (n=5-6) in control (C) and diabetic (D) arteries. Some tissues were treated with the RhoK inhibitor, Y-27632 prior to stimulation with PE. Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.6), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to the corresponding basal group. + $p<0.05$ compared to the corresponding control group. # $p<0.05$ compared to the corresponding PE-treated group (Two-way ANOVA followed by Newman Keul`s post hoc test).

Figure 3.23

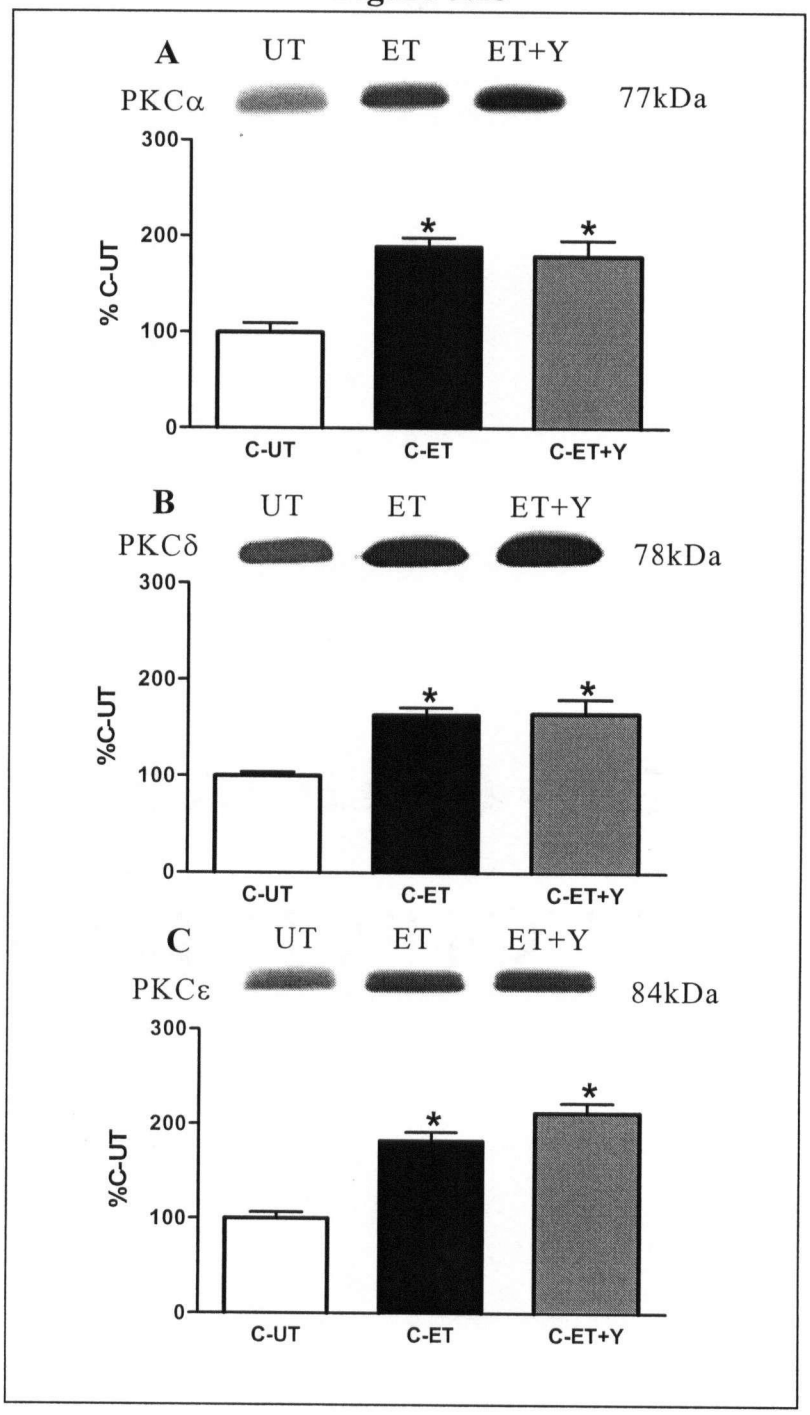


Figure 3.23 Particulate levels of PKCα (A), δ (B) and ε (C) in the absence (UT) and presence of ET-1 (n=5) in control (C) arteries. Some tissues were treated with the RhoK inhibitor, Y-27632 prior to stimulation with ET-1. Arteries were ground in EGTA buffer (for composition see Results - Section 2.2.6), centrifuged and 20 μg of total protein was loaded onto 11% polyacrylamide gels for separation of proteins. * $p<0.05$ compared to the corresponding basal group (One-way ANOVA).

3.4 ROLE OF TKs AND $cPLA_2$ IN ENHANCED CONTRACTILE RESPONSES OF MESENTRIC ARTERIES FROM DIABETIC RATS TO α_1-AR STIMULATION

3.4.1 Effect of TKs and $cPLA_2$ Inhibitors on Contractile Responses to PE and ET-1

Contractile responses to PE in mesenteric arteries from diabetic rats were significantly enhanced as compared to the corresponding controls (Fig. 3.24.B, 3.25.B, 3.26.B and Table 3.4). TK inhibitors, tyrphostin A23 and genistein were used at concentrations at which they had no significant effect on KCl induced contractions (Fig. 3.24.A and 3.25.A). In control arteries, tyrphostin A23 (10μM for 30min.) did not affect the PE response (Fig. 3.24.B). Genistein (10μM for 30min.) significantly shifted the PE concentration response curve without affecting the Rmax (Fig. 3.25.B, Table 3.4). Neither inhibitor had any significant effect on diabetic arteries. The inhibitors were unable to abrogate the significant difference in Rmax between control and diabetic arteries.

The $cPLA_2$ inhibitor, $AACOCF_3$ was used at a concentration at which it did not have a significant effect on the contractile responses to KCl (Fig. 3.26.A). $AACOCF_3$ (10μM for 30min.) did not significantly affect the pD_2 or the Rmax to PE in either control or diabetic arteries (Fig. 3.26.B, Table 3.4). The diabetic Rmax remained significantly greater than the control in the presence of the inhibitor.

The contractile responses to ET-1 were not significantly different between control and diabetic arteries (Fig. 3.27, 3.28, 3.29 and Table 3.4). Tyrphostin A23, genistein and $AACOCF_3$, did not have any significant effect on the ET-1 concentration-response curves in either control or diabetic arteries.

Figure 3.24.A

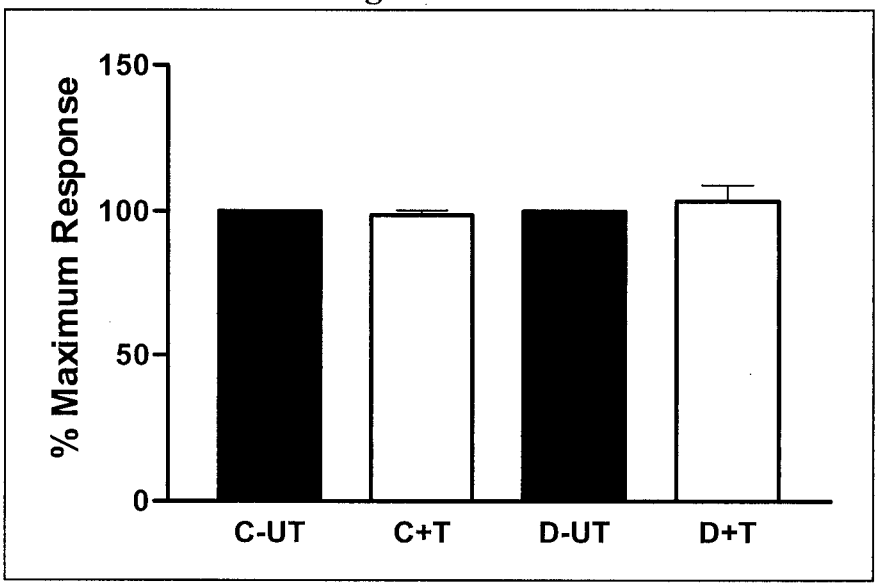


Figure 3.24.B

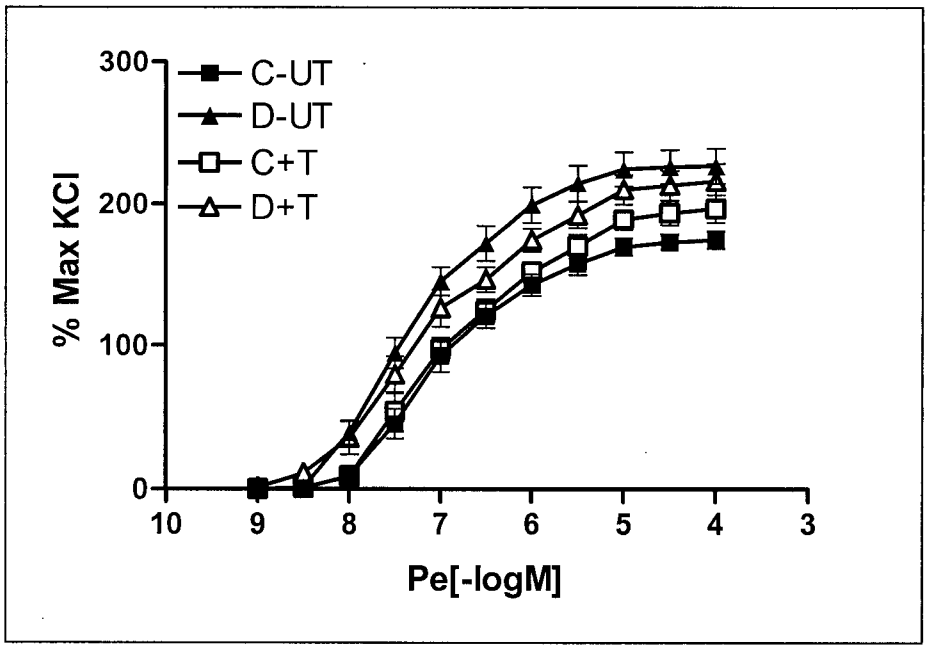


Figure 3.24 Effect of the TK inhibitor, Tyrphostin A23 (T, 10μM for 30min) on maximum KCl contractions (A, n=6) and PE concentration response curves (B, n=6) in control (C) and diabetic (D) mesenteric arteries. The response to KCl was 0.7 ± 0.02g in the control mesenteric arteries.

