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ENDOTHELIN RESPONSE AND REACTIVE OXYGEN IN CORONARY ARTERY SMOOTH MUSCLE

BY

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ABSTRACT

Endothelin-1, the most potent endogenous vasoconstrictor peptide known, plays a key role in regulating coronary artery vascular tone. Reactive oxygen species generated during cardiac ischemia-reperfusion cause several types of damage to cardiovascular tissues, in particular to the intracellular Ca^{2+}-regulating mechanisms. *This thesis explores the endothelin-1 receptor types in coronary artery smooth muscle and their signal transduction pathways and the effects of reactive oxygen on them.*

Endothelin-1 mediated contraction of de-endothelialized pig coronary artery rings. There are two types of endothelin receptors known: ET_A and ET_B. Using ET_A and ET_B receptors selective agonists and antagonists, the contraction mediated by ET_B- receptors was approximately 20% and the remaining was due to ET_A receptors. Ca^{2+} pools mobilized by the two receptors were similar except that the ET_B receptor activation utilized more of the intracellular Ca^{2+} pool than the ET_A activation. ^{125}I-ET-1 binding to microsomes isolated from smooth muscle of this artery also showed ET_A and ET_B binding sites with most binding occurring at the ET_A sites. Thus, the endothelin-1 induced vasoconstrictor response in pig coronary artery smooth muscle involves both ET_A and ET_B-receptors with ET_A being predominant.

Pretreating the artery with hydrogen peroxide inhibited the subsequent contraction upon ET_A or ET_B receptor activation. However, the ET_B-mediated contraction was significantly ($p < 0.05$) sensitive to peroxide ($IC_{50} = 0.3 \pm 0.08$ mM) than the ET_A receptor mediated contraction ($IC_{50} = 1 \pm 0.3$ mM). Pretreating smooth muscle cells cultured from

pig coronary artery with 0.3 mM hydrogen peroxide inhibited the endothelin-1 induced increase in $[Ca^{2+}]_i$ by more than 95 %. Thus the exposure to reactive oxygen damaged the ET_B receptor mediated contractions preferentially, possibly as a result of a Ca^{2+}-independent component in ET_A receptor mediated contractions.

ATP-dependent azide-insensitive oxalate-stimulated Ca^{2+}-uptake in permeabilized smooth muscle cells cultured from pig coronary artery exhibited the expected kinetic properties of the sarcoplasmic reticulum (SR) Ca^{2+}-pump. Under optimum conditions, inositol 1,4,5 trisphosphate (IP_3) released up to 65% of $^{45}Ca^{2+}$-loaded into the SR. Pretreating the cells with hydrogen peroxide or superoxide did not affect the IP_3-dependent Ca^{2+}-release but inhibited the Ca^{2+}-uptake in the SR. Peroxide was equipotent in inhibiting $^{45}Ca^{2+}$-loading into IP_3-sensitive and IP_3-insensitive Ca^{2+} pools but superoxide inhibited loading only into the IP_3-sensitive pool indicating that the SR Ca^{2+} pump in vascular smooth muscle cells is heterogenous.

These results indicate that both ET_A and ET_B receptors are involved in ET-1 mediated contraction in smooth muscle pig coronary artery, with similar Ca^{2+} utilization pathways but the ET_A receptors may also induce contraction in part by a Ca^{2+}-independent mechanism. The peroxide pretreatment damages the SR Ca^{2+} pump and this leads to a diminished contraction by endothelin-1, with the exception of the Ca^{2+}-independent mechanism(s) associated with ET_A receptor activation which may be resistant to peroxide. This Ca^{2+}-independent mechanism provides a potential therapeutic target for diseases where ET_A plays a major role. The second major finding is the heterogeneity of SR Ca^{2+}

pool which can also be used in designing pharmacological agents specific to a distinct component of the SR Ca^{2+} pool.

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LIST OF ABBREVIATIONS

AngII	Angiotensin II
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Ionized calcium
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
$CaCl_2$	Calcium Chloride
CaM	Calmodulin
cAMP	Cyclic 3' 5' adenosine monophosphate
cADP-ribose	Adenosine 5'-cyclic diphosphoribose
cDNA	Complementary deoxyribonucleic acid
CPA	Cyclopiazonic acid
DAG	Diacylglycerol
DTT	Dithiothreitol
EDTA	Ethylene-diamine tetra-acetic acid
EGTA	Ethyleneglycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ET	Endothelin
GTP	Guanosine triphosphate
^{125}I-ET-1	Ionidined endothelin-1
IP_3	Inositol 1,4,5 trisphosphate
IP_3R	Inositol 1,4,5 trisphosphate receptor
K^+	Ionized potassium
KCl	Potassium chloride
kDa	kilo-Dalton
KH_2PO_4	Potassium dihydrogen orthophosphate
M	Molar
$MgCl_2$	Magnesium Chloride
mm	Millimetre
min	minute
mN	MilliNewton
mRNA	messenger ribonucleic acid
MLCK	Myosin light chain kinase
Na^+	Ionized sodium
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
nm	Nanometre
NO	Nitric Oxide
PLC	Phospholipase C

PIP2	Phosphatidylinositol 4,5 bisphosphate
PKC	Protein kinase C
PKC-ϵ	Protein kinase C-epsilon
P-myosin	Phosphorylated myosin
PMSF	Phenylmethylsulfonyl Fluoride
PSS	Physiological saline solution
ROS	Reactive oxygen species
ROCC	Receptor operated Ca^{2+} channels
Sec	Second
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} pump
SEM	Standard error of the means
SR	Sarcoplasmic reticulum
RyR	Ryanodine receptor
U	Unit
VOCC	Voltage operated Ca^{2+} Channels
X	Xanthine
XO	Xanthine Oxidase

CHAPTER ONE

INTRODUCTION

Coronary arteries are the primary blood supply to the heart and hence they are essential for its survival. A major controlling factor of coronary blood flow is the coronary artery vascular tone which is regulated via several mechanisms (Ganz, Braunwald, 1997). Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is the main mechanism for regulating the coronary vascular tone (Burnstock, 1993; Daniel et al., 1995). $[Ca^{2+}]_i$ is also regulated through several mechanisms. Thus, any dysfunction in the Ca^{2+}-regulating mechanisms can ultimately lead to a development of cardiovascular ischemia. Several neurotransmitters and hormones are involved in the Ca^{2+}-regulating mechanisms in the arteries. The most potent vasoconstrictor known is the peptide endothelin (ET) (Yanagisawa et al., 1988). The signal transduction of ET-mediated contraction of the coronary artery smooth muscle depends on extracellular Ca^{2+} entry and Ca^{2+} mobilization from intracellular Ca^{2+} pools (Sudjarwo et al., 1995; Yoshida et al., 1994). The major insult during cardiac ischemia and reperfusion is due to the damage to Ca^{2+} regulating mechanisms by reactive oxygen species (ROS) (Grover, Samson, 1988; Grover et al., 1995; Roveri et al., 1992). *The aim of the present study is to investigate the ET-1 responses involved in the Ca^{2+} mobilization pathways in pig coronary artery smooth muscle and to examine the effects of ROS on them.* The Introduction will first outline the anatomy of the coronary arteries, the regulation of coronary blood flow and the structure

and function of the vascular smooth muscle. Since ET signal transduction involves Ca^{2+}-mobilization, the second focus will be on the mechanisms which regulate intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. The focus then shifts to endothelin structure, biosynthesis, receptor types and signal transduction pathways. Finally, the nature and sources of reactive oxygen and their role in cardiac ischemia and reperfusion will be briefly reviewed. This will lead to the rationale for this study and the specific hypotheses to be tested.

1.1. Coronary Arteries:

1.1.1 Anatomical Outline of the Coronary Arteries:

The left and right coronary arteries (*corona* in Latin means crown) are the main blood suppliers to the heart in all mammals. Both left and right coronary arteries originate from the aortic sinuses of the ascending aorta and run in the coronary groove (sulcus) which encircles the base of ventricles like a crown . The right coronary artery gives several branches that supply the right atrium, right ventricular, inter-ventricular septum, sinoatrial and atrioventricular nodes, and atrioventricular bundles. The left coronary artery supplies most of the left ventricle and atrium, and the inter-ventricular septum. The left artery is also branched, with the anterior inter-ventricular being the largest branch that supplies both the ventricular and the inter-ventricular septum. This branch is the one used in our study and it is one of the most common sites of occlusion during cardiac ischemia (Moore, 1992).

The coronary arteries can also be divided according to their diameter into epicardial arteries (>400 μm in diameter), intramural arteries or prearteriolar (between 100

and 400 μm), and arterioles (less than 100 μm) which ultimately form the subendocardial plexus. Functionally these arteries can be classified into conductive arteries with almost no resistance to the blood flow, and resistive arteries which regulate the myocardial perfusion (Grover et al., 1987).

1.1.2 Regulation of the Coronary Blood Flow:

For its energy, the heart relies mainly on the oxidation of substrates derived from the coronary blood flow. Depending on the energy requirements, the coronary blood flow can vary considerably (Loiselle, 1987). The coronary blood flow depends on the driving arterial pressure (aortic-left ventricular diastolic pressure), the diastolic time, and the vascular tone which determine the resistance of the coronary bed (Ganz, Braunwald, 1997). Of these factors, the vascular tone is one of the most important in regulating coronary blood flow. There are several mechanisms that control the vascular tone such as metabolic, myogenic, autoregulatory, extravascular forces, and neural control.

1.1.2.1. Metabolic Regulation: Any change in the oxygen supply to the heart can lead to modification of the coronary resistance via various mediators such as adenosine, endothelin, nitric oxide , prostaglandins, CO_2, and H^+. The importance of the endothelium in regulating vascular tone has been realized since the discovery of endothelium-derived relaxing factor (nitric oxide) and endothelium-derived constricting factor (endothelin) (Furchgott, Vanhoutte, 1989; Palmer et al., 1988; Yanagisawa et al., 1988). Nitric oxide is synthesized in the endothelium by an endothelial nitric oxide synthase which is activated by Ca^{2+}-calmodulin (Lamas et al., 1992). Nitric oxide

formation can be stimulated by many agents such as thrombin, serotonin, histamine, and by shear stress (Vanhoutte, 1989). The role of endothelin in regulating vascular tone will be discussed in detail in *Section 1.4.*

1.1.2.2. Autoregulation of Coronary Tone: Autoregulation is the ability of the coronary artery to stabilize the myocardial perfusion in spite of changes in the driving pressure within the normal range. Above and below the normal range of the aortic pressure, the coronary tone will sharply decrease or increase (Ganz, Braunwald, 1997). The physiological mechanisms of autoregulation involve vasodilatory effects of nitric oxide as well as the myogenic properties of the smooth muscle. The arteriolar smooth muscle can contract in response to the increase in intramural pressure which helps the blood flow to return to normal despite the increase in resistance. The myogenic mechanisms are more important in arterioles than in larger arteries especially in the subendocardial regions (Harrison et al., 1988).

1.1.2.3. Physical extravascular forces: The force of the systolic pressure is strong enough, especially in the left ventricle, to compress the intra-myocardial vessels. Thus most of the coronary blood flow supply to the ventricles occurs during diastole. Even a backflow of the blood occurs at the peak of systole especially in intramural or small epicardial vessels (Chilian, Marcus, 1985). The extravascular force is formed from intra-cavity pressure of the left ventricle systole, and vascular narrowing caused by muscular compression (Ganz, Braunwald, 1997).

1.1.2.4. Neural Control of coronary tone: The coronary arteries are innervated by sympathetic and parasympathetic nerves which play an important role in regulating their vasomotor tone. The sympathetic innervation results of direct vasocontriction which is mediated via α and β_2 adrenergic receptors. However, this direct vasoconstriction is opposed by a vasodilatation due to an increase in cardiac metabolism secondary to sympathetic increase of the heart rate and myocardial contraction (Young MA, et al., 1987; Schlant R, et al., 1998). An increase in the sympathetic activity in ischemic patients can lead to greater vascular resistance and a reduction in coronary blood flow (Murray et al., 1984). The parasympathetic supply to the coronary bed is mediated via the vagus nerve which releases acetylcholine. The vagus stimulation usually results in direct vasodilatation, but this vasodilation is overwhelmed by vasoconstriction caused by a reduction in myocardial metabolism. It should be noted, however, that the effect of acetylacholine on the coronary arteries is species dependent (Young MA, et al 1986; Schlant R, et al., 1998). Thus, the sympathetic and parasympathetic innervation to the coronary arteries may play only secondary roles in regulating its vascular tone.

1.1.3 Role of endothelium in the regulation of vascular tone:

Endothelium plays a major role in integrating metabolic, flow-mediated and neural pathways for regulation of vascular tone. This endothelium vascular tone regulation is achieved, in part, by production of vasodilatory or vasoconstrictor agents (e.g. NO, ET-1) in response to various stimuli (Luscher, Tanner, 1993). These agents can act directly or indirectly on the blood vessels. For example, acetylcholine has a double action: a direct

vasoconstrictor action on smooth muscle in the absence of the endothelium, and an endothelium-mediated vasodilatory action (Vanhoutte, Cohen, 1984). Acetylcholine stimulates the endothelium to produce nitric oxide which relaxes the smooth muscle (*Section 1.1.2.2*). In a normal artery, the indirect vasodilatory action is predominant. However, in atherosclerosis, damage to the endothelium in epicardial or resistance arteries can impair the vascular tone (Weidinger et al., 1990). The impairment results in a vasoconstriction either from the decrease in nitric oxide release and/or from the unopposed action of the endothelium-dependent vasodilator (e.g. acetylcholine) on the smooth muscle. This endothelial dysfunction may lead to an increase in vascular tone and subsequently to elevated vascular resistance which reduces the coronary blood flow, thus causing ischemia (Vita et al., 1992).

1.2. Smooth muscle in coronary artery:

Smooth muscle is distributed in various tissues such as the muscular wall of the blood vessels, lung, and different visceral organs including the alimentary canal and urogenital tract. There are several recent reviews on the ultrastructural features of smooth muscle (Gabella, 1990; Somlyo, Somlyo, 1992). In spite of the structural similarities among various smooth muscles, there are many differences based on their locations and functions. The focus of this section is to outline the structure and function of the vascular smooth muscle.

1.2.1. Smooth muscle structure:

A smooth muscle cell has an elongated fusiform shape which is circular or oval in

transverse section. The arrangement of the smooth muscle cells varies with their location. For instance, in large arteries the cells are arranged circularly or helically, while in small vessels they are arranged in a curve around the lumen and sometimes in a full circle (Gabella, 1979b). The length of the cells also varies significantly. The cells in arterioles may be as short as 20 μm and those in visceral smooth muscle may be as long as 1000 μm (Daniel et al., 1983). In general, vascular smooth muscle cells have highly corrugated surfaces which results in high surface-to-volume ratios compared to visceral smooth muscle. The cell packing density of smooth muscle is usually small compared to skeletal muscle. This low cell density results in larger extracellular space. Cells may be connected through intercellular junctions such as gap junctions (Gabella, 1979). The following is a brief description of key structures in a smooth muscle cell.

1.2.1.1. Plasma membrane: The plasma membrane or plasmalemma in smooth muscle is not different from other cell types. It acts as a semipermeable membrane to maintain the distinct environments between intracellular and extracellular spaces. The plasma membrane is by no means a homogenous structure. It consists of morphologically distinct areas which may differ in their functions. These areas include: areas with numerous vesicles invagination called *caveolae* that increase the cell surface area up to 70% (Gabella, 1976), and a *dense area* with electron dense materials that attach to the cytoplasmic side of the membrane especially at the tapering ends, which anchor the thin contractile filaments to the plasma membrane (Daniel et al., 1983).

1.2.1.2 Sarco/Endoplasmic reticulum: The sarco/endoplasmic reticulum (SR/ER) in

vascular smooth muscle cells differs in its organization from that in the skeletal or cardiac muscle. In general, SR is a network of tubular systems which are not directly connected to the extracellular space. Based on appearance, there are two types of SR: rough (granular) SR which has ribosomes attached to it and smooth SR lacking ribosomes (Gabella, 1979a). The rough SR is involved in protein synthesis while the smooth SR is important in regulating $[Ca^{2+}]_i$. The smooth SR may be close to the plasma membrane or located somewhat deeper. The sub-plasma membrane and deeper SR may play slightly different roles in regulating intracellular Ca^{2+}. The role of SR in regulating $[Ca^{2+}]_i$ is discussed in *Section1.3.1.* The total volume of SR in smooth muscle cells is determined as a percentage of the cytoplasmic volume, and varies considerably among different types of smooth muscle cells. It can be as high as 5% in rabbit aortic smooth muscle and as low as 1.5% in guinea pig taenia coli. (McGuffee, Bagby, 1976).

1.2.1.3 Mitochondria: The mitochondria may be localized peripherally close to the plasma membrane or centrally near the poles of nucleus. A mitochondrion has characteristic ultrastructural features that can easily be recognized under an electron microscope. The mitochondrial volume of a cell varies for different smooth muscle cells - being 7-8 % of the cell volume in rat aorta and renal artery and 3.5-4 % in guinea pig taenia coli (Gabella, 1976; Osborne-Pellegrin, 1978). The main function of the mitochondria is to generate ATP via oxidation-reduction metabolism. Mitochondria may also accumulate Ca^{2+} under non-physiological conditions. However, recent evidence suggests that there also may be some physiological role for the mitochondria in Ca^{2+}

mobilization (Gunter, Gunter, 1994; Gunter et al., 1998), 426}, this will not be discussed here further.

1.2.1.4 Contractile apparatus: The contractile apparatus in smooth muscle consists of thick, thin, and intermediate filaments. These filaments are made of myosin, actin plus tropomyosin, and desmin or vimentin respectively. The diameter of these filaments vary as follows: 15, 6-8, and 10 nm respectively. The sliding filament model proposed for contraction of skeletal muscle also is applicable to the smooth muscle. The tension in smooth muscle is generated from a change in the overlap between the thick and thin filaments resulting from a cross-bridge interaction (Shoenberg, Needham, 1976). The regulation of smooth muscle contraction will be discussed in *Section 1.2.2.*

Myosin is a hexameric protein which consists of two heavy chains and four light chains. Each heavy chain is a 200 kDa protein. Two light chains are 20kDa proteins and the other two are 16 kDa. Each heavy chain has a tail region embedded in the thick filament and a head portion which contains an active site for ATP hydrolysis (Stull et al., 1991). The light chains are located in each head and regulate ATPase activity. Actin, in contrast, is a smaller protein (42 kDa). It activates Mg^{2+}-ATPase activity of myosin under certain conditions (e.g. low ionic strength solution). The thin filaments are anchored to the dense bodies of the plasmalemma and thus form a part of the cytoskeleton (Somlyo et al., 1983). The ratio of thin to thick filaments is greater in smooth muscle (10-15:1) than in skeletal muscle (2:1) (Hartshorne, 1980). Within the various smooth muscles, the arterial muscle contains approximately double the amount of actin compared to non-arterial

muscle (50 mg/g vs 27.5 mg/g cell wet weight respectively) (Murphy et al., 1977).

1.2.1.5 Extracellular matrix: Collagen is the main protein of the extracellular space in smooth muscle. Smooth muscle has a higher collagen content than skeletal muscle (Gabella, 1979). Besides providing a cytoskeletal support, collagen fibrils may act as Ca^{2+} binding sites. However its role in regulating extracellular Ca^{2+} during muscle contraction and relaxation is not understood. The basal lamina is another extracellular structure that covers the entire surface of the smooth muscle cell. It is approximately 20 nm in thickness, and it is separated from the plasma membrane by an electron-lucent space. Although the basal lamina is not well defined, it contains glycoproteins and other glycoaminoglycans (Ross, 1975). The basal lamina and the plasma membrane together may regulate ion fluxes across the cell membrane.

1.2.1.6 Cell to cell junctions: In smooth muscle, the main types of connections between adjacent cells are through gap junctions, intermediate junctions, and close oppositions. Gap junctions (nexus) are present in most smooth muscles, and act as a link between adjacent cells to communicate and exchange information (Daniel et al., 1983).

1.2.2 Regulation of smooth muscle contraction:

Myosin phosphorylation is a key step in initiating the actin-myosin cross bridge cycle of the smooth muscle in response to neuronal or hormonal stimulation. Myosin light chain kinase, cAMP-dependent protein kinase A or protein kinase C can all phosphorylate myosin, however activation by myosin light chain kinase appears to be the predominant pathway (Nishizuka, 1992; Nishizuka, Nakamura, 1995; Walsh et al., 1994; Walsh et al.,

1996). Myosin light chain kinase can be activated by Ca^{2+}-calmodulin and hence one can think of pathways for the regulation of smooth muscle contraction as being Ca^{2+}-dependent or Ca^{2+}-independent.

Four Ca^{2+} ions bind to each calmodulin molecule and induce a conformational change that allows it to activate myosin light chain kinase. The ternary complex of 4 Ca^{2+}-calmodulin-myosin light chain kinase catalyzes the phosphorylation of Ser-19 on the two myosin 20 kDa light chains (Walsh et al., 1994). This phosphorylation reaction initiates the myosin head-cycling (cross-bridge cycle) with actin filaments and leads to the development of the contractile force. Smooth muscle relaxation can occur by reversing the phosphorylation reaction by decreasing $[Ca^{2+}]_i$ which leads to Ca^{2+} dissociation from calmodulin and subsequently inactivates the myosin light chain kinase (Kitazawa et al., 1989). A myosin light chain phosphatase dephosphorylates the myosin light chain to complete the cycle. Thus, $[Ca^{2+}]_i$ plays a major role in contracting smooth muscle via the myosin light chain kinase pathway.

Many factors may produce Ca^{2+} sensitization or desensitization of myosin phosphorylation. The Ca^{2+} sensitization mechanisms are not completely understood but they may involve guanosine triphosphate (GTP) binding proteins. Phenylephrine can induce contraction in permeabilized vascular smooth muscle strips with subthreshold concentrations of Ca^{2+}. This response was potentiated with GTP or GTPγS and inhibited with GDPβS (Kitazawa et al., 1989; Kitazawa et al., 1991). Ca^{2+} desensitization can occur upon exposing the cells to a high Ca^{2+} concentration. The mechanisms of this

desensitization may involve activation of Ca^{2+}/Calmodulin-dependent kinase II. Ca^{2+}/Calmodulin-dependent kinase II can catalyze myosin light chain kinase phosphorylation which leads to a decreased Ca^{2+}/Calmodulin-dependent kinase II affinity of the myosin light chain kinase (Walsh et al., 1994).

Vascular smooth muscle can also contract following agonist stimulation (e.g. endothelin) via Ca^{2+}-independent mechanisms. The main mechanism suggested involves the Ca^{2+}-independent isoforms of protein kinase C (Oriji, Keiser, 1996; Shimamoto et al., 1992b). These mechanisms are discussed in *Section 1.4.4.2.*

1.3. Intracellular Ca^{2+} regulation:

Intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ is critical in regulating vascular smooth muscle tone as discussed in *Section 1.2.2.1.* In resting smooth muscle, $[Ca^{2+}]_i$ is in the range of 50-200 nM and it increases during a contraction. In contrast, the extracellular $[Ca^{2+}]$ is approximately 1 mM and a high concentration of Ca^{2+} is also sequestered in the SR. Several transporters and channels in the plasma membrane and SR are implicated in the precise control of $[Ca^{2+}]_i$ (Himpens et al., 1995; Horowitz et al., 1996). The focus of this section is on the role of SR in regulating $[Ca^{2+}]_i$ either through Ca^{2+}-sequestration by the SR/ER Ca^{2+}-pump (SERCA pump) or through Ca^{2+} release via the IP_3 and ryanodine sensitive channels.

1.3.1. SR/ER Ca^{2+}-pump:

As discussed in *Section 1.2.1.2,* SR is an enclosed membrane network that functions as an internal Ca^{2+} store. The SR membrane contains a high density of Ca^{2+}-

pump molecules which transport Ca^{2+} from the cytosol to the SR lumen. This Ca^{2+}-pump helps to maintain a Ca^{2+} gradient across the SR membrane and to decrease cytosolic [Ca^{2+}] during muscle relaxation. The Ca^{2+}-pump hydrolyzes ATP and uses the energy of this hydrolysis for Ca^{2+} transport. The pump molecule exists in two conformational states: E1 and E2. Ca^{2+} binds to E1 and initiates its phosphorylation with the formation of a β-aspartyl phosphate intermediate (E-P). A transition then occurs from E1 to E2 where the affinity for Ca^{2+} decreases by more than 1000-fold, releasing Ca^{2+} into the SR lumen (Missiaen et al., 1991).

The SR Ca^{2+} pump is encoded in the SERCA gene family (Sarco/Endoplasmic Reticulum Ca^{2+} pump) which consists of three genes: SERCA1, 2, and 3. The SERCA pump structure has been characterized mainly using the adult fast twitch skeletal muscle pump, but the high degree of homology between different isoforms suggests similar conformations for all the SERCA pumps (Lompre et al., 1994). Fast twitch muscle expresses SERCA1. The SERCA2 gene is expressed in slow-twitch skeletal muscle, cardiac muscle, smooth muscle and non-muscle cells, while SERCA3 is expressed mainly in intestine, lung and spleen and vascular endothelium. Alternative splicing of SERCA2 transcripts creates mRNAs which differ only in the 3'-end region . These mRNAs encode two SERCA2 proteins: SERCA2a and SERCA2b. SERCA2a is expressed mainly in cardiac and slow twitch skeletal muscles while SERCA2b is expressed in most other tissues including smooth muscle (Missiaen et al., 1991).

SERCA2 regulation in smooth muscle may occur via the low molecular weight

protein phospholamban or by direct phosphorylation of the pump molecule (O'Donnell, Owen, 1994). Phospholamban inhibits SERCA2 pump activity and phosphorylation of phospholamban reverses this inhibition. Phospholamban can be phosphorylated by cAMP-dependent protein kinase, protein kinase C or by Ca^{2+}/calmodulin kinase II. Ca^{2+}/calmodulin kinase II can also phosphorylate ser-38 of SERCA2 directly to produce its activation (Dealfonzo et al., 1996; Eggermont et al., 1988).

1.3.2 Ca^{2+} release from SR :

Ca^{2+} accumulated in SR in vascular smooth muscle can be released upon stimulation with various agonists including endothelins. Two types of Ca^{2+} channels are known to be present in the SR of different cells: IP_3-dependent and ryanodine sensitive channels. The IP_3-dependent channels (also termed IP_3-receptors or IP_3R) play a more important role in the signal transduction in smooth muscle cells, whereas the ryanodine sensitive channels whose opening may be triggered by cADP-ribose or by Ca^{2+} play a larger role in skeletal and cardiac muscle (Marks, 1997). The focus of this section is on the structure and function of IP_3-dependent channels.

1.3.2.1 IP_3 -induced Ca^{2+} release: An activation of phospholipase C upon agonist stimulation of a receptor hydrolyzes phosphotidylinositol 4,5-bisphosphate (PIP_2) to give IP_3 and diacylglycerol. IP_3 acts as a second messenger to release Ca^{2+} from the SR by opening IP_3 channels in the SR plasma membrane, while diacylglycerol mainly activates protein kinase C (Berridge, 1993).

IP_3R channels are tetramers with each subunit being ~300kDa. Three forms of

IP_3R have been reported based on cDNA cloning: IP_3R1, IP_3R2, and IP_3R3. Vascular smooth muscle expresses mainly IP_3R1 (Marks et al., 1990). The IP_3R protein has three domains: a ligand binding site near the – terminus, a coupling domain which links the IP_3 binding site to the Ca^{2+} release activation channel, and a C-terminal region with 6 putative transmembranous domains. There are also two putative tyrosine phosphorylation sites - one adjacent to the IP_3 binding site and the other near the C-terminus (Mignery et al., 1990). The phosphorylation of IP_3R can occur either by itself or via the protein kinases A, C, G, and by Ca^{2+} calmodulin-dependent kinase II (Ferris et al., 1991; Komalavilas, Lincoln, 1994; Nakade et al., 1994). Tyrosine phosphorylation was recently shown to activate IP_3 Ca^{2+} channels in the brain and T-cells (Jayaraman et al., 1996). However, its role in vascular smooth muscle cells is yet to be determined.

$[Ca^{2+}]_i$ has a biphasic effect on Ca^{2+} release via IP_3R1. Below 300 nM, Ca^{2+} stimulates the release of Ca^{2+}, and above this concentration it inhibits the release (Ehrlich et al., 1994). Two main mechanisms have been suggested to explain the change in sensitivity of the IP_3-induced Ca^{2+} release. In the first mechanism, the induction of Ca^{2+} release by IP_3 increases with increasing luminal $[Ca^{2+}]$ (Missiaen et al., 1992). The exact mechanism for sensing luminal Ca^{2+} is not clear, since there is no Ca^{2+} binding domain in the IP_3R facing the SR lumen. However, proteins such as calsequestrin or calreticulum have been suggested to mediate this Ca^{2+} sensing pathway. The second mechanism is based on a heterogeneity of the IP_3R molecules within the same cell causing the variation in sensitivity (Berridge, 1993). The heterogeneity may occur due to the expression of

different IP_3R molecules within the same cell. Alternative splicing or post-translational modifications such as phosphorylation or autophosphorylation may produce these different types of molecules (Hirose, Iino, 1994).

1.3.2.2 Ca^{2+} induced Ca^{2+} release (Ryanodine sensitive channels): In skeletal muscle, extracellular Ca^{2+} entry via voltage operated Ca^{2+} channels increases cytosolic Ca^{2+} which in turn triggers release of Ca^{2+} from the SR. This phenomenon is termed Ca^{2+} induced Ca^{2+} release (Fabiato, 1983). The Ca^{2+} induced Ca^{2+} release regulates the $[Ca^{2+}]_i$ and muscle contraction more profoundly in cardiac and skeletal muscle compared to the smooth muscle. The Ca^{2+} induced Ca^{2+} release channels can pharmacologically opened by cADP-ribose, caffeine, or the plant alkaloid ryanodine (Berridge, 1993; Galione et al., 1993). Ryanodine sensitive channel receptors (RyRs) are twice the size of the IP_3R. They are tetrameric, each subunit being ~560 kDa. Three forms of RyRs have been reported in vascular smooth muscle: RyR1, RyR2, and RyR3. In smooth muscle, the Ca^{2+} induced Ca^{2+} release has been reported in very few studies (Saida, K., 1981; Saida, K, 1982). It is initiated by membrane depolarization or agonist-receptor interaction. The role of the Ca^{2+} induced Ca^{2+} release mechanism in the smooth muscle cells under physiological conditions is still not well defined (Marks A. et al., 1997).

1.4 Endothelins:

1.4.1 Endothelin Structure:

Endothelins (ET) are a family of potent vasoactive polypeptides which are synthesized primarily in endothelial cells (Yanagisawa et al., 1988). Mammalian tissues

contain three isoforms of endothelins: ET-1, ET-2 and ET-3. These isoforms also have different vasoconstrictor potencies: ET-1 > ET-2 > ET-3. Of all the ET isoforms, ET-1 is shown to be primarily released from the endothelial cells and has an important role in regulating vascular function. The various isoforms are similar in structure but are encoded by three distinct genes (Inoue et al., 1989). The amino acid sequences of ET-1, ET-2 and ET-3 are shown in Fig. 1. ET-1 contains 21 amino acids of which residues 1, 3, 11 and 15 are cysteines. Cysteine 1 forms a disulfide bridge with 15 and cysteine 3 with 11. The ET-1 molecule has a hydrophobic tail and a terminal tryptophan residue. ET-2 and ET-3 also have the same number of amino acids and resemble ET-1 in their structures. However, ET-2 has a total of 2 amino acids substituted at positions 6 and 7 while ET-3 has a total of 6 amino acids substitutions at residues 2, 4, 5, 6, 7 and 14. Sarafotoxin, a toxic polypeptide produced by *Atractspis engaddensis,* has a similar structure as ET-1 and also elicits a potent agonist response in the presence of ET-1 receptors (Wollberg et al., 1988).

