A COMPARATIVE STUDY OF ENDOTHELIN AND SARAFOTOXIN ACTION IN VASCULAR AND NON-VASCULAR SMOOTH MUSCLE

EKONG ITO EKONG ETA

A COMPARATIVE STUDY OF ENDOTHELIN AND SARAFOTOXIN ACTION IN VASCULAR AND NON-VASCULAR SMOOTH MUSCLE

BY

EKONG ITO EKONG ETA. B.M., B.Ch (Nig.)

A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements for the degree
of Master of Science

Faculty of Medicine
Memorial University of Newfoundland
Spring 1991

St. John's Newfoundland

ISBN 0-315-82663-0

Canada

This thesis is dedicated to my parents and to my son, Ito.

ABSTRACT

The hypothesis that endothelin (ET-1) is the endogenous ligand for "sarafotoxin receptors" (SRTX receptors) was examined. The actions of ET-1 and SRTX-b were compared with those of the well characterized vasoconstrictor, norepinephrine (NE). The rationale was to identify and compare the transduction-effector mechanisms of these peptides versus NE in vascular and non-vascular smooth muscles.

In this study, the rat aorta and anococcygeus muscles were used as representatives of vascular and non-vascular smooth muscle, respectively. The role of extracellular Ca^{2+} was studied by omitting Ca^{2+} from the physiological saline solution, and the contribution of voltage-operated Ca^{2+} channels (VOCCs) assessed by determining the effects of nifedipine. In addition, the effects of ryanodine, which interferes with the release of cellular Ca^{2+}, was also studied. The roles of arachidonic acid products were determined by studying the effects of the cyclooxygenase inhibitor, indomethacin, and the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). The contractions elicited by ET-1 and SRTX-b (10 nM), or NE (1 μM) were approximately equieffective in terms of tension developement and correspond to EC_{50} values, and these concentrations were thus used throughout the study. In Ca^{2+}-free Krebs, the three agonists generated approximately similar levels of tone in the aorta and the anococcygeus corresponding to 18 and 5 % of the maximum response, respectively. Nifedipine, 10 μM, significantly inhibited responses to ET-1 in both the aorta and anococcygeus; the responses to SRTX-b and NE were, however, not significantly affected in either tissue. A combination of 10 μM ryanodine and nifedipine caused near complete inhibition of response to ET-1 in the aorta and also significantly

reduced the response to both ET-1 and NE in the anococcygeus. The lipoxygenase inhibitor, NDGA, inhibited the response to ET-1 in the aorta and ET-1 and NE in the anococcygeus muscle. The cyclooxygenase inhibitor, indomethacin, however, had no effect on the responses to any of the three agonists in either the aorta or anococcygeus. At concentrations greater than 30 nM both ET-1 and SRTX-b induced myogenic activity in normally quiescent anococcygeus muscle. As determined by the loss of myogenic activity, the tissues recovered more rapidly from SRTX-b than ET-1, with complete recovery apparent after 2.62 ± 0.85 and 5.22 ± 0.06 h, respectively. Omitting Ca^{2+} from the Krebs solution reduced recovery times to 1.62 ± 0.2 and 2.4 ± 0.51 h, respectively.

In conclusion, the studies performed indicate that a number of cell signalling processes are activated by ET-1 and SRTX-b in smooth muscle and this could account for the varied responses. The suggestion that ET-1 solely acts on the 'SRTX receptor,' as proposed by Kloog and Sokolovsky (1989), is probably too simplistic. Results from this study also indicate that tissue variability does exist and, indeed, subclasses of ET/SRTX receptors have been inferred by others (Yanagisawa and Masaki, 1989b; Nayler, 1990).

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to Dr. C.R. Triggle for his unflinching support and supervision throughout the programme.

Gratitude is also expressed to members of the supervisory committee, Dr. A. Rankin and Dr. V. Richardson, for patience and understanding for the duration of the programme.

Informal discussions with Dr. Reza Tabrizchi and Dr. Ayo Adeagbo were most useful and greatly appreciated.

I would like to acknowledge the financial support of the Medical Research Council of Canada and Faculty of Medicine of Memorial University.

Finally, I thank my wife, Asibong, for her steadfastness.

Portions of this thesis have been published in a peer reviewed Journal:

Eta, E. and Triggle, C.R. (1991) A comparative study of endothelin and sarafotoxin action in vascular and non-vascular smooth muscle. Neurochem. Int. 18, 559-564.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

G-Proteins	guanine nucleotide binding protein
GTP	guanosine triphosphate
TM2	transmembrane domain 2
DAG	diacylglycerol
GMP	guanosine monophosphate
cAMP	cyclic adenosine monophosphate
IP_3	inositol 1,4,5-trisphosphate
ATPase	adenosine triphosphatase
GTPase	guanosine triphosphatase
GTP	guanosine triphosphate
ATP	adenosine triphosphate
EDRF	endothelium-derived relaxing factor
EDHF	endothelium-derived hyperpolarizing factor
L-NMMA	N^G-monomethyl-L-arginine
VIC (ET-4)	vasoactive intestinal contractor (endothelin-4)
mRNA	messenger ribonucleic acid
TGF-ß	transforming growth factor ß
c-myc	cellular oncogene myc
c-fos	cellular oncogene fos
$p21^{ras}$	GTP hydrolysis stimulating oncogenic protein
Kd	dissociation constant
Ki	$Ki = \frac{1}{K_0}$ (dissociation constant of a competitive inhibitor)
DHP	dihydropyridine

NE	norepinephrine
i.v.	intravenous
AVP	arginine vasopressin
PGH_2	Prostaglandin H_2
MRV	mesenteric resistance vessels
NEP	neutral endopeptidase
ANOVA	Analysis of variance (one-way)
CICR (Sα)	calcium induced calcium release (α stores)
IICR (Sß)	inositol trisphosphate induced calcium release (ß stores)
EDCF	endothelium-derived contracting factor
PA	phosphatidic acid
PO_4^{3-}	phosphate
PLC	phospholipase C
PLA_2	phospholipase A_2
NDGA	nordihydroguaiaretic acid
ET-1	endothelin-1
SRTX-b	sarafotoxin-b
PC	phosphatidylcholine
VOCC	voltage operated Ca^{2+}-channel
SHR	spontaneously hypertensive rat
PIP_2	phosphatidylinositol 1,4-bisphosphate
PGI_2	Prostaglandin I_2
G	glycine (Gly)
A	alanine (Ala)
V	valine (Val)
L	leucine (Leu)

I	isoleucine (Ile)
F	phenylalanine (Phe)
P	proline (Pro)
S	serine (Ser)
T	threonine (Thr)
C	cysteine (Cys)
M	methionine (Met)
W	tryptophan (Trp)
Y	tyrosine (Tyr)
D	aspartic acid (Asp)
N	asparagine (Asn)
E	glutamic acid (Glu)
Q	glutamine (Gln)
K	lysine (Lys)
R	arginine (Arg)
H	histidine (His)
Rb^{+}	Rubidium
Sr^{2+}	Strontium
TMM	Tunica muscularis mucosa

Chapter 1

INTRODUCTION

1.1. Vascular tone.

The control of vascular tone has continued to be an enigma to physicians and medical researchers alike, the plurality of antihypertensives attest to this. Whatever its cause,and theories abound, the characteristic hemodynamic feature of hypertension is a sustained elevation in total peripheral vascular resistance, with variable cardiac output (Lund-Johansen, 1977). Control of resistance and capacitance functions in peripheral circulation has been shown to be dependent on the tone of vascular smooth muscle (Mellander and Johansson, 1968). Vascular tone is harmoniously controlled at various levels; extrinsically by neural and humoral inputs and intrinsically by the properties of structural components of the smooth muscle. Consequently, hypertension may be viewed as a failure of circulatory regulation by one or more of these mechanisms (Brody *et al.*, 1980). This view is not shared by Folkow (1990) who emphasizes that structural changes of resistance vessels are the key site of disturbance in essential hypertension. Nonetheless, considerable evidence points to alterations, at the cellular level, in the regulation of vascular tone as at least a contributing factor to the etiology of hypertension (Mulvany, 1989; Triggle, 1984). Thus, studies of the various influences that can affect the level of vascular smooth muscle tone are central to furthering our understanding of not only normal control processes but also changes that may prove to be the basis for hypertensive disease.

Since this thesis concerns the pharmacology of a family of potent vasoconstrictor peptides, the endothelins, the introduction will focus on

processes that regulate vascular tone. Thus, it is apparent that vascular smooth muscle is exposed to a wide array of stimuli ranging from mechanical to chemical, which may be excitatory or inhibitory, and that vascular tone at any point in time is the algebraic sum of responses to these inputs. Most, if not all, of these events or stimuli are mediated by receptor activation, subsequent signal transduction and resultant physiologic response which, in this case, would be contraction or relaxation.

Receptors for neurotransmitters such as norepinephrine and acetylcholine are no longer abstract concepts as it has become possible to biochemically solubilize and isolate them and, with molecular biological techniques, determine their amino acid sequence. We now know that these receptors are membrane proteins with distinct configurations. The wide array of stimuli earlier mentioned arrive at the receptor either as mechanical signals, such as stretch, which in the intact organism may be a rise in intravascular distending pressure, or electrical stimuli applied directly or induced by chemicals (e.g. potassium chloride or by a wide variety of neurotransmitters ranging from simple amino acids, such as glycine, to polypeptide hormones and the well known neurotransmitters acetylcholine and catecholamines).

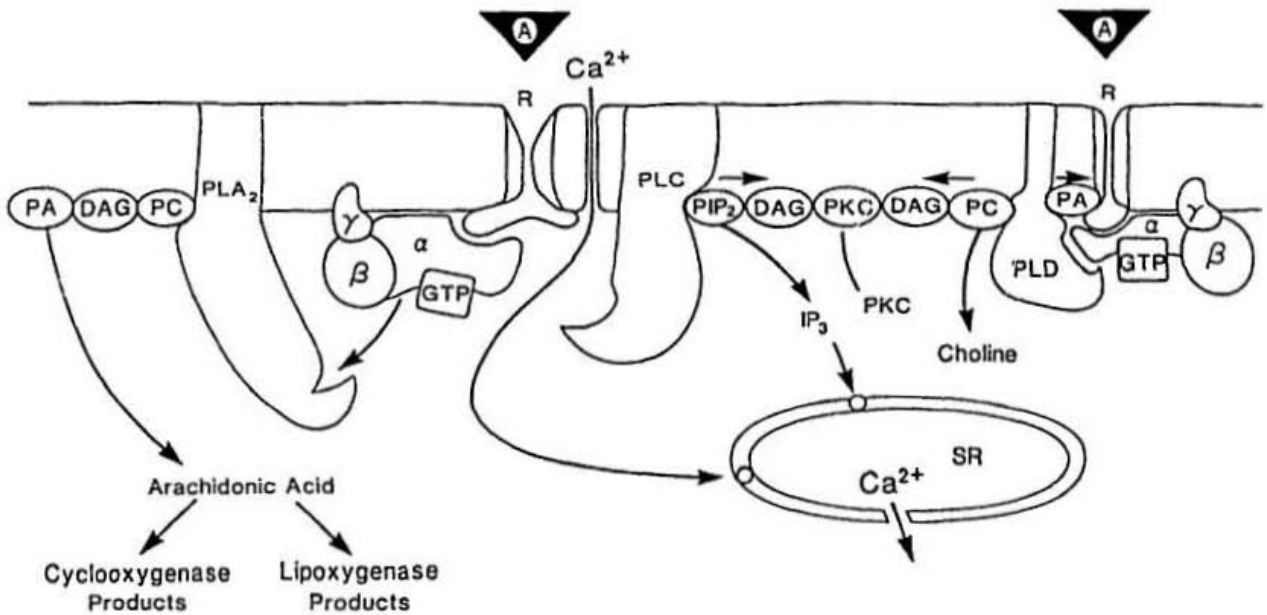
The first step of receptor activation involves the binding of a given ligand to its receptor with a resultant conformational change. The conformational change in the membrane receptor induces a high affinity state for agonist in the presence of guanine nucleotides (Cassel and Selinger, 1976). It was later shown that the guanine nucleotides were bound to certain proteins now simply referred to as G-proteins or guanine nucleotide binding proteins (DeLean *et al.*, 1980). It is becoming clear that plasma membrane receptors belong to structurally related families such as the G protein

coupled receptors or ligand-gated ion channels (Schofield and Abbott, 1989). Indeed, nine different genes have been found to code for G proteins (Freissmuth *et al.*, 1989). The G-protein is a trimer made up of α, β and γ subunits which are necessary for interaction with receptor, but the dissociation of subunits is required for activation. There appears to be a consensus of opinion that the α subunit, which binds the activated receptor and GTP, effects the transduction process to the amplifier enzyme system in an agonist-dependent manner (see Figure 1). The presence and preponderance of these amplifier enyzmes appear to be tissue dependent and their activation is agonist dependent. Thus, the activation of adenylate cyclase and the resultant increase in cAMP in cardiac muscle leads to increased contractile ability whereas in smooth muscle the increase in cAMP leads to relaxation (Sharma and Bhalla, 1989).

The second messengers produced by the amplifier enzyme phospholipase C viz, DAG and IP_3, act interdependently. Inositol 1,4,5-trisphosphate (IP_3) induces the cell to mobilize still another messenger, calcium ions (Ca^{2+}) from endoplasmic (sarcoplasmic) reticulum or calcisome depending on the cell (Berridge, 1985). In general, the second messengers bind to regulatory components of a protein kinase, an enzyme that activates or inhibits a cellular response such as contraction or secretion by adding phosphoryl(PO_3^{2-}) groups to particular proteins. Calcium binds to a family of proteins including calmodulin and troponin C. In turn, calcium-calmodulin activates a protein kinase. Activation of protein kinase C (PKC) requires Ca^{2+} and phosphatidylserine, and diacylglycerol increases the affinity of PKC for these activators. Protein kinase C selectively phosphorylates serine and threonine residues. It is now generally believed that the major mechanism of

Figure 1: Schematic representation of receptor activation by agonist and some post receptor events.

A, agonist; R, receptor; α, β, γ, subunits of GTP binding protein; GTP, guanosine triphosphate; PLA_2, phospholipase A_2; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PC, phosphatidylcholine; PA, phosphatidic acid; IP_3, inositol 1,4,5-trisphosphate; SR, sarcoplasmic reticulum.


A
A
Ca^{2+}
R
R
PA
DAG
PC
PLA_2
PLC
PIP_2
DAG
PKC
DAG
PC
PA
PLD
α
γ
β
GTP
GTP
β
IP_3
PKC
Choline
SR
Ca^{2+}
Arachidonic Acid
Cyclooxygenase Products
Lipoxygenase Products

regulation of contraction is through binding of Ca^{2+} to calmodulin and of the Ca^{2+}-calmodulin complex to myosin light chain kinase, followed by myosin light chain phosphorylation that permits activation of myosin ATPase by actin (Kamm and Stull, 1985).

The initiation of contraction is brought about by a transient rise in cytosolic calcium when a cell is stimulated by an external signal. The Ca^{2+} enters through channels that are selective or relatively selective for this ion and simultaneous release from intracellular stores aids in the transient rise of cytosolic calcium. Some channels are voltage-dependent and open when the action of a neurotransmitter leads to changes in the transmembrane voltage differences that normally exist across the membrane ; and others open when a hormone or neurotransmitter interacts with a cell surface receptor that is linked to channels known as receptor operated Ca^{2+}-channels (Carafoli and Penniston, 1985). This rise in calcium is thought to be responsible for the transient or initial contraction resulting from the cascade of kinase activation leading to muscle contraction with relaxation resulting when a fall of cytosolic calcium concentration occurs. The on/off switch role for Ca^{2+} may be overridden during a sustained contraction (Rasmussen, 1989). During a sustained or tonic contraction, the intracellular Ca^{2+} concentration does rise as predicted but only transiently and then it falls back to basal levels within a minute or so even though active tension is maintained (Morgan and Morgan, 1984; Silver and Stull, 1982). Rasmussen (1986) postulates that, during the sustained phase, contraction is dependent on extracellular Ca^{2+} in spite of the fact that its concentration is no longer elevated. During a sustained contraction, it appears that a specific Ca^{2+}-sensitive enzyme, protein kinase C, becomes associated with the plasma membrane and in this

location is responsive to hormonally induced changes in the rate of plasma membrane Ca^{2+} influx rather than changes in Ca^{2+} concentration (Shearman *et al.*,1989). Thus, it seems that there is altered sensitivity of contractile proteins in the presence of activated protein kinase C at basal Ca^{2+} levels.

The transient rise in intracellular calcium and DAG together cause protein kinase C to associate with plasma membrane. Unlike IP_3, DAG remains in the membrane; as long as the DAG content of the membrane remains high, protein kinase C remains associated with the membrane (Rasmussen, 1989). Rasmussen (1989) thus concluded that the transient release of Ca^{2+} from calcisomes and the migration of PKC from cytosol to plasma membrane are the hallmark of the initial stages of a sustained cellular response to an extracellular signal. However, the recent finding by Sunako *et al.* (1990) that sustained high levels of diacylglycerol (DAG) do not directly correlate with increasing or sustained tension questions the singular role of DAG in the sustained cellular response. In a comparison of measured levels of DAG in response to angiotensin II and endothelin-1, Sunako *et al.* (1990) observed that a sustained high level of DAG induced by angiotensin was accompanied by a loss of tension whereas a decrease in the level of DAG was associated with increasing tension for endothelin-1.

The key feature of the Rasmussen model (Rasmussen, 1989) of sustained cellular response is the operation of two temporally distinct branches of the calcium messenger-system: a calmodulin branch active during the initial phase of response, in which the transient, IP_3 -induced rise in the cytosolic concentration of calcium acts on calmodulin-dependent protein kinases to alter the phosphorylation of one subset of cellular proteins, and a PKC branch, in which the rise in calcium concentration in the submembrane domain

acts on plasma membrane-associated PKC to alter the phosphorylation of a different subset of cellular proteins involved in mediating the second, sustained phase of cellular response. This model further postulates that the physical distance between the plasma membrane location of activated PKC and the contractile proteins can be bridged by kinase cascades, one phosphorylating the other until contractile protein phosphorylation is achieved. The evidence in support of this model is that there is phosphorylation of two high molecular-weight proteins associated with smooth muscle contraction, desmin and caldesmon, which are localized in domains of the cell that are remote from the site of PKC action. In conclusion, Rasmussen noted that Ca^{2+} appears to operate as an intracellular messenger only during brief cellular responses or during the initial phases of sustained responses. Furthermore, a sustained response phase is dependent on a calcium signal generated in a restricted region of the cell membrane by an increase in rate of Ca^{2+} cycling across the membrane. Rasmussen maintains that this submembrane calcium signal acts on calcium sensitive, plasma membrane-associated transducers to generate other signals. In essence, it is the messengers generated by the transducers, rather than calcium, that convey information from the cell surface to the cell interior. It is,however, apparent that much remains to be learned about the kinase cascades and the separate control of calcium cycling and the plasma membrane association of PKC.

The plant alkaloid ryanodine has proved to be a useful probe for studying the roles of intracellular Ca^{2+} stores in initiating and sustaining muscle contraction (Jenden and Fairhurst, 1969). Most studies with ryanodine have involved investigations of intracellular calcium stores in skeletal and

heart muscle (Besch, 1985; Lakatta *et al.*, 1985; Sutko *et al.*, 1985). More recently, Hwang and van Breemen (1987) have used ryanodine to examine intracellular calcium stores in vascular smooth muscle. Caffeine has also been used in similar studies (Leijten and van Breemen, 1984; Sato *et al.*, 1988). The extent to which the different calcium stores in the plasma membrane, sarcoplasmic reticulum and mitochondria of smooth muscle can be examined may help to elucidate the nature of the defect in Ca^{2+} alluded to in the studies by Shibata *et al.* (1975) and Kwan (1985) concerning changes in vascular smooth muscle function in hypertension.

Recent studies of endothelial-vascular smooth muscle interaction, however, suggest that changes in endothelial cell function may be of importance (Lüscher, 1990). The endothelium forms an interface between circulating humoral agents and vascular smooth muscle which, for a long time was thought to serve only barrier functions with the primary role of regulating the passage of varied substances between blood and tissues. The endothelium can also act as an endocrine organ in response to stimuli and release prostaglandins, growth factors, relaxing factors and constricting substances, all of which have potential to modulate growth or reactivity of the underlying smooth muscle (Loeb and Peach, 1989).

An increase in blood pressure has the potential to alter many of the normal smooth muscle-endothelium reactions. Endothelial damage potentially can impair important metabolic functions of the endothelial cell layer, thereby allowing exposure of the vessel wall to factors with which it normally does not come into contact (Loeb and Peach,1989). Pressure induced changes also have the potential to alter the ability of the endothelium to release smooth muscle conditioning factors or to alter responsiveness of the

smooth muscle cells and vascular nerve terminals to substances or signals derived from the endothelium (Loeb and Peach, 1989). Golby and Beilin (1972) first proposed that endothelial injury resulted from an elevation in blood pressure. Subsequently, Huttner *et al.* (1973) clearly demonstrated that endothelial permeability to horseradish peroxidase and ferritin was increased in animals made hypertensive by either aortic coarctation or catecholamine infusion when compared to normotensive controls. The changes in permeability were associated with structural changes in the endothelium such as opening of intercellular gaps and nuclear pinching. As permeability increases, the access of vasoactive blood borne substances to smooth muscle is also enhanced (Loeb and Peach, 1989).

In addition to acting as a barrier to blood borne vasoactive agents, either as an actual physical barrier or as a metabolic barrier, the endothelium can synthesize and release compounds which can increase as well as decrease blood pressure(see review by Lüscher,1990). Although the ultimate control of blood pressure is the result of a myriad of competing signals, an alteration in the synthesis, storage, release or response to locally acting, tonically released endothelium-derived substances could prove catastrophic to the delicate balance of feedback loops controlling vasoactive hormone release, neuronal activity levels and central reflexes. The homeostatic mechanisms normally controlling blood pressure could be disturbed enough to produce or exacerbate hypertension (Loeb and Peach, 1989). It is thus apparent that studies of the pharmacology of the factors released from endothelial cells may prove to be profitable with respect to furthering our understanding of the cellular basis for the increased peripheral resistance associated with essential hypertension.

1.2. Endothelium-derived relaxing factors.

As noted above, certain functions have been ascribed to the endothelium but the non-recognition of the crucial role it could play in modulating the functions of vascular smooth muscle, in spite of its strategic anatomical location is, perhaps, surprising. Fortuitous as some may claim, it took the keen sense of observation of Furchgott and Zawadzki (1980) to indicate to vascular physiologists and pharmacologists that there is more to endothelium than just a physical barrier.

The ability of acetylcholine and other muscarinic agonists to produce marked vasodilatation in various vascular beds in vivo was well established many years ago (see review by Furchgott, 1988). It has also been demonstrated that acetylcholine and other muscarinic agonists could produce relaxation of isolated perfused or superfused blood vessels contracted by stimulation of adrenergic nerves (Rand and Varma, 1970; Steinsland *et al.*, 1973; Vanhoutte, 1974). Thus, it was to be expected that muscarinic agonists should produce relaxation of spontaneous or drug-induced contractions of isolated preparations of blood vessels (see review by Furchgott, 1988). This was not the case until 1980 when Furchgott and Zawadzki noted that the non-relaxation of their helical strip preparations was due to the dissection technique which denuded the vessels of endothelium. When this procedure was modified so that damage to the endothelial cell layer was avoided, the resulting helical strips exhibited good relaxation in response to low concentrations of acetylcholine. It was demonstrated by light microscopy, and also by scanning electron microscopy (Furchgott *et al.*, 1981), that there is a direct relationship between the extent of relaxation of aortic preparations by acetylcholine and the reaction of endothelial cells retained.

One hypothesis to explain the obligatory role of endothelial cells in the relaxation of rabbit aorta by acetylcholine was that this agent, acting on the muscarinic receptor in these cells, stimulated them to release a substance that, in turn, acts on smooth muscle cells in the vessel media to activate relaxation. In testing this hypothesis, cascade perfusion and superfusion procedures have yielded positive results (Griffith *et al.*, 1984; Forstermann *et al.*, 1984; Rubanyi *et al.*, 1985) and the "sandwich" mounts of Furchgott and Zawadzki (1980) have been consistently positive. Thus was born the concept of endothelium-derived relaxing factor (EDRF). The nature of EDRF has been a subject of controversy ranging from the number of possible EDRFs to chemical characterization. There seem to be two candidates so far for the relaxation induced by endothelium. One of them has been named endothelium-derived relaxing factor (EDRF: Furchgott & Zawadzki, 1980) and the other has been named endothelium-derived hyperpolarizing factor (EDHF) (Taylor *et al.*, 1988). EDRF is said to increase tissue cyclic GMP concentrations and produces an electrically-silent relaxation, whereas EDHF produces transient hyperpolarization associated with opening of ^{86}Rb-permeable K^+-channels. This event may serve to initiate relaxation processes and to close any open voltage dependent Ca-channels (Taylor *et al.*, 1988). The findings of Feletou and Vanhoutte, 1988; Chen and Suzuki, 1990; Miller and Vanhoutte (1989) confirm this postulate. Marshall and Kontos (1990), in a review of endothelium-derived relaxing factors, have noted that several suggestions, including those that EDRF is a product of lipoxygenase metabolism of arachidonate (Peach *et al.*, 1985), a product of cytochrome P-450 oxygenase (Singer *et al.*, 1984) or a carbonyl-containing compound (Griffith *et al.*, 1984), have been disproved. Most recently, evidence has

been gathered suggesting that EDRF generated by acetylcholine in blood vessels, and by bradykinin in cultured endothelial cells, is nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). This view is supported by pharmacological similarities between nitric oxide and EDRF (Ignarro *et al.*, 1987), by inhibition of action of both nitric oxide and EDRF by various pharmacological blocking agents such as hemoglobin and methylene blue (Martin *et al.*, 1984), and inhibition of production of both nitric oxide and EDRF from arginine by N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989).

In spite of such compelling data,there has been no unanimity of opinion. Some investigators have been able to identify pharmacological differences between nitric oxide and EDRF (Long *et al.*, 1987); other investigators have shown that the amount of nitric oxide released by acetylcholine from vessels or by bradykinin from endothelial cells is not sufficient to explain the observed vasodilation (Myers *et al.*, 1989); finally, other investigators have been unable to find release of nitric oxide by electron-spin resonance techniques coupled with the use of spin traps (Rubanyi *et al.*, 1989). That EDRF may be a nitric oxide containing compound that is much more active in inducing vasodilation than nitric oxide itself has been suggested. Nitrosothiols have properties that render them appropriate candidates for this purpose (Myers *et al.*, 1989; Wei and Kontos, 1990).

The obligatory role of endothelial cells for the manifestation of acetylcholine-induced relaxation extends to many arteries in a variety of species including dog, cat, guinea-pig and humans (Furchgott and Zawadzki, 1980; Furchgott *et al.*, 1981; Cherry *et al.*, 1982), the unexplained exceptions being the basilar artery of the dog and coronary artery of the pig

(Katusic *et al.*, 1984; Shimokawa *et al.*, 1987).

The endothelium-dependent responsiveness to acetylcholine (or to other vasoactive agents) as noted by Vanhoutte (1989) is not fixed, as it can be modulated chronically by hemodynamic variables or hormonal status (Miller *et al.*, 1986; Criscione *et al.*, 1989). Chronic modulation by hemodynamic variables may explain why, in animals and in humans, the endothelium-dependent responses to acetylcholine (and of the endothelium-dependent dilators) are considerably blunted in peripheral veins compared to arteries (De Mey and Vanhoutte, 1982). It is important to note that acetylcholine does not circulate in blood and in most tissues innervated by cholinergic nerves, acetylcholinesterase, an enzyme that is remarkably effective in rapidly destroying the cholinergic transmitter, is present. Hence, to date, no evidence has been obtained that acetylcholine released from cholinergic nerves can reach endothelial cells in amounts sufficient to evoke endothelium-dependent relaxations. However, substances circulating in the blood would be potential candidates for triggering endothelium-dependent responses. Indeed, endothelial cells, at least in large blood vessels carrying oxygenated blood, contain α_2-adrenergic receptors that, when activated, can evoke endothelium-dependent relaxation of the underlying smooth muscle (Cocks and Angus, 1983; Miller and Vanhoutte, 1985; Vanhoutte and Miller, 1989). Hence, it is likely that α_2-adrenergic endothelium-dependent effects of catecholamines contribute to vasodilator effects in the coronary circulation or the splanchnic bed (Vanhoutte, 1989).

Finally, it has been known for decades that, when flow increases through large arteries, dilatation ensues ("flow-induced vasodilatation") (see review by Vanhoutte,1989). It is now established that this phenomenon is

endothelium-dependent and can be attributed to EDRF caused by an increase in shear stress. Whether this is secondary to release of autacoid (acetylcholine, ATP, serotonin or substance P) by certain selective endothelial cells or to direct activation of all endothelial cells by shear stress is uncertain (Vanhoutte, 1989).

1.3. Contracting factors.

While searching for possible heterogeneity of endothelium-dependent relaxation to acetylcholine, adenosine diphosphate, arachidonic acid and thrombin, it was discovered that endothelial cells, when exposed to certain stimuli, not only produced vascular dilator(s) (Furchgott, 1983; Vanhoutte *et al.*, 1986), but also vasoconstrictors - so called endothelium-derived contracting factor(s)(EDCF) (De Mey and Vanhoutte, 1982; 1983). At least two different types of EDCFs have been identified (Vanhoutte and Katusic, 1988). One type is a rapid onset, hypoxia induced, cyclooxygenase inhibitor resistant EDCF; a rapid relaxation ensues when normoxia returns. This substance is still unidentified. The time course of action makes it unlikely that the endothelium-derived contracting factor(s) involved are peptides released from hypoxic endothelial cells (Rubanyi and Vanhoutte, 1985; Vanhoutte *et al.*, 1989), since the response to hypoxia is faster than peptide induced contraction and can be prevented by a calcium antagonist. Indeed, endothelin is not stored in endothelial cells (Yanagisawa *et al.*, 1988b). A second type of EDCF is sensitive to cyclooxygenase inhibition, and its release is induced by a variety of stimuli, including arachidonic acid, acetylcholine, calcimycin (A23187), sudden stretch in cerebral arteries of dog, aorta of (SHR) spontaneously hypertensive rat, canine systemic and pulmonary veins (Miller and Vanhoutte, 1985; Katusic *et al.*, 1987; De Mey and

Vanhoutte, 1983). Recent studies (see Lüscher, 1990) suggest that an alteration in the production of the constrictor PGH_2 may be a contributing factor to the increase in the peripheral resistance associated with essential hypertension. A third EDCF is the peptide endothelin, a factor originally associated with the supernatant of cultured endothelial cells which produces a long lasting contraction. The endothelin-induced contraction is very difficult to reverse and it is resitant to cyclooxygenase inhibition but is attenuated by trypsin (Gillespie *et al.*, 1986; Yanagisawa *et al.*, 1988a).

In canine systemic and pulmonary veins, arachidonic acid augmented contractions evoked by norepinephrine; the augmentation was not observed in preparations in which the endothelium had been mechanically removed (De Mey and Vanhoutte, 1982). This observation was the first demonstration that the presence of endothelial cells can augment, rather than depress, contractile response of isolated blood vessels. The facilitation by arachidonic acid of the responses of the endothelium of systemic and pulmonary veins was prevented by inhibitors of the enzyme cyclooxygenase but not by inhibitors of prostacyclin synthetase, thromboxane synthetase or lipoxygenase (Miller and Vanhoutte, 1985). These findings indicate that venous endothelial cells could metabolize arachidonic acid into a vasoconstrictor prostanoid, other than prostacyclin and thromboxane A_2.

Most recently, the fact that attempts to bioassay the endothelium-derived contracting factor released by acetylcholine have failed, indicates that a very labile substance such as an endoperoxide intermediate or oxygen-derived free radical must be involved or that the substance is only released towards the abluminal side of the blood vessel wall (Lüscher, 1990). Scavengers of oxygen-derived radicals, such as superoxide dismutase do prevent the

endothelium-dependent contractions to acetylcholine in the cerebral blood vessels but not in the rat thoracic aorta (Auch-Schwelk *et al.*, 1989). It is pertinent to note that the relaxation of perfused canine femoral artery by acetylcholine consists of transient and sustained components. The transient component is observed with lower concentrations of acetylcholine and appears to be caused by activation of endothelial M_1-muscarinic receptors; the sustained component is seen with higher concentrations of the cholinergic transmitter and can be attributed to stimulation of M_2-muscarinic receptors (Rubanyi *et al.*, 1987). The transient, but not the sustained, response is inhibited by quinacrine and metyrapone, which suggest that the former is mediated by a metabolite of arachidonic acid (Rubanyi and Vanhoutte, 1987). Conversely, high concentrations of catecholamines inhibit the sustained but not the transient phase of the response to acetylcholine (Rubanyi *et al.*, 1985). The endothelium-dependent contractions to acetylcholine occur with higher concentrations of acetylcholine than those needed to release EDRF (Lüscher and Vanhoutte, 1986). Thus, with higher concentrations of acetylcholine, EDRF is still released, however, release of the contracting factor(s) by higher concentration of the cholinergic transmitter blunts the relaxant effect with a preponderant contractile response.

The recent review by Lüscher (1990) sums up the current trend of thought, notably that a contracting factor other than endothelin, namely prostaglandin H_2, may be involved in defective endothelial cell function in hypertension. Nevertheless, a role for endothelin has not been ruled out.

1.3.1 Endothelin.

Evidence for a vasoconstrictor endothelial peptide came from studies on cultured bovine endothelial cells, whose supernatant had contractile

properties on bovine pulmonary, pig and rabbit coronary arteries (O'Brien and McMurtry, 1984; Hickey *et al.*, 1985; Gillespie *et al.*, 1986). The existence of a new vasoconstrictor peptide synthesized by the endothelium was postulated after ruling out the possibility that angiotensin or substance P could mediate those effects (Gillespie *et al.*, 1986).

The contractile peptide was isolated and purified from cultured cells of porcine aortic endothelium by Yanagisawa *et al.* (1988a). Endothelin was identified as a 21-amino acid, potent vasoconstrictor peptide and since it was reported that there were at least three endothelin genes in the human genome, the products of its expression were designated ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989). More recently, ET-4 (vasoactive intestinal contractor - VIC) has been isolated from the mouse (Saida *et al.*, 1989) and expressed predominantly in the intestine, raising the possibility of tissue specific ET variants. ET-1 is identical to the form originally isolated from porcine endothelial cells (Itoh *et al.*, 1988), whereas the amino acid sequence of ET-3 is identical to that found in the rat genome (Yanagisawa *et al.*; 1988b; Inoue *et al.*, 1989). Therefore, it was thought that many mammalian species, including pig, dog, rat and human produce the three isopeptides of the endothelin family (Yanagisawa and Masaki, 1989a, b).

1.3.2 Regulation of synthesis and release of endothelin.

A peptide of 203 amino acids is the precursor molecule for endothelins. These pro-hormones demonstrate species and isopeptide-specific differences in amino acid sequence (Simonson and Dunn, 1990a). In addition to containing the mature ET peptide, the prepro-precursors contain a cysteine-rich, ET-like region (15 residues) that is highly conserved (Yanagisawa *et al.*, 1988a; Itoh *et al.*, 1988). The biological significance of differences in amino acid

sequence between the prepro species, and of the presence of an ET-like peptide within the prepro ET, are unclear.

In cultured endothelial cells, prepro ET-1 is proteolytically cleaved to form a 38 (human)- or 39 (porcine)-amino acid big ET (Shinmi *et al.*, 1989). This is subsequently processed to mature ET-1 by the putative 'endothelin converting enzyme' (Yanagisawa *et al.*, 1988a). Yanagisawa and Masaki (1989a) have suggested that endoproteolysis may provide an important site for pharmacological intervention.

It appears that vascular endothelial cells do not produce ET-2 or ET-3; only ET-1, or its mRNA, can be detected in endothelial cells in culture supernatant (Yanagisawa and Masaki, 1989a). The expression of ET-2 has not yet been convincingly demonstrated in any tissue. Shinmi *et al.*(1989) have demonstrated that ET-3 immunoreactive material is present in porcine brain homogenate. In addition, endothelin and related peptides can be synthesized and secreted from renal epithelial cells (Shichiri *et al.* 1989) and intestinal cells (Saida *et al.* 1989) of non-endothelial origin.

Most recent studies (MacCumber *et al.*, 1989; MacCumber *et al.*, 1990; Yoshizawa *et al.*, 1990) show that ET isopeptides are differentially expressed only in specific tissues, which suggest that tissue-specific factors control the rate of ET gene expression. Above cited investigators demonstrated transcripts for both prepro ET-1 and prepro ET-3 in adult rat lung, kidney, eye and brain, whereas both ET-3 and ET-1 were abundantly expressed in the lung. mRNA transcripts for VIC (ET-4) have been found in the murine intestinal tract (Saida *et al.*, 1989). In the rat kidney, ET-1 was expressed predominantly in the medullary vasa recta (MacCumber *et al.*, 1989). The inability to demonstrate ET-1 gene expression in some adult tissues could

reflect the relative insensitivity of Northern analysis to detect rare transcript or poor hybridization of ET-1 and ET-3 probes with noncomplementary tissue specific ET isoforms (Simonson and Dunn, 1990a).

An important aspect of endothelin biology is the failure to demonstrate, in situ, endothelium-dependent vasoconstriction mediated by endothelin. It has been speculated that ET-1 from endothelium is regulated at the level of peptide synthesis but not at the level of the mechanisms of release (Yanagisawa and Maskai, 1989a, b). The induction of ET-1 mRNA and/or peptide by various chemical stimuli has been studied mainly in cultured endothelial cells. The growing list of physiological stimuli that can increase ET-1 production includes thrombin (Yanagisawa *et al.*, 1988a); transforming growth factor ß (TGF-ß) (Kurihari *et al.*, 1989); angiotensin II (Emori *et al.*, 1989); [Arg]vasopressin (Emori *et al.*, 1989); fluid dynamic shear stress (Yoshizumi *et al.*, 1989). Thrombin, angiotensin II and vasopressin stimulate phospholipase C activity in endothelial cells leading to the formation of the second messengers inositol 1,4,5-trisphosphate (which mobilizes Ca^{2+} from intracellular storage sites) and 1,2 diacylglycerol (which stimulates protein kinase C). Shear stress also increases intracellular free Ca^{2+} concentration in cultured endothelial cells , both by stimulating the influx of extracellular Ca^{2+} and mobilizing intracellular Ca^{2+} (Ando *et al.*, 1988). Indeed, ET-1 mRNA and peptide are also induced by Ca^{2+} ionophores and phorbol esters (Emori *et al.*, 1989; Yanagisawa *et al.*, 1989). These observations are consistent with the idea that production of ET-1 in endothelial cells can both be regulated by intracellular Ca^{2+} and protein kinase C - that is, possibly by PI turnover signalling in endothelial cells.

The 5' promoter region of human ET-1 gene contains several elements

responsive to 12-O-tetradecanoylphorbol 13-acetate which are found in other genes that can be induced by phorbol esters (Inoue *et al.*, 1989). It also contains the nuclear factor-1-binding elements that have recently been recognized to be involved in response to TGF-ß. Whether these potential regulatory DNA elements in the ET-1 gene are actually involved in the regulation of ET-1 production by agents such as those listed above will be determined by promoter mapping studies. The level of ET-1 mRNA in endothelial cells may be controlled not only by transcriptional regulation but also by post transcriptional regulation of mRNA degradation. Half-life studies using the transcription inhibitor actinomycin D have revealed that ET-1 mRNA is extremely labile, having an intracellular half-life of about 15 minutes (Yanagisawa *et al.*, 1989). This rapid degradation is specific for ET-1 mRNA, since ß-actin mRNA has a much longer half-life (10-20 h) in the same cells. Both porcine and human ET-1 mRNA possess several 'AUUUA' sequences in the 3' non-translated regions. These AU motifs have been recognized to be involved in highly selective mRNA destabilization and are found in mRNAs encoding certain transiently expressed cytokines, growth factors and nuclear proto-oncogene products (usually involved in programming of cellular growth and differentiation). It is conceivable that ET-1 may also belong to this class of signal molecules.

1.3.3 Pharmacological effects of endothelin.

Endothelin has been described as the most potent vasoconstrictor known (Yanagisawa *et al.*, 1988a). The contractile activity of the different endothelins in ranked order is: ET-2>ET-1≥ET-4>ET-3 (Yanagisawa *et al.*, 1989; Rodman *et al.*, 1989; Saida *et al.*, 1989).

ET-1 provokes strong sustained contractions in isolated vascular smooth

muscle preparations in almost all animal species and vascular regions examined including microvessels (Brain, 1989). Other vascular effects of ET-1 include the stimulation of release of eicosanoids and endothelium-dependent relaxing factor (EDRF) from perfused vascular beds (de Nucci *et al.*, 1988).

The additional finding that endothelin acts not only on vascular but non-vascular tissues (Yanagisawa and Masaki, 1989b), has raised questions about its role in the control of vascular tone. ET-1 has a wide spectrum of pharmacological effects on non-vascular tissues viz: contraction of airway (Uchida *et al.*, 1988), intestinal smooth muscle (deNucci *et al.*, 1988; Borges *et al.*, 1989), uterine smooth muscle (Borges *et al.*, 1989; Eglen *et al.*, 1989), cardiac chronotropic and inotropic actions (Ishikawa *et al.*, 1988a, b) and stimulation of atrial natriuretic peptide release (Fukuda *et al.*, 1988), inhibition of ouabain sensitive Na^+/K^+ ATPase (Zeidel *et al.*, 1989) blockade of the antidiuretic effect of vasopressin in vivo (Goetz *et al.*, 1988), modulation of catecholamine release from sympathetic termini and adrenomedullar chromaffin cells (Tabuchi *et al.*, 1989; Boarder and Mariott (1989), and the stimulation of aldosterone release in adrenal glomerulosa cells (Cozza *et al.*, 1989). Evidence suggestive of a neuromodulatory role has been adduced (Jones *et al.*, 1989; Kokesi *et al.*, 1989). The wide spectrum of target tissues and of species non-specificity imply that ET-1 might have wide ranging actions in diverse organs. In cultured mesangial cells (Badr *et al.*, 1989; Simonson *et al.*, 1989), and vascular smooth muscle cells (Komuro *et al.*, 1988), ET-1 is a potent mitogen and stimulates c-myc and c-fos proto-oncogene expression resulting in hyperplasia, suggesting a possible role for ET in vascular remodelling or atherosclerosis (Simonson and Dunn; 1990b).

1.3.4 Homology between ET-1 and SRTX-b.

Perhaps the most perplexing finding has been the discovery of a strong identity between endothelins and sarafotoxin. Sarafotoxins, as characterized by Kochva *et al.* (1982), Weiser *et al.* (1984) and Takasaki *et al.* (1988), are potent vasoconstrictor, cardiotoxic peptides from the venom of the burrowing asp (Atractaspis engaddensis), which cause severe coronary spasm and ECG changes in snake bite victims. The similarity between these two peptides led Graur *et al.* (1988/1989) to examine their evolutionary trends, and Kloog and Sokolovsky (1989) have hypothesized that the biological activity of endothelin and sarafotoxin are mediated via a common receptor. Indeed, they suggest that endothelins are endogenous ligands for 'sarafotoxin receptors' (SRTX receptors).

Sarafotoxins (SRTX-a, -b and -c) and more recently -d (Bdolah *et al.*, 1989) are a group of 21-amino acid residue cardiotoxic peptides isolated from the venom of Atractaspis engaddensis, which are rich in cysteine and show sequence identity to the mammalian endothelins ET-1, ET-2, ET-3 and ET-4. Eight naturally occurring peptides of the endothelin/sarafotoxin 'family' are now known (Fig. 2). All eight peptides contain 21 amino acids which possess Cys1, Cys3, Cys11 and Cys15 residues, with disulfide bonds between Cys3 and Cys11 and between Cys1 and Cys15 (Yanagisawa *et al.*, 1988b, Takasaki *et al.*, 1988). Reduction of the disulfide bonds of the SRTXs (Kloog *et al.*, 1988), and of the endothelins (Kimura *et al.*, 1988), results in a marked loss of binding and activity. Also common to these peptides is the hydrophobic carboxyl terminal tail His16-Trp21. Cleavage of Trp21 from ET-1 resulted in marked loss of vasoconstrictive ability (Kimura *et al.*, 1988). Moreover, even though Glu17 and Val 19 of SRTX peptides are replaced by Leu17 and Ile19

respectively in endothelin peptides, the size and hydrophobic nature of the carboxy terminal tail are preserved. This replacement, however, appears to be of little functional significance. For example, ^{125}I-iodinated SRTX-b and ET-1 possess indistinguishable binding properties and both stimulate PI (phosphatidyl inositide) hydrolysis in various regions of the rat brain and also in the atrium (Ambar *et al.*, 1989). SRTX-b and ET-1 also exert almost identical effects on the cardiovascular system, namely coronary vasoconstriction, which is manifested electrocardiographically in mice by elevation of S-T segment, induction of atrioventricular block and induction of positive inotropic and chronotropic effects (Yanagisawa *et al.*, 1988a, Takasaki *et al.*, 1988; Wollberg *et al.*, 1988; Ru Hu *et al.*, 1988; Ishikawa *et al.*, 1988a, b). Furthermore, cross-desensitization between ET-1 and SRTX-b induced changes in phosphoinositide turnover in neurons has also been demonstrated (Lin *et al.*, 1989).

It is also interesting to note that in all endothelin/SRTX peptides, the N-terminal sequence is Cys1-Ser2/Thr2-Cys3; SRTX-a, SRTX-b, ET-1, ET-2 and ET-4 (VIC) contain Ser2, while SRTX-c, SRTX-d and ET-3 contain Thr2. Takasaki *et al.* (1988) argue that it seems unlikely that the lower toxicity of the latter three peptides compared to the former, and the vasodilatory activity of both SRTX-c and ET-3, are due to substitution of the threonine for serine in position 2 on the grounds that the properties of these two residues are rather similar. Thus, the most important differences between the various peptides of endothelin/sarafotoxin 'family' reside within the sequence of the inner loop Cys3-Cys11. All the peptides possess Glu10, and, except for SRTX-c, they have Asp8-Lys9. Therefore, the sequence at position 4-7 represents the variable region of the endothelin/SRTX peptide 'family'

(Kloog and Sokolovsky, 1989). Thus, unlike the 'constant' C-terminal tail of these peptides, their N-terminal sequences are variable. Another interesting difference between the various endothelin/SRTX peptides involves their net charges which appear to derive from variations in the loop created by their disulfide bonds. For instance, SRTX-a and SRTX-b each have two positive and three negative charges and ET-1 and ET-2, with one positive and two negative charges would all have a net charge of -1 within the loop; SRTX-c (with four negative charges) would have a net charge of -4, and ET-3 (with two positive and two negative charges) would have a net charge of 0. As SRTX-b and ET-1 have similar vasoconstrictive and cardiotoxic effects, which are different from those of SRTX-c and ET-3, it seems that an overall net change of -1 within the Cys3-Cys11 loop is required for biological activity (Kloog and Sokolovsky, 1989). The absence of this single net charge in SRTX-c and ET-3 is, aside from their common Cys1-Thr2-Cys3, another common feature of these two peptides which may contribute to their vasodilatory activity. However, the marked difference between the intraloop charges of SRTX-c (-4) and ET-3 (0) may not explain the differences in potencies and/or mechanism of binding, and second messenger systems utilized by SRTX-c and ET-3 versus ET-1 or SRTX-b (Kloog and Sokolovsky, 1989). It should be stressed that SRTX-d differs from SRTX-b in two substitutions, which apparently do not affect binding in rabbit aorta, but do result in considerably lower lethality and vasoconstrictor potency (EC_{50} values of 90 nM and 5 nM, respectively; Bdolah *et al.*, 1989). From the functional point of view it is interesting that SRTX-c and SRTX-d are the least toxic. Although a lower binding affinity to heart and brain membranes, and lower phosphoinositide hydrolysis and vasoconstricting activities have been claimed for SRTX-c (Takasaki *et al.*,

1988; Kloog *et al.*, 1988), SRTX-d has been shown to have similar binding characteristics to SRTX-b but less efficacy as a vasoconstrictor (Bdolah *et al.*, 1989). According to Graur *et al.* (1988/1989), the suggestion that the C-terminal 'tail' is essential for vasoconstrictor activity (Yanagisawa *et al.*, 1988b) seems less likely as this tail differs between the two groups (mammal versus snake), but it is virtually identical within each group with the exception of SRTX-d which has an ET-like tail. For instance, SRTX-b and ET-1 are both potent vasoconstrictors, and differ in their C-terminal 'tails', while SRTX-b and SRTX-c on the one hand, and ET-1 and ET-3 on the other, which have similar tails differ in their vasoconstricting powers by an order of magnitude (Yanagisawa *et al.*, 1988b; Wollberg *et al.*, 1988).