Figure 3.25.A

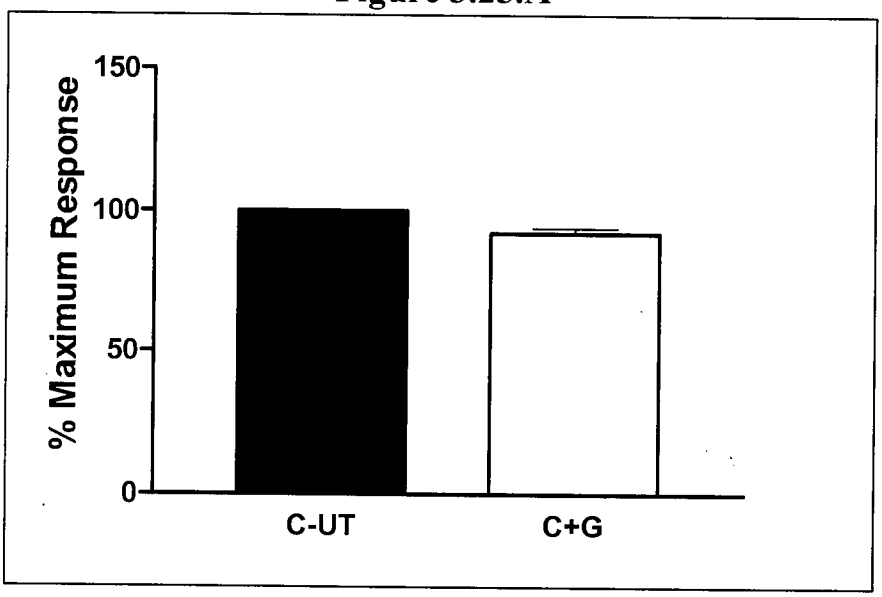


Figure 3.25.B

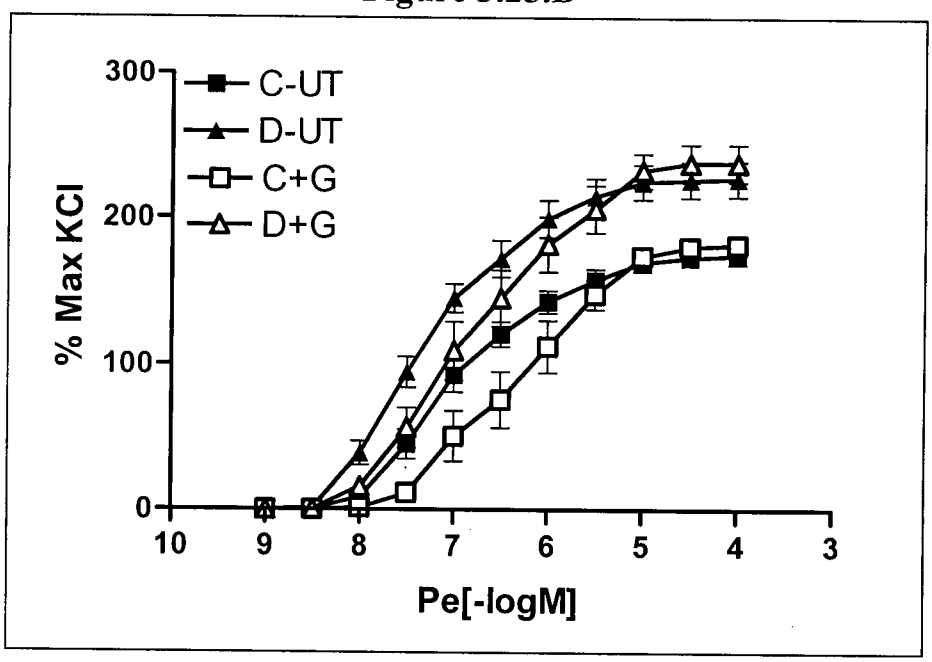


Figure 3.25 Effect of the TK inhibitor genistein (G, 10μM for 30min) on maximum KCl contractions (A, n=4) and PE concentration response curves (B, n=6) in control (C) and diabetic (D) mesenteric arteries. The response to KCl was 1.0 ± 0.02g in the control mesenteric arteries.

Figure 3.26.A

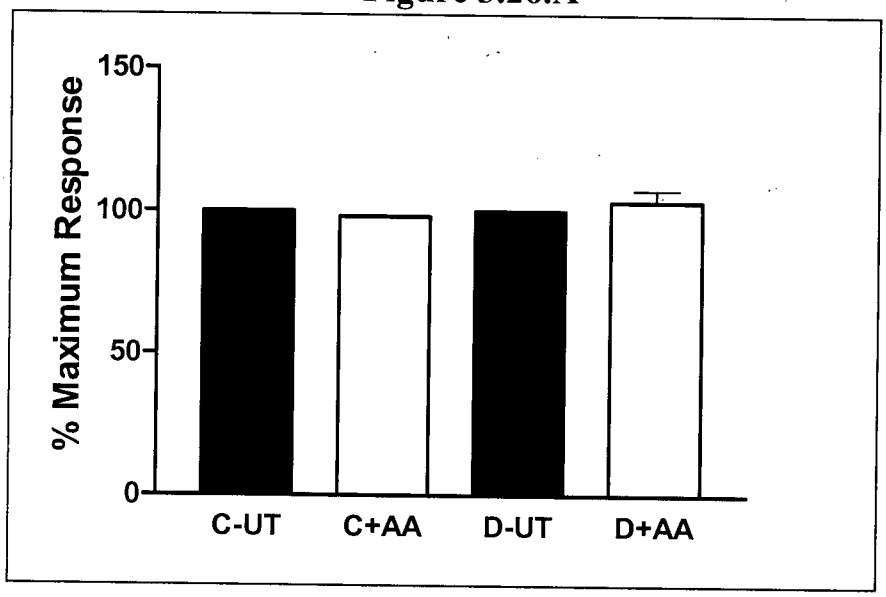


Figure 3.26.B

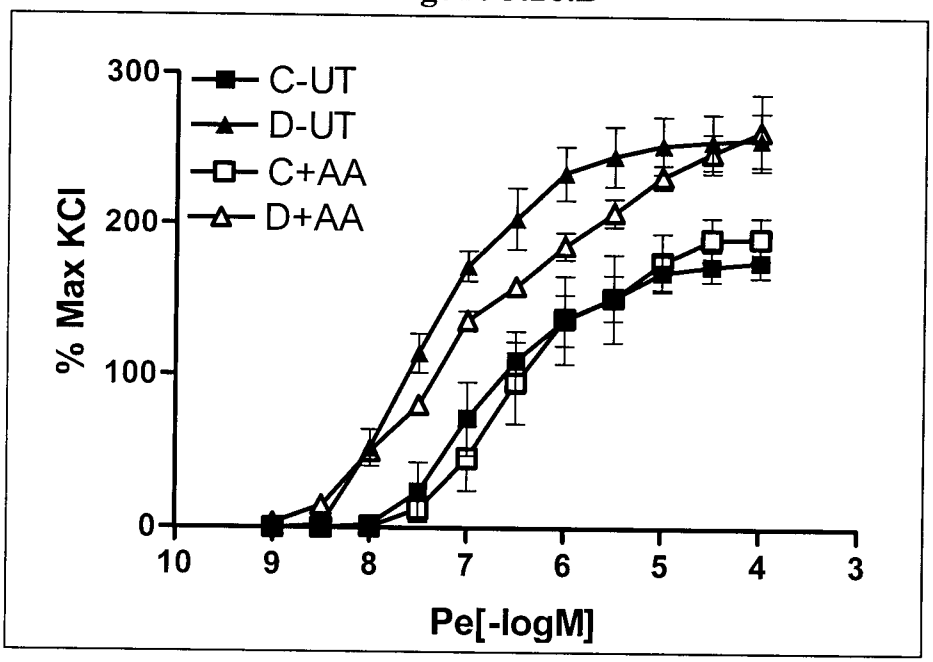


Figure 3.26 Effect of the $cPLA_2$ inhibitor $AACOCF_3$ (AA, 10μM for 30min) on maximum KCl contractions (A, n=3) and PE concentration response curves (B, n=4) in control (C) and diabetic (D) mesenteric arteries. The response to KCl was 0.6 ± 0.02g in the control mesenteric arteries.

Figure 3.27

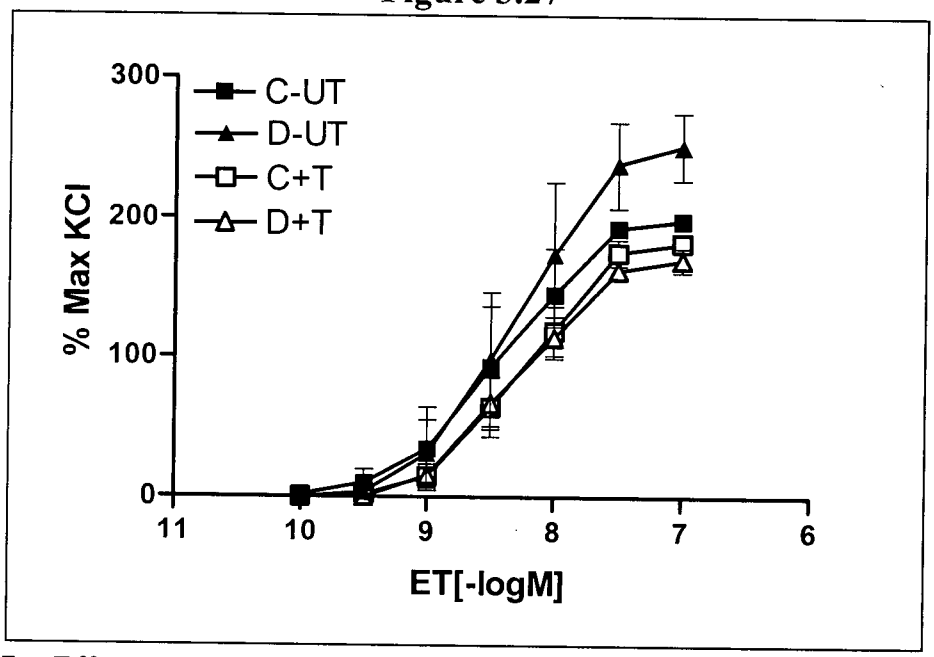


Figure 3.27 Effect of the TK inhibitor Tyrphostin A23 (T, 10μM for 30min) on ET-1 concentration response curves (n=3) in control (C) and diabetic (D) mesenteric arteries.

Figure 3.28

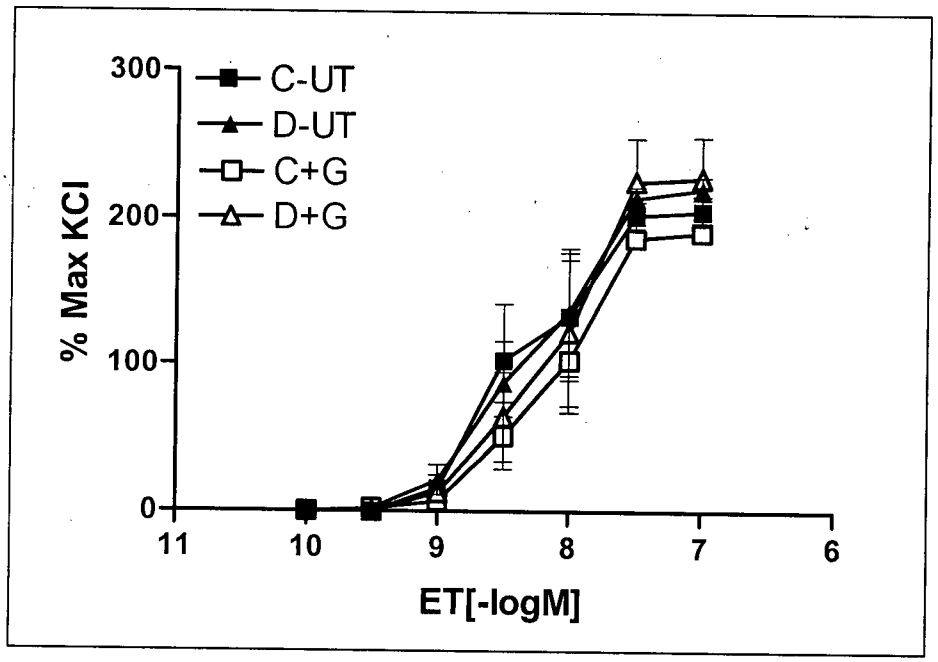


Figure 3.28 Effect of the TK inhibitor genistein (G, 10μM for 30min) on ET-1 concentration response curves (n=3) in control (C) and diabetic (D) mesenteric arteries.