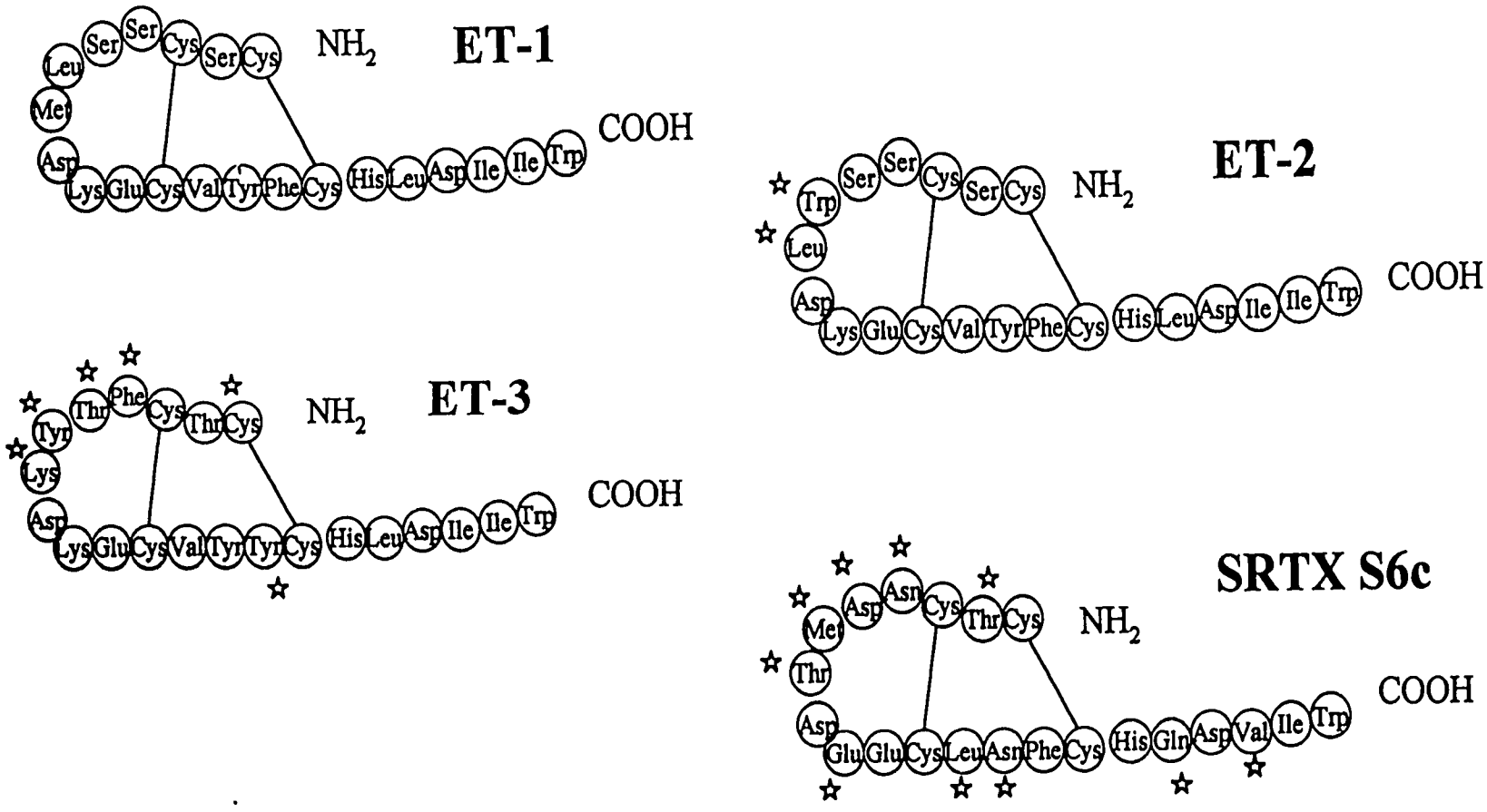


Fig. 1 <u>The structure of ET-1, ET-2, ET-3 and sarafotoxin (S6c):</u> the star sign indicates the amino acids which differ in the various ET isoforms compared to those in ET-1. Modified from Gray, Webb, 1996.

1.4.2 Biosynthesis of Endothelin-1:

Endothelins are synthesized in many tissues as preprohormone and then processed to form the active protein. ET-1 mRNA encodes a 203 amino acid preproendothelin (Pollock et al., 1995). A proteolytic cleavage, by an endopeptidase, converts the large preproendothelin to a 39 amino acid precursor peptide termed big ET-1. The big ET-1 is 100 times less active than ET-1 as a constrictor of isolated vascular tissue. Endothelin converting enzyme converts big endothelin to the 21 amino acid long mature ET-1. The enzyme is a neutral metalloprotease and is inhibited by phosphoramidon. The availability of endothelin converting enzyme in the endothelial cells may be rate limiting in the ET-1 formation. Endothelin converting enzyme has recently been purified, its cDNA cloned and characterized (Xu et al., 1994). However, little is known about the formation of the other ET isoforms.

1.4.3 Endothelin Receptor Types:

Studies on the effects of different ET isoforms on arterial blood pressure have revealed that there are two types of ET receptor binding sites on vascular smooth muscle and endothelial cells, named ET_A and ET_B (Moreland, 1994). There is also evidence for an ET_c receptor (Karne et al., 1993) however this evidence is weak and will not be discussed further. ET_A receptors present only in smooth muscle and mediate vasoconstriction. ET_B receptors, on the other hand, present in endothelium and smooth muscle. ET_B receptors in smooth muscle mediate vasoconstriction while the ET_B receptors in endothelium activate the endothelial NO synthase, thereby producing

vasodilatation (Hirata et al., 1993). The development of many specific agonists and antagonists has led to further characterization of these receptor types (Warner et al., 1994). The affinities of the ET isoforms as well as selected agonists and antagonists to both the receptor subtypes are shown in Table 1.

ET_A and ET_B receptors are present in vascular tissue, kidney, myocardium, lung, prostate and brain (Moreland, 1994). The distribution of these receptors varies among different tissues and with various species. It has also been shown that ET receptors may be up and down regulated during various pathological conditions. The cDNAs encoding human ET_A and ET_B receptors have been cloned (Adachi et al., 1991). The deduced amino acid sequences and locations of key sites in ET_A and ET_B receptors have also been identified. For more details see reference (Adachi et al., 1993).

Table 1. The relative affinities of various ET agonists and antagonists (peptide or non-peptide) to ET_A and ET_B receptors (Gray, Webb, 1996):

ET_A agonists	ET_A antagonists	ET_B agonists	ET_B antagonists
Peptide			
None	• BQ 123, IC_{50} ET_A = 7.3 nM ET_B = 18000 nM • BQ 518, IC_{50} ET_A = 1.2 nM ET_B = 55000 nM	• IRL 1620, IC_{50} ET_A = 16 pM ET_B = 200 pM • ET-3, IC_{50} ET_A = 4.5 nM ET_B = 70 pM	• BQ788, IC_{50} ET_A = 1300 nM ET_B = 1.2 nM • BQ 017, IC_{50} ET_A = 3.8 nM ET_B = 0.8 nM
Non-peptide			
	• PD 155080, IC_{50}, ET_A = 7.4 nM ET_B = 4500 nM		• Ro 46-8443, IC_{50}, ET_A = 7000 nM ET_B = 40 nM

1.4.4 Endothelin Signal Transduction:

Both ET_A and ET_B receptors are coupled to G-proteins which activate multiple pathways leading primarily to an increase in cytosolic [Ca^{2+}] (Gardner et al., 1992; Van Renterghem et al., 1988). The following discussion will focus on the ET_A receptor subtype which is predominant in vascular smooth muscle and whose signal transduction has been characterized to a greater extent than that for the ET_B receptor. ET binding to ET_A receptor in vascular smooth muscle produces vasoconstriction by a complex but well

coordinated signal transduction pathway as shown in Fig.2. The vasoconstrictor effect of ET may be accompanied by an increase in $[Ca^{2+}]_i$ or it may occur independently of such an increase (Oriji, Keiser, 1996; Shimamoto et al., 1992b).

1.4.4.1. ET_A-mediated Ca^{2+}-mobilization: ET_A activation produces an increase in $[Ca^{2+}]i$ by releasing Ca^{2+} from the SR and by extracellular Ca^{2+} entry. The activated ET_A receptor binds a G-protein, Gαq or Gα11 subunits and these, in turn, activate phospholipase C (PLC) (Sokolovsky, 1993). The activated receptor induces a dissociation of the G-protein to release the Gα subunits which activate the isozyme PLC-β1. Gα subunits hydrolyze GTP to GDP before interacting with PLC-β1. The activated PLC-β1 then hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1993). IP_3 acts as a second messenger to release Ca^{2+} via IP_3 sensitive Ca^{2+} channels located in the SR which may contribute to the contraction. The ET_A mediated contraction also depends on Ca^{2+} influx from the extracellular sites, and this Ca^{2+} influx may occur via voltage sensitive L-type Ca^{2+} channels and other pathway(s) whose exact nature are not well defined. However, a major route of Ca^{2+} influx appears to be through voltage sensitive L-type Ca^{2+} channels (Goto et al., 1989). What induces membrane depolarization is unresolved. Some investigators suggest that ET activates nonspecific cation channels while others suggest that ET-1 induces a chloride current which may contribute to the depolarization of the cell membrane and to Ca^{2+} influx (Chen, Wagoner, 1991; Van Renterghem, Lazdunski, 1993). Alternative mechanisms which have been suggested to explain the Ca^{2+} influx include, the Na^+/ Ca^{2+} exchanger, protein kinase

C mediated phosphorylation of the L-type Ca^{2+} channel or of an intermediate protein, or of receptor operated Ca^{2+} channels, or inhibition of adenylyl cyclase, or activation of the Na^{+}/H^{+} exchanger (Gardner et al., 1992; Hubel, Highsmith, 1995; Meyer-Lehnert et al., 1989; Shimamoto et al., 1992a).

1.4.4.2 Ca^{2+}-independent mechanisms: Vascular smooth muscle can contract following stimulation with ET-1 and other agonists without an increase in $[Ca^{2+}]_i$. The main suggested mechanisms involve activation of a protein kinase C isoform which is Ca^{2+}-independent or increased Ca^{2+} sensitivity of the contractile apparatus (Shaw et al., 1997; Shimamoto et al., 1992a; Simonson, Herman, 1993; Sudjarwo et al., 1995).

The protein kinase C activator phorbol ester induces slow developing but sustained contraction of vascular smooth muscle without increasing $[Ca^{2+}]_i$ or myosin phosphorylation (Jiang, Morgan, 1987; Sudjarwo, Karaki, 1995). The contraction is abolished by the protein kinase C inhibitors 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) or calphostin C. There are many isoenzymes of protein kinase C. The isoenzyme PKC-ϵ may be responsible for the Ca^{2+}-independent contractions in ferret aortic smooth muscle (Khalil, Morgan, 1992). The possible steps may involve: agonists binding to a receptor, receptor binding to G-proteins and activation of phospholipase C or D to produce diacyglycerol which in turn activates PKC-ϵ (Walsh et al., 1996). PKC can phosphorylate the thin filament protein caldesmon via the mitogen-activated protein (MAP) kinase cascade. Protein kinase C can also phosphorylate the thin filament protein calponin on ser-175 which can consequently mediate a Ca^{2+}-independent contraction.

Ca^{2+}/calmodulin protein kinase II was also shown to phosphorylate calponin. Calponin inhibits the actin-activated myosin ATPase and the phosphorylation of calponin reverses this inhibition (Adam et al., 1995; Winder, Walsh, 1990; Winder et al., 1993). Dephosphorylation by type 2A protein phosphatase restores the inhibitory property of calponin (Winder et al., 1992).

An increase in Ca^{2+} sensitivity of the contractile apparatus of rat aortic strips has also been reported in the absence of any PKC activation (Hori M., et al. 1993). In this study the activity of PKC was down regulated by prolonged treatment with phorbol esters. This down regulation of PKC inhibited the Ca^{2+} sensitization produced by phorbol ester but not by receptor agonists (e.g. ET-1 and prostaglandin $F_{2\alpha}$). This suggested the presence of two distinct pathways for Ca^{2+} sensitization in this tissue. Later, however, it was shown that atypical PKCs are not down regulated with phorbol esters (Hori M., and Karaki, H., 1998). Furthermore, inhibitors for atypical PKCs inhibit the Ca^{2+} sensitization induced by receptors agonists (Jensen PE. et al., 1996). Thus, Ca^{2+} sensitization by receptor agonists may depends on atypical PKC isoforms activation. It remains to be determined how ET-1 may contract vascular smooth muscle by Ca^{2+}-independent pathways with and without PKC activation.

Fig. 2 The ET-1 signal transduction pathways in vascular smooth muscle cells : L-type VOCC, voltage operated Ca^{2+} channels; ROCC, receptor operated Ca^{2+} channels; PLC, phospholipase C ; DAG, diacylglycerol; PKC, protein kinase C; CaM, calmodulin; MLCK, myosin light chain kinase; IP_3, Inositol 1,4,5 trisphosphate; SERCA, Sarco/Endoplasmic Reticulum Ca^{2+} pump; P-myosin, phosphorylated -myosin

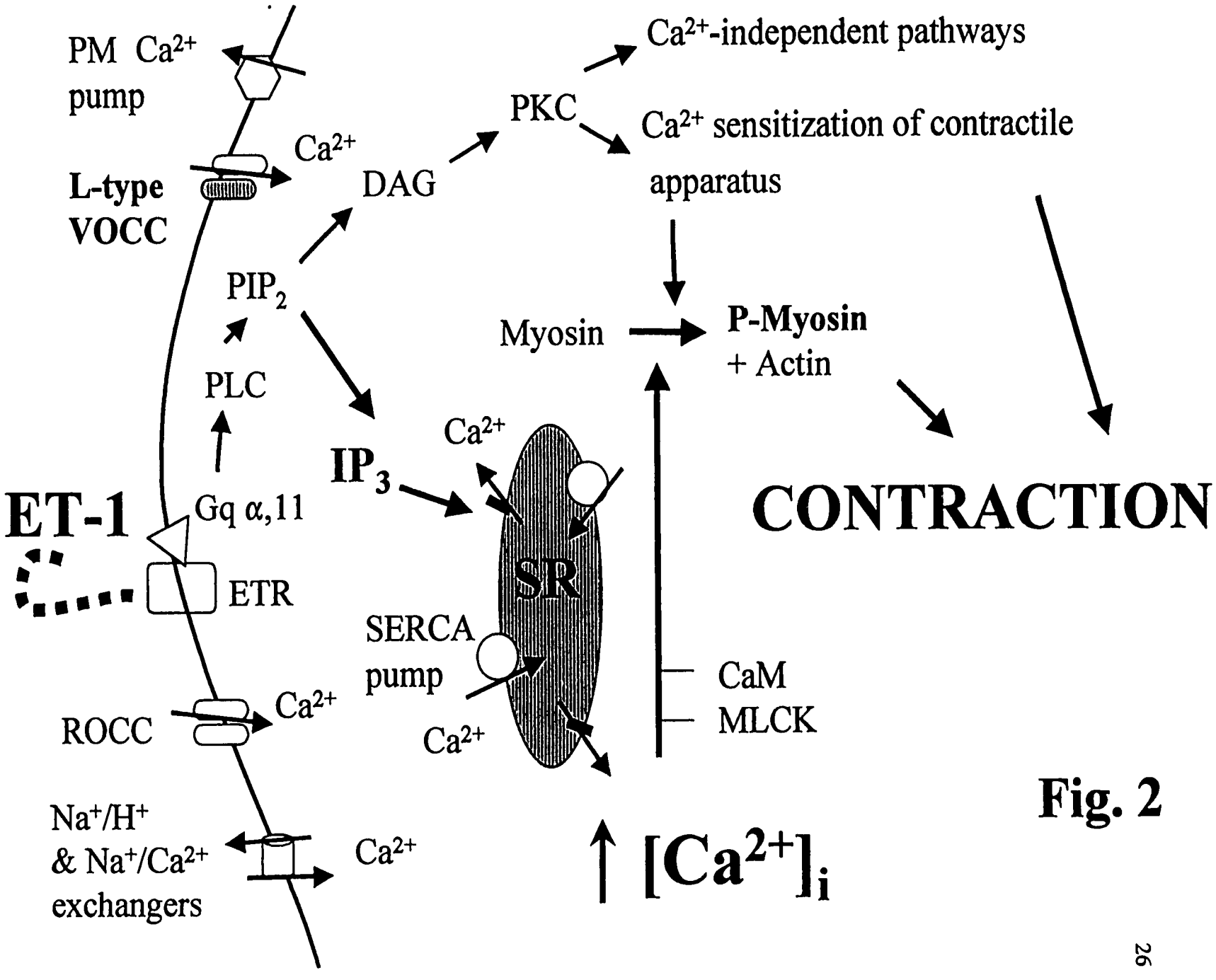

PM Ca^{2+} pump
Ca^{2+}
L-type VOCC
DAG
PKC
Ca^{2+}-independent pathways
Ca^{2+} sensitization of contractile apparatus
PIP_2
Myosin
P-Myosin + Actin
PLC
IP_3
Ca^{2+}
ET-1
Gq α,11
CONTRACTION
SR
ETR
SERCA pump
CaM
MLCK
Ca^{2+}
ROCC
Ca^{2+}
Na^+/H^+ & Na^+/Ca^{2+} exchangers
Ca^{2+}
$[Ca^{2+}]_i$

Fig. 2

1.5 Reactive Oxygen species (ROS)

1.5.1 Nature and formation of the ROS:

ROS are often loosely referred to as free radicals or oxygen free radicals. A ROS is a chemical compound that has an unpaired electron in an outer orbit of the oxygen atom. ROS are intermediates and by-products of cellular aerobic metabolism . For example superoxide radical ($\cdot O_2^-$) is produced from reduction of O_2 to H_2O. In the Haber-Weiss reaction, superoxide can reduce transition metals such as Fe^{3+} or Cu^{3+} (Goldhaber, Weiss, 1992).

$$\cdot O_2^- + M^{n+} \rightarrow O_2 + M^{(n-1)+}$$

Superoxide can be easily dismutated to hydrogen peroxide spontaneously or by endogenous superoxide dismutase. In the Fenton reaction, hydrogen peroxide can react with the reduced transition metal ions to form hydroxyl radical ($\cdot OH$):

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + \cdot OH + OH^-$$

ROS cause peroxidation of several lipids and proteins inside the cell . The active unpaired electron of the ROS is implicated in its cytotoxicity. The unstable electron can be donated to the cellular lipid and/or protein to form peroxy derivatives. This oxidation reaction can lead to changes in the nature of the lipids or to direct oxidation of sulfhydryl groups of proteins which ultimately result in several biological dysfunctions (e.g. damage cell membranes, and /or loss of enzyme activity, and /or damage to ion channels and amino acids and pumps) (Elmoselhi et al., 1994; Grover, Samson, 1989; Grover et al., 1992; Kilgore, Lucchesi, 1993; Kim, Akera, 1987).

1.5.2 ROS sources and cellular defense mechanisms against ROS:

Under physiological conditions ROS are generated mostly in the mitochondria where oxidation-reduction reactions occur and the metal containing cytochromes are present. In addition, Fe^{3+} formed due to the break down of hemoglobin in red blood cells also can catalyze the production of ROS (Dunford, 1987). Since in pathological conditions increased levels of ROS were demonstrated, other additional sources were suggested such as: neutrophils , endothelial cells, and as by products of arachidonic acid metabolism. The inflammatory reaction due to ischemia for instance, leads to an increase in the neutrophil infiltration . Several ROS are produced and stored in granules within the cytoplasm of the neutrophil and they are released upon inflammatory reactions which occur during ischemia (Granger et al., 1986; Kilgore, Lucchesi, 1993; Kim, Akera, 1987). Endothelial cells contain the enzyme xanthine oxidase which forms superoxide when it reacts with hypoxanthine and oxygen. Endothelial cells can also be a source of oxidative stress through the production of the reactive radical, nitric oxide ($NO^{\cdot}$), which is highly cytotoxic by itself or via the formation of other radicals (Zweier et al., 1988). During ischemia, the increase in $[Ca^{2+}]_i$ leads to the activation of some isoforms of protein kinase C which in turn stimulate arachidonic acid metabolism and produce ROS. An increase in oxygen tension during reperfusion following an ischemia is a major source of ROS (Kilgore, Lucchesi, 1993).

Many endogenous ROS scavenging mechanisms are present in the cell. These eliminate ROS and defend the cells from any cytotoxic effects of ROS (Lilley, Gibson,

1997). Superoxide dismutases (SODs) can breakdown superoxide to hydrogen peroxide which can then be decomposed by catalase. Reducing agents such as glutathione and ascorbic acid as well as enzymes to regenerate them play important roles in scavenging ROS (Petty et al., 1994).

1.5.3 Effect of ROS on coronary artery function:

Both *in vitro* and *in vivo* studies have shown that there is an increase in the concentration of ROS, such as hydrogen peroxide and superoxide during cardiac ischemia and reperfusion. The increase in ROS levels can lead to cardiac dysfunction and arrhythmia (Goldhaber, Weiss, 1992). One of the major injuries to cardiac and smooth muscle cells is an impairment of the Ca^{2+}-regulating mechanisms. It can result in intracellular Ca^{2+} overload. $[Ca^{2+}]_i$ overload may be due to a reduction in Ca^{2+} removal from the cell by the SR-Ca^{2+} pump, the mitochondria, the sarcolemmal Ca^{2+} pump, or the Na^{+}-Ca^{2+} exchanger (Grover, Samson, 1988; Grover et al., 1992; Kaneko et al., 1989; Kilgore, Lucchesi, 1993). Prolonged exposure to ROS damages the intracellular Ca^{2+} homeostasis of the smooth muscle. One of the proteins most sensitive to ROS is the SR-Ca^{2+} pump, which accumulates Ca^{2+} into the SR (Grover et al., 1992). The consequences of damage to this pump on the endothelin response in this tissue remain to be explored.

1.6. Rationale for the Model and the Proposed Experiments:

The increase in ROS levels during cardiac ischemia and reperfusion may cause several cardiovascular injuries. These injuries are particularly important in patients undergoing percutaneous transluminal coronary angiography (PTCA) and thrombolytic treatment following myocardial infarction (Grech et al., 1996). The injuries to the Ca^{2+}-regulating mechanisms in cardiac myocytes have been investigated extensively (Kaneko et al., 1989; Kilgore, Lucchesi, 1993; Kim, Akera, 1987). However, damage to the Ca^{2+}-regulating mechanisms in vascular tissues which are exposed first to the increased levels of ROS has not been examined. The mobilization of $[Ca^{2+}]_i$ in smooth muscle cells is critical in regulating vascular tone as well as in the development of many cardiovascular diseases. ROS damage the SR Ca^{2+}-pump in vascular smooth muscle. This damage affects responses to agents such as angiotensin II which mobilize the SR Ca^{2+}-pool. However, there are some discrepancies in these data. For example in de-endothelialized pig coronary artery the contractions induced by angiotensin II seem to be more sensitive to peroxide than expected from damage to the SR Ca^{2+}-pump (Grover et al., 1995). Furthermore, the angiotensin II induced contractions are extremely small in this tissue (Grover et al., 1994). It is not known whether there is a heterogeneity in the smooth muscle Ca^{2+}-pool, or if angiotensin II mobilizes only a small component of it. ET-1 induces a larger sustained vasoconstriction than angiotensin II in this tissue. Thus, ET-1 mediated contractions may mobilize $[Ca^{2+}]_i$ pools to a greater extent than angiotensin II does, via several pathways. Therefore, to resolve these issues, we examined the effects of

ET-1 on this artery. However, the action of ET-1 itself is quite complex.

1.6.1. The Model: To obtain the best results in our study, human coronary artery would be the ideal model. However, one can not get large amounts of human tissues and hence several animal models were developed as an alternative. We chose the pig coronary artery for the following reasons: (a) the gross anatomy of coronary artery and its anastomosis is similar in the two species (Patterson, Kirk, 1983), (b) many agents produce similar responses in pigs and humans (Davenport et al., 1995b; Elmoselhi, Grover, 1997), (c) we can routinely obtain large number of hearts from a slaughter house, (d) there has been substantial progress in terms of knowledge of the mechanisms in this model, (e) the studies on angiotensin II, which are the basis of this study, were also conducted in this model (Grover et al., 1995), (f) the anterior descending branch of the left coronary artery is one of the most common sites for arterial occlusion during myocardial infarction (Patterson, Kirk, 1983).

The rationale for using endothelins is that ET-1 is the most potent endogenous vasoconstrictor peptide known. In preliminary experiments, ET-1 gave a large contraction after angiotensin II; however, angiotensin II did not produce an additional contraction after ET-1. Therefore, ET-1 may use additional sources of Ca^{2+} compared to angiotensin II and the effects of ROS damage on these may be important.

1.6.3 Proposed Hypotheses and Experiments:

The overall theme of my thesis is that *exposing coronary artery smooth muscle to ROS damages its ET-1 mediated contractions.* The ET-1 induced contraction has been

shown to be mediated via ET_A and ET_B receptors in various vascular smooth muscles. The ET_A receptors were shown to be predominant in most of these tissues (Pollock et al., 1995). ET-1 induced contraction is mainly due to an increase in $[Ca^{2+}]_i$ although other pathways such as Ca^{2+} sensitization of contractile apparatus and Ca^{2+}-independent mechanisms may also play a role of ET-1 mediated contraction (Gardner et al., 1992; Van Renterghem et al., 1988). The increase in $[Ca^{2+}]_i$ may be due to Ca^{2+} released from intracellular Ca^{2+} stores via IP_3 -dependent Ca^{2+} channels, Ca^{2+} entry from extracellular stores via L-type Ca^{2+} channels and other pathways (Goto et al., 1989). However, the proportion of each of these pathways in ET-1 mediated contraction is not known. Furthermore, ROS have been shown to readily damage the SR Ca^{2+} pump and AngII induced contraction in pig coronary artery smooth muscle (Grover et al., 1992; Grover et al, 1995). However, their effects on ET-1 mediated contraction including IP_3-induced Ca^{2+} release in vascular smooth muscle has not been reported. Here, I have divided the overall theme of my thesis into four hypotheses. These hypotheses and the experiments proposed to test them are as follows:

Hypothesis 1. In pig coronary artery smooth muscle the ET_A-mediated contraction is more predominant than the ET_B-mediated contraction.

I tested this hypothesis by examining contractile response of de-endothelialized coronary artery rings to selective agonists and antagonists for ET_A and ET_B receptors. I also examined the effects of several agents on the ^{125}I-ET-1 binding to microsomes isolated from this tissue.

Hypothesis 2: A large part of the ET-1 induced contraction depends on Ca^{2+}-released from the SR.

I tested this hypothesis by examining contractions due to ET_A or ET_B receptor-activation under the following conditions: in Ca^{2+}-containing solutions, in Ca^{2+}-containing solutions with a voltage operated Ca^{2+} channel blocker, and in Ca^{2+}-free solutions. I also monitored the increase in $[Ca^{2+}]_i$ in smooth muscle cells cultured from this artery.

Hypothesis 3: ROS pretreatment of the smooth muscle cells cultured from pig coronary artery damages primarily the sequestration of Ca^{2+} into the SR and it has little effect on Ca^{2+} release from the SR or the ET-receptor binding.

I tested this hypothesis by treating cultured smooth muscle cells with peroxide or superoxide, permeabilizing them and then examining the $^{45}Ca^{2+}$-uptake into the SR and the IP_3-induced Ca^{2+} release from it. I also examined the effects of treating microsomes with peroxide and superoxide on the ET-1 binding to ET_A and ET_B receptors.

Hypothesis 4: ROS pretreatment of pig coronary artery smooth muscle damages mainly the SR Ca^{2+} release dependent component of the ET-mediated contraction.

I tested this hypothesis by pretreating the de-endothelialized arteries with peroxide and superoxide before examining their contractions by ET_A and ET_B-receptor activation in Ca^{2+}-containing and in Ca^{2+}-free solutions.

CHAPTER TWO

EXPERIMENTAL METHODS

2.1 Contractility Experiments:

This section contains methods for tissue dissection, measurement of isometric contractions, various solutions to study the effects of $[Ca^{2+}]$ and treatment with peroxide.

Fresh pig hearts were brought from a slaughter house in ice cold physiological saline solution (PSS). The hearts were kept in this solution until the dissection. The PSS contained the following in mM: 136.9 NaCl, 9.9 glucose, 9.9 Hepes-NaOH pH 7.4, 4.96 KCl, 1.97 $CaCl_2$, 0.47 $MgCl_2$. Slices of the ventricle containing the left descending coronary artery were removed and placed in Krebs' solution bubbled with 95% O_2 and 5% CO_2. The Krebs' solution contained the following in mM: 115 NaCl, 5 KCl, 22 $NaHCO_3$, 1.7 $CaCl_2$, 1.1 $MgCl_2$, 1.1 KH_2PO_4, 0.03 EDTA, and 7.7 glucose at pH 6.8. The tissues were pinned in a tray containing the bubbled Krebs' solution at 20-23 °C and the left descending coronary artery was dissected. The endothelium was removed by passing a piece of cotton thread through each artery.

Each artery was cut into 3 mm long rings which were mounted in an organ bath at 37°C. A tension of 3 g was applied to each ring and after 30 min the tension was readjusted to 3 g again. This tension was determined to be optimum in initial experiments. The removal of endothelium was frequently tested by the loss of relaxation to bradykinin.

After the tissues had been equilibrated at the applied tension, their contraction was monitored by adding 4 M stock KCl to attain a final concentration of 60 mM. The contraction produced by 60 mM KCl at the beginning of each experiment was used as a control. The tissues were then washed 4 times and incubated in Krebs' solution for 45 min before any effects of the agonists or antagonists were monitored. When Ca^{2+} free solutions were used, the tissues were washed 4 times in a Krebs' solution with 0.2 mM EGTA substituted for $CaCl_2$ and then incubated in the same solution for another 45 min. At the end of each experiment, the tissues were blotted and weighted. All the peptides used for the contraction were dissolved in ice-cold water, divided in small aliquots and stored at -70°C. Just before use, an aliquot was dissolved in the Krebs'solution. The force of contraction produced by each agent was expressed as mN or as a percentage of the force of contraction elicited by 60 mM KCl.

In the ROS treatment experiments, the tissues were incubated at the specified concentrations of peroxide for 30 min and then washed 4 times and incubated in Krebs' solution with or without Ca^{2+} for 45 min before measuring the effects of any agent. This protocol avoided any direct effects of peroxide on the tissues or assay solution, but measured the long term damage of peroxide on the tissues.

2.2 Ca^{2+}-uptake and -release in permeabilized cells

2.2.1 Smooth muscle permeabilization:

Pig coronary artery smooth muscle cells were isolated, cultured in 200 cm^3 flasks in passage four as described and characterized in the literature (Samson et al., 1991). The flasks were rinsed with PSS at 37°C. The cells were then trypsinized for 10 min in a solution containing 0.05% trypsin and 0.53 mM EDTA. The trypsinization was stopped by adding 20 ml PSS containing 10% fetal calf serum and 0.03% soybean trypsin inhibitor. The cells were centrifuged at 20-23°C at 500 rpm for 20 min. The cell pellets were washed with the permeabilizing solution (containing in mM: KCl 165, $MgCl_2$ 0.4, Na-azide 5, DTT 1, EGTA 1, morpholino-propane sulphonate-Na 20 at pH 6.8) without saponin and then suspended in it. The permeabilizing solution containing saponin was then added. The final cell count was 10^7/ml and the saponin concentration was 250 μg/ml. The cells were incubated with saponin at 25°C for 15 min and then placed on ice. The permeabilization of cells was tested by mixing a small volume of the cells with an equal volume of 0.4 % Trypan blue and the proportion of the stained cells was determined using a haemocytometer.

2.2.2 Ca^{2+}-Uptake Experiments:

The permeabilized cells were used to characterize sequestration of $^{45}Ca^{2+}$ by the SR Ca^{2+}-pump. This sequestration is dependent on $MgATP^{2-}$, stimulated by oxalate, and it

is also insensitive to azide which inhibits $^{45}Ca^{2+}$-uptake by the mitochondria. Typically 20 μl of permeabilized cell suspension containing 30-60 μg protein was added to a Ca^{2+}-uptake solution containing in mM: KCl 100, $CaCl_2$ 0.85, DTT 1, azide 5, ATP-Na 5, creatinine phosphate 20, $MgCl_2$ 5, EGTA-Na 1, imidazole-HCl 30 at pH 6.8 (pH at 37°C), oxalate 5 and creatinine phosphokinase (40 units/ml). Ca^{2+}-buffering computations were carried out using pH, temperature and ionic strength corrections as described previously but using a Ca^{2+}-EGTA association constant of $1.58x10^{11}$ M which we determined using the fluorescence dye Fluo 3. Under the previous Ca^{2+}-uptake solution $[Ca^{2+}] = 10$, $[MgATP^{2-}] = 4500$, $[Mg^{2+}] = 500$, and $[ATP] = 500$ μM. Various $[Ca^{2+}]$ were used by varying [EGTA], and $[MgATP^{2-}]$ by varying [ATP] and $[Mg^{2+}]$. The reaction mixtures were incubated typically at 37°C for 60 min and then filtered through 0.45 μm nitrocellulose filters under suction. The filters, under suction, were washed with 3x5 ml of a chilled solution containing in mM: 0.5 EGTA, 250 sucrose and 30 imidazole-HCl pH 6.8. The amount of $^{45}Ca^{2+}$ trapped in the filters was determined by scintillation counting. Several variations of this protocol were used as described in the Results.

2.2.3 Ca^{2+} Release Experiments:

In order to examine the IP_3-induced release of $^{45}Ca^{2+}$ from the SR, the permeabilized cells were first loaded with $^{45}Ca^{2+}$ and then diluted with a solution containing IP_3 to determine how much $^{45}Ca^{2+}$ was retained in the SR. The *standard Ca^{2+}*

loading was carried out at 37°C for 18 min, by incubating 50 μl of a suspension of permeabilized cells (100-150 μg protein plus 7500 units/ml of creatinine phosphokinase) with 100 μl of a Ca^{2+}-uptake solution containing in mM: 100 KCl, 0.1 $CaCl_2$, a trace amount of $^{45}CaCl_2$, 1 DTT, 0.5 Na-azide, 1.2 ATP, 1.5 $MgCl_2$, 10 creatinine phosphate and 30 imidazole-HCl at pH 6.8 at 37°C. An equal volume of the *standard release solution* was then added. The standard release solution contained in mM: 300 KCl, 30 imidazole, 0.192 EGTA. Routinely, the release with 50 μM IP_3 or 10 μM A23187 in the standard solution was also examined. Ca^{2+}-buffering calculations were carried out using pH, temperature and ionic strength corrections as described in Section 2.4.2. Under these conditions adding the standard release solution to the standard loading solution gave $[Ca^{2+}] = 0.1$ μM. The reaction mixture was filtered through 0.45 μm nitrocellulose filters under suction after 30 sec of adding the release solution. The filters were washed 3 times with 5 ml of chilled wash solution. The wash solution contained in mM: EGTA 0.5, sucrose 250 and imidazole-HCl 30 at pH 6.8. Using scintillation counting the amount of ^{45}Ca trapped in the filters was measured. Modifications of these conditions were used in some experiments as specified in the Results.