1.3.5 Endothelin and sarafotoxin receptors.

The existence of four endothelin peptides also raises the possibility of existence of multiple endothelin receptor subtypes (Yanagisawa and Masaki, 1989b; Saida *et al.*, 1989). However, in cultured rat aortic smooth muscle cells, a single class of saturable, high affinity binding sites for [^{125}I]ET-1 has been described (Hirata *et al.*, 1988b). There is no unanimity in classes or subtypes of receptors; thus Watanabe *et al.*(1989) have defined two distinct types of ET receptors on chick cardiac membranes. Kloog *et al.* (1989), using homogenates of rat atria, aorta, uterus, cerebellum, caudate and putamen, indicated heterogeneity of ET/SRTX receptor and adduced evidence for three receptor subtypes. More than one receptor has been proposed in blood vessels: a receptor with high affinity for ET-1 may mediate the vasoconstrictor response in vascular smooth muscle cells, whereas a receptor with higher affinity for ET-3 may be involved in the endothelin-induced release of EDRF from endothelial cells. Binding studies with synthetic

sarafotoxin suggest the existence of a heterogeneous population of vascular receptors for endothelins and sarafotoxins (Hirata *et al.*, 1989) since the K_i value for SRTX is apparently greater than the apparent K_d value for the vascular ET receptors. Furthermore, Ambar *et al.* (1989) have demonstrated marked differences in the affinities of ^{125}I-SRTX for its binding sites in various tissues of the rat. Activation of endothelin receptors is associated with an increase of phosphoinositide turnover except in cultured vascular smooth muscle cells (Hirata *et al.*, 1988b), suggesting the existence of a specific receptor-phospholipase C system. This system can also be stimulated equally by sarafotoxins (Lin *et al.*, 1989; Kloog and Sokolovsky, 1989; Hirata *et al.*, 1989). The fact that stimulation of phospholipase C by ET-1 in rat aortic cells involves a pertussis toxin-insensitive G-protein allows for the speculation that the receptors for endothelin belong to a G-protein coupled superfamily (Yanagisawa and Masaki, 1989a). Furthermore, Tabrizchi and Triggle (1990) have provided in vivo evidence from pithed rats that, in vascular smooth muscle, a component of the response to ET results from activation of a pertussis toxin-sensitive G-protein that is coupled to a receptor operated calcium channel and/or non-specific cation channel.

Autoradiographic studies have demonstrated the existence of endothelin receptors not only in vascular tissue, but also in rat brain, kidney, lung, adrenal gland and intestine (Jones *et al.*, 1989; Kokesi *et al.*, 1989). Long lasting effects of endothelin appear to be directly related to the nature of the interaction with the receptor(s)and. Thus, the dissociation of radiolabelled endothelin has been shown be very slow, with 85% of the initial cell-bound radioactivity remaining after 2 hours (Hirata *et al.*, 1988b).

Galron *et al.* (1989) have studied ET-1 and ET-3, SRTX-b and SRTX-c

binding in intact cells and homogenates of rat heart myocytes and have demonstrated higher affinities for ET-1 and SRTX-b than ET-3 and SRTX-c. With cross-linking techniques Sugiura *et al.* (1989) were also able to distinguish ET-binding site proteins from the α_1,ß and γ subunits of the L-type Ca-channel. Furthermore, Ambar *et al.* (1989), using competitive binding studies in rat atria and brain, had inferred receptor subtypes for ET-1 and SRTX-b but suggested that the two share a common binding site.

Immunological evidence from antibodies against ET-1 and SRTX-b reveal low cross-reactivity between the two peptides (Fleminger *et al.*1989). Fleminger *et al.*(1989) argued that this low reactivity reflects the fact that the antibodies recognize the variable sequence found within amino acids 4-7. This hypothesis was confirmed by CNBr cleavage of the methionyl residue at position 6 in SRTX-b and at position 7 in ET-1 (Fleminger *et al.*, 1989). On the other hand, the binding properties, as well as the ability to induce phosphoinositide hydrolysis, were very similar in the modified and native peptides, indicating that, despite cleavage of the peptide bond, the biologically active conformation responsible for either binding or phosphoinositide hydrolysis is retained. Probably this retention of activity reflects the importance of the two disulfide bonds. It thus appears that neither the argument for a role of the charge of(-1) on the peptides loops of ET-1 and SRTX-b , nor similarities or dissimilarities in the C-terminal 'tail' of the two groups of peptides (ET/SRTX) can adequately explain their contractile or biochemical properties. Perhaps the only characteristic distinguishing the weak from strong contractile peptides is threonine at position 2. However, even the presence of threonine at position 2 does not confer differing binding properties for the potent contractile SRTX-b versus

the weakly contractile SRTX-c (Bdolah *et al.*, 1989). In this context, the findings of Graur *et al.* (1988/89) must be emphasized as there is a strict conservation of the C-terminal amino acid sequence in the mammalian versus snake peptide except for SRTX-d which has Ile-Ile-Trp of the mammalian group at position 19, 20 and 21, respectively. Nonetheless,the pharmacological activities of SRTX-d and ET-1 differ. It is also interesting to note that the C-terminal hexapeptide, endothelin (16-21), differentiates between endothelin receptors in the rat aorta and guinea pig bronchus, where it is neither an agonist nor antagonist in the former but a full agonist in the latter (Maggi *et al.*, 1989). Cleavage of Trp21 from ET-1 and SRTX-b resulted in marked loss of vasoconstrictive ability (Kimura *et al.*, 1988; Nakajima *et al.*, 1989). If vasoconstrictive ability is so dependent on Trp21, it follows that all eight peptides should be equieffective since all have Trp21; but this is not the case. The common factor of Trp21 and variable potencies of the eight peptides lend credence to suggestions of Kloog and Sokolovsky (1989) that the variability of the C-terminal tail is of little functional significance. The recent report by Galron *et al.* (1990), that different pathways are utilized by endothelin and sarafotoxin subsequent to phosphoinositide hydrolysis in rat myocytes, lends credence to our hypothesis that the different seven amino acid sequence between ET-1 and SRTX-b may lead to different cell binding and/or utilization of messenger systems in different tissues. Indeed, such dissimilarities between ET-1 and SRTX-b have already been reported by Goetz *et al.* (1989) for the effects of the two peptides on renal blood flow. This latter observation provides evidence for the report that there are two specific receptors in the rat kidney mesangial cells (Sugiura *et al.*, 1989) .

1.3.6 Mechanisms of action of the endothelins/sarafotoxins.

In the original paper of Yanagisawa *et al.* (1988a), it was suggested that endothelin may act directly on membrane ion channels. This hypothesis was supported by the similarity between the structure of endothelin and those of the α-scorpion toxins, which are known to bind to the tetrodotoxin-sensitive Na-channels. Based on the sensitivity of endothelin contraction to nicardipine, and the fact that Na- and Ca-channels belong to the same family of voltage-dependent membrane ion channels as those sensitive to the α-scorpion toxins (Tanabe *et al.*, 1987), they proposed that endothelin could represent the endogenous ligand for L-type calcium channels. Additional support for this hypothesis was obtained with the observation that, using patch clamp techniques, ET-1 activated calcium influx currents through dihydropyridine voltage-sensitive Ca-channels (Goto *et al.*, 1989). In addition, it has been reported that, in the rat aorta and portal vein, the contraction and ^{45}Ca uptake induced by endothelin, but not those caused by α-scorpion toxin or veratridine, were insensitive to tetrodotoxin, ω-conotoxin and Na removal, whereas nifedipine, nitrendipine, verapamil, nickel and Ca-free medium inhibited these processes (Borges *et al.*, 1989; Eglen *et al.*, 1989).

There is, however, experimental evidence that contradicts this hypothesis. Indeed, Auguet *et al.* (1988) also observed that, in the rat aorta, the vasoconstrictor effects of endothelin and also the dihydropyridine Bay K 8644, an agonist at the L-type Ca-channel, were different, and Bay K 8644, but not endothelin, required a partial pre-depolarization stimulation in order to evoke contractions. Furthermore, the effects of endothelin were comparatively insensitive to nifedipine, nitrendipine, verapamil, diltiazem

or gallopamil, whereas those of Bay K 8644 were markedly reduced (Auguet *et al.*, 1988; Criscione *et al.*, 1989). In the rabbit aorta, the endothelin mediated contraction was insensitive to nifedipine, nicardipine and verapamil (Ohlstein *et al.*, 1989), and in the mesenteric artery and jugular and mesenteric veins of this animal, nicardipine blocked Bay K 8644 evoked contractions but did not affect those produced by endothelin (D'Orleans-Juste *et al.*, 1989). It has been reported that the hyperpolarization elicited by the K^+-channel opener, cromakalin, reduced endothelin-mediated contractions in rat aortic strips (Criscione *et al.*, 1989) Dichlorobenzamil, a blocker of Na^+-Ca^{2+} exchange, also inhibited endothelin-mediated contractions; amiloride, had no effect (Criscione *et al.*, 1989). The effects of dichlorobenzamil may, however, reflect the non-selective inhibition of endothelin-activated cation channels and not result from inhibition of the Na^+-Ca^{2+} exchange system (Van Renterghem *et al.*, 1989; Criscione *et al.*, 1989). The electrophysiological studies of Wallnöfer *et al.* (1989) and Van Renterghem (1989) have been most illuminating in elucidating the mechanism of action of endothelin in comparison to other agonists. Van Renterghem (1989) proposes that the electrophysiologic effect of ET-1 involves three types of channels. The first electrophysiological effect of ET-1 in spontaneously active cells is a transient hyperpolarization, during which the spontaneous electrical activity of the cell stops. It is followed by depolarization and a recovery of the electrical activity with a higher frequency. In cells with no spontaneous activity, ET-1 also produces a hyperpolarization followed by a depolarization during which a spiking activity is triggered. In the presence of a 1,4-dihydropyridine (DHP), ET-1 still produces a depolarization following a transient hyperpolarization, but the spiking activity is abolished. The

transient outward current induced by ET-1 was characterized as a K^+ current. It is due to the opening of Ba^{2+} and charybdotoxin-sensitive Ca^{2+}-activated K^+ channels which are also involved in the spontaneous activity. The inward current activated by ET-1 reversed near -1 mV and is due to the opening of Ca^{2+} permeable non-selective cation channels. Van Renterghem *et al.* (1989) concluded that ET-1 changes the intracellular Ca^{2+} concentration by two different mechanisms: (i) it liberates Ca^{2+} from internal stores (presumably through IP_3 production) and (ii) it activates a non-selective cation channel in the plasma membrane that is permeable to Ca^{2+}. The depolarization induced by the opening of the non-selective cation channels brings the membrane potential level near the threshold for L-type Ca^{2+} channel activation and thereby eventually produces a spiking activity. Consequently, substantial amounts of Ca^{2+} then flow into the cells via L-type Ca-channels. The authors surmised that it is therefore not surprising that blockers of L-type Ca^{2+} channels can eliminate a significant component of the ET-induced contraction. The remaining contraction component observed in the presence of channel blockers may be due to : (a) Ca^{2+} liberated from internal stores, (b) Ca^{2+} flowing through non-selective cation channels and (c) to a putative stimulation of protein kinase C by diacylglycerol. Wallnöfer *et al.* (1989) have shown that in the mesenteric resistance vessels (MRVs) of the rat, ET-1 acts as a full contractile agonist, giving tonic contractions equivalent to those seen with NE and arginine vasopressin (AVP), but it produces only about 35% of the membrane depolarization (-7 mV) produced by NE or AVP (-20 mV). The reasons underlying this difference have been amply explained by Van Renterghem *et al.* (1989). The authors concluded that the degree of depolarization caused by ET-1 would not alone induce enough Ca^{2+} entry through

voltage-sensitive Ca^{2+} channels to yield tension (12 mV depolarization with 15 mM K^+ gave no tension). Hence, ET-1 must evoke other cellular effects in addition to membrane depolarization in order to activate the MRVs. Indeed, the sequential or temporal spacing of events, as proposed by Van Renterghem *et al.* (1989), seem to provide the missing link to the electrophysiologic study by Wallnöfer *et al.* (1989). However, Hay (1990), in examining the mechanism of ET-1-induced contractions of the rat aorta in comparison with the guinea pig trachea, concluded that ET-1-induced contractions of the rat aorta were more sensitive to the effects of incubation in a Ca^{2+}-free medium, or with nicardipine or staurosporine, suggesting that differences exist in the relative mechanisms whereby ET-1 produces contraction in different tissues.

1.3.7 Pharmacokinetics of endothelin.

Pharmacokinetic studies with endothelin are few. Despite its long lasting vascular effects, the half-life of this peptide in the plasma is very short; less than 2 minutes (Pernow *et al.*, 1989). This has been attributed to a quick extraction of the peptide from plasma during pulmonary circulation (de Nucci *et al.*, 1988) or by renal, splenic and skeletal muscle clearance (Pernow *et al.*, 1989). In rats, i.v. injected ^{125}I-labeled ET-1 was rapidly eliminated from the blood stream, and the administered radioactivity distributed chiefly to the parenchyma of the lungs, kidney and liver (Shiba *et al.*, 1989). This rapid decay rate was virtually unchanged even when a pressor amount (1-2 nMol. Kg^{-1}) of cold ET-1 is co-administered with the radiolabeled tracer. Nevertheless, the pressor response usually lasts more than one hour. The extremely slow dissociation of ET-1 from its receptors and/or other cellular components in vascular smooth muscle (Hirata *et al.*,

1988b) may at least partly account for the discrepancy between the time-course for elimination of the exogenously applied peptide from the circulating blood and that for the pressor effect of the peptide. Within a few minutes of i.v. injection of ^{125}I-labeled ET-1, a major fraction of radioactivity was found in lungs, kidneys, liver and spleen (Kokesi *et al.*, 1989; Shiba *et al.*, 1989). Moreover, HPLC analysis of the injected ^{125}I-labeled ET-1 remaining in the circulating blood indicated that its chemical form was not significantly altered in the blood stream (Pernow et al., 1989; Shiba *et al.*, 1989). ^{125}I-labeled ET-1 also appears to be very stable in heparinized whole blood in vitro at 37°C. Thus, the rapid elimination of ET-1 from the circulation may be due to trapping of the intact peptide by the parenchymal organs. More recently, Sokolovsky et al.(1990) have shown that incubation of endothelins (ETs) with bovine kidney neutral endopeptidase (NEP) resulted in a selective two step degradation with loss of biochemical activity. The first step was the "nicking" of the Ser5-Leu6 bond, followed by cleavage at amino-terminal side of Ile19. The" nicked" peptide exhibited biochemical activities comparable to those of intact peptide - i.e., binding to the ET-receptor, induction of inositol phospholipid hydrolysis and toxicity. The twice-cleaved product, however, was inactive. On the other hand, the sarafotoxins (SRTXs) were more resistant to NEP than were the ETs: the $t_{½}$ for ET-1 was -1 hour while it was -4 hours for SRTX-b and even higher for SRTX-c. The authors concluded that the results from these in vitro findings may indicate a regulatory role for NEP (or similar enzymes) in the physiological inactivation of ETs. This might also help to explain why, under physiological conditions, ETs may be less toxic than SRTXs. Neutral endopeptidase is said to be widely distributed in the body, occurring in

specific structures in the central nervous system, kidney, lung and intestines as well as in neutrophils and fibroblasts, but its concentration in vascular endothelial cells is very low. The trapping of radiolabeled ET-1 in certain organs, and the availability of neutral endopeptidase at a relatively high concentration may not just be a coincidence but a physiologic localization of substrate and enzyme for optimal degradation. Despite their close biochemical and receptor binding properties, this may also be a noteworthy difference between SRTX-b and ET-1. It may well be that the structural differences could account for the differences in pharmacokinetics of the two peptides. Basal concentration of ET-1 in healthy human plasma seems to vary from one laboratory to another. Ando *et al.* (1989) reported a value of 0.6±2 fmol/mL (mean±S.D.), Cernacek and Stewart (1989) reported 0.26±0.24 pg/mL or 104±94 fmol/L and Suzuki *et al.* (1989) reported 1.5±0.5 pg/mL (range 0.7-2.4 pg/ml) for females. In disease states such as cardiogenic shock, levels of 3.65±1.14 pg/mL have been measured (Cernacek and Stewart, 1989). Hopefully, more data on the pharmacokinetics of endothelin and sarafotoxin will become available and perhaps, with standardization of methods, the "normal" plasma concentration of ET will be determined.

In summary, research with endothelin has left us with more questions than answers. Notably, what are the physiologic roles of endothelin? Is endothelin the cause or the effect of a pathologic state? Are the signal transduction pathways of ET-1 and SRTX-b the same? Finally, is endothelin a 'hormone' or autacoid of self-destruction?

1.4. Objectives.

The preceding literature review indicates general agreement on the following areas:

(i) Endothelin acts on a wide spectrum of target tissues and lacks species specificity. Furthermore, endothelin does not originate from endothelium alone.

(ii) Although ET-1 and SRTX-b show biochemical and binding characteristics, the existence of receptor subtypes allows for the possibility of these two peptides binding to distinct receptors and utilizing different signal transduction pathways.

The overall objectives of the thesis were : A) to compare the actions of endothelin-1 (ET-1) and sarafotoxin S6b (SRTX-b) with those of a well characterized agonist, norepinephrine (NE) and B) identify and compare the transduction effector mechanisms utilized by these peptides with NE. Experiments were designed to answer the following questions:

1) Are the contractile mechanisms induced by these peptides and norepinephrine the same in vascular and non-vascular smooth muscles?

2) Do ET-1 and SRTX-b interact with the same receptor?

Chapter 2

MATERIALS AND METHODS

2.1. Animals.

Male Sprague-Dawley rats (225-250 g) were purchased from Canadian Hybrid Farms, Halifax, Nova Scotia. These animals were used for all *in vitro* studies mentioned in the thesis.

The rats were housed in the animal care facility of the Faculty of Medicine, Memorial University of Newfoundland, under minimal disease conditions, three rats to a cage. The rooms had a 12-hour light, 12-hour dark, light cycle with controlled humidity and temperature.

At all times, the animals had free access to food (Purina Rat Chow from Charles River Inc., Montreal) and water.

2.2. Choice of tissue.

The preceding literature review illustrates that a wide range of tissues have been used in the study of endothelin and sarafotoxin. The major objective of this study was to determine whether endothelin and sarafotoxin shared the same membrane receptor-transduction system in smooth muscle. It was decided that a comparison of a vascular to a non-vascular preparation be made. The rat anococcygeus muscle and oesophageal tunica muscularis mucosa (TMM) were chosen as representatives of the non-vascular smooth muscle, and the portal vein and thoracic aorta for vascular smooth muscle. The tissue had to meet the following criteria, to be considered suitable:

(i) resilience of tissue in long experimental protocol;

(ii) repeated use following recovery from exposure to given agonists;

(iii) consistency of results; and

(iv) sensitivity in terms of response at low concentrations.

2.3. Tissue preparation.

The rats were killed by a sharp blow to the head and exsanguinated by cervical transection. The thoracic aorta, portal vein, oesophagus and anococcygeus muscle were isolated and placed in warm (37°C) Krebs solution continuously bubbled with oxygen/carbon dioxide (95% O_2 / 5% CO_2). Adherent connective tissue was carefully removed from the aorta, care being taken not to stretch the preparation. Any residual clot was removed from the lumen of the aorta with fine forceps before cutting the artery into 3-4 mm ring segments (Goldberg and Triggle, 1977). These rings were suspended between triangular shaped arterial hooks in a 10 ml organ bath containing Krebs solution, at pH 7.4, double jacketed to maintain a temperature of 37°C and bubbled with 95% O_2 /5% CO_2 continuously throughout the duration of experiments. One hook was anchored to a fixed point in the organ bath while the other was connected to the force-displacement transducer.

The portal vein was dissected free of adherent tissue, incised along its length to form a strip, and tied at both ends with silk (Sutter and Ljung, 1977). The vein was then mounted in an organ bath for isometric force measurements in a similar manner as described above for the aorta.

The paired anococcygeus muscles were dissected free of connective tissue and each tied at its origin and insertion before removal from the animal for suspension in the organ bath with conditions identical as for the vascular preparations (modified from Gillespie, 1972).

The tunica muscularis mucosa (TMM) of the oesophagus was set up as described by Bieger and Triggle (1985) and suspended in an organ bath with conditions identical to those described for the other tissues.

All the tissues were set up at a preload tension of 0.5 g, except for the aortic ring preparation whose preload tension was set at 2 g. The choice of pre-load tension was based on previously published evidence as to the optimal conditions for these tissues. The tissues were allowed one hour to attain equilibrium under resting tension. Isometric tension was recorded on a Beckman R611 Dynograph using Grass FT 03C force displacement transducers.

2.4. Experimental protocol.

Study 1: Tissue screening.

This study was performed using all four isolated preparations. During equilibration, Krebs buffer was changed every 15 minutes until the tissue maintained a stable baseline under the specified resting tension. Generally this took one hour to achieve. The tissues were always prepared in pairs except for portal vein which had to be isolated from two rats (attempts at using two halves from one rat had produced repeated experimental failures). Each tissue was exposed to NE and, using a concentration range of 10^{-9} M to 10^{-4} M, a cumulative concentration-response curve was constructed. The tissues were washed every 10 minutes until a return to baseline was achieved following which the tissues were allowed 30 minutes of recovery with change of Krebs buffer every 15 minutes. After recovery, each tissue was exposed to ET-1 and, using a range of 10^{-12} M to 10^{-7} M, a cumulative concentration-response curve was constructed. The tissues were washed every 10 minutes until a return to baseline was achieved. Since, with SRTX-b, recovery was rarely complete on a given day, the same protocol was repeated on other experimental dates. From these experiments it was possible to determine the threshold concentration of response (sensitivity) of each tissue and determine EC_{50} values for the different agonists as well as their recovery

time in physiologic buffer (Krebs). In the case of the oesophagus preparation, a muscarinic agonist, cis-dioxolane (CD) was used to test the viability of the TMM before determining the effectiveness of NE as a spasmolytic agent.

Study 2: Role of extracellular calcium (Ca^{2+}).

Anococcygeus muscle, which showed most resilience, was initially used for this study. All other conditions earlier stated in Study 1 were applicable except the omission of Ca^{2+} from the Krebs buffer. A pair of anococcygeus muscle was obtained from each rat and one of each pair was used as a control in which the Krebs buffer had Ca^{2+} at normal concentration. Three concentration response curves were constructed beyond the threshold value of NE for the tissues. Following recovery from NE, ET and SRTX-b concentration- response curves were constructed under identical experimental conditions.

Further examination of the role of extracellular Ca^{2+} involved the use of nifedipine (calcium channel antagonist). In these experiments, anococcygeus and aorta, having met the earlier defined criteria, were used for the study. The protocol involved the initial challenge of a pair of tissues with NE, ET-1 and SRTX-b at concentrations equivalent to the EC_{50} values, which had been earlier determined, in normal Krebs buffer followed by repeated washing of tissues until a return to baseline was achieved. The tissues were allowed 30 minutes to recover with a change of Ca^{2+}-free Krebs buffer every 15 minutes. Subsequently, ET-1 or SRTX-b 10^{-8} M (10 nM) or 10^{-6} M NE was added to the organ bath and a Ca^{2+} concentration-response curve was constructed. The tissues were again washed every 10 minutes with Ca^{2+}-free Krebs buffer until a return to baseline was achieved and then they were

allowed another 30 minutes to recover. The next stage involved the addition of nifedipine (calcium channel blocker) at a concentration of 10 μM to all the organ baths. These tissues were allowed one hour incubation in nifedipine before repeating Ca^{2+} concentration-response curve in the presence of 1 μM NE, 10 nM ET-1 and 10nM SRTX, respectively. Since nifedipine was dissolved in absolute ethanol, control experiments to examine the effect of this solvent were performed.

Study 3: Role of extracellular and intracellular calcium (Ca^{2+}).

Anococcygeus muscles and rings from thoracic aorta were used in this protocol in pairs. All previous experimental procedures were maintained except that Ca^{2+} was omitted from the Krebs buffer as was the case for examination of extracellular calcium. 10 nM ET or 10nM SRTX-b or 1 μM NE were added to each organ bath and if any contractions occurred in the Ca^{2+}-free medium, they were allowed to reach a stable plateau, following which a Ca^{2+} concentration response curve was constructed. Tissues were washed with Ca^{2+}-free Krebs every 10 minutes until a return to baseline was achieved and allowed another 30 minutes for recovery. Ryanodine, which interferes with the release of cellular Ca^{2+}, was added to each organ bath to achieve a concentration of 10 μM in Ca^{2+}-free Krebs buffer. The tissues were allowed one hour incubation in ryanodine before repeating a Ca^{2+} concentration response curve in the presence of either ET-1, SRTX-b or NE. Subsequently, they were washed with Ca^{2+}-free Krebs every 10 minutes until a return to baseline was achieved, following which ryanodine 10 μM was added and the tissues allowed to incubate for one hour. Finally, nifedipine 10 μM was added and the tissues allowed a further one hour incubation and a Ca^{2+} concentration response curve constructed in the presence of ET-1, SRTX-b and

NE.

In order to address the possibility that ryanodine is more effective in depleting intracellular Ca^{2+} in the presence of an agonist, further experiments using ryanodine alone, ryanodine with caffeine, and ryanodine concurrently with an agonist in a Ca^{2+}-free Krebs were designed and run essentially using the same experimental protocol.

Further experiments were conducted to examine the effect of other intracellular Ca^{2+} depletors, namely repeated agonist stimulation with NE, or a combination of repeated agonist stimulation followed by ryanodine incubation, before construction of a Ca^{2+} concentration-response curve. To further evaluate the role of Ca^{2+} in the contractile process of ET-1, SRTX-b and NE, anococcygeus muscle responses to 10 nM ET-1 and SRTX-b and 1 μM NE were measured in normal Krebs, Ca-free Krebs, and following ryanodine treatment in normal Krebs.

Additional evaluation of the role of Ca^{2+} in the contractile process involved the use of the VOCC opener Bay K8644. In these sets of experiments, a depolarizing (subcontractile) level of K^+ (15 mM) in a Krebs buffer, was used. Isotonicity of Krebs buffer was achieved by substitution of Na^+ at equivalent concentration. Concentration-response curves to Bay K 8644 were constructed for anococcygeus muscle and aorta. The tissues were washed every 10 minutes until recovery was achieved and allowed a further 30 minutes for complete recovery. Subsequently, 1 nM ET or SRTX-b or 100 nM NE were added to the respective organ baths and concentration response curves to Bay K 8644 repeated.

Study 4: Role of arachidonic acid metabolites.

Paired anococcygeus muscles and rings of thoracic aorta were used for

this study. They were isolated and placed in Ca^{2+}-free Krebs buffer with experimental conditions identical to those previously applied. Ca^{2+} concentration-response curves were constructed in the presence of 10 nM ET-1 and SRTX-b and 1 μM NE, respectively. The tissues were washed with Ca^{2+}-free Krebs buffer every 10 minutes until recovery to baseline was achieved following which a further 30-minute interval was allowed for complete recovery. 10 μM indomethacin or 10 μM NDGA was added to each organ bath and tissues were incubated therein for one hour. Ca^{2+} concentration-response curves in the presence of the three agonists were constructed.

Further experiments were also designed in order to address the question of specificity of NDGA. In these sets of experiments, anococcygeus and thoracic aortic rings were again used. A control experiment with 50 mM K^+ in Ca^{2+}-free Krebs was designed and Ca^{2+} concentration-response curves constructed without and with NDGA. The three agonists ET-1, SRTX-b and NE were examined in normal Krebs buffer by construction of agonist concentration-response curves without and with NDGA. As with other experiments, and in order to aid agonist dissociation, the tissues were washed with Ca^{2+}-free Krebs after the initial concentration-response curves.

Study 5: Cross-desensitization.

Paired anococcygeus muscles and thoracic aortic rings were used for this study. Each pair of tissues was placed in a 10 ml organ bath with normal Krebs solution and maintained under identical experimental conditions, as earlier defined. A pair of aortic rings and anococcygeus muscle were exposed to NE and another set to KCl. After the initial concentration-response curves to these two agonists, the tissues were washed every 10 minutes until recovery to baseline was achieved, then allowed another 30 minutes for full

recovery. Threshold concentrations of ET-1 or SRTX-b (1 nM) were added to one organ bath with one of a pair while its complementary unit served as a control . Tissues were allowed to incubate for 1 hour following the addition of either ET-1 or SRTX-b and then concentration-response curves to NE and KCl were constructed.

2.5. Composition of buffers (Krebs bicarbonate buffer, adapted from Janis and Triggle, 1973) [mM].

Normal Krebs buffer (mM): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 12.5; Dextrose 11.1.

Ca^{2+}-free Krebs was made by omission of $CaCl_2$ from the above with the addition of 0.01 mM EGTA.

High K^+ Krebs (mM) NaCl 107.7; KCl 15; $CaCl_2$ 0; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 12.5; Dextrose 11.1.

Very High K^+ Krebs (mM) NaCl 72.7; KCl 50; $CaCl_2$ 0; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 12.5; Dextrose 11.1.

All constituents were reagent grade and were dissolved in deionized distilled water.

2.6. Drugs and chemicals.

The following agents were used: endothelin-1 and sarafotoxin (S6b) (Peptides International, Louisville, KY).

NE, indomethacin and NDGA were purchased from Sigma Chemical Co.,(St. Louis, MO).

Ryanodine and cis-dioxolane (Research Biochemicals Incorporated, Natick, MA).

Caffeine was obtained from J.T. Baker Co., Phillipsburg, NJ.

Nifedipine was a generous gift from Bayer, Leverkusen, W. Germany.

Ryanodine, nifedipine, indomethacin and NDGA were prepared as stock solutions of 10^{-2} M in absolute ethanol; 10 μL of stock solution added to 10 ml organ bath brought the bath concentration to 10^{-5} M.

All other reagents, of analar grade, were dissolved in distilled water and where appropriate, pH adjustments for increased solubility were made with NaOH or HCl.

2.7. Statistics.

The EC_{50} values were obtained by graphically calculating the concentration of agonist producing 50% of its maximum response (Fleming, Westfall, DeLaLande and Jellet, 1972).

Where indicated, results are expressed as mean ± SEM (standard error of the mean) calculated by the computer statistical programme of Gustafson, T.L. Statistical significance was determined by Student's t test (one-tailed) for paired samples or analysis of variance (ANOVA) for independent samples using the above computer programme. Differences by the 't'-test or ANOVA were considered to be significant if $p<0.05$. The sample size (n value) is indicated in all tables.

CHAPTER 3

RESULTS

Study 1: Tissue screening

Due to the failure of the TMM from the rat oesophagus to respond to either ET-1 or SRTX-b (3 trials),the TMM was omitted from further studies. Of the remaining three tissues under consideration, the portal vein (Figure 4), showed great sensitivity in two parameters - frequency and amplitude of its spontaneous myogenic response - the responses were rather erratic (Figure 3). It also was omitted from further studies.

The anococcygeus and aortic preparations met the defined criteria and were subsequently used for all the in vitro work described in this thesis. In choosing anococcygeus muscle as the non-vascular smooth muscle of choice, the following useful properties, or characteristics, as enumerated by Goyal (1984) were considered viz :-

(i) It consists entirely of smooth muscle cells arranged in parallel bundles to form a sheet, so that there is a minimal diffusion barrier for drug access or ion exchange studies.

(ii) The muscle is represented bilaterally so that control and test preparations can be taken from the same animal.

Anatomically, this muscle is associated with the intestine but differs in a number of aspects from intestinal smooth muscle. Intestinal smooth muscle possesses spontaneous myogenic activity and is inhibited by stimulation of sympathetic nerves and excited by the stimulation of parasympathetic nerves (Goyal, 1984). The anococcygeus muscle, by contrast, has a high stable resting membrane potential of -60 mV, shows no spontaneous activity, either

electrical or mechanical, and is excited by sympathetic nerves but inhibited by a non-cholinergic, non-adrenergic pathway. The nature of this inhibitory transmitter is unknown (Creed and Gillespie 1973; Creed, Gillespie and Muir, 1975).

Time-dependent responses were assessed by constructing four concentration-response curves to NE over a period of four hours. A loss of tone of about 15% of initial maximal response was noted (Table 1). In order to determine the optimal concentration range for these tissues and obtain EC_{50} values, concentration-response curves to NE, ET-1 and SRTX-b were constructed. The results of these experiments are graphically presented (Figure 5), which does not show the full concentration range of agonists examined because the descending limb of the hyperbola has been deleted for neatness. It was evident that concentrations of NE beyond 10^{-5}M or 3×10^{-8}M for ET-1 and SRTX-b were detrimental to tissue responsiveness and resulted in a loss of tension or subsequent non-response to these agonist . Furthermore 10^{-7}M (0.1 μM) of ET-1 or SRTX-b induced myogenic-like activity in normally quiescent anococcygeus muscle which rendered the tissues unusable for subsequent experiments in normal Krebs buffer (Figure 6). As determined by the loss of myogenic activity, the tissues recovered more rapidly from SRTX-b than ET-1 with complete recovery apparent after 2.62±0.85 and 5.22±0.06 hours respectively. Omitting Ca^{2+} from the Krebs solution significantly ("t"-test) reduced recovery times to 1.62±0.2 and 2.40±0.51 hours respectively (Table 2). Thus, a Ca^{2+}-free Krebs buffer was used to wash tissues whenever ET-1, or SRTX-b, was used in an experimental protocol to hasten tissue recovery from effects of the peptides. In order to avoid the induction of myogenic activity by ET-1 or SRTX-b, concentrations of 30 nM, or greater, were rarely

used in subsequent experiments. Indeed 10 nM ET-1, or SRTX-b, which corresponds approximately to the EC_{50} values of these peptides was used, unless otherwise stated. Nifedipine, at 10 μM, had no effect on the peptide-induced myogenic activity. Generally, contractile responses to ET-1 and SRTX-b seemed insensitive to nifedipine in the nano-low micromolar range.

Study 2: Role of extracellular Ca^{2+}

Control responses elicited by ET-1 and SRTX-b (10 nM) or NE (1 μM) were approximately equieffective in terms of tension developed (Figure 7). In Ca^{2+}-free Krebs, the three agonists generated approximately similar levels of tone in the aorta and anococcygeus muscle, corresponding to 18% and 5% of maximum response in the controls respectively (Table 3). Muscle tone generated by the peptides even in the absence of external Ca^{2+}, was maintained in contrast to that with NE (Figure 8). Nifedipine was used in the experiments designed to determine the role of VOCCs in regulating the entry of extracellular Ca^{2+}. Nifedipine (10 μM) did significantly reduce by "t"-test only (not by ANOVA) the responses to ET-1, but not to SRTX-b and NE in the aorta (Table 4 ; see also Figure 9a,b and c). In the anococcygeus, 10 μM nifedipine significantly reduced the maximal response to ET-1 (Fig. 10a), but responses to SRTX-b (Fig. 10b) and NE (Fig. 10c) were not significantly reduced. However, it is noteworthy that the decrease in sensitivity for ET-1, SRTX-b and NE was more than that observed in the aorta. The persistence of nifedipine in the tissues was assessed by repeating Ca^{2+} concentration-response curves in the presence of a stated agonist concentration after washing every 10 minutes for one hour following the return to baseline. In the aorta, recovery from nifedipine in the presence of ET-1 and SRTX-b did not occur. The response of the aorta to NE did not show recovery after

nifedipine application, although the maximum contractile response, post nifedipine washout, approached the control maximum, but, as with the two peptides there was a decrease in sensitivity as evident by the shift of the curve to the right (Figure 11a, b and c). In the anococcygeus, recovery from nifedipine in the presence of the three agonists exhibited a similar pattern (see Figure 12a, b and c). As a result of the incomplete recovery from nifedipine, tissues which had been exposed to nifedipine were not used for further investigations.

As summarized in Table 3 , it is evident that simple exposure to a Ca^{2+}-free medium drastically reduced contractile responses to ET-1, SRTX and NE in both the aorta and the anococcygeus. To further evaluate the role of Ca^{2+} in the contractile processes of ET-1, SRTX-b and NE, the responses to 10 nM ET-1 and SRTX-b and 1 μM NE were measured in normal Krebs, Ca^{2+}-free Krebs, and after treatment with ryanodine for 1 hour followed by re-introduction of normal Krebs (Figure 13). Anococcygeus tissues, which had been previously exposed to normal Krebs but were switched to a Ca^{2+}-free Krebs immediately before agonist exposure, demonstrated significantly reduced responses to all three agonists with the inhibition of responses to ET-1 and SRTX-b being greater than that observed for NE. Incubation for one hour in a Ca^{2+}-free Krebs plus 10 μM ryanodine, followed by re-introduction of normal Krebs and agonist, led to an enhancement of the responses to ET-1 and SRTX-b. The effect on the SRTX-b response was significantly greater than that for ET-1. The response to NE was not different from the control. Figure 14 illustrates the effect of the above protocol in the aorta. In the aorta, the control responses of the peptides in normal Krebs did not differ significantly from each other but each was significantly different from the NE response. In

Ca^{2+}-free Krebs, the responses to ET-1 and NE were not significantly different from their control responses in normal Krebs, whereas SRTX-b in Ca^{2+}-free Krebs demonstrated a significantly reduced response compared to its control. Like anococcygeus, the response of the aorta to NE in a Ca^{2+}-free Krebs was significantly higher than the responses to either ET-1 or SRTX-b. In contrast to studies with the anococcygeus, the post ryanodine responses to ET-1, SRTX-b and NE in the aorta did not differ significantly from their controls.

Study 3: Role of extracellular versus intracellular Ca^{2+}

Experiments were conducted to further compare and evaluate the role of extracellular versus intracellular Ca^{2+} in the contractile response to ET-1, SRTX-b and NE. After obtaining the control concentration-response curves to Ca^{2+} in the presence of the appropriate agonist, tissues were washed and incubated in 10 μM ryanodine for one hour following which the Ca^{2+} concentration-response curves were repeated. Responses to ET-1 in the anococcygeus were significantly enhanced by ryanodine treatment alone (Figure 15a, b and c). Subsequently, the tissues were again subjected to ryanodine treatment in the presence of 10 μM nifedipine in light proof chambers and Ca^{2+} concentration-response curves were again repeated in the presence of appropriate agonist. Responses to ET-1 and NE were significantly reduced in the anococcygeus but less so for SRTX-b which was not significantly reduced (Figure 15a, b and c). In the aorta, with a combination of ryanodine and nifedipine treatment, there was marked and significant inhibition of the contractile response to ET-1 and NE but not to SRTX-b (Figure 16a, b and c; see also Table 4). The discrepancy arising from visual assessment of concentration response curves (Figures 15 and 16 (a, b and c)) against Table

4 is the result of presentation of data derived from % inhibition of control maximal response subjected to statistical analysis. Further exploration of the pools of intracellular Ca^{2+}-utilized by the agonist was necessitated, since ryanodine alone did not entirely deplete the cell of the intracellular Ca^{2+} utilized for contraction. Thus, responses to NE even after incubation of both aorta and anococcygeus with ryanodine were maintained . Patel and Triggle (1986) had demonstrated intracellular Ca^{2+} depletion by repeated stimulation of the rat tail artery in Ca^{2+}-free Krebs with NE. Repeated stimulation with NE can partially deplete the intracellular stores of Ca^{2+} from both the anococcygeus and aorta, however, there is a Ca^{2+}-pool which resists depletion even after an extended, and repeated, stimulation with NE for 2-3 hours (Figure 17a and b). Figures 18 and 19 (a, b and c) demonstrate the response characteristics for NE depletion which, when compared to ryanodine depletion (Figures 15 and 16 (a,b and c)), were similar, except that ryanodine was more effective in reducing responses to SRTX-b when used with nifedipine. Further attempts to address the question of which intracellular Ca^{2+} pools are sensitive to ryanodine and/ or NE, involved experiments in which tissues had been repeatedly stimulated with NE and then incubated in ryanodine for one hour. The results from studies with the anococcygeus (Figure 20a, b and c) maintained a pattern similar to those for ryanodine alone (Figure 16a, b and c). The results from studies with the aorta, Figure 21a, b and c,were identical to those obtained following Ca^{2+} depletion with NE alone(Figure 19a, b and c). It has been suggested that the effectiveness of ryanodine in depleting sarcoplasmic reticulum Ca^{2+} is enhanced by the simultaneous presence of an agonist. In the anococcygeus, the maximal contractile responses to SRTX-b after ryanodine treatment alone or

ryanodine plus caffeine or concurrent application of ryanodine plus SRTX-b were apparently enhanced when compared to the control responses (Figure 22a, b and c). However, evaluation of the three treatments by ANOVA showed that these differences were not significantly different from each other. Figure 23a, b and c illustrates the response obtained from anococcygeus with 10 nM ET-1. Evaluation of the three treatments by ANOVA showed these differences were not significant. In the aorta, treatment with ryanodine, or ryanodine plus caffeine ,again, resulted in no significant differences from the respective controls (Figure 24a , b and c). Similarly in the aorta, with ET-1 as the agonist, the differences were not significant when examined by ANOVA as three independent variables (Figure 25a, b and c).

Since nifedipine was only partially effective at inhibiting Ca^{2+} entry, the further elucidation of routes of entry of extracellular Ca^{2+} were necessary. For this purpose, a known L-type calcium channel opener Bay K 8644 was used with a partially depolarizing (15 mM K^{+}) Krebs buffer to explore the effects of ET-1, SRTX-b and NE. Figure 26a, b and c shows the response in anococcygeus to Bay K 8644 without, and with, sub-threshold concentrations of 1 nM ET-1 and SRTX-b and 100 nM NE. An apparent enhanced contractile response to the three agonists was noted but these differences were not statistically evaluated for significance primarily because the responses in the anococcygeus in the presence of Bay K 8644 (see Figure 27) were not concentration-dependent. Figure 28a, b and c demonstrates the enhancement of Bay K 8644 responses in the aorta in the presence of the three agonists under examination.

Study 4: Role of arachidonic acid metabolites

The role of the production of arachidonic acid metabolites in response

to ET-1, SRTX-b and NE was explored by comparing control Ca^{2+} concentration response curves in the presence of 10 nM ET-1, 10 nM SRTX-b or 1 μM NE in the absence of or following a one hour incubation with the cyclooxygenase inhibitor, indomethacin(10 μM). Figure 29 and 30 show no enhancement of the responses to the three agonists. There were no significant differences from controls (Table 4).

A further elucidation of the role of agonist-induced arachidonic acid metabolite production involved the use of the lipoxygenase inhibitor, NDGA. In the anococcygeus preparation (Figure 31a, b and c), the responses to ET-1 and NE, but not SRTX-b, were significantly reduced by 10 μM NDGA (Pohl *et al.*, 1987). In the aorta (Figure 32a, b and c), the responses to SRTX-b and NE were not significantly reduced by NDGA, whereas NDGA significantly reduced the responses to ET-1.

The issue of specificity of inhibition produced by NDGA was addressed by designing experiments which compared the inhibition by NDGA of the concentration-dependent contractile responses to Ca^{2+} in the presence of ET-1, SRTX-b or NE versus 50 mM K^+. Figures 33a and b demonstrate that, in the anococcygeus preparation, a significant inhibition of the NE was achieved (Table4). A similar, but insignificant pattern of inhibition by NDGA of K^+ induced contractions was noted (Figure 33a and b). Figures 34a and b demonstrate that the reduction of the contractile response by NDGA was not noted in the aorta with either NE or K^+ induced contractions.

Study 5: Cross-desensitization to agonist

Experiments were designed in order to evaluate whether desensitization of the anococcygeus and the aorta occurred following exposure to subthreshold concentration of either SRTX-b or ET-1. Figures 35a and b illustrate the

effects of the prior exposure of the anococcygeus to a sub-contractile concentration, 1 nM of SRTX-b, and 1nM of ET-1, respectively, on concentration-response curves to NE. Prior exposure to SRTX-b (Figure 35a), but not ET-1 (Figure 35b), significantly ("t"-test), reduced subsequent responses to NE. Figures 35c and d illustrate the effects of the same procedure on the concentration-response curves to KCL and indicate that the presence of ET-1 but not SRTX-b, significantly enhanced the responses to KCL. In the aorta (Figures 36a - d) neither the prior exposure to 1 nM SRTX-b nor 1 nM ET-1 resulted in a significant ("t"-test) desensitization of the responses of the tissues to subsequent concentration-response curves to either NE or KCL. However, significant ("t"-test) enhancement was noted for KCL concentration-response curves following exposure to 1 nM SRTX-b or 1 nM ET-1 (Figures 36c and d), and also for NE concentration-response curves following exposure to 1 nM SRTX-b (Figure 36a), but not to 1 nM ET-1 (Figure 36b) .

Figure 2: Amino acid sequences of endothelins and sarafotoxins. (See list of abbreviations for alternative single letter abbreviations for amino acids.)

Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp ET-1

Cys-Ser-Cys-Ser-Ser-Trp-Leu-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp ET-2

Cys-Thr-Cys-Phe-Thr-Tyr-Lys-Asp-Lys-Glu-Cys-Val-Tyr-Tyr-Cys-His-Leu-Asp-Ile-Ile-Trp ET-3

Cys-Ser-Cys-Asn-Ser-Trp-Leu-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp ET-4

Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp SRTX-a

Cys-Ser-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Val-Ile-Trp SRTX-b

Cys-Thr-Cys-Asn-Asp-Met-Thr-Asp-Glu-Glu-Cys-Leu-Asn-Phe-Cys-His-Gln-Asp-Val-Ile-Trp SRTX-c

Cys-Thr-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Ile-Ile-Trp SRTX-d

1 5 10 15 20

Figure 2 Amino acid sequence of endothelins and sarafotoxins

Figure 3: Illustration of the erratic response of the rat portal vein to ET-1, SRTX-b and NE (n = 6).

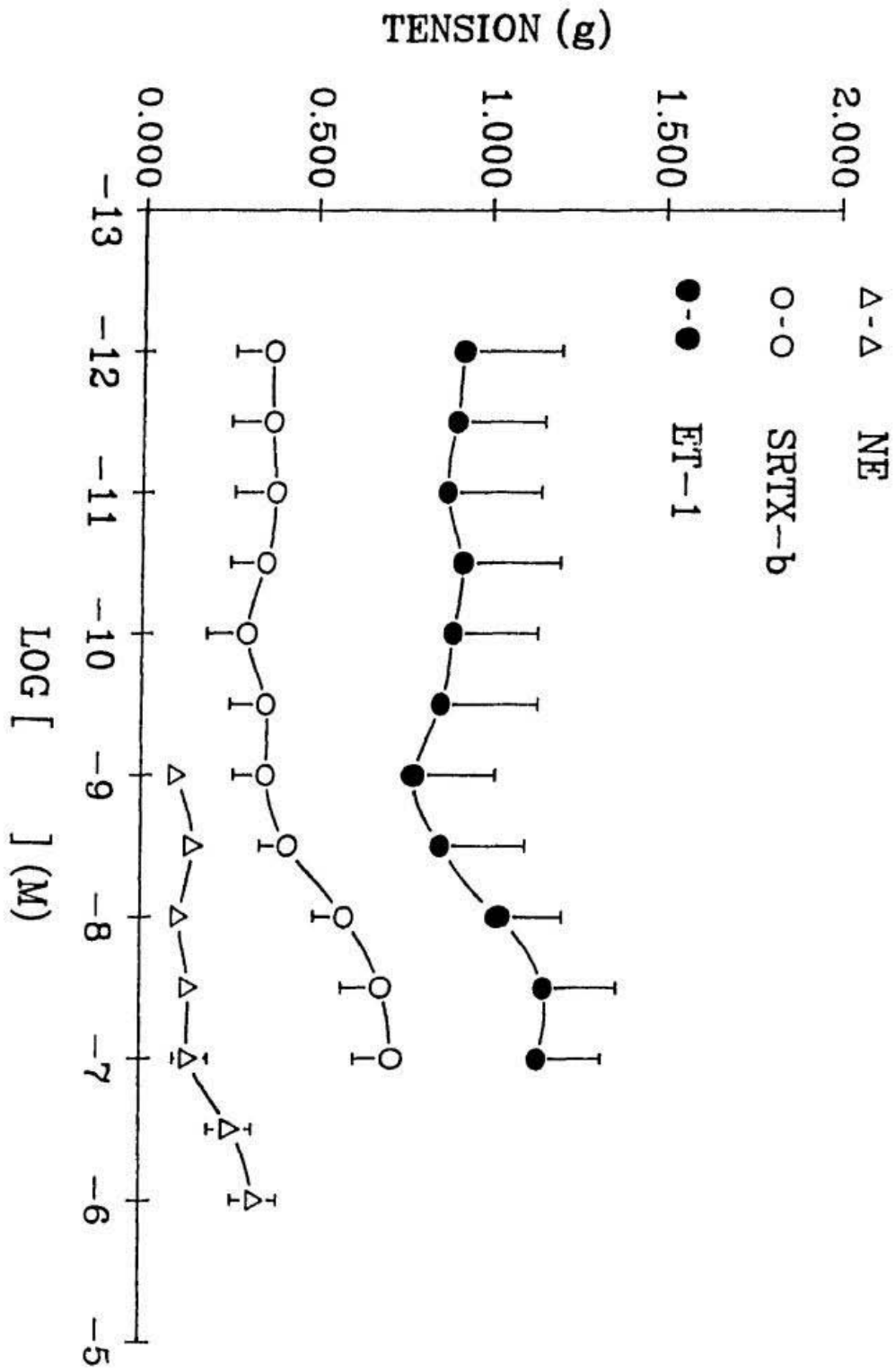

TENSION (g)
2.000
1.500
1.000
0.500
0.000
-13
-12
-11
-10
-9
-8
-7
-6
-5
LOG [ ] (M)
Δ-Δ NE
O-O SRTX-b
●-● ET-1

Figure 4: Illustrates the response of rat portal vein to SRTX-b with changes in frequency and amplitude of response at agonist concentrations as low as 10^{-10} M.

TISSUE TENSION (g)
2
1
SRTX-b
-9.5
-9
-8.5
-8

Figure 5: Concentration-response curves for ET-1, SRTX-b and NE with the anococcygeus muscle in normal Krebs buffer. Points are the mean (± S.E.M.) of n=6. EC_{50} values were obtained from these curves.