Figure 3.29

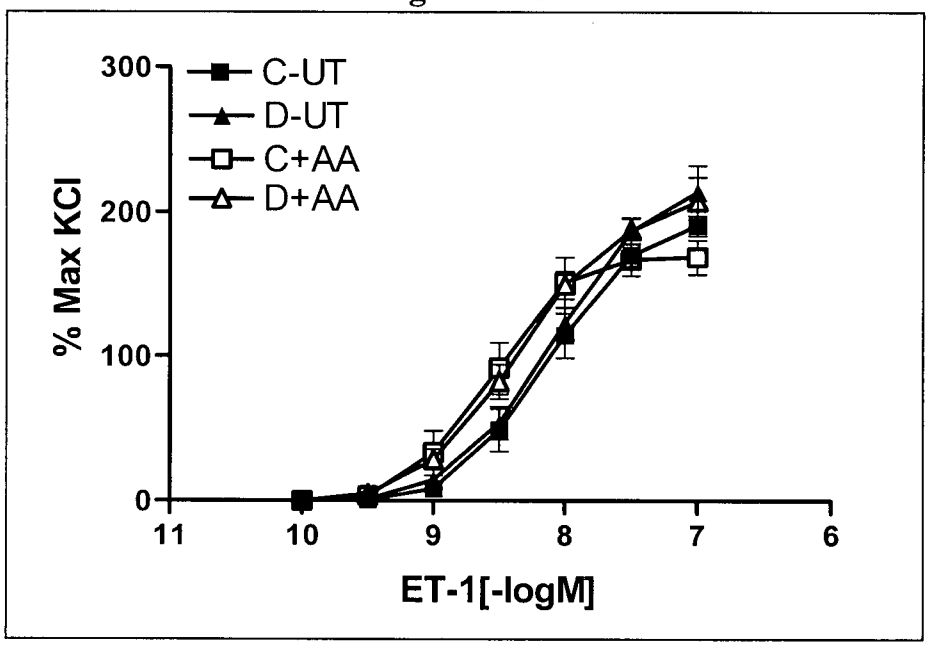


Figure 3.29 Effect of the cPLA$_2$ inhibitor AACOCF$_3$ (AA, 10μM for 30min) on ET-1 concentration response curves (n=4) in control (C) and diabetic (D) mesenteric arteries

TABLE 3.4

Effect of TKs and $cPLA_2$ Inhibitors on PE-Induced Contractions

	CONTROL		DIABETIC	
	Rmax (% Max KCl)	pD_2 (- log EC_{50})	Rmax (% Max KCl)	pD_2 (- log EC_{50})
PHENYLEPHRINE				
Untreated	168.2 ± 5.9	7.0 ± 0.1	218.8 ± 12.2*	7.3 ± 0.1
Genistein	182.0 ± 7.4	6.4 ± 0.2	229.4 ± 10.7*	6.8 ± 0.2
Untreated	168.2 ± 5.9	7.0 ± 0.1	218.8 ± 12.2*	7.3 ± 0.1
Tyrphostin A23	185.5 ± 7.4	7.0 ± 0.1	211.5 ± 6.2*	7.2 ± 0.2
Untreated	170.6 ± 9.7	6.8 ± 0.2	249.7 ± 18.9*	7.4 ± 0.1
$AACOCF_3$	195.0 ± 14.1	6.3 ± 0.3	236.4 ± 14.5*	6.9 ± 0.2
ENDOTHELIN-1				
Untreated	201.2 ± 9.8	8.4 ± 0.2	220.6 ± 9.8	8.3 ± 0.2
Genistein	192.9 ± 8.8	8.2 ± 0.2	230.6 ± 25.5	8.2 ± 0.2
Untreated	235.4 ± 16.3	8.3 ± 0.1	244.9 ± 16.3	7.1 ± 0.1
Tyrphostin A23	215.3 ± 21.9	8.1 ± 0.1	231.4 ± 12.4	8.2 ± 0.1
Untreated	217.5 ± 12.2	8.1 ± 0.2	242.1 ± 26.3	8.1 ± 0.1
$AACOCF_3$	182.2 ± 11.7	8.6 ± 0.1	220.2 ± 16.0	8.3 ± 0.1

* $p<0.05$ compared to corresponding control value

Two–way ANOVA followed by Newman-Keuls post-hoc test.

4. DISCUSSION

4.1 OVERVIEW

Epidemiological and pathological data have shown that diabetes is an independent risk factor for the development of CVD in both men and women (McGill *et al.*, 1998; Wilson *et al.*, 1998). Diabetic women tend lose their inherent protection against development of CVD (Brezinka *et al.*, 1994; Wilson *et al.*, 1998). Vascular diseases, such as coronary, cerebrovascular and peripheral arterial disease, are major causes of disability and death in diabetic patients (Nesto, 2004) and the processes that lead to the changes in the peripheral vascular system in diabetes have been the subject of intense investigation. A better understanding of the underlying mechanisms leading to vascular dysfunction could unmask new strategies to decrease the incidence of cardiovascular morbidity and mortality in diabetic patients.

One of the most common findings in both experimental models of diabetes and human diabetic subjects is functional changes in the sensitivity of the vascular smooth muscle to vasoactive substances (Kamata *et al.*, 1992). Structural and functional deterioration of large and small blood vessels leading to abnormalities in vascular reactivity can contribute significantly to the development of CVD in diabetes (McVeigh *et al.*, 1996). Abnormalities in the diabetic vasculature can compromise blood flow in a number of organs such as the kidney, retina, peripheral arteries and nerves (as reviewed by Koya & King, 1998). Enhanced vascular reactivity in diabetes is observed in the absence of autonomic neuropathy and hypertension (Christlieb *et al.*, 1976; Drury *et al.*, 1984; Tuck *et al.*, 1990), which suggests that it is due to hyperglycemia-associated biochemical changes and can contribute to the eventual development of cardiovascular dysfunction in diabetes (Weidmann *et al.*, 1985a; Weidmann *et al.*, 1985b; Tuck *et al.*, 1990).

The purpose of this study was to characterize the biochemical abnormalities underlying enhanced vasoconstrictor responses of mesenteric arteries from STZ-diabetic rats, a widely used rodent model of Type 1 diabetes. Rats injected with STZ demonstrate many of the features observed in patients suffering from uncontrolled Type 1 diabetes (as reviewed by Tomlinson *et al.*, 1992). Histological studies have shown that STZ-induced toxicity is primarily observed in the pancreatic β-cells and not in any other organs (Junod *et al.*, 1967).

Moreover prolonged exposure of tissues to STZ is unlikely since the serum half-life of the drug is reported to be 15 minutes (Rossini *et al.*, 1977). Most of the abnormalities observed in the cardiovascular system in STZ-diabetic rats are prevented by correction of hyperglycemia with insulin treatment (Fein *et al.*, 1981; Tahilani *et al.*, 1983; MacLeod, 1985; Hopfner *et al.*, 1998). Therefore it can be concluded that the cardiovascular changes observed in the STZ-diabetic rat are due to metabolic derangements such as hyperglycemia, hyperlipidemia and hypoinsulinemia rather than due to a direct effect of STZ.

4.2 CONTRACTILE RESPONSES TO α_1-AR STIMULATION

It has been frequently reported that arteries from Type 1 diabetic animal models exhibit enhanced reactivity to α_1-AR stimulation. Contractile responses to NE have been shown to be significantly elevated in aorta (Nobe *et al.*, 2002) and renal artery (Inazu *et al.*, 1991) from STZ-induced diabetic rats as well as in the tail artery from alloxan-induced rats (Fiol de Cuneo *et al.*, 1988) and carotid artery from alloxan-induced rabbits (Agrawal *et al.*, 1987). Similarly PE- and methoxamine-induced contractions were significantly elevated in STZ-induced diabetic rat aorta (Otter et al., 1994; Zhu *et al.*, 2001) and alloxan-induced rabbit carotid artery (Agrawal *et al.*, 1987). Contractile responses to PE and NE were also significantly elevated in aorta obtained from STZ-induced (Nangle *et al.*, 2003) and alloxan-induced (Abe *et al.*, 2003) diabetic mice respectively. Animal models of Type 2 diabetes have also been shown to exhibit enhanced contractile responses to α_1-AR stimulation. For example PE-induced contractions were significantly elevated in aorta and mesenteric artery of ob/ob mice (a spontaneous mouse model of NIDDM characterized by obesity, hyperglycemia and hyperinsulinemia) (Piercy & Taylor, 1998; Okon *et al.*, 2003) and in cremaster muscle arterioles of Otsuka-Long-Evans-Tokushima fatty (OLETF) rats (a rat model of NIDDM that naturally develops insulin resistance) (Yoshida *et al.*, 2003). Small mesenteric arteries from diabetic (db/db) mice (a model of Type 2 diabetes also) exhibited significantly elevated contractions to PE (Pannirselvam *et al.*, 2003, Pannirselvam *et al.*, 2005).

However not all studies have reported enhanced α_1-AR vasoconstrictor responses in arteries from Type 1 diabetic animal models. There have been reports of depressed contractile responses to NE in aorta (Pfaffman *et al.*, 1982; Head *et al.*, 1987) and methoxamine in perfused mesenteric arterial bed (MAB) (Misurski *et al.*, 2001) obtained from STZ-induced

diabetic rats. Some investigators have reported unchanged vasoconstrictor responses to α_1-AR agonists in diabetic arteries (Beenan *et al.*, 1996; Kam *et al.*, 1996; Torffvit *et al.*, 1997). The discrepancy between the different studies could be due a number of factors such as differences in the species and strains of animals used, the diabetogenic agent used, the duration of diabetes or the presence of endothelium in some studies.

Our lab has reported that aorta, mesenteric and caudal arteries from STZ-diabetic rats exhibit significantly enhanced contractile responses to α_1-AR stimulation as compared to age and gender matched controls. In the present study, maximum contractile responses to NE and PE in mesenteric arteries from STZ-diabetic rats were also significantly enhanced. However increased sensitivity to α_1-AR stimulation (as evidenced by significantly greater pD_2 values) was not always observed in the diabetic mesenteric arteries. This is also consistent with previously published work from our lab as significantly greater NE pD_2 values in diabetic arteries were observed in some studies (Harris & MacLeod, 1988; Weber & MacLeod, 1994) but not in others (Abebe & MacLeod, 1990). Enhanced contractile responses to NE and PE have been reported frequently in the mesenteric vasculature from diabetic rats. Vasoconstrictor responses and sensitivity to NE were significantly enhanced in the MAB (Yousif *et al.*, 2003; Yousif *et al.*, 2004) and mesenteric resistance arteries (3^{rd} branch) (Van Buren *et al.*, 1998) from STZ-diabetic Wistar rats, respectively. Vascular sensitivity to PE was significantly elevated in mesenteric arterioles (3^{rd} and 4^{th} branch) from STZ-diabetic rats (Ishikawa *et al.*, 2004) whereas maximum responses to PE were significantly greater in terminal branches of ileal mesenteric arteries from STZ-induced Sprague Dawley rats (Dresner *et al.*, 1997).

Our lab has shown that contractile responses to NE in mesenteric arteries are predominantly mediated by the α_1-AR (Abebe *et al.*, 1990). This is also true for contractile responses to PE, since prazosin (an α_1-AR antagonist) significantly attenuated these contractions, whereas yohimbine (an α_2-AR antagonist) and timolol (a β-AR antagonist) had no effect on these responses (data not shown).

While α_1-AR-mediated vasoconstrictions have been reported to be increased, decreased or unchanged in isolated arteries from STZ-diabetic rats, many studies have shown that pressor responses to vasoactive agents like NE and methoxamine are depressed *in vivo* (Hebden et al., 1987; Makino & Kamata, 1998). A number of reasons have been suggested to contribute

to this observation. Hyperglycemia-induced impairment of sympathetic function (Maeda *et al.*, 1995a; Maeda *et al.*, 1995b) can reduce the pressor responses to α_1-AR agonists. Makino and Kamata (1998) suggested that depressed pressor levels to methoxamine in STZ-diabetic rats were due to alterations in prostanoid turnover, that is increased production of PGI_2 and decreased formation of TXA_2. A number of studies have shown that the inducible isoform of nitric oxide synthase (iNOS) is activated in diabetic tissues (Smith *et al.*, 1997; Bardell & MacLeod, 2001; Cheng *et al.*, 2004a; Ishikawa *et al.*, 2004) and it could play a significant role in cardiovascular dysfunction in diabetes. Cheng and Pang (2004b) showed that the maximum pressor response and mean circulatory filling pressure (index of body venous tone) in response to NE was significantly attenuated in 3-week STZ-diabetic rats. Selective inhibition of iNOS by 1400W restored arterial and venous constriction in response to NE administration. It was also shown that NE-induced left ventricular contractility *in vivo* was attenuated in 3-week STZ-diabetic rats and treatment with 1400W resulted in partial restoration of the depressed cardiovascular contractile function in response to NE (Cheng *et al.*, 2004a).