In order to examine the effects of peroxide pretreatment, the cells ($2x10^6$ cells/ml) were suspended in a solution containing in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, and 10 HEPES-Na pH 7.4 plus different concentrations of hydrogen peroxide. The cells

were pretreated with hydrogen peroxide for 30 min at 37°C. Catalase (30000 U/ml) and 10% fetal calf serum were added to quench the reaction and the cells were centrifuged to remove peroxide, catalase and the serum. The cell pellets were permeabilized and then used for the Ca^{2+} uptake and release experiments. For treating the cells with superoxide, a xanthine + bovine milk xanthine oxidase system was used. Bovine milk xanthine oxidase was purified and assayed as described previously (Grover, Samson, 1988). The cells were treated with 0.3 mM xanthine + different concentrations of xanthine oxidase +1 unit/ml of catalase (to quench hydrogen peroxide formed upon spontaneous dismutation of superoxide) for 30 min at 37°C and used for the permeabilization. The conversion of xanthine to uric acid was determined at 22-24°C in a reaction mixture containing the following: 50 mM K-phosphate pH 7.4, 0.3 mM xanthine along with the enzyme. One enzyme unit equals the amount of enzyme which can convert 1 μmol of xanthine to uric acid in 1 min under these conditions. A value of 11000 $M^{-1}cm^{-1}$ was used for a difference between the extinction coefficients of xanthine and uric acid (Grover, Samson, 1988).

2.3. Binding Experiments:

Binding experiments were conducted using ^{125}I-labelled ET-1 (Amersham) binding to freshly isolated microsomes from pig coronary artery smooth muscle in order to determine the proportions of ET_A and ET_B receptors present in this tissue and the effects of ROS on them.

In order to prepare the microsomes, 30 pig hearts were dissected per experiment to obtain smooth muscle from the left anterior coronary arteries. The dissected smooth muscle layer was placed in a solution containing in mM: 250 sucrose + 50 imidazole (HCl to pH 6.4) + 1 PMSF and 2 DTT. The smooth muscle was chopped, passed through a sieve-press with 1 mm holes under 2000 psi pressure and homogenized using a Polytron PT20 at a setting of 5. The homogenate was centrifuged for 10 min at 4°C. Powdered KCl was added gradually while stirring to attain a final concentration of 0.7 M. After 10 min of stirring at 0°C, the homogenate was centrifuged for 45 min at 140,000 g. The pellet was suspended in 50 mM Tris-HCl pH 7.4 and used in the binding experiment.

The ^{125}I-ET-1 binding was carried out using the freshly isolated microsomes in binding buffer solution containing in mM: 50 Tris-HCl pH 7.4, 0.5 PMSF, 1 1,10-phenanthroline, 1 EDTA, 0.01 $CaCl_2$, 0.01 Mg Cl_2, and 0.1% BSA. A trace amount of ^{125}I- ET-1 (~ 4 pM/ml) was added to the binding buffer solution and then the solution was divided into three parts. The first part was used to determine total binding and hence nothing else was added to it, second part for monitoring non-specific binding and hence a saturating concentration of unlabelled ET-1 (200 nM) was added to it, and the third part was used to determine the binding of ET-1 to either ET_A and ET_B receptor types and a saturating concentration of unlabelled BQ123 (250 nM) was added to it. The binding reaction was started by adding 200 μl from different parts of the radioactive solutions to

50 μl from the microsomes (~ 1 mg/ml) in each sample. The samples were incubated in a slow shaking water bath at 25°C for 4 h. The binding reaction was terminated by adding 5 ml of ice-cold Hepes-Tris buffer (pH 7.4) containing 0.3% BSA in each tube and the content was filtered immediately by vacuum suction through a GF/F glass filter which had previously been soaked in Hepes/Tris buffer. Each filter was then washed three times with ice-cold Hepes/Tris buffer, and the radioactivity on the filter was measured using a gamma counter. Specific binding was computed as total binding minus the mean value of non-specific binding. The binding to ET_B sites was determined as the binding in the presence of BQ123 minus the non-specific binding. Binding to the ET_A sites was the total binding minus binding in the presence of BQ123.

The isolated microsomes were treated either with specified concentrations of peroxide or with a superoxide generating system for 30 min at 37°C. The superoxide generating system contained 0.3 mM xanthine + different concentration of xanthine oxidase and catalase (100 U/ml) to decompose the hydrogen peroxide formed upon dismutation of superoxide. The microsomes were then centrifuged at 500,000 g for 15 min. The pellets were rinsed superficially with 50 mM Tris/HCl buffer (pH 7.4) and then resuspended in it. Two xanthine oxidase preparations were used in these experiments - a purified bovine milk xanthine oxidase or a commercial bacterial xanthine oxidase. The mean value of the specific binding in the absence of XO was taken as 100% and all

the other values were expressed relative to it.

2.4. $[Ca^{2+}]_i$ measurements:

$[Ca^{2+}]_i$ measurements were carried out to determine the effects of ROS pretreatment on ET-1 induced $[Ca^{2+}]_i$ -transients in smooth muscle cells cultured from pig coronary artery. Cultured cells were formed on glass cover slips. The glasses coated with the cells were rinsed 3 times in 2 mM $CaCl_2$-Maklouf buffer. The 2 mM $CaCl_2$-Maklouf buffer contained the following in mM:115 NaCl, 25 Hepes, 12 glucose, 5.8 KCl , 2.2 KH_2PO_4, 1 $CaCl_2$, 0.6 $MgCl_2$. The cells were placed in the same medium for 0 or 300 μM hydrogen peroxide for 30 min at 37°C. The peroxide was removed by rinsing the cover glasses 3 times in the same buffer and the cells loaded with Fluo-3 AM (acetoxy-methyl ester) in the above buffer as described previously. After the loading, the cover glasses were placed in 2 ml of the 1 mM $CaCl_2$-Maklouf buffer solution in a stirred thermostated cuvette at 37°C for fluorescence measurement using SPEX fluorolog 112 model. The fluorescence was monitored at excitation and emission wavelengths of 490 and 530 nm, respectively. A typical experiment consists of the following steps: monitoring fluorescence for 60 -120 s until it became stable, examining the effect of 50 nM ET-1 and then calibrating the fluorescence as described previously (Grover et al., 1995).

2.5. Protein estimation:

Protein estimation was carried out in 96-well microtiter plates using Bradford reagent . Typically 2,10 and 20 μl of a protein sample were pipetted per well in triplicates. Bovine serum albumin was used as a standard. Bradford reagent was diluted 5-fold in water and 250 μl was added per well. Absorbance of the plates was monitored using a microplate reader with a 595 nm narrow band pass filter.

2.6. Data Analysis:

All the values given are means ± SEM of the indicated number of replicates. Null hypotheses were tested using Student's t-test and p values of < 0.05 were considered to negate the null hypotheses. Except where specified, the curve fitting for hyperbolic and non-hyperbolic kinetics was carried out using FigP (Biosoft, USA) for fitting curves by nonlinear regression into the equation:

$v=(V_{max}.S^n)/(K_{0.5}{}^n+S^n)$

Here v is the reaction velocity, S is the substrate concentration, V_{max} is the maximal velocity, $K_{0.5}$ is the affinity constant and n is the Hill-coefficient. Correlation coefficients (r) were also determined using FigP by linear regression of the data where specified. The t values $(r(N-2)^{0.5} / (1-r^2)^{0.5}$, N-2 equals the degrees of freedom) calculated from these coefficients were used to determine the confidence limits to test null hypotheses. Data for the competition binding were analysed using GraphPad Prism, version 2 (GraphPad

Software, San Diago, CA, USA).

2.7. Materials:

Bacterial xanthine oxidase, BQ788, Probenecid, EGTA, A23187, ATP and phosphocreatinine were purchased from Sigma Chemical Company (St. Louis, MO, USA). IP_3 was from RBI and creatinine phosphokinase was obtained from Boeringer Manaheim Canada (Laval, Quebec). ET-1, BQ123, IRL1038, and IRL1620 were purchased from the American Peptide Company (Sunnyvale, CA, USA). ^{125}I-ET-1 was from Mandel Scientific Company (Guelph, ON, Canada). GF/F filters were from Schleicher& Schnell (New Hampshire, USA). Fluo-3/AM, and 4-Br-A23187 were from TEFLABS (Texas Fluorescence Laboratories), Austin, Texas, USA. Bradford reagent was purchased from Biorad. All the other reagents were of analytical grade and purchased from standard commercial sources.

CHAPTER THREE

RESULTS

In order to examine the effect of ROS on intracellular Ca^{2+} mobilization of the ET-1 response in coronary artery smooth muscle, we first characterized the ET-1 signal transduction pathways at four different organizational levels, and then examined the effects of hydrogen peroxide and superoxide on these pathways. Each of the four levels are presented here in a separate section as follows: (3.1) ET-1-mediated contraction in de-endothelialized coronary artery; (3.2) ET-1 induced increase of cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ in cultured smooth muscle cells; (3.3) ET-1 binding to ET_A and ET_B receptors in isolated microsomes from coronary arteries; and (3.4) IP_3-induced Ca^{2+} release in permeabilized smooth muscle cells.

3. 1 Effect of peroxide on ET-1-mediated contractions in de-endothelialized coronary artery:

Since ET-1-induced contraction in smooth muscle may be mediated via ET_A and ET_B receptors, we first characterized the ET_A and ET_B components of the ET-1 mediated contraction, and then identified the different Ca^{2+} pathways for each receptor type. Having done this, we examined the effects of treating the artery rings with hydrogen peroxide on ET_A-, and ET_B-mediated contraction in Ca^{2+}-containing and Ca^{2+}-free medium. Several agonists and antagonists have been developed to characterize ET-1

receptor types. In our study we used the following compounds: ET-1, a non-selective agonist for ET_A and ET_B receptors; IRL1620, a selective agonist for the ET_B receptors; BQ123, a selective antagonist for ET_A receptors; and BQ788, a selective antagonist for the ET_B receptors. We also conducted some experiments with BQ1038 which was meant to be an ET_B antagonist, but this agent was subsequently withdrawn by the company. The results with BQ1038 are not included here.

3.1.1 Characterization of ET_A and ET_B receptor mediated contraction:

3.1.1.1 Concentration-dependent responses of ET-1 and IRL1620:

To determine the components of the smooth muscle contraction mediated by ET_A and ET_B receptors in de-endothelialized coronary artery rings, we carried out concentration-dependent experiments for both ET-1 and IRL1620 (Fig.3). The force for all the contractions was expressed in milli-Newton (mN) and sometimes as a percentage of force obtained with 60 mM KCl. The de-endothelialized artery rings contracted in a graded manner by increasing concentrations of ET-1 with an $EC_{50} = 5.5 \pm 0.8$ nM. The maximum contraction was obtained using 50 nM ET-1 and it was 44.8 ± 4 mN. Thus, this concentration of ET-1 was used in all subsequent experiments. IRL1620 also contracted the de-endothelialized artery rings with an $EC_{50} = 13.3 \pm 3$ nM. The maximum contraction was 11.1 ± 1 mN and it was obtained using 100 nM IRL1620. The absolute value for maximum contraction of KCl was 45.5 ±3.4 mN (n = 75). ET-1 and IRL1620 maximum contractions were also recorded as a percentage of KCl contraction as 98.6 ± 9% (n = 10), and 24.8 ± 2.7% (n = 18) respectively. These results indicate that both ET_A and ET_B

receptors contribute to mediating the contraction in coronary artery smooth muscle, but with different efficacy.

3.1.1.2 Effects of BQ123 and BQ788 on ET-1-, and IRL1620-mediated contraction:

To identify the component of the ET-1 contraction mediated by the ET_A receptors, we examined the inhibition and relaxation effects of BQ123 (ET_A antagonist) on the ET-1-mediated contraction. The pA_2 value of BQ123 is reported to be 7.4 (Ihara et al., 1992). As expected, in the presence of 5 μM BQ123, 50 nM ET-1 gave only 7.7 ± 0.5 mN or 16.8 ±1 % of the KCl contraction. In another set of experiments, after adding 5 μM BQ123 at the peak of the sustained ET-1 contraction, only 9.5 ± 1.4 mN or 20.6 ±3% of the ET-1-mediated contraction remained at the end [Fig.4 A, B]. Thus the ET_A receptors mediated contraction accounts for approximately 80% of ET-1 mediated contraction according to both ET-1 inhibition and relaxation experiments.

Similarly, the ET_B component of the ET-1-mediated contraction was determined using BQ788 (ET_B antagonist) inhibition on the IRL1620- (ET_B agonist) mediated contraction. Since the contraction by IRL1620 was not sustained, the relaxation experiments could not be carried out. The pA_2 value reported for BQ788 is 8.8 (Ishikawa et al., 1994). As expected, in the presence of 200 nM BQ788, 87± 4% of the contraction produced by 100 nM IRL1620 was inhibited (Fig.5 A, B).

Thus, these results indicate that approximately 80% of ET-1 induced contraction is due to activation of the ET_A receptors and 20% is mediated by the ET_B receptors.

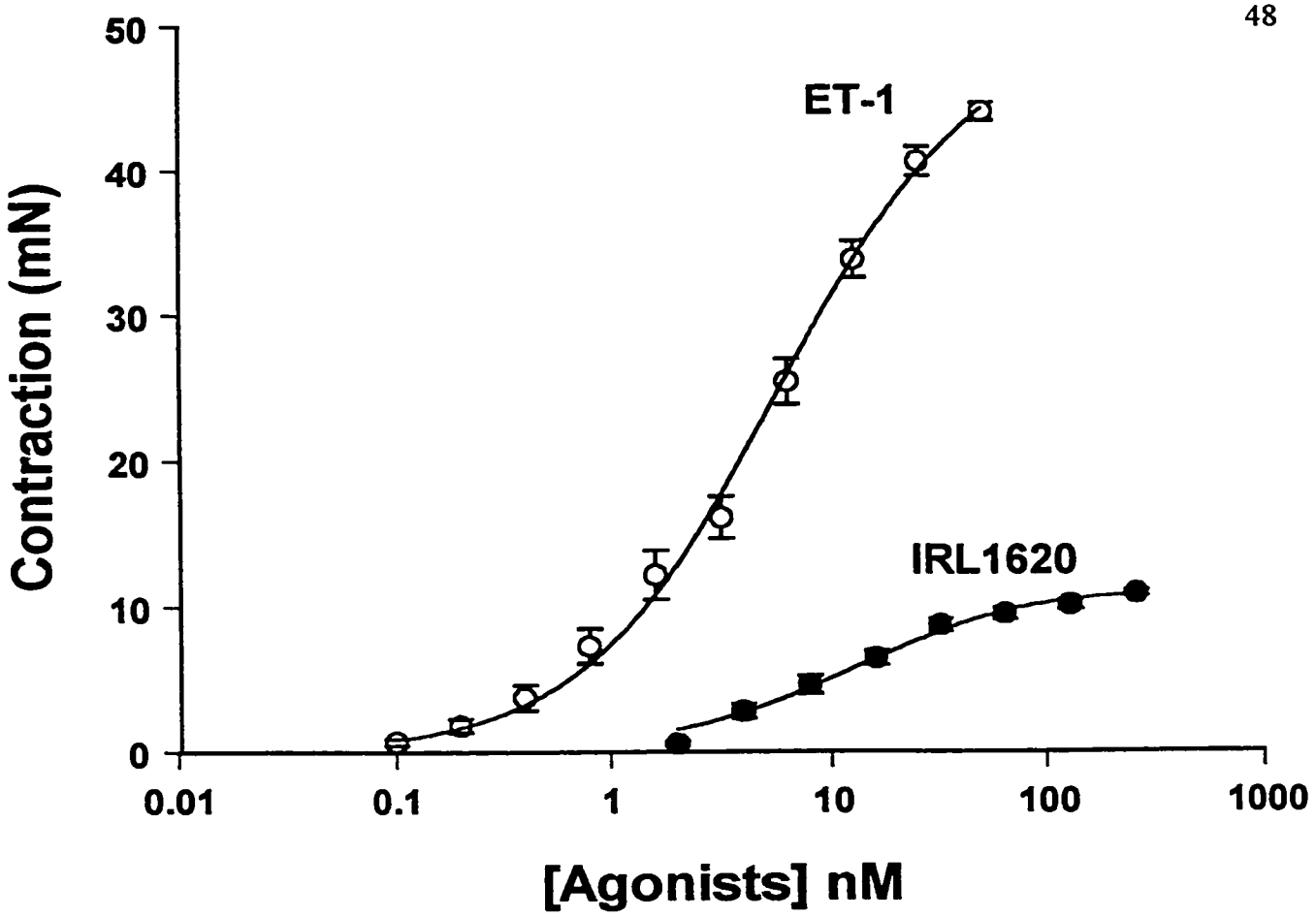


Fig.3 ***Concentration response curves for ET-1 and IRL1620 in de-endothelialized pig coronary artery rings :*** The maximum response of ET-1 was 44.8 ± 4 mN or 98.6 ± 9 of KCl contraction (n = 10) with EC_{50} = 5.5 ± 0.8 nM. While maximum contraction of IRL1620 was 11.1 ± 1 mN or 24.5 ± 2.7% of KCl contraction (n= 18) with EC_{50} = 13.3± 2.8 nM. The values shown are mean ± SEM of 4 experiments. The data were analyzed following a single site equation for fitting curves using FigP (Biosoft, USA). Taken from Elmoselhi et al, 1997.

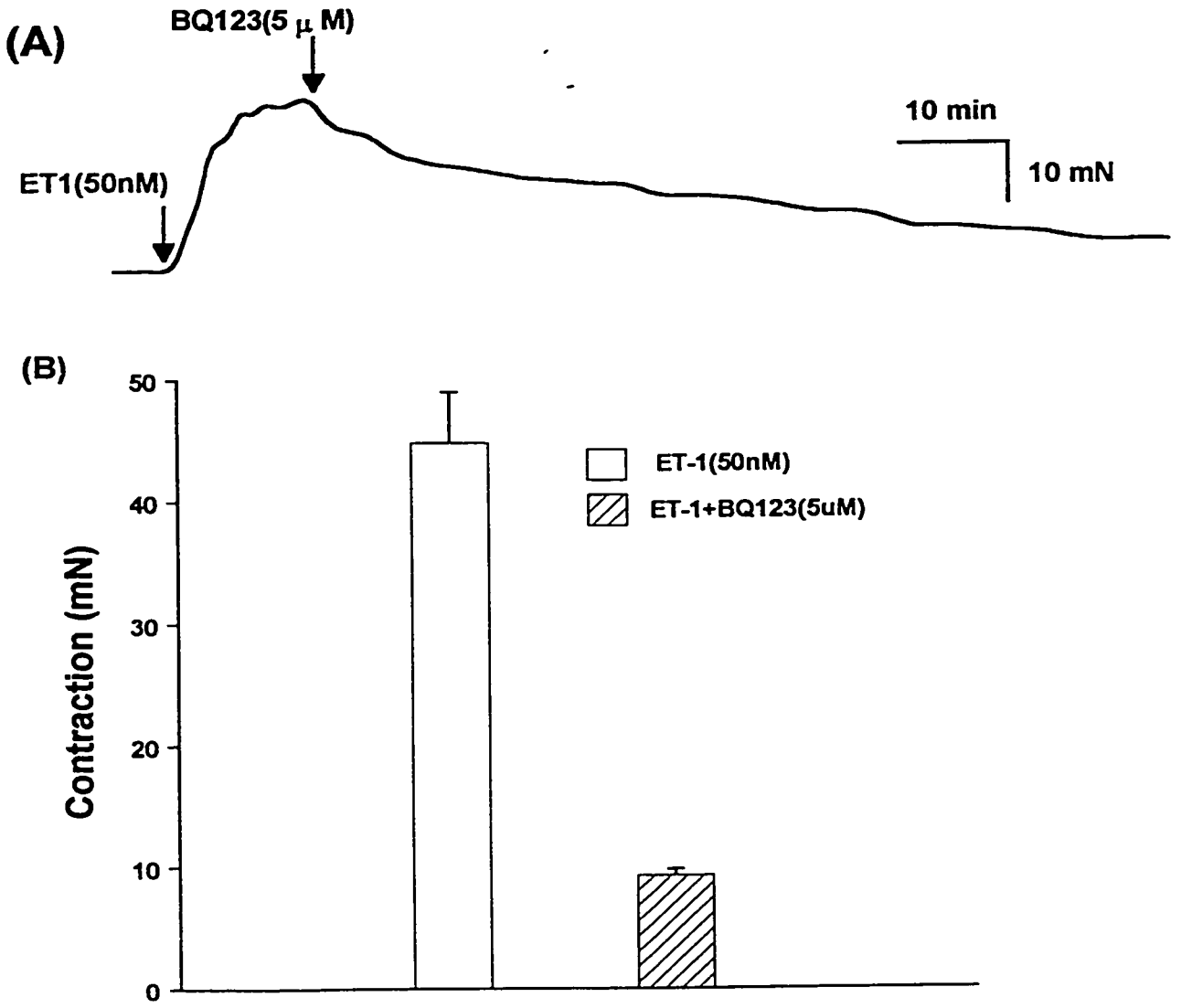
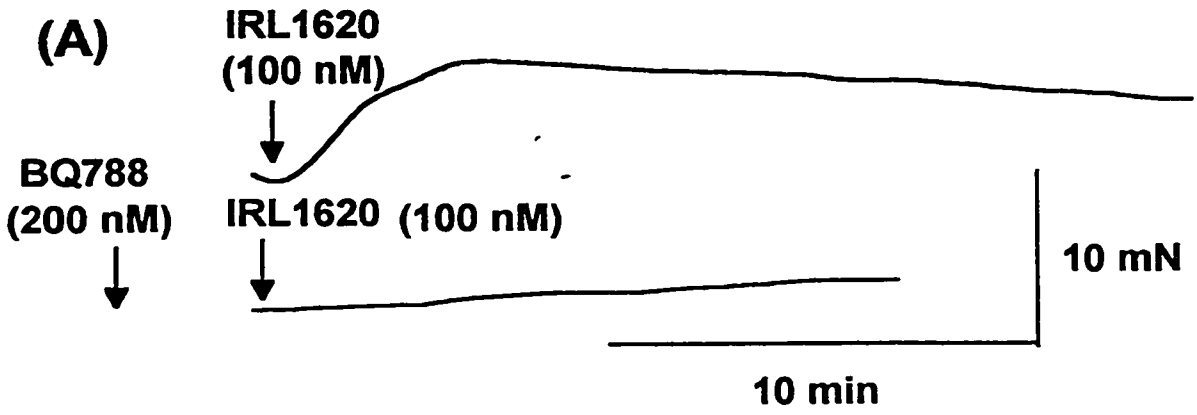


Fig. 4 ***The effect of BQ123 (5μM) on ET-1 (50nM) precontracted de-endothelialized pig coronary artery rings:*** (A) typical tracing of the relaxation of the ET-1 mediated contraction by BQ123 over 90 min, (B) after adding BQ123, 23.4 ± 6% of the ET-1 contraction remained. The values are the mean ± SEM of the data of 3 experiments. Taken from Elmoselhi et al, 1997.



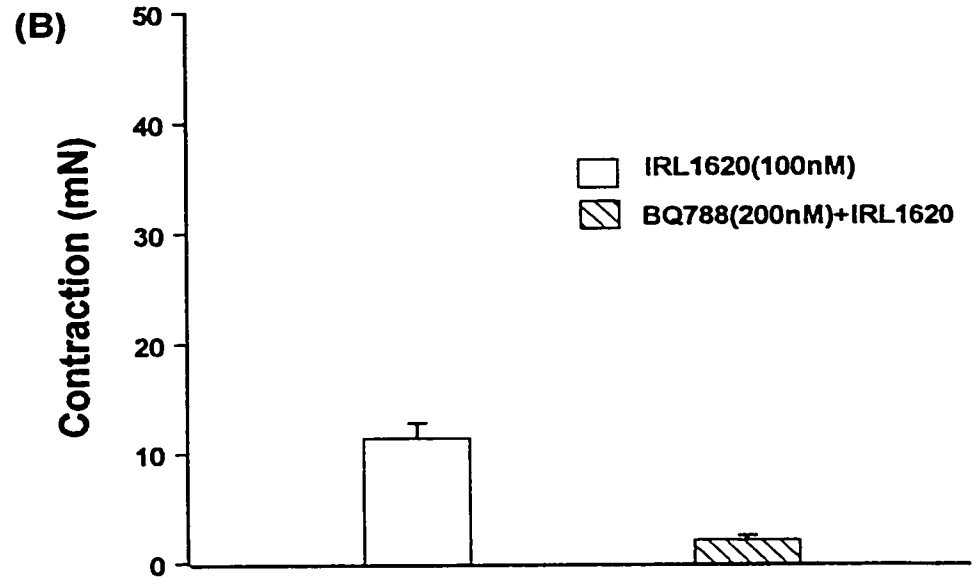


Fig. 5 ***The effect of BQ788 (200nM) on the IRL1620 (100nM) mediated contraction in de-endothelialized pig coronary artery rings:*** (A) typical tracing of IRL1620-mediated contraction. More than 85% of the IRL1620-mediated contraction was inhibited by BQ788, (B) BQ788 inhibited 87± 4% of IRL1620-mediated contraction expressed as mN. The values are expressed as the mean ± SEM of the data of 3 experiments. Taken from Elmoselhi et al, 1997.

3.1.2 Ca^{2+} pathways of ET-1-, and IRL1620-mediated contraction:

ET-1 mediates vasoconstriction mainly by increasing $[Ca^{2+}]_i$ and by other mechanisms such as protein kinase C and tyrosine kinase activation. ET-1 stimulates $[Ca^{2+}]_i$ increase via intracellular Ca^{2+} release through opening of the IP_3-induced Ca^{2+} channels on the sarcoplasmic reticulum, and also via Ca^{2+} entry from extracellular spaces through the L-type Ca^{2+} channels and via other undefined routes (Pollock et al., 1995). However, the contribution of each pathway to the ET-1-mediated contraction is not known. To assess Ca^{2+} mobilization due to ET_A-, and ET_B-mediated contraction, we monitored the ET-1 and IRL1620 induced contractions in a Ca^{2+}-containing solution where the increase in $[Ca^{2+}]_i$ can occur via both Ca^{2+} entry and intracellular Ca^{2+} release. We also measured ET-1 and IRL1620 responses in Ca^{2+}-containing solution in the presence of nitrendipine which blocks the L-type Ca^{2+} channel component of Ca^{2+} entry pathways. We further examined the ET-1 and IRL1620 responses in Ca^{2+}-free medium, containing 0.2 mM EGTA to chelate extracellular Ca^{2+}, thus only the contribution by the intracellular Ca^{2+} release would be observed.

3.1.2.1 Ca^{2+} mobilization in ET-1-mediated contraction vs AngII-mediated contraction:

In preliminary experiments, we compared the ET-1-, vs angiotensin II- (AngII) mediated contractions as an indication for the relative contribution to Ca^{2+} mobilization. AngII is another vasoactive peptide that mediates contraction of smooth muscle and has been used in our laboratory and others to investigate intracellular Ca^{2+} mobilization and to test the effects of different agents on this mobilization. As shown in Fig.6, after maximum

contraction had been obtained by adding 50 nM ET-1 two times, the addition of 0.5 μM AngII did not produce any further significant contraction 0.3 ± 0.2 mN (n= 4). However, after the maximum contraction obtained by AngII by adding 0.5 μM AngII twice, 50 nM ET-1 contracted the artery rings close to their full response 39 ± 9 mN (n = 4). Furthermore, it has been shown previously that AngII produces only a very small contraction when the tissues are placed in a Ca^{2+} free medium containing EGTA (Grover et al., 1994). In contrast, ET-1 produced approximately 20% of its response in the Ca^{2+} free medium compared to the Ca^{2+} containing medium. A possible explanation of this result is that ET-1 mobilizes a larger portion of the intracellular Ca^{2+} stores than AngII. This property of the ET-1 response was one of the main reasons for its selection to characterize its Ca^{2+} mobilization pathways and examine the effects of ROS on it.

3.1.2.2 ET-1-mediated contraction in different [Ca^{2+}] containing media:

In Ca^{2+} containing medium the maximum ET-1-mediated contraction was 44.8 ± 4 mN (n=75). In the presence of 10 μM nitrendipine ET-1 mediated contraction was 24.3 ± 3 mN (n=14), which indicates that approximately half the ET-1 mediated contraction could be abolished by blocking the L-type Ca^{2+} channels (Fig.7). In Ca^{2+} free medium containing EGTA, however, ET-1-mediated contraction was 6.1 ± 0.7 mN (n=14) indicating that only a small portion of the total ET-1-mediated contraction was due to the mobilization of intracellular Ca^{2+} stores (Fig.7). The pEC_{50} values of the ET-1 were similar in the three conditions (8.2±0.1, 8.2±0.1, and 8.1± 0.2 respectively) suggesting that the same receptor types were involved in the different Ca^{2+} pathways. Thus the relative

contribution of the EGTA-sensitive and insensitive components was independent of the concentrations of the agents used. BQ123 (5 μM) relaxed 88 ± 2 % of the precontracted artery rings with 50 nM ET-1 in Ca^{2+} free medium, indicating that most of the ET-1-mediated contraction in Ca^{2+} free medium occurred via the activation of the ET_A receptors. These results indicate that ET_A -mediated contraction uses mainly extracellular Ca^{2+} pools (~ 85%) via the L-type Ca^{2+} channels and other undefined route(s), as well as a small contribution (~ 15%) from intracellular Ca^{2+} pools.

3.1.2.3 Ca^{2+} pathways utilized by IRL1620 -mediated contraction:

The IRL1620-mediated contraction was 11.1 ± 1 mN in the Ca^{2+} containing medium. In the Ca^{2+} containing medium with nitrendipine IRL1620-mediated contraction was 7.5 ±0.9 mN and 5.2 ± 1 mN was obtained in the Ca^{2+} free medium (Fig. 8). Furthermore, BQ788 inhibited more than 85% of IRL1620-mediated contraction in both Ca^{2+} containing medium and Ca^{2+} free medium, while BQ123 did not induce any significant inhibition. Thus, the ET_B mediated contraction uses the same Ca^{2+} pathways as ET_A, but it uses a greater proportion of the intracellular Ca^{2+} stores compared to those used by the ET_A receptors.

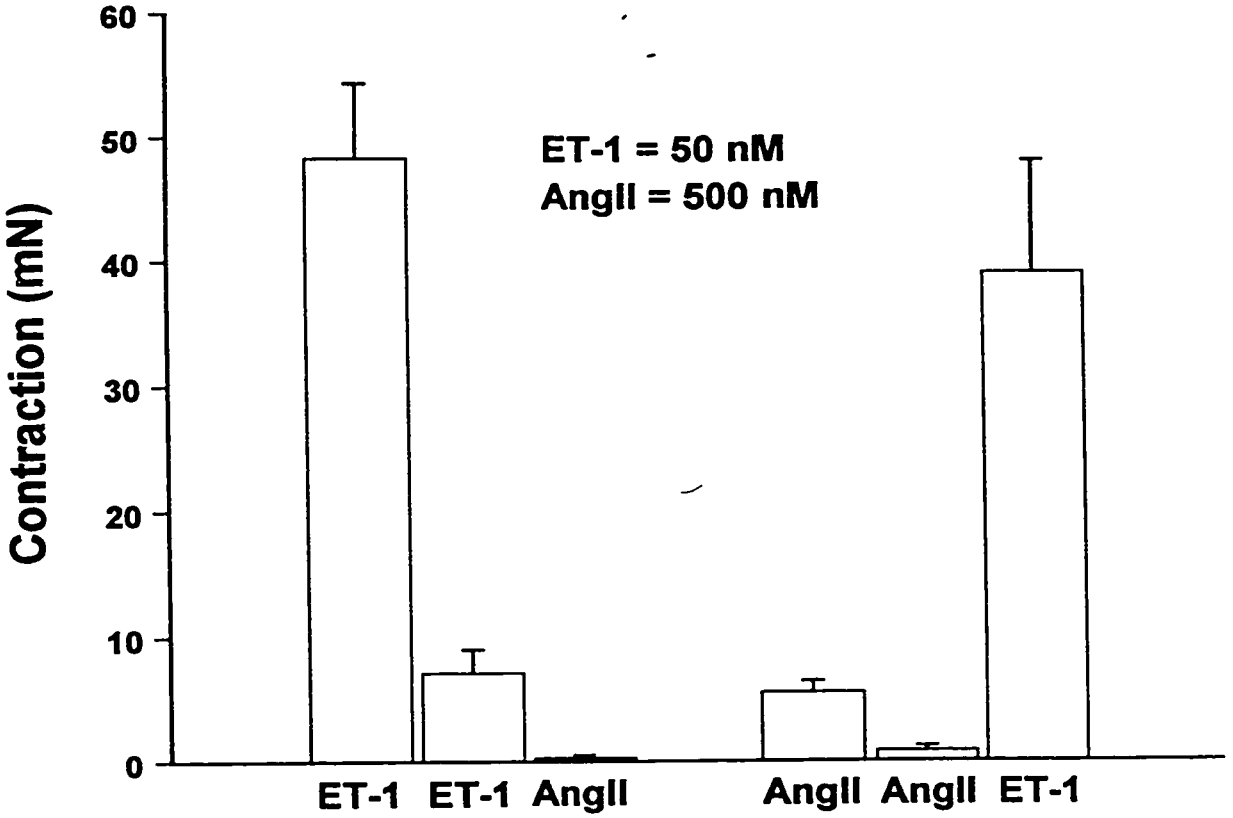


Fig. 6 ***ET-1- vs AngII- mediated contractions in de-endothelialized pig coronary artery rings:*** Contractions obtained upon consecutive additions of ET-1 (50 nM) twice, then AngII (0.5 μM) or AngII (0.5 μM) twice, then ET-1 (50 nM): AngII addition gave almost no contraction following ET-1-mediated contraction 0.3 ± 0.2 mN (n= 4), while ET-1 addition gave almost its full contraction when it was added following AngII 39 ± 9 mN (n= 4).