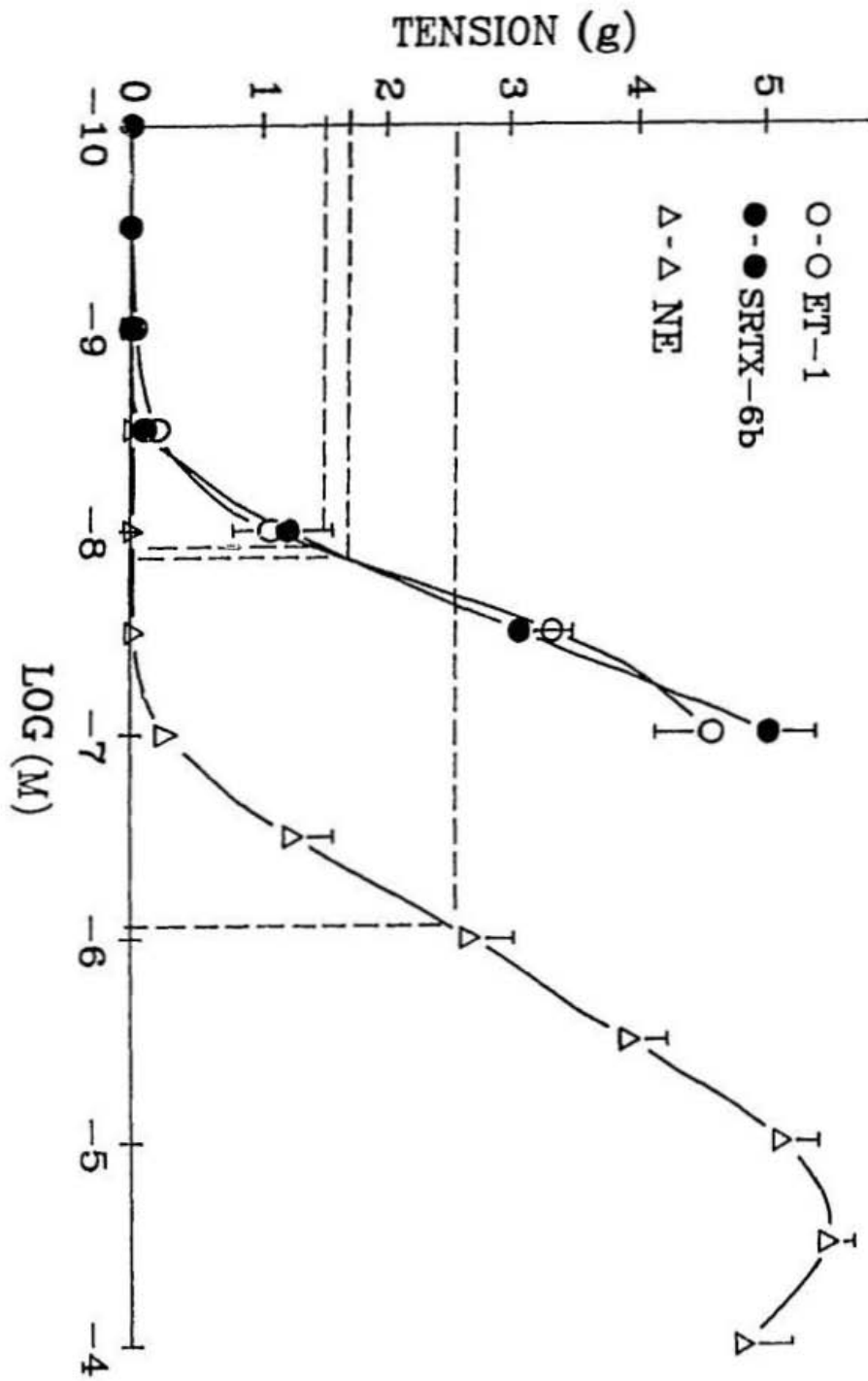

TENSION (g)
0
1
2
3
4
5
6
O-O ET-1
●-● SRTX-6b
Δ-Δ NE
−10
−9
−8
−7
−6
−5
−4
LOG (M)

Figure 6: Induction of myogenic activity in the anococcygeus muscle following washout of A) 0.1 μM ET-1; B) 0.1 μM SRTX-b with normal Krebs buffer.

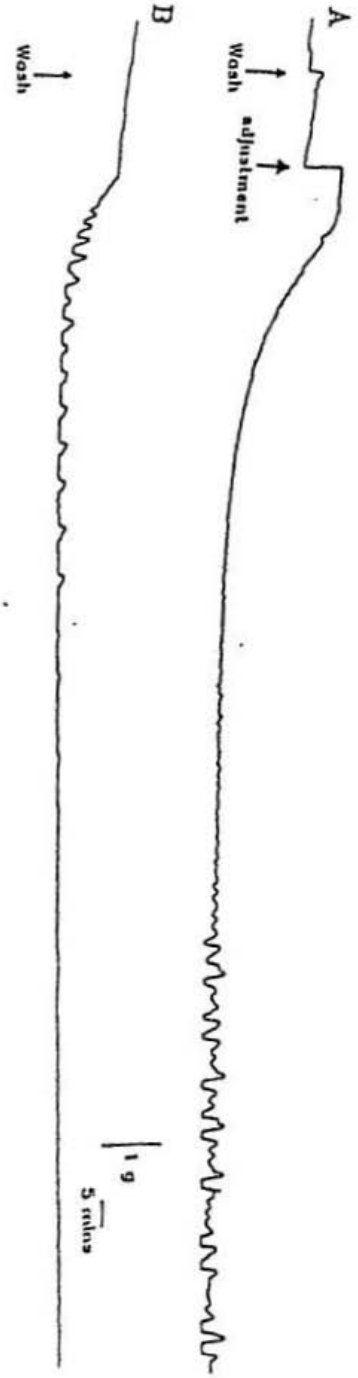

A
Wash
adjustment
B
Wash
1 g
5 mins

Table 1: Time-dependent variation in the response of the anococcygeus muscle to four concentration-response curves obtained to NE over a four hour period.

log conc. (M)	1	2	3	4	hours
-7.5	.06	.06	.08	.08	
-7.0	.41	.30	.31	.40	
-6.5	1.21	.81	.91	.98	
-6.0	2.63	2.03	2.08	2.13	
-5.5	4.70	3.83	3.65	3.5	
-5.0	7.23	6.7	5.6	5.65	
-4.5	8.12	8.0	6.8	6.88	

Tissue tension is measured in grams.

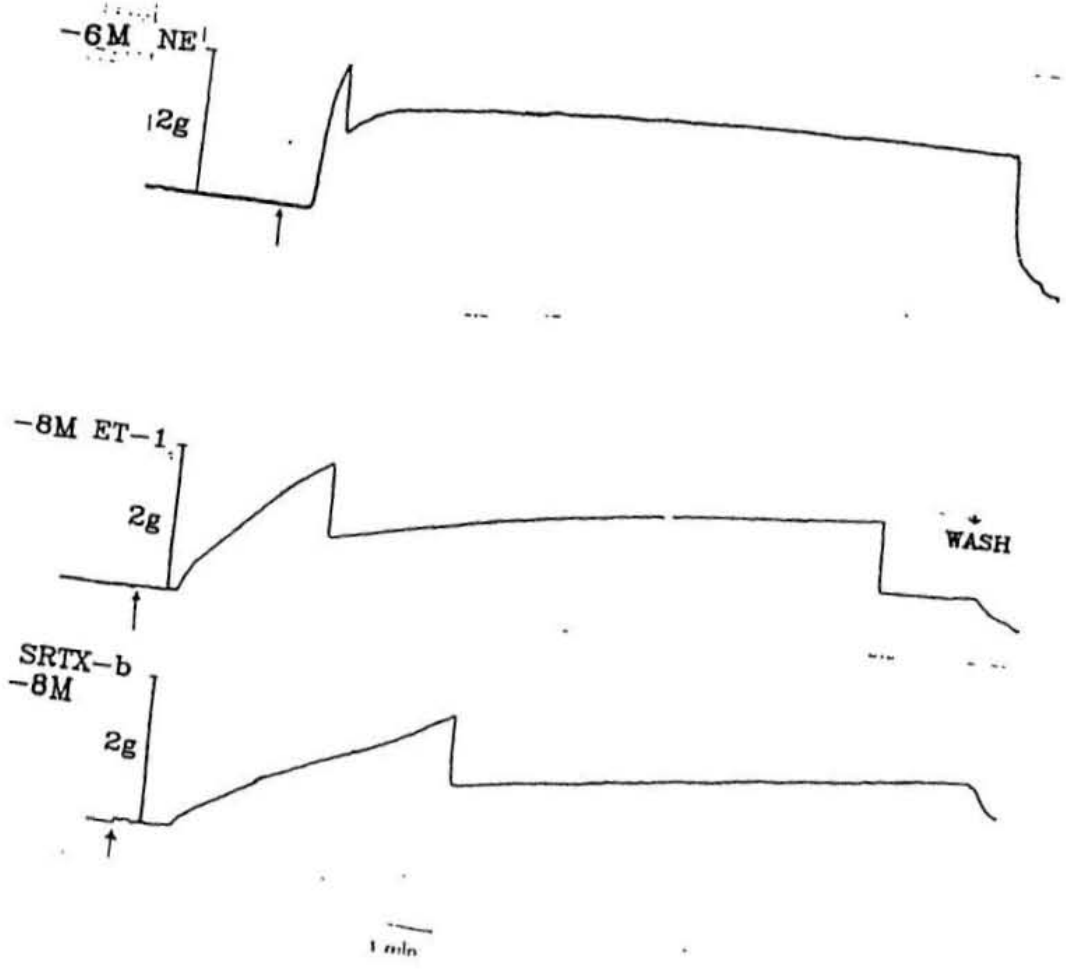
Table 2: Effect of Ca^{2+}-omission on recovery time (hours) of the anococcygeus muscle from peptide-induced myogenic activity.

	Normal Krebs (2.5 mM Ca^{2+})	Ca^{2+}-free Krebs
ET-1 (n = 4)	* 5.20 ± 0.66	* 2.40 ± 0.50
SRTX-b (n = 4)	2.62 ± 0.55	1.62 ± 0.20

Time values are Means (± S.E.M.)
* Denote significant differences (ANOVA) between ET-1 and SRTX-b. $p < 0.5$.

Figure 7: Illustrates the response to equieffective concentrations of ET-1, SRTX-b and NE at their approximate EC_{50}s in : (a) anococcygeus and (b) aorta. Chart speed throughout this study was maintained at 0.25mm/sec.

A



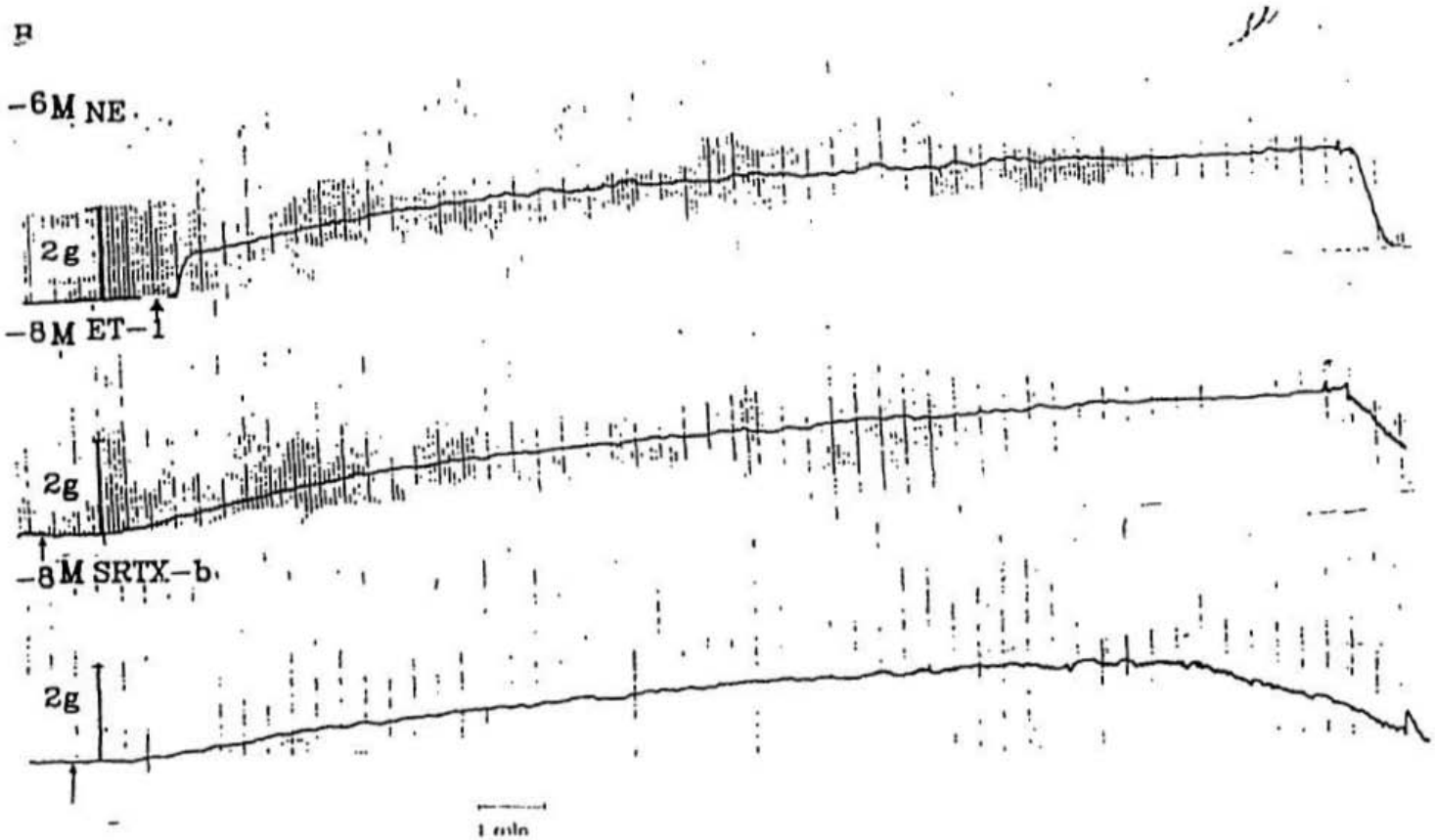

B
-6M NE
2g
-8M ET-1
2g
-8M SRTX-b
2g
1 min

Table 3: Effect of removal of extracellular Ca^{2+} on contractile responses of anococcygeus and aorta to ET-1, SRTX-b and NE.

	Anococcygeus	Aorta
ET-1 (10 nM)	5.16 ± 0.81	17.0 ± 3.0
SRTX-b (10 nM)	4.98 ± 0.70	15.3 ± 1.6
NE (1 μM)	5.92 ± 1.50	21.0 ± 2.3

Values are means of percentage maximum control responses (e.g. in the presence of extracellular Ca^{2+}) ± S.E.M. (n = 4)

Table 4. The responses to ET-1, SRTX-S6b and NE as compared to untreated tissue in normal Krebs buffers. Mean ± S.E.M.

Rat aorta	Ca^{2+}-free	Nifedipine	Ryanodine	Nifedipine and Ryanodine	Indomethacin	NDGA
ET-1	↓ 83 ± 3	↓ *35 ± 4	↓ 26 ± 17	↓ **92 ± 4	↑ 7 ± 2	↓ *29 ± 9
SRTX	↓ 84.7 ± 1.7	↓ 24 ± 13	↑ 8 ± 5	↓ 61 ± 20	↓ 1.7 ± 1.7	↓ 9 ± 6
NE	↓ 78.3 ± 2.3	↓ 17 ± 3	↓ 3 ± 5	↓ *38 ± 8	↓ 2 ± 6	↓ 50 ± 20
Rat Anococcygeus						
ET-1	↓ 94.8 ± 0.8	↓ *49 ± 5	↑ *35 ± 8	↓ *46 ± 11	↑ 17 ± 6	↓ *31 ± 10
SRTX	↓ 95.1 ± 0.7	↓ 32 ± 11	↑ 15 ± 8	↑ 32 ± 11	↑ 14 ± 7	↓ 8 ± 8
NE	↓ 94.1 ± 1.1	↓ 21 ± 11	↑ 44 ± 19	↓ *62 ± 8	↑ 0.5± 9	↓ **63 ± 8

All data derived from % inhibition of control maximal response.

* Denotes significant difference ($p<0.05$) assessed by one-way analysis of variance

* Denotes significant difference ($p<0.05$) from control response as assessed by Student's 't' test for paired observation.

n = 4 except in (NDGA column) where n = 5

[ ] of agonist and treatment applied in each column is given in the methods section.

Figure 8: Illustration of the maintained tone induced by ET-1 and SRTX-b compared to the non-sustained tone induced by NE in Ca^{2+}-free Krebs buffer.

A) Anococcygeus

B) Aorta

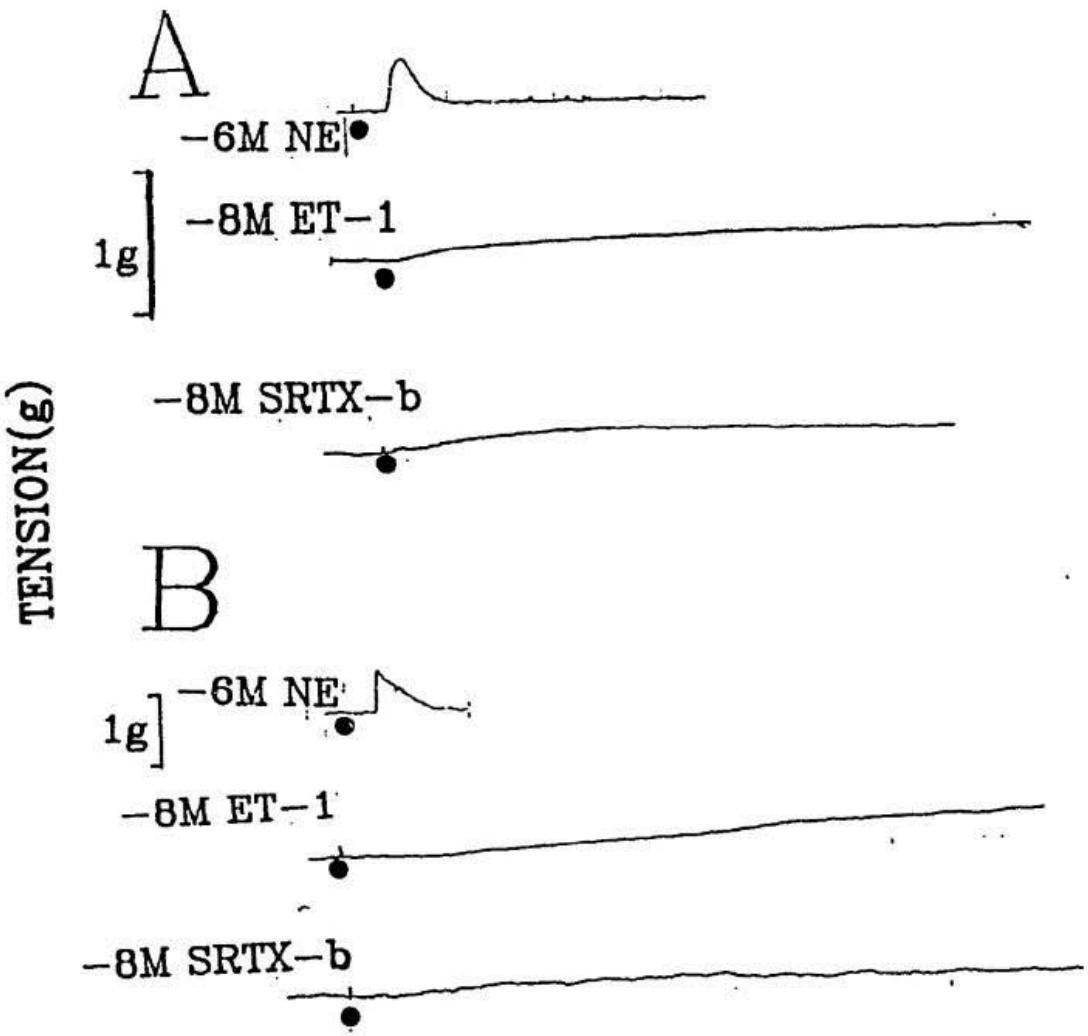
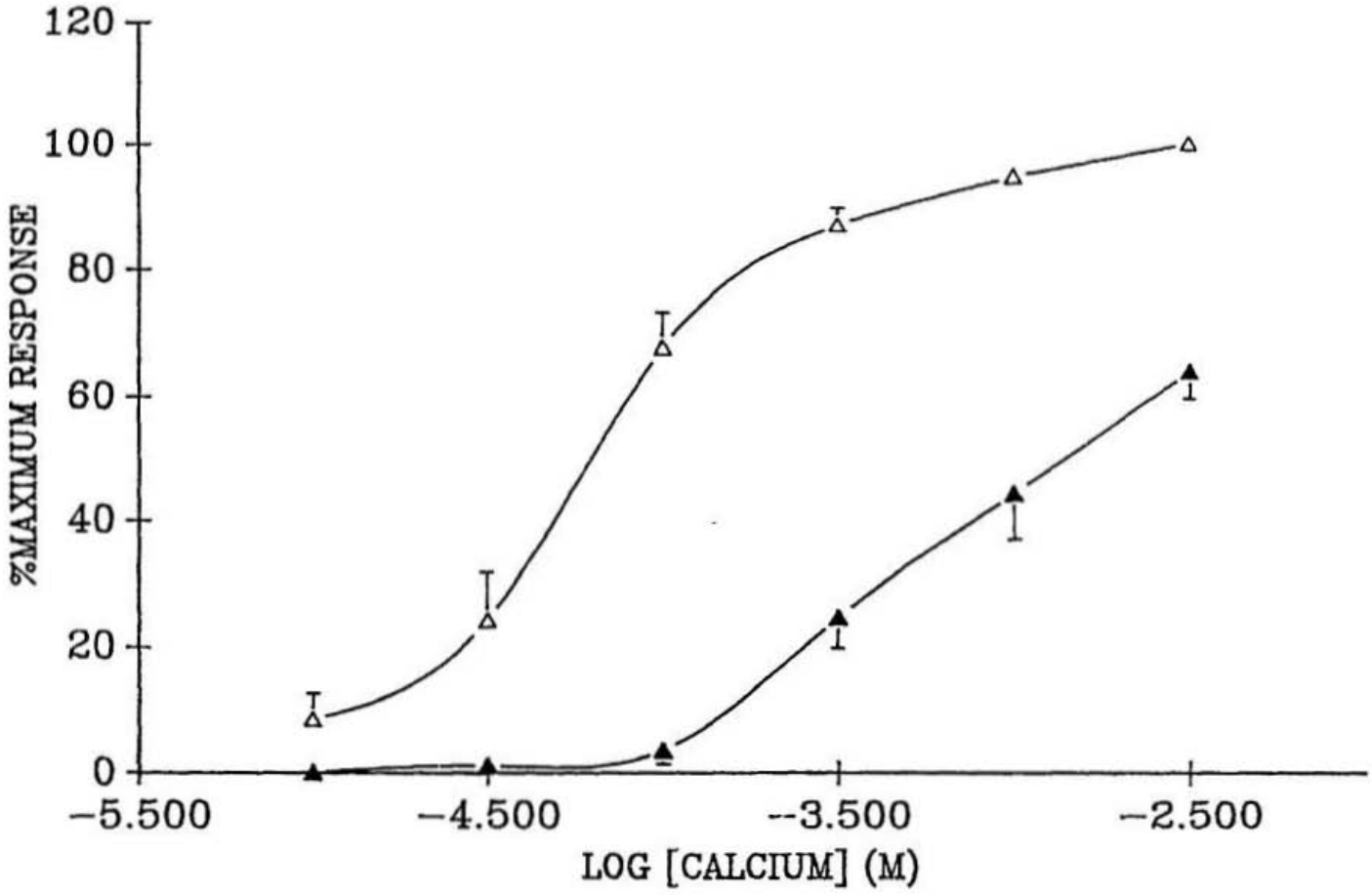
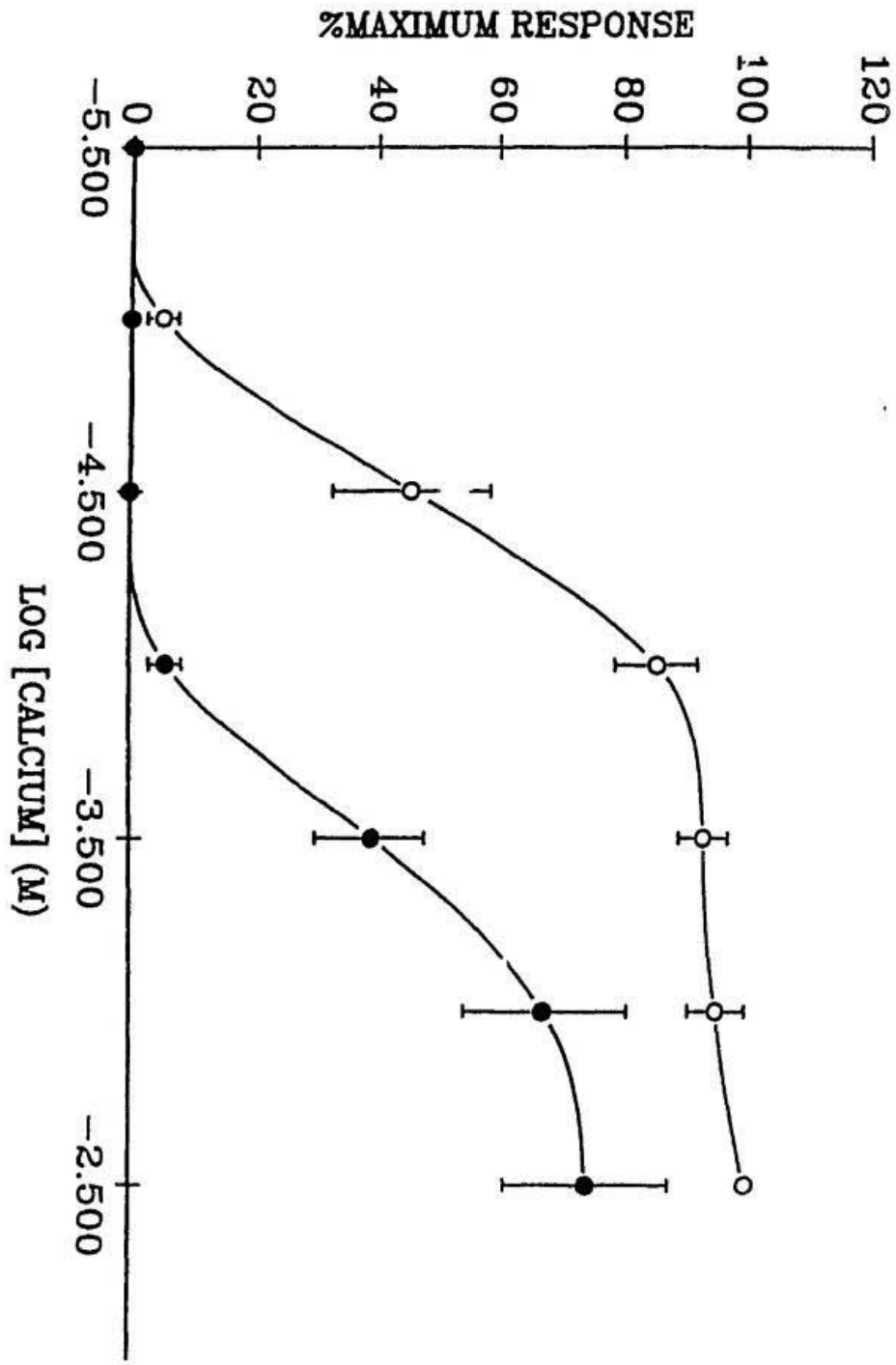

A
−6M NE
1g
−8M ET−1
−8M SRTX−b
TENSION(g)
B
1g
−6M NE
−8M ET−1
−8M SRTX−b

Figure 9a, b and c: Effect of 10 μM nifedipine on Ca^{2+} concentration-response obtained in the aorta in the presence of: a) 10 nM ET-1; b) 10 nM SRTX-b; and c) 1 μM NE. Open and closed symbols represent control and nifedipine treatment, respectively ± S.E.M. (n = 4)

(a)


120
100
80
60
40
20
0
%MAXIMUM RESPONSE
-5.500
-4.500
--3.500
-2.500
LOG [CALCIUM] (M)


%MAXIMUM RESPONSE
0
20
40
60
80
100
120
-5.500
-4.500
-3.500
-2.500
LOG [CALCIUM] (M)

(b)

(c)

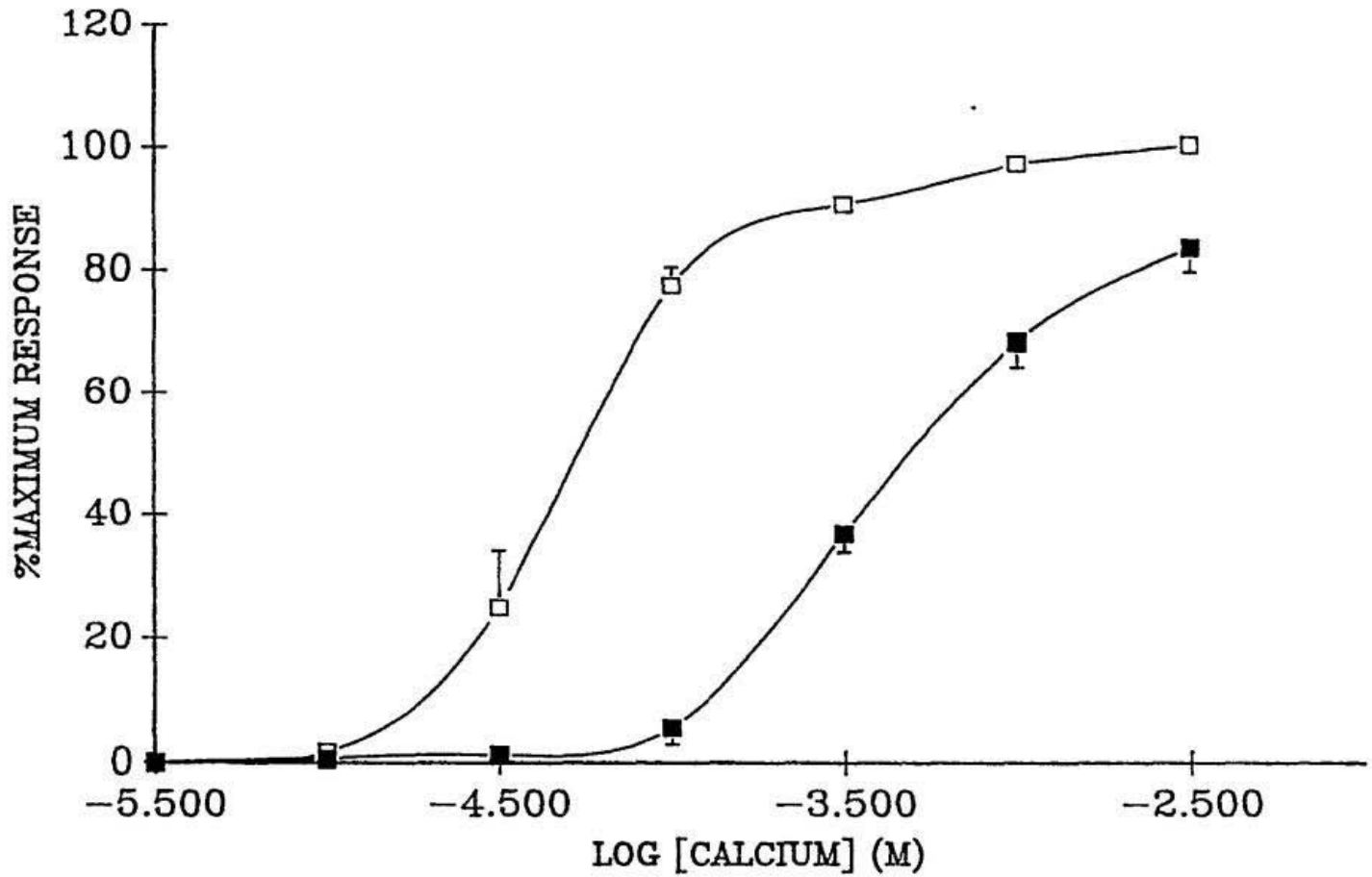
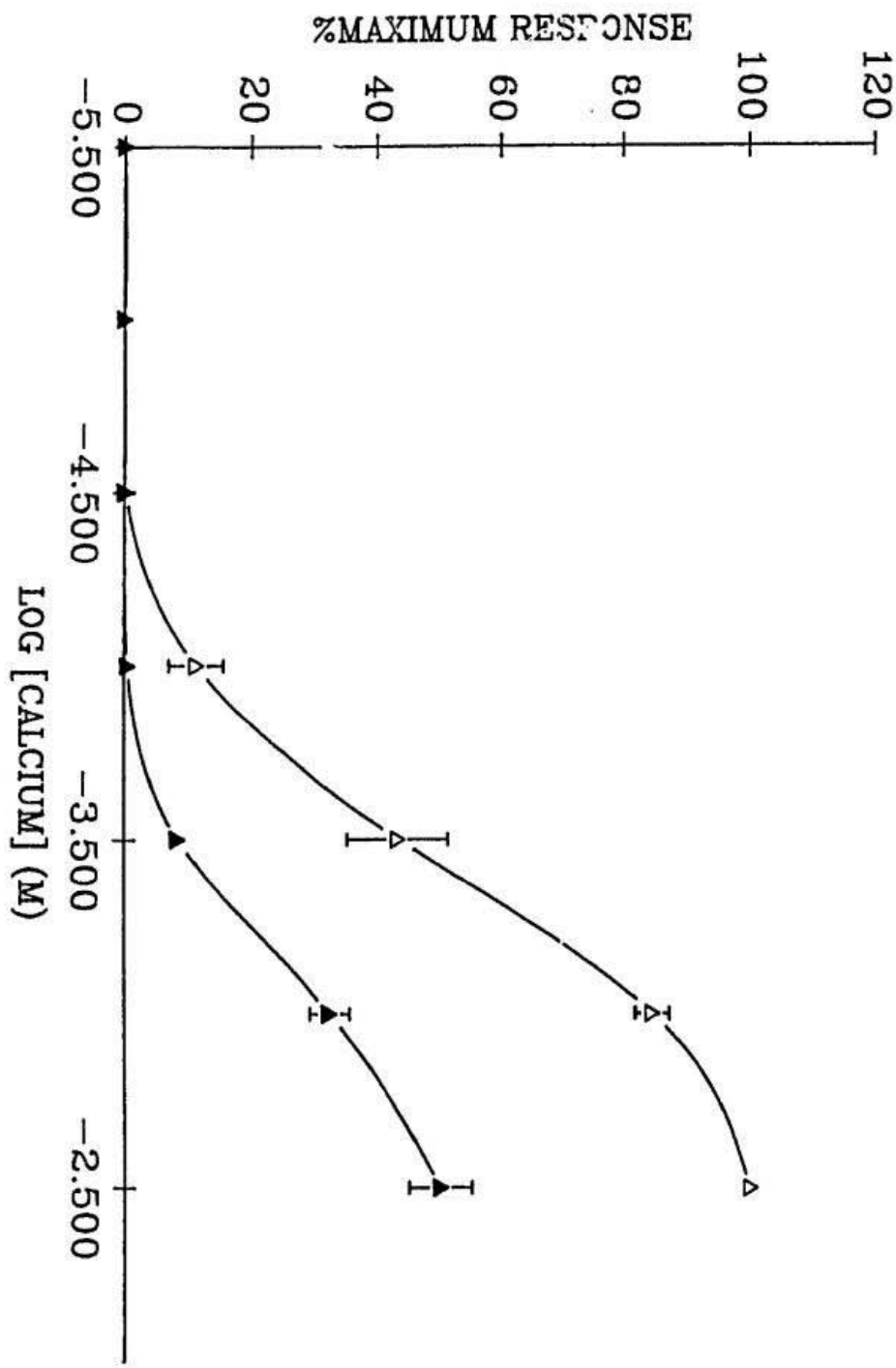
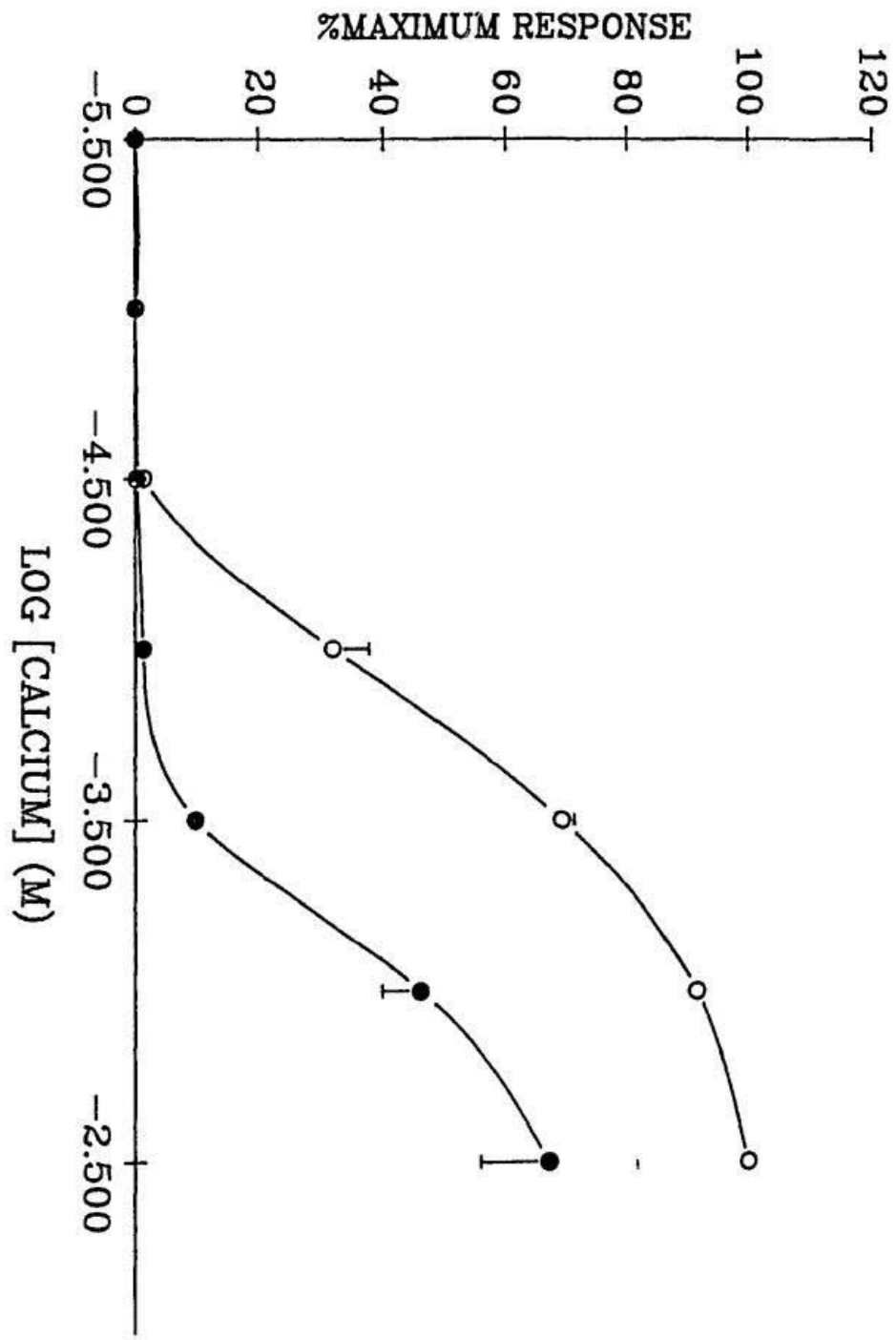

120
100
80
60
40
20
0
%MAXIMUM RESPONSE
−5.500
−4.500
−3.500
−2.500
LOG [CALCIUM] (M)

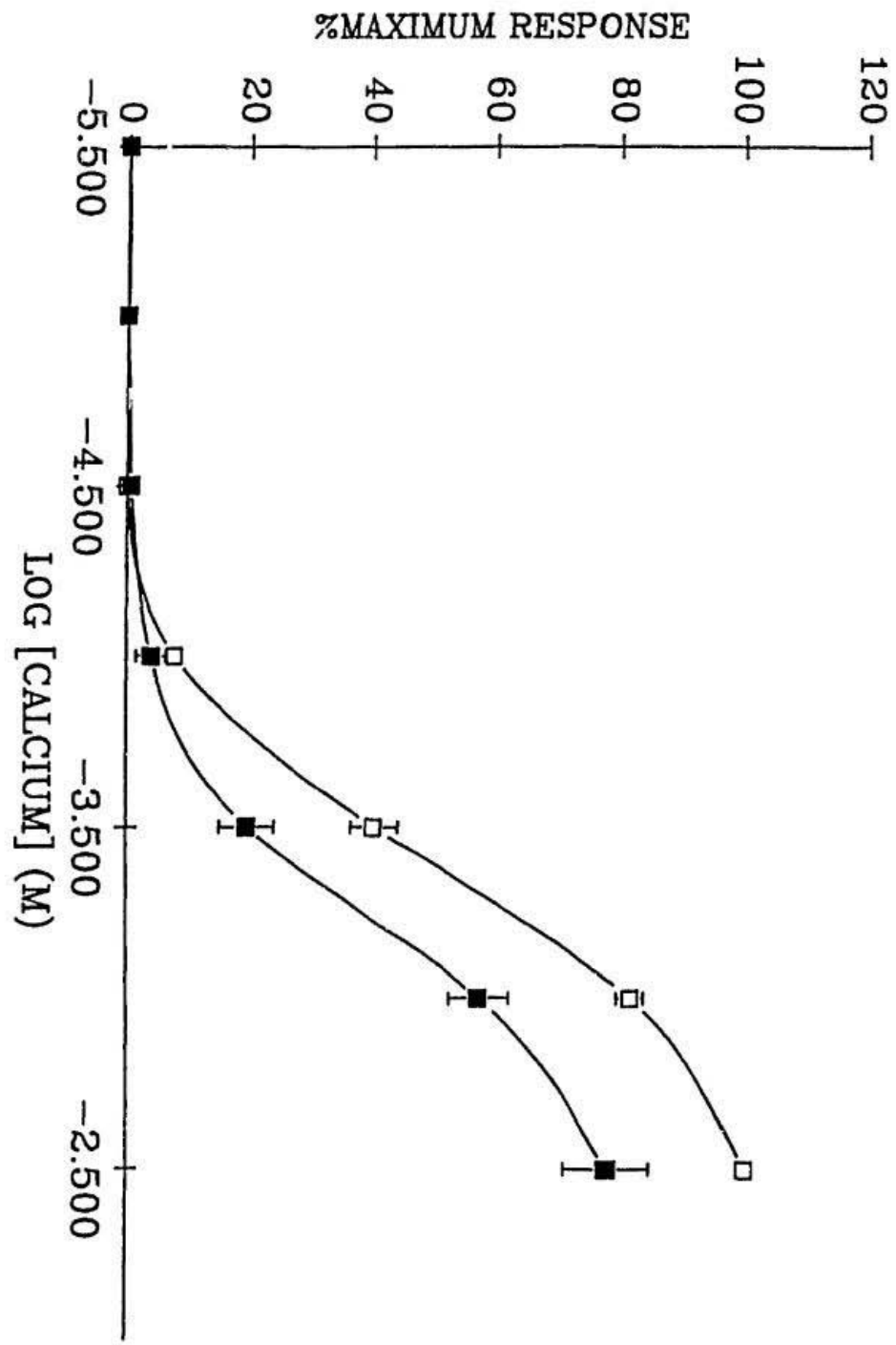
Figure 10a, b and c: Effect of 10 μM nifedipine on Ca^{2+} concentration-response obtained in the anococcygeus in the presence of: a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Open and closed symbols represent control and nifedipine treatment, respectively ± S.E.M.. (n = 4)


%MAXIMUM RESPONSE
0
20
40
60
80
100
120
-5.500
-4.500
-3.500
-2.500
LOG [CALCIUM] (M)

(a)

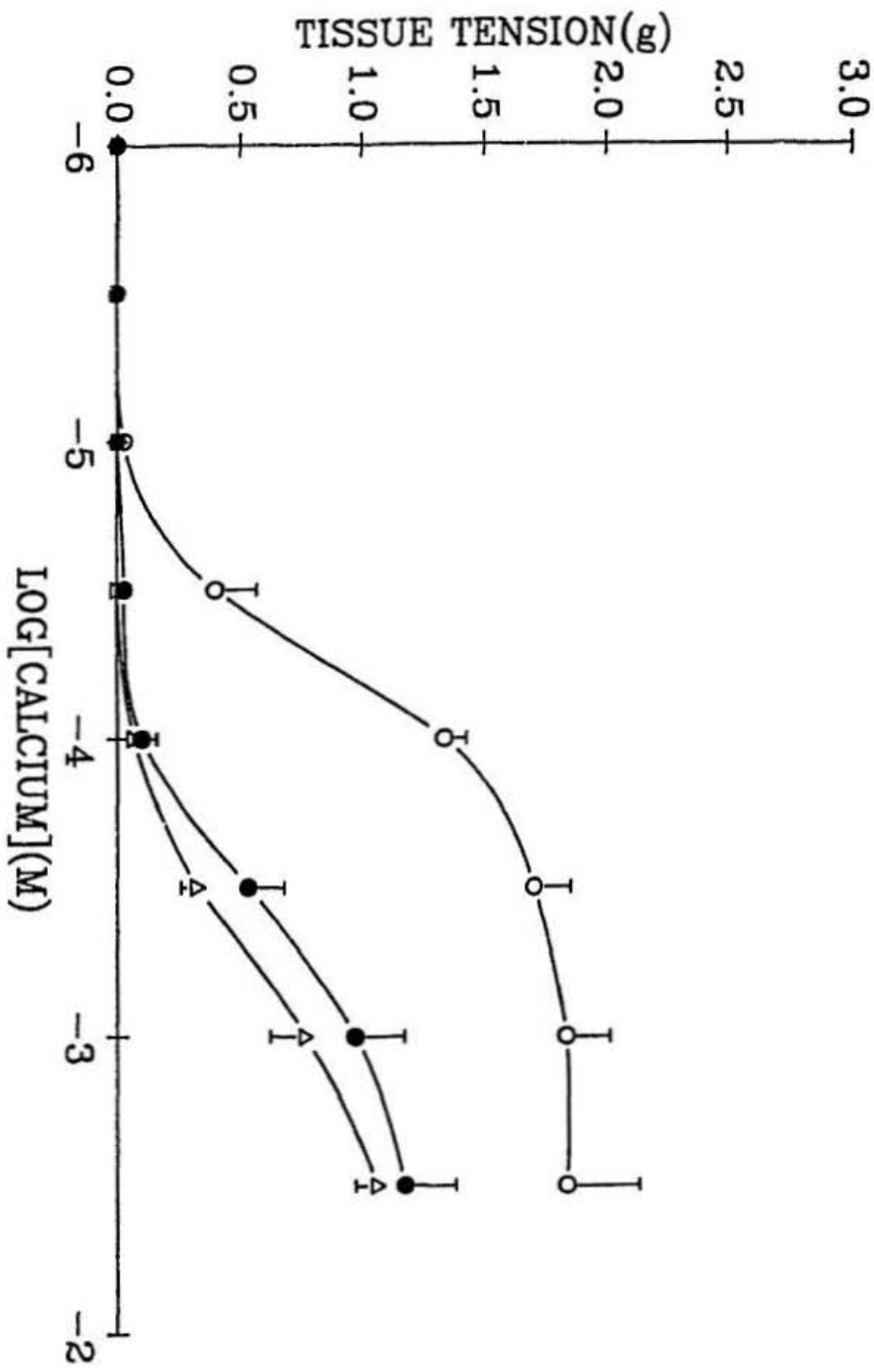

%MAXIMUM RESPONSE
0
20
40
60
80
100
120
-5.500
-4.500
-3.500
-2.500
LOG [CALCIUM] (M)

(b)

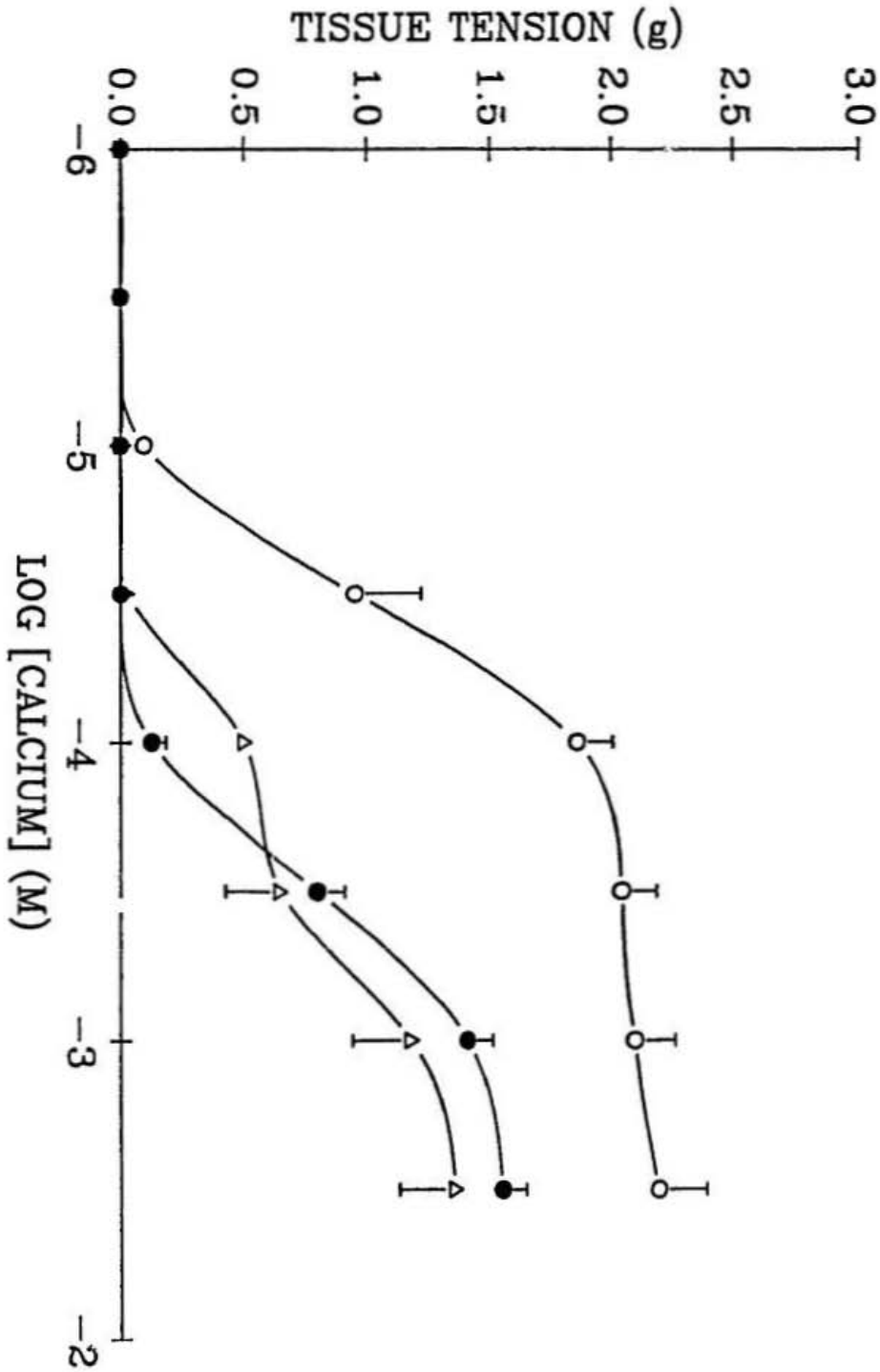

%MAXIMUM RESPONSE
0
20
40
60
80
100
120
-5.500
-4.500
-3.500
-2.500
LOG [CALCIUM] (M)