4.3 CONTRACTILE RESPONSES TO ET-1

A number of studies have reported that ET-1-induced contractions are enhanced in arteries from Type 1 diabetic animal models. For example, aorta from STZ-induced diabetic rats (Hopfner et al., 1998; Hattori *et al.*, 1999; Murat *et al.*, 1999) and carotid and basilar arteries from alloxan-induced diabetic rabbits (Alabadi *et al.*, 2004; Llorens *et al.*, 2004) exhibited significantly elevated contractile responses to ET-1. Coronary arteries isolated from Sprague-Dawley rats made diabetic by partial pancreatectomy (Tickerhoof *et al.*, 2003) also exhibited enhanced vasoreactivity to ET-1. However in other studies, contractile responses of aorta from STZ-diabetic rats to ET-1 have been reported to be depressed (Fulton *et al.*, 1991; Hodgson & King, 1992; Tada *et al.*, 1994; Utkan *et al.*, 1998). In one study vasoconstriction to ET-1 was unchanged in perfused hindquarters of STZ-diabetic rats (James & Hodgson, 1995). Reasons for these variable results are not completely understood but could be due to the differences in the methodologies used to measure vasocontractile responses. It has also been shown that there are regional differences in vascular responses to ET-1 (Gardiner *et al.*, 1989), which could lead to variable data in vessels isolated from different vascular beds.

In the mesenteric vasculature from diabetic rats there have been reports of enhanced (Yousif *et al.*, 2003; Yousif *et al.*, 2004) as well as depressed (Makino & Kamata, 2000a; Misurski *et al.*, 2001) contractile responses to ET-1. In our studies, contractile responses to ET-1 (both Rmax and pD_2) were not significantly different in mesenteric arteries from control and diabetic animals, which is consistent with another study reporting similar results (Arikawa *et al.*, 2001). One explanation for the variability between our study and those quoted above could be the preparation used, as we conducted our experiments on the superior mesenteric artery while the perfused MAB was used by the other investigators.

4.4 BASAL LEVELS OF PKC ISOFORMS

Increased levels and activity of specific isoforms of PKC occur in the cardiovascular system in diabetes, and evidence suggests that this may contribute to the cardiovascular dysfunction associated with this condition (as reviewed by Meier & King, 2000; Way *et al.*, 2001). The isoform that has most frequently been found to be altered in the vasculature is $PKC\beta_2$. For instance, an increase in total PKC activity was found to be associated with increased particulate levels of $PKC\beta_2$ but not $PKC\alpha$ in aorta from STZ-diabetic rats (Inoguchi *et al.*, 1992). Increased total levels of $PKC\beta_2$ were reported in coronary arteries (Tickerhoof *et al.*, 2003) and in femoral and small mesenteric arteries (Wigg *et al.*, 2004) from diabetic rats. There was significant upregulation of $PKC\beta_2$ at the transcriptional and translational levels in aorta from STZ-diabetic pigs (Guo *et al.*, 2003). Consistent with these studies, we detected a two-fold increase in total $PKC\beta_2$ levels in superior mesenteric arteries from diabetic rats. There was also a smaller but significant increase in total $PKC\varepsilon$ levels and particulate levels of this isoform were also enhanced in diabetic tissues. However, there was no change in expression of $PKC\alpha$ or δ in the diabetic arteries. This contrasts with a recent report of increased expression of $PKC\alpha$ and β_2, but not $PKC\varepsilon$, in diabetic coronary arteries (Tickerhoof *et al.*, 2003). Whether this reflects a difference due to the longer duration of diabetes in the present study, or to differences between arteries is not clear.

The significant involvement of $PKC\beta_2$ in hyperglycemia-mediated cardiovascular dysfunction was strongly suggested by experiments using LY333531, an inhibitor specific for $PKC\beta$ (Jirousek *et al.*, 1996). Enhanced $PKC\beta_2$ activation was shown to contribute to diabetes-induced cardiomyopathy, as treatment with LY333531 significantly improved

functional and morphological abnormalities associated with cardiomyopathy in diabetic mice (Wakasaki *et al.*, 1997; Takeishi *et al.*, 1998). Renal function and abnormalities in the retinal circulation in diabetic rats were also ameliorated by treatment with LY333531 (Ishii *et al.*, 1996). Treatment with LY333531 prevented impairment of endothelium-dependent vasodilatation caused by experimental hyperglycemia in intact, healthy humans (Beckman *et al.*, 2002). Similarly in STZ-induced diabetic rats, LY333531 produced significant improvement in abnormalities associated with neuronal function, neural tissue perfusion and endothelium-dependent vasodilatation (Cotter *et al.*, 2002). In the latter study PE-induced contractile responses were not enhanced in the MAB and LY333531 treatment had no effect on contractility. But in another study, significantly greater sensitivity to PE in aorta from STZ-diabetic rats was normalized following treatment of rats with LY333531 (Nangle *et al.*, 2003).

Similar to the results of our study, PKCε levels have previously been shown to be increased in particulate fractions of diabetic tissues such as the rat kidney (Kang *et al.*, 1999), glomeruli (Babazono *et al.*, 1998) mesangial cells (Shiba *et al.*, 1993) and cardiomyocytes (Malhotra *et al.*, 1997), suggesting enhanced activation of this isoform. However the precise role of this isoform in hyperglycemia-induced cardiovascular dysfunction is not clearly understood. PKCε has been shown to participate in hyperglycemia-induced enhancement of endothelial cell permeability (Hempel *et al.*, 1997). In addition, Malhotra et al (1997) suggested that activation of PKCε might participate in the cardiac dysfunction seen in chronic diabetes.

A number of mechanisms have been proposed to lead to hyperglycemia-induced PKC activation. The *de novo* synthesis of DAG is significantly enhanced in diabetes as a result of which glucose is directly metabolized to DAG (Inoguchi *et al.*, 1994) (discussed in Introduction, section 1.10). In addition to this, it has been suggested that accumulation of AGEs leading to activation of AGE receptors can lead to activation of PKC (Li *et al.*, 1996). STZ-diabetic rats were shown to exhibit enhanced PKC activation in the retina, glomeruli and mesenteric artery after 8 and 24 weeks of induction of diabetes (Osicka *et al.*, 2001). Aminoguanidine, an agent that leads to inhibition of AGEs, partially prevented activation of PKC in the retina and completely inhibited PKC activation in the mesenteric artery from 8-week diabetic rats. In 24-week STZ-diabetic rats, it completely prevented PKC activation in

the retina, glomeruli and mesenteric artery. These effects were observed in the absence of any significant effects on blood glucose levels (Osicka *et al.*, 2001). Similarly AGEs were shown to lead to enhanced activation of PKCα in cultured human mesangial cells leading to increased collagen mRNA expression (Kim *et al.*, 2001). These changes were inhibited by treatment of the cells with aminoguanidine.

Increased activation of PKC has also been suggested to arise from AG II-induced activation of AT_1 receptors (Malhotra *et al.*, 1997). Mesenteric arteries from STZ-diabetic rats were shown to exhibit significantly elevated levels of angiotensin-converting enzyme (ACE), suggesting that the activity of renin-angiotensin system and tissue levels of AG II were increased (Cooper *et al.*, 1994). Ramipril, an ACE inhibitor, prevented increases in PKC activation in the retina, glomeruli and mesenteric artery in 8 and 24-week STZ-diabetic rats (Osicka *et al.*, 2001). Treatment of STZ-diabetic rats with L-158,809, an AG II receptor antagonist, prevented translocation of PKCε in cardiomyocytes (Malhotra *et al.*, 1997).

4.5 BASAL LEVELS OF RhoK ISOFORMS

Dysregulation of RhoA/RhoK expression and/or activity has been shown to participate in vascular pathophysiologies. For example, RhoA activity was significantly increased in a number of different models of hypertension, such as stroke-prone spontaneously hypertensive rats (SHR), renal hypertensive rats and deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Seasholtz *et al.*, 2001; Seko *et al.*, 2003; Moriki *et al.*, 2004).

We found no evidence for increased expression of RhoK isoforms in diabetic mesenteric arteries. However, increased expression and/or activity of components of the RhoA/RhoK pathway have been reported in other vascular tissues from Type I and II diabetic animals. For example, upregulation of basal RhoA expression and activity was found in basilar arteries from STZ-diabetic rats (Miao *et al.*, 2002a). Increased expression of RhoA (membrane) and ROCK II (cytosol) in the corporal tissue of STZ-diabetic Sprague-Dawley rats was suggested to be the reason for erectile dysfunction in this model (Bivalacqua *et al.*, 2004). Over expression of ROCK I was proposed to mediate increased contractility to ET-1 in corpus cavernosum smooth muscle from chronic diabetic rabbits (Chang *et al.*, 2003). In aortic smooth muscle cells isolated from Goto-Kakizaki rats (a highly inbred strain of Wistar rats that spontaneously develop Type II diabetes), the basal RhoA activity and membrane levels were significantly greater when compared to the corresponding controls (Sandu *et al.*, 2000).

4.6 PARTICIPATION OF PKC IN α_1-AR AND ET-1 RECEPTOR STIMULATION

Selectivity of PKC Inhibitors

Although there are a large number of inhibitory compounds for PKC, there are very few that exhibit selectivity for PKC alone or for individual PKC isoforms. Inhibitors of PKC are broadly classified into groups according to their site of interaction with the PKC molecule (Way *et al.*, 2000):

1) Inhibitors of the regulatory domain, that target the phospholipid or DAG-binding site
2) Inhibitors of the catalytic domain, that target the substrate binding or ATP-binding site.

Calphostin C is a non-isoform selective PKC inhibitor that acts by inhibiting the regulatory domain of the PKC molecule, competing at the binding site for diacylglycerol and phorbol esters (Kobayashi *et al.*, 1989). Ro-318220 and rottlerin on the other hand inhibit PKC by acting on the catalytic domain, competing at the ATP binding site. Ro-318220, a non-isoform selective PKC inhibitor, belongs to the family of bisindolylmaleimides derived from the microbial alkaloid staurosporine (Wilkinson *et al.*, 1993). Although less potent than the parent compound, Ro-318220 shows more selectivity for PKC compared to other kinases. Rottlerin (mallotoxin) is a naturally occurring product derived from *Mallotus philippinesis.* It inhibited the activity of PKCδ with an IC_{50} of 3μM while the other isoforms were at least one order of magnitude less sensitive to this inhibitor (Gschwendt *et al.*, 1994). The IC_{50} of the different PKC isoforms increased in the following order $\delta > \alpha, \beta, \gamma > \eta, \zeta, \varepsilon$ (Gschwendt *et al.*, 1994). The selectivity of rottlerin for PKCδ has been controversial. Davies et al (2000) found that even at a concentration of 20μM rottlerin had no inhibitory effect on activity of PKCα and δ. Nonetheless, rottlerin has continually been used as a PKC inhibitor with relative selectivity for the δ isoform (De Witt *et al.*, 2001; Nishizawa *et al.*, 2003; Jin *et al.*, 2004). De Witt et al (2001) showed by immunohistochemical analysis that PMA- and AG II-induced localization of PKCδ in feline pulmonary arterial smooth muscle cells was decreased by treatment with rottlerin. Nishizawa et al (2001) showed by Western blotting that PKCδ and α were translocated to the particulate fraction in canine basilar arteries on development

of angiographic vasospasm, and that the translocation of only PKCδ was inhibited by rottlerin. Calphostin C, Ro-318220 and rottlerin have been frequently used to investigate the role of PKC in contractile processes in vascular smooth muscle (Dallas & Khalil, 2003; Nishikawa *et al.*, 2003; Tan *et al.*, 2004).