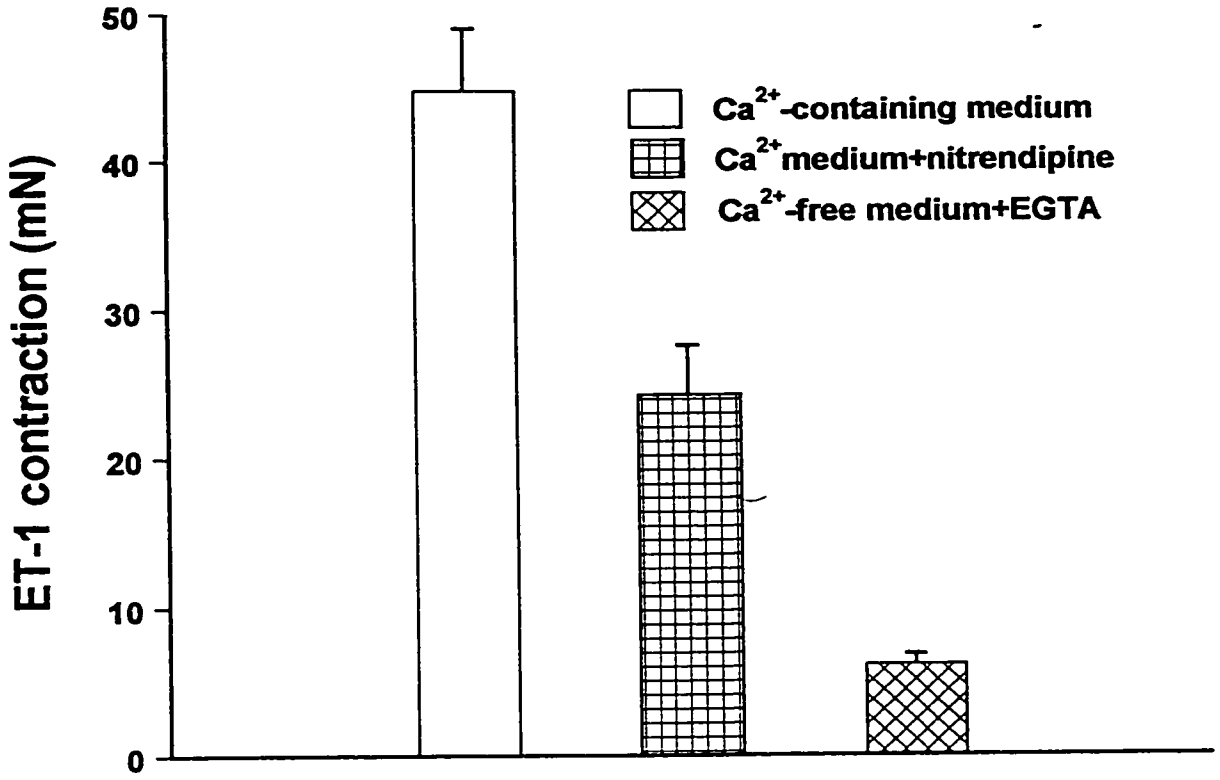


Fig.7 ***ET-1-mediated contraction of de-endothelialized pig coronary artery rings in different Ca^{2+} containing media:*** ET-1-mediated contraction in Ca^{2+}-containing medium (n= 75), Ca^{2+} containing medium + 10 μM nitrendipine (n= 14), and in Ca^{2+}-free medium (n= 14). The ET-1-mediated contractions were 44.8 ± 4, 24.3± 3, and 6.1± 0.7 mN respectively. The values are the mean ± SEM of the data of 9 experiments. Taken from Elmoselhi et al, 1997.

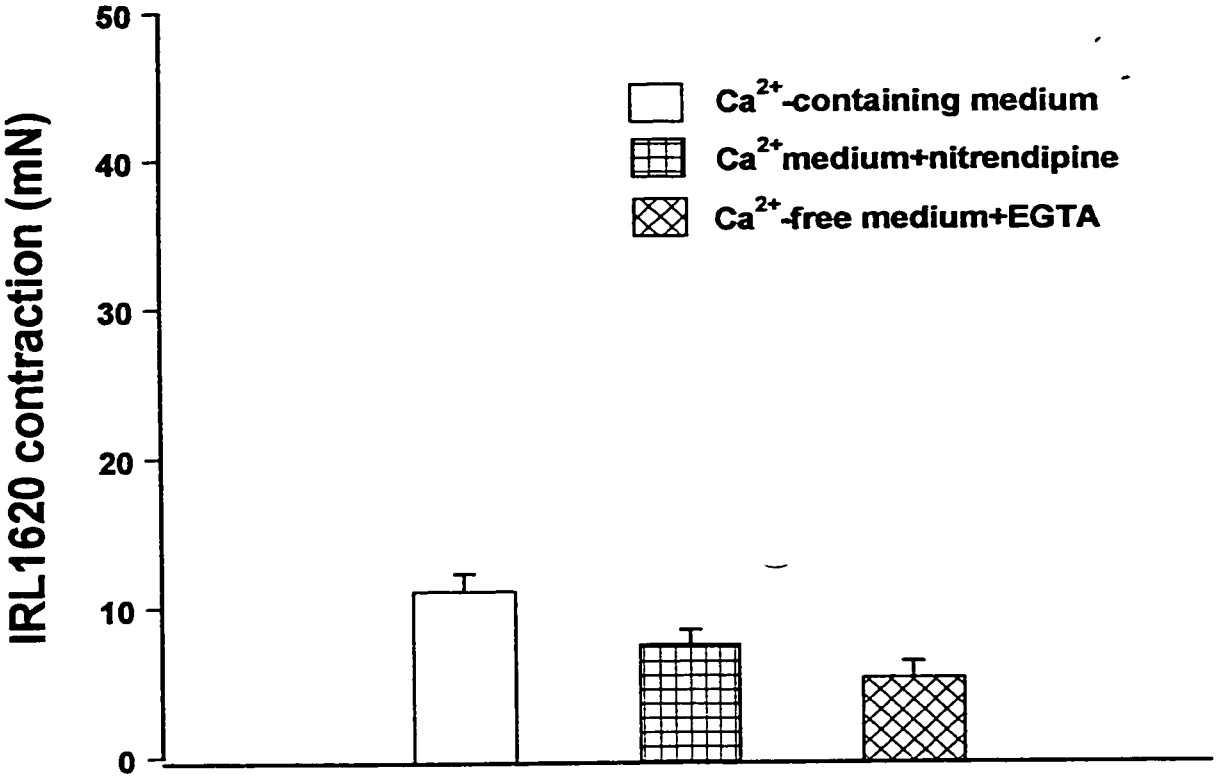


Fig.8 ***IRL1620-mediated contraction of de-endothelialized pig coronary artery rings in different Ca^{2+} containing media:*** IRL1620-mediated contraction in Ca^{2+}-containing medium, Ca^{2+} containing medium + 10 μM nitrendipine, and in Ca^{2+}-free medium. The IRL1620 contraction were 11.1 ±1, 7.5± 0.9, and 5.2± 1 mN respectively. The values are the mean ± SEM of the data of 9 experiments. Taken from Elmoselhi et al, 1997.

3.1.3 Effects of peroxide on ET-1-, and IRL1620-mediated contractions:

In these experiments the de-endothelialized coronary artery rings were treated with different concentrations of hydrogen peroxide for 30 min at 37°C. After the peroxide treatment the tissues were washed with normal Krebs's solution in order to recover, incubated in either Ca^{2+} containing medium or Ca^{2+} free medium for at least 45 min, and then the contraction induced by either ET-1 or IRL1620 was monitored. This protocol was used to avoid any immediate effects of peroxide on the tissues and assay solutions, but instead measured the long term damage of peroxide in the vascular smooth muscle. Maximum responses of ET-1 (50 nM) and IRL1620 (100 nM) were monitored after peroxide pretreatment by using a saturating concentrations of each agonist.

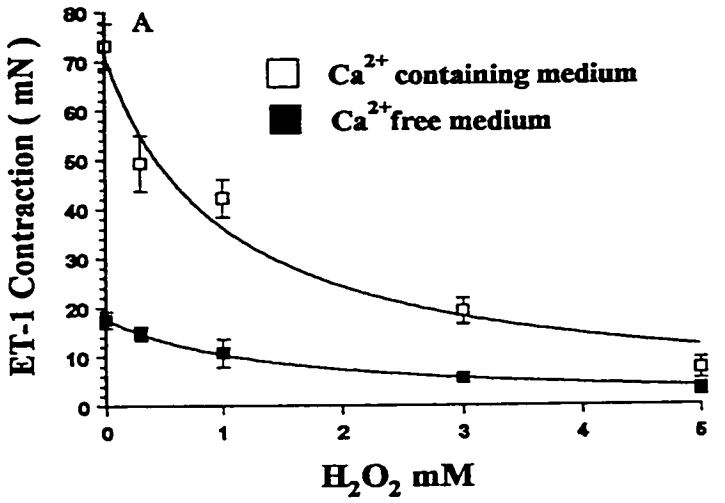
3.1.3.1 Effects of hydrogen peroxide on ET-1 induced contraction in Ca^{2+}-containing and Ca^{2+}-free medium:

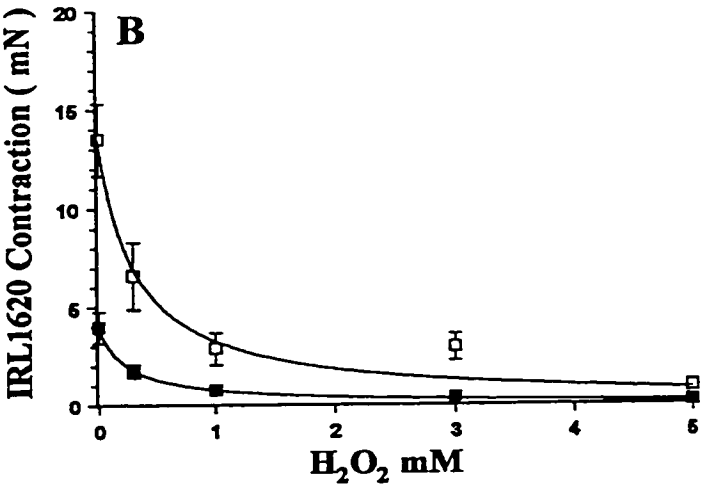
Peroxide concentrations were varied from 0.3 to 5 mM. For each peroxide concentration the mean ± SEM of the ET-1-mediated contraction of at least 12 replicates was calculated. ET-1 mediated contractions (50 nM) were inhibited in both Ca^{2+}-containing and Ca^{2+}-free medium by peroxide with IC_{50} of 1.0 ± 0.3 and 1.4 ±0.1 mM respectively (Fig.9A). There was no significant difference between these two values ($p > 0.05$). This indicates that ET-1 mediated contraction in vascular smooth muscle is resistant to damage by peroxide. However, the question here is which step in the ET-1-mediated contraction signal transduction pathway is resistant to peroxide.

3.1.3.2 Effect of peroxide on IRL1620 contraction in Ca^{2+}-containing and Ca^{2+}-free medium:

The effects of peroxide were examined on IRL1620-mediated contractions. The IRL1620-mediated contractions (100 nM) were more sensitive to damage by peroxide compared to those induced by ET-1 with IC_{50} of 0.32 ± 0.08 and 0.25 ± 0.01 mM in both Ca^{2+}-containing and Ca^{2+}-free medium respectively (Fig. 9B). These two values did not differ significantly from each other ($p > 0.05$). However, the IC_{50} values for peroxide treatment for IRL1620 were significantly lower than those obtained with ET-1 ($p < 0.05$) (Fig.7 C). The differences in the sensitivity between the ET_A-, and the ET_B-mediated contraction to peroxide in Ca^{2+} containing medium are consistent with the results on the Ca^{2+} pathways (3.1.2) suggesting that the ET_A mediated contraction may use different pathways than that of the ET_B . These may be protein kinase C and/or tyrosine kinase and /or change in Ca^{2+} sensitivity to contractile proteins (Oriji, Keiser, 1996; Sudjarwo, Karaki, 1995). These pathways, which could be involved in ET-1 mediated contraction are however resistant to damage by peroxide. Furthermore, the difference in the peroxide sensitivity between ET_A-, and ET_B -mediated contraction in Ca^{2+} free medium may suggest that the contractions mediated by these receptors may utilize different compartments of the intracellular Ca^{2+} stores. This may indicate that the ER is heterogeneous as has been suggested in a later section (3.4.4).

Fig. 9. ***Effect of hydrogen peroxide on ET-1-, and IRL1620-mediated contraction of coronary artery smooth muscle in Ca^{2+}-containing and Ca^{2+}-free medium :*** Each point shown is the mean ± SEM of the contraction of 10-20 tissues except the data point as 0 mM peroxide which represents 20 -30 tissues. **A:** The ET-1-mediated contractions (50 nM) were inhibited in Ca^{2+}-containing and Ca^{2+}-free medium with IC_{50} of 1.0 ±0.3 and 1.4 ± 0.1 mM respectively. The two values of the IC_{50} did not differ significantly ($p > 0.05$). **B:** IRL1620-mediated contractions (100 nM) were inhibited in Ca^{2+}-containing and Ca^{2+}-free medium with IC_{50} of 0.32 ±0.08 and 0.25 ± 0.01 mM respectively. The two values of the IC_{50} did not differ significantly ($p > 0.05$). **C:** Comparison of IC_{50} values for peroxide inhibition of ET-1 and IRL1620 mediated contractions showed that IC_{50} for IRL1620 was much lower than ET-1 ($p < 0.05$) in both Ca^{2+}-containing and Ca^{2+}-free medium.


A
Ca2+ containing medium
Ca2+free medium
ET-1 Contraction (mN)
H2O2 mM


B
IRL1620 Contraction (mN)
H2O2 mM

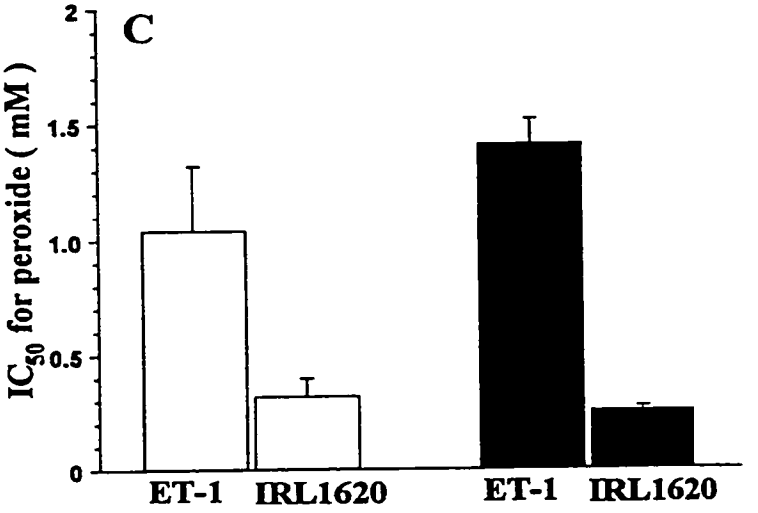

C
IC50 for peroxide (mM)
ET-1
IRL1620
ET-1
IRL1620

Fig. 9

3.2 Effect of peroxide and superoxide on ET-1 binding to its receptors in pig coronary artery smooth muscle

3.2.1 Characterization of ET-1 receptor subtypes:

First we characterized the binding to ET_A and ET_B receptors using ^{125}I-labeled ET-1 and by competition with unlabeled ET-1, BQ123, IRL1620, and BQ788 and then we examined the effects of peroxide on the two receptor types. ET-1 competes for binding to both ET_A and ET_B receptors, BQ123 competes for the ET_A sites, and IRL1620 and BQ788 inhibit binding to ET_B receptors. The results on binding to microsomes isolated from fresh smooth muscle dissected from the artery are shown in Fig.10. This figure shows that the unlabeled ET-1 and BQ123 displaced most of labeled ET-1 binding with a K_i value of 7.6 x 10^{-9} and 33.1 x10^{-8} M respectively. While IRL1620 and BQ788 displaced only a small proportion of the labeled ET-1 binding sites with K_i values of 8.4 x 10^{-11} and 3.3 x 10^{-9} M respectively. These results confirm that the predominant ET receptor type present in this tissue is ET_A and that ET_B receptors account for approximately 15-20 % of the total ET-1 binding sites. We also determined ET-1 binding to the smooth muscle cells cultured from this artery but these cells contained only the ET_A receptors (data not shown). This is consistent with a previous report that porcine coronary artery smooth muscle cells lose ET_B receptors upon culturing (Ihara et al., 1991).

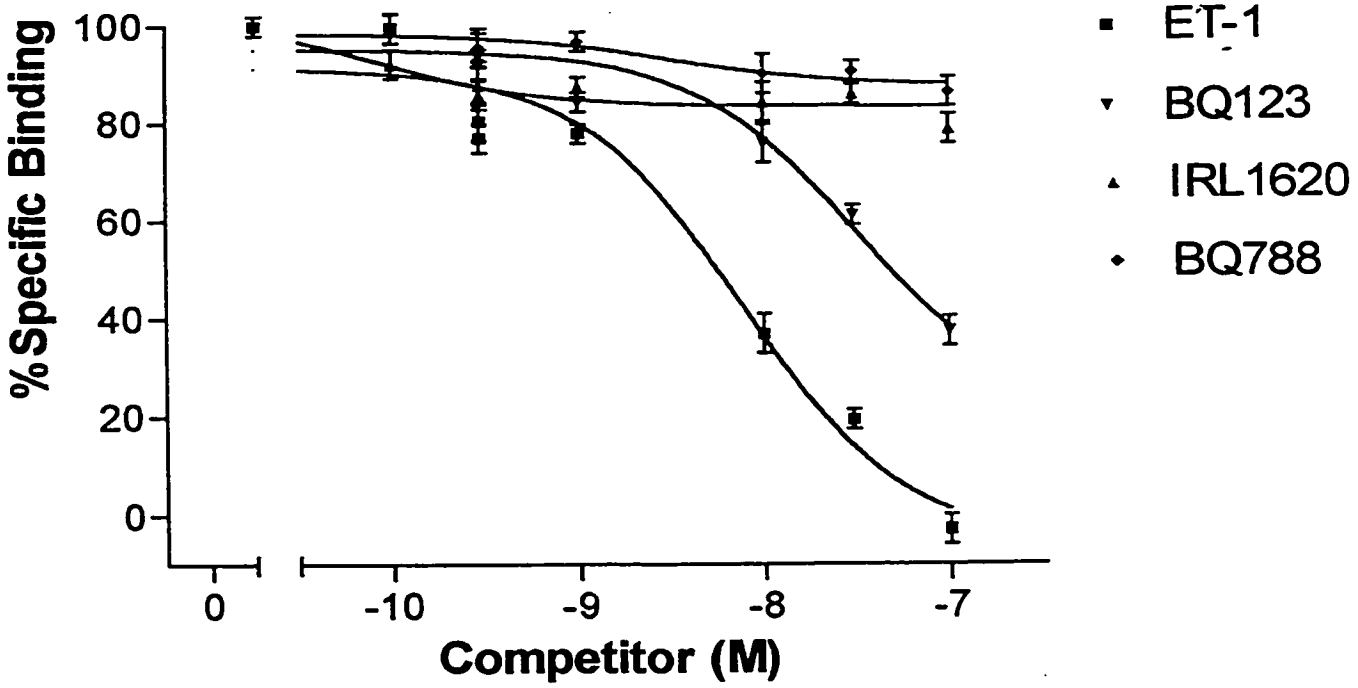


Fig.10 ***Competition binding of ^{125}I-labeled ET-1 to ET_A and ET_B receptors in isolated microsomes from pig coronary artery:***
The competition was conducted with unlabeled ET-1, BQ123, IRL1620, and BQ788 . ET-1 competes for binding to both ET_A and ET_B receptors, BQ123 competes for ET_A sites, and IRL1620 and BQ788 inhibit binding to ET_B receptors. The unlabeled ET-1 and BQ123 displaced most of labeled ET-1 binding with a K_i value of 7.6×10^{-9} and 33.1×10^{-8} M respectively. While IRL1620 and BQ788 displaced only a small proportion of the labeled ET-1 binding sites with K_i values of 8.4×10^{-11} and 3.3×10^{-9} M respectively. Each data point is the mean ± SEM of 4-8 replicates. The experiment was repeated twice and similar results were obtained. The fitting of the competition binding and K_i values were analyzed as a single or double site model using GraphPad software.

3.2.2 Effect of peroxide on ET-1 receptor inding sites:

To determine the effects of peroxide on ET-1 receptors (ET_A and ET_B), we pretreated the isolated microsomes with different concentrations of peroxide (0 to 10 mM) for 30 min at 37 C and then diluted them several fold in the binding solution. Peroxide pretreatment using concentrations up to 10 mM did not affect either BQ123-sensitive or BQ123-insensitive binding (Fig.11). In another experiment peroxide pretreatment did not affect the binding with either IRL1620 or BQ788 (data not shown). These results indicate that ET_A and ET_B receptors are resistant to damage by hydrogen peroxide.

3.2.3 Effect of superoxide on ET-1 receptor inding sites:

In order to examine the effect of superoxide on ET-1 receptors in isolated microsomes from coronary artery smooth muscle, we used 0.3 mM xanthine (X) + different concentration of xanthine oxidase (XO), and 85 U/ml catalase to decompose any peroxide formed from superoxide. In the first set of experiments the XO used was isolated from bovine milk which resulted in an increase of the ET-1 binding. After control experiments showed that bovine milk XO alone has the same effect, we used a commercial XO isolated from microsomal sources and this had no effect on ET-1 binding.

3.2.3.1 Effect of bovine milk XO + X on ET-1 binding to ET_A and ET_B receptor types:

As shown in figure 12, the bovine milk XO + X pretreatment of isolated microsome from pig coronary artery smooth muscle increases the ET-1 binding to ET_A receptors (BQ123-sensitive) and ET_B receptors (BQ123-insensitive) in a dose dependent manner by using different concentrations of XO prepared from bovine milk (0, 30, 100,

and 1000 mU) + 0.3 mM X . Figure 12 (A) shows an increase of the total ET-1 binding, non-specific binding using excess of unlabelled ET-1 (200 nM), and ET-1 binding when 250 nM BQ123 (a selective ET_A antagonist) was added. To further illustrate this effect on each ET-1 receptor type, Figure 12 (B) shows an approximate 200% increase at 1000 mU XO in the specific binding (% control) of the BQ123-sensitive component (ET_A receptors). Figure 12 (C) shows an approximate 270% increase at 1000 mU XO in BQ123-insensitive component which represents ET_B receptors. It should be noted that the baseline was taken as 100% at 0 mU XO. We repeated the experiment twice and the same results were obtained. The question at this point is whether the increase in ET-1 binding to its receptors is due to the effect of superoxide or something else.

3.2.3.2 Effect of bovine milk XO alone on ET-1 binding to ET_A and ET_B receptor types:

We conducted the same ET-1 binding experiments after treating the isolated microsomes with either a different concentration of XO alone (0, 100, and 1000 mU) or XO + 0.3 mM xanthine. There was an increase in ET-1 binding to both ET_A (BQ123-sensitive) and ET_B (BQ123-insensitive) receptor types with increasing [XO] in both treatments as shown in Fig. 13 (A and B). However, there was no significant difference between the pretreatment with XO alone and XO + X in all samples ($P > 0.05$). This result indicates that the increase in ET-1 binding to ET_A and ET_B receptors is not due to superoxide generation, but rather due to a possible impurity in the bovine milk XO preparation.

3.2.3.3 Effect of bacterial XO + X on ET-1 binding to ET_A and ET_B receptor types:

Pretreating the isolated microsomes from coronary artery smooth muscle with different concentrations of bacterial XO (0, 30, 100, and 1000 mU) + 0.3 mM xanthine had no significant effect on ET-1 binding to either ET_A or to ET_B receptor types ($P > 0.05$). Figure 14 (A) shows no significant effect of increasing concentration of bacterial XO on ET_A receptors (BQ123-sensitive). Furthermore by increasing bacterial XO up to 1000 mU, no significant effect was observed on ET-1 binding to ET_B receptors (BQ123-insensitive) as shown in Fig. 14 (B). We repeated the experiment twice and the same results were obtained. These results indicate that ET-1 binding to ET_A and ET_B are resistant to damage by superoxide generated from xanthine and XO obtained from bacterial sources.

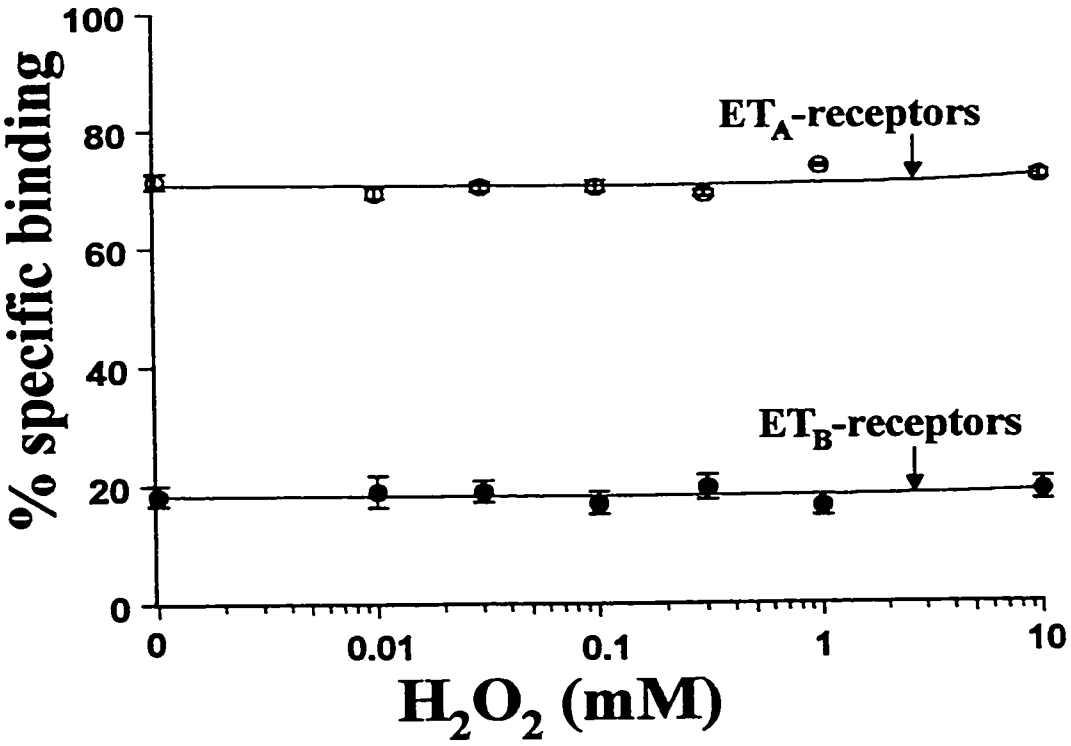
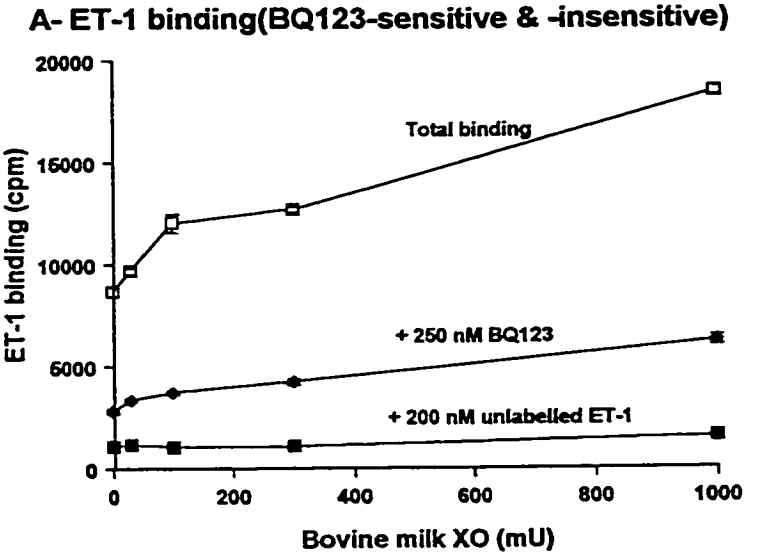
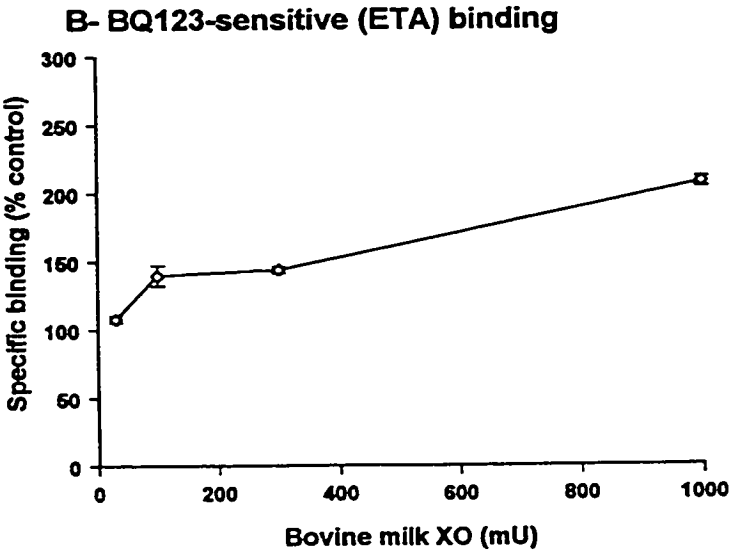


Fig.11 ***Effect of hydrogen peroxide on binding of ^{125}I-ET-1 to ET_A and ET_B receptors in isolated microsomes from pig coronary arteries:*** The isolated microsomes were treated with peroxide for 30 min at 37 °C and then washed to remove peroxide and then used for binding. Up to 10 mM peroxide did not affect ET-1 binding to either BQ123-sensitive (ET_A) or BQ123-insensitive (ET_B). Each data point represents the mean ± SEM of 6 replicates. The experiment was repeated producing similar results.

Fig 12. ***Effect of 0.3 mM xanthine + different concentration of XO prepared from ovine milk on ET-1 inding to its receptor in isolated microsomes from pig coronary arteries:*** **A:** This figure shows the increase in ET-1 total binding, non-specific binding (200 nM unlabelled ET-1), and ET-1 binding after adding 250 nM BQ123 (a selective ET_A antagonist). **B:** This figure shows the increase in specific binding of the BQ123-sensitive component which represents ET-1 binding to ET_A receptors. **C:** This figure shows the increase in the specific binding of ET-1 to the BQ123-insensitive component (ET_B receptors).