(c)

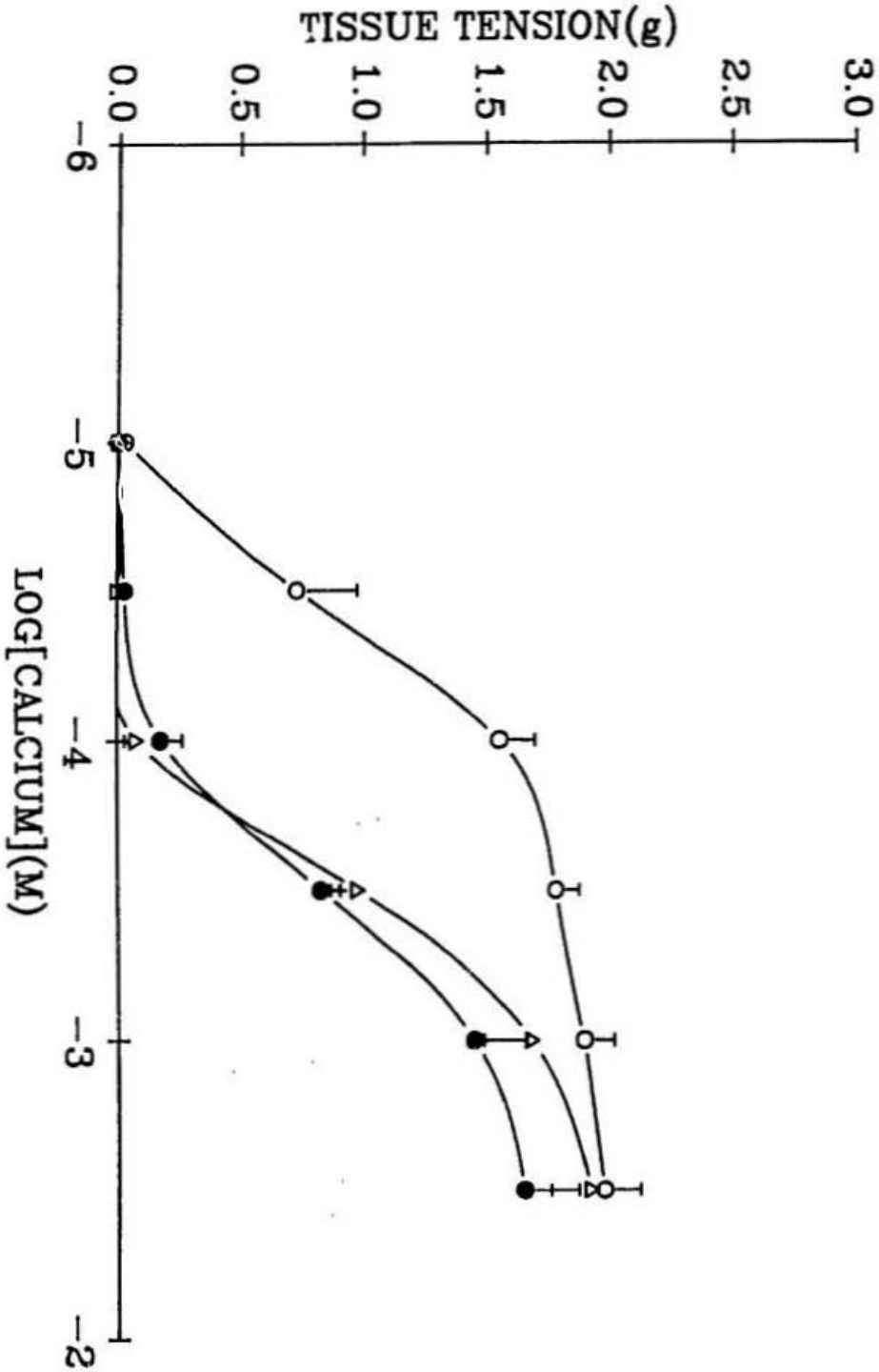
Figure 11a, b and c: Illustration of the persistent effect of 10 μM nifedipine on Ca^{2+} concentration-response obtained in the aorta in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O-O, and in the presence of nifedipine by filled symbols, e.g. ●-● and post-nifedipine by Δ-Δ. (n = 4)



(a)

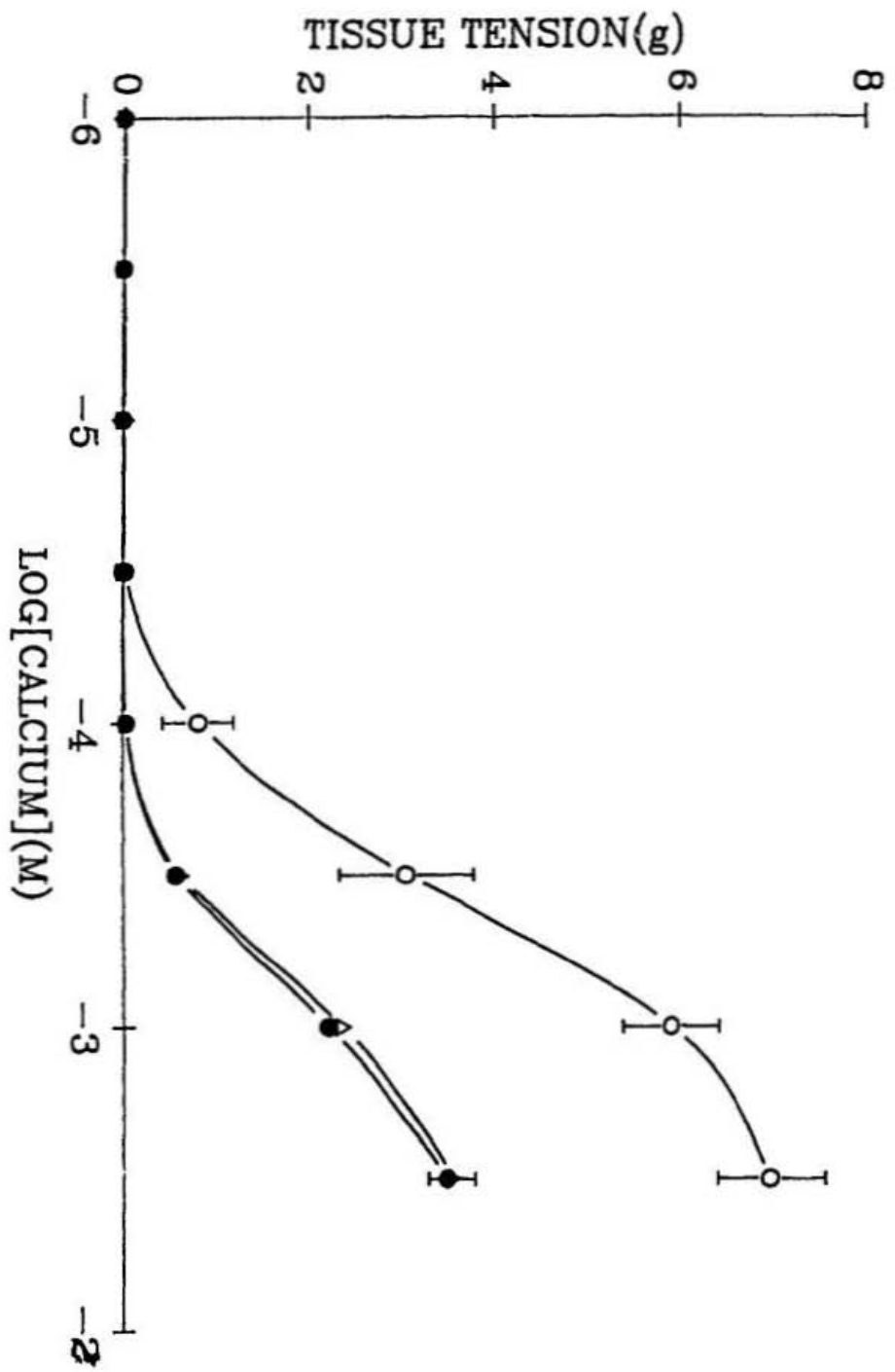


(b)

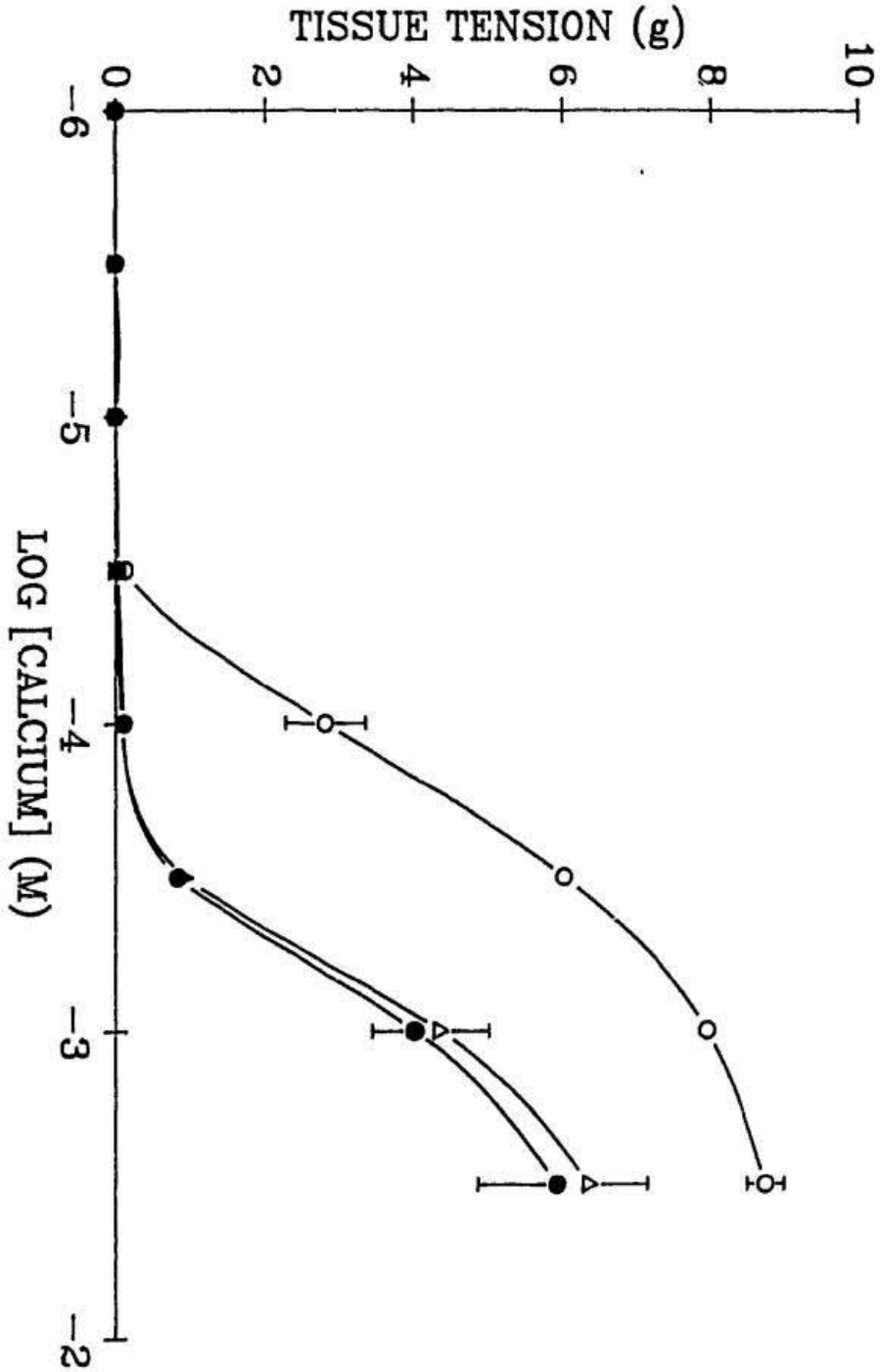


(c)

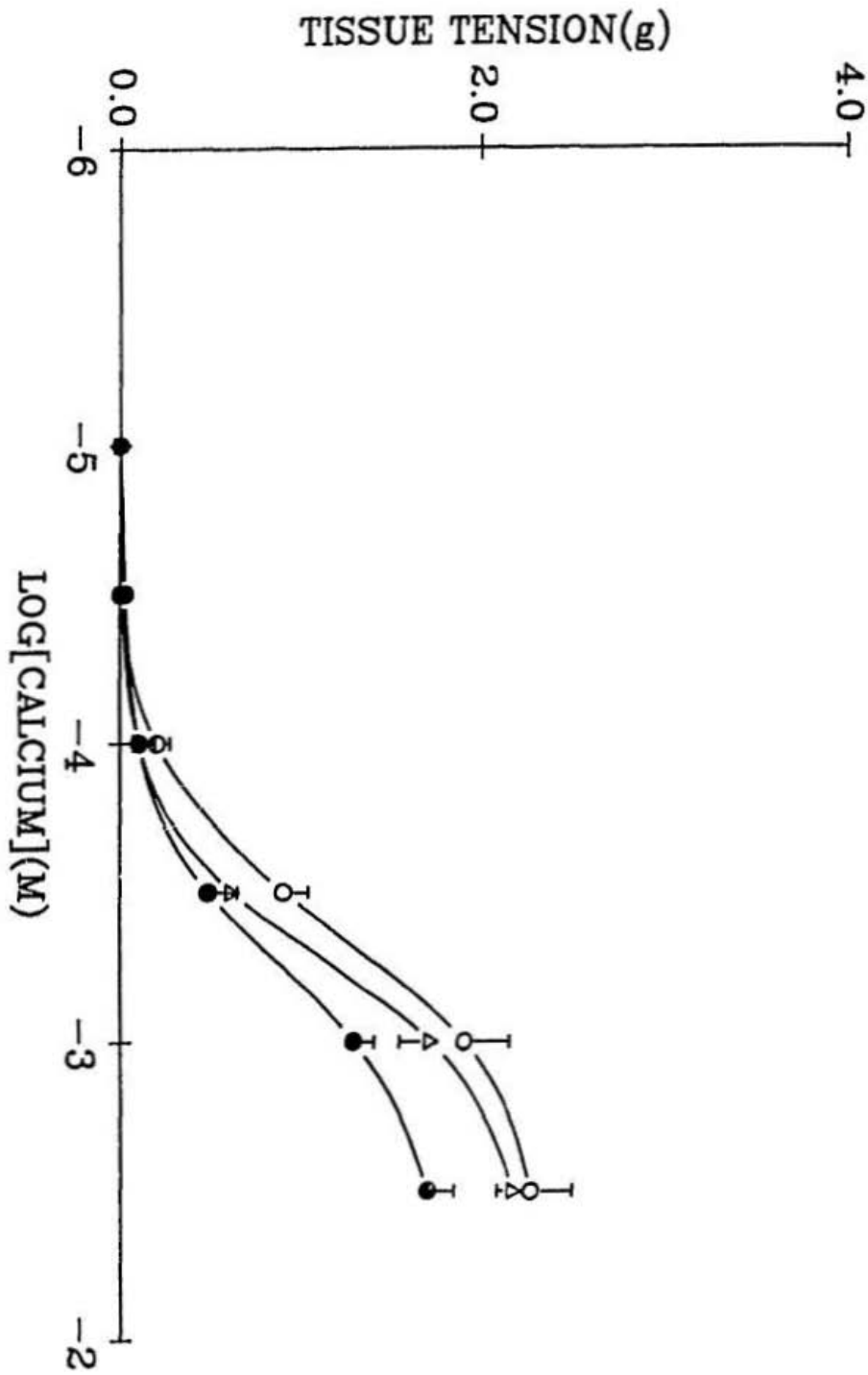
Figure 12a, b and c: Illustration of the persistent inhibition following 10 μM nifedipine application on the Ca^{2+} concentration-response curve obtained in the anococcygeus in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O-O, and in the presence of nifedipine by filled symbols, e.g. ●-● and post-nifedipine by Δ-Δ. (n = 4)



(a)



(b)



(c)

Figure 13: A comparison of the contractile effects induced by 10 nM ET-1, SRTX-b and 1 μM NE in the rat anococcygeus preparation in normal Krebs  and Ca^{2+}-free Krebs  . The Krebs solution was switched from normal to Ca^{2+}-free Krebs just before agonist addition. The effects of ryanodine  were studied by incubating tissues in Ca^{2+}-free Krebs and 10 μM ryanodine for one hour followed by re-introduction of normal Krebs and agonist. Values represent means ± (S.E.M.) n = 6. Statistical differences between means were assessed by one-way ANOVA.

* Denote significant differences ($p < .05$)

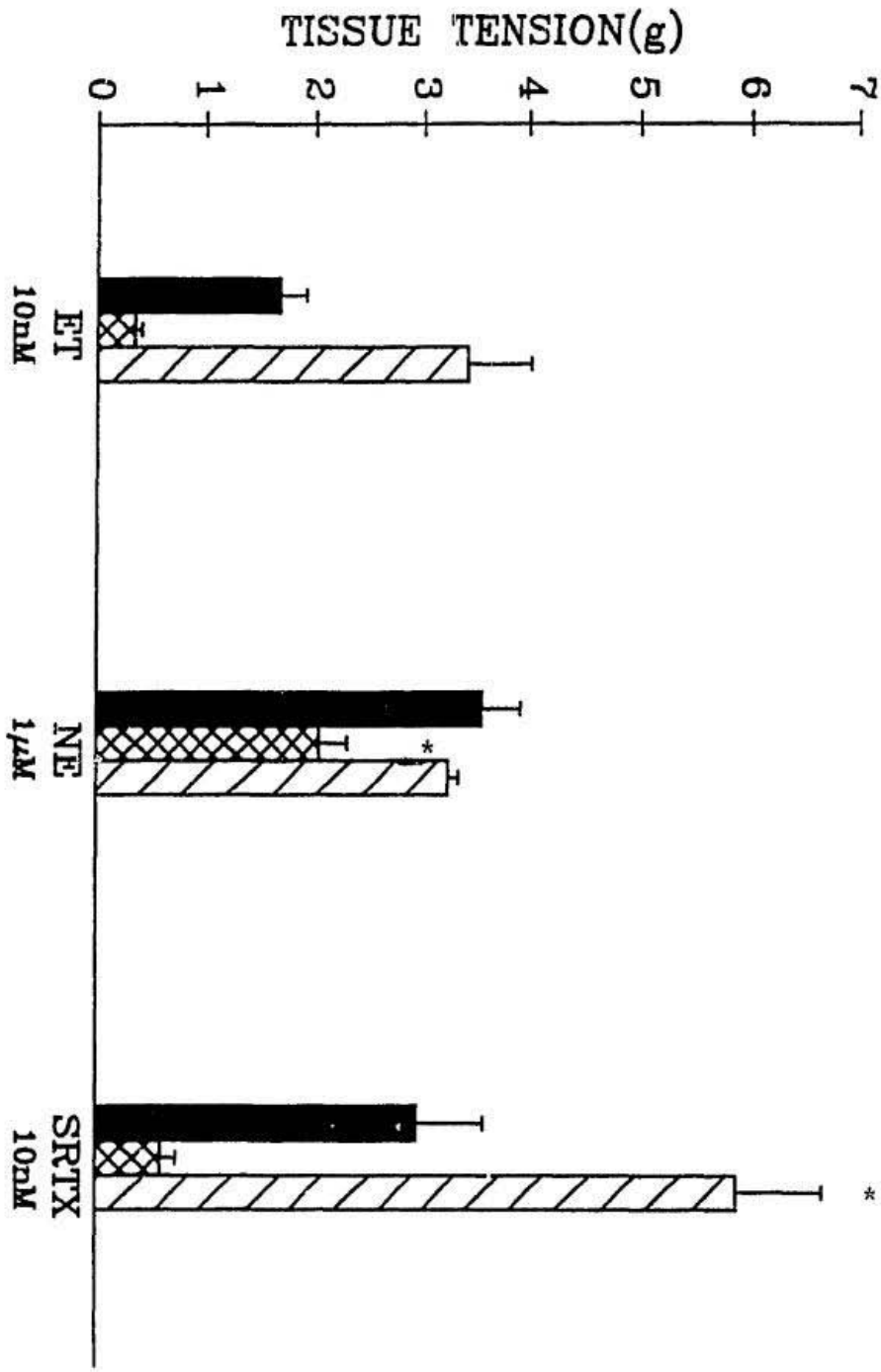

TISSUE TENSION(g)
0
1
2
3
4
5
6
7
10nM
ET
1μM
NE
10nM
SRTX
*
*

Figure 14: A comparison of the contractile effects induced by 10 nM ET-1, SRTX-b and 1 μM NE in the rat aortic ring preparation in normal Krebs  , Ca^{2+}-free Krebs  . The Krebs solution was switched from normal to Ca^{2+}-free just before agonist addition. The effects of ryanodine  were studied by incubating tissues in Ca^{2+}-free Krebs + 10 μM ryanodine for one hour followed by re-introduction of normal Krebs and agonist. Values represent means ± (S.E.M.). (n = 6)

Statistical differences between means were assessed by one-way ANOVA.

* Denote significant differences ($p < .05$)

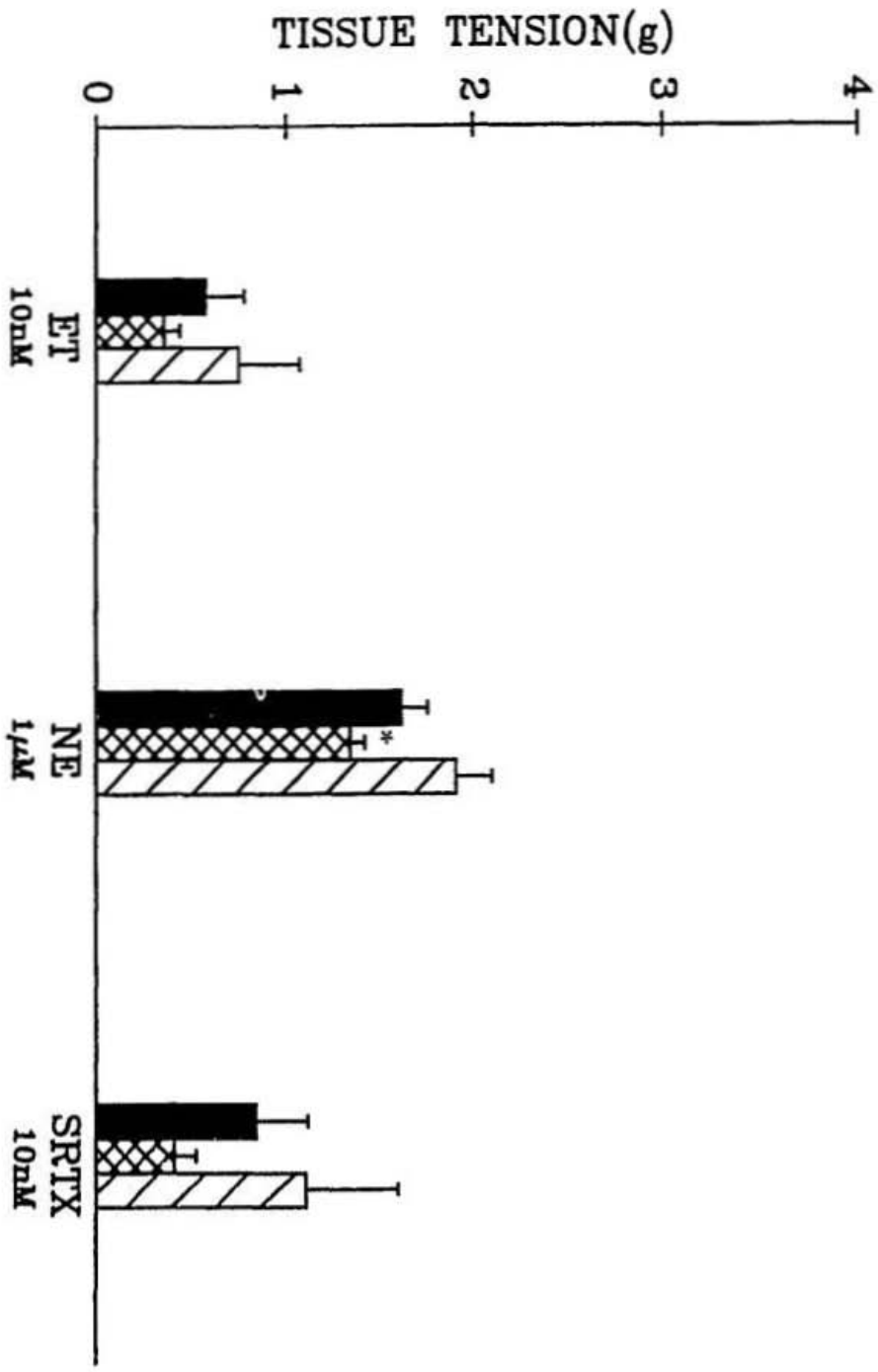
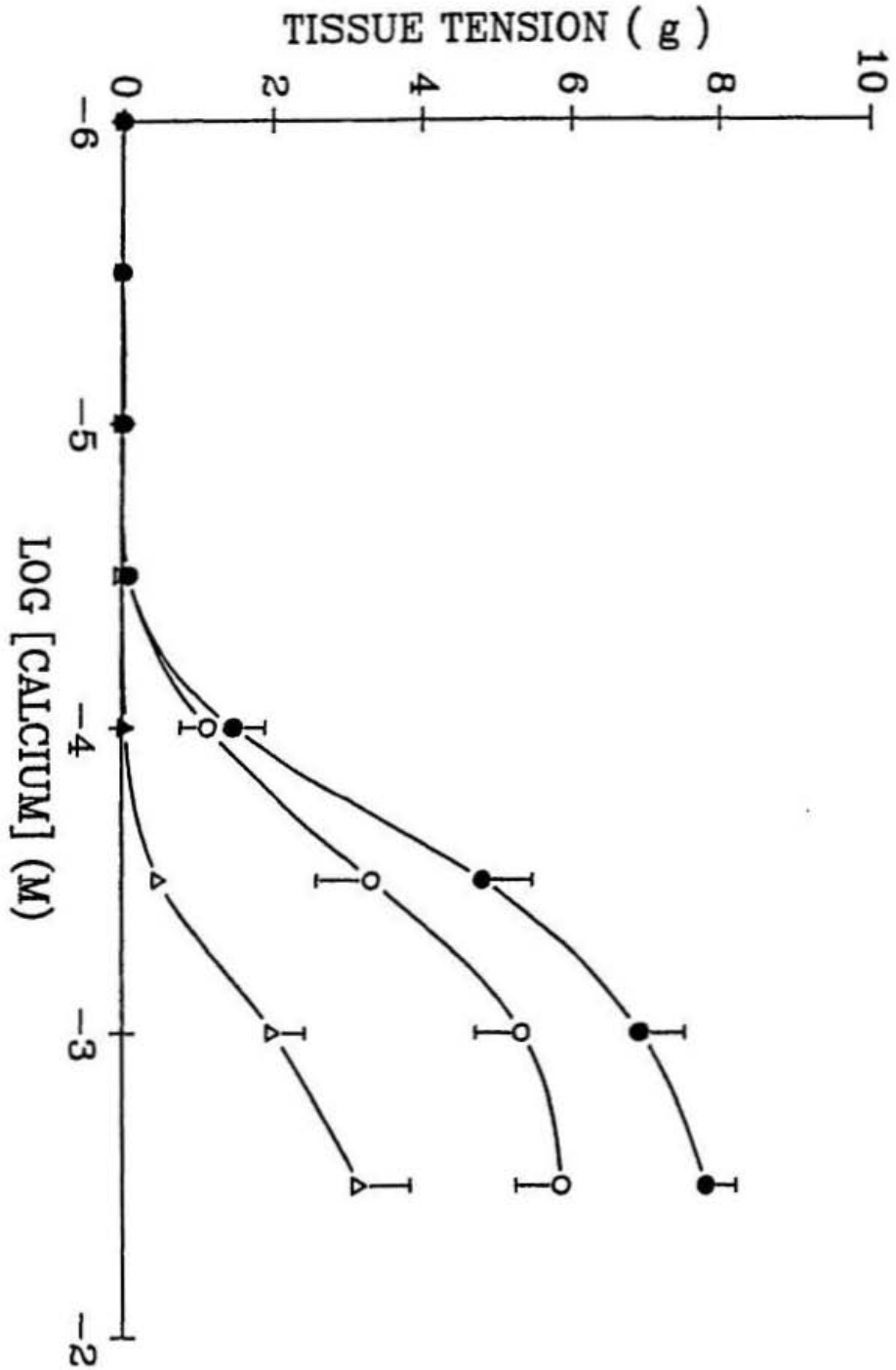
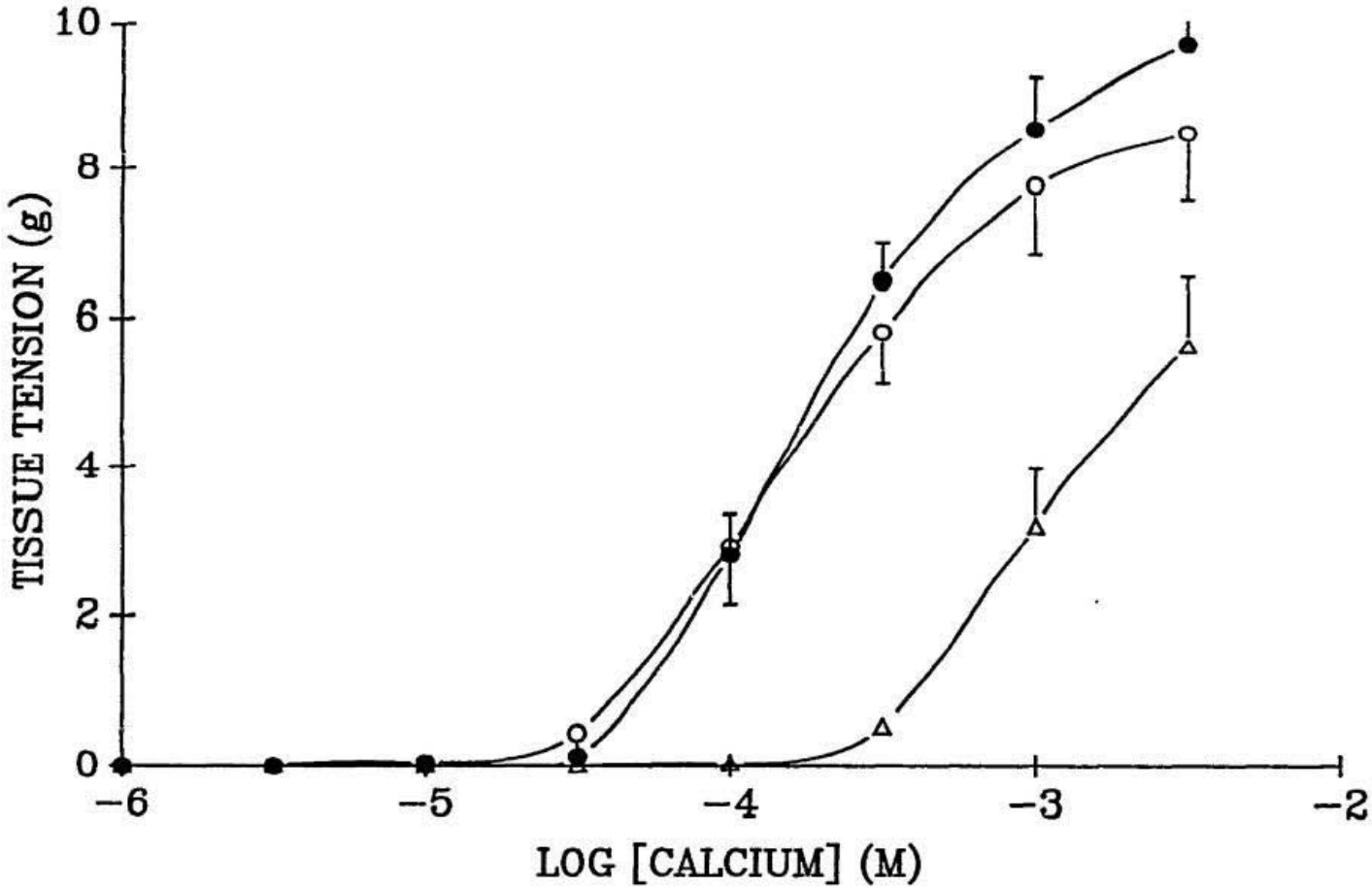

TISSUE TENSION(g)
0
1
2
3
4
10nM ET
1μM NE
10nM SRTX
*

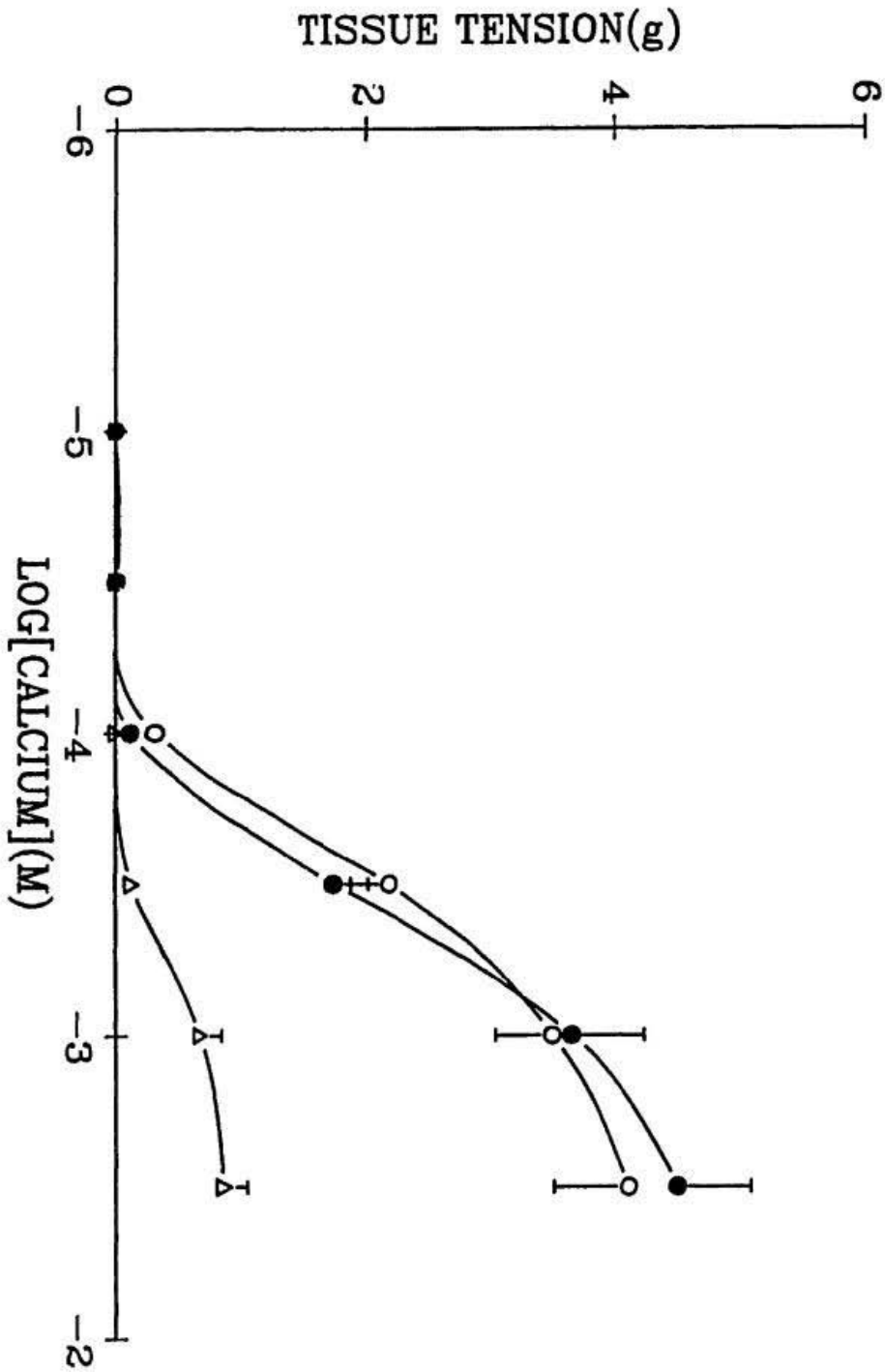
Figure 15a, b and c: Effect of 10 μM ryanodine on Ca^{2+} concentration-response curves obtained in the anococcygeus in the presence of a) 10 nM ET-1; b) 10 nM SRTX-1; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O-O, and in the presence of ryanodine by filled symbols, e.g. ●-●, and, in the presence of 10 μM ryanodine + nifedipine by Δ-Δ. (n = 4)



(a)

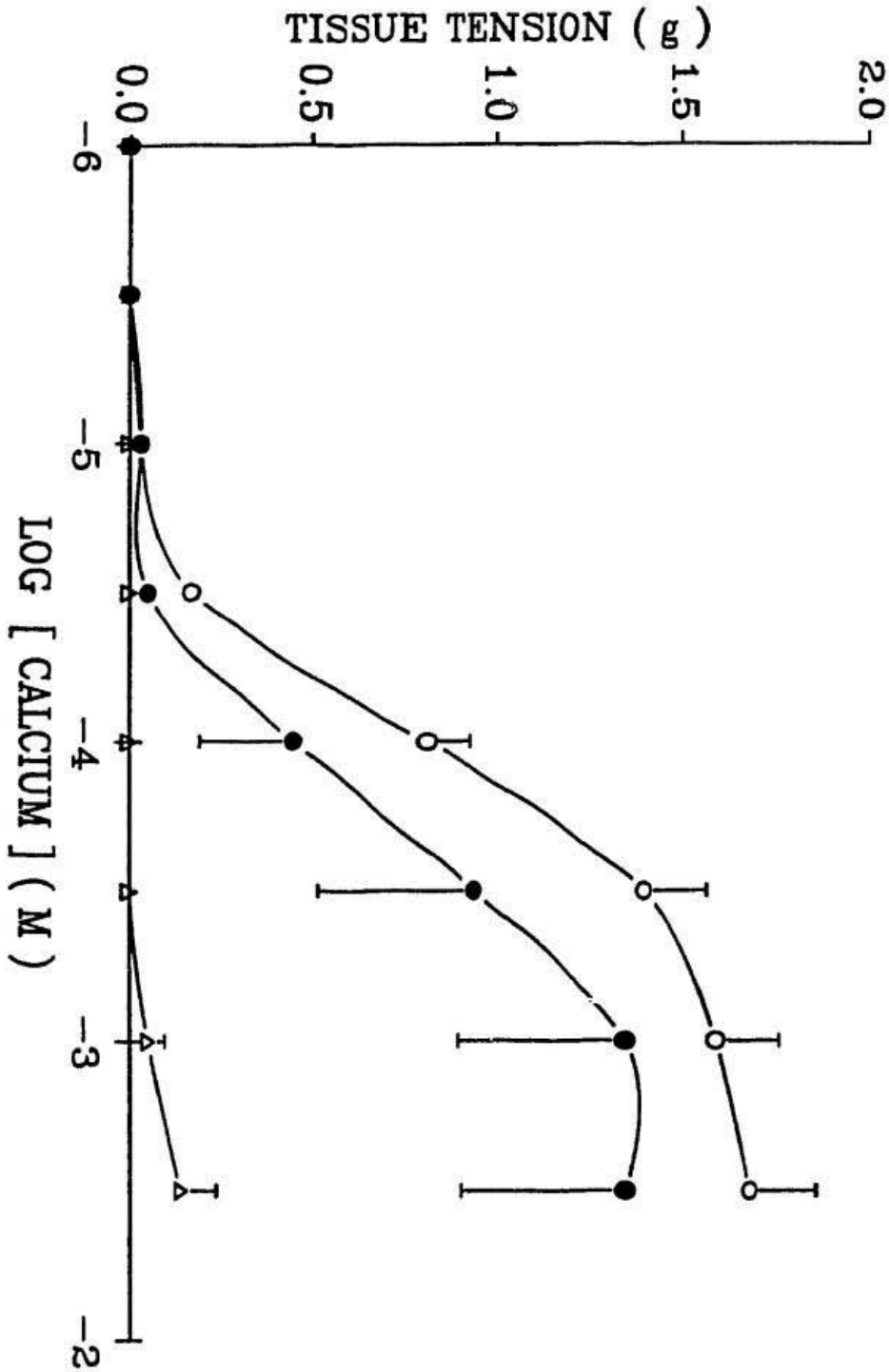
(b)



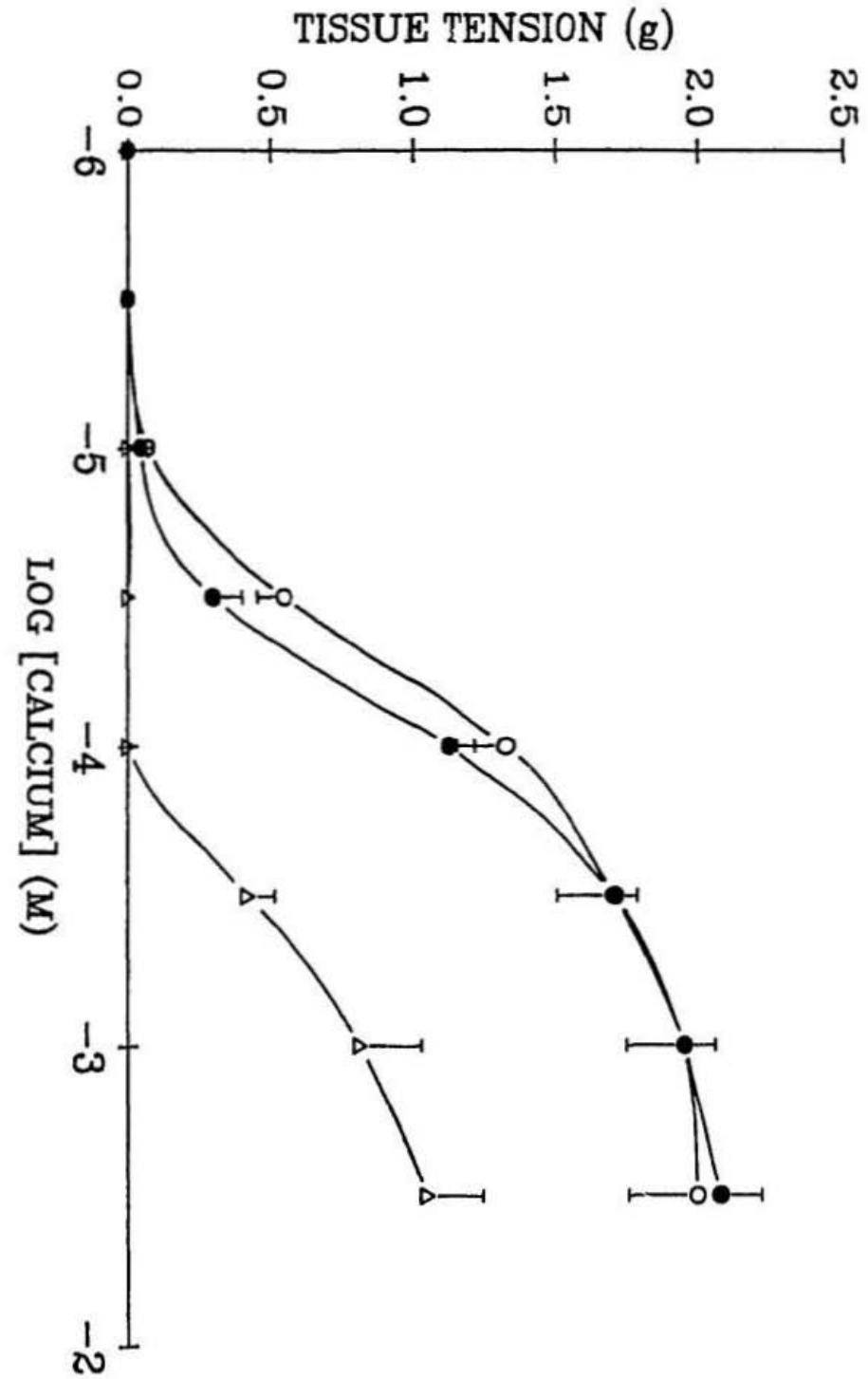


(c)

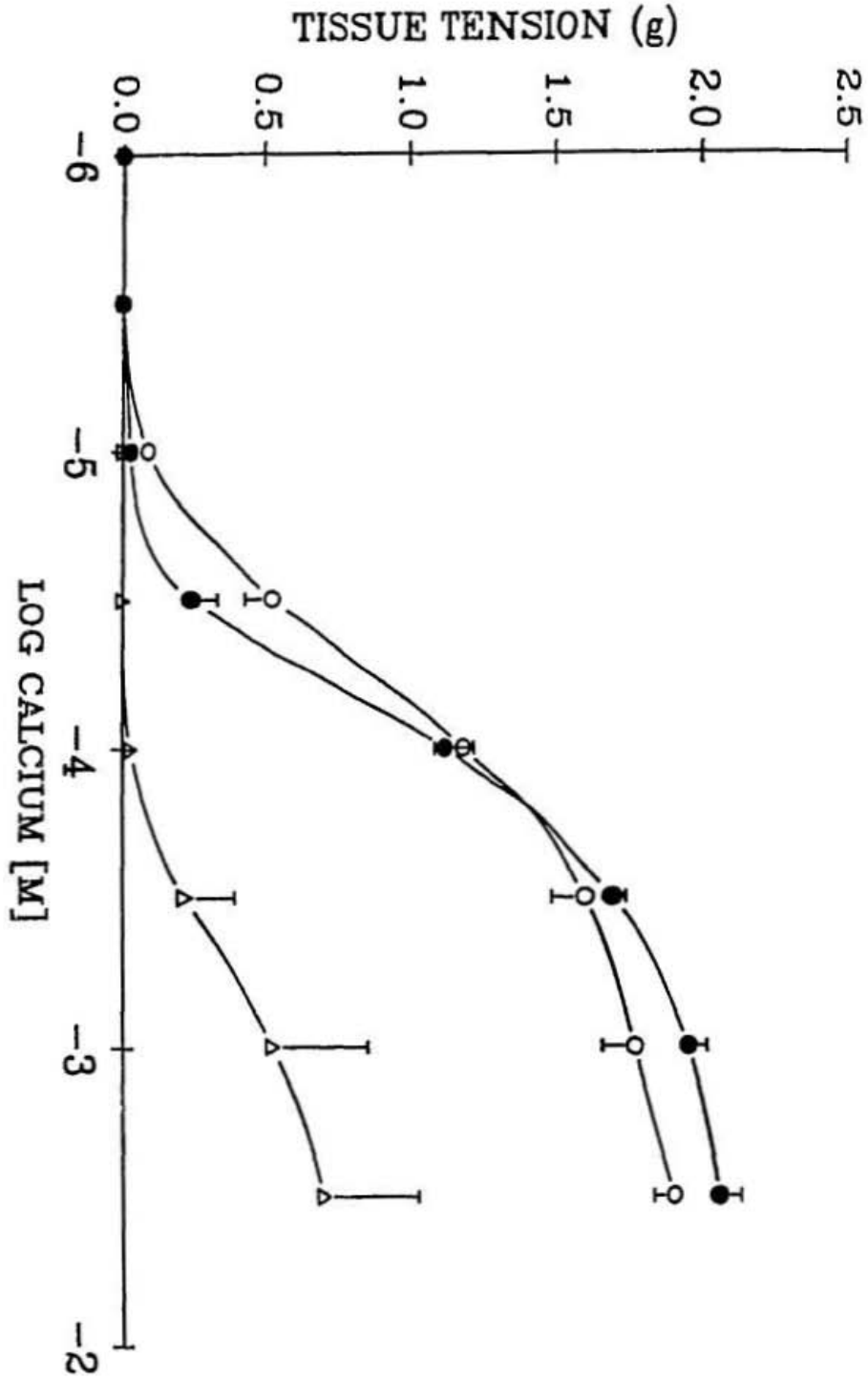
Figure 16a, b and c: Effect of 10 μM ryanodine on Ca^{2+} concentration-response obtained in aorta in the presence of a) 10 nM ET-1; b) 10 nM SRTX-1; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O-O, and, in the presence of ryanodine, by filled symbols, e.g. ●-●, and, in the presence of 10 μM ryandoine + nifedipine by Δ-Δ. (n = 4)



(a)



(b)



(c)

Figure 17 (a and b): Illustration in : a) anococcygeus and b) aorta that repeated stimulation with NE, in a Ca^{2+}-free Krebs buffer can reduce the tissue response to a certain basal level beyond which further attenuation is not possible.