In order to establish selectivity, the effects of various concentrations of the PKC inhibitors on KCl induced contractions were investigated at in control and diabetic mesenteric arteries. Ro-318220 and calphostin C, used at a concentration of 3μM, had no significant effect on the contractile responses to K^+-depolarization, suggesting that their inhibitory effects were not due to nonspecific actions. However, at a concentration of 10μM, rottlerin produced a small but significant attenuation of KCl-induced contractions in mesenteric arteries from control, not diabetic, rats suggesting that some of its actions might arise from non-specific inhibition of the contractile process. At the same time, Ro-318220 abolished the contractile response of mesenteric arteries to PdBu, suggesting that it is effectively blocking the activation of PKC.

Pharmacological Inhibition and Translocation of PKC in Response to α_1-AR Stimulation

Treatment of mesenteric arteries from control rats with Ro-318220 and calphostin C produced a small decrease in the NE pD_2 values but had no effect on the Rmax to this agonist. However, rottlerin had no effect on NE responses. NE produced only a small translocation of PKCε, with no change in PKCα or δ. The non-isoform selective PKC inhibitors had similar effects on PE-induced contractions in control arteries. However, like Ro-318220 and calphostin C, rottlerin also produced a small decrease in the sensitivity to PE. Unlike NE, PE produced no detectable translocation of any PKC isoform. The fact that the PKC inhibitors did not have a prominent effect on contractile responses to α_1-AR agonists, and that agonist-induced translocation of PKC isoforms was not very pronounced suggests that PKC does not play an important role in α_1-AR mediated vasoconstriction in mesenteric arteries from control rats.

Our results are consistent with other studies in which the PKC inhibitors did not significantly affect PE or NE-induced contractions in mesenteric arteries (Sasaki *et al.*, 1998; Fetscher *et al.*, 2001). However at the same time other studies conducted on the mesenteric vasculature have implicated PKC in Ca^{2+} sensitization and contraction in response to α_1-AR stimulation

(Buus *et al.*, 1998; Shirasawa *et al.*, 2003). The reasons for the discrepancy between these studies is not clear but could be due differences in the selectivity of the PKC inhibitors used, differences in experimental animals or in the methodologies used to measure vasocontractile responses.

While PKC may not contribute to a large extent to the contractile responses of control mesenteric arteries to NE or PE, it appears to mediate the enhanced contractile responses of diabetic mesenteric arteries to α_1-AR stimulation. Both Ro-318220 and calphostin C produced greater inhibition of NE-induced contractions in diabetic than control arteries, and normalized the significant difference in Rmax values found in the absence of antagonist. However, treatment of diabetic arteries with rottlerin produced only a small decrease in NE pD_2 values and the Rmax continued to be significantly greater than the control arteries in the presence of the inhibitor. The enhanced contractile responses to NE were associated with translocation of PKCα and ε (greater than in mesenteric arteries from control rats), suggesting that the enhanced NE-induced contractile responses may be mediated by increased activation of these isoforms. Consistent with the functional data obtained with rottlerin, there was no detectable translocation of PKCδ in response to NE in diabetic arteries.

As was the case with NE, Ro-318220 and calphostin C were able to normalize the significantly greater PE-induced contractions in diabetic arteries. However there was a difference in the effects of rottlerin with respect to the α_1-AR agonists in diabetic mesenteric arteries. Rottlerin normalized contractile responses to PE in diabetic tissues and abolished the significant difference in Rmax existing prior to inhibitor treatment. The enhanced contractile responses to PE were associated with translocation of PKCα, δ and ε. The significant translocation of PKCδ in response to PE is consistent with the effect of rottlerin on contractile responses to this agonist.

The greater inhibitory effect of rottlerin on PE-induced contractions and the significant translocation of PKCδ in response to PE suggests that PKCδ may play a more prominent role in PE-, than in NE-induced contractions in diabetic arteries. The reasons for this difference are not clear. It is possible that there are differences in the α_1-AR subtype activated by PE or NE as a result of which the signal transduction pathways activated vary between the agonists. It has also been suggested that the magnitude and efficiency of pharmacomechanical

coupling varies between different agonists (Somlyo & Somlyo, 1968). The relative importance of PKCδ in PE and NE-induced contractile responses in diabetic mesenteric arteries requires further investigation.

There have been some studies investigating the role of PKC in α_1-AR-mediated responses in diabetic tissues. For example, in one study significantly elevated NE responses in aorta from STZ-induced diabetic rats were normalized following treatment with staurosporine (Kawasaki, 1997). Normalization of significantly elevated α_1-AR responses in mesenteric arteries from diabetic rats by pharmacological inhibition of PKC has been previously reported by our lab (Abebe & MacLeod, 1990) and has been confirmed in this study. To the best of our knowledge, this is the first study that demonstrates enhanced α_1-AR-mediated translocation of specific PKC isoforms in diabetic tissues. The elevated contractile response to α_1-AR agonists in diabetic arteries may be mediated by increased activation of one or more of these isoforms. This may result from increased production of DAG as PIP_2 breakdown was shown to be enhanced in response to a maximum concentration of NE in mesenteric arteries from diabetic rats (Abebe & MacLeod, 1991a; Abebe & MacLeod, 1991b).

Pharmacological Inhibition and Translocation of PKC in Response to ET-1 Receptor Stimulation

The PKC inhibitors produced a much greater attenuation of ET-1 than PE or NE-induced contractions in mesenteric arteries from control rats. Ro-318220 and calphostin C reduced the ET-1 Rmax by more than 50% while rottlerin decreased it by 33%. The significant inhibition produced by the PKC inhibitors together with the much greater translocation of all three PKC isoforms by ET-1 suggests that under normal circumstances, the ET-1 signaling pathway may be more effectively coupled to PKC than the α_1-AR pathway.

In contrast to the α_1-AR agonists, there was no evidence for an enhancement of the contractile response to ET-1 in diabetic mesenteric arteries, nor was there any indication that the contractile response of diabetic mesenteric arteries to ET-1 was more dependent on PKC than that of control arteries, since both Ro-318220 and calphostin C produced a similar magnitude of block of contractile responses to ET-1 in control and diabetic mesenteric arteries, and ET-1 produced similar translocation of PKCα and ε. PKCδ translocation was

actually lower in response to ET-1 in diabetic than in control arteries, although the reasons for this are not clear at this point.

The results of our study are in contrast to previous reports in aorta (Hattori *et al.*, 1995; Kawasaki, 1997) and coronary (Tickerhoof *et al.*, 2003) arteries of diabetic rats, which indicated that enhanced vasoconstrictor responsiveness to ET-1 could be blocked by inhibition of PKC. This could be due to the development of tissue-specific changes in reactivity to ET-1 during diabetes, as a result of which different diabetic vascular tissues respond variably to ET-1-induced vasoconstrictor actions (Gardiner *et al.*, 1989).

Differences in the effects of diabetes on contractile responses to stimulation of GPCRs in different arteries have previously been reported. For example, contractile responses to U-46619 and ET-1 were unchanged in the perfused hindquarters of 6-week STZ-diabetic rats whereas 5-HT-induced contractions were attenuated (James & Hodgson, 1995). Similarly in mesenteric resistance vessels from 40-week STZ-diabetic rats sensitivity to NE was significantly enhanced but contractile responses to serotonin and vasopressin were unchanged (Van Buren *et al.*, 1998).

4.7 PHOSPHORYLATION OF CPI-17

PKC mediated phosphorylation of CPI-17 has been shown to participate in contractile responses to GPCR agonists such as PE (Hayashi *et al.*, 2001), histamine (Eto *et al.*, 2001) and ET-1 (Kitazawa *et al.*, 2003). Evidence suggests that CPI-17 is the most important downstream mediator of the Ca^{2+} sensitizing effects of PKC (as reviewed by Somlyo & Somlyo, 2000). For instance, it was shown that in vascular smooth muscle, demembranation with Triton-X-100 resulted in significant depletion of PKC and CPI-17, with a corresponding loss in Ca^{2+} sensitization of contraction and MLC phosphorylation in response to PKC activators (Kitazawa *et al.*, 1999). This was restored by addition of exogenous PKC and CPI-17 and not by the former alone. CaP, CaD, myosin and MLC were shown to be much less efficient substrates for PKC when compared to CPI-17. The importance of CPI-17 for G-protein/PKC-mediated Ca^{2+} sensitization in vascular smooth muscle was also established by another elegant study conducted by Kitazawa and colleagues (Kitazawa *et al.*, 2004). In this study they showed that smooth muscle (such as artery, gizzard and small intestine) from chickens was deficient in CPI-17, and consequently contraction and Ca^{2+} sensitization in response to GTPγS, PdBu, histamine, PE and ET-1 were significantly attenuated when

compared to `CPI-17-rich` tissues from rabbits and pigeons. Addition of recombinant CPI-17 to permeabilized chicken smooth muscle resulted in restoration of PdBu-and GTPγS-induced Ca^{2+} sensitization.

There is not a lot of information about how the expression and/or activation of CPI-17 vary with disease states associated with vascular dysfunction, such as diabetes. However in one study the role of CPI-17 in gastrointestinal (GI) motility disorders such as inflammatory bowel disease, Crohn`s disease and ulcerative colitis was investigated (Ohama *et al.*, 2003). In this study, long-term treatment of ileal muscle from Wistar rats with interleukin-1β (a pro-inflammatory cytokine that plays an integral role in GIT disorders) resulted in attenuated agonist-induced contraction and this was associated with a decrease in total levels and phosphorylation of CPI-17.

In the present study, there was no detectable increase in phosphorylation of CPI-17 in response to stimulation with NE in mesenteric arteries from control rats, which is consistent with a minor role for PKC in α_1-AR-mediated responses. However in contrast, treatment of diabetic arteries with NE produced an almost three-fold increase in CPI-17 phosphorylation and this increase was blocked by the same concentration of calphostin C (3 μM) that normalized the contractile response of diabetic mesenteric arteries to NE. These data suggest that the significantly greater contractile responses to NE in diabetic arteries arise from PKC-dependent phosphorylation and activation of CPI-17. While all PKC isoforms have been shown to bind to CPI-17 *in vitro*, PKCα, δ and ε have the greatest ability to phosphorylate and activate it (Eto *et al.*, 2001; Zemlickova *et al.*, 2004). Although RhoK has also been shown to phosphorylate and activate CPI-17 (Koyama *et al.*, 2000), it seems unlikely that it is doing so in mesenteric arteries from control or diabetic rats. This is because in spite of evidence of participation of RhoK in α_1-AR-mediated responses in both control and diabetic mesenteric arteries, there was no detectable phosphorylation of CPI-17 in control arteries and calphostin C treatment completely blocked CPI-17 phosphorylation in diabetic tissues.

It has not been experimentally demonstrated how activated PKC in the membrane fraction can phosphorylate CPI-17, which is located in the cytosol. Eto et al (2001) suggested that CPI-17 lies close to the plasma membrane where activated PKC is present and can readily phosphorylate it. Once phosphorylated, CPI-17 can diffuse to the myofilaments to inhibit MLCP. A similar idea was put forward by Ruegg (1999) who suggested that CPI-17 shuttles

between membrane-bound active PKC and MLCP. It is also possible that activated PKC in the membrane may be in rapid equilibrium with cytosolic PKC. In the cytosolic compartment the quantity of activated PKC may be low but could still be sufficient to cause CPI-17 phosphorylation (Ruegg, 1999).