A- ET-1 binding(BQ123-sensitive & -insensitive)
ET-1 binding (cpm)
20000
15000
10000
6000
0
Total binding
+ 250 nM BQ123
+ 200 nM unlabelled ET-1
0
200
400
600
800
1000
Bovine milk XO (mU)


B- BQ123-sensitive (ETA) binding
Specific binding (% control)
300
250
200
150
100
50
0
0
200
400
600
800
1000
Bovine milk XO (mU)

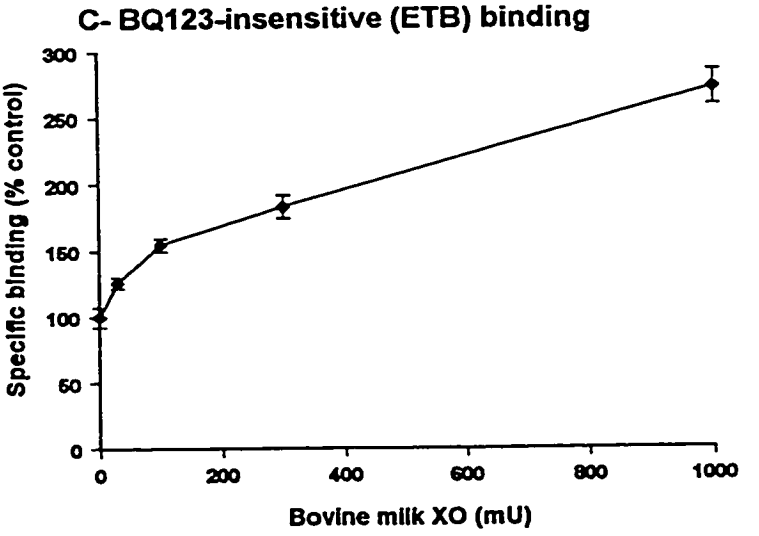

C- BQ123-insensitive (ETB) binding
Specific binding (% control)
300
250
200
150
100
50
0
0
200
400
600
800
1000
Bovine milk XO (mU)

Fig. 12

Fig. 13. ***ET-1 inding to ET_A and ET_B in isolated microsomes of pig coronary arteries in the presence or a sence of 0.3 mM xanthine:***

A control experiment showing the increase in ET-1 binding by increasing [XO], but no significant difference in the effect of bovine milk XO alone and XO +X on both the ET-1 binding to BQ123 sensitive component (ET_A receptors) Fig. 13 **(A)**, and BQ123-insensitive component (ET_B receptors) Fig. 13 **(B)**.

Fig.13

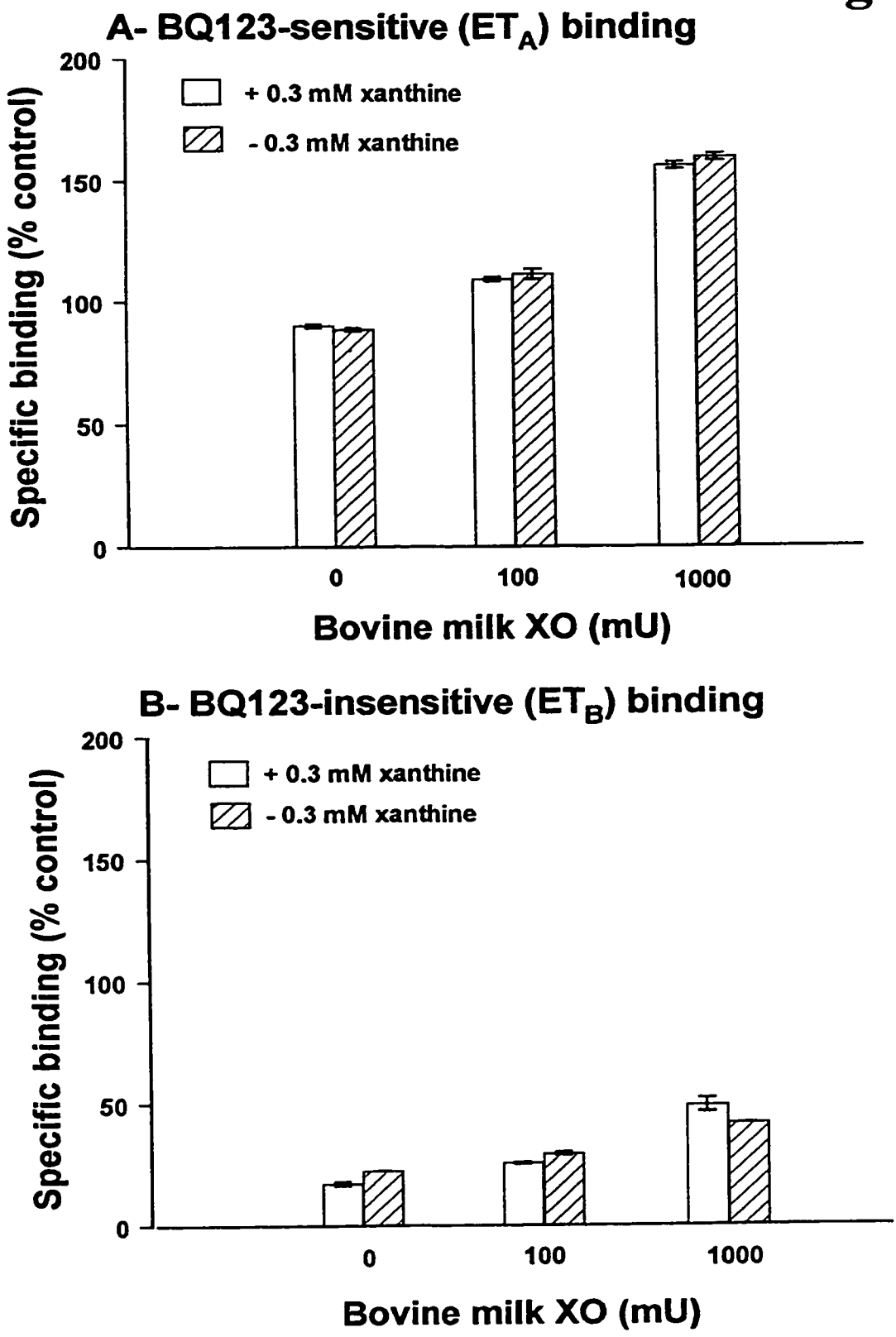
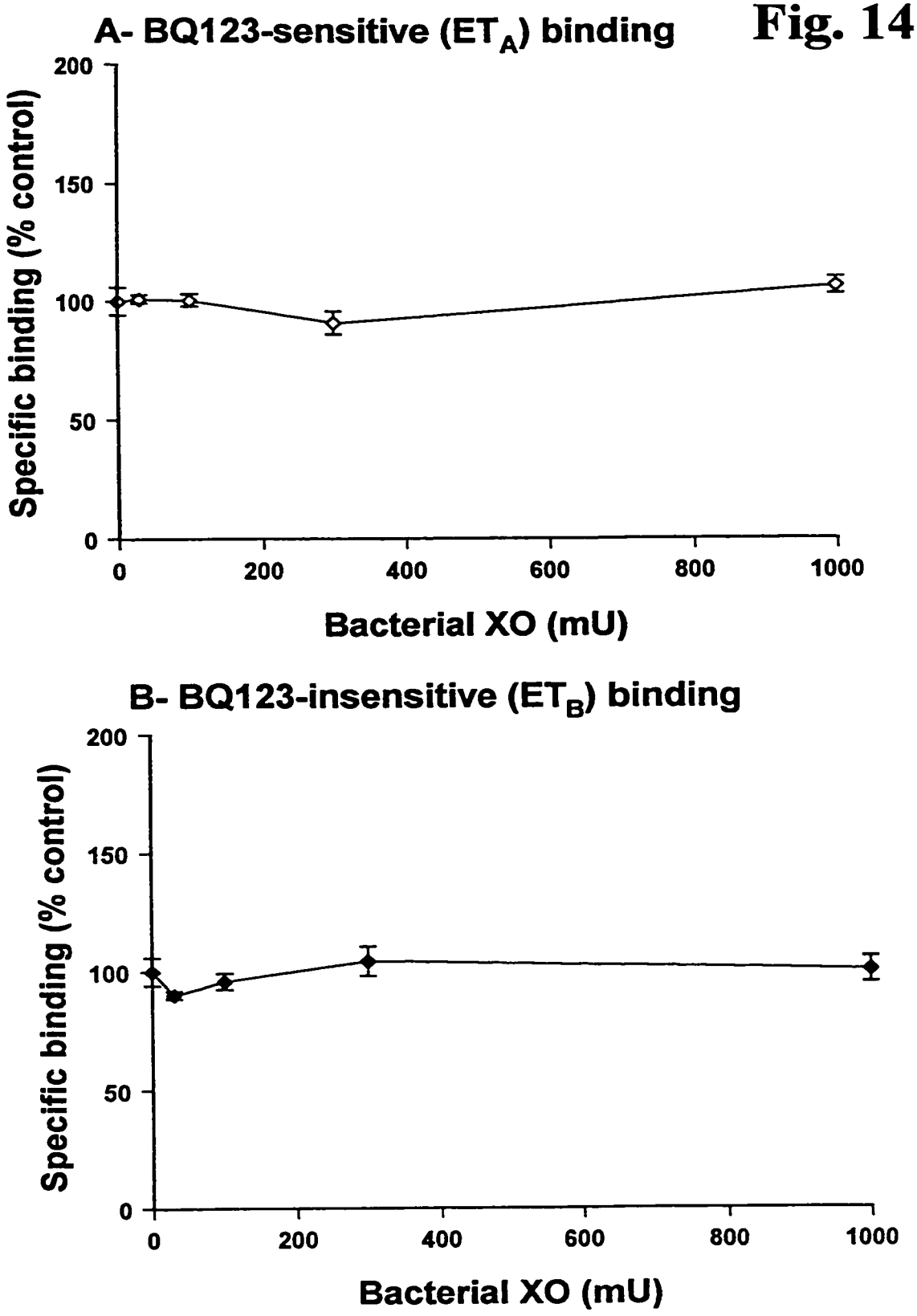

A- BQ123-sensitive (ET_A) binding
+ 0.3 mM xanthine
- 0.3 mM xanthine
Specific binding (% control)
200
150
100
50
0
0
100
1000
Bovine milk XO (mU)
B- BQ123-insensitive (ET_B) binding
+ 0.3 mM xanthine
- 0.3 mM xanthine
Specific binding (% control)
200
150
100
50
0
0
100
1000
Bovine milk XO (mU)

Fig. 14. ***Effect of 0.3 mM xanthine + different concentrations of XO prepared from a acterial source on ET-1 inding to its receptors in isolated microsomes from pig coronary arteries:***

A: This figure shows no significant effect of increasing XO on ET-1 binding to the BQ123-sensitive component (ET_A receptors). **B:** This figure also shows that increasing bacterial XO did not alter ET-1 binding to the BQ123-insensitive component (ET_B receptors).

Fig. 14


A- BQ123-sensitive (ET_A) binding
Specific binding (% control)
200
150
100
50
0
0
200
400
600
800
1000
Bacterial XO (mU)
B- BQ123-insensitive (ET_B) binding
Specific binding (% control)
200
150
100
50
0
0
200
400
600
800
1000
Bacterial XO (mU)

3.3 Effect of peroxide on cytosolic [Ca^{2+}] in cultured smooth muscle cells of pig coronary artery

Smooth muscle cells cultured from pig coronary artery were pre-incubated with Fluo-3 AM for 40 min at 22°C and then the cytosolic [Ca^{2+}] was measured when the cells were challenged with ET-1. Following peroxide treatment, SMCs were washed four times with 2 mM $CaCl_2$-Maklouf buffer to avoid peroxide interference with assay solution, before loading the cells with Fluo-3 and measuring the $[Ca^{2+}]_i$ increase.

3.3.1 Effect of peroxide on ET-1 induced increase in $[Ca^{2+}]_i$:

We examined the effects of peroxide on the ET-1 induced increase in $[Ca^{2+}]_i$ in smooth muscle cells cultured from the coronary artery. These cells showed a transient increase in $[Ca^{2+}]_i$ when challenged with 50 nM ET-1. Treating the cells with 300 μM hydrogen peroxide for 30 min at 37°C before loading them with the Ca^{2+}-sensitive dye inhibited this increase by greater than 95% (Fig.15). This indicates that ET_A induced increase in $[Ca^{2+}]_i$ is very sensitive to damage by peroxide, unlike the contraction mediated by the ET_A receptors which was resistant to peroxide with IC_{50} = 1.2 ± 0.2 mM. As the ET_B receptors were absent in the cultured cells, we could not test for their induced increase of the $[Ca^{2+}]_i$. The level of Fluo-3 loading was also monitored in control and peroxide treated cells. The loading as monitored by the fluorescence did not differ significantly ($p > 0.05$) between the control (41 ± 2 kcps) and the treated cells (42 ± 2.7 kcps). Therefore, the possibility that a reduction of the Fluo-3 loading into the cells contributed to any other results was eliminated.

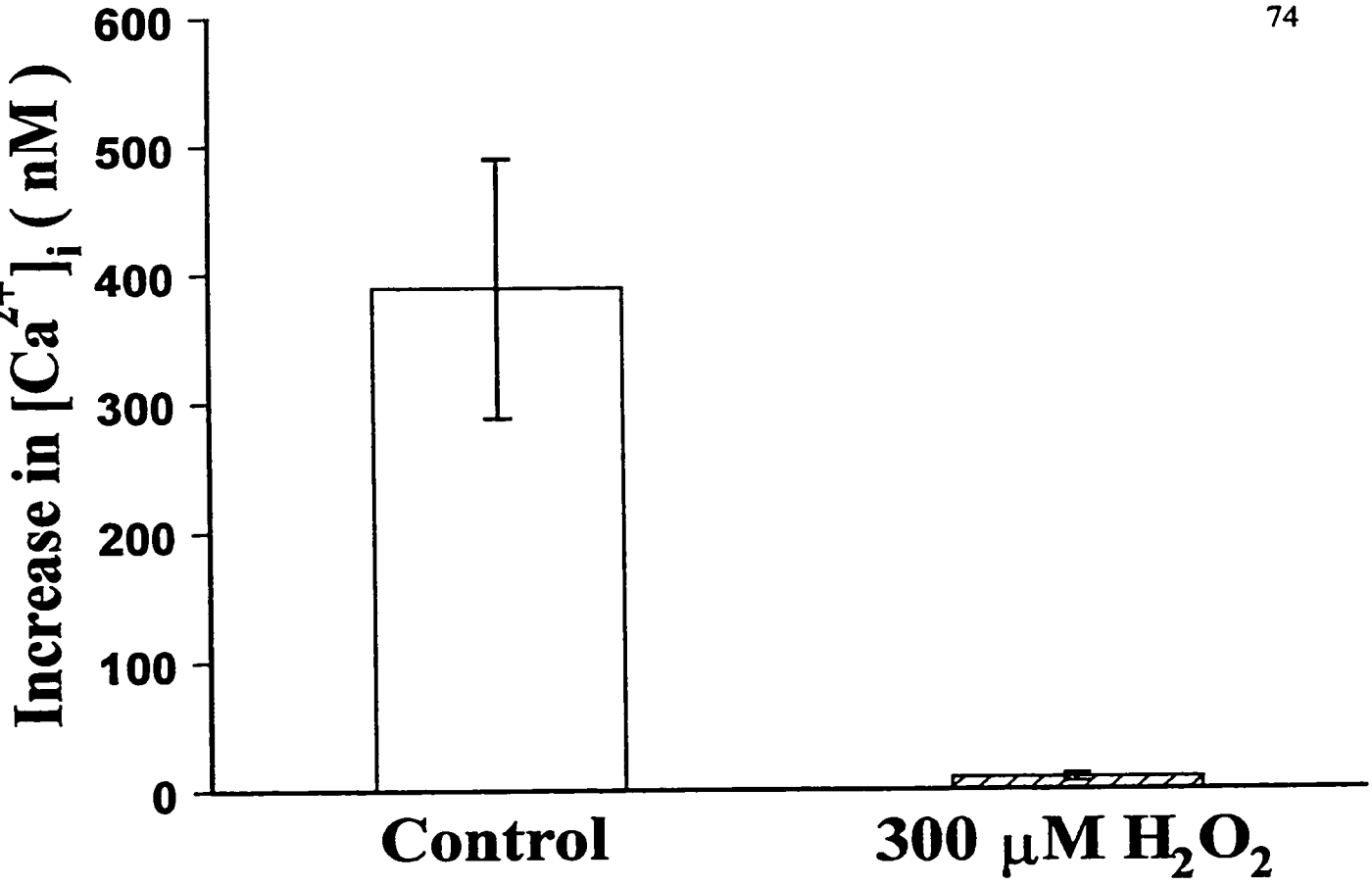


Fig. 15 ***Effect of peroxide on ET-1 induced $[Ca^{2+}]_i$ increase in cultured smooth muscle cells from pig coronary artery:*** Cultured smooth muscle cells from pig coronary artery were treated with peroxide for 30 min at 37°C, washed to remove peroxide and then loaded with Fluo-3 for $[Ca^{2+}]_i$ measurements. Hydrogen peroxide (300 μM) inhibited greater than 95% of ET-1 induced increase $[Ca^{2+}]_i$: Mean ± SEM from 21 samples was shown.

3.4 Sarcoplasmic reticulum (SR) Ca^{2+}-uptake and IP_3-induced Ca^{2+} release in permeabilized smooth muscle of pig coronary artery

3.4.1 Optimization of permea ilization condition:

In initial experiments the smooth muscle cells cultured from pig coronary artery were permeabilized at various cell densities and different concentrations of saponin and their ability to exclude Trypan blue was examined. Based on these experiments it was determined that it was the ratio of cells to saponin which was critical. At 25°C in 15 min, 250 μg/ml saponin permeabilized >95 % of the cells at a density of 10^7/ml. All the subsequent permeabilization experiments were carried out under these conditions.

3.4.2 Characterization of the Ca^{2+}-uptake y SR:

Several experiments were carried out to optimize the conditions of the Ca^{2+}-uptake. In these experiments, it was determined that the creatinine phosphate-creatinine kinase ATP-regenerating system provided more reproducible results than the NADH-pyruvate enol phosphate-pyruvate kinase-lactate dehydrogenase systems. Fig. 16 represents a time course of the Ca^{2+}-uptake, in the presence of 5 mM Na^+-azide to inhibit the mitochondrial Ca^{2+}-uptake, with or without $Mg^{2+}ATP^{2-}$, creatinine phosphate-creatinine kinase ATP-regenerating system, and ionomycin. Fig. 16 shows that the Ca^{2+}-uptake stimulated by $Mg^{2+}ATP^{2-}$ was very low in the absence of oxalate or the ATP-regenerating system, and that this was blocked by the Ca^{2+}-ionophore ionomycin. Fig. 16 also shows that in the presence of $Mg^{2+}ATP^{2-}$, the ATP-regenerating system and oxalate, the uptake was linear for up to 120 min. In subsequent experiments, the Ca^{2+}-uptake at 60

min was used as the initial velocity of the uptake.

Fig. 17 shows the $[Ca^{2+}]$-dependence of the SR Ca^{2+}-uptake. In repeated experiments the K_m value for Ca^{2+} was found to be 0.20±0.03 μM. Fig. 18 is the saturation curve for the pump for $Mg^{2+}ATP^{2-}$. In repeated experiments the K_m value for Mg^{2+} was 400±34 μM.

Fig. 19 shows a summary of data from 5 experiments on the pH-dependence of the Ca^{2+}-uptake. The optimal pH for the Ca^{2+}-uptake was 6.2. In order to determine if the permeabilized cells hydrolyzed phosphate esters differently in this pH range, the hydrolysis of P-Nitrophenylphosphatase (PNPP) was examined. In two experiments using different batches of cells, PNPP hydrolysis by the permeabilized smooth muscle cells was 66±15 % higher at pH 6 than at pH 6.8. Thus, the PNPP hydrolysis was consistent with the presence of an acid phosphatase activity in the permeabilized cell preparations.

Fig. 20 shows that the SR Ca^{2+}-uptake by the permeabilized smooth muscle cells was inhibited by thapsigargin and cyclopiazonic acid (CPA). Either agent caused a complete inhibition; however, thapsigargin (IC_{50} = 0.14±0.02 μM) was a more potent inhibitor than CPA (IC_{50} =0.56±0.04 μM). A summary of the characteristics of the SR Ca^{2+} pump in coronary artery smooth muscle in permeabilized cells, and in isolated membranes is presented in Table 2.

Table 2. ***Summary of the properties of the SR Ca^{2+}-pump in coronary artery smooth muscle (Elmoselhi et al., 1995):***

	Permeabilized Cells	Isolated Membranes
Ca^{2+} K_m μM	0.20 ± 0.03	0.58 ± 0.05
$MgATP^{2-}$ K_m μM	400 ± 34	812 ± 83
pH Optimum	6.2	6.4- 6.8
Thapsigargin IC_{50} (μM)	0.126 ± 0.016	-
IC_{50} for CPA (μM)	0.564 ± 0.037	-

Note: The values for the isolated membranes are from reference (Grover et al., 1995). There are no comparable data available for inhibition by thapsigargin or CPA. However, in various other studies it was shown that thapsigargin is a more potent inhibitor than CPA (Misquitta et al., 1996).

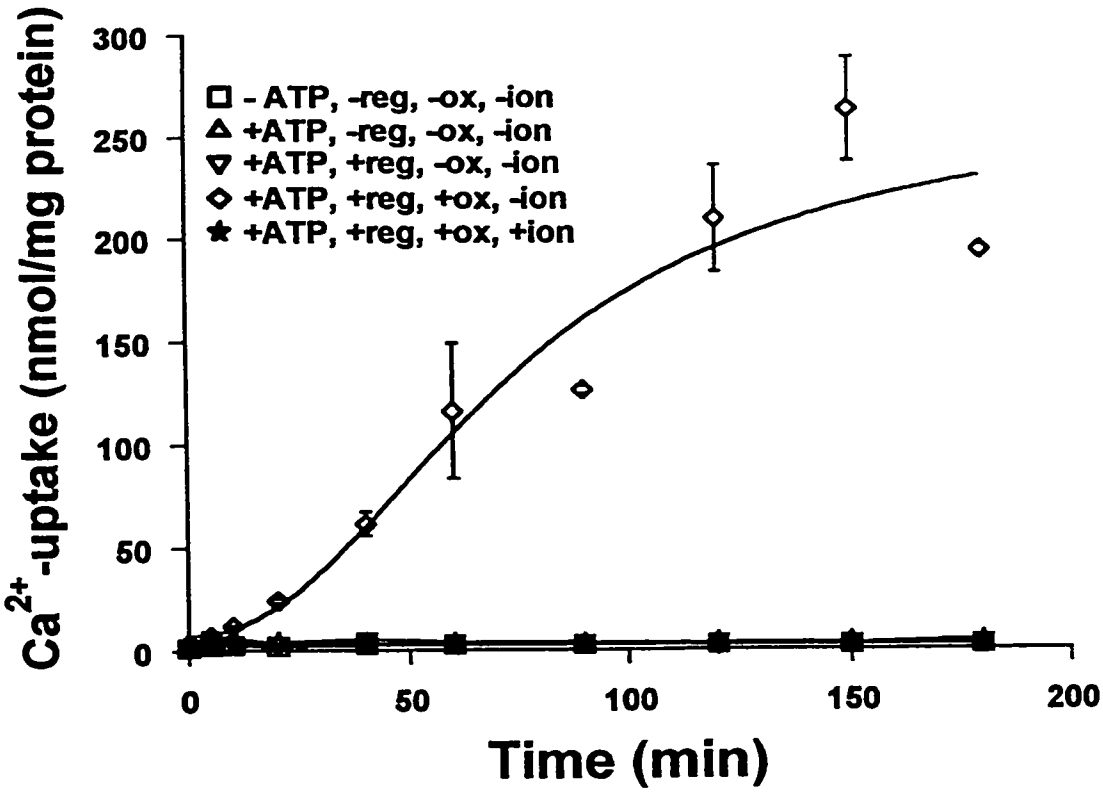


Fig. 16 ***Time course of SR Ca^{2+}-uptake in permeabilized smooth muscle cells cultured from pig coronary artery:*** The Ca^{2+}-uptake reactions were carried out at saturating substrate concentrations as described in the Experimental Methods. Values at each time point are mean±SEM of 4 replicates. Taken from Elmoselhi et al, 1995.

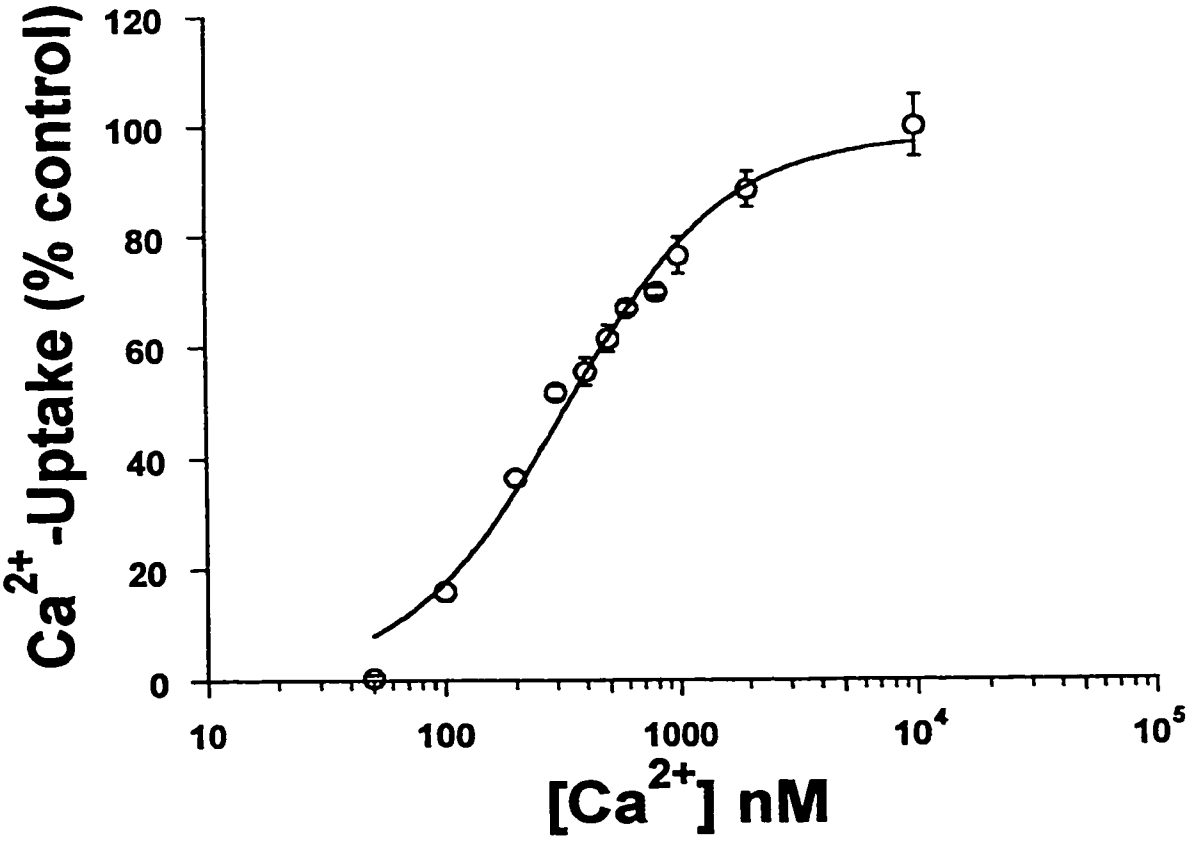


Fig. 17 ***Ca^{2+}-concentration dependence of SR Ca^{2+}-uptake in permeabilized smooth muscle cells cultured from pig coronary artery:*** $[Ca^{2+}]$ was varied by varying the amount of EGTA. Concentrations of all the other substrates were saturating. Only the oxalate-stimulated component of the ATP-dependent azide insensitive uptake is shown. The values are mean±SEM of 9 replicates. Taken from Elmoselhi et al, 1995.

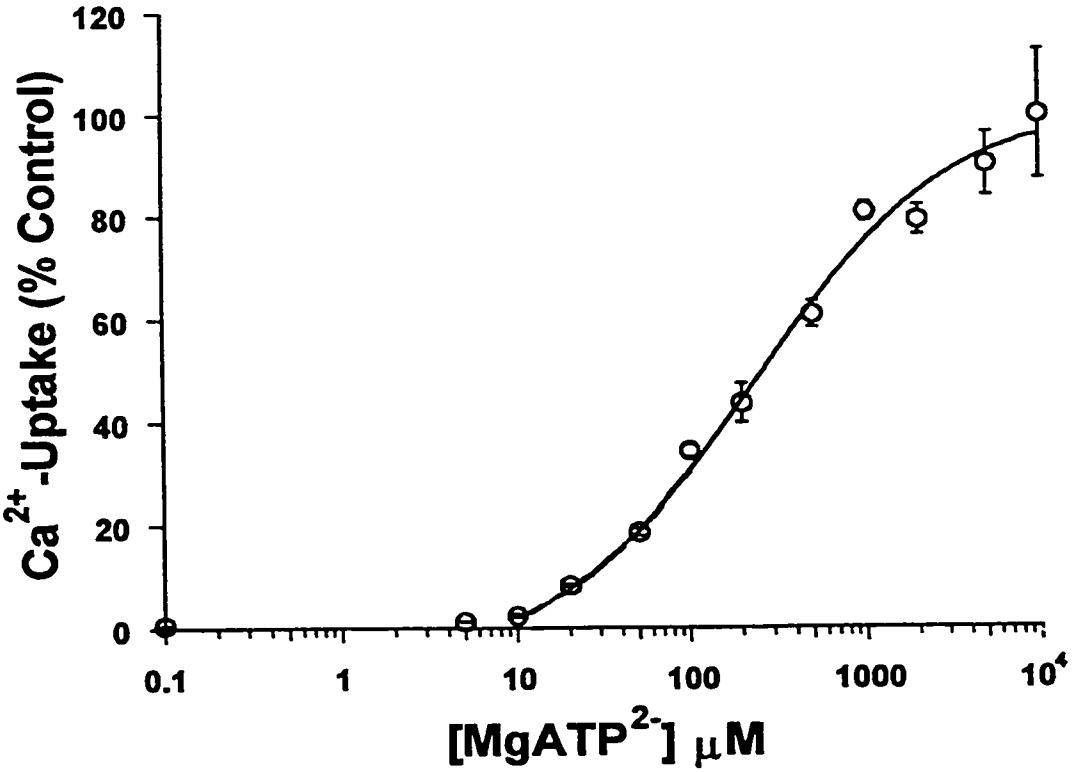


Fig. 18 ***$Mg^{2+}ATP^{2-}$ concentration dependence of SR Ca^{2+}-uptake in permeabilized smooth muscle cells cultured from pig coronary artery:*** $[MgATP^{2-}]$ was varied and concentrations of all the other substrates were saturating. Only the oxalate-stimulated component of the ATP-dependent azide insensitive uptake is shown. The values are mean±SEM of 9 replicates. Taken from Elmoselhi et al, 1995.

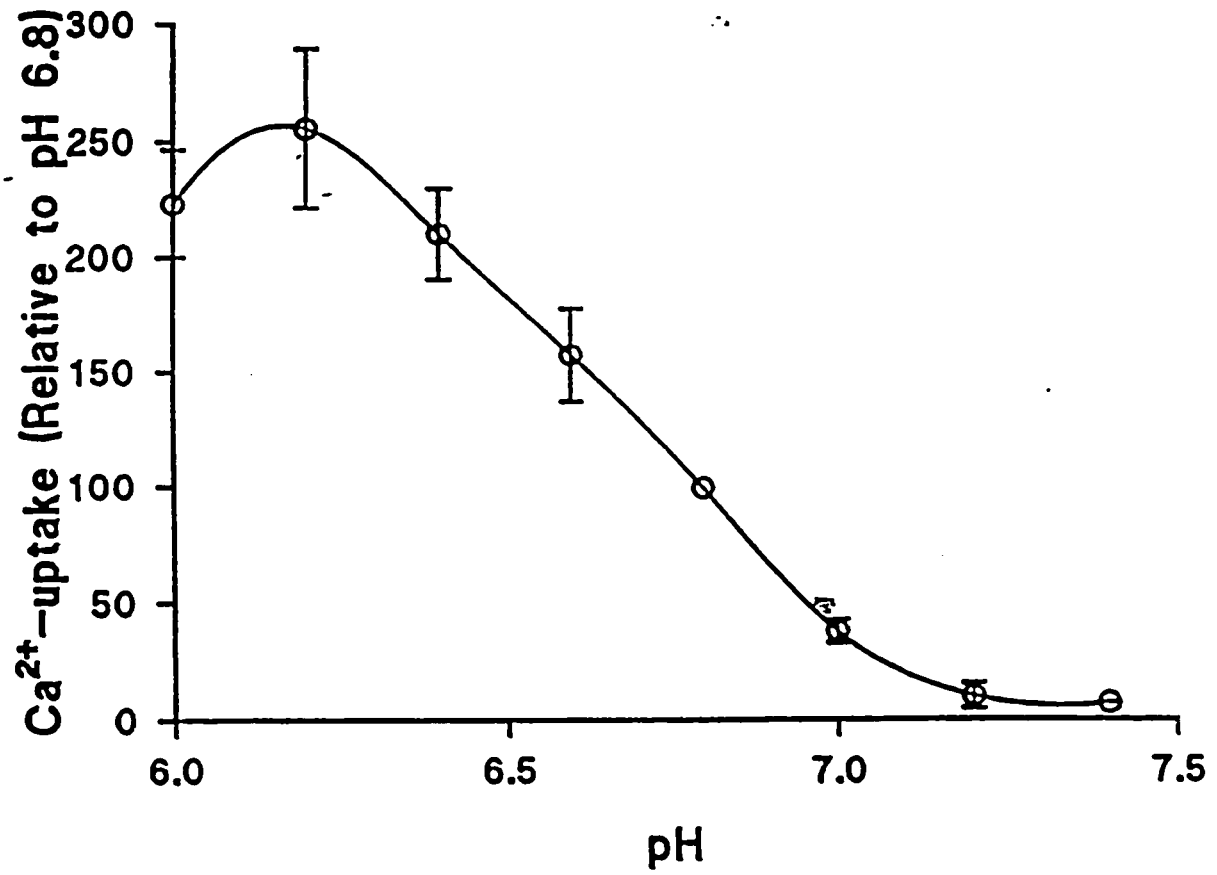


Fig. 19 ***Effect of pH on SR Ca^{2+}-uptake in permeabilized smooth muscle cells cultured from pig coronary artery:*** Ca^{2+}-uptake was carried out in reaction mixtures containing 6.66 mM $MgCl_2$ and 4.75 to 5.5 mM ATP so as to yield 4.5 mM $MgATP^{2-}$ at each pH. EGTA concentrations were also varied so as to yield 10 μM Ca^{2+} at each pH. Only the oxalate-stimulated component of the ATP-dependent azide insensitive uptake is shown. The values are mean±SEM of 12-30 replicates pooled from 5 experiments in each of which Ca^{2+}-uptake was normalized taking the oxalate-stimulated component of the uptake at pH 6.8 to be 100%. Taken from Elmoselhi et al, 1995.