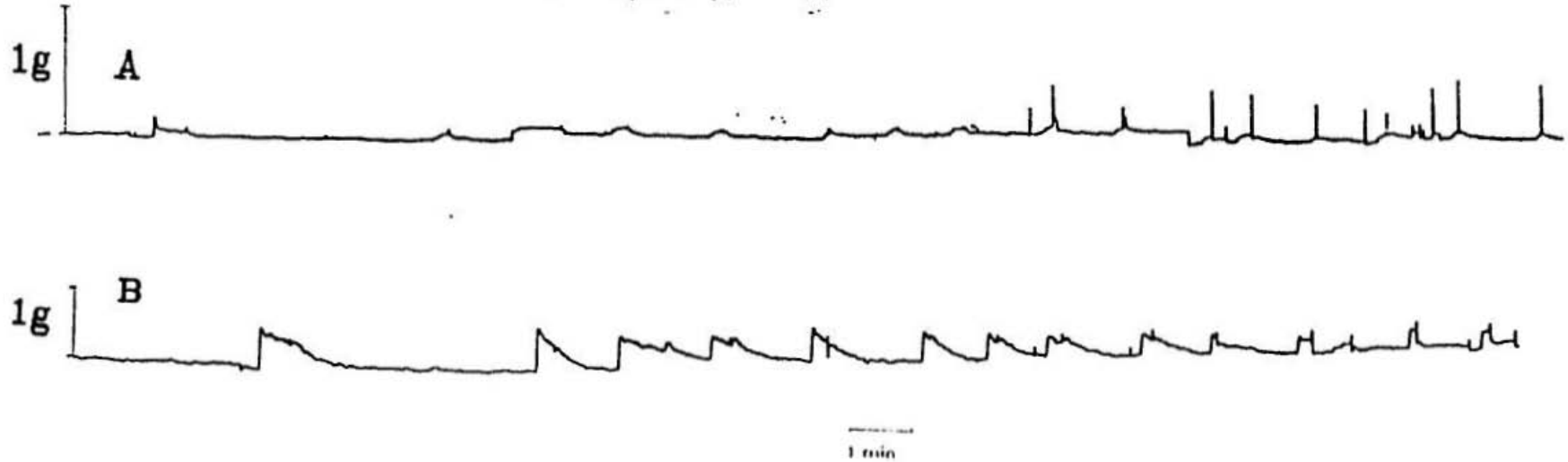
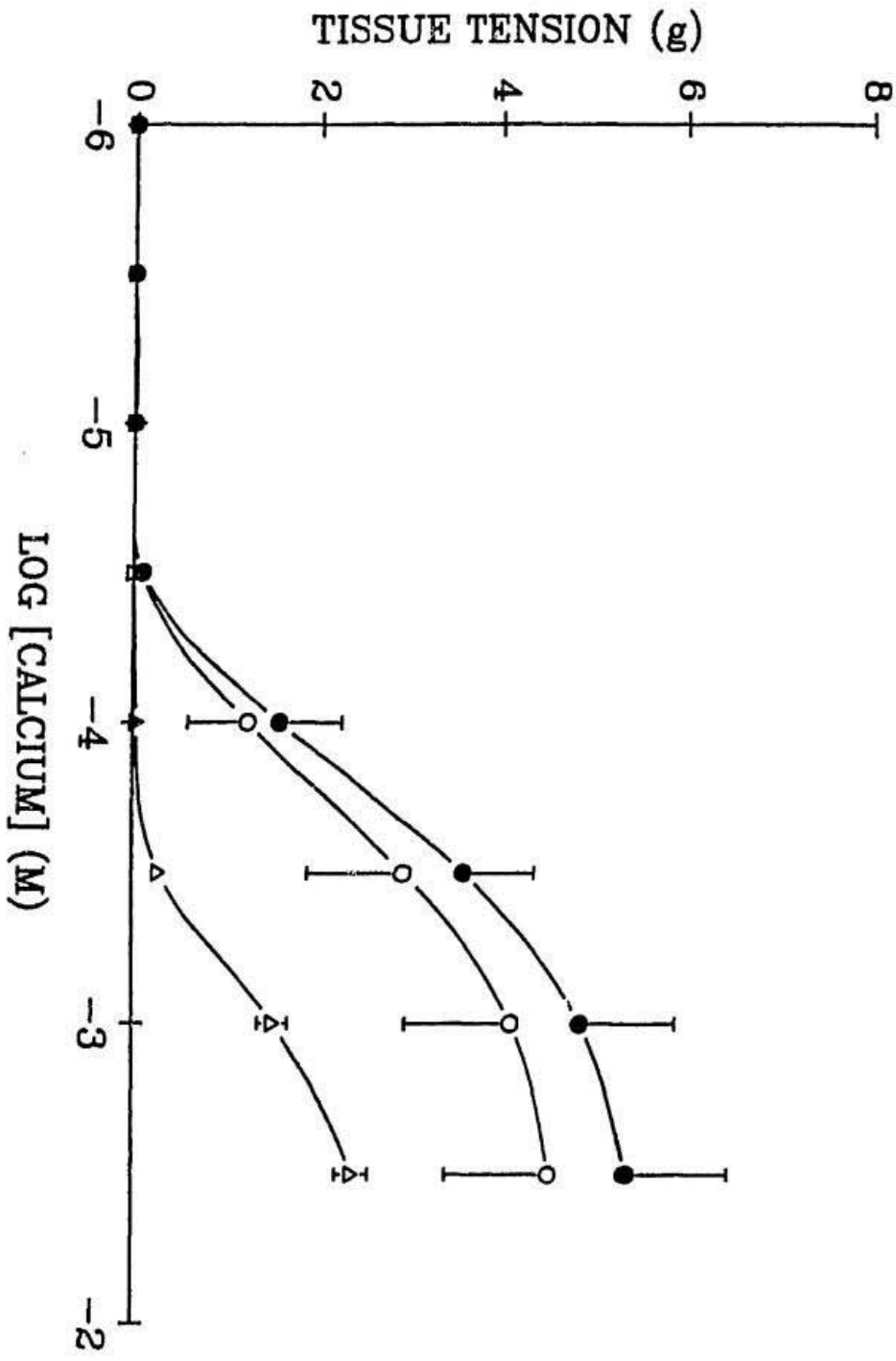
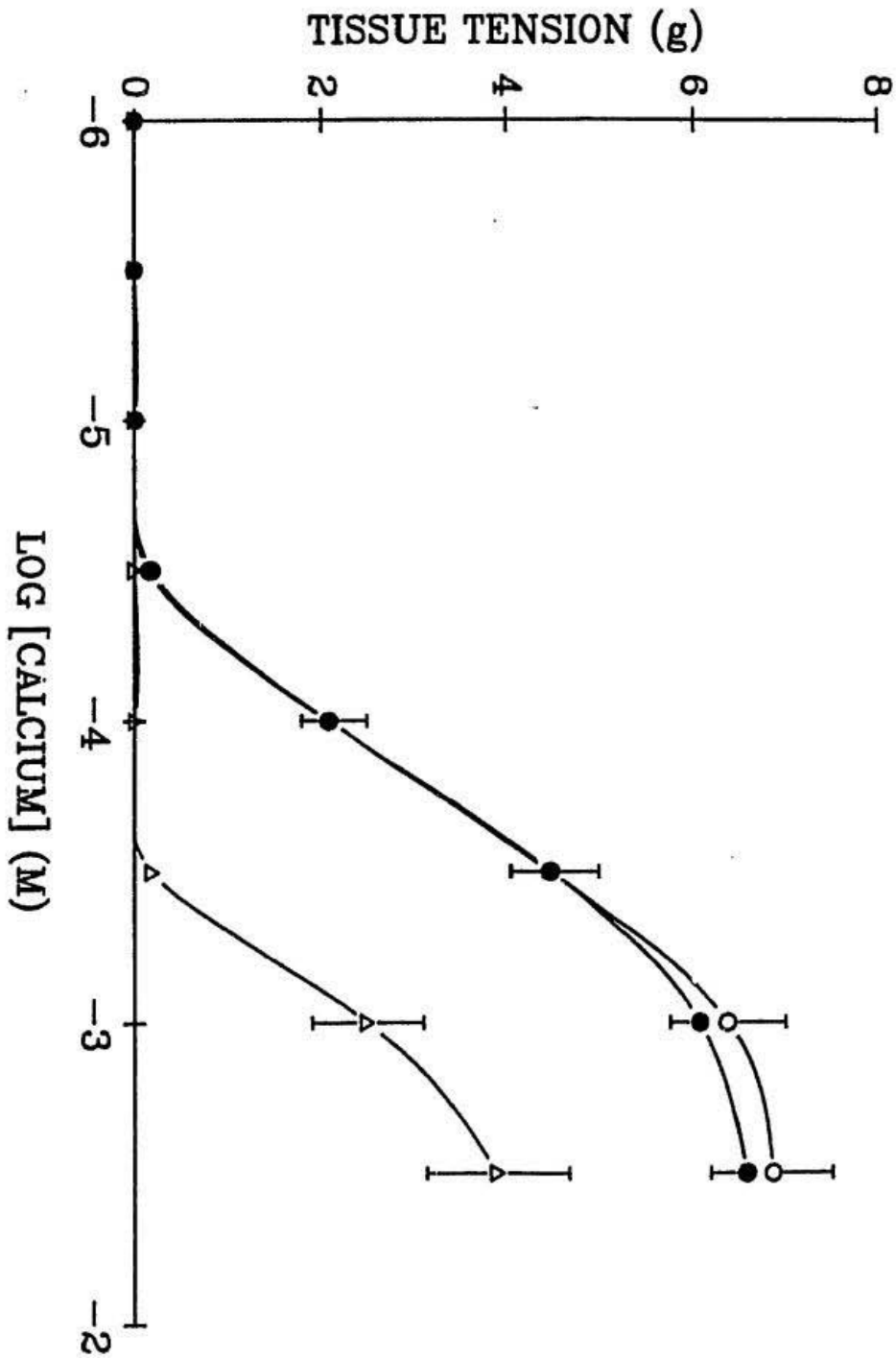

1g
A
1g
B
1 min

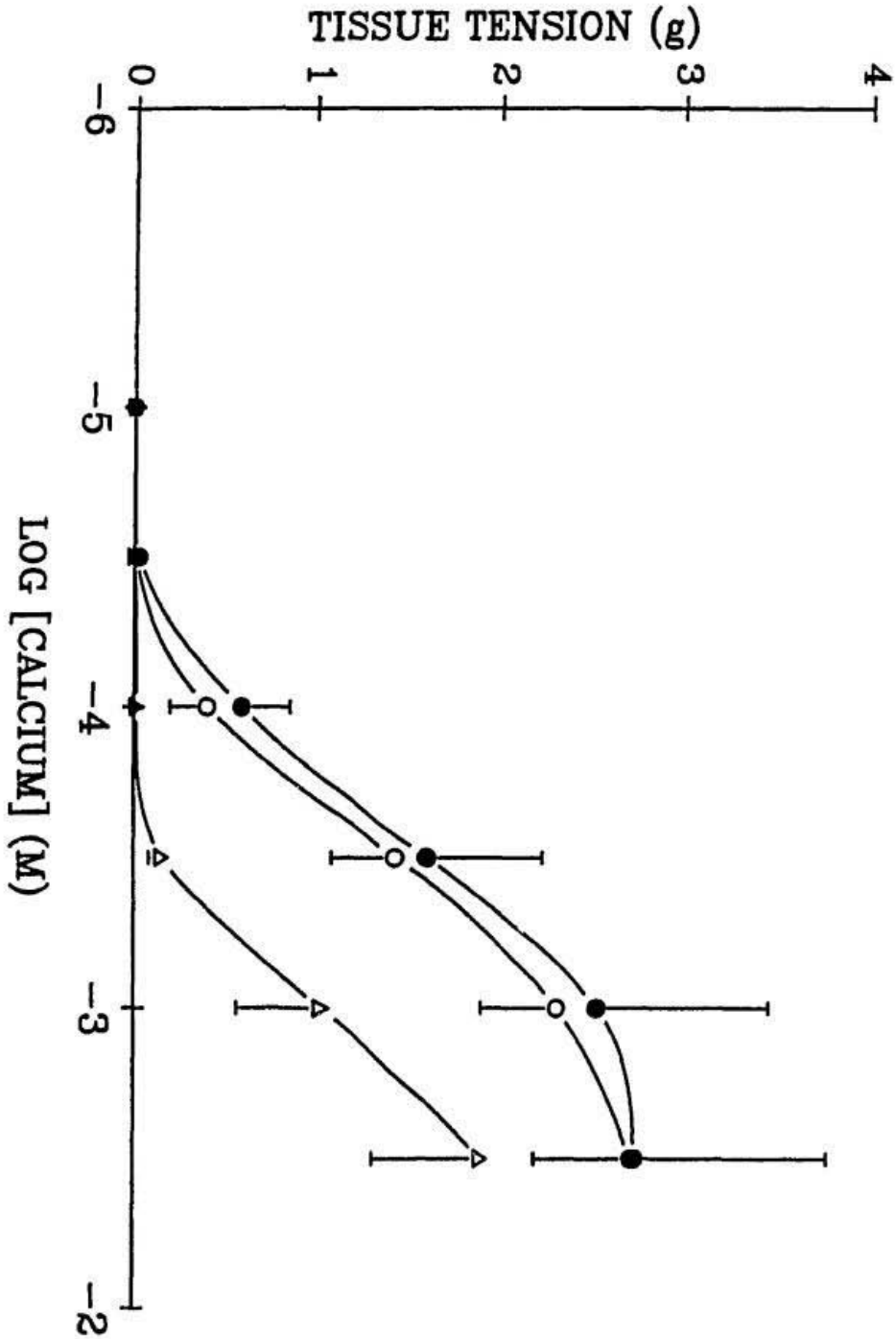
Figure 18a, b and c: Effect of NE-induced Ca^{2+} depletion on calcium concentration-response obtained in anococcygeus in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O-O, and after NE depletion by filled symbols, e.g. ●-● and, in the presence of 10 μM nifedipine, response is by Δ-Δ. (n = 4)



(a)

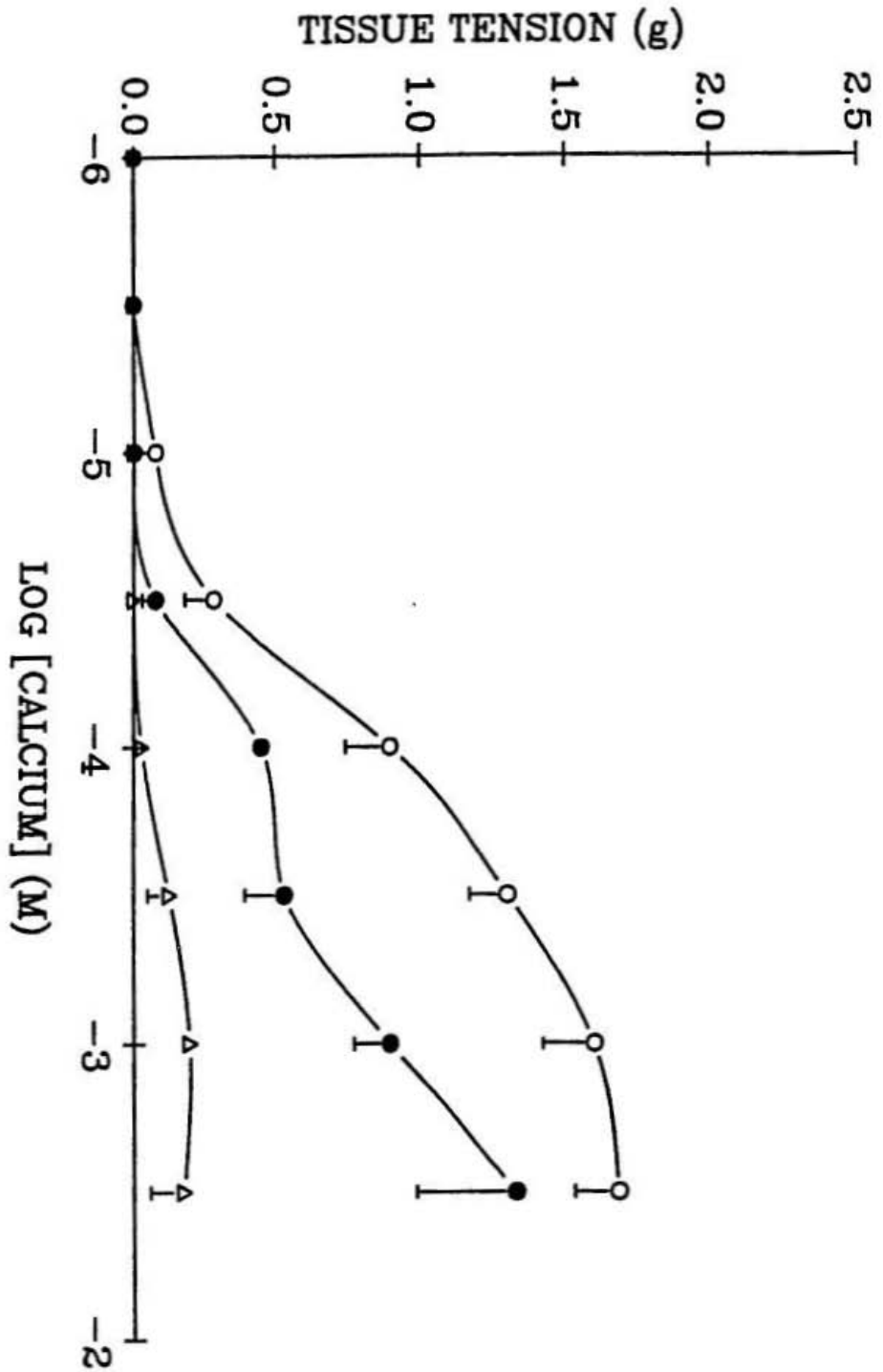


(b)

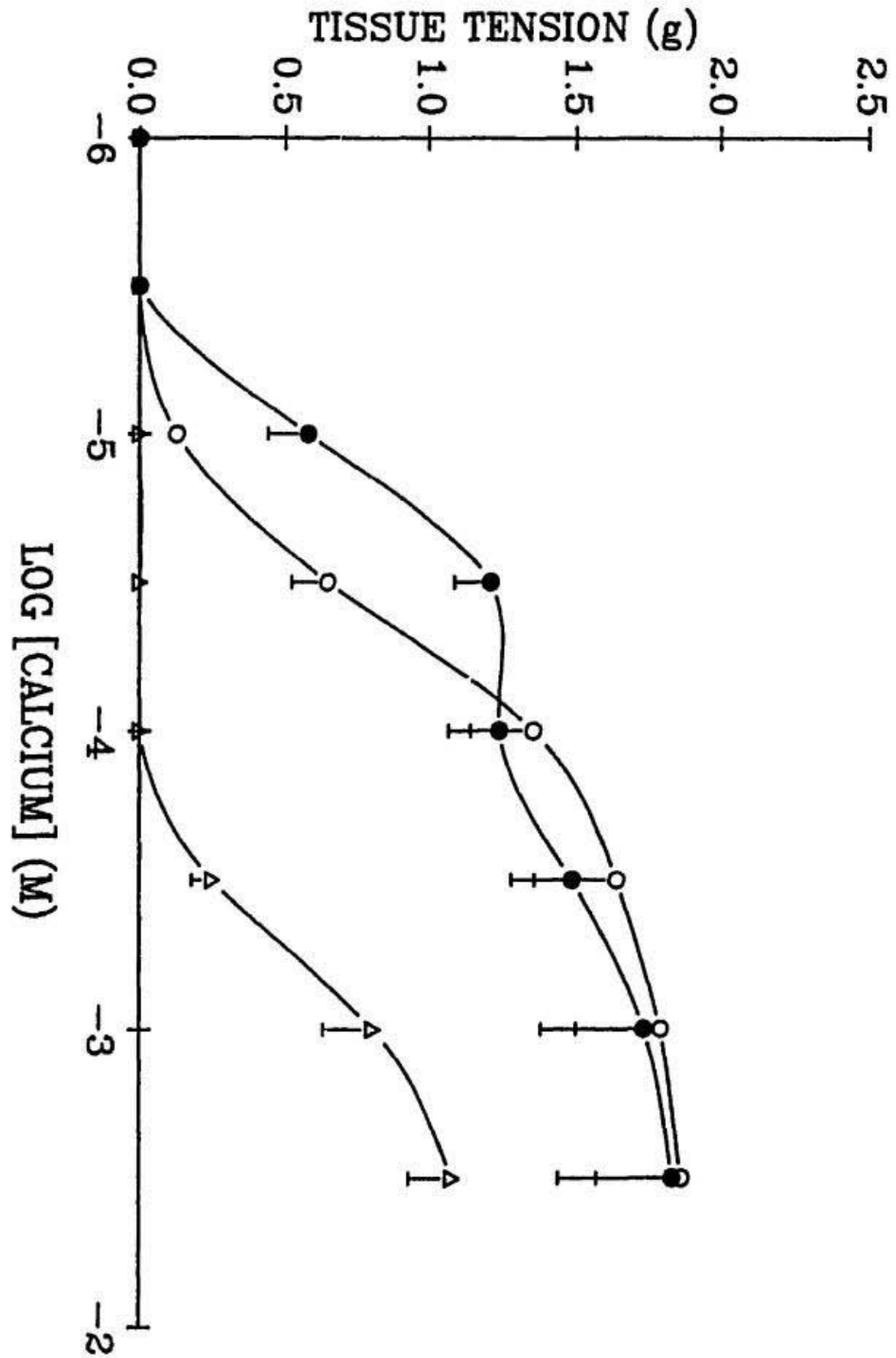


(c)

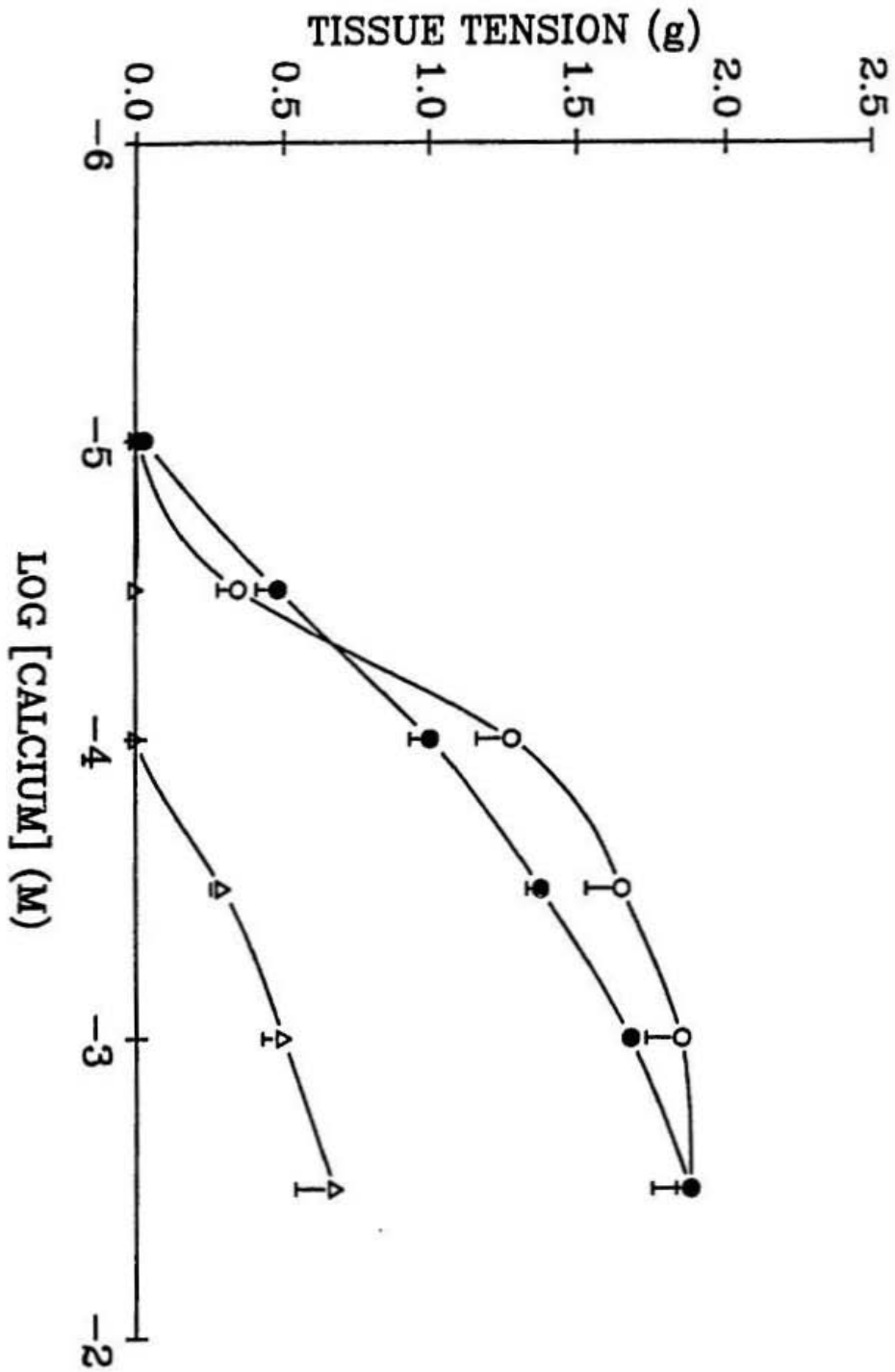
Figure 19a, b and c: Effect of NE-induced Ca^{2+} depletion on Ca^{2+} concentration-response curves obtained in the aorta in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O–O and after NE depletion by filled symbols, e.g. ●–● and, in the presence of 10 μM nifedipine, by Δ-Δ. (n = 4)



(a)

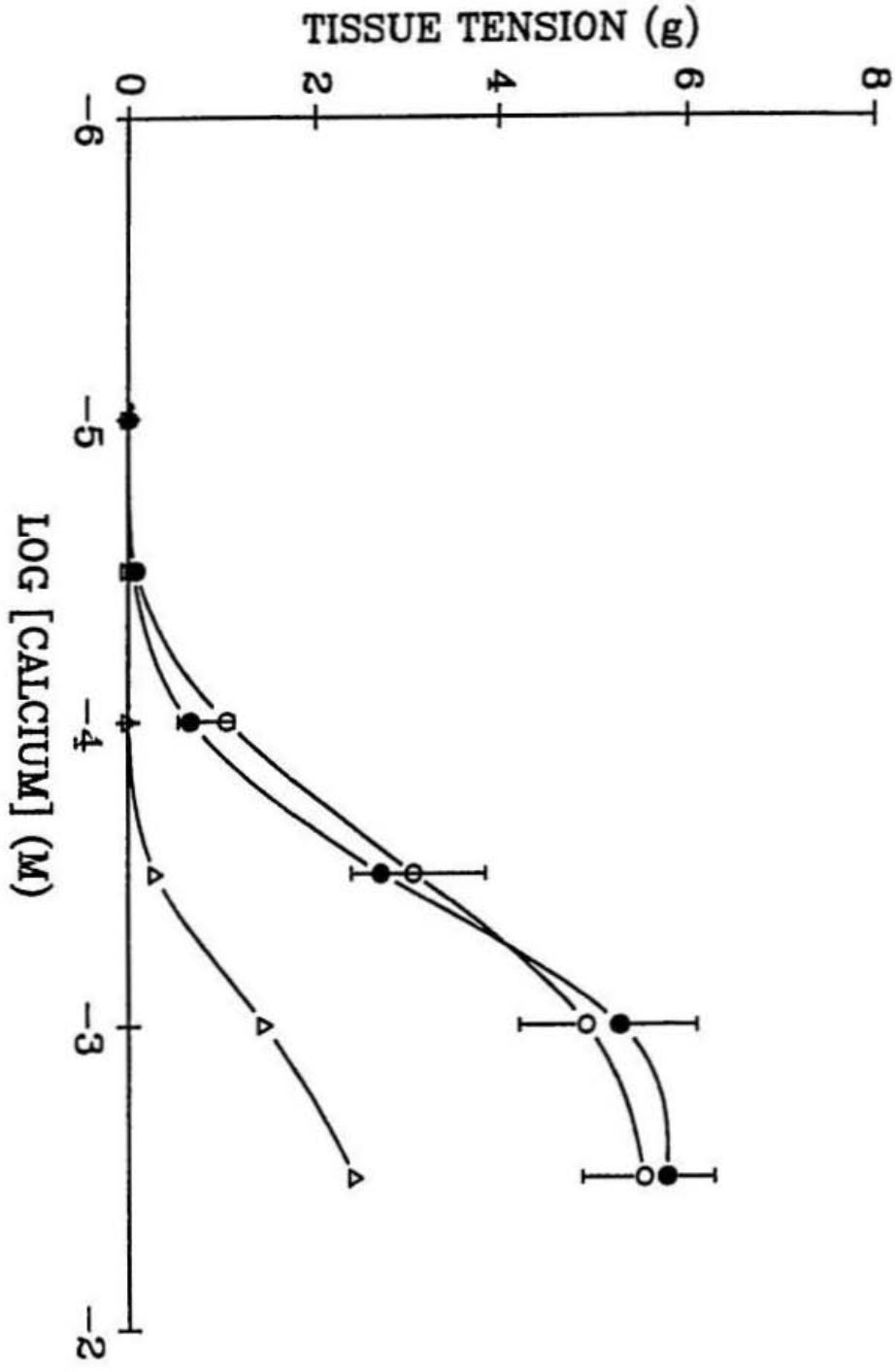


(b)

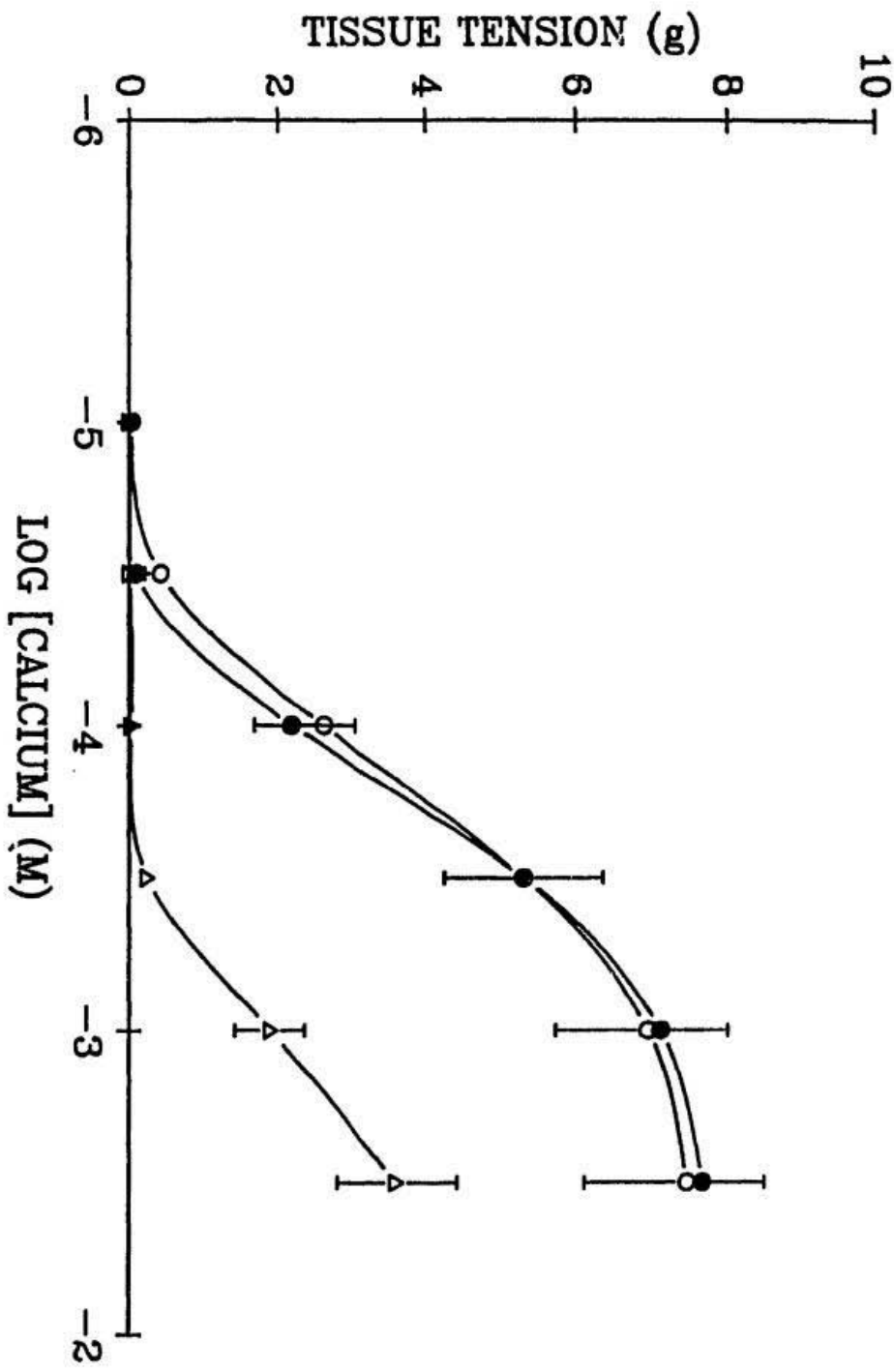


(c)

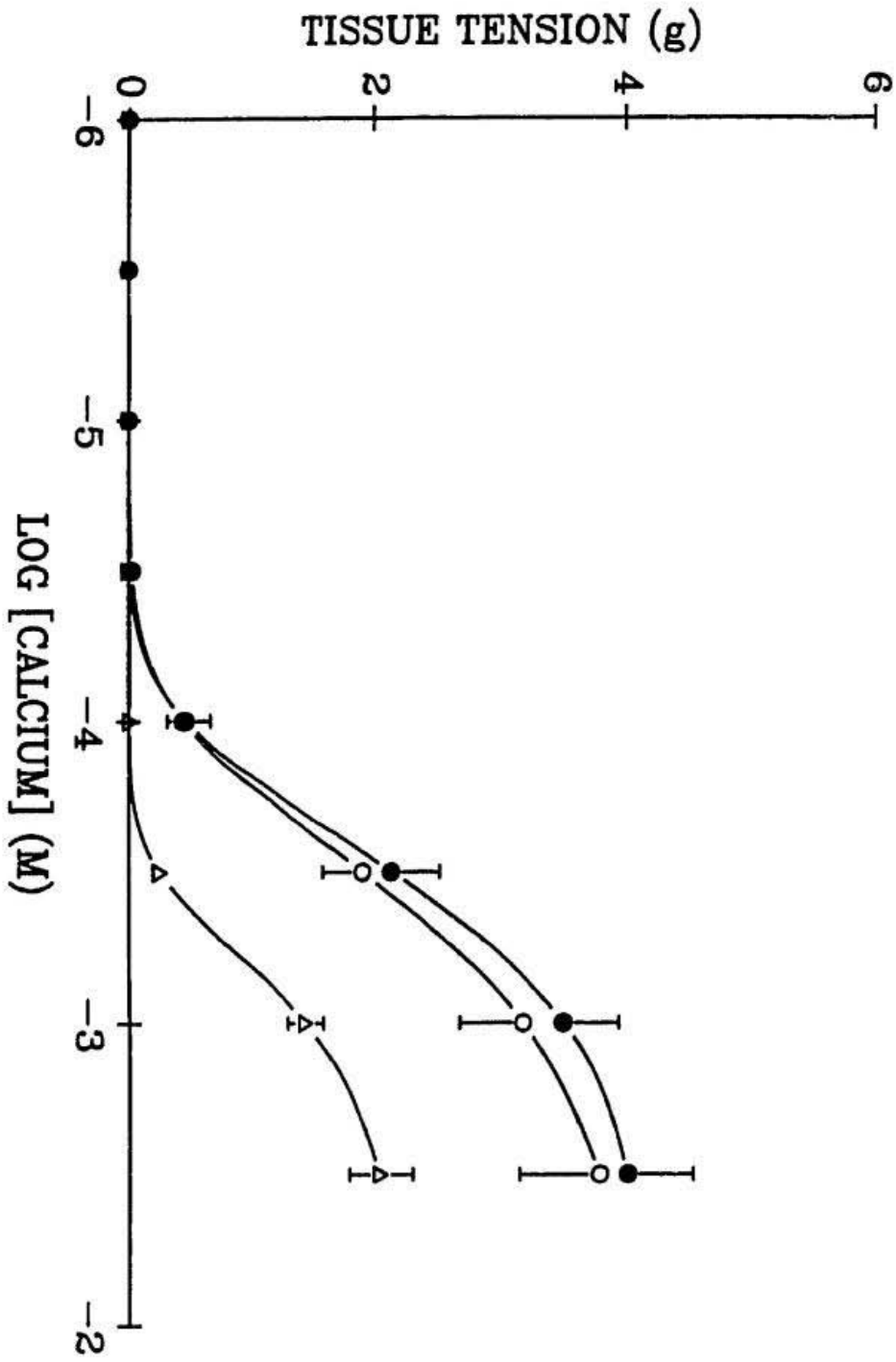
Figure 20a, b and c: Effect of sequential NE and ryanodine induced Ca^{2+} depletion on Ca^{2+} concentration-response curves obtained in the anococcygeus in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O–O and after NE and ryanodine depletion by filled symbols, e.g. ●–● and, in the presence of 10 μM nifedipine, by Δ–Δ. (n = 4)



(a)

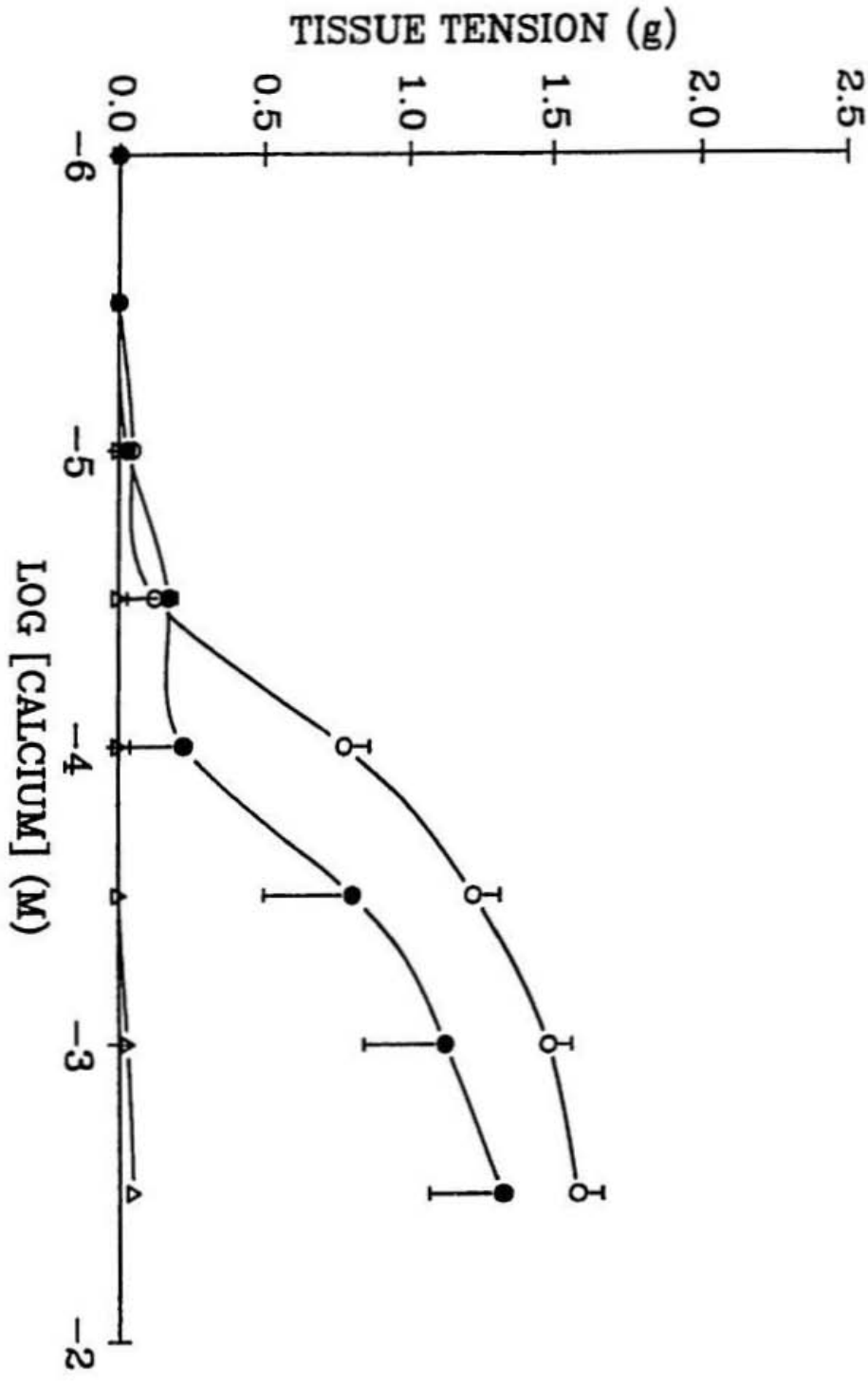


(b)

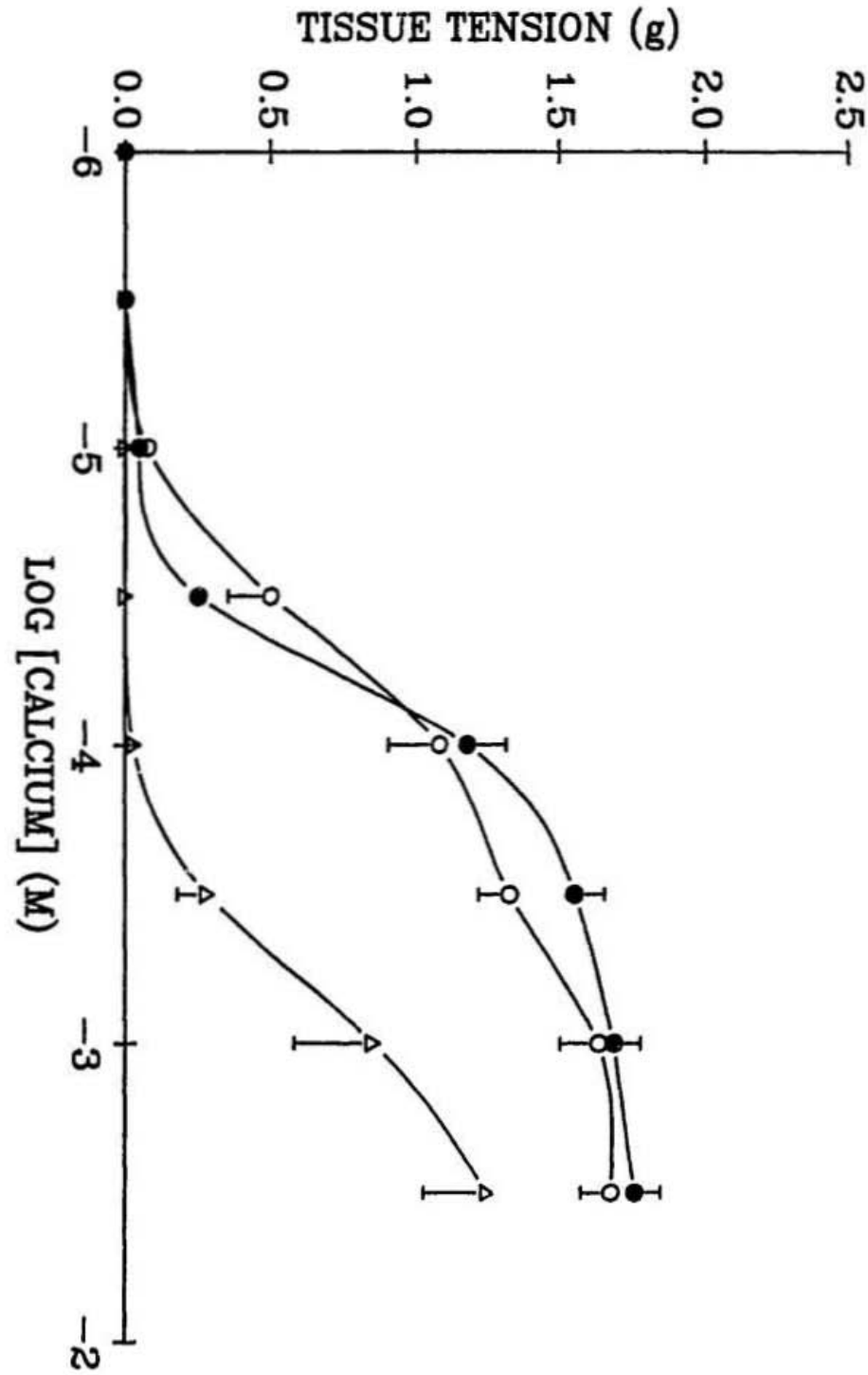


(c)

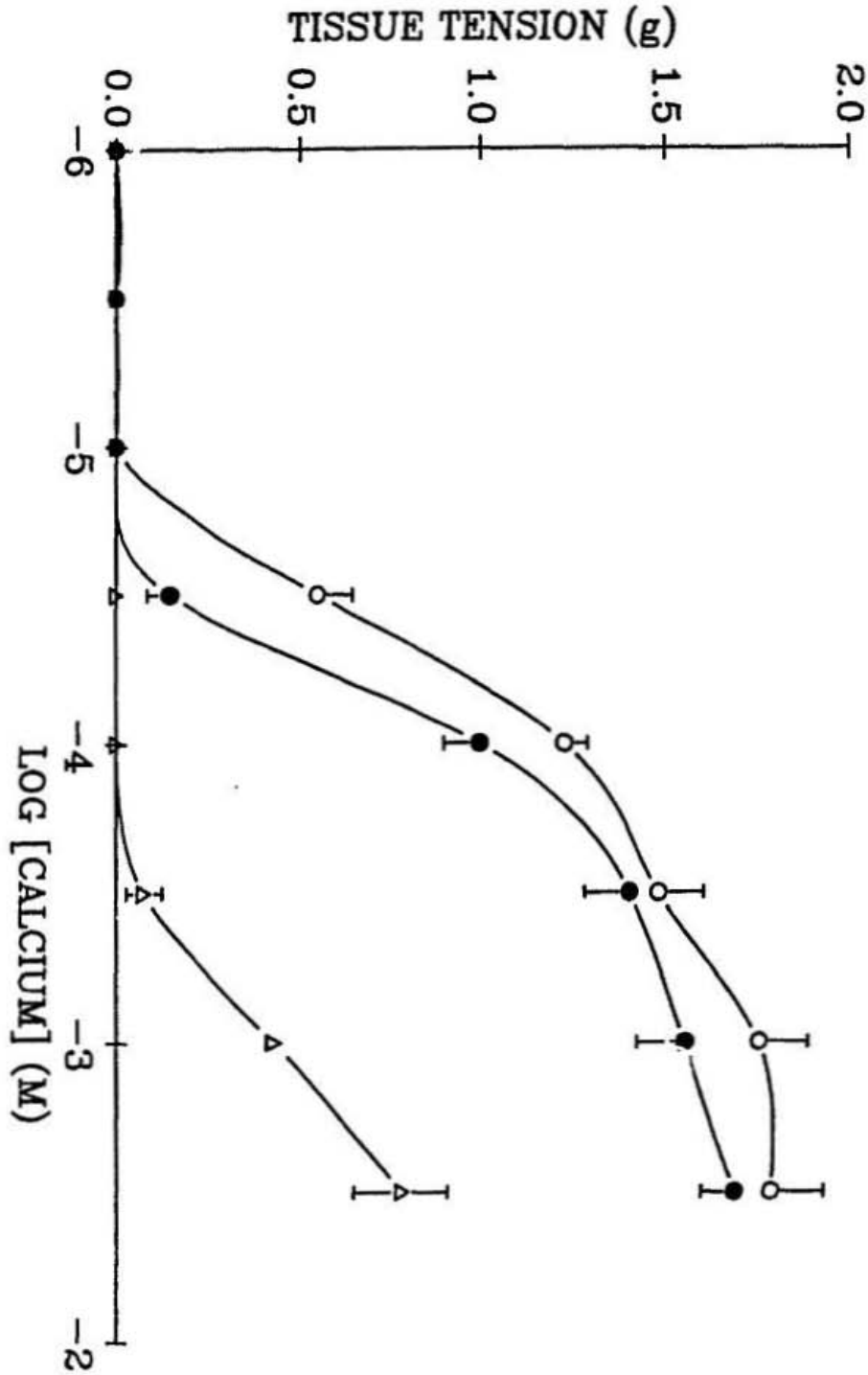
Figure 21a, b and c: Effect of sequential NE and ryanodine Ca^{2+} depletion on Ca^{2+} concentration-response curves obtained in the aorta in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols, e.g. O–O and after NE and ryanodine-induced depletion by filled symbols, e.g. ●–● and, in the presence of 10 μM nifedipine, by Δ-Δ. (n = 4)



(a)