4.8 PARTICIPATION OF RhoK IN α_1-AR AND ET-1 RECEPTOR STIMULATION

Selectivity of RhoK Inhibitors

Most of the RhoK inhibitors that are used in cardiovascular studies, such as Y-27632, H-1152 and HA-1077 act by inhibiting the ATP-binding site in the catalytic domain of the RhoK molecule (Takami *et al.*, 2004). There are some agents that act by inhibiting the substrate-binding site in the catalytic domain but they are usually characterized by a high degree of hydrophilicity (Takami *et al.*, 2004). The ATP-competitive agents on the other hand are less hydrophilic and accordingly exhibit good membrane permeability and absorption. Introduced by Narumiya and colleagues, Y-27632 (K_i = 0.2 – 0.3 μM) selectively inhibits both isoforms of RhoK approximately 200 times more potently than cPKCs and PKA and 2000 times more potently than MLCK (Uehata *et al.*, 1997; Ishizaki *et al.*, 2000). H-1152 is a novel RhoK inhibitor (K_i = 0.0016 μM) (Sasaki *et al.*, 2002) that has been shown to have a better inhibitory profile than its parent compound HA-1077 and to be approximately 10 times more potent than Y-27632 at inhibiting RhoK (Shum *et al.*, 2003). H-1152 inhibits RhoK approximately 400 times more potently than PKA, and 6000 times more potently than cPKCs and MLCK (Sasaki *et al.*, 2002).

Y-27632 and H-1152, used at concentrations of 1μM and 0.1μM respectively, had no effect on contractile responses of mesenteric arteries to K^+- depolarization, suggesting that their effects were not due to non-specific actions on smooth muscle. Two independent studies have reported the nPKCs, δ (Eto *et al.*, 2001) and ε (Uehata *et al.*, 1997) to be sensitive to inhibition by Y-27632. We therefore investigated the effect of this inhibitor on direct activation of PKC with PdBu and found that the concentration of Y-27632 used in this investigation had no significant effect on contractile responses of control or diabetic mesenteric arteries to PdBu.

Pharmacological Inhibition and Translocation of RhoK

The significant shift in the PE concentration-response curve produced by both the RhoK inhibitors in mesenteric arteries from control rats is consistent with a role for this pathway in contractile responses to α_1-AR stimulation in this tissue. This is supported by the observation that PE increased levels of both ROCK I and II in the particulate fraction, and this was abolished by Y-27632. Other studies conducted on the rat mesenteric vasculature have also implicated RhoK in Ca^{2+} sensitization and contraction in response to α_1-AR stimulation (VanBavel *et al.*, 2001; Altmann *et al.*, 2003). However in the rabbit mesenteric artery, contractile responses to PE were unaffected by inhibition of RhoA by C3 exoenzyme (Sasaki *et al.*, 1998). This suggests that coupling of the RhoA/RhoK pathway to α_1-AR-mediated Ca^{2+} sensitization mechanisms could differ depending on the species being investigated.

The role of the RhoA/RhoK pathway in abnormal vascular reactivity in Type 2 DM has been investigated in a few studies. In vascular smooth muscle cells isolated from diabetic Goto-Kakizaki rats, thrombin increased RhoK activity and this was associated with an increase in MYPT phosphorylation and inhibition of MLCP (Begum *et al.*, 2000). This was proposed to be partly due to the loss of insulin-mediated inhibition of RhoK. Very recently it was reported that RhoK mediated endothelial dysfunction in cerebral arterioles isolated from db/db mice (a genetic model of Type 2 DM) by enhancing basal superoxide levels, probably via inhibition of nitric oxide synthase (Didion *et al.*, 2005). However whether the RhoA/RhoK pathway also contributes to abnormal vasoconstrictor responses in animal models of Type 1 DM has not yet been investigated.

Our results show that the RhoK inhibitors, like the PKC inhibitors, produced a greater attenuation of PE responses in mesenteric arteries from diabetic rats and eliminated the significant difference in contractile responses of control and diabetic arteries to PE. In addition to significant translocation of ROCK I, there is greater translocation of ROCK II in response to PE in diabetic than in control arteries, suggesting that increased activation of RhoK contributes to the enhanced contractile response of diabetic arteries to α_1-AR stimulation.

The RhoA/RhoK pathway has been implicated in ET-1-induced Ca^{2+} sensitization and contraction in vascular smooth muscle such as the rabbit aorta (Sakurada *et al.*, 2001) and basilar artery (Miao *et al.*, 2002b), human mammary artery (Batchelor *et al.*, 2001) and gerbil spiral modiolar artery (Scherer *et al.*, 2002). While there is not a lot of information regarding

the role of this pathway in abnormal vasoconstrictor responses to ET-1 in diabetes, in one study significantly elevated ET-1-induced vasoconstriction in corpus cavernosum smooth muscle from alloxan-induced diabetic rabbits was suggested to be mediated by RhoK, specifically ROCK I (Chang *et al.*, 2003). However the results of this study were based on pharmacological inhibition of RhoK by Y-27632 and measurement of total ROCK I expression, while ET-1-induced activation or translocation of ROCK I were not determined.

In the present investigation no participation of RhoK in contractile responses of control and diabetic mesenteric arteries to ET-1 was found, since concentrations of the antagonists that significantly blocked the PE response had no effect on responses to ET-1. The lack of effect of RhoK inhibitors on ET-1-induced contraction is consistent with the lack of detectable translocation of RhoK isoforms following stimulation with this agonist in both control and diabetic arteries. Our results are similar to a recent report showing that ET-1-induced vasoconstriction in the rat mesenteric artery bed was resistant to RhoK inhibition by Y-27632 and HA-1077 (Buyukafsar *et al.*, 2004). In another study, sub-threshold concentrations of ET-1 induced potentiation of NE responses in perfused rat mesenteric arteries and RhoK was suggested to participate in this process (Matsumura *et al.*, 2001). However, the role of RhoK in ET-1-induced contractions was not determined in that investigation.

The results of the present investigation suggest that α_1-ARs couple more efficiently to the RhoK than the PKC pathway, while ET-1 receptors couple more effectively to the PKC pathway in mesenteric arteries from control rats. Receptor agonists have been shown to couple to different Ca^{2+} sensitization pathways in a particular vascular tissue. Variations in the contribution of the PKC and RhoK signaling pathways to contractile responses to stimulation of different receptors have been reported. For instance, in porcine coronary artery, the Ca^{2+} sensitizing effect of carbachol was suggested to be primarily due to RhoA-independent tyrosine phosphorylation, with no participation of PKC whereas that of ET-1 was proposed to be mediated by PKC and RhoA-dependent and -independent tyrosine phosphorylation, based on pharmacological inhibition of these pathways (Sato *et al.*, 2000a). In another study also conducted on the porcine coronary artery, RhoK was shown to play a more prominent role in U-46619-induced Ca^{2+} sensitization than PKC (Nobe & Paul, 2001). RhoK was also shown to participate to a greater extent in NE- and AG II-induced contraction when compared to PKC in the rat renal artery (Bauer & Parekh, 2003). This may be due to

variations in the ability of agonists and receptors to activate G-proteins of the $G_{q/11}$ and $G_{12/13}$ families (Gohla *et al.*, 2000).

4.9 K^+ DEPOLARIZATION-INDUCED RhoA/RhoK ACTIVATION

There is evidence to show that K^+ depolarization of vascular smooth muscle can sensitize the contractile proteins to Ca^{2+}, and this process has been shown to involve the activation of RhoA/RhoK (Mita *et al.*, 2002; Sakamoto *et al.*, 2003; Urban *et al.*, 2003). For example, stimulation of rabbit femoral artery with KCl resulted in significant translocation of ROCK II to the caveolae, which are invaginations of the cell membrane (Urban *et al.*, 2003) and KCl significantly increased activities of both RhoA and RhoK in airway smooth muscle (Janssen *et al.*, 2004). This activation is Ca^{2+} dependent since removal of extracellular Ca^{2+} or use of Ca^{2+} channel blockers abolishes KCl-induced RhoA/RhoK activation (Sakurada *et al.*, 2003; Janssen *et al.*, 2004). It is unlikely that in our studies K^+-depolarization is associated with RhoK activation because the concentration of Y-27632 that blocked PE-induced translocation of ROCK I and II did not have a significant effect on KCl-induced contractions in both control and diabetic mesenteric arteries. It is possible that activation of the RhoA/RhoK pathway by depolarization takes place only in some smooth muscles. Somlyo and Somlyo (2003) suggested that participation of the RhoA/RhoK pathway in KCl-induced contractions might represent constitutively active RhoA and/or RhoK or activation of this pathway due to very high $[Ca^{2+}]_i$, since translocation of RhoK was evident only at 30 μM free Ca^{2+} or more (Gong *et al.*, 1997).

4.10 AGONIST-INDUCED CHANGES IN CYTOSOLIC (SOLUBLE) LEVELS OF PKC AND RhoK ISOFORMS

In our study, we have seen varying effects of α_1-AR agonists and ET-1 on particulate levels of PKC and RhoK isoforms (discussed in detail in section 4.6 and 4.9). However the agonist-induced increases in particulate levels of these isoforms were not associated with corresponding decreases in the cytosolic fractions (data not shown). This been previously reported for both PKC and RhoA/RhoK. For instance, PE induced significant translocation of PKCε in cardiomyocytes but there was no detectable decrease in soluble fractions of this isoform (Puceat *et al.*, 1994; Wang *et al.*, 2003). Similarly, oxyhemoglobin-mediated cerebral vasoconstriction was associated with significant increases in particulate levels of

PKCα and ε and RhoA but the corresponding soluble fractions were not significantly decreased (Wickman *et al.*, 2003). Reactive oxygen species induced by treating rat aortic rings with xanthine-xanthine oxidase also resulted in a significant increase in RhoA particulate levels without any detectable decrease in the soluble fraction (Jin *et al.*, 2004), while stimulation of smooth muscle cells from rabbit colon with ACh resulted in a significant increase in RhoA and ROCK II in the particulate fraction without any significant changes in the soluble fraction (Patil *et al.*, 2004). A possible explanation for these observations is the greater amount of protein present in the soluble as compared to the particulate fraction, as a result of which decreases in soluble protein levels of PKC or RhoK are relatively difficult to detect as compared to increases in the particulate levels (Henry *et al.*, 1996).

4.11 INTERACTION OF PKC AND RhoK SIGNALING PATHWAYS

It has been suggested that interaction between the different kinase pathways activated on stimulation of GPCRs may be a key signaling event of Ca^{2+} sensitization of the contractile apparatus in vascular smooth muscle (Sasaki *et al.*, 1998; Martinez *et al.*, 2000). Crosstalk between the RhoK and PKC pathways has been demonstrated to occur in a number of cell systems. For example, translocation and activation of PKC in mammalian endothelial and bronchial epithelial cells was blocked by inhibition of the Rho GTPases, RhoA and/or Cdc42 (Hippenstiel *et al.*, 1998). Conversely in another study, PKC inhibition or depletion blocked RhoK activation in cultured human umbilical vein endothelial cells (Barandier *et al.*, 2003). RhoA association with PKCα in the membrane fraction was essential for optimal enhancement of AP-1 transcriptional activity in Jurkat T cells (Chang *et al.*, 1998). A direct interaction between PKCα and RhoA, demonstrated in an *in vitro* assay, was shown to lead to the activation of the former (Slater *et al.*, 2001). Stimulation of rabbit rectosigmoid smooth muscle cells with ACh resulted in translocation and significant complexing of RhoA and PKCα in the membrane fraction (Bitar *et al.*, 2002). Two studies have investigated interactions between the PKC and RhoK pathway in intact artery preparations. PKC and RhoK appeared to be acting along the same intracellular signaling pathway leading to interleukin β-induced coronary artery vasospasm in a porcine model (Kandabashi *et al.*, 2003). Since PdBu-induced contraction was significantly attenuated by hydroxyfasudil (a RhoK inhibitor) and RhoA was significantly translocated to the membrane fraction on stimulation with the phorbol, PKC was suggested to be located upstream of RhoK. Similarly,

in another study conducted on the rabbit aorta, fasudil (a RhoK inhibitor) inhibited 12-deoxyphorbol 13-isobutyrate-induced contraction and phosphorylation of MLC_{20}, also suggesting that PKC is located upstream of RhoK in that artery (Shimomura *et al.*, 2004).