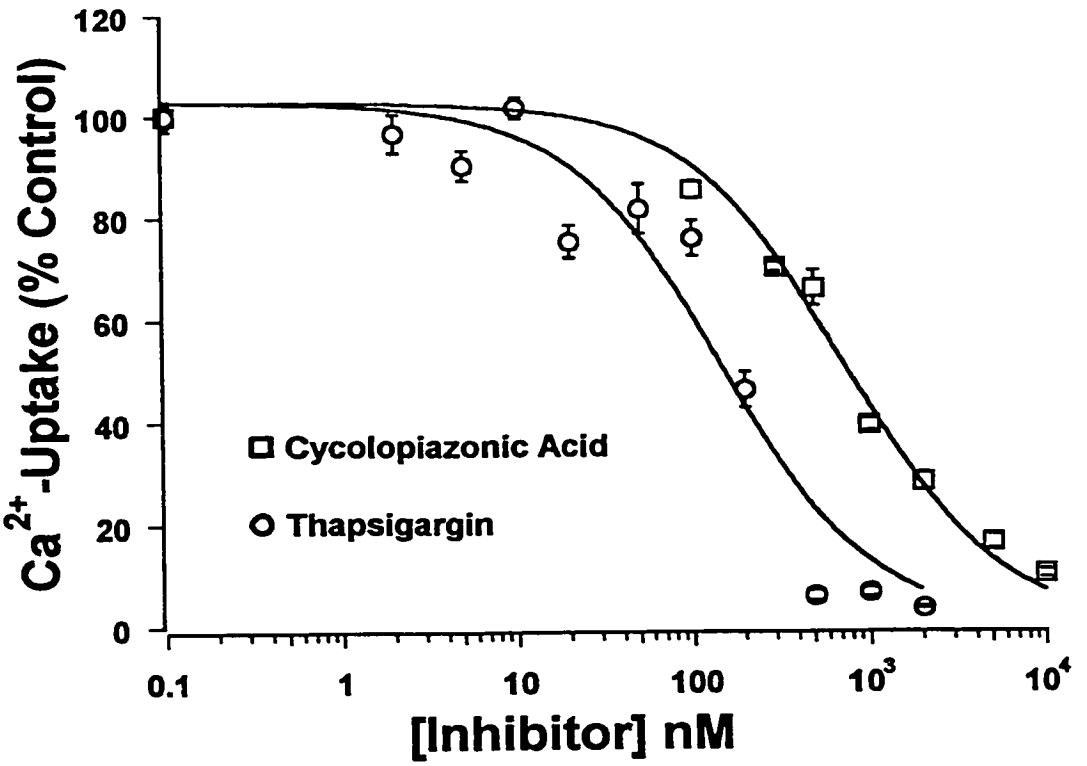


Fig. 20 ***Inhibition of SR Ca^{2+}-uptake by CPA and thapsigargin in permeabilized smooth muscle cells cultured from pig coronary artery:*** [CPA] and [thapsigargin] were included in the Ca^{2+}-uptake solutions containing saturating concentrations of all the substrates. Taken from Elmoselhi et al, 1995.

3.4.3 Characterization of the IP_3-induced Ca^{2+} release:

Following optimization of the conditions of the SR Ca^{2+}-uptake in the permeabilized cells, we further characterized the IP_3 -induced Ca^{2+} release to be able to examine the effects of peroxide and superoxide on them. The permeabilized cells were loaded with $^{45}Ca^{2+}$ using the standard loading solution for 18 min at 37°C, diluted in the release buffer at 37°C so that the final [Ca^{2+}] was 0.1 μM, and the reaction mixture was filtered 30 sec later. Less than 5% of the $^{45}Ca^{2+}$ was retained when 10 μM of the Ca^{2+} ionophore A23187 was included in the release medium. In a given experiment, the Ca^{2+} uptake under the standard conditions was defined as the value of Ca^{2+}-retained minus the value of the Ca^{2+}-retained when samples were diluted in the presence of A23187. All the values of Ca^{2+} retained are presented as a percentage of the Ca^{2+} uptake under standard conditions in the same experiment and the values of the ionophore insensitive component have already been subtracted.

Since the release via ion channels usually has a very low temperature dependence, this property of the IP_3-induced Ca^{2+} release was examined. After loading with $^{45}Ca^{2+}$, the permeabilized cells were diluted with or without 50 μM IP_3 at 0 or 37°C and then filtered. The dilution without IP_3 resulted in a marginally higher value of the Ca^{2+} -retained at 0°C than at 37°C but the values of the Ca^{2+}-retained with IP_3 were comparable at the two temperatures (Fig. 21A). The values of the IP_3-induced release were 37±2 and 31±3 %, at 0° and 37°C respectively , and these did not differ significantly ($p > 0.05$). This temperature independence indicated that most likely IP_3 activated a Ca^{2+} channel rather

than a metabolic pathway.

Figure 21B shows the time course of the IP_3-induced Ca^{2+} release. The IP_3-induced Ca^{2+} release was very rapid and nearly complete in 15-30 sec. Therefore, the release time of 30 sec was used in the rest of the experiments. Fig. 21C shows the effect of varying $[IP_3]$ on the Ca^{2+} release. Increasing the $[IP_3]$ increased the IP_3-induced Ca^{2+} release with an EC_{50}= 2.7±1.0 μM and the maximum IP_3-induced Ca^{2+} release was approximately 45±8%. In all the subsequent experiments, we used a saturating concentration (50 μM) of IP_3 to obtain the maximum IP_3-sensitive Ca^{2+} release.

Fig 21D shows the pH dependence of the IP_3-induced Ca^{2+} release. The IP_3-induced Ca^{2+} release at pH 6.8 was 25±2 % and did not differ significantly ($p > 0.05$) from the corresponding values at pH 6.4 or 7.1 but it was significantly ($P < 0.05$) higher than the IP_3-induced Ca^{2+} release of 10±2 % observed at pH 7.4. The IP_3-induced Ca^{2+} release was determined at pH 6.8 in the remainder of the experiments.

Fig. 21E shows the effect of varying $[Ca^{2+}]$ in the release medium on the IP_3-induced Ca^{2+} release. In this experiment, the permeabilized cells were loaded using the standard loading solution and the subsequent release was examined in solutions containing the specified $[Ca^{2+}]$. The maximum IP_3-induced Ca^{2+} release (44±6%) was obtained at 0.1 μM Ca^{2+} and the minimum (19±3%) at 10 μM. Thus, the IP_3-induced Ca^{2+} release was inhibited by high $[Ca^{2+}]$ in the release medium with an inhibition constant of 0.61 μM. The IP_3-insensitive Ca^{2+} uptake, however, increased slightly with increasing $[Ca^{2+}]$ with an apparent activation constant of 0.43 μM.

Fig. 21. ***Characterization of the IP_3-induced Ca^{2+} release in permea ilized smooth muscle cells cultured from pig coronary artery:***

A: Effect of temperature: The skinned cells were loaded at 37°C for 18 min using the standard uptake solution and then diluted in solutions at 0 or 37°C for the release experiment. **B:** Time course: The time shown is that between the filtration and the dilution of the samples after the completion of the Ca^{2+} uptake. **C:** $[IP_3]$- dependence. **D:** Effect of pH. **E:** Effect of $[Ca^{2+}]$ in the release medium. Standard loading conditions were used for A-E. Where not specified, standard release conditions were used: $[Ca^{2+}]$ in the release medium = 0.1 μM, release temperature = 37°C, release time = 30 sec, release medium pH = 6.8 and $[IP_3]$ = 50 μM. The values shown are mean ± SEM of 8-12 replicates except for the pH-dependence experiment where the data were pooled from 3 separate experiments and the total number of replicates is 12-24. See Experimental Methods for data normalization and other details. Taken with permission from (Elmoselhi et al., 1996).

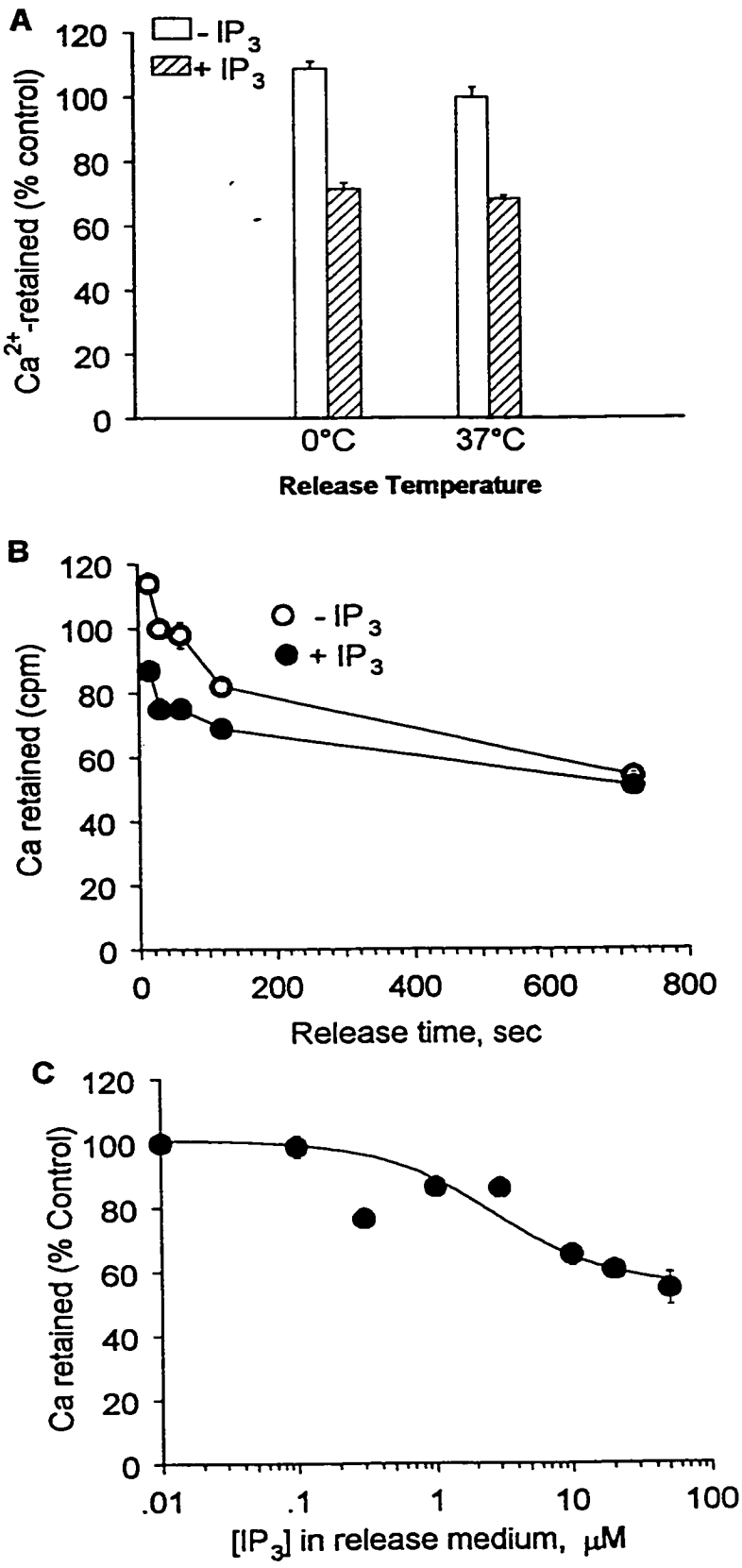

A
- IP3
+ IP3
Ca2+-retained (% control)
120
100
80
60
40
20
0
0°C
37°C
Release Temperature
B
- IP3
+ IP3
Ca retained (cpm)
120
100
80
60
40
20
0
0
200
400
600
800
Release time, sec
C
Ca retained (% Control)
120
100
80
60
40
20
0
.01
.1
1
10
100
[IP3] in release medium, μM

Fig. 21 A-C

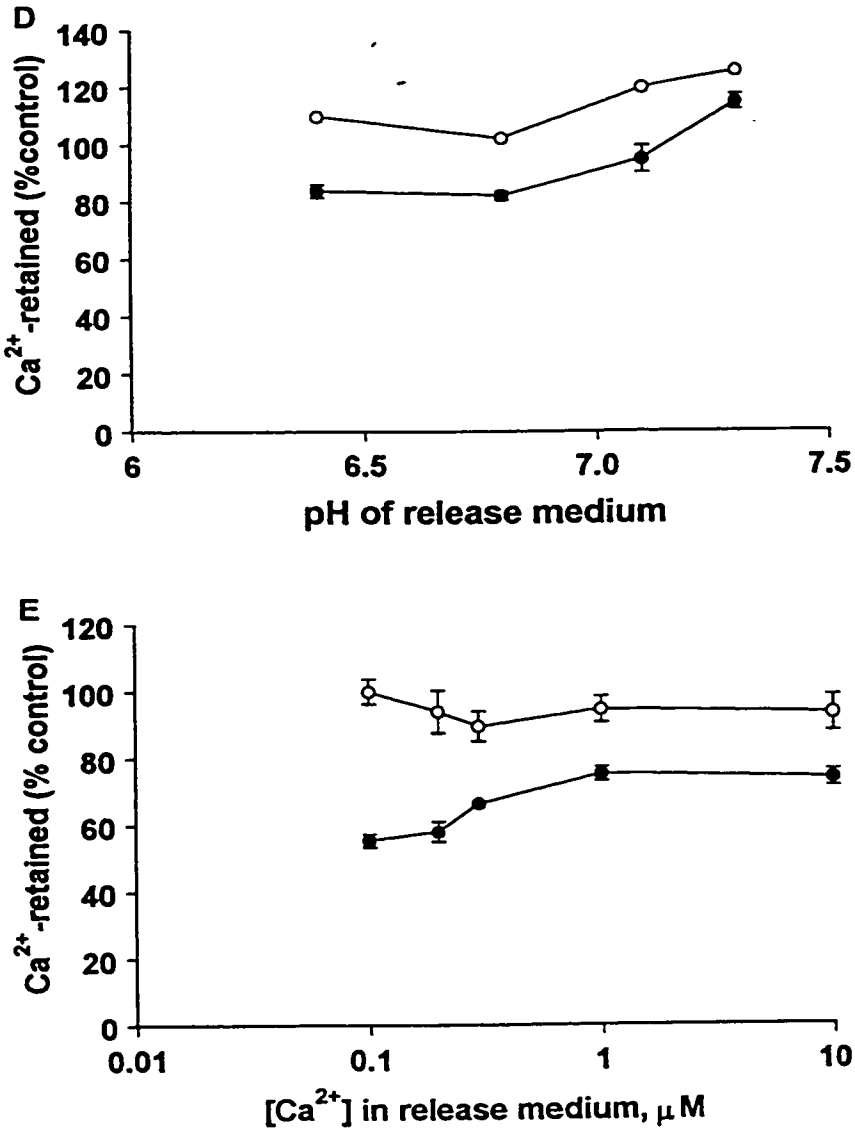

D
140
120
100
80
60
40
20
0
Ca^{2+}-retained (%control)
6
6.5
7.0
7.5
pH of release medium
E
120
100
80
60
40
20
0
Ca^{2+}-retained (% control)
0.01
0.1
1
10
$[Ca^{2+}]$ in release medium, μM

Fig. 21 D-E

3.4.3.1 Effect of Ca^{2+} load on IP_3-induced Ca^{2+} release: To examine the effect of Ca^{2+} load on the IP_3-induced Ca^{2+} release, we varied the Ca^{2+} load either by using different loading times or by varying [Ca^{2+}] in the loading solution. The term Ca^{2+} load signified the total Ca^{2+} uptake which was not released upon dilution in EGTA (used to lower the [Ca^{2+}] in the medium), but was released with the Ca^{2+} ionophore A23187 under the conditions described in the Experimental Methods. The total Ca^{2+} retained was considered here as a sum of two Ca^{2+} pools: an IP_3 insensitive pool which is not released in the presence of 50 μM IP_3 and an IP_3 sensitive Ca^{2+} pool (or IP_3-induced Ca^{2+} release component) which represented the difference between the total uptake and the IP_3 insensitive Ca^{2+} pool. Fig. 22 shows the effects of varying the loading times. When the permeabilized smooth muscle cells were loaded using the standard Ca^{2+} uptake medium for 0.5, 1, 3 or 18 min, respective values of the total Ca^{2+}-uptake were 24±1, 38±2, 103±6 and 100±2 % of the standard uptake. The corresponding mean values for the Ca^{2+} loading into the IP_3-insensitive pool were 15, 23, 57 and 71%, respectively and those for the IP_3-sensitive Ca^{2+}-pool were 9, 15, 45 and 29%, respectively. Thus the uptake into both the IP_3-insensitive, and the IP_3-sensitive pools increased with time for the initial 3 min. This relationship is further demonstrated in the inset in Fig. 22 which is a plot of loading into each of the two pools vs. the total Ca^{2+}-uptake (total Ca^{2+} load).

In Fig. 23, the amount of Ca^{2+} taken up by the permeabilized smooth muscle cells was varied by loading at different [Ca^{2+}]. The release was then examined in a medium containing 0.05 μM Ca^{2+}. The values of Ca^{2+} loads were 55±1, 61±2, 77±1, 94±1 and

100±2 % of the standard Ca^{2+} uptake when [Ca^{2+}] in the loading solutions were 0.05, 0.1, 0.2, 1 and 10μM, respectively. The corresponding mean values for loading into the IP_3-insensitive Ca^{2+}-pools were 25, 29, 38, 51 and 70 %, respectively and those for the IP_3-sensitive Ca^{2+}-pool were 31, 33, 38, 43 and 30%, respectively. Thus, there is a positive correlation between the Ca^{2+} loading into the IP_3-insensitive pool and the total Ca^{2+} load, but not for loading into the IP_3-sensitive Ca^{2+} pool and the total Ca^{2+} load (inset in Fig. 23). Further analysis of the data showed that whereas the loading into the IP_3-sensitive Ca^{2+} pool remained somewhat constant, the loading into the IP_3-insensitive Ca^{2+} pool increased with [Ca^{2+}] with a $K_{0.5}$ of 0.12 μM.

Fig. 22. ***Effect of loading time on the IP_3-induced Ca^{2+} release in permea ilized smooth muscle cells cultured from pig coronary artery:*** The permeabilized cells were loaded using the standard loading solution for specified amounts of time before examining the Ca^{2+} release. The standard release conditions were then employed. The IP_3-insensitive component was defined as the Ca^{2+}-retained after 30 sec in the presence of 50 μM IP_3 minus the Ca^{2+}-retained upon dilution in 10 μM A23187. The IP_3-sensitive component is the IP_3-induced Ca^{2+}-release and it was determined as the difference between the total uptake and the IP_3-insensitive component. Inset: Relationship between the IP_3-sensitive and the insensitive Ca^{2+} pools and the Ca^{2+} uptake (Ca^{2+} load). For the IP_3-insensitive component line, the slope = 0.631±0.109 and the correlation coefficient = 0.971 indicate that there was a significant ($p < 0.05$) positive correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded. For the IP_3-sensitive component line, the slope = 0.369±0.109 and the correlation coefficient = 0.922, indicate that there was a significant ($p < 0.05$) positive correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded. The values shown are mean±SEM of the data pooled from 3 separate experiments and the total number of replicates is 18-32. All data obtained in each experiment were normalized to the control (A23187 sensitive Ca^{2+}-retained in the skinned cells after loading under the standard conditions for 18 min followed by incubation with the standard release solution without IP_3 for 30 sec). See Experimental Methods for data normalization and other details. Taken with permission from (Elmoselhi et al., 1996).

Fig. 22

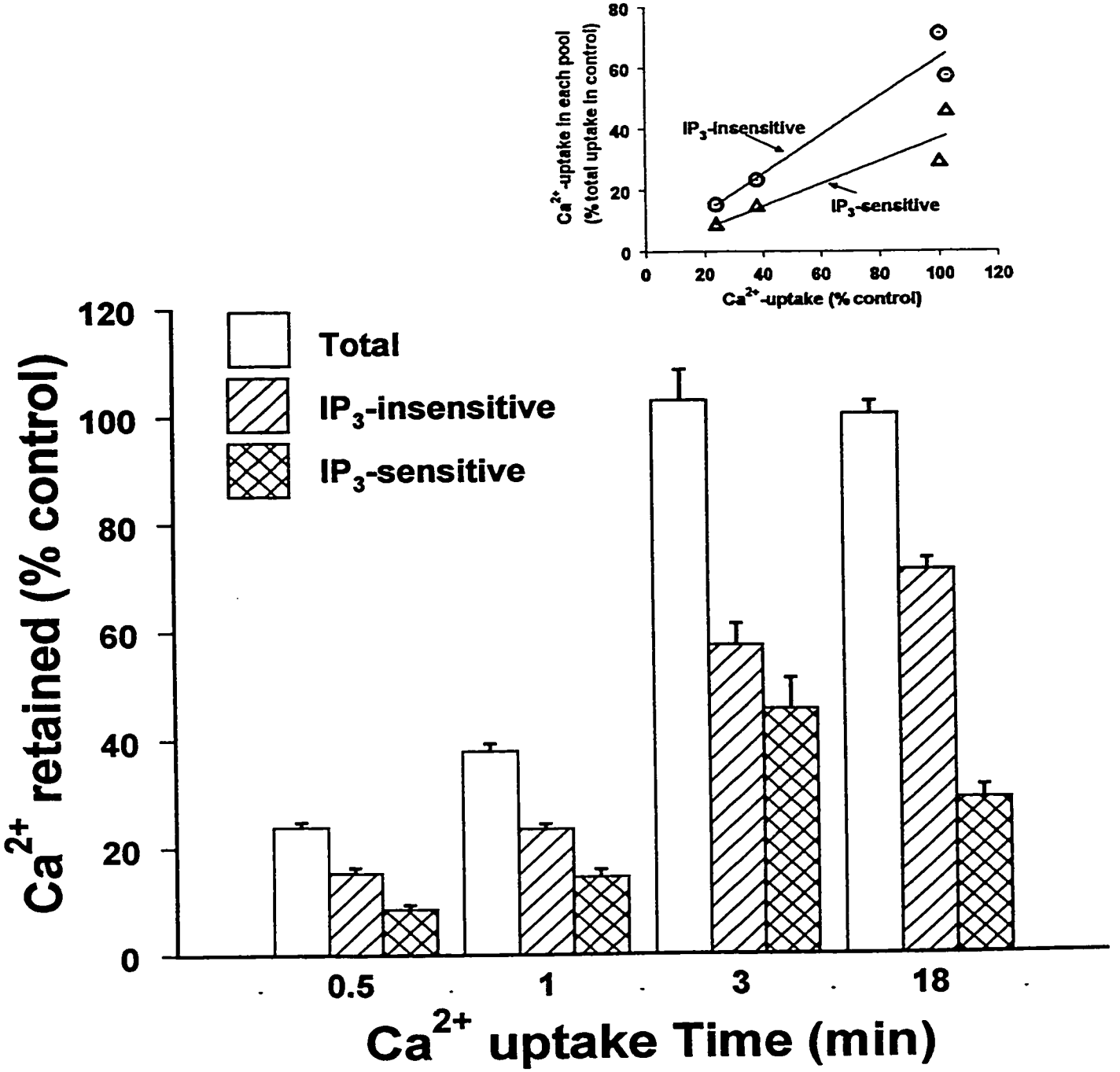
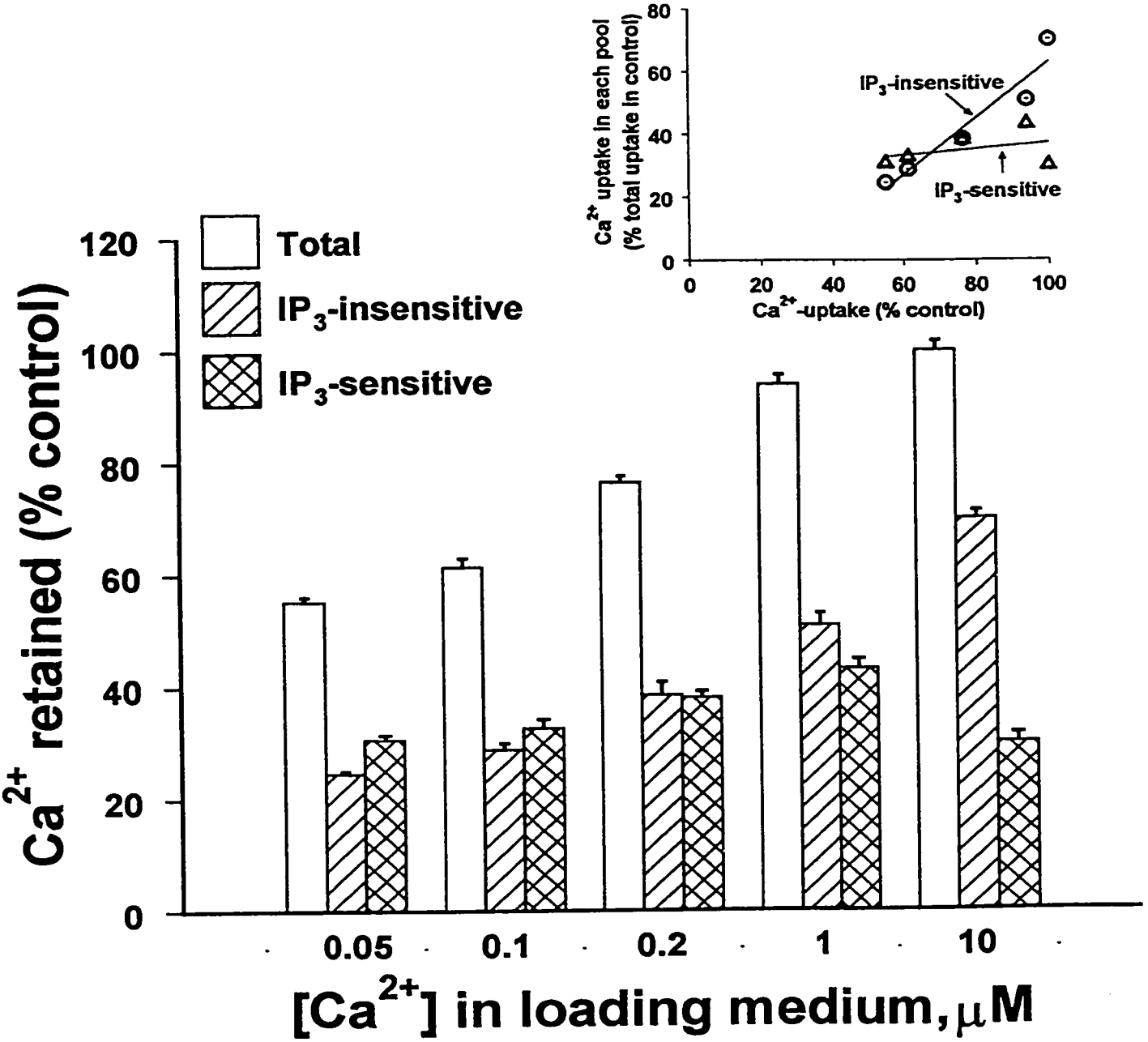

Ca2+ retained (% control)
120
100
80
60
40
20
0
Total
IP3-insensitive
IP3-sensitive
0.5
1
3
18
Ca2+ uptake Time (min)
Ca2+-uptake in each pool (% total uptake in control)
80
60
40
20
0
IP3-insensitive
IP3-sensitive
0
20
40
60
80
100
120
Ca2+-uptake (% control)

Fig. 23. ***Effect of [Ca^{2+}] in the loading solution on the IP_3-induced Ca^{2+} release in permea ilized smooth muscle cells cultured from pig coronary artery:*** The permeabilized cells were loaded for 18 min using loading solutions containing the same amount of $^{45}Ca^{2+}$ but varying amounts of EGTA so as to yield the specified [Ca^{2+}]. The samples were then diluted so as to obtain 0.05 μM Ca^{2+} and filtered 30 sec later. The IP_3-insensitive component was defined as the Ca^{2+}-retained after 30 sec in the presence of 50 μM IP_3 minus the Ca^{2+}-retained upon dilution in 10 μM A23187. The IP_3-sensitive component was the IP_3-induced Ca^{2+}-release and it was determined as the difference between the total uptake and the IP_3-insensitive component. Inset: Relationship between the IP_3-sensitive and the insensitive Ca^{2+} pools and the Ca^{2+} uptake (Ca^{2+} load). For the IP_3-insensitive component line, the slope = 0.899±0.154 and the correlation coefficient = 0.971 indicate that there was a significant ($p < 0.05$) positive correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded. For the IP_3-sensitive component line, the slope = 0.101±0.154 and the correlation coefficient = 0.353 indicate that the correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded was not significant ($p > 0.05$). The values shown are mean±SEM of the data pooled from 3 separate experiments and the total number of replicates is 12-24. All data obtained in each experiment were normalized to the control (A23187 sensitive Ca^{2+}-retained in the skinned cells after loading under the standard conditions for 18 min followed by incubation with the standard release solution without IP_3 for 30 sec). Taken with permission from (Elmoselhi et al., 1996).

Fig. 23


Total
IP3-insensitive
IP3-sensitive
Ca2+ retained (% control)
120
100
80
60
40
20
0
0.05
0.1
0.2
1
10
[Ca2+] in loading medium, μM
Ca2+ uptake in each pool (% total uptake in control)
Ca2+-uptake (% control)
IP3-insensitive
IP3-sensitive

3.4.4 Effect of peroxide and superoxide on IP_3-induced Ca^{2+} release:

The cultured smooth muscle cells from pig coronary artery were pretreated with different concentrations of peroxide (0 to 0.3 mM) for 30 min and then washed to remove the peroxide. The cells were then permeabilized and the effects of peroxide pretreatment on the Ca^{2+} uptake and the IP_3-induced Ca^{2+} release were examined. This protocol was used to avoid any interference between reactive oxygen species and any of the reagents used during permeabilizing, Ca^{2+} loading or Ca^{2+} release. This protocol has the further advantage that it monitors only the irreversible effects of the reactive oxygen species. The pretreatment with 0, 0.1 and 0.3 mM peroxide resulted in Ca^{2+} uptake values of 100±1, 58±3 and 32±2%, respectively (Fig. 24). The corresponding mean values for loading into the IP_3-insensitive Ca^{2+}-pool were 75, 42 and 23%, respectively and those for the IP_3-sensitive Ca^{2+}-pool were 25, 16 and 9%, respectively. Thus, there was a positive correlation between the loading into the IP_3-insensitive, or the sensitive Ca^{2+} pools and the total Ca^{2+}-uptake indicating that peroxide inhibited loading into either pool. Further analysis showed that the K_i values for peroxide inhibition of the Ca^{2+} uptake into the IP_3-insensitive (0.140±0.007 mM) and IP_3-sensitive (0.165±0.008 mM) pools did not differ significantly ($p > 0.05$), i.e., peroxide was equipotent in inhibiting Ca^{2+} uptake into the two pools.

In another set of experiments, the effects of superoxide were examined. The smooth muscle cells cultured from pig coronary artery were pretreated with 0.3 mM xanthine + different concentrations of xanthine oxidase (0 to 0.3 units/ml) + 1 unit of

catalase (to decompose any peroxide formed from superoxide) for 30 min and then washed to remove the superoxide. The cells were then permeabilized and the effects of superoxide pretreatment on the Ca^{2+} uptake and the IP_3-induced Ca^{2+} release were examined. Pretreatment with 0, 0.1 and 0.3 units/ml of xanthine oxidase resulted in Ca^{2+} uptake values of 100±1, 76±1 and 62±1%, respectively (Fig. 25). The corresponding mean values for loading into the IP_3-insensitive Ca^{2+}-pool were 43, 46 and 44%, respectively and those for the IP_3-sensitive Ca^{2+}-pool were 57, 29 and 18%, respectively. Thus, there was no correlation between the Ca^{2+} loading into the IP_3-insensitive pool and the total Ca^{2+} uptake, but the Ca^{2+} loading into the IP_3 sensitive pools correlated positively with the total Ca^{2+}-uptake indicating that superoxide inhibited loading into the IP_3-sensitive but not the IP_3-insensitive Ca^{2+} pool. The K_i value for loading into the IP_3-sensitive pool was 0.35±0.08 units/ml. Thus, both superoxide and peroxide inhibited the Ca^{2+} uptake into the IP_3-sensitive pool but only peroxide inhibited Ca^{2+} loading into the IP_3-insensitive pool.