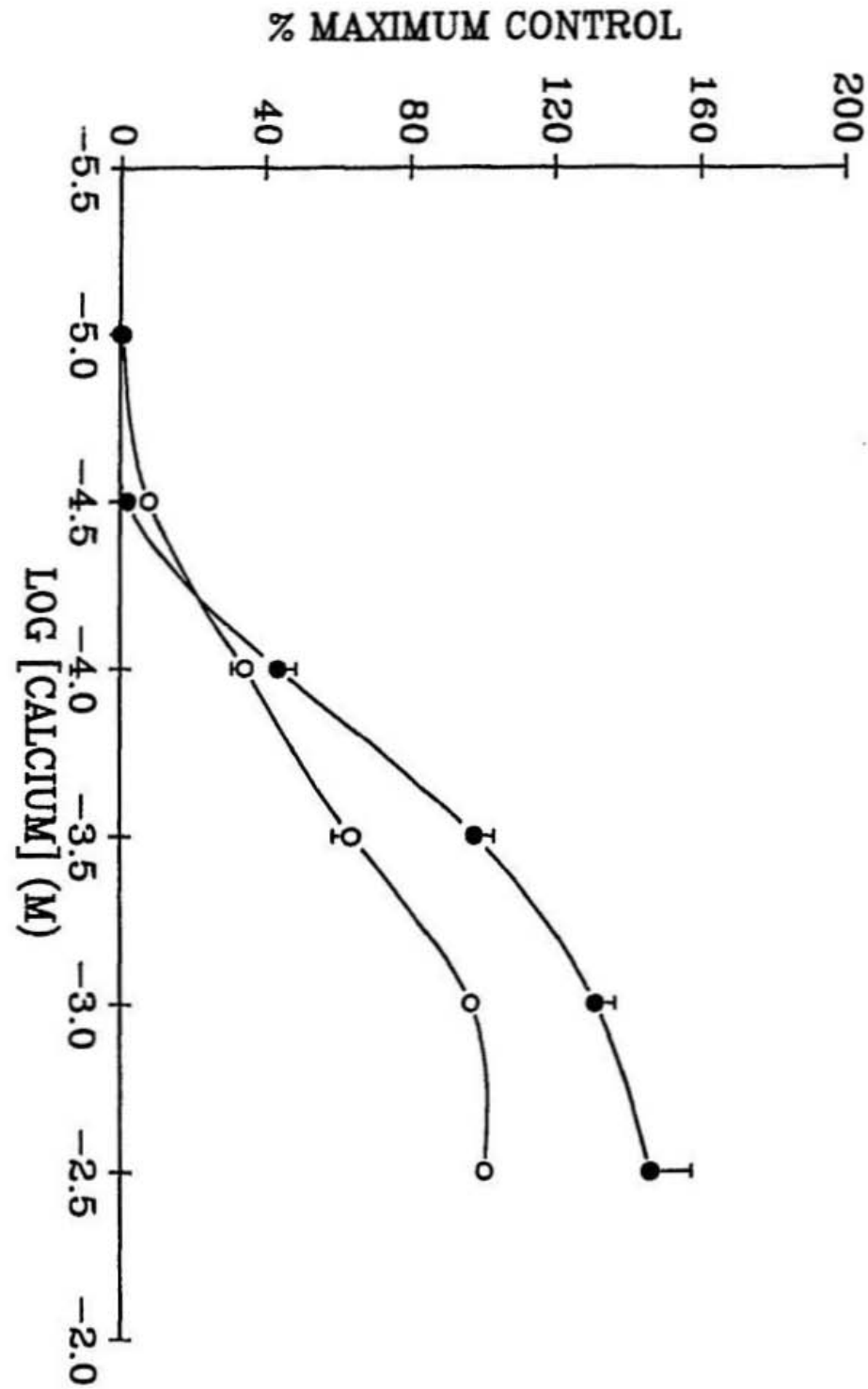


(b)

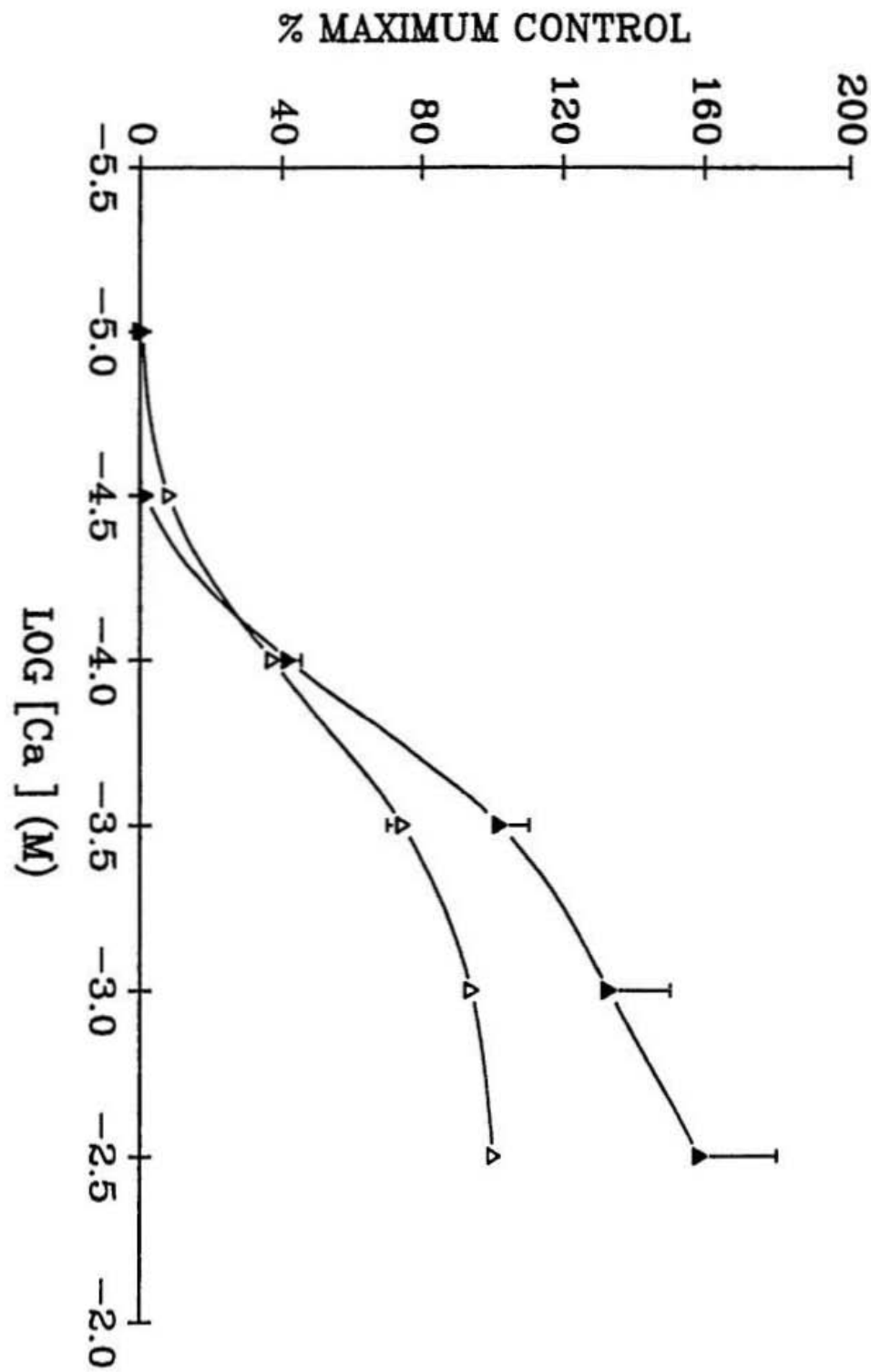


(c)

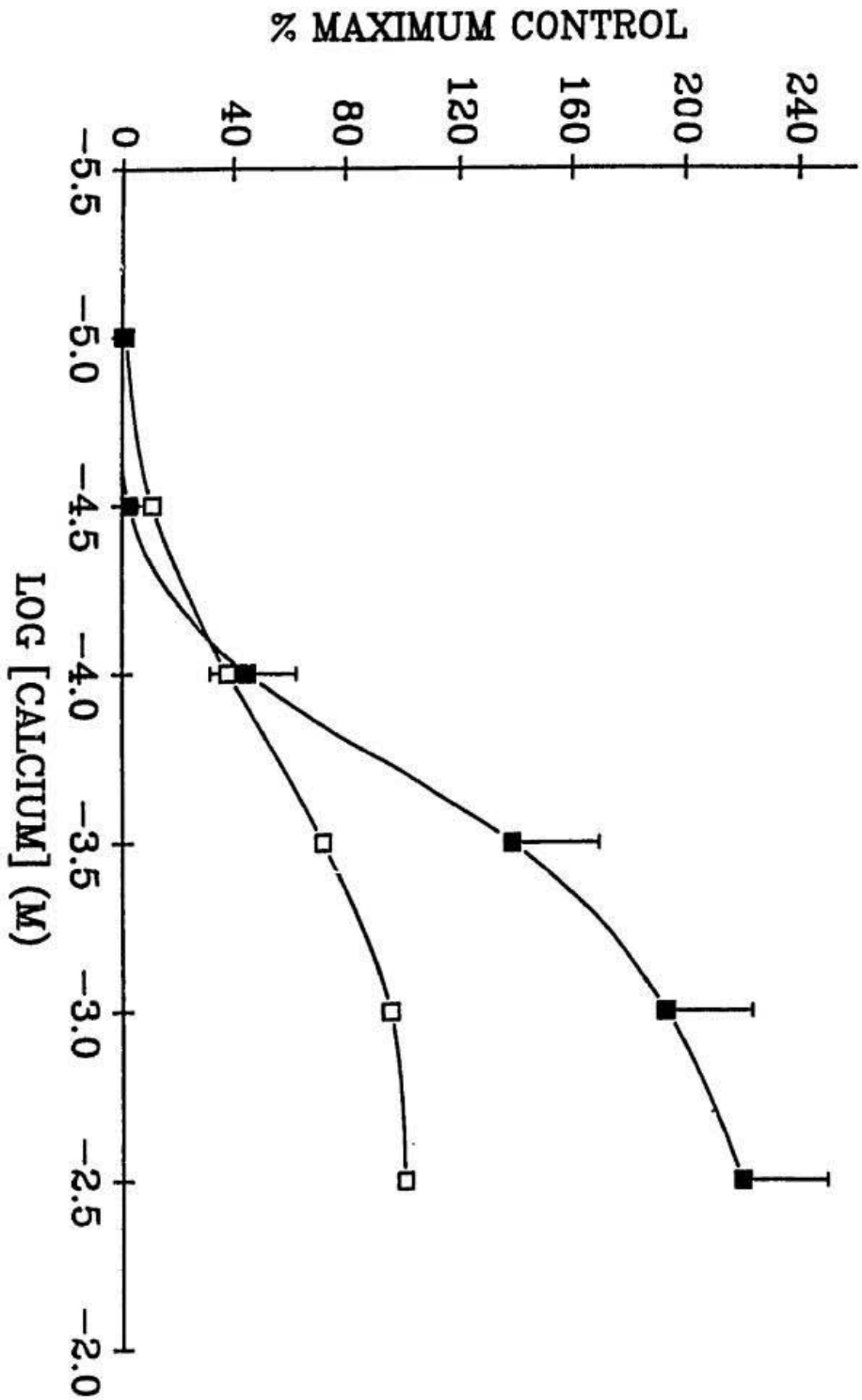
Figure 22a, b and c: Comparative effects of a) 10 μM ryanodine; b) 10 μM ryanodine plus 0.1 mM caffeine; c) 10 μM ryanodine + 10 nM SRTX-b respectively on Ca^{2+} concentration-response curves obtained in the anococcygeus. Control curves ± S.E.M. are represented by open symbols e.g. O–O and after treatment by a, b and c above, by filled symbols e.g. ●–● . Note that 10 nM SRTX-b is administered after Ca^{2+} depletion in a and b. (n = 4)


% MAXIMUM CONTROL
0
40
80
120
160
200
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
LOG [CALCIUM] (M)

(a)

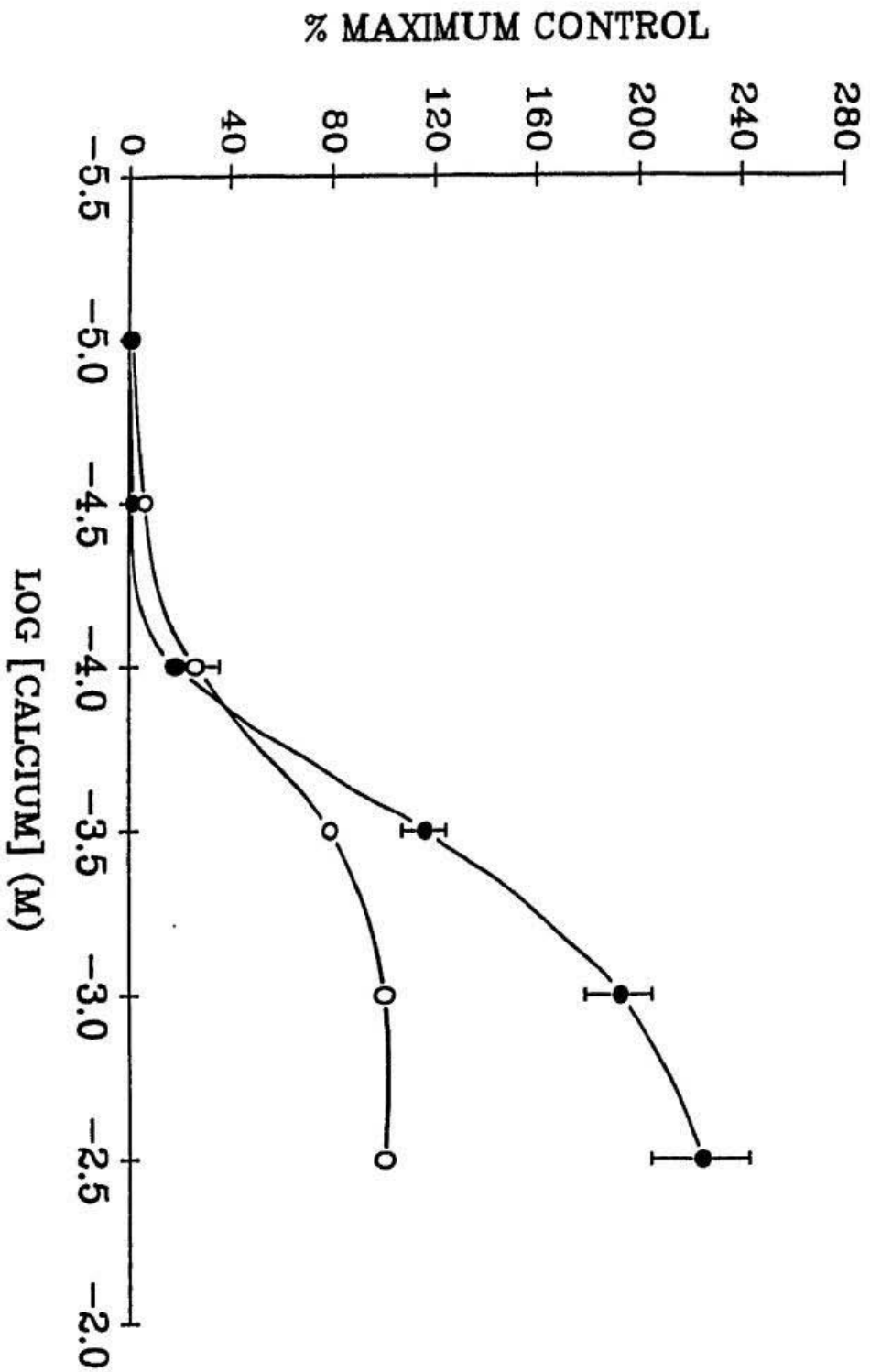

% MAXIMUM CONTROL
0
40
80
120
160
200
LOG [Ca] (M)
−5.5
−5.0
−4.5
−4.0
−3.5
−3.0
−2.5
−2.0

(b)


% MAXIMUM CONTROL
0
40
80
120
160
200
240
LOG [CALCIUM] (M)
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0

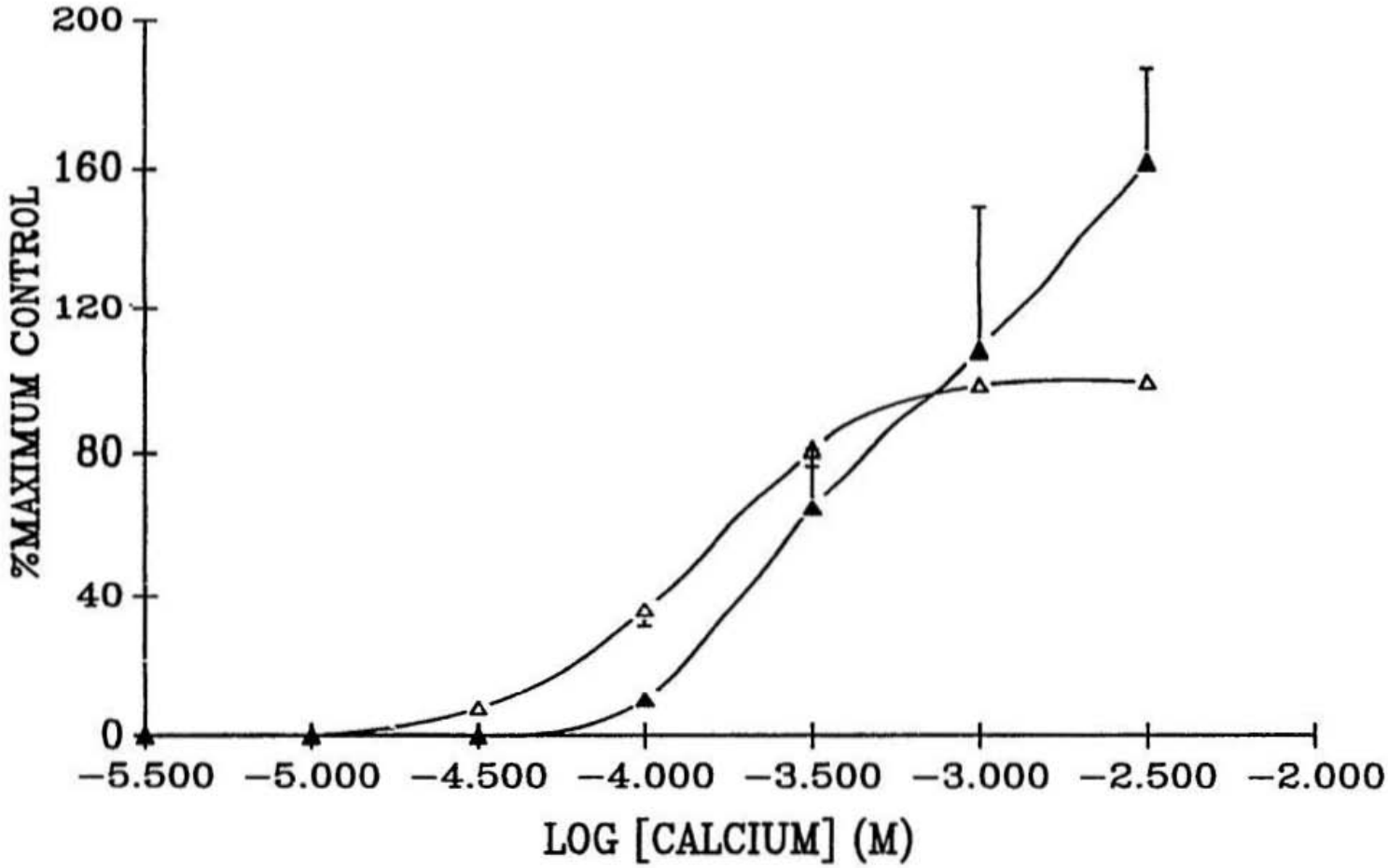
(c)

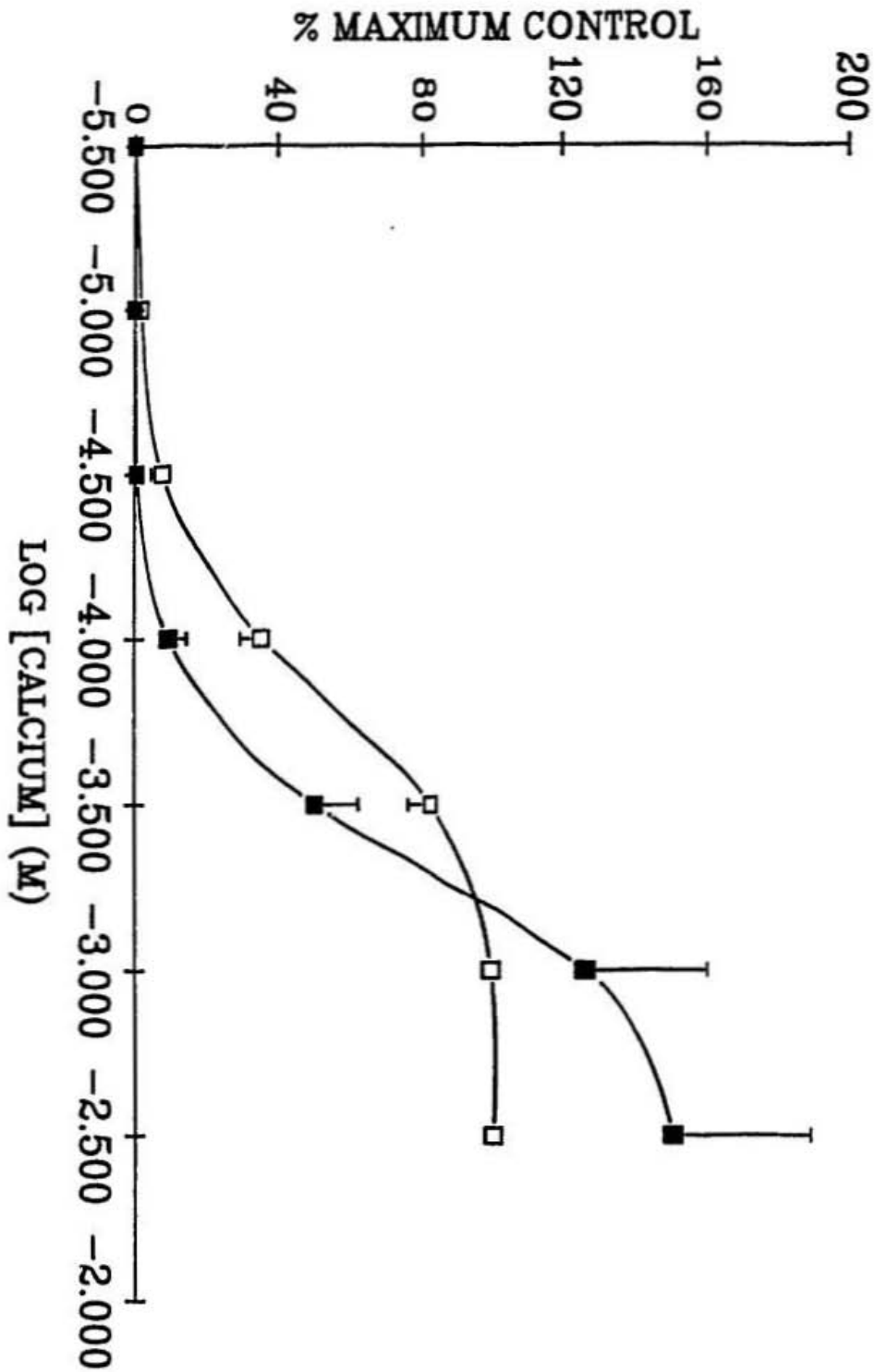
Figure 23a, b and c: Comparative effects of a) 10 μM ryanodine; b) 10 μM ryanodine plus 0.1 mM caffeine; c) 10 μM ryanodine + 10 nM ET-1 concurrently on Ca^{2+} concentration-response obtained in the anococcygeus. Control curves ± S.E.M. are represented by open symbols e.g. O-O and after treatment by a, b and c above, by filled symbols e.g. ●-● . Note that 10 nM ET-1 was administered after Ca^{2+} depletion in a and b. (n = 4)


% MAXIMUM CONTROL
0
40
80
120
160
200
240
280
LOG [CALCIUM] (M)
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0

(a)

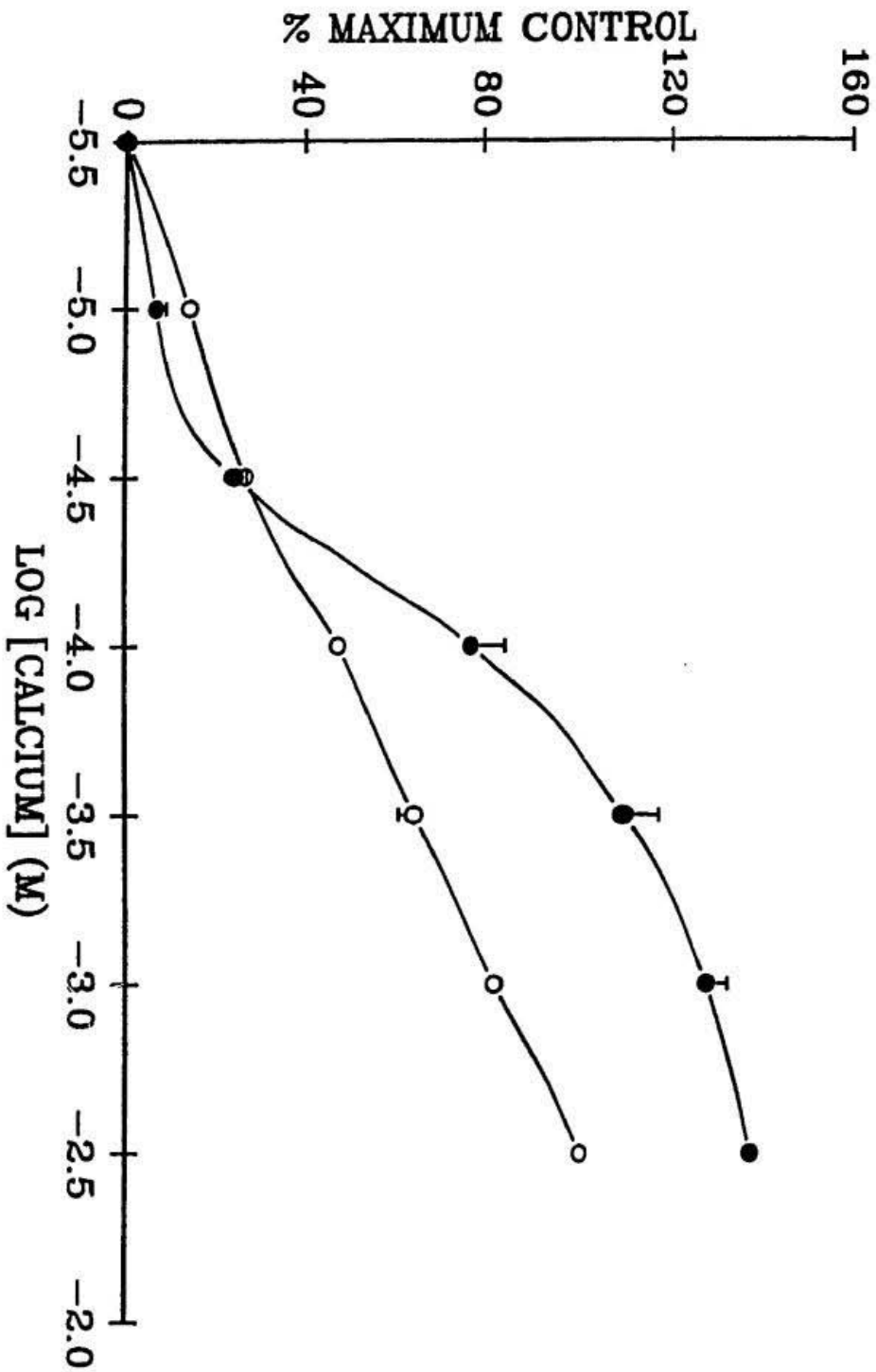
(b)


200
160
120
80
40
0
%MAXIMUM CONTROL
−5.500
−5.000
−4.500
−4.000
−3.500
−3.000
−2.500
−2.000
LOG [CALCIUM] (M)

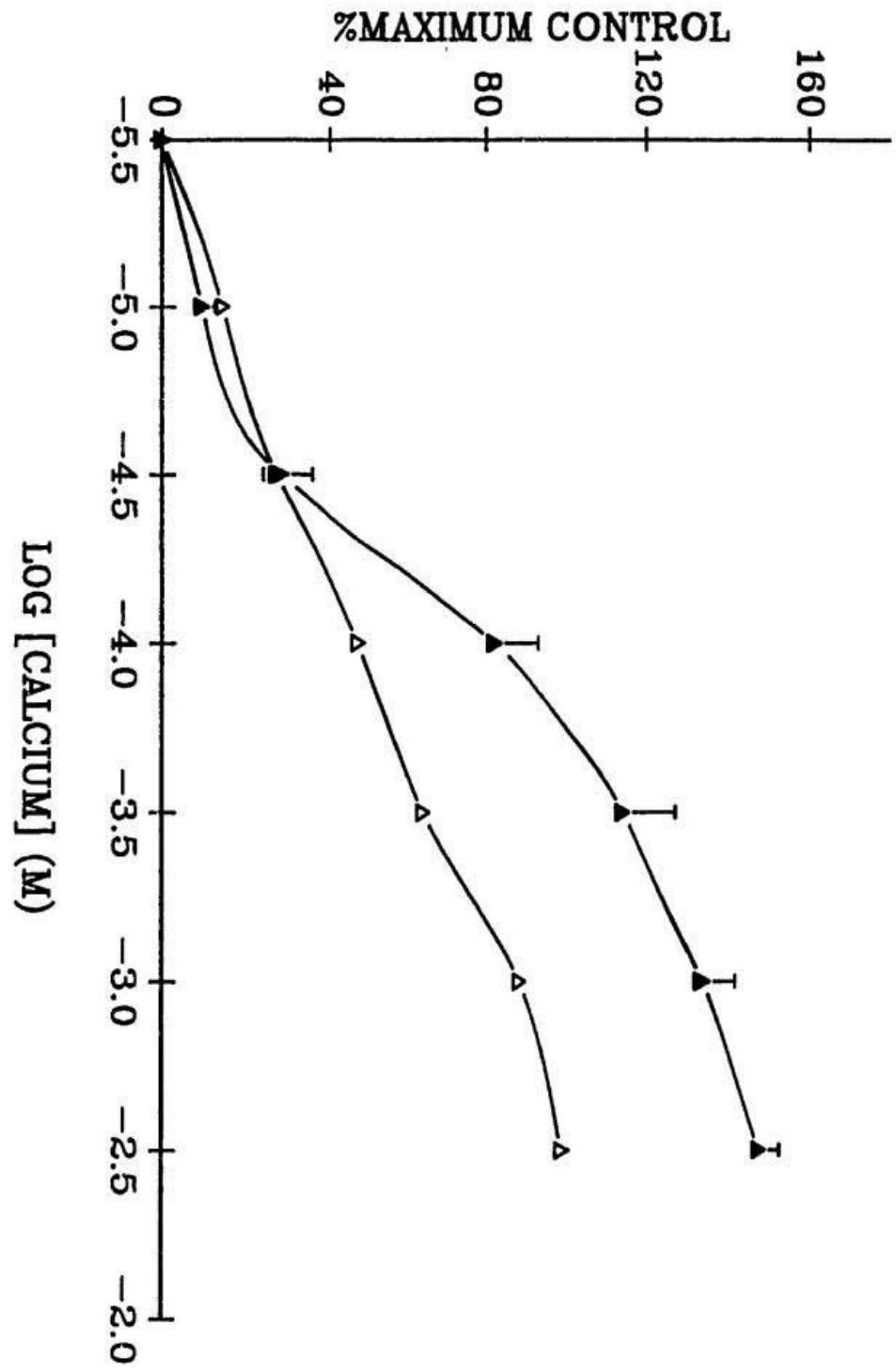

% MAXIMUM CONTROL
0
40
80
120
160
200
-5.500
-5.000
-4.500
-4.000
-3.500
-3.000
-2.500
-2.000
LOG [CALCIUM] (M)

(c)

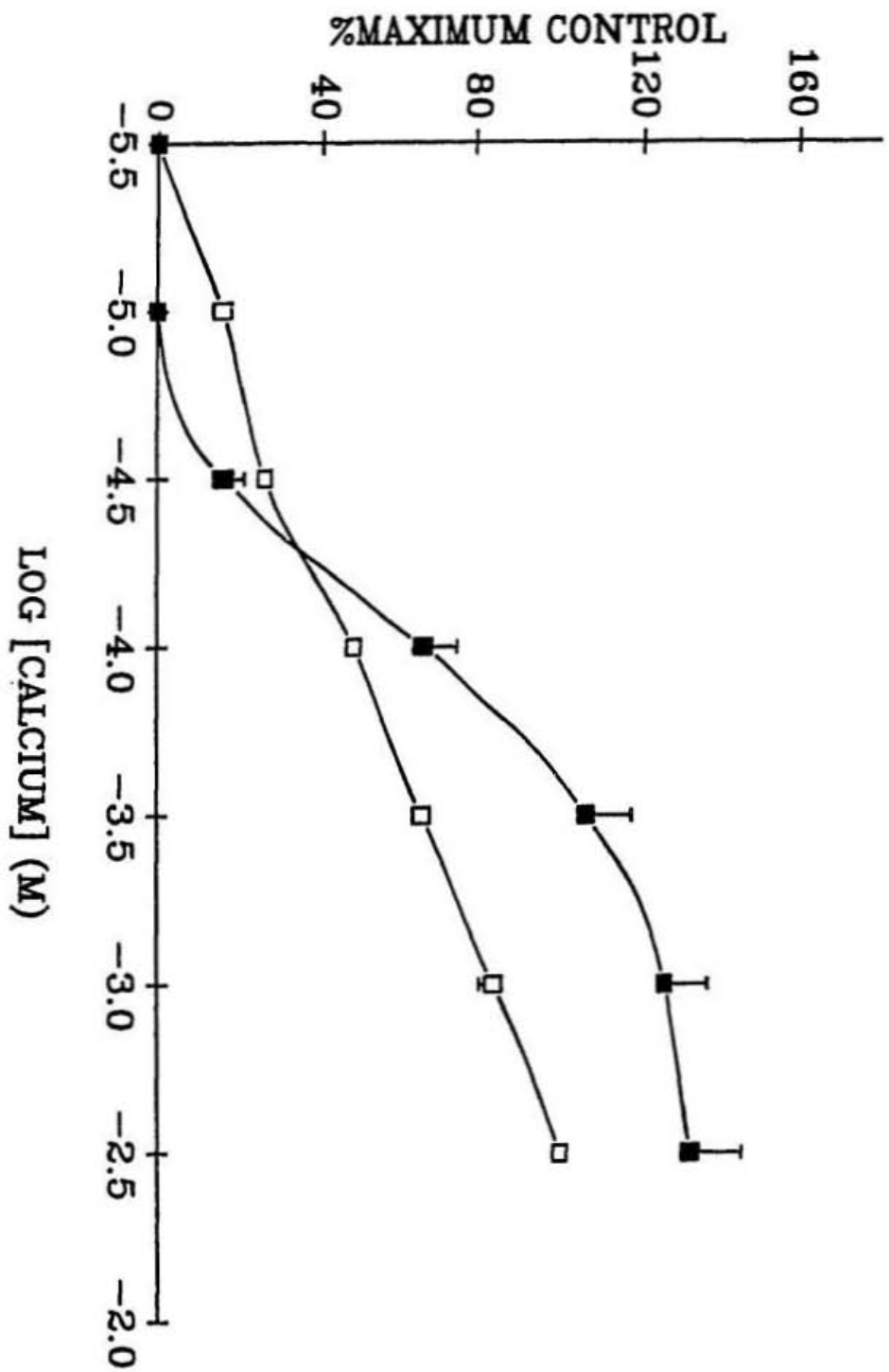
Figure 24a, b and c: Comparative effects of: a) 10 μM ryanodine; b) 10 μM ryanodine plus 0.1 mM caffeine; c) 10 μM ryanodine + 10 nM SRTX-b concurrently on Ca^{2+} concentration-response curves obtained in the aorta. Control curves ± S.E.M. are represented by open symbols e.g. O–O and after treatment by a, b and c above, by filled symbols e.g. ●–●. Note that 10 nM SRTX-b was administered after Ca^{2+} depletion in a and b. (n = 4)


% MAXIMUM CONTROL
0
40
80
120
160
LOG [CALCIUM] (M)
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0

(a)

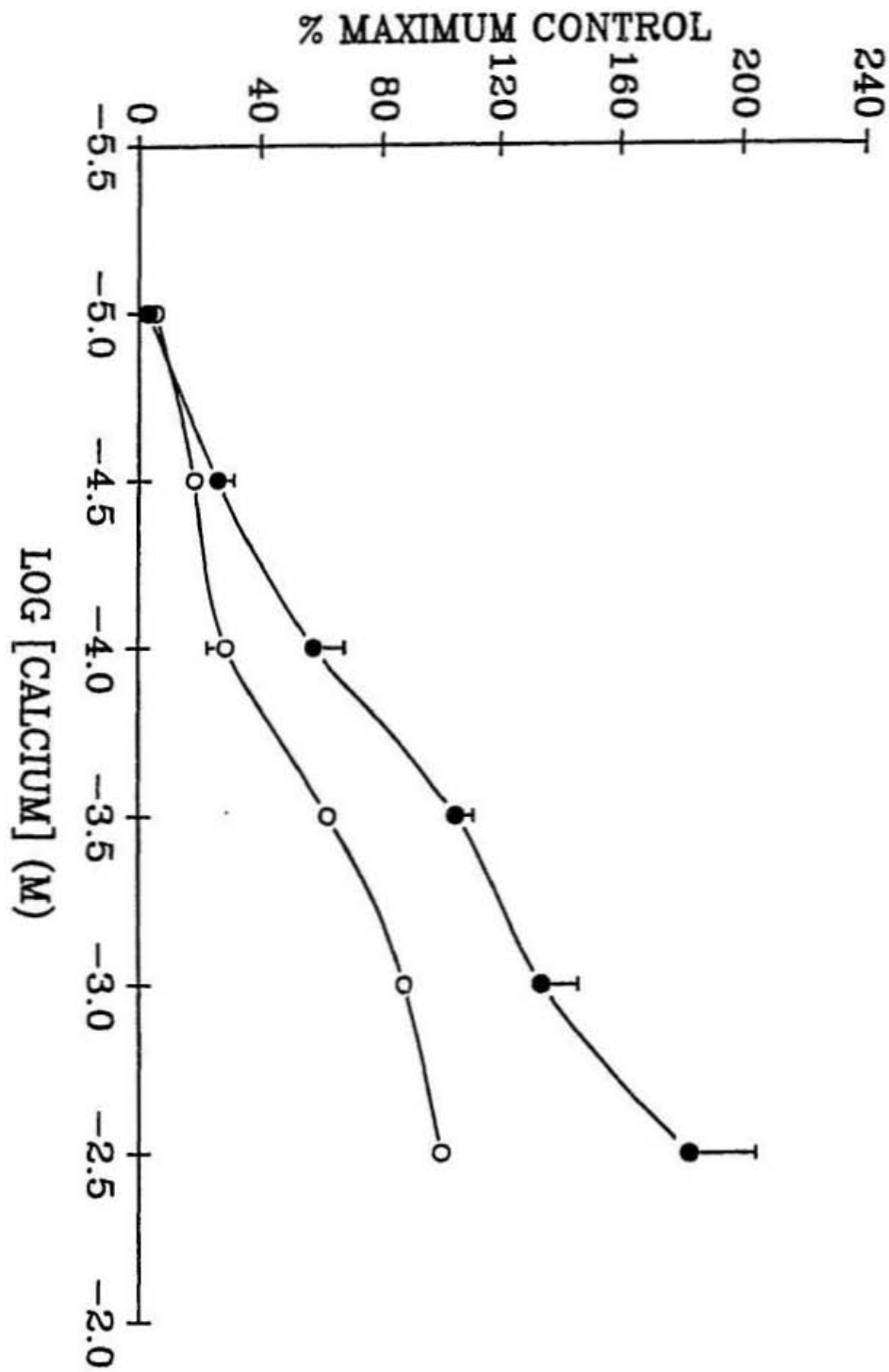

%MAXIMUM CONTROL
0
40
80
120
160
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
LOG [CALCIUM] (M)

(b)

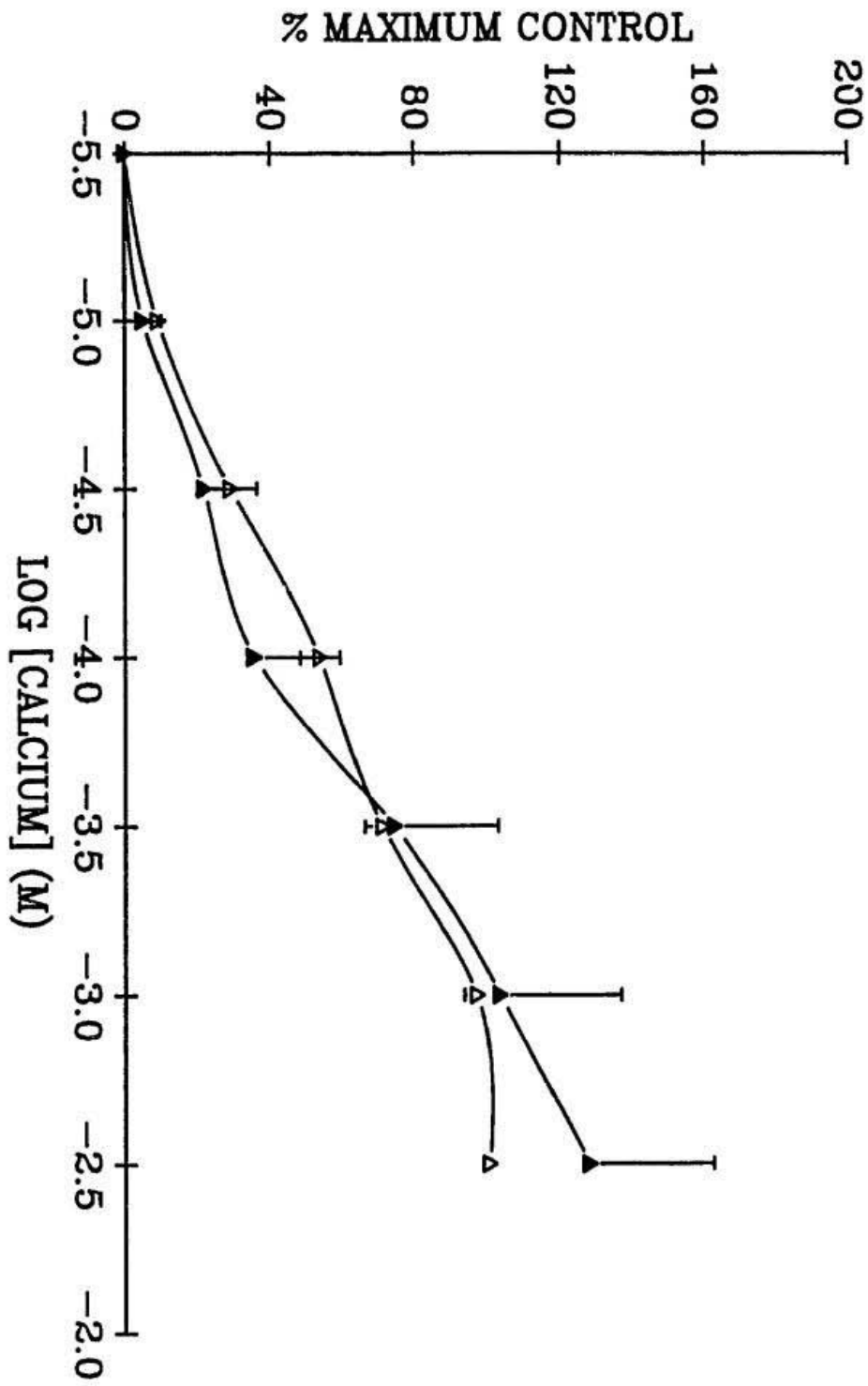

%MAXIMUM CONTROL
0
40
80
120
160
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
LOG [CALCIUM] (M)

(c)

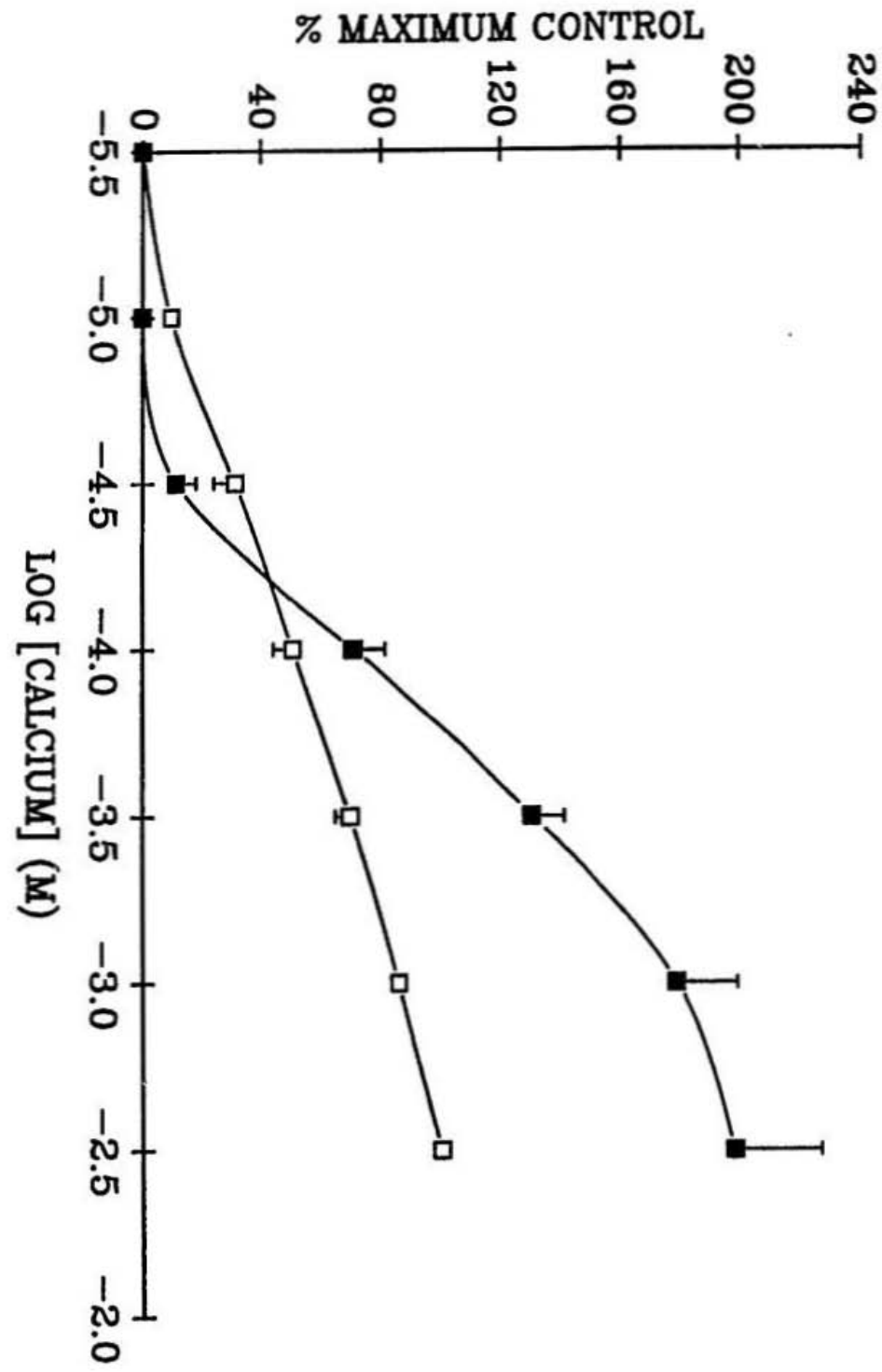
Figure 25a, b and c: Comparative effects of a) 10 μM ryanodine; b) 10 μM ryanodine plus 0.1 mM caffeine; c) 10 μM ryanodine + 10 nM ET-1 concurrently on Ca^{2+} concentration-response curves obtained in the aorta. Control curves ± S.E.M. are represented by open symbols e.g. O-O and after treatment by a, b and c above, by filled symbols e.g. ●–●. Note that 10 nM SRTX-b was administered after Ca^{2+} depletion in a) and b). (n = 4)


% MAXIMUM CONTROL
240
200
160
120
80
40
0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
LOG [CALCIUM] (M)

(a)

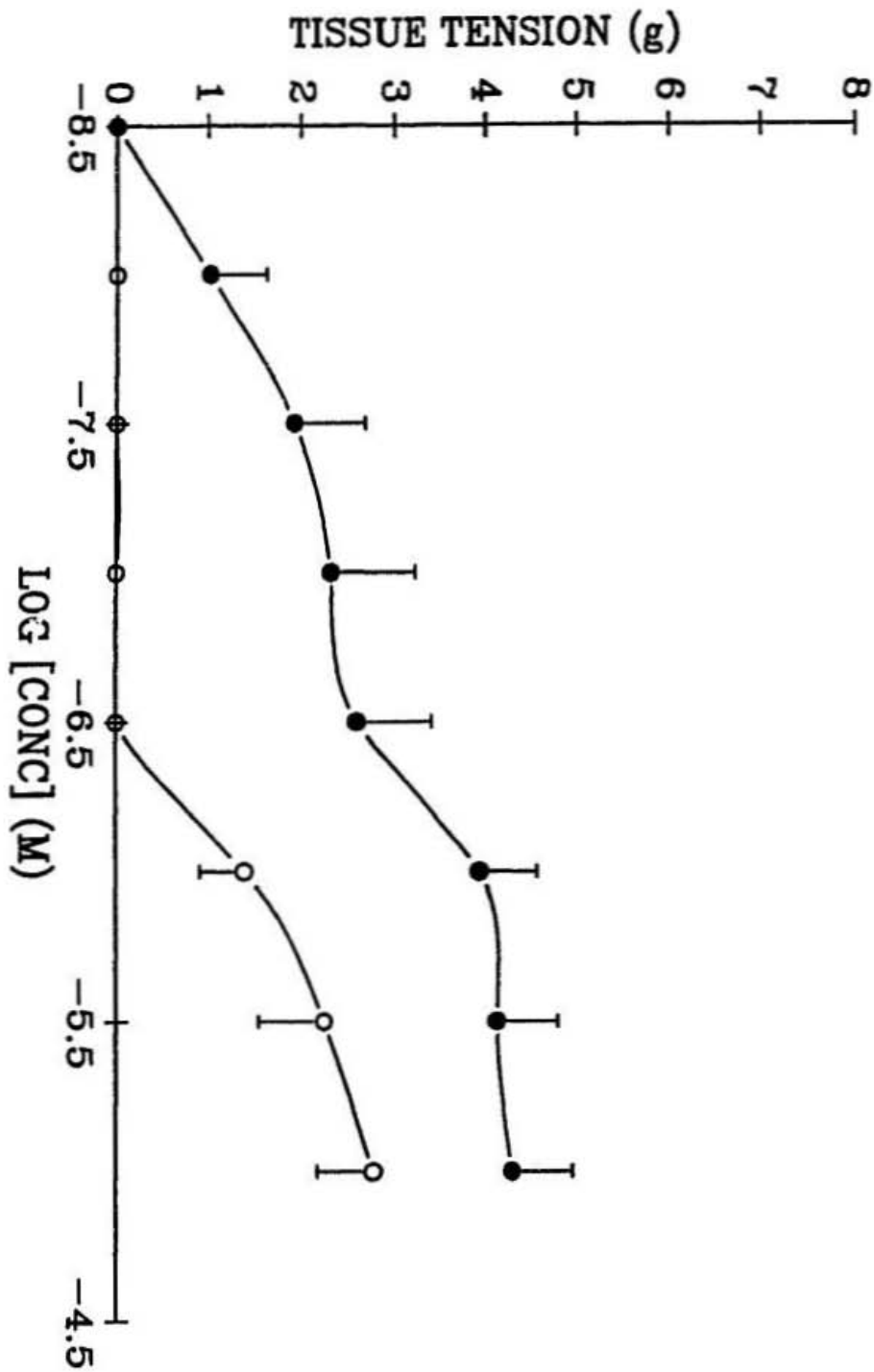

% MAXIMUM CONTROL
0
40
80
120
160
200
LOG [CALCIUM] (M)
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0