In our studies, PKC but not RhoK appeared to participate in the unchanged contractile responses of diabetic mesenteric arteries to ET-1, so we reasoned that RhoK might be upstream of PKC on stimulation of α_1-ARs. This possibility is supported by the finding that a concentration of Y-27632 that completely inhibited PE-induced RhoK translocation in both control and diabetic mesenteric arteries, also completely prevented PE-induced translocation of PKC isoforms in diabetic mesenteric arteries. We do not believe that this is due to direct inhibition of PKC because:

1) The concentration of Y-27632 used in this investigation had no significant effect on contractile responses of control or diabetic mesenteric arteries to direct activation of PKC with PdBu.
2) Y-27632 had no inhibitory effect on contractile responses to ET-1, which are markedly reduced by inhibition of PKC.
3) Y-27632 was completely without effect on the ET-1 induced translocation of PKC α, δ and ε.

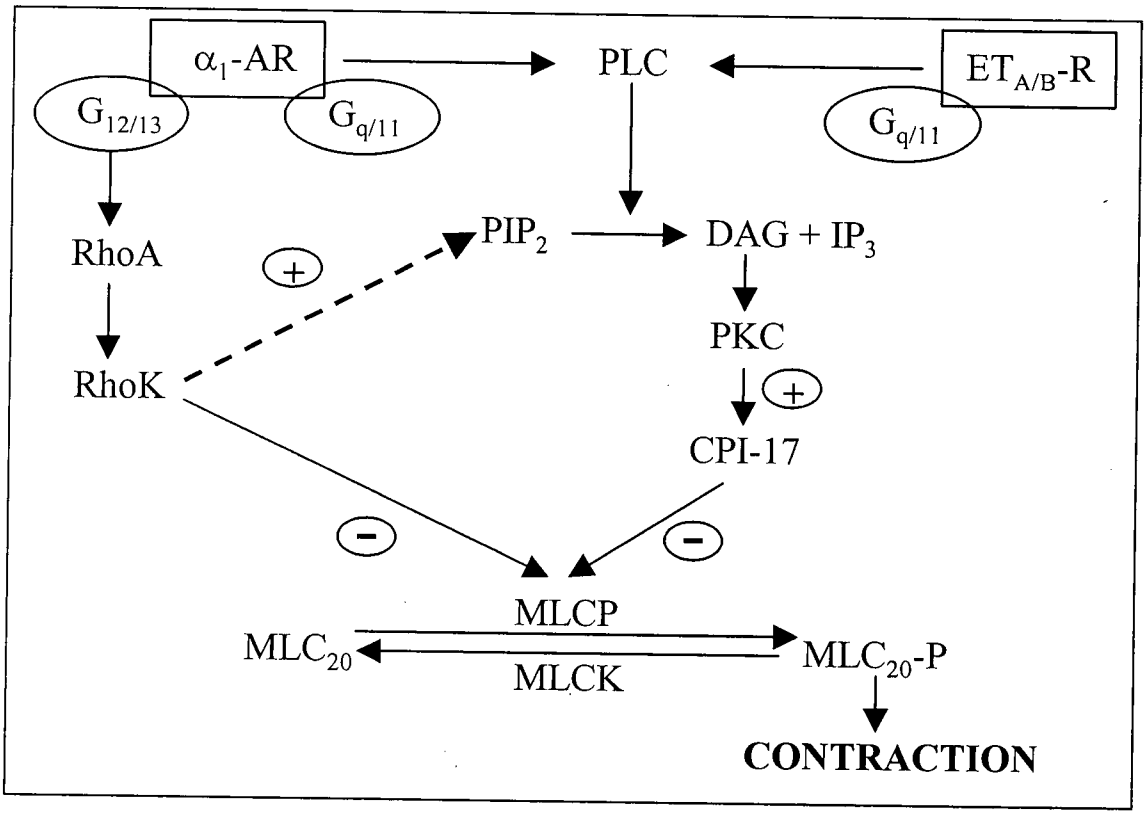
This suggests that the effects of Y-27632 on the PE-induced translocation of PKC isoforms do not arise from direct inhibition of PKC by this antagonist, but result from the inhibition by Y-27632 of PE-induced increases in PKC activity.

At the present time we have no direct evidence to suggest how increased activation of RhoK might lead to increased activation of PKC in mesenteric arteries from diabetic rats. However, our lab has found that α_1-AR stimulated phosphoinositide hydrolysis is also enhanced in diabetic arteries, since breakdown of PIP_2, and production of total inositol phosphates and IP_3 are all increased (Abebe & MacLeod, 1991a; Abebe & MacLeod, 1991b; Abebe & MacLeod, 1992). There is evidence that small GTPases of the Rho family especially RhoA associate with phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to regulate the production of PIP_2 (Ren & Schwartz, 1998). PIP5Ks exist in multiple isoforms, which can be divided into type-I and type-II on the basis of their biochemical and chromatographic profiles (as reviewed by Anderson *et al.*, 1999). Type-I PIP5Ks phosphorylate phosphatidylinositol 4-phosphate (PI4P), which is the final step in PIP_2 synthesis *in vivo* and therefore regulate supply of the

substrate for PLC. It was found that in HEK-293 cells, type-I PIP5K isoforms were positively regulated by RhoA resulting in enhanced PIP_2 synthesis (Weernink *et al.*, 2004). In Cos-7 cells stimulation of GPCRs by thrombin led to the activation of type-I PIP5K and its translocation to the cell membrane and this was dependent on GTP-bound RhoA (Chatah & Abram, 2001). The RhoA-induced increase in PIP5K activity in HEK-293 cells was mediated by RhoK as treatment with HA-1077 prevented the increment in PIP_2 cellular levels (Oude Weernink *et al.*, 2000). Since PIP_2 levels in the cell are limited and are rapidly depleted on sustained agonist stimulation, RhoA/RhoK mediated regulation of this phospholipid has been suggested to have significant implications on PKC signaling by receptors coupled to PLC (Sah *et al.*, 2000). However the regulation of inositol lipid kinases by RhoA and RhoK have been investigated in relation to their effects on the actin cytoskeleton, as the Rho family have been shown to be key regulators of the cytoskeletal organization (Etienne-Manneville & Hall, 2002), and it is not clear whether a similar pathway exists in vascular smooth muscle.

The signal transduction pathways in mesenteric arteries from STZ-diabetic rats as suggested by the results of our study are summarized in Figure 4.1. The dashed line shows the proposed interaction between the PKC and the RhoK pathway. We hypothesized that the enhanced activation of RhoK, especially ROCK II, mediates the increased participation of PKC in α_1-AR-induced vasocontractile responses by increasing cellular PIP_2 levels in diabetic mesenteric arteries. This explanation is speculative at present since we do not have experimental evidence to support it. Whether RhoK contributes to the regulation of PIP_2 production requires further investigation.

Figure 4.1



The proposed signal transduction pathways activated in response to stimulation of α_1-AR and ET-1 receptors in diabetic mesenteric arteries.

Abbreviations: **α_1-AR** – alpha$_1$ adrenoceptor, **CPI-17** – PKC potentiated inhibitor protein of 17kDa, **DAG** – diacylglycerol, **ET-1** – endothelin-1, **IP_3** – inositol trisphosphate, **MLC_{20}** – regulatory light chains of myosin, **MLC_{20}-P** – phosphorylated regulatory light chains of myosin, **MLCK** - myosin light chain kinase, **MLCP** – myosin light chain phosphatase, **PIP_2** – phosphatidylinositol bisphosphate, **PKC** – protein kinase C, **PLC** – phospholipase C, **RhoK** – Rho kinase

4.12 POTENTIAL MECHANISMS LEADING TO ENHANCED RhoK PARTICIPATION IN α_1-AR-MEDIATED VASOCONSTRICTION IN MESENTERIC ARTERIES FROM DIABETIC RATS

There is no experimental evidence concerning the mechanisms responsible for enhanced participation of RhoK in PE-induced vasoconstriction in mesenteric arteries from diabetic rats at present. However a number of possible mechanisms can be proposed. For instance, α_1-ARs in vascular smooth muscle have been shown to couple to the $G_{12/13}$ G-protein, which regulate the activity of RhoA/RhoK (as reviewed by Sah *et al.*, 2000). It has been suggested that an increase in the number of G-proteins or in the efficiency of their coupling to the receptor can result in enhanced vasocontractile responses (Raymond, 1995). A number of studies have shown that diabetes can affect G-protein function and expression. For instance, G_i G-protein levels were decreased in diabetic hepatocytes (Bushfield *et al.*, 1990; Caro *et al.*, 1994) and its functioning was compromised in diabetic adipocytes (Green & Johnson, 1991). Although previous studies from our lab have shown that levels of $G_{q/11}$ and $G_{i2/3}$ G-proteins and their coupling efficiency to α_1-ARs in diabetic arteries are not altered, it is possible that there are differences in $G_{12/13}$ G-protein functioning and/or expression in mesenteric arteries from diabetic rats. Increased levels and/or coupling efficiency of this G-protein to α_1-ARs could explain the enhanced participation of RhoK to PE-induced vasoconstriction in diabetic arteries.

It is also possible that chronic diabetes is associated with abnormalities in regulators of Rho GTPases such as RhoGEFs and RhoGDI, which are positive and negative modulators of RhoA, respectively. Ying et al (2004) showed that RhoGEF (PDZ-RhoGEF, p115 RhoGEF and LARG) expression levels were significantly elevated in aorta from stroke-prone spontaneously hypertensive rats (SHRSP), and this was suggested to contribute to enhanced vasoconstrictor responsiveness in hypertension. Since RhoGEFs facilitate activation of RhoA and hence regulate signaling via GPCRs, it is possible that overexpression of these regulatory proteins could increase PE-induced RhoK activation in mesenteric arteries from diabetic rats. Another possible explanation for enhanced participation of RhoK in PE-induced vasoconstriction in diabetic arteries is hyperglycemia-induced generation of ROS. ROS have been suggested to be important modulators of vascular tone and some studies have shown

that even after removal of the endothelium, ROS can affect vascular smooth muscle contractility (Auch-Schwelk *et al.*, 1989; Rodriguez-Martinez *et al.*, 1998; Yang *et al.*, 1999). This has been suggested to be via Ca^{2+}-independent stimulation of signal transduction pathways such as the MAP kinase or TK pathway (Jin *et al.*, 1991; Yang *et al.*, 1999; Pelaez *et al.*, 2000). In a recent study, Jin et al (2004) showed that ROS induced activation of the RhoA/RhoK pathway in rat aorta. ROS have been suggested to participate in vascular abnormalities in DM (Ceriello *et al.*, 1993) and some studies have demonstrated a role for ROS in the development of enhanced α_1-AR responses in diabetic animal models (Chang et al., 1993, Kanie & Kamata, 2000). It is possible that enhanced oxidative stress in mesenteric arteries from chronic diabetic rats in our study mediates increased α_1-AR vasoconstriction via enhanced activation of RhoK.

4.13 PHARMACOLOGICAL INHIBITION OF TKs AND $cPLA_2$

Functional studies implicating TKs in GPCR-mediated contractile processes have used low molecular weight TK inhibitors that are categorized depending on their mechanism of action. One class of TK inhibitors acts by interacting with the ATP-binding site; the most commonly used inhibitor from this class is the naturally occurring quercetin derivative, genistein. The other class of inhibitors, which includes the synthetic analogues of erbstatin, the tyrphostins, acts by interacting with the substrate-binding site (Casnellie, 1991). Inactive analogues for each class of inhibitors have also been developed, namely daidzein and tyrphostin A1.