Fig. 24. ***Effect of peroxide pretreatment on SR Ca^{2+} uptake and the IP_3-induced Ca^{2+} release in permea ilized smooth muscle cells cultured from pig coronary artery:*** The cells were pretreated with the specified concentrations of peroxide for 30 min, centrifuged to remove the peroxide, permeabilized, loaded with $^{45}Ca^{2+}$ using standard loading conditions, diluted in the standard release solution and filtered 30 sec later. The IP_3-insensitive component was defined as the Ca^{2+}-retained after 30 sec in the presence of 50 μM IP_3 minus the Ca^{2+}-retained upon dilution in 10 μM A23187. The IP_3-sensitive component was the IP_3-induced Ca^{2+}-release and it was determined as the difference between the total uptake and the IP_3-insensitive component. Inset: Relationship between the IP_3-sensitive and the insensitive Ca^{2+} pools and the Ca^{2+} uptake (Ca^{2+} load). For the IP_3-insensitive component line, the slope = 0.758 ± 0.007 and the correlation coefficient > 0.999 indicating that there was a significant ($p < 0.05$) positive correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded. For the IP_3-sensitive component line, slope = 0.242 ± 0.007 and correlation coefficient >0.999 indicating that there was a significant ($p < 0.05$) correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded. The values shown are means ± SEM of the data pooled from three separate experiments and the total number of replicates is 12-24. All data obtained in each experiment were normalized to the control (A23187 sensitive Ca^{2+}-retained in the skinned cells not treated with peroxide after loading under the standard conditions for 18 min followed by incubation with the standard release solution without IP_3 for 30 sec). Taken with permission from (Elmoselhi et al., 1996).

Fig. 24

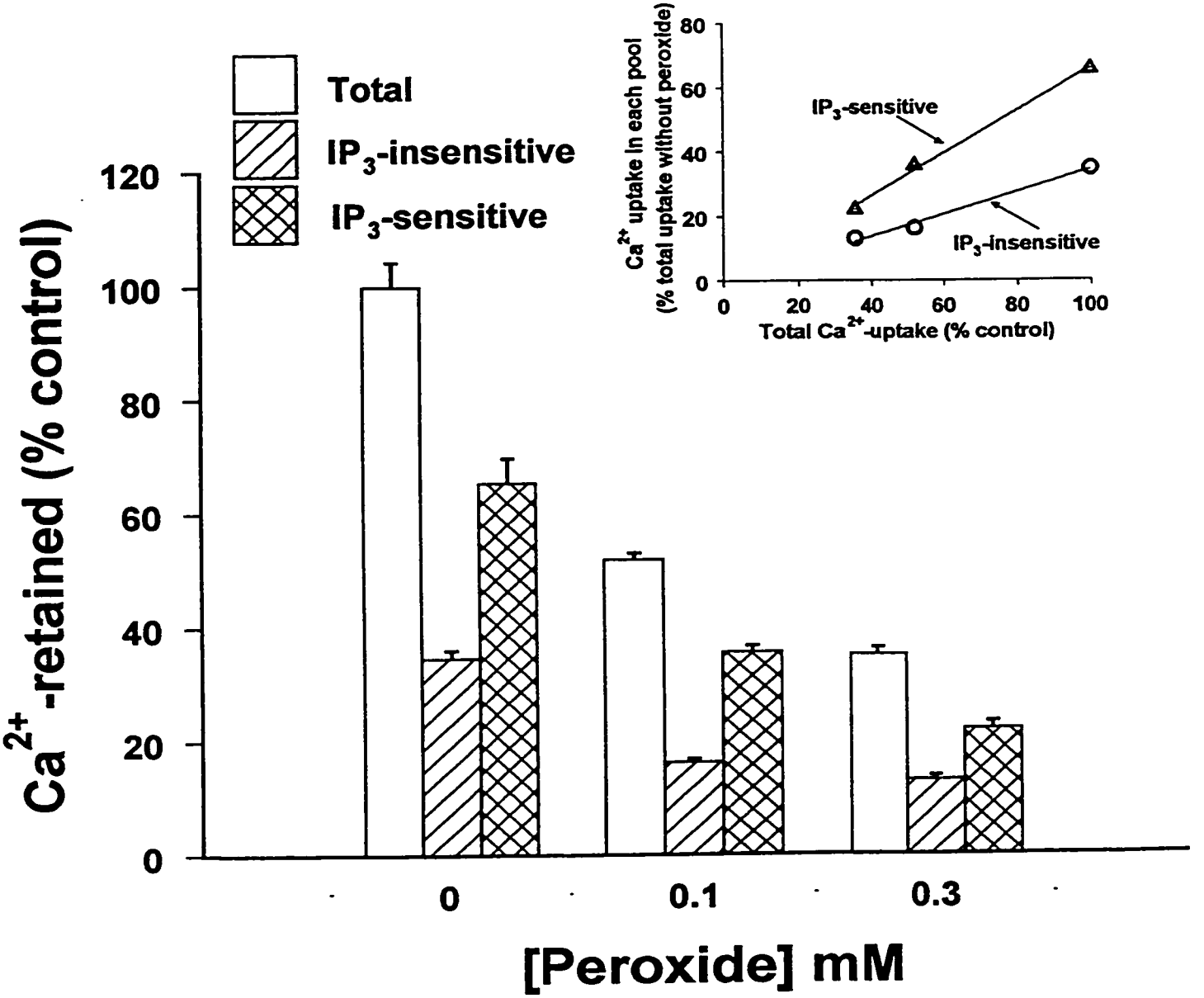
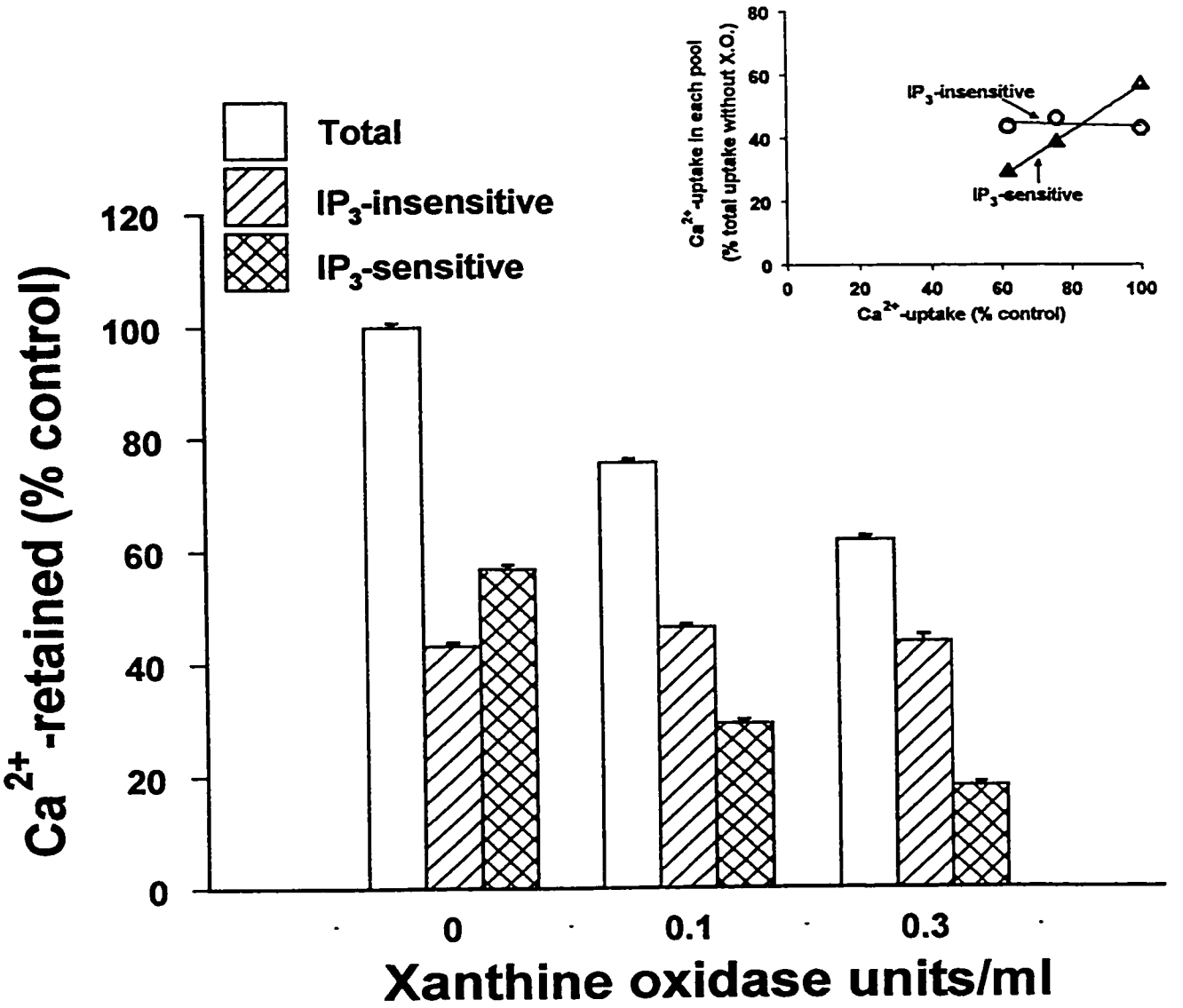

Total
IP3-insensitive
IP3-sensitive
Ca2+-retained (% control)
120
100
80
60
40
20
0
0
0.1
0.3
[Peroxide] mM
Ca2+ uptake in each pool (% total uptake without peroxide)
80
60
40
20
0
IP3-sensitive
IP3-insensitive
0
20
40
60
80
100
Total Ca2+-uptake (% control)

Fig. 25. *Effect of superoxide pretreatment on SR Ca^{2+} uptake and on the IP_3-induced Ca^{2+} release in permea ilized smooth muscle cells cultured from pig coronary artery:* The cells were pretreated with 0.3 mM xanthine + specified concentrations of xanthine oxidase + 1 unit/ml of catalase for 30 min, washed to remove the superoxide, permeabilized, loaded with $^{45}Ca^{2+}$ using standard loading conditions, diluted in the standard release solution and filtered 30 sec later. The IP_3-insensitive component was defined as the Ca^{2+}-retained after 30 sec in the presence of 50 μM IP_3 minus the Ca^{2+}-retained upon dilution in 10 μM A23187. The IP_3-sensitive component was the IP_3-induced Ca^{2+}-release and it was determined as the difference between the total Ca^{2+} uptake and the IP_3-insensitive component. Inset: Relationship between the IP_3-sensitive and the insensitive Ca^{2+} pools and the Ca^{2+} uptake (Ca^{2+} load). For the IP_3-insensitive component line, the slope = 0.726 ± 0.173 and the correlation coefficient > 0.999 indicating that there was a significant ($p < 0.05$) positive correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded. For the IP_3-sensitive component line, the slope = 0.029 ± 0.085 and the correlation coefficient = 0.321 indicating that the correlation between the Ca^{2+}-retained in this pool and the total amount of Ca^{2+} loaded was not significant ($p > 0.05$). The values shown are SEM of the data from one experiment with 12 replicates. Repeating the experiment gave results with similar conclusions. All data obtained in each experiment were normalized to the control. Taken with permission from (Elmoselhi et al., 1996).

Fig. 25


Total
IP3-insensitive
IP3-sensitive
Ca2+-retained (% control)
120
100
80
60
40
20
0
0
0.1
0.3
Xanthine oxidase units/ml
Ca2+-uptake in each pool (% total uptake without X.O.)
Ca2+-uptake (% control)
IP3-insensitive
IP3-sensitive

CHAPTER FOUR

DISCUSSION

The objective of this study was to determine how ROS affect ET-1 responses in pig coronary artery smooth muscle. The key experimental findings in our Results were as follows:

1. The ET-1 mediated contraction in de-endothelialized coronary artery rings occurred via both ET_A receptors (ET_A)~80% and ET_B receptors (ET_B) ~20%.

2. Both receptor types utilized similar intracellular Ca^{2+} pathways in their contraction, however ET_B utilized the intracellular Ca^{2+} pool to a greater extent than ET_A.

3. ET_B mediated contraction was more sensitive to damage by hydrogen peroxide compared to ET_A mediated contraction.

4. ET-1 induced increase in $[Ca^{2+}]_i$ was almost completely damaged by peroxide in smooth muscle cells cultured from pig coronary artery.

5. Unlike ET_B, the ET_A mediated contraction involved Ca^{2+}-independent mechanism(s) that had a high resistance to peroxide damage.

6. Microsomes isolated from pig coronary artery contained ET_A and ET_B receptors with ET_A being the predominant one.

7. The concentrations of hydrogen peroxide or superoxide which affected Ca^{2+}-mobilization had no effects on the ET-1 binding to ET_A or ET_B receptors. Initial

experiments conducted using bovine xanthine oxidase to generate superoxide had to be discarded as this preparation interfered with ET-1 binding to isolated microsomes from pig coronary artery. This showed a possible impurity of xanthine oxidase prepared from bovine milk and also the importance of running control experiments.

8. Permeabilized smooth muscle cells cultured from pig coronary artery had properties of the ATP-dependent azide-insensitive oxalate-stimulated Ca^{2+}-uptake similar to those described for the SR Ca^{2+}-pump in isolated membranes from the same artery.

9. A large part of the Ca^{2+}-accumulated in the permeabilized cells could be released with IP_3 under optimum conditions.

10. ROS did not affect the IP_3-dependent Ca^{2+}-release although they inhibited the SR Ca^{2+}-pump. There was a heterogeneity in the inhibition of the SR Ca^{2+}-pump with ROS.

The Discussion will focus on the validity of the methods used, comparison of our observations with the literature, conceptual synthesis of the various findings, the importance of these results in understanding contractile pathways in the coronary artery, and the clinical implications of these results to cardiac ischemia and reperfusion.

4.1. Validity of the Methods Used:

ET-1 responses were monitored in pig coronary artery smooth muscle as: effects of contraction on de-endothelialized artery rings, ET-1 binding to isolated microsomes, effects on $[Ca^{2+}]_i$ in cultured cells, and Ca^{2+}-uptake and release using permeabilized cells.

4.1.1. Contractility Experiments: The effects of ET-1 and other agents were monitored on isometric *contractions of de-endothelialized artery rings*. This method of contractility measurement has the following advantages: (i) one can rapidly monitor the effects of various compounds on many tissues, (ii) monitoring de-endothelialized artery rings, allowed for ruling out any endothelial-mediated actions, (iii) the tissues can be used with or without endothelium depending on whether one wants to examine the tissue-tissue interactions or just the effects on the smooth muscle, and (iv) it allows for extrapolation of these results to *in vivo* conditions and yet allows for dissection of the underlying mechanisms. The disadvantages of using tissue rings in a functional assay are: (i) a lack of humoral and neuronal feed back. One can use an open chest model (Daniel, Kwan, 1983; Granger et al., 1986), a whole animal model which allows for monitoring of integrated actions but this presents difficulties in dissecting out mechanisms; (ii) the best way for data normalization is not clear. We measured the force of contraction in mN and used rings of uniform lengths from a specific region of the artery. However, one can normalize the effects of an agent as: mN/mg tissue, mN/mm artery area, or percent response obtained with KCl. Each method has its own advantages and pitfalls. Therefore, when comparing the proportions of receptor types inducing contraction in the artery rings using different agonists and antagonists, the data were analyzed in mN tension as shown in the results and also as percent KCl (not shown) and the conclusions were independent of

the method of normalization. There was considerable variability in the responses since the hearts obtained were from unknown animals with variation in size, age and sex. Also there was a time lapse for transportation of the hearts from the slaughter house to the laboratory. We have tried to minimize this variability by keeping the hearts on ice or in ice-cold PSS solutions.

4.1.2. Pharmacological Agents Used: In characterizing ET-1 receptor subtypes, we used three *selective agonists and antagonists*. Cyclic pentapeptide BQ123 has a high selectivity and affinity to ET_A receptors (approximately 800 times higher affinity to ET_A than to ET_B receptors) (Ihara et al., 1992). The selectivity of this highly potent and water soluble compound has been demonstrated in pharmacological, biochemical and *in vivo* studies in both animal and human models. In this study, BQ123 inhibited ET-1-induced contractions and also relaxed arteries precontracted with ET-1. These results indicated that the contribution of ET_A receptors was approximately 80% of the ET-1-mediated contraction. These results were confirmed using IRL1620 which is a highly selective agonist for ET_B receptors and BQ788 which is a highly selective antagonist for ET_B receptors (Takai et al., 1992). Thus, the results on the proportion of ET_A and ET_B receptor-mediated contractions are the same using BQ123, IRL1620 and BQ788.

4.1.3. Binding Experiments: ET-1 receptors were also characterized by *direct binding of ^{125}I-ET-1 to microsomes* isolated from fresh coronary artery smooth muscle. In

competition binding experiments unlabeled ET-1 and BQ123 displaced most of the ET-1 binding sites while unlabeled IRL1620 and BQ788 displaced only a small proportion of the ET-1 binding sites in coronary artery isolated microsomes. Thus the direct binding studies confirmed the results obtained in the contractility experiments.

4.1.4. $[Ca^{2+}]_i$ Measurements and the Use of Cultured Cells:

An increase in $[Ca^{2+}]_i$ in response to ET-1 is a major intermediary event in smooth muscle contraction (Pollock et al., 1995). In this study, $[Ca^{2+}]_i$ measurements were made using the fluorescence probe Fluo 3 which can be trapped inside the cells and whose fluorescence efficiency depends on $[Ca^{2+}]$ (Rijkers et al., 1990). Alternative approaches include the use of other fluorescence probes such as Fura2 and proteins such as aquorin (Abe et al., 1995; Goldman et al., 1990). An ideal experiment would have used a method suitable for measuring $[Ca^{2+}]_i$ continuously while also monitoring contraction of the tissue (Tameyasu, 1998). However, equipment for such experiments was not available. The method used here allowed for monitoring $[Ca^{2+}]_i$ only in cultured cells. The major disadvantage of using the cultured cells was that these cells had changed to a phenotype which expressed only the ET_A receptors. This problem was detected in a preliminary experiment in this study and has also been reported previously. However, these cells retain the smooth muscle phenotype according to the following criteria: i) the cell protein reacts with α-actin smooth muscle antibodies in Western blots to show an α-actin band

(45kDa), and with SERCA2b selective antibodies which give 115kDa bands, (ii) the cells retain the typical spindle shape of the smooth muscle cells, and (iii) all the cells react with the anti-α-actin antibodies using immunohistochemical staining (Elmoselhi et al., 1995). Therefore, the results of the $[Ca^{2+}]_i$ measurements may be useful for studying the mechanisms involved in ET-1 response.

4.1.5. IP_3-induced Ca^{2+} Release Study: Release of Ca^{2+} by IP_3 from the SR is a key step in the signal transduction of ET-1 mediated contraction. In this study, the characteristics of the IP_3-induced release were examined using a permeabilized cell preparation. Permeabilization with saponin has been used previously to examine $^{45}Ca^{2+}$ accumulation in the SR of other cells (Saida, Nonomura, 1978). It allows low molecular weight materials such as ATP and IP_3 to freely enter the cells and access the various organelles so that one can examine the effects of these agents on Ca^{2+} entry and/or release from them. In order to examine the release of $^{45}Ca^{2+}$ stored in the SR, we permeabilized the cells and "loaded" them with $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ therefore entered the SR via the SR Ca^{2+} pump present in these cells. The loading solution contained azide to inhibit any $^{45}Ca^{2+}$ accumulation in mitochondria, an ATP-regenerating system to fuel the $^{45}Ca^{2+}$ accumulation and oxalate to enhance its accumulation. This procedure was preferable to using isolated microsomes because cell homogenization and ultracentrifugation can damage the cellular organization which was not desired. However, the main pitfall in using the permeabilized cells is that

one can not distinguish between the SR Ca^{2+} pools from the nuclear ones, since both SR Ca^{2+} pump and IP_3 channels have been reported in nuclear membranes (Gerasimenko et al., 1996).

4.1.6. Treatment with ROS:

The ideal model to examine the effect of ROS on the different parameters of the coronary artery function in ischemia/reperfusion is in a human subject following the onset of myocardial infarction and after reopening the coronary artery obstruction. One can obtain tissues from the affected subjects and study the various parameters of interest and also measure the ROS levels by either electron paramagnetic resonance spectroscopy or tissue chemiluminescence (Grech et al., 1996). However, since it is grotesque to do so, the ideal model can not be used. One alternative is to use animal models in similar experiments (Daniel, Kwan, 1983). However, a large amount of tissue needed in these experiments makes this choice exorbitantly expensive. Therefore, the *in vitro* studies are needed to arrive at the mechanisms and animal studies to examine their implications. Studies of the latter type are being conducted in other laboratories.

Throughout the present study tissues or cells were treated with the ROS for 30 min at 37°C, the tissues or the cells were washed with normal buffer and then used for the various measurements. The advantage of this protocol is that it avoids any immediate effect of the ROS on the tissues or on the assay solutions, and it measures only the long

term damage of the parameter studied. The concentrations of peroxide and superoxide used were higher than those attained in physiological or pathological conditions due to two main reasons. First to emphasize a specific observation or phenomenon using an extreme condition of exposing these tissues or cells to a high concentration of ROS. Second, during cardiac ischemia and reperfusion in *in vivo* situation the tissues and cells are exposed to ROS concentrations for longer than the time scale of the experiments performed here.

Superoxide is very unstable; however, it can be produced using a regenerating system consisting of xanthine plus xanthine oxidase which has been used previously in several studies (Grover, Samson, 1988; Grover et al., 1995; Nunes et al., 1997). Since this system requires both xanthine and xanthine oxidase, one can use either component alone to test for any other effects or impurities. Since superoxide can readily dismutate to produce peroxide which has been shown to damage the SR Ca^{2+}-pump (Grover, Samson, 1987), the superoxide regenerating system used in our experiments always contained excess catalase to decompose any peroxide formed during the course of the incubation. Thus any effects reported here are due to superoxide only. Another control that could have been carried out was to use superoxide dismutase. However, this was not necessary since superoxide generated using bacterial xanthine oxidase did not affect the ET-1 receptor binding. Several commercial xanthine oxidases contain impurities which inhibit

the SR Ca^{2+}-pump without generating superoxide (Grover, Samson, 1987). A purified bovine milk xanthine oxidase preparation was shown not to inhibit the pump in the absence of xanthine. However, this preparation activated the ET-1 receptor binding. Possible explanations for this increase in the ET-1 binding by the bovine milk xanthine oxidase preparation include the presence of latent receptors or an increase in retention of the membranes due to an aggregation of the ET-1 receptors during binding. Bacterial xanthine oxidase, which did not affect ET-1 receptor binding has not yet been tested for its effects on the SR Ca^{2+}-pump. However, the exact reason of this artefact is not known. Due to this complication, further experiments using superoxide in ET-1 mediated contraction were not conducted.

Thus all the experiments were conducted using appropriate controls and wherever possible more than one method was used in this study.

4.2. Comparison of the Results with the Literature: This section compares our results with those in the literature on ET-1 receptor types, Ca^{2+} mobilization, properties of the SR Ca^{2+}-pump and IP_3 -dependent Ca^{2+} release, contractile pathways, effects of ROS on contraction and effects of ROS on Ca^{2+} pumps and channels.

4.2.1. ET-1 Receptor Types:

The contractility experiments using de-endothelialized artery rings and the direct binding experiments demonstrate that smooth muscle in pig coronary artery contains ET_A

(~80%) and ET_B (~20%) receptors. These results are consistent with a previous report on porcine de-endothelized coronary artery using the endothelins ET-1 and ET-3, the former being more selective for ET_A and the latter for ET_B (Ihara et al., 1991). The predominance of ET_A receptors has also been reported in human coronary artery (Davenport et al., 1995b; Davenport et al., 1995a). In contrast to smooth muscles in the blood vessels, the endothelium consistently expresses ET_B receptors only whose activation releases nitric oxide thereby producing smooth muscle relaxation (Karaki et al., 1993).

4.2.2. Ca^{2+} Mobilization:

ET-1 mediated vasoconstriction in various smooth muscles is a complex multi-step physiological process. Increase in $[Ca^{2+}]_i$, nevertheless, is a common feature in all signal pathways of smooth muscle contraction. ET-1 causes an increase in $[Ca^{2+}]_i$ via both Ca^{2+} release from intracellular stores and Ca^{2+} entry from extracellular spaces (Pollock et al., 1995). The Ca^{2+} release occurs mainly via IP_3 sensitive channels and possibly Ca^{2+} induced Ca^{2+} release. The Ca^{2+} entry from extracellular Ca^{2+} stores occurs via L-type Ca^{2+} channels and other undefined pathways (Goto et al., 1989; Van Renterghem, Lazdunski, 1993). Our results suggest that ET_A and ET_B receptors share similar Ca^{2+} mobilization pathways as reported by others. However, since changes in $[Ca^{2+}]_i$ in Ca^{2+}-containing and Ca^{2+}-free solutions were not conducted in this study or in the literature, this suggestion remains to be explored further. In several *in vitro* studies, ET-1 application to smooth muscle results

in transient increase in $[Ca^{2+}]_i$ and a subsequent sustained increase in $[Ca^{2+}]_i$. The initial transient increase in $[Ca^{2+}]_i$ depends on Ca^{2+} release from intracellular stores while the subsequent sustained increase in $[Ca^{2+}]_i$ is due to extracellular Ca^{2+} entry from extracellular Ca^{2+} stores (Pollock et al., 1995). Similarly in this study, cultured smooth muscle cells showed both phases of $[Ca^{2+}]_i$ increase. However, in Ca^{2+}-free solution only the initial transient increase was observed (Data not shown). These results are consistent with the previous results on coronary artery smooth muscle using angiotensin II and CPA. The maximum increase in $[Ca^{2+}]_i$ with AngII and CPA was shown to be 103± 8 and 142± 23 nM respectively (Grover et al., 1995). Also, in renal arteriolar and aortic cultured smooth muscle cells, the initial peak response of Ang. II (10^{-7} M) increased $[Ca^{2+}]_i$ from 50 to 240 nM and from 57 to 201 nM, respectively. However, a sustained phase was followed at 50-60% of this initial peak response (Zhu Z et al, 1996).

4.2.3.Properties of SR Ca^{2+} in Different Tissue Preparations: The kinetic parameters for the SR Ca^{2+}-pumps are shown in Table 1 and were obtained using permeabilized cells in both this study and those reported previously using isolated membranes from pig coronary artery smooth muscle. For permeabilized cells, the K_m value for Ca^{2+} was 0.20 ± 0.03 μM and it was comparable with that in the isolated membranes which was 0.58 ± 0.05 μM (Grover, Samson, 1986). However, the two studies used slightly different values for the binding constant for Ca-$EGTA^{2-}$ (Grover et al., 1982). K_m values for

$MgATP^{2-}$ for the SR Ca^{2+}-pump using permeabilized cells (400±34 μM) or using membranes (812±83 μM) in the previous study were not very different. The inhibition of the SR Ca^{2+}-pump has been examined in contractility experiments using pig coronary artery rings and using isolated membranes in tissues other than coronary artery . In these studies, and in the present study using permeabilized cells, thapsigargin was a more potent inhibitor than CPA (Lytton et al., 1991; Misquitta et al., 1996; Uyama et al., 1993). The pH optimum of the SR Ca^{2+}-pump using isolated membranes was reported to be 6.4 to 6.8. However, in the present study the pH optimum was 6.2. It can not be ascertained whether or not this property is unique to this preparation because in other studies using vascular smooth muscle permeabilized cells the pH optimum of the Ca^{2+}-uptake was not reported (Stout, 1991; Suematsu et al., 1984). Some of the possible explanations for this discrepancy follow but at present it is difficult to distinguish between the different possibilities:

(a) The permeabilized cells contain acid phosphatase - an enzyme localized in the cytosol and the plasma membranes (Kwan, Ito, 1987). Therefore, more creatinine phosphate was hydrolysed at the acidic pH values. At the lower pH values, this hydrolysis produced higher concentrations of inorganic phosphate which, in turn, acted as a Ca^{2+}-precipitating agent and thereby reduced Ca^{2+}-efflux. The isolated membranes, which are enriched in SR, contain a lower ratio of plasma membrane to SR membranes and almost

no soluble fractions (Grover et al., 1985). Hence at the lower pH values, they may not generate the inorganic phosphate as rapidly. Thus the pH optimum for the permeabilized cells may result from a complexity of the pump pH optimum and the artefactual decrease in the Ca^{2+}-efflux.

(b) The isolated membrane preparation does not contain nuclei but the permeabilized preparation does. Also, the nuclei contain a Ca^{2+}-pump similar to the SR Ca^{2+}-pump (Stout, 1991) but the pH optimum of the Ca^{2+}-uptake by nuclei is not known.

(c) The plasma membrane components released due to saponin treatment modified the pH profile of the SR Ca^{2+}-pump.

(d) The pH profile using the isolated membranes reported previously (Grover, Samson, 1986) was artefactual due to the process of membrane isolation and/or due to change of cellular components in isolated membrane compared to the permeabilized preparation.

Thus, with the exception of the pH optimum, the properties of the SR Ca^{2+}-pump in the permeabilized cultured cell preparation are similar to those reported using the isolated membranes. However, the permeabilized cells have the advantage that they contain intact cellular organelles which are lost during the membrane isolation.

4.2.4. Properties of IP_3-induced Ca^{2+} Release in Our Results and Others:

The IP_3-induced Ca^{2+} release from the coronary artery permeabilized smooth muscle cells occurred rapidly at 0 or 37°C, consistent with the release being due to an

opening of an ion channel and not by a transport process involving a metabolic pathway. The EC_{50} value for IP_3 was 2.7±1.0 μM and the IP_3-induced Ca^{2+} release was inhibited by exposure to high [Ca^{2+}] in the release medium. These properties are consistent with those reported for the IP_3-induced Ca^{2+} release from other vascular smooth muscles (Ehrlich, Watras, 1988). The amount of Ca^{2+}-released with IP_3 did not exceed 65% of the total ionophore sensitive Ca^{2+} uptake. However, the nature of the IP_3-insensitive Ca^{2+}-pool is not known. In a preliminary experiment, deoxy-IP_3 or didedoxy IP_3 was observed to release approximately the same proportion of Ca^{2+} as did IP_3 (data not shown). Thus degradation of IP_3 is not a likely reason for the incomplete release. It is possible that the remainder of the Ca^{2+} can be released either with other agents in an IP_3-dependent (e.g., with IP_4 or GTP) or IP_3-independent manner (e.g., ryanodine) (Gill et al., 1992; Sturek et al., 1992; Wagner-Mann et al., 1992). Submaximal concentration of IP_3 can release only a fraction of the available Ca^{2+} in the SR. This property of the IP_3-induced Ca^{2+} release has been presented as the "quantal hypothesis" (Parys et al., 1995; Patel, Taylor, 1995; Patel, Taylor, 1995). It has been suggested that two mechanisms can account for this quantal release. An all-or-none mechanism which proposed the existence of multiple Ca^{2+} stores with different IP_3 sensitivity (Lino et al., 1993). A specific concentration of IP_3 will induce total release of certain stores but not others. The seconds mechanism that has been proposed is the steady-state mechanism which suggests that all the stores are equally

sensitive to IP_3 but their sensitivity is regulated by the luminal Ca^{2+} concentration (Parys et al., 1993). The IP_3 would release Ca^{2+} from all the stores until luminal Ca^{2+} drops to a certain threshold . Thus, the extent of the IP_3-sensitive Ca^{2+} release from the SR is limited not only by [IP_3] but also by the amount of Ca^{2+} load. The proportion of Ca^{2+} released via the IP_3 channels should increase with increasing Ca^{2+} load (Irvine, 1990; Missiaen et al., 1992; Parys et al., 1993; Short et al., 1993). In the present study, varying the load by using different loading times showed that the proportion of the loaded Ca^{2+} released via the IP_3-induced pathway was constant. However, when the load was varied by using different [Ca^{2+}] in the loading medium, an inverse relationship was obtained between the extent of loading and the proportion of the loaded Ca^{2+} release via the IP_3-induced pathway. Our results do not support the steady-state mechanism of the "quantal hypothesis", since the proportion released did not decrease with the decreasing load, and in fact, it may have been greater at lower loads when the loading was performed by using different [Ca^{2+}].

4.2.5. Contractile Pathways:

This section focuses on the use of extracellular Ca^{2+} entry and intracellular Ca^{2+}-pools for force generation by endothelin and other pharmacological agents.

The ET-1 mediated contraction in de-endothelialized coronary artery rings occurred by ET_A (~80%) and ET_B (~20%) receptor activation. Several studies have reported similar

findings using smooth muscle from coronary artery and other blood vessels (Pierre, Davenport, 1998; White et al., 1993). However, in jugular vein ET-3 was equipotent with ET-1, and BQ123 did not inhibit the ET-1 induced contractions, indicating that in this tissue ET_B receptors predominate (Sumner et al., 1992). Furthermore, in the human coronary artery, a heterogeneity of the endothelin receptors within the different segments has been suggested (Godfraind, 1993). Thus, ET-1 receptor subtype densities in the smooth muscle vary considerably in different blood vessels and even in different segments of the same vessel. Whether these changes depend on the force of the blood flow or on the function of the blood vessels and what role they play in regulating the vessel functions under physiological and pathological conditions, is yet to be determined.