(b)

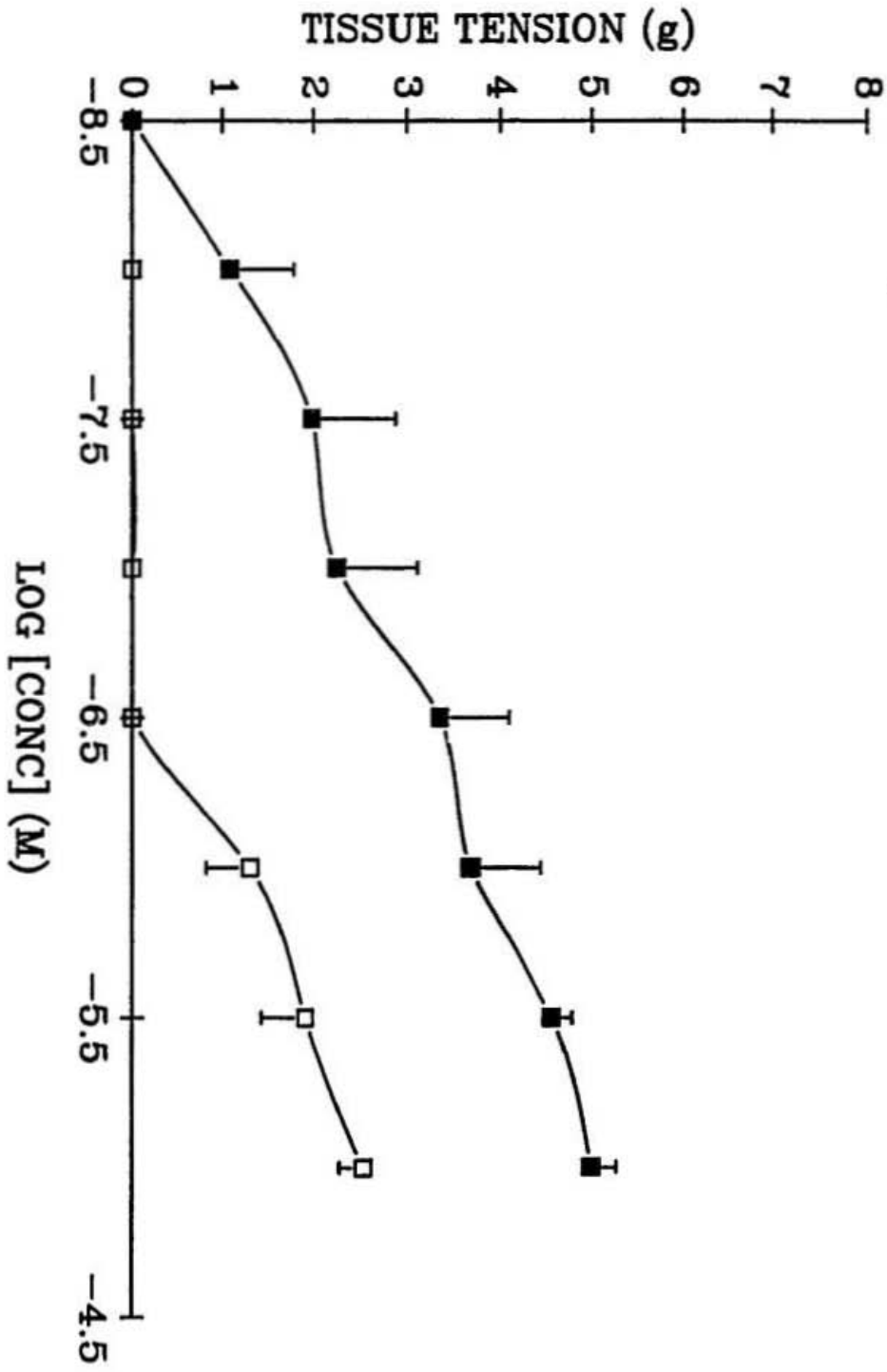

% MAXIMUM CONTROL
0
40
80
120
160
200
240
LOG [CALCIUM] (M)
−5.5
−5.0
−4.5
−4.0
−3.5
−3.0
−2.5
−2.0

(c)

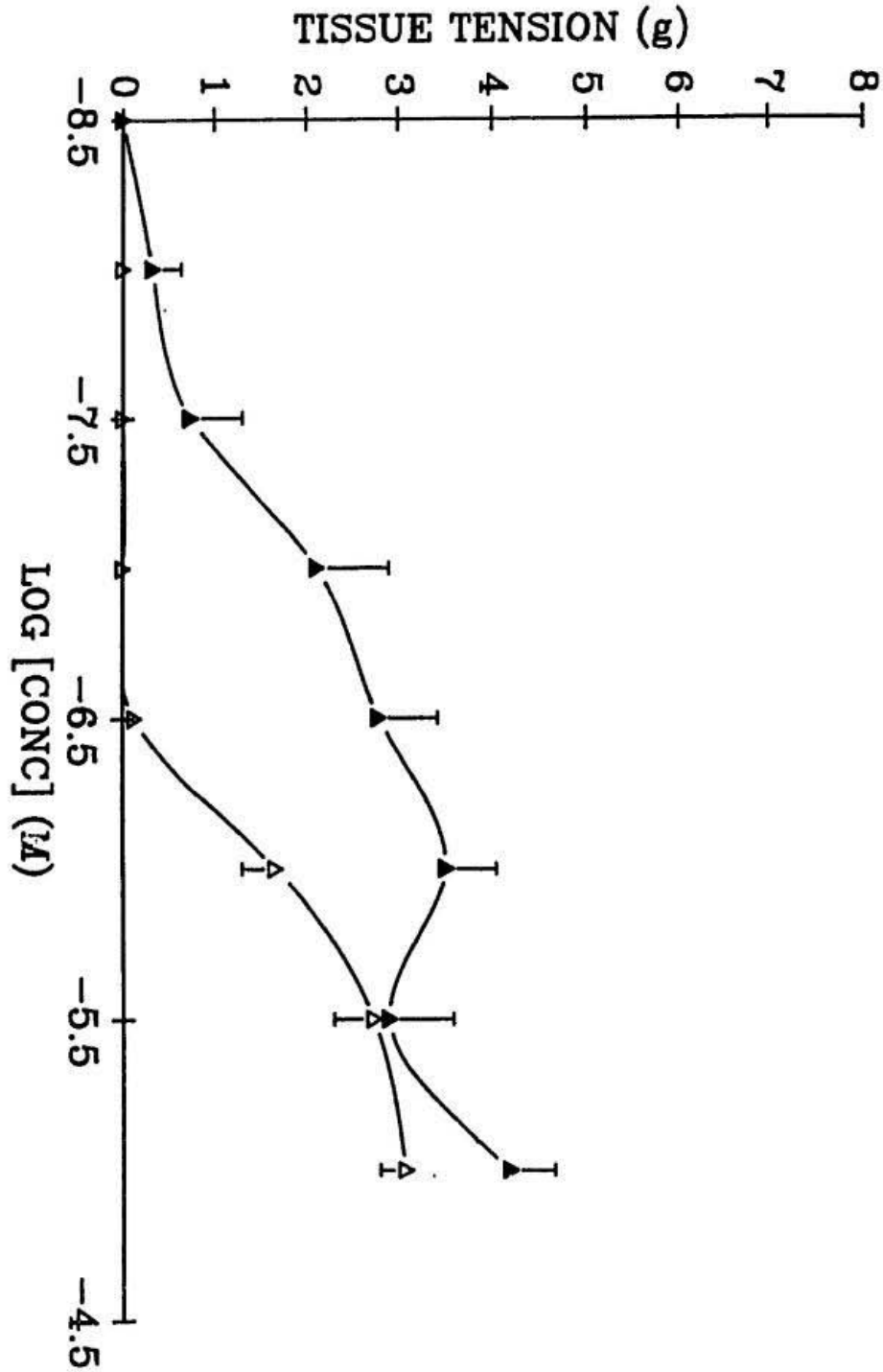
Figure 26a, b and c: Effect of a) 1 nM ET-1; b) 1 nM SRTX and c) 100 nM NE on the Bay K 8644 concentration-response curve obtained in anococcygeus. Control curves ± S.E.M. are represented by open symbols e.g. O-O and after application of a, b and c above, by filled symbols e.g. ●-●. (n = 4)



(a)



(b)



(c)

Figure 27: Illustration of the concentration-independent response of the anococcygeus (a) in contrast to the concentration-dependent response of the aorta (b) to Bay K 8644.

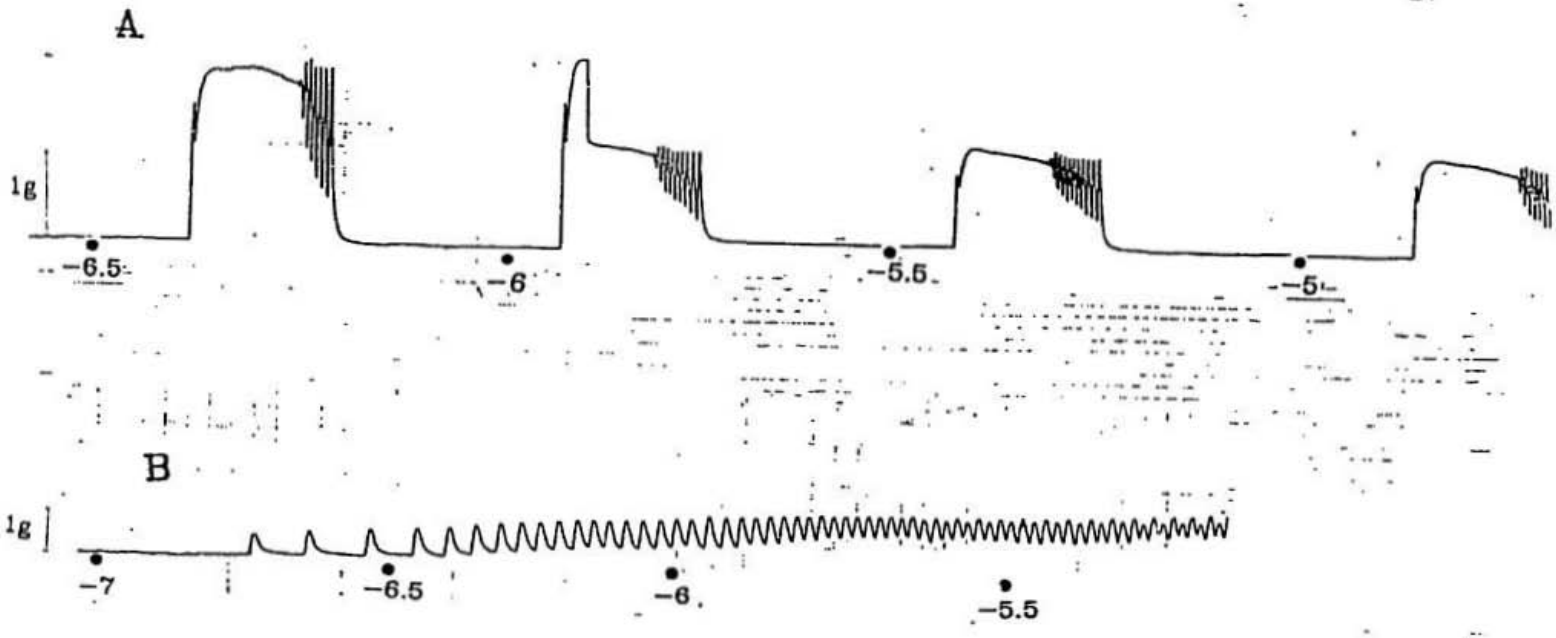
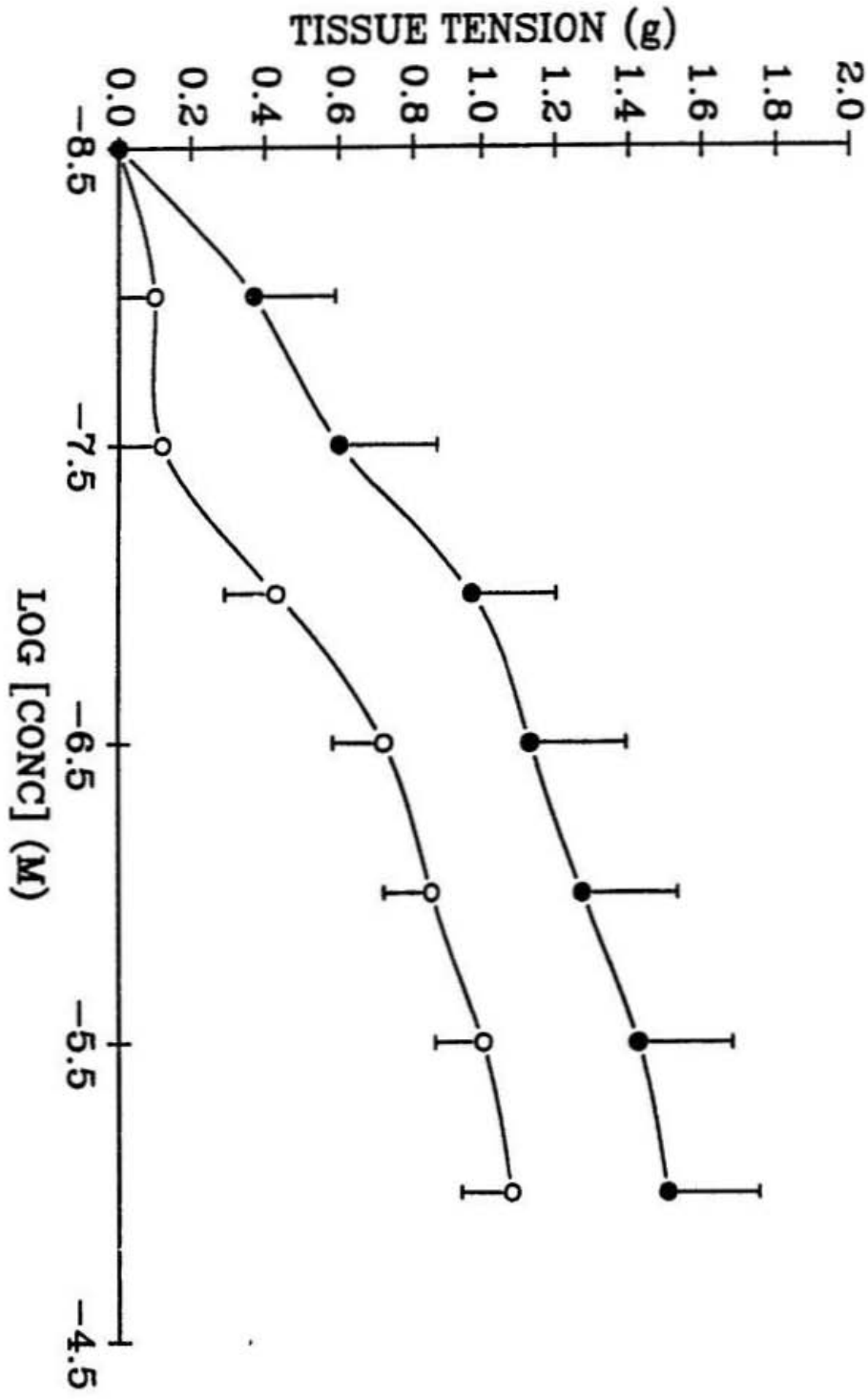
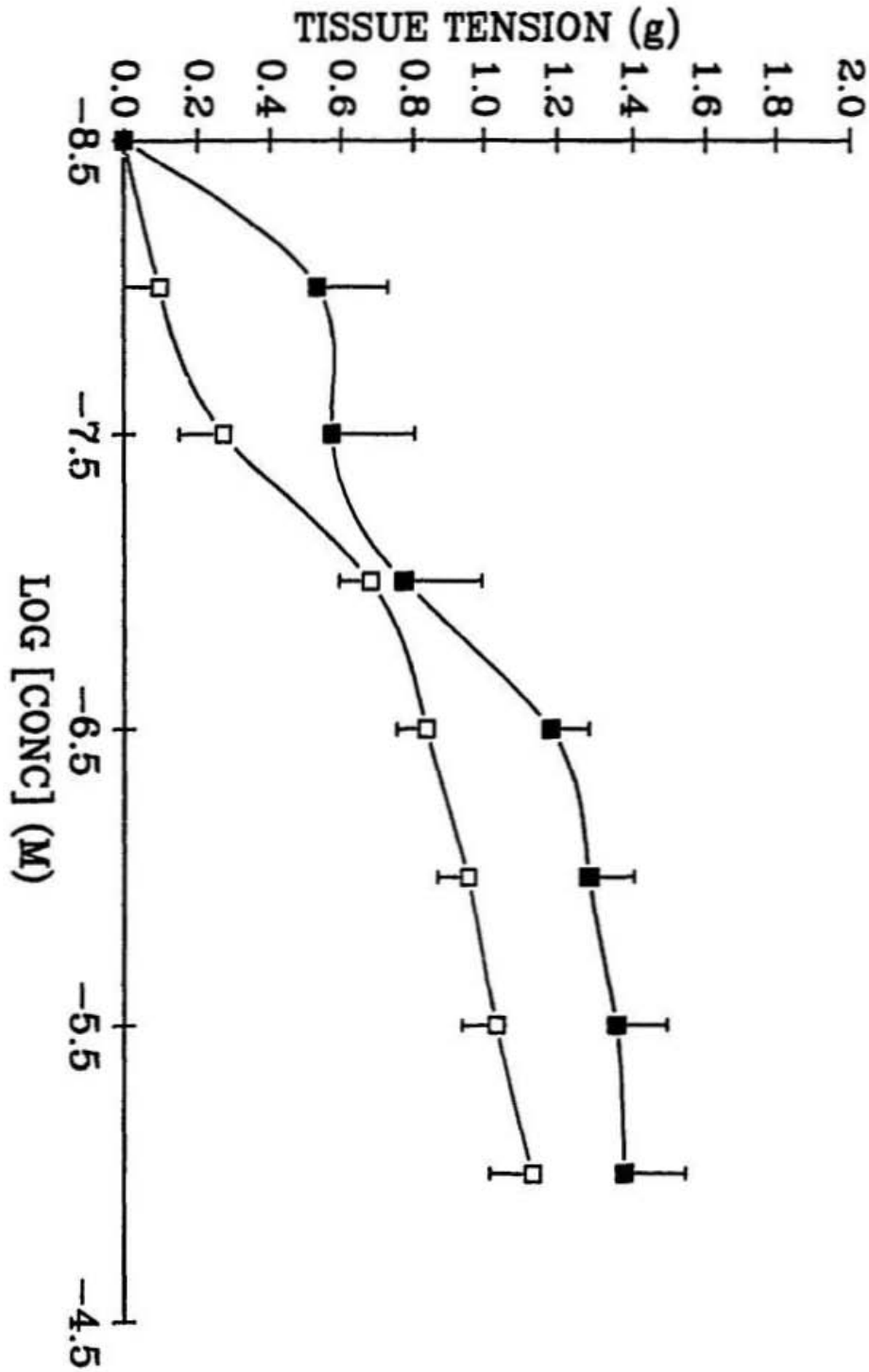

A
1g
-6.5
-6
-5.5
-5
B
1g
-7
-6.5
-6
-5.5

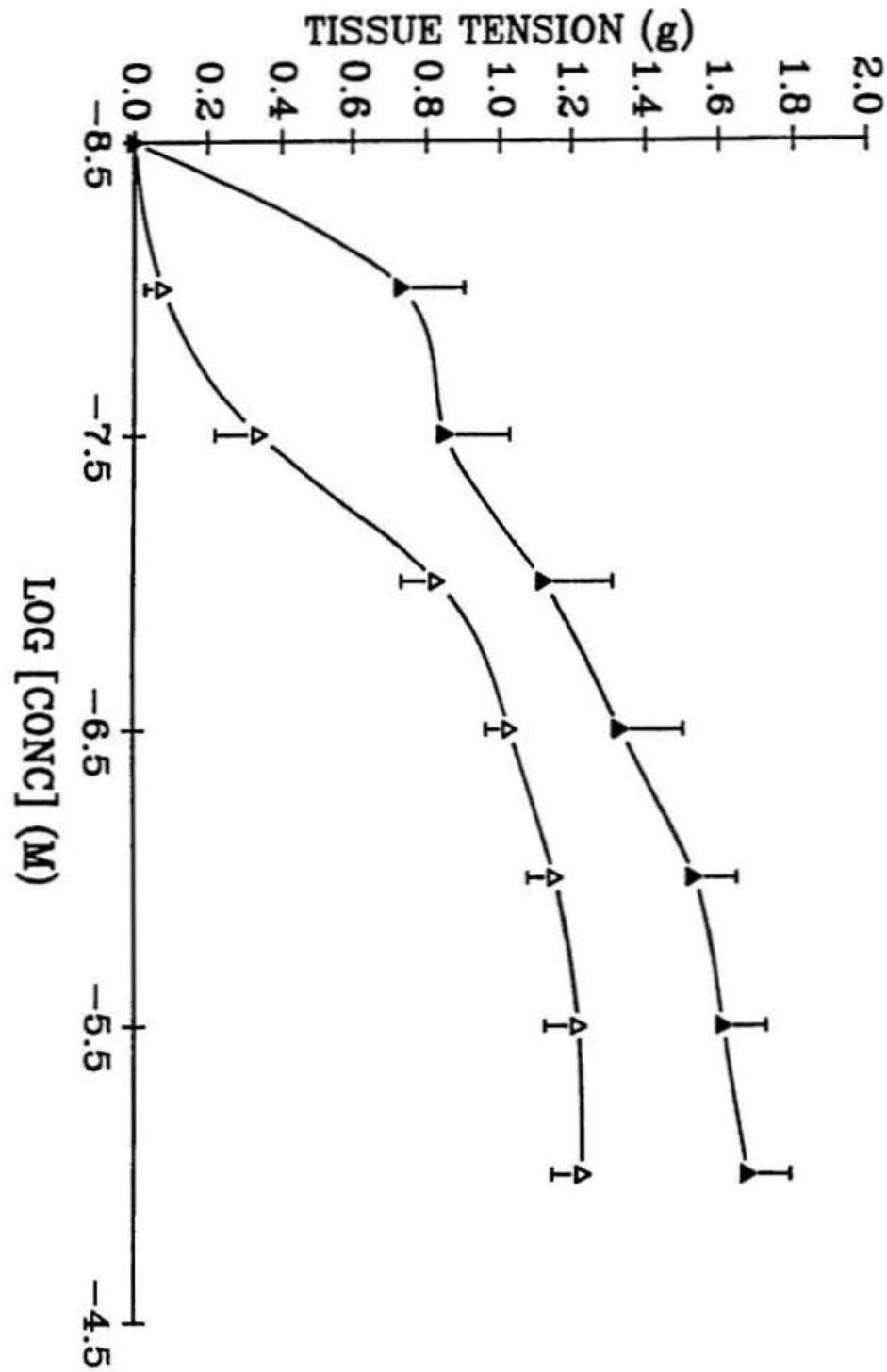
Figure 28a, b and c: Effect of the presence of a) 1 nM ET-1; b) 1 nM SRTX-b and c) 100 nM NE on Bay K 8644 concentration-response curves obtained in the aorta. Control curves ± S.E.M. are represented by open symbols e.g. O-O and after application of a, b and c above by filled symbols e.g. ●-● . (n = 4)


TISSUE TENSION (g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
−8.5
−7.5
−6.5
−5.5
−4.5
LOG [CONC] (M)

(a)

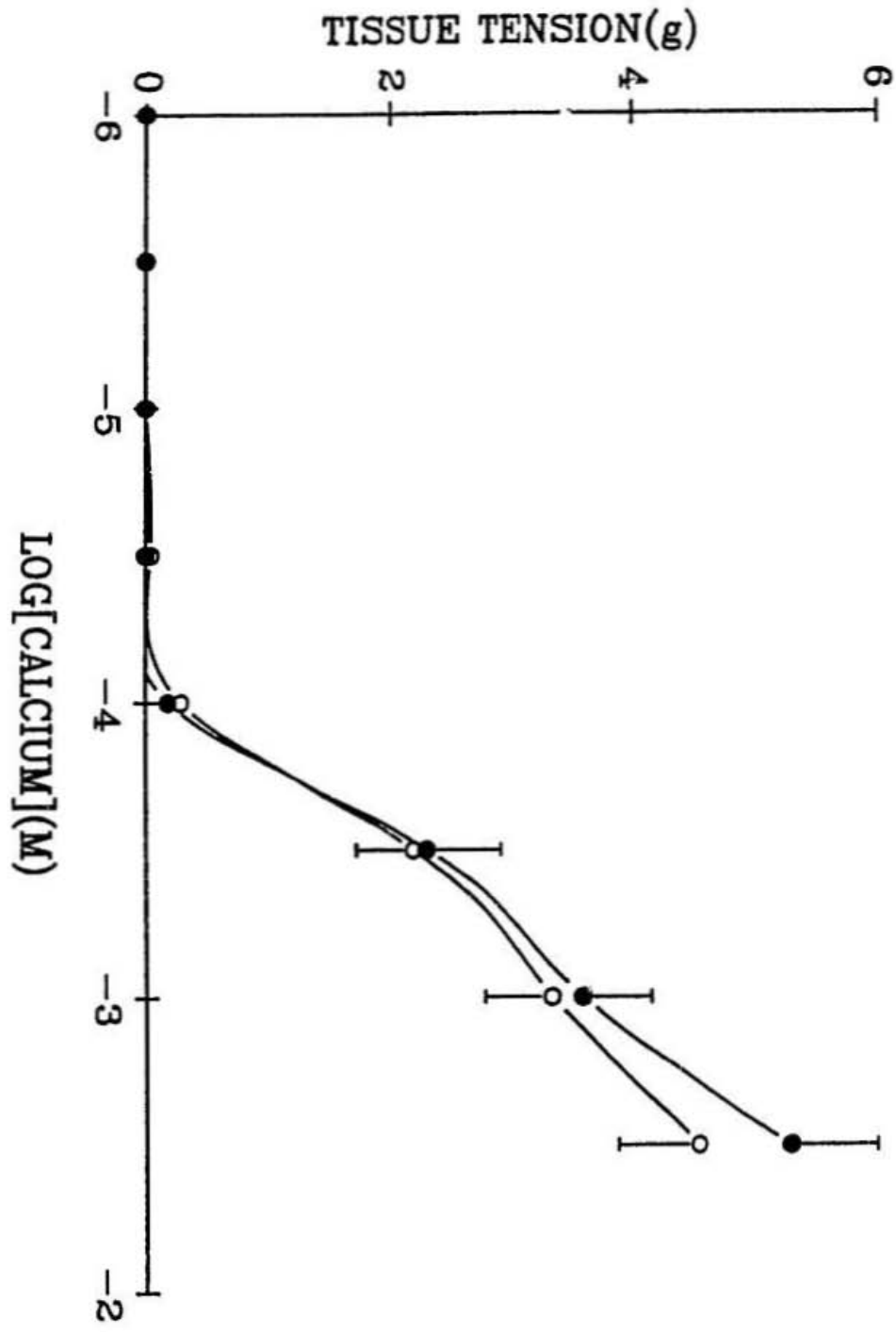

TISSUE TENSION (g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
−8.5
−7.5
−6.5
−5.5
−4.5
LOG [CONC] (M)

(b)

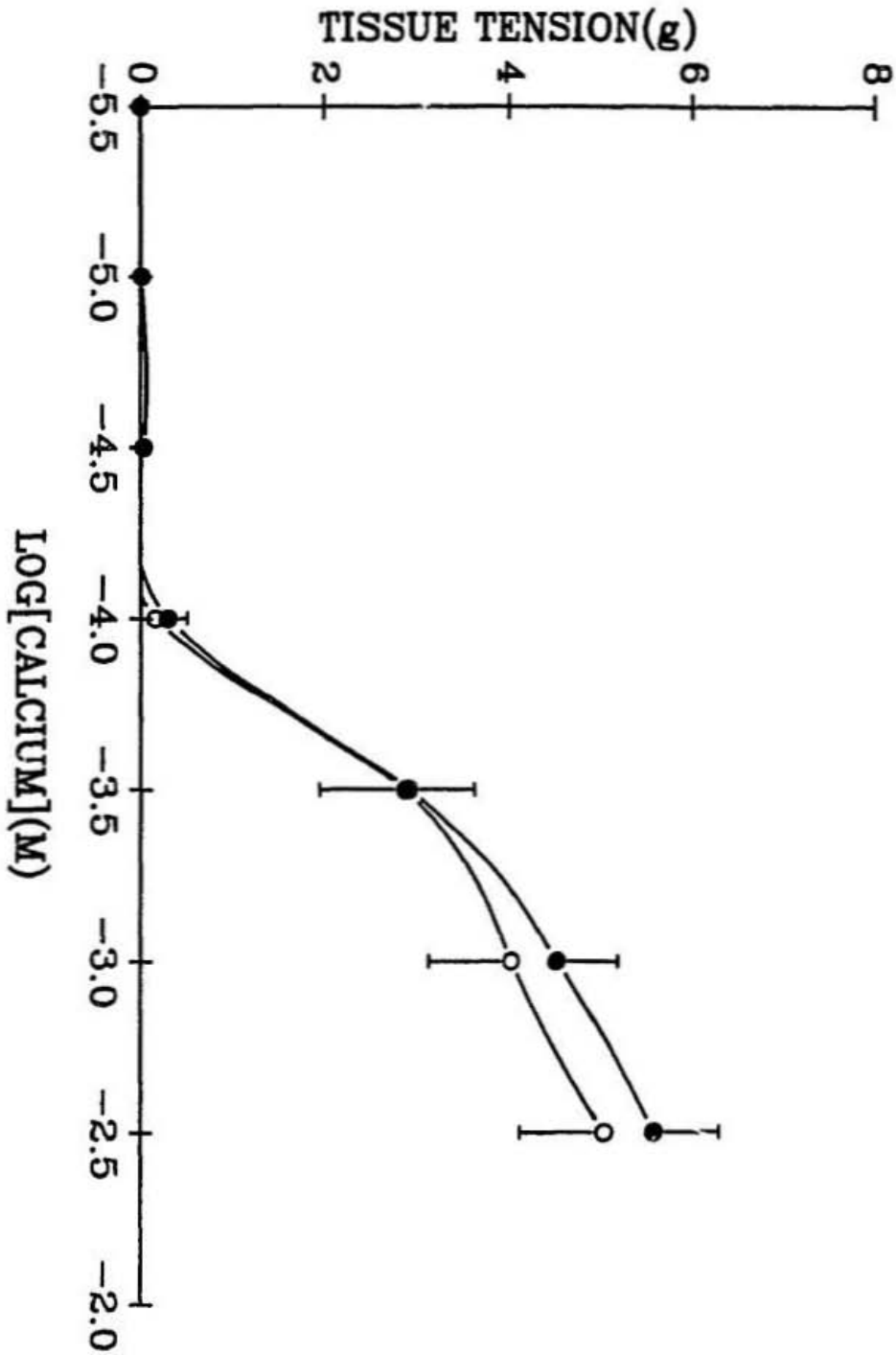

TISSUE TENSION (g)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−8.5
−7.5
−6.5
−5.5
−4.5
LOG [CONC] (M)

(c)

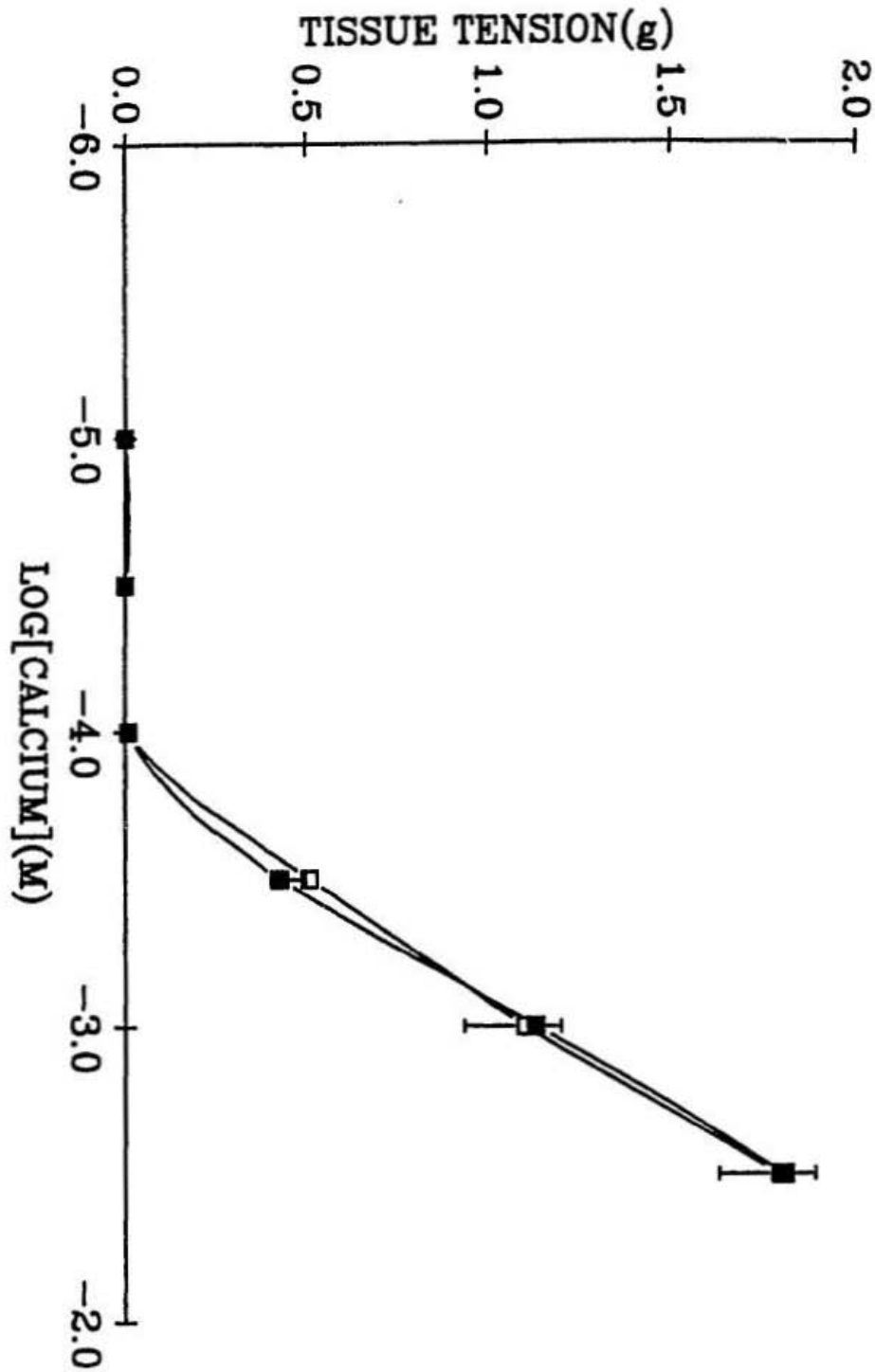
Figure 29a, b anc c: Effect of 10 μM indomethacin on the Ca^{2+} concentration-response curve obtained in the anococcygeus in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b ; and c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols e.g. O-O and after application of indomethacin by filled symbols e.g. ●-●. (n = 4)



(a)

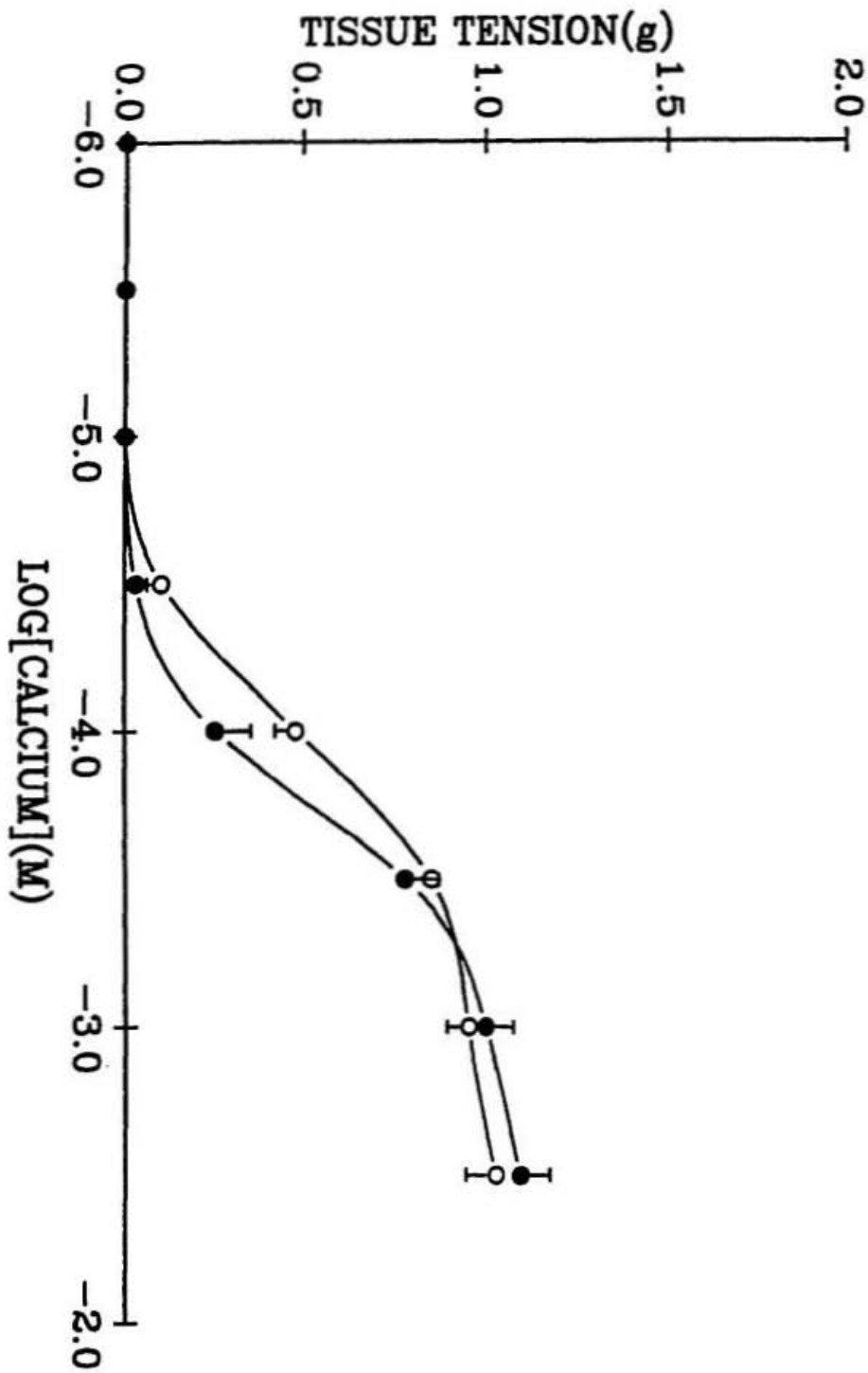


(b)

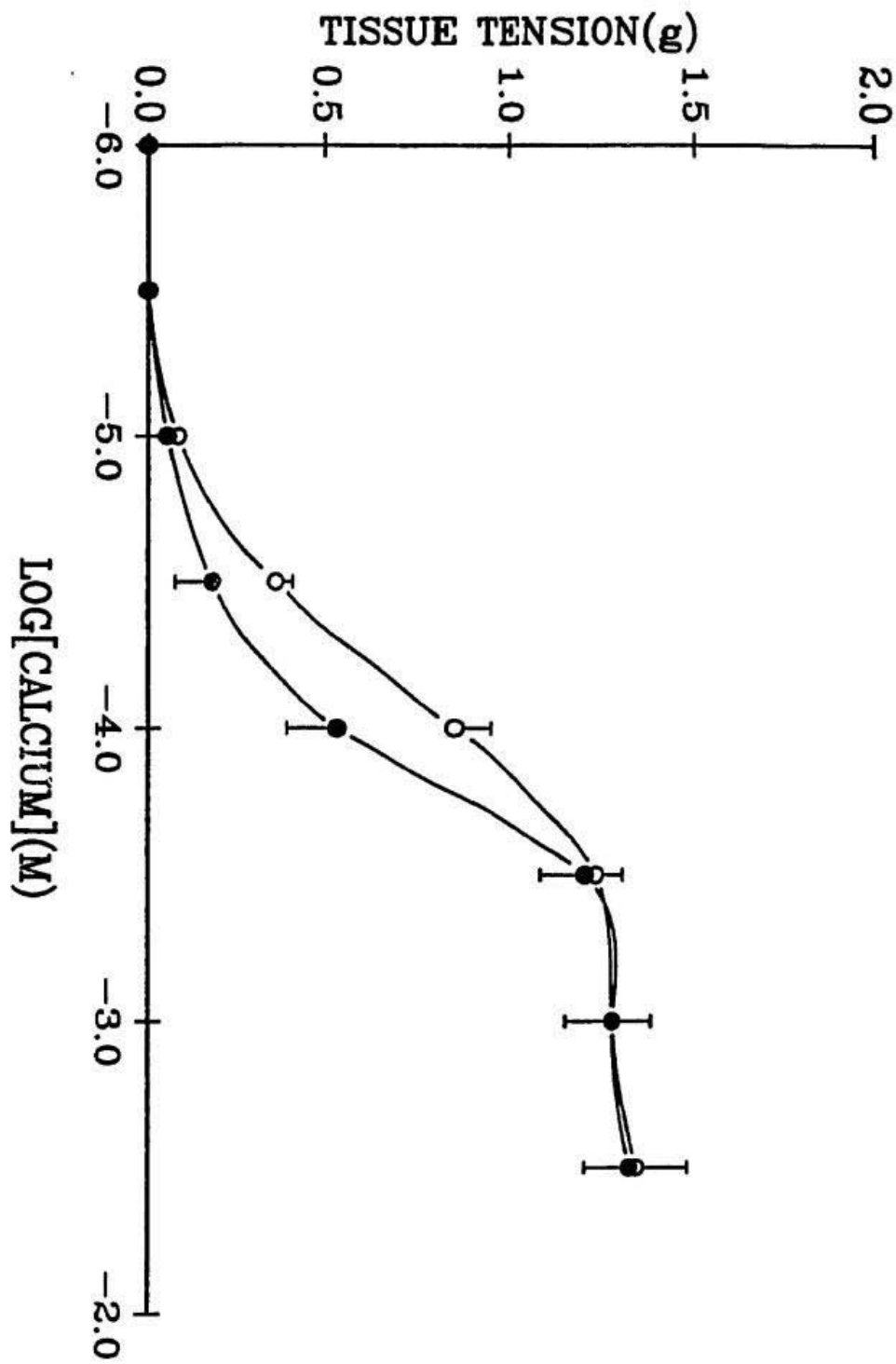


(c)

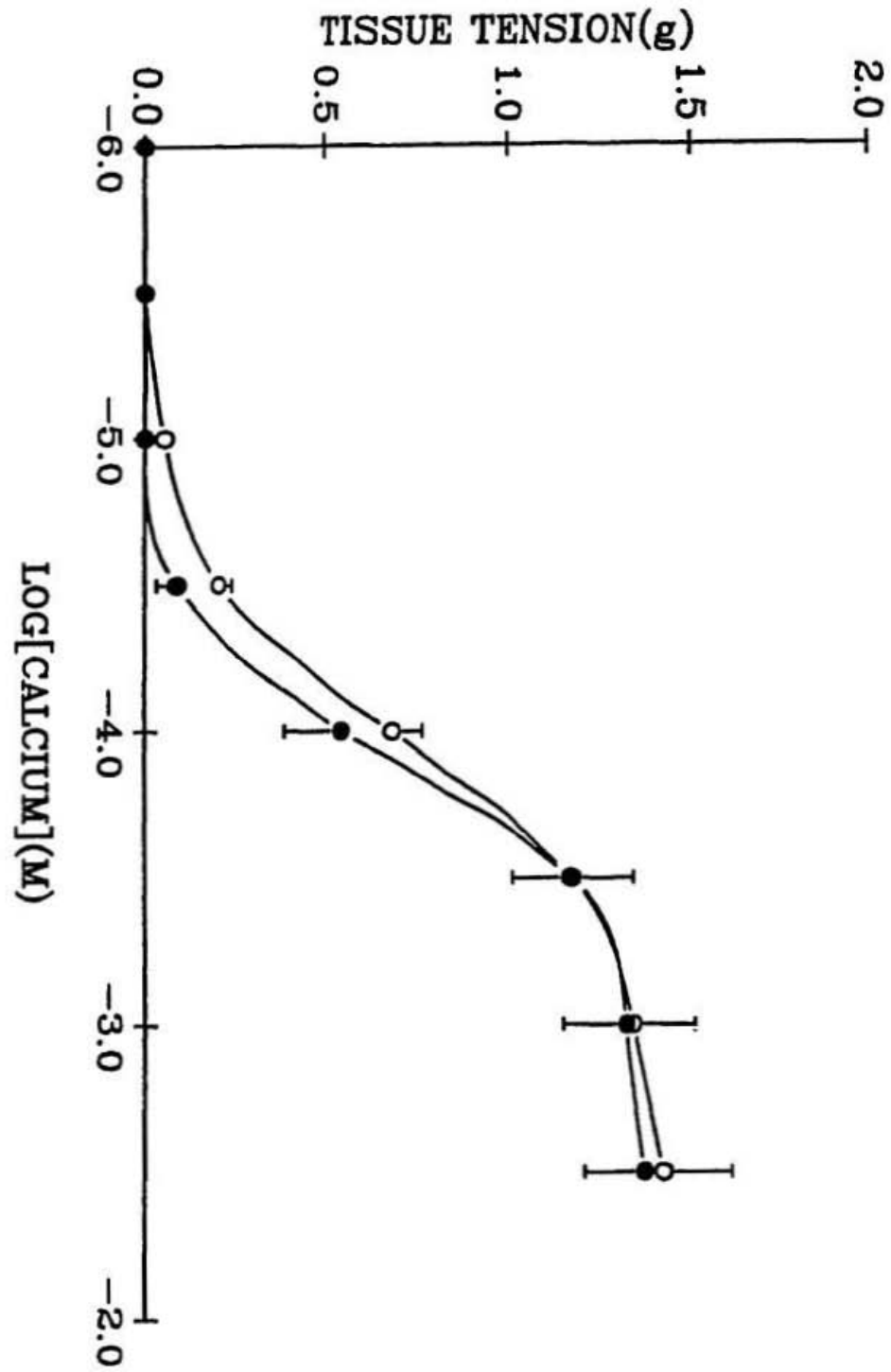
Figure 30a, b and c: Effect of 10 μM indomethacin on the Ca^{2+} concentration-response curve obtained in the aorta in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b, and c) 1 μM NE. Control curves ± S.E.M. are represented by open symbols e.g. O–O and after application of indomethacin by filled symbols e.g. ●–● . (n = 4)



(a)

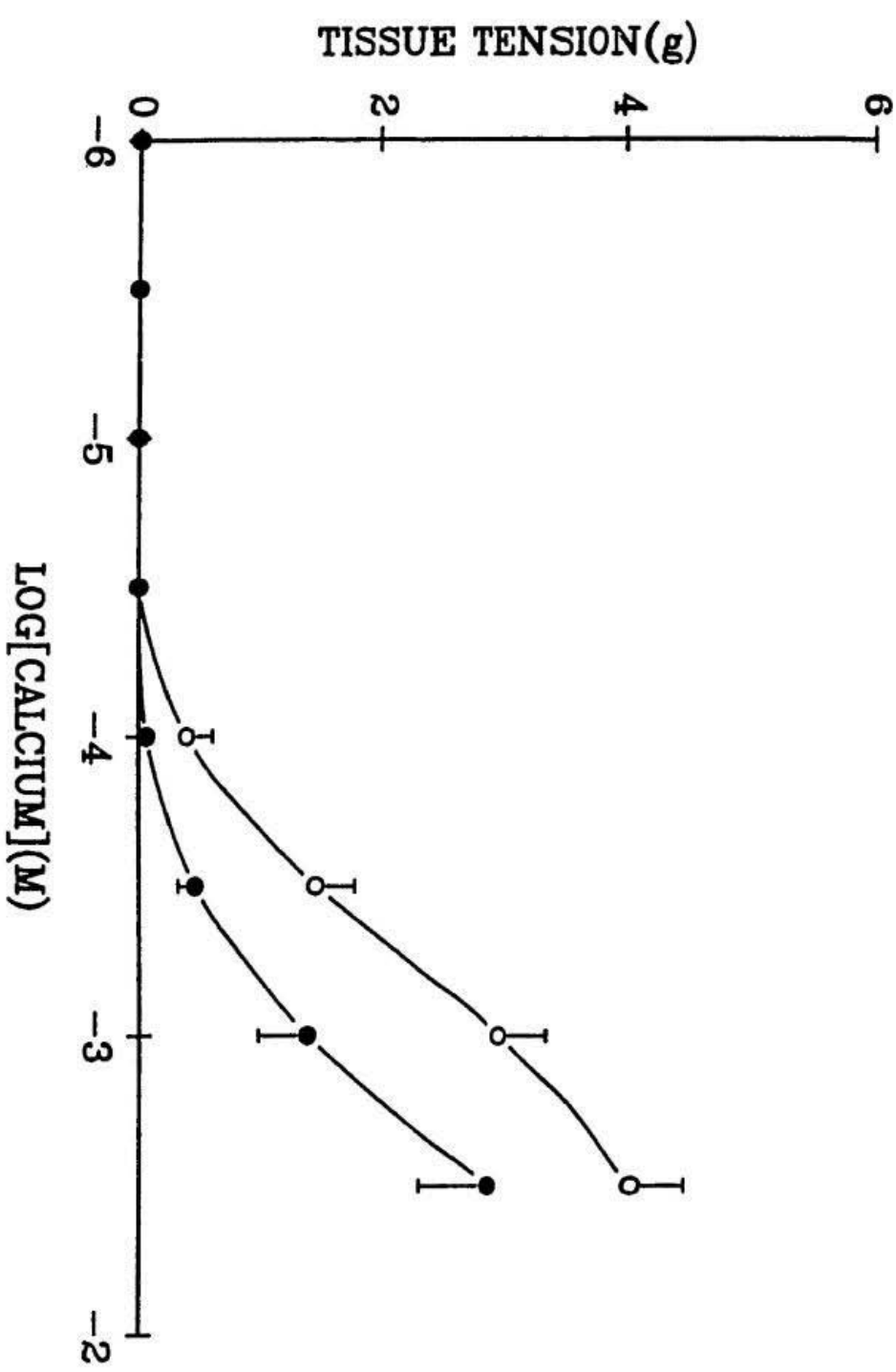


(b)

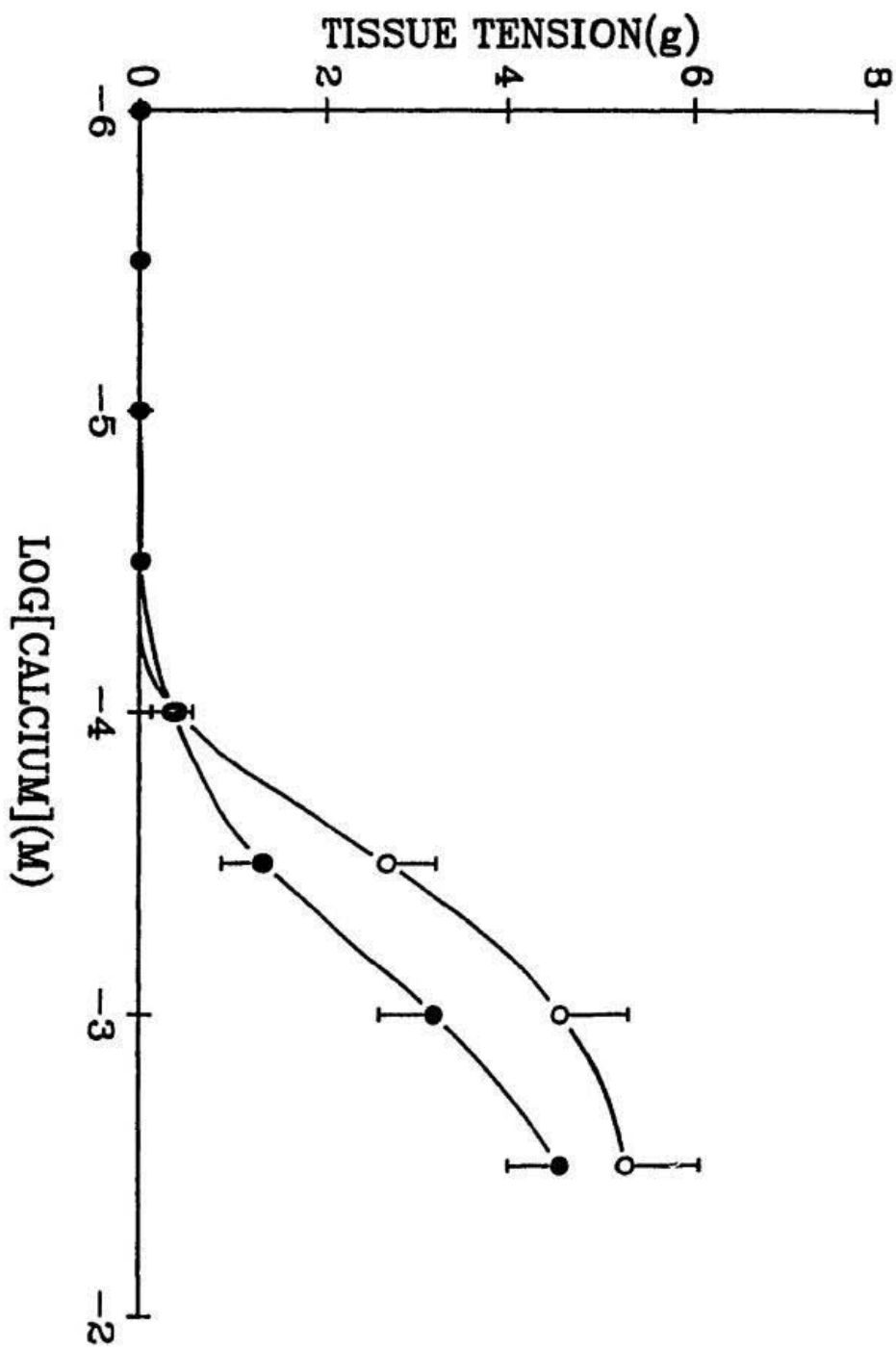


(c)

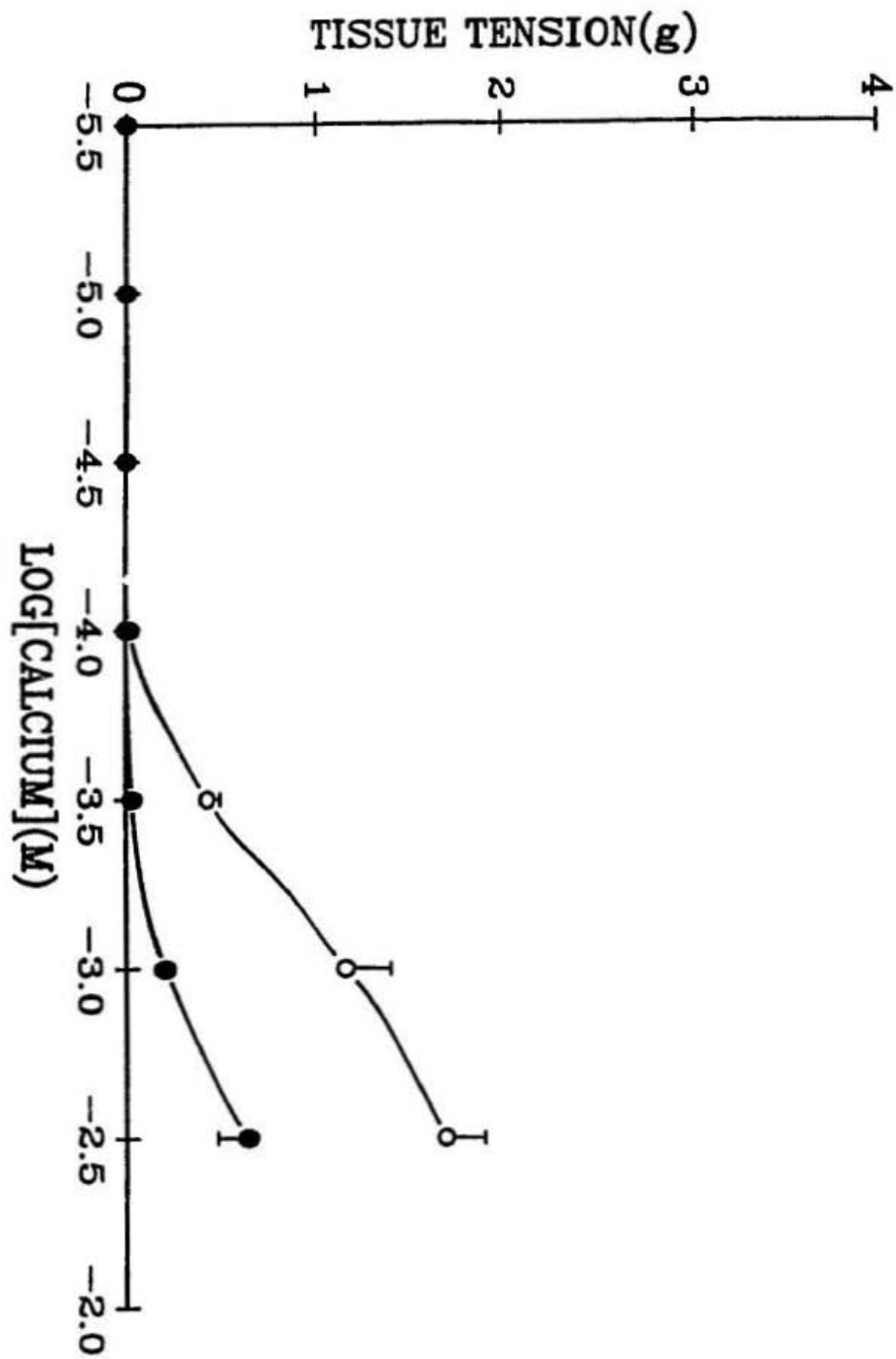
Figure 31a, b and c: Effect of 10 μM NDGA on the Ca^{2+} concentration-response curves obtained in the anococcygeus in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; and c) 1 μM NE . Control curves ± S.E.M. are represented by open symbols e.g. O-O and after application of NDGA by filled symbols e.g. ●-●. (n = 4)



(a)



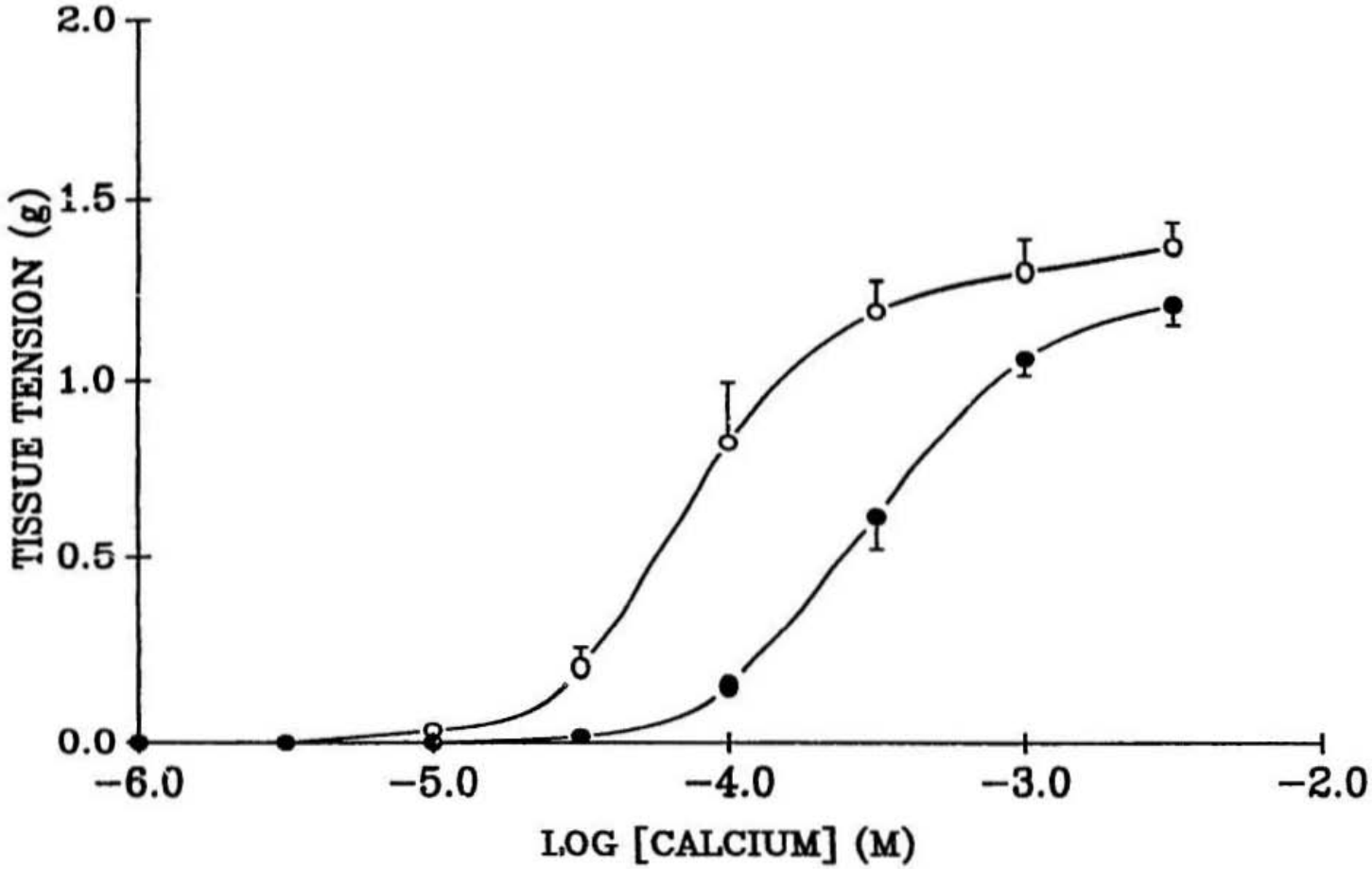
(b)

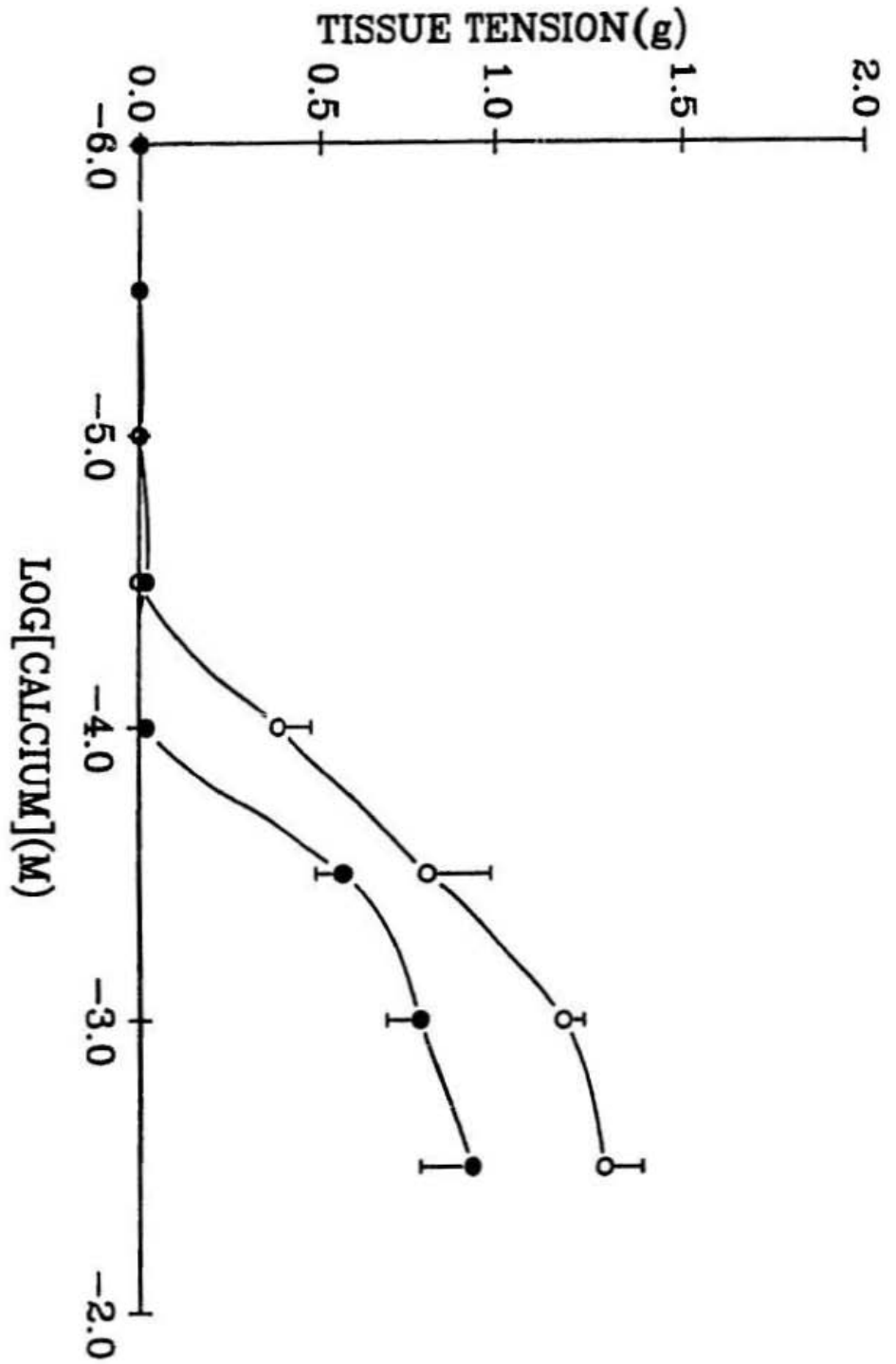


(c)

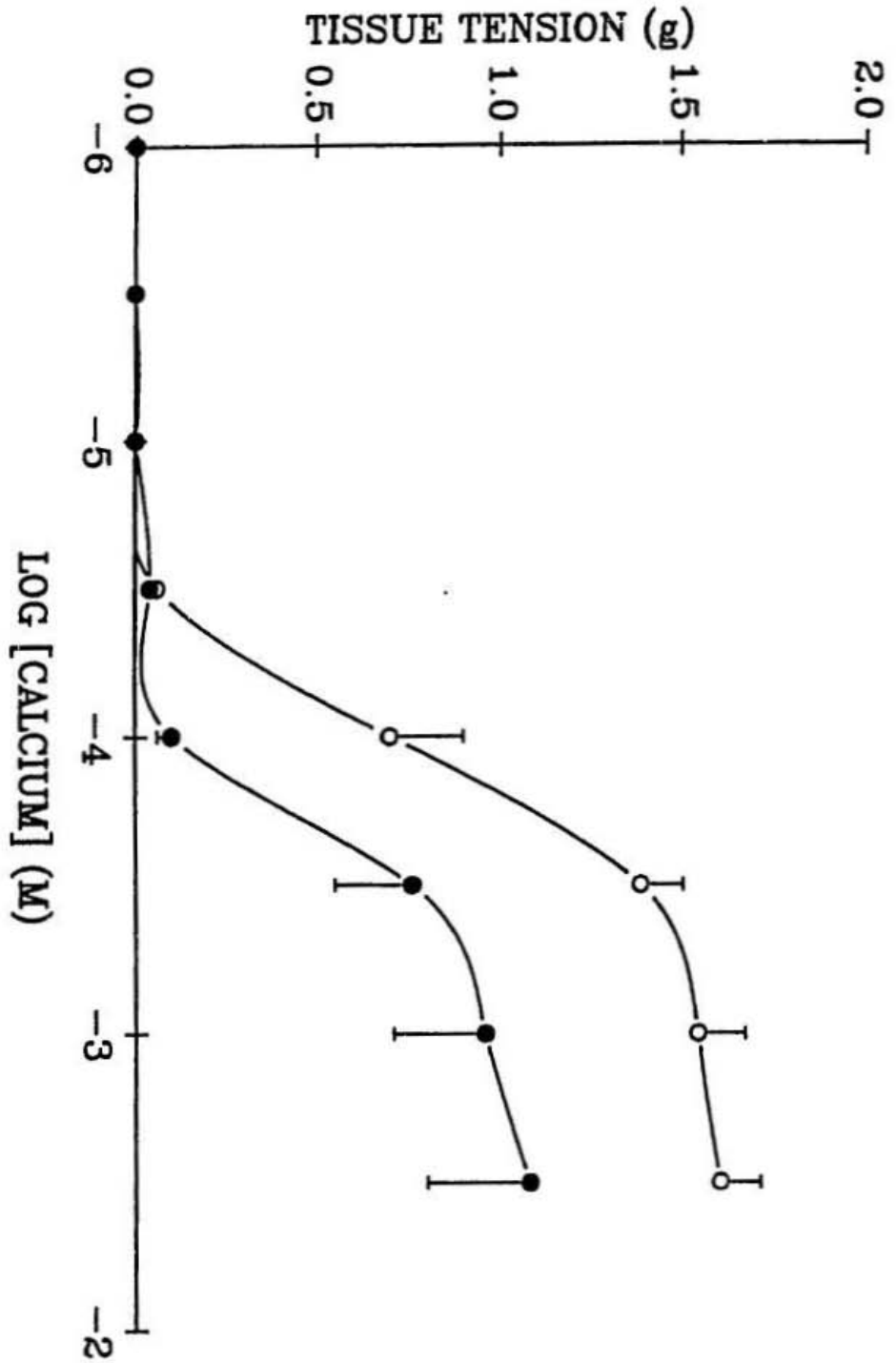
Figure 32a, b and c: Effect of 10 μM NDGA on the Ca^{2+} concentration-response curves obtained in the aorta in the presence of a) 10 nM ET-1; b) 10 nM SRTX-b; c) 1 μM NE . Control curves ± S.E.M. are represented by open symbols e.g. O–O and after application of NDGA by filled symbols e.g. ●-● . (n = 5)

(b)





(a)

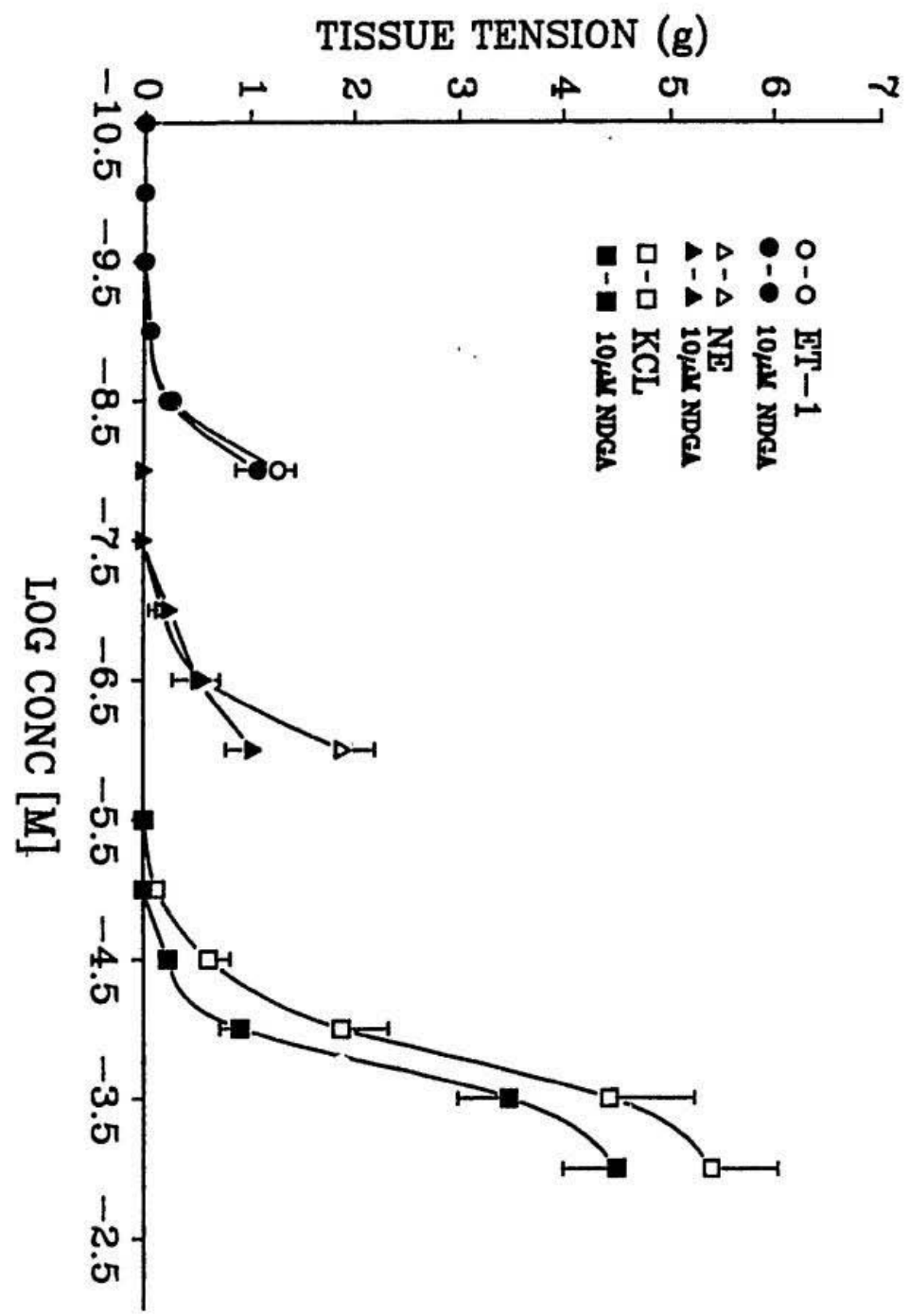


(c)

Figure 33 (a and b): a) Effect of 10 μM NDGA on agonist concentration-response curves in the anococcygeus to:- i) ET-1 O–O ; ii) NE Δ–Δ; iii) Ca^{2+} concentration-response curves in the presence of 50 mM K^+ □–□.

b) Effect of 10 μM NDGA on agonist concentration-response curves in the anococcygeus to :- i) SRTX-b ▽–▽; ii) NE O–O; iii) Ca^{2+} concentration-response curves in the presence of 50 mM K^+ □–□.

Control curves ± S.E.M. are represented by open symbols, e.g. O–O and after the application of NDGA by filled symbols, e.g. ●–●. (n = 6)


TISSUE TENSION (g)
0
1
2
3
4
5
6
7
LOG CONC [M]
−10.5
−9.5
−8.5
−7.5
−6.5
−5.5
−4.5
−3.5
−2.5
o-o ET−1
●-● 10μM NDGA
△-△ NE
▲-▲ 10μM NDGA
□-□ KCL
■-■ 10μM NDGA

(a)

(b)

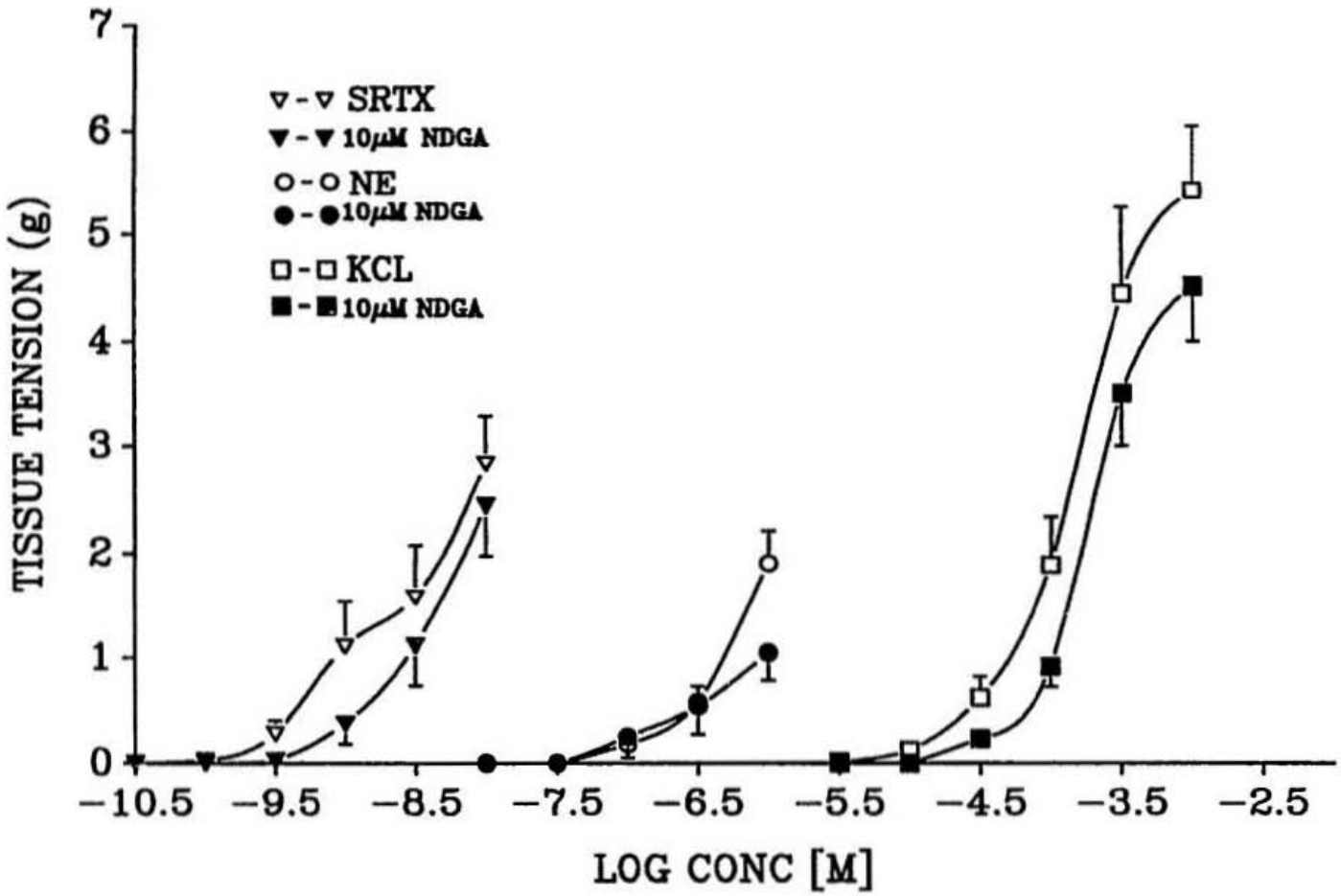
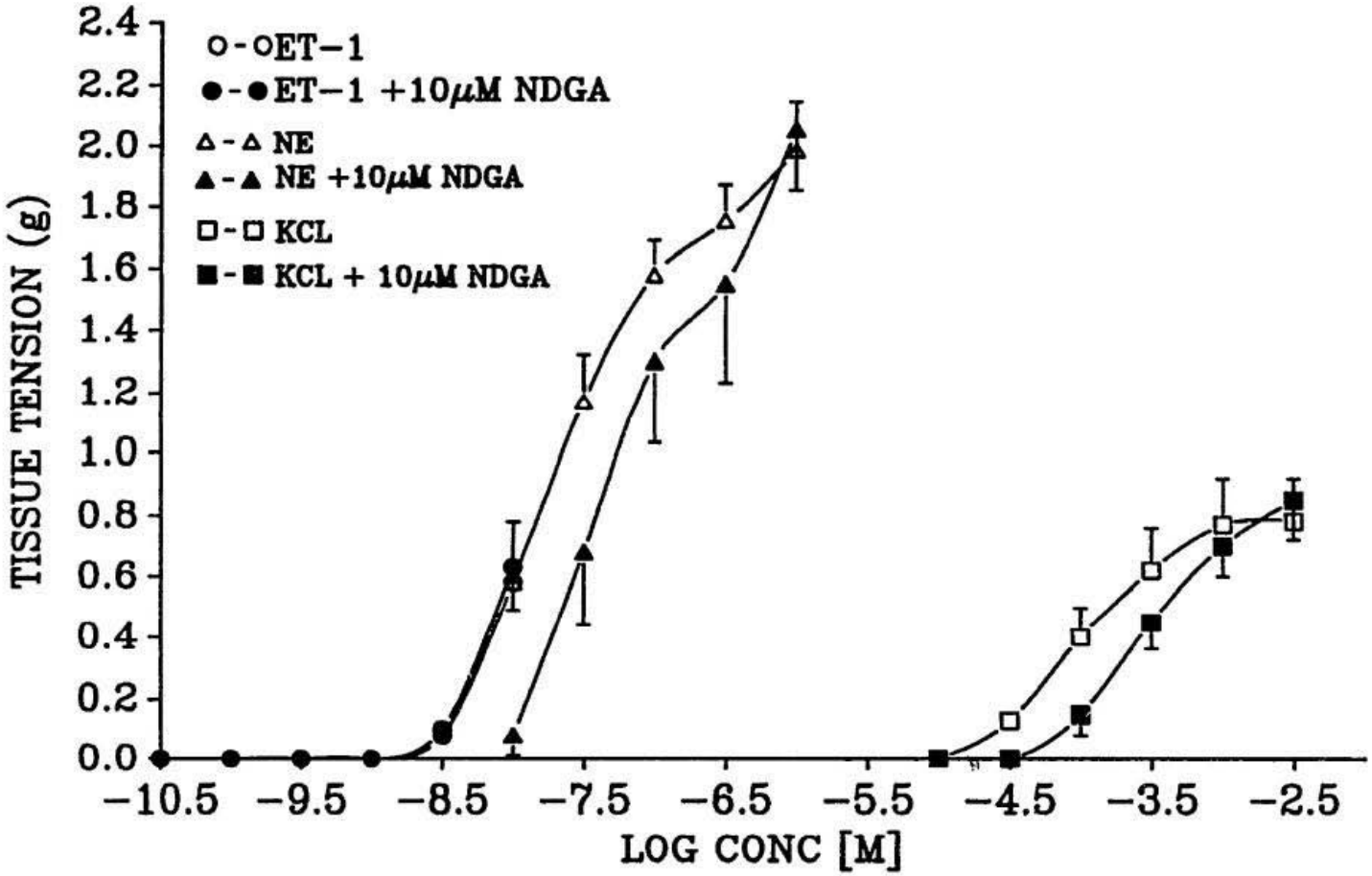

TISSUE TENSION (g)
7
6
5
4
3
2
1
0
▽-▽ SRTX
▼-▼ 10μM NDGA
○-○ NE
●-● 10μM NDGA
□-□ KCL
■-■ 10μM NDGA
−10.5
−9.5
−8.5
−7.5
−6.5
−5.5
−4.5
−3.5
−2.5
LOG CONC [M]

Figure 34 (a and b): a) Effect of 10 μM NDGA on agonist concentration-response curves in the aorta to :- i) ET-1 O-O; ii) NE Δ-Δ; iii) Ca^{2+} concentration-response curves in the presence of 50 mM K^+ □-□ .

b) Effect of 10 μM NDGA on agonist concentration-response curves in the aorta to :- i) SRTX-b ▽-▽ ; ii) NE Δ-Δ; iii) Ca^{2+} concentration-response curves in the presence of 50 mM K^+ □-□ .

Control curves ± S.E.M. are represented by open symbols, e.g. O-O and after application of NDGA by filled symbols, e.g. ●-● . (n = 6)

(a)


o-oET−1
●-●ET−1 +10μM NDGA
△-△ NE
▲-▲ NE +10μM NDGA
□-□ KCL
■-■ KCL + 10μM NDGA
TISSUE TENSION (g)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−10.5 −9.5 −8.5 −7.5 −6.5 −5.5 −4.5 −3.5 −2.5
LOG CONC [M]

(b)

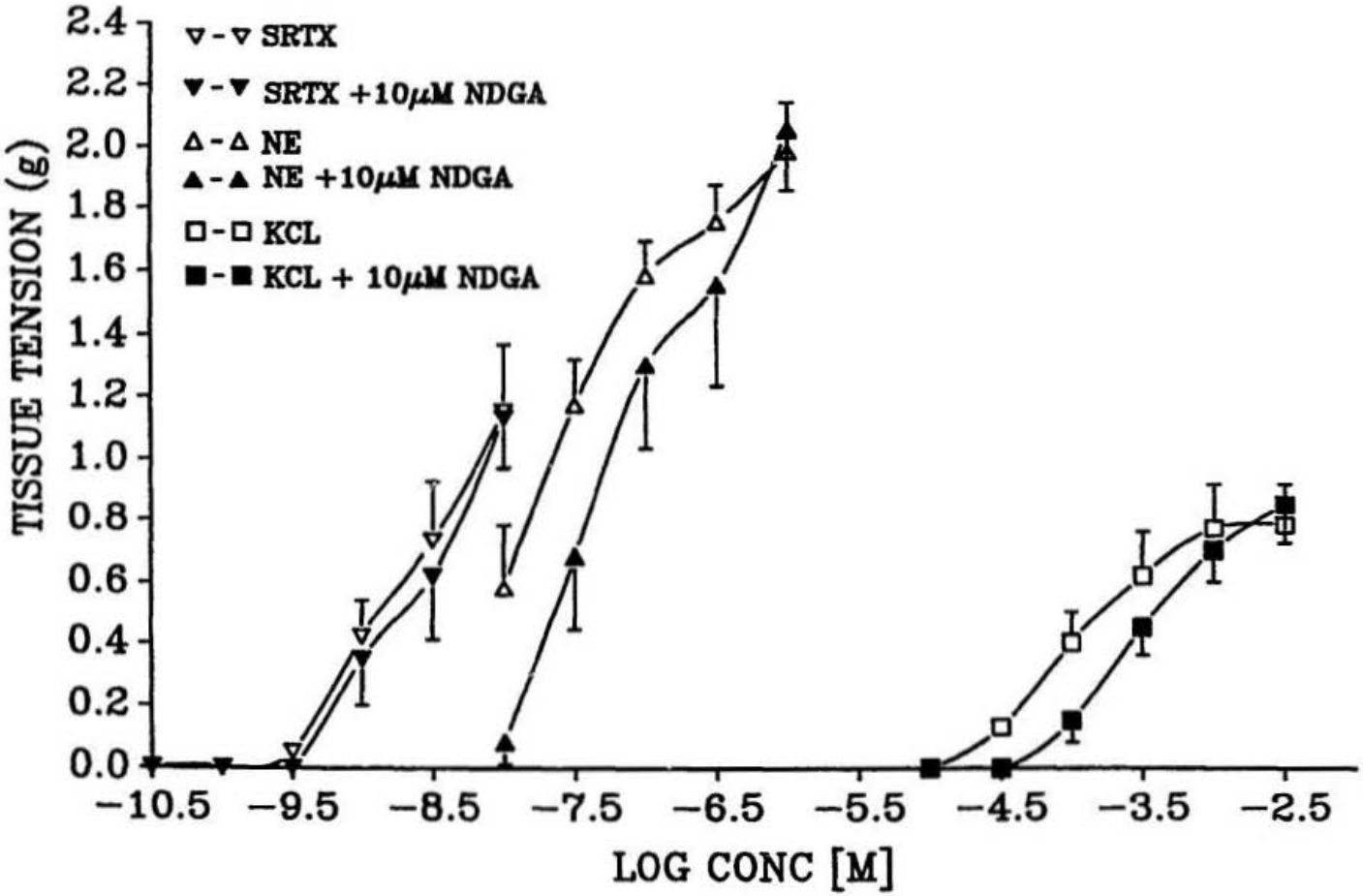
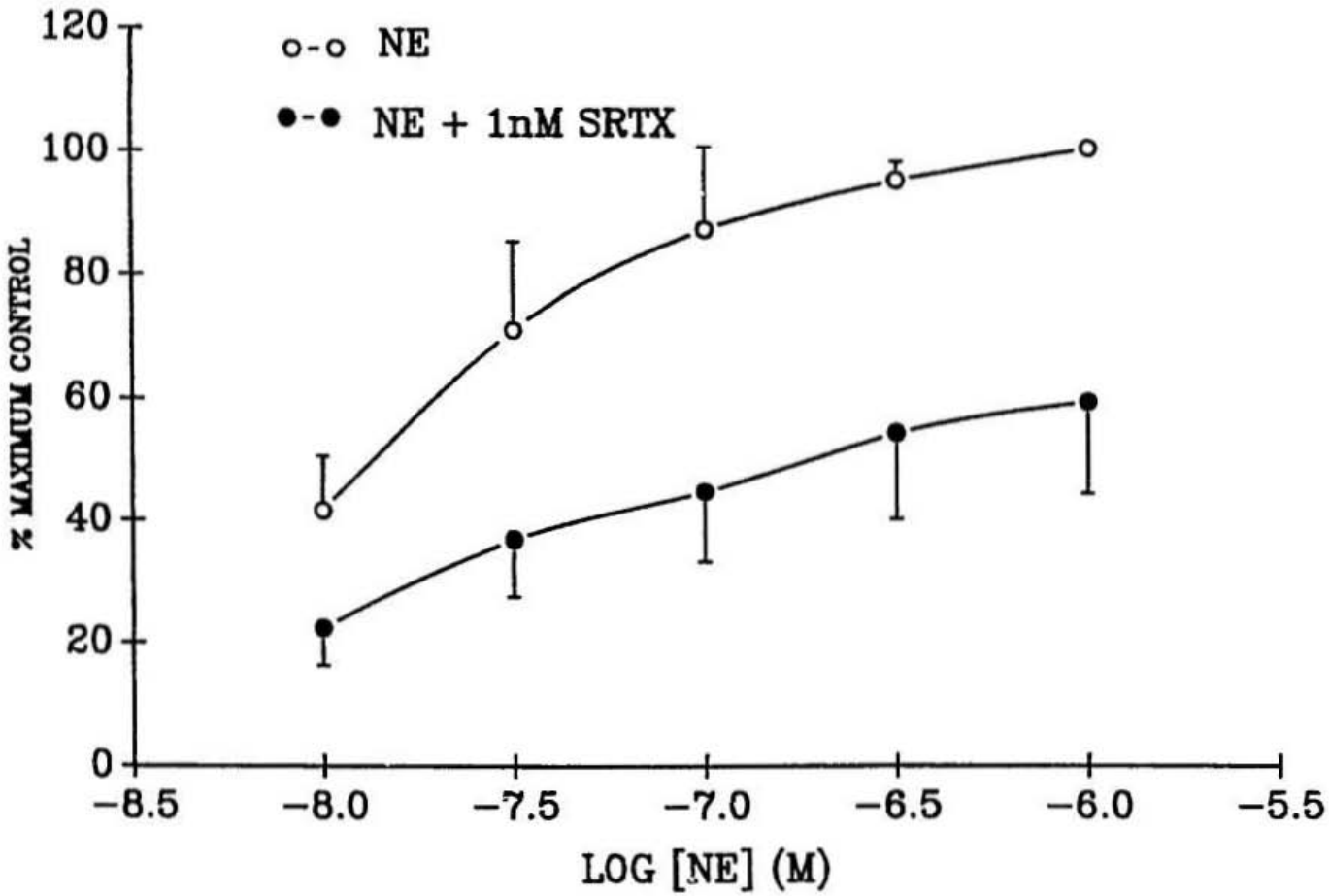
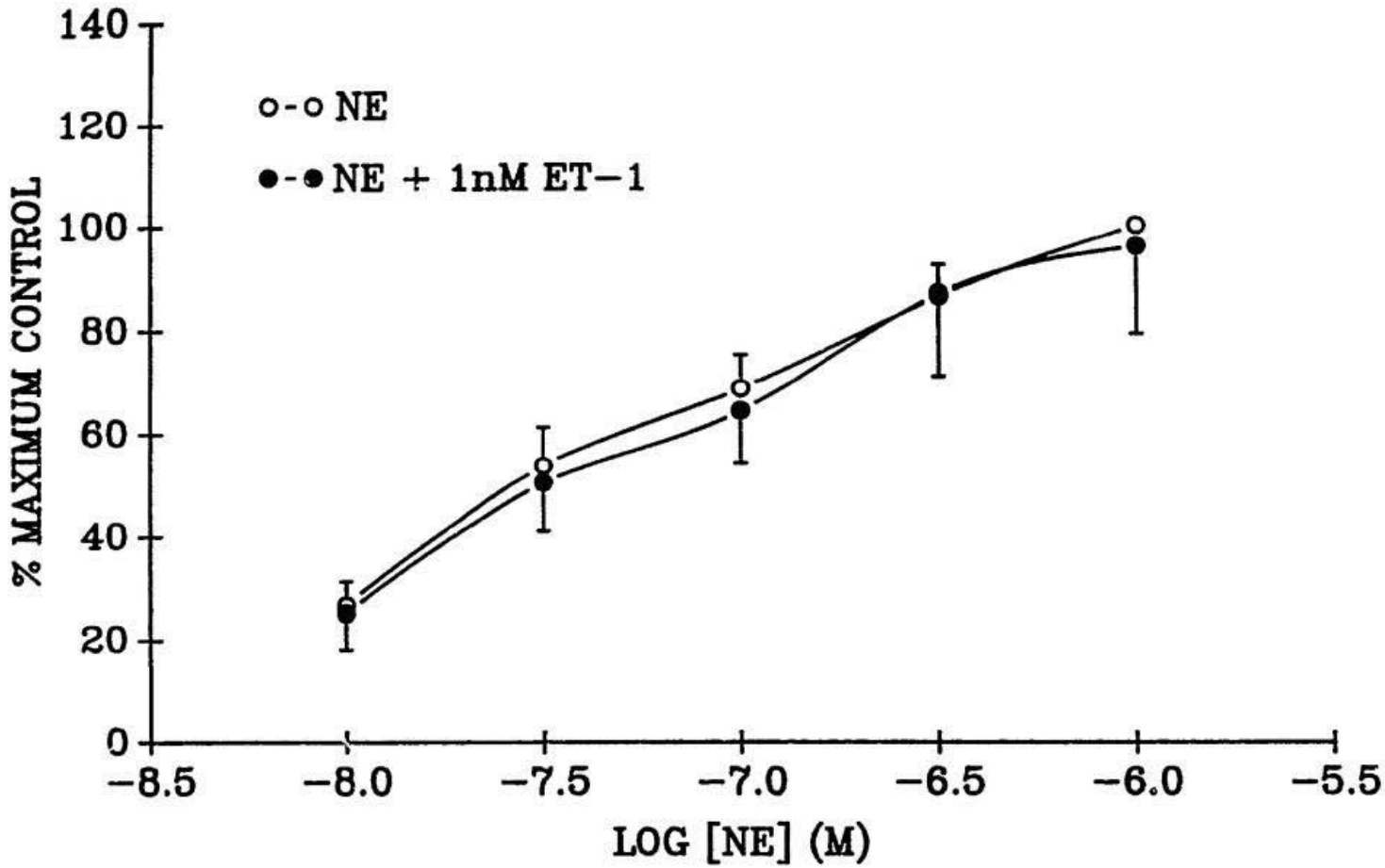

▽-▽ SRTX
▼-▼ SRTX +10μM NDGA
△-△ NE
▲-▲ NE +10μM NDGA
□-□ KCL
■-■ KCL + 10μM NDGA
TISSUE TENSION (g)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−10.5
−9.5
−8.5
−7.5
−6.5
−5.5
−4.5
−3.5
−2.5
LOG CONC [M]

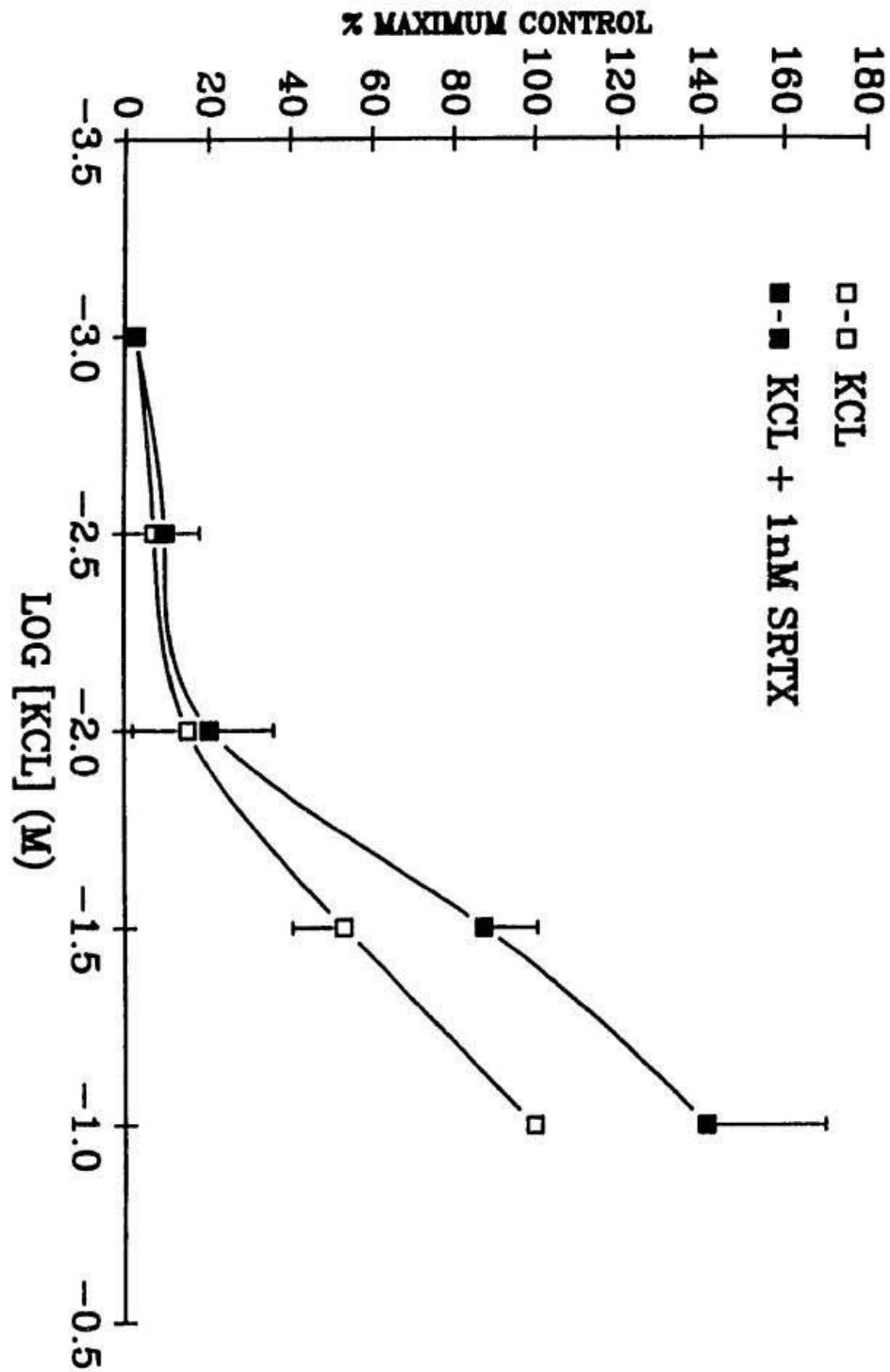
Figure