TKs have been implicated in GPCR-mediated contractile responses in the mesenteric vasculature. Arginine vasopressin-induced contractions were significantly attenuated in resistance mesenteric arteries from 6-week, but not 21-week, spontaneously hypertensive rats by treatment with tyrphostin A23 (Endemann *et al.*, 2002). NE induced tyrosine phosphorylation of a number of different proteins in small mesenteric arteries from adult female Sprague Dawley rats and this was inhibited by treatment with tyrphostin A23 (Ward *et al.*, 2002). In the same vessels, tyrphostin A23 significantly attenuated ET-1-induced contractions (Ohanian *et al.*, 1997). In rat mesenteric resistance arteries, genistein and tyrphostin A23 produced a concentration-dependent (1μM – 100μM) relaxation of NE-induced contractions (Toma *et al.*, 1995). In rabbit permeabilized mesenteric artery, histamine- and PE-induced Ca^{2+} sensitization was significantly attenuated by genistein and tyrphostin A25 (Sasaki *et al.*, 1998). However there are also reports of TKs not participating

in GPCR-mediated contractions in mesenteric arteries. For example in rat mesenteric resistance arteries, U-46619-induced contractions were unaffected by tyrphostin A25 (Bolla *et al.*, 2002) and in permeabilized rabbit mesenteric arteries genistein did not affect ET-1-induced Ca^{2+} sensitization (Sato *et al.*, 2000a).

The lack of effect of TK inhibitors in our studies could be due differences in the preparation used, as most of the studies described above have utilized the resistance arteries in the mesenteric vasculature whereas we have used the superior mesenteric vessels. There could also be differences in coupling of TKs to GPCRs depending on species and gender of the experimental animal (Sato *et al.*, 2000a). The concentration of genistein and tyrphostin A23 used in the present investigation (10μM) has been shown to attenuate agonist-induced responses in vascular smooth muscle (Filipeanu *et al.*, 1995; Toma *et al.*, 1995; Fetscher *et al.*, 2001; Endemann *et al.*, 2002). At this concentration, genistein and tyrphostin A23 had no significant effect on KCl-induced contractile responses, suggesting that their effects were not due to non-specific actions. At higher concentrations they significantly attenuated KCl-mediated responses (data not shown). It has been suggested that tyrphostins may require up to 16 hours to attain their optimal inhibitory effect in intact cells (Lyall *et al.*, 1989). However in most studies the effects of TK inhibitors in intact vessels have been investigated following an incubation period of 10 - 30 minutes (Toma *et al.*, 1995; Fetscher *et al.*, 2001).

There is not a lot of information regarding the role of TKs in abnormal vasoconstrictor responses in Type I diabetic animal models. However in one investigation, insulin-mediated potentiation of NE-induced contraction in rabbit facial artery was suppressed by pretreatment with genistein (10 μM) (Henrion & Laher, 1994). In our studies TK inhibition did not normalize α_1-AR mediated responses in mesenteric arteries from diabetic rats. This preliminary evidence suggests that TKs do not participate in abnormal vasoconstrictor responses in diabetic tissues. However more experimental evidence, such as measurement of PE-induced tyrosine phosphorylation, is required before the role of TKs in GPCR-mediated responses in diabetic tissues can be determined.

$cPLA_2$ has been frequently implicated in GPCR-mediated responses using $AACOCF_3$, which has been suggested to be a selective $cPLA_2$ inhibitor (Street *et al.*, 1993; Li & Cathcart, 1997). In the perfused rat MAB, $AACOCF_3$ significantly attenuated histamine- and ACh-induced decreases in perfusion pressure in response to cirazoline (Adeagbo & Henzel, 1998).

This inhibition was due to attenuation of $cPLA_2$-mediated production of AA, which in turn led to release of endothelium derived hyperpolarizing factor (EDHF). $AACOCF_3$ significantly attenuated NE- (Labelle & Polyak, 1998) and ET-1-induced (Trevisi *et al.*, 2002) AA release in rat tail artery. In rat aorta, $AACOCF_3$ significantly decreased ET-1-induced prostacyclin production (Oriji *et al.*, 1996). NaF, a nonselective activator of heterotrimeric G-proteins, increased AA and prostacyclin production in bovine aortic endothelial cells and this was inhibited by treatment with $AACOCF_3$ (Rosenstock *et al.*, 1996).

The concentration of $AACOCF_3$ used in the present investigation (10 μM) has been used to selectively inhibit $cPLA_2$ in vascular smooth muscle (Xia et al., 1995; Labelle & Polyak, 1998; Silfani & Freeman, 2002). At this concentration, $AACOCF_3$ had no significant effect on KCl-induced contractile responses, suggesting that its effects were not due to non-specific actions. The fact that $AACOCF_3$ had no effect on PE and ET-1-induced contractile responses in mesenteric arteries from control rats suggests that $cPLA_2$ is not activated in response to these agonists in our preparation. However it is not possible to rule out a role of other classes of PLA_2 in α_1-AR and ET-1 receptor-mediated responses in superior mesenteric arteries. This can be investigated using BEL, an inhibitor suggested to be selective for $iPLA_2$ (Balsinde & Dennis, 1998), LY311727, an inhibitor suggested to be selective for $sPLA_2$ (Schevitz *et al.*, 1995) or non-isoform selective PLA_2 inhibitors like quinacrine (Mukherjee *et al.*, 1994) or ONO-RS-082 (Billah & Anthes, 1990).

The role of $cPLA_2$ in abnormal vasoconstrictor responses in Type I diabetes has not been investigated in detail. However there is evidence to show that AA-induced production of prostaglandins can affect vascular responses. For example vasodilator responses to bradykinin were attenuated in the isolated perfused kidney of STZ-induced diabetic rats and this was due to decreased renal PLA_2 activity and attenuated release of prostaglandin E_2 (Quilley *et al.*, 1992). Similarly in diabetic rat aorta, NE- and U-46619-induced prostacyclin production was significantly attenuated due to decreased PLA_2 activity (Jeremy *et al.*, 1987). In our studies, $AACOCF_3$ did not normalize significantly elevated PE responses in mesenteric arteries from diabetic rats, suggesting that $cPLA_2$ does not participate in the enhanced α_1-AR-induced contractions in diabetic tissues. The role of $cPLA_2$ and AA in

GPCR-mediated Ca^{2+} sensitization and contraction in diabetic arteries is controversial and requires further investigation.

5. SUMMARY AND CONCLUSIONS

1) Contractile responses of endothelium-denuded mesenteric arteries from diabetic rats to NE were enhanced, but were normalized by the PKC inhibitors, Ro-318220 and calphostin C. In contrast, no change in contractile responses of diabetic arteries to ET-1 could be detected, and PKC inhibition attenuated ET-1 responses to a similar extent in both control and diabetic tissues. A maximum concentration of NE produced a small translocation of PKCε in control arteries, but a significant translocation of PKCα and a much larger translocation of PKCε in diabetic arteries. ET-1 increased translocation of PKCα, δ and ε in both control and diabetic arteries and the degree of translocation was no greater in diabetic tissues. NE significantly enhanced CPI-17 phosphorylation in diabetic, but not control, arteries and this was blocked by PKC inhibition. These data suggest that increased activation of the PKC/CPI-17 pathway mediates the enhanced contractile response to NE in mesenteric arteries from diabetic rats. On the other hand, although PKC appears to contribute to a greater extent to ET-1 than to NE-induced contractions in control mesenteric arteries, no evidence was found for increased activation of this pathway in response to ET-1 in mesenteric arteries from diabetic rats. This suggests that significantly elevated NE-induced contractions result from a selective change in α_1-AR-mediated signaling in the diabetic arteries.

2) The selective RhoK inhibitors, Y-27632 and H-1152, produced greater inhibition of contractile responses to PE in diabetic than control mesenteric arteries and normalized the difference between them. Contractile responses to ET-1 were not affected by RhoK inhibition. A maximum concentration of PE produced significant translocation of ROCK I and II that was inhibited by Y-27632 in both control and diabetic arteries. The PE-induced translocation of ROCK II was significantly greater in diabetic tissues. ET-1 produced no detectable translocation of RhoK isoforms. These data suggest that activation of RhoK, especially ROCK II, in addition to PKC, is required for the enhanced vasoconstrictor responses of diabetic mesenteric arteries to α_1-AR stimulation. On the other hand, RhoK does not appear to contribute to contractile responses to ET-1 in either control or diabetic mesenteric arteries.

3) PE produced significant translocation of PKC α, δ and ε in diabetic but not control arteries. Y-27632 blocked the translocation of these isoforms in diabetic arteries but had no effect on ET-1 induced translocation of PKC isoforms in control arteries. These data suggest that there is an interaction between the RhoK and PKC pathways on stimulation of α_1-ARs in diabetic mesenteric arteries and that increased activation of RhoK may mediate enhanced activation of PKC. Although increased participation of PKC in enhanced contractile responses of diabetic arteries to stimulation of GPCRs has been proposed, this is the first demonstration to our knowledge that RhoK may also be required and may be functioning upstream of PKC.

4) Preliminary pharmacological data suggests that TKs and $cPLA_2$ do not participate in PE or ET-1 induced responses in either control or diabetic mesenteric arteries.

6. FUTURE DIRECTIONS

1) It would be helpful to identify the α_1-AR subtype (α_{1A}, α_{1B} or α_{1D}) that is involved in mediating the enhanced contractile responses to NE and PE in diabetic arteries. While selective agonists are only available for the α_{1A}-AR (A-61603 and oxymetazoline), selective antagonists for every subtype are available (e.g. WB 4101 and KMD 3213 for α_{1A}-AR, CEC for α_{1B}-AR and BMY 7378 for α_{1D}-AR). These could be used in tissue bath contraction studies to identify the subtype mediating enhanced α_1-AR responses in diabetic arteries. The number of the different subtypes and their binding affinity to PE/NE can be determined by saturation binding assays using the selective antagonists for the different subtypes.

2) To investigate which subtype is mediating the enhanced activation of RhoK and PKC following α_1-AR stimulation in diabetic arteries, the effect of subtype-selective antagonists on PE-induced translocation of RhoK and PKC isoforms should be determined.

3) We have proposed that RhoK mediates enhanced PKC activation in response to α_1-AR stimulation in diabetic arteries via increased activation of PIP5K. In order to confirm this we need to determine if increased activation of RhoK results in enhanced stimulation of PIP5K activity and if this promotes α_1-AR-mediated phosphoinositide hydrolysis and DAG production in diabetic mesenteric arteries.

4) Agonist-induced translocation of PKC and RhoK isoforms is regarded as an index of their activation. To further confirm a role for PKC and RhoK in enhanced α_1-AR vasoconstriction in diabetic mesenteric arteries, direct activity measurements can be carried out for PKC (Kanashiro & Khalil, 2001) and RhoK (Murthy *et al.*, 2003) following stimulation with NE/PE. Activity should be measured in the absence and presence of selective inhibitors to confirm specificity of the assay

5) The mechanism of RhoK-mediated Ca^{2+} sensitization in control and diabetic mesenteric arteries should be determined. This can be done by measuring MYPT phosphorylation (if any) at Thr 696 and Thr 850 following α_1-AR stimulation. Since CPI-17 also mediates MYPT phosphorylation and inhibition, the effect of RhoK inhibition on the degree of phosphorylation of MYPT should also be investigated.

6) The reasons for the enhanced PE-induced RhoK activation in diabetic arteries are speculative at present (discussed in section 4.13). In order to investigate the underlying mechanisms, levels of $G_{12/13}$ G-protein and its interaction with α_1-ARs in diabetic mesenteric arteries can be determined by Western blotting and competition binding experiments, respectively. To determine if increased expression of RhoGEFs is mediating enhanced PE-induced RhoK activation in diabetic mesenteric arteries, its expression levels can be measured by RT-PCR and Western blotting. In order to determine if increased levels of ROS are contributing to the agonist-induced RhoK activation in diabetic mesenteric arteries, the effect of antioxidants such as superoxide dismutase (SOD) and catalase on PE-induced translocation of RhoK isoforms in diabetic arteries can be investigated. If ROS such as superoxide and hydrogen peroxide, are mediating enhanced PE-induced activation of RhoK, the antioxidants should abolish the increased translocation of ROCK I and II in diabetic tissues.

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