In this study, the contractions of the artery by ET-1 and IRL1620 depended on the mobilization of intracellular Ca^{2+}-pools and on extracellular Ca^{2+}-entry via the L-type channels and other pathways. Even though the contractions produced by IRL1620 were smaller, the relative utilization of the intracellular Ca^{2+}-pools in these was larger than for the contractions with ET-1. The contraction induced by IRL1620 in our experiments was not sustained as in the case of the ET-1 mediated contraction, which may imply that its contraction depends more on Ca^{2+} release from intracellular stores than on Ca^{2+} entry. The utilization of intracellular Ca^{2+}-pools by the ET_A mediated pathway reported vary approximately in the range of 10-35% in different tissues and is thus comparable to the

proportion in this study (Gardner et al., 1992; Fareh et al., 1996; Furuya et al., 1994). However, there are no reports on the utilization of intracellular Ca^{2+}-pools by the ET_B receptors. Several agonists such as histamine, epinephrine, and angiotensin II have been used to examine different parameters of the cellular Ca^{2+} mobilization. However, ET-1, as shown by others as well as in our results, is more potent constrictor than any other (Touyz et al., 1996). Thus, one possibility is that ET-1 mobilizes more cellular Ca^{2+} pathways than the other agonists. Another possibility is that the ET-1 mediated contraction involves Ca^{2+}-independent pathways as will be discussed later in this chapter.

4.2.6. Effects of ROS on ET-1 Mediated Contraction: Pretreating the de-endothelialized pig coronary artery rings showed that ET_B mediated contraction was more sensitive to damage by peroxide than the ET_A mediated contraction. The inhibition of ET_A mediated contraction occurred in both Ca^{2+} containing and in Ca^{2+} free solutions with IC_{50}= 1 and 1.4 mM respectively. While the inhibition of the ET_B-mediated contractions occurred with IC_{50}=0.32 and 0.25 mM in both Ca^{2+}-containing and Ca^{2+}-free solutions respectively. Sensitivity to peroxide of contraction by 60 mM KCl, angiotensin and CPA has been reported previously using this tissue (Grover et al., 1995). Compared to the contractions obtained with ET_A-receptors, the membrane depolarization dependent contractions due to 60 mM KCl are extremely resistant to peroxide, which indicates the resistance of the contractile apparatus to peroxide. Peroxide damage to contractions due to CPA have IC_{50}

values similar to those obtained for the ET_B receptor mediated contraction in this study while contractions due to angiotensin II are even more sensitive (Grover et al., 1995). These results indicated that ET_B mediated contraction may utilize similar Ca^{2+} pools as for CPA and angiotensin II mediated contractions. It is emphasized that in this study the effects of preincubating arteries on the contractions were examined. There are several studies where contractions in the presence of peroxide or superoxide have been reported and these can not be directly compared (Yasuda et al., 1994; Iesaki et al., 1996).

4.2.7. Effects of ROS on SR Ca^{2+} Pumps and Channels:

The effects of peroxide and superoxide can be explained using a model (Fig. 26) in which Ca^{2+} is loaded actively into two types of pools: the release from one is IP_3-dependent and from the other it is IP_3-independent. In this model, peroxide treatment inhibits loading into both types of pools while that with superoxide damages only the loading into the IP_3-sensitive Ca^{2+}-pool. It is the Ca^{2+} pump and not the IP_3-channel which is damaged by ROS. One can suggest models in which the IP_3 channels are damaged and not the Ca^{2+}-loading. If the IP_3 channels were to be damaged so that they could not be opened with IP_3, this would not cause a decrease in the Ca^{2+}-loading. Such a model does not apply since an inhibition of the loading was observed. However, based on Ca^{2+}-flux studies alone, it is difficult to distinguish between a model in which the damaged IP_3 channel remains open even without IP_3, thus decreasing the loading into the

IP_3-sensitive pool vs. a model in which the pump is inhibited. In another study examining the effects of peroxide on the same cells but using the Ca^{2+}-dependent formation of a 115 kDa acylphosphate protein rather than Ca^{2+}-fluxes, it was shown that 0.1 mM peroxide inhibited the Ca^{2+}-pump by 36.7% (Grover et al., 1995). These results are in agreement with an inhibition of 38 to 42% expected from the inhibition constants for peroxide for loading into the IP_3-insensitive and the IP_3-sensitive pools of 0.140 to 0.165 mM obtained in the present study. This is in accordance with the proposed model in which the Ca^{2+} loading is impaired but not the IP_3 channel. The model in which reactive oxygen damages the pump and not the IP_3 channels is also consistent with experiments on bovine thoracic aorta in which IP_3-dependent Ca^{2+} channels were not damaged by superoxide (Suzuki, Ford, 1992).

In isolated SR vesicles from pig coronary artery, the IC_{50} of ATP-dependent azide-insensitive oxalate-stimulated Ca^{2+}-uptake was found to be 1.5-5 μM. (Grover et al., 1992). Thus the SR Ca^{2+}-pump in the permeabilized cells was less sensitive to peroxide than the isolated membranes, consistent with the presence of catalase and other scavenging mechanisms in intact cells. Furthermore, the experiments with the isolated membranes were conducted in the presence of azide which inhibits catalase but the work with the intact cells could not be carried out using azide. The effect of peroxide on Ca^{2+}-accumulation is due to an inhibition of the Ca^{2+}-pump rather than an increased membrane

permeability because peroxide inhibits the Ca^{2+}-dependent formation of 115 kDa acylphosphate formation (Grover et al., 1992). ROS also depress phospholamban-sensitive SR Ca^{2+}, Mg^{2+}-ATPase and Ca^{2+}-uptake in isolated vesicles from cardiac muscle (Morris, Sulakhe, 1997). Thus even though cardiac muscle expresses the Ca^{2+}-pump isoform SERCA2a and the coronary artery expresses SERCA2b, peroxide damages the pump in both tissues (Grover, Khan, 1992; Grover et al., 1997). In patch clamp studies, in intact myocytes from ventricular guinea pig loaded with FURA -2, peroxide treatment showed a marked inhibition of the caffeine-induced $[Ca^{2+}]_i$ (Goldhaber, Weiss, 1992). However, in saponin permeabilized rat ventricular trabeculae , peroxide and hypochlorous acid reduced the amount of Ca^{2+} release from SR, but superoxide did not (MacFarlane et al., 1994). It is not clear whether these results were due to an inhibition of Ca^{2+} loading into the SR, or inhibition of Ca^{2+} release from it. In any event, these results are consistent with the idea of heterogeneity of the SR Ca^{2+}-pumps.

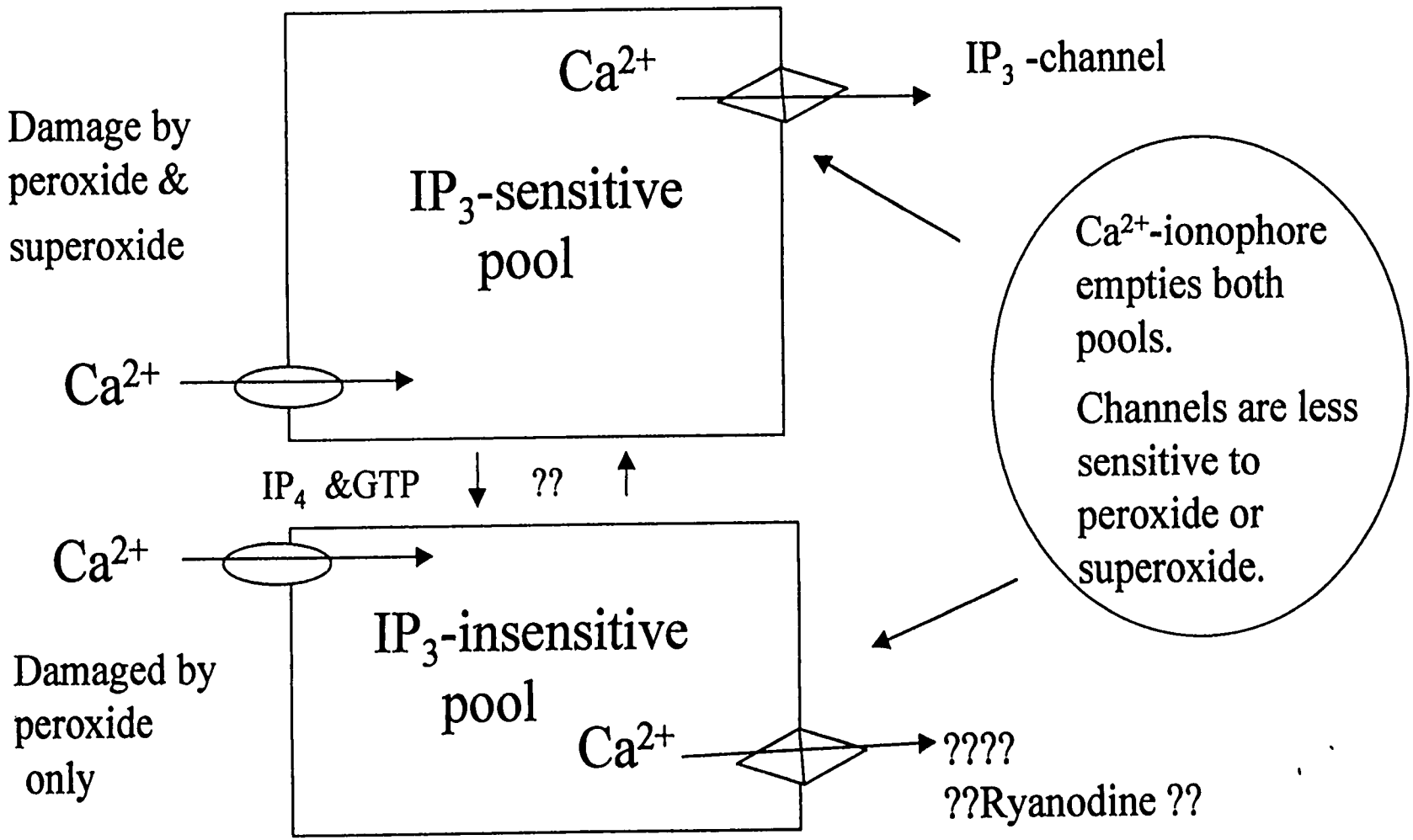


Fig. 26 A model of IP_3-sensitive, and -insensitive Ca^{2+} pools. Taken from Elmoselhi et al, 1996.

4.3. Conceptual Synthesis of the Various Findings:

The proportion of ET_A receptors (~80%) and ET_B receptors (~20%) which contribute to the ET-1 mediated contraction is similar using both experimental approaches: a receptor binding of isolated microsomes and contractility to artery rings. However, ET_B mediated contractions utilized the intracellular Ca^{2+} pool to a greater extent than ET_A. An attempt was made to determine if this was directly related to the Ca^{2+} mobilization; however, these experiments were not successful as the cultured smooth muscle cells used for Ca^{2+} monitoring did not contain ET_B receptors. The observations related to the damage by peroxide treatment were as follows: ET_B mediated contraction was more sensitive to damage by hydrogen peroxide compared to ET_A mediated contraction; ET-1 induced increase in $[Ca^{2+}]_i$ was almost completely damaged by peroxide in smooth muscle cells cultured from pig coronary artery; unlike ET_B, the ET_A mediated contraction involved Ca^{2+}-independent mechanism(s) that had a high resistance to peroxide damage, the concentrations of hydrogen peroxide or superoxide which affected Ca^{2+}-mobilization had no effects on the ET-1 binding to ET_A or ET_B receptors; permeabilized smooth muscle cells cultured from pig coronary artery had properties of the ATP-dependent-azide insensitive-oxalate stimulated Ca^{2+}-uptake similar to those described for the SR Ca^{2+}-pump in isolated membranes; ROS did not affect the IP_3-dependent Ca^{2+}-release although they inhibited the SR Ca^{2+}-pump. The differences in the effects of peroxide and superoxide

on Ca^{2+} loading into IP_3-sensitive and insensitive pools were discussed in *section 4.2.7.* The focus in this section will be on correlating the Ca^{2+}-mobilization, ROS-sensitivity and contractility data on ET_A and ET_B-mediated pathways into a coherent model.

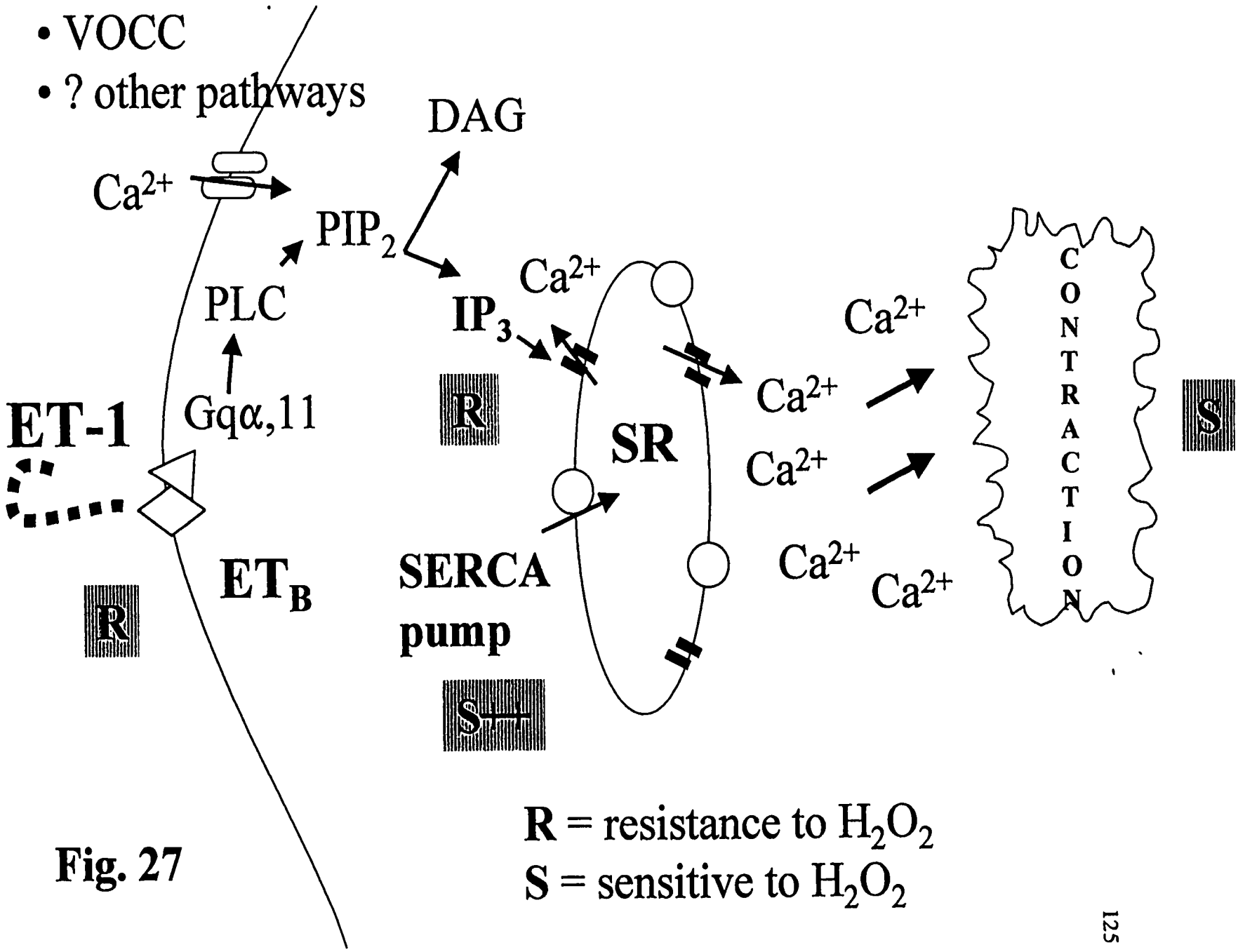
ET_A and ET_B mediated contractions in various smooth muscles may utilize multiple pathways (Little et al., 1992). Peroxide inhibited both ET_A and ET_B mediated contractions in the denuded coronary artery rings but the ET_B mediated contraction was more sensitive to damage by peroxide than the ET_A mediated contraction. Peroxide treatment did not damage binding to ET_A or ET_B sites but it readily inhibited the ET-1 induced increase in $[Ca^{2+}]_i$ and it damaged the SR Ca^{2+} pump in smooth muscle cells. Therefore, the effect of peroxide on the ET_B mediated contraction is consistent with the greater dependence of the ET_B mediated contraction on the release of Ca^{2+} from SR. This explanation forms the basis of the model for the ET_B-mediated contraction outlined in Fig. 27. Then the lower peroxide sensitivity of the ET_A-mediated contraction can be explained in a model similar to the ET_B model except that an additional peroxide insensitive Ca^{2+}-independent pathway is also involved as shown in Fig. 28. Possibilities for this pathway may include PKC, a tyrosine kinase or other unknown pathways which may alter the Ca^{2+}-sensitivity of the contractile apparatus. Thus the ET_A and ET_B receptor activation may share some common pathways but not others. Other reports have also suggested differences between the signal pathways of ET_A and ET_B (Henry, 1993; Pang et al., 1998;

Saita et al., 1997; Saita et al., 1997). The exact contribution of the different pathways to the ET-1 mediated contraction in smooth muscle has not yet been reported. In rabbit saphenous vein, the protein kinase C inhibitor calphostin C decreased both efficacies and potencies of ET-1 and ET-3 suggesting that PKC is involved in the ET_A and ET_B mediated contractions in this tissue (Sudjarwo, Karaki, 1995). However, the role of PKC may be greater in the ET_A-mediated contraction than in the ET_B-pathway (Gray et al., 1994). The PKC mediated pathway, most probably ϵ isoform of protein kinase C, does not appear to be Ca^{2+}-dependent (Walsh et al., 1996; Shimamoto et al., 1992a). Thus, the peroxide damage of the ET-1 response may be related to cellular Ca^{2+}- regulating mechanisms, in particular, the sarcoplasmic reticulum Ca^{2+} pump as shown in other reports (Morris, Sulakhe, 1997).

Thus the results of this study can be summarized as follows: pig coronary artery smooth muscle contains a larger proportion of ET_A receptors than ET_B; both ET-1 receptor types depend on extracellular Ca^{2+}-entry and mobilization of SR Ca^{2+}; peroxide readily damages SERCA2 pumps; and ET_A mediated contraction involves an additional Ca^{2+}-independent pathway which is not affected by peroxide.

Fig. 27. ***The Effect of hydrogen peroxide on the signal transduction pathways of ET_B mediated contraction in pig coronary artery smooth muscle.***

A summary model of the peroxide susceptibility of various signal pathways of ET_B mediated contraction. ET-1 binding to ET_B and IP_3 channels was resistant to damage by peroxide. ET_B-mediated contraction was responsible for approximately 20% of the ET-1 mediated contraction in denuded coronary artery, and utilized more $[Ca^{2+}]_i$ than ET_A. The ET_B mediated contraction was more sensitive to damage by peroxide compared to ET_A mediated contraction. SR Ca^{2+}-pump was also very sensitive to damage by peroxide.



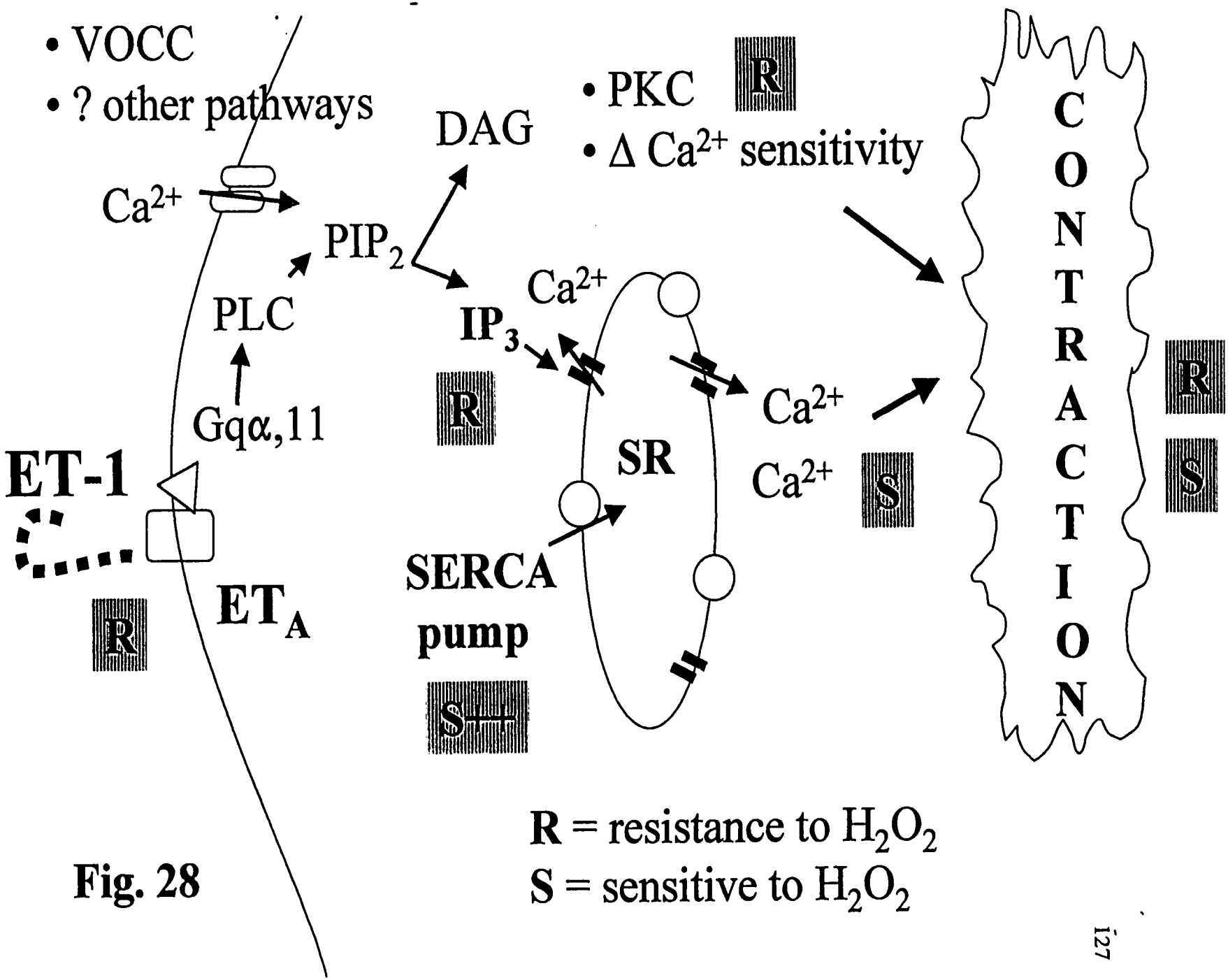
R = resistance to H_2O_2

S = sensitive to H_2O_2

Fig. 27

Fig. 28. ***The Effect of hydrogen peroxide on the signal transduction pathways of ET_A mediated contraction in pig coronary artery smooth muscle.***

A summary model of the peroxide susceptibility of various signal pathways of the ET_A mediated contraction. ET-1 binding to ET_A, IP_3 channels, and suggested Ca^{2+}-independent pathways were all resistant to peroxide. ET_A-mediated contraction was responsible for approximately 80% of the ET-1 mediated contraction in denuded coronary artery, and utilized less $[Ca^{2+}]_i$ than ET_B. The ET_A mediated contraction was less sensitive to damage by peroxide compared to ET_B mediated contraction. SR Ca^{2+}-pump was very sensitive to damage by peroxide as well as the ET-1 induced increase in $[Ca^{2+}]_i$. Thus, a Ca^{2+}-independent mechanism was suggested in mediating ET_A mediated contraction such as protein kinase C-ϵ (PKC-ϵ) and/or tyrosine kinase (TK), and/or change in the Ca^{2+} sensitivity to the contractile apparatus.



R = resistance to H_2O_2

S = sensitive to H_2O_2

Fig. 28

4.4. Implications in Cardiac Ischemia/Reperfusion:

There are three major findings in this study: heterogeneity of SR Ca^{2+} pools, differences in the Ca^{2+}-dependence of the ET_A and ET_B mediated contractions and the effects of peroxide on the ET_A and ET_B mediated contractions.

4.4.1. Heterogeneity of SR Ca^{2+} Pools:

The effects of peroxide and superoxide were explained using a model (Fig.24) in which Ca^{2+} is loaded actively into two types of pools: the release from one is IP_3-dependent and from the other it is IP_3-independent. In this model, peroxide treatment inhibits loading into both types of pools while that with superoxide damages only the loading into the IP_3-sensitive Ca^{2+}-pool. Several investigators have proposed that the SR should be considered as three compartments according to its proximity to the plasma membrane: superficial SR, intermediate and deep SR (van Breemen et al., 1995). Further, since the outer nuclear membrane also contains the same type of SR Ca^{2+}-pump, another possible source of the observed heterogeneity in the Ca^{2+} pump may reflect nuclear vs. SR distribution. It has recently been suggested that the Ca^{2+} pump isoform SERCA2b can also be chaperoned by calreticulin to specific molecular complexes (John et al., 1998). The implications of the heterogeneity in the SR may include differential utilization of each Ca^{2+} pool in response to individual pharmacological agents and differential loss of these pools in response to ROS during ischemia-reperfusion and/or percuteneous transluminal

coronary angioplasty (PTCA).

4.4.2. Differences in the Ca^{2+}-dependence of ET_A and ET_B Mediated Contractions:

The lower peroxide sensitivity of the ET_A-mediated contraction was explained in a model in which both ET_A and ET_B-mediated contractions depended on extracellular Ca^{2+} entry and SR Ca^{2+}-pool mobilization and an additional peroxide insensitive Ca^{2+}-independent pathway was also involved in the ET_A contraction only, (Figs. 25 and 26). The importance of this finding is twofold. First, if such a pathway is invoked during ET_A-mediated contraction, it is likely that the same signal transduction pathway is also available for action of other agents. This would allow a co-ordinated modulation of the coronary tone in response to various stimuli. The second implication is that this pathway presents additional targets for therapeutic interventions. For example during cardiac failure, ET_A receptors are up-regulated in a rat model (Picard et al., 1998). If this pathway is known, one can counteract the effects of the increased ET_A receptor activation not only by ET_A antagonists but also by others pharmacological agents. For instance, it was discussed that protein kinase C activation may be involved. If so, selective inhibitors of protein kinase C may be used alone or in a combination therapy with ET_A antagonists.

4.4.3. Effects of Peroxide on the ET_A and ET_B Mediated Contractions:

The ROS induced damage of the Ca^{2+} regulating mechanisms in cardiac tissues may ultimately lead to cardiac dysfunction and arrhythmia. However, very little is known

about the effects of ROS on the vascular tissues, which is the one that is exposed first to ROS. It is likely that the processes which are most sensitive to ROS will be the first target for damage during cardiac ischemia-reperfusion. In order to assess the relative importance of the effects of peroxide on ET_A and ET_B receptor activation in pig coronary artery smooth muscle, this section compares these effects with those on various other functions of this tissue. Fig. 29 summarizes the sensitivity of various Ca^{2+} processes to inactivation by hydrogen peroxide in pig coronary artery. The SR Ca^{2+}-pump appears to be the most sensitive to damage by peroxide in pig coronary artery. On the other hand, ET_A-receptor mediated contraction is the least sensitive which may be due to its Ca^{2+}-independent mechanism(s). Also the IP_3 channels, L-type Ca^{2+} channels and Na^+/K^+ pump are relatively insensitive to peroxide. Therefore, it appears likely that the damage to the ET-1 mediated contractions plays only a secondary role in cardiac ischemia-reperfusion .

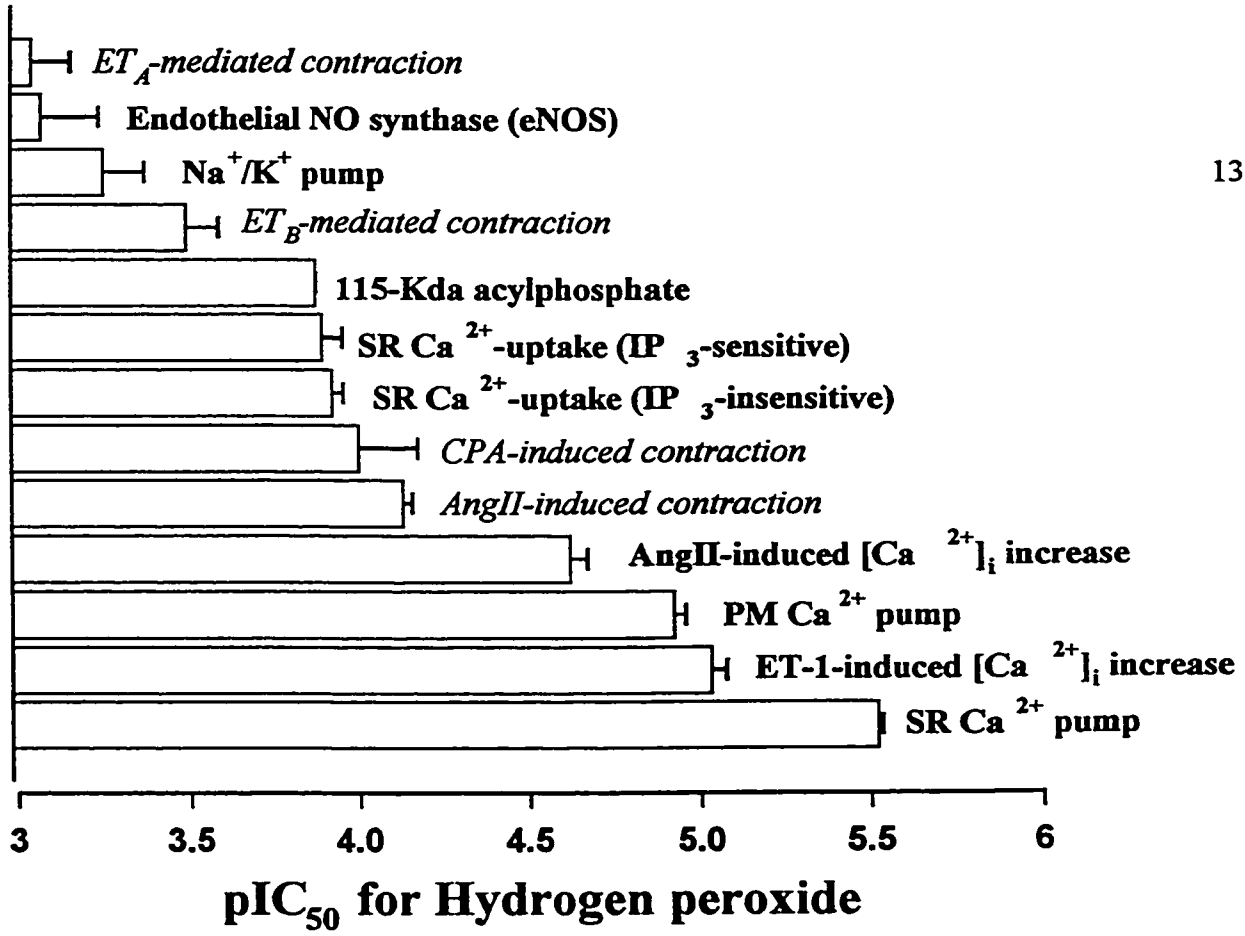


Fig. 29 ***Summary of the effects of hydrogen peroxide on various parameters of pig coronary artery smooth muscle:*** pIC_{50} is -Log of peroxide concentration that inhibits 50% of the given parameter. Thus, the higher the pIC_{50}, the more sensitive the parameter to hydrogen peroxide. From all parameters examined, the SR Ca^{2+} pump is the most sensitive to damage by peroxide in the coronary artery smooth muscle, and ET_A mediated contraction is the least sensitive to peroxide. The values of ET_A-mediated contraction, ET_B-mediated contraction, and ET-1 induced increase in $[Ca^{2+}]_i$ are taken from the present study; SR Ca^{2+}-uptake (IP_3-sensitive and -insensitive) from Elmoselhi A. et al, 1996. eNOS from Shah K. et al, 1998; CPA and Ang.II mediated contractions, AngII induced increase in $[Ca^{2+}]_i$, and 115 kDa acylphosphate level from Grover A. et al, 1995; Na^+/K^+ pump from Elmoselhi A. et al, 1994; Plasma membrane (PM) and sarcoplasmic reticulum (SR) Ca^{2+}-uptake from Grover A. et al, 1992.

4.5. Conclusions:

The results of this study can be summarized as follows: pig coronary artery smooth muscle contains a larger proportion of ET_A receptors than ET_B; The effects of both the receptor types depend on extracellular Ca^{2+}-entry and mobilization of SR Ca^{2+}; peroxide and superoxide can readily damage the SERCA2 pump but not the IP_3 channels; and ET_A -mediated contraction involves an additional Ca^{2+}-independent pathway which is not affected by peroxide. From these results three major concepts are invoked and can be suggested as follows: heterogeneity of SR Ca^{2+} pools, differences in the Ca^{2+}-dependence of ET_A and ET_B mediated contractions and the effects of peroxide on the ET_A and ET_B mediated contractions. The concepts of heterogeneity of intracellular Ca^{2+}-pools and Ca^{2+}-independent contractions may be therapeutically useful. Finally, it is likely that the damage to the ET-1 mediated contractions plays only a secondary role in cardiac ischemia-reperfusion.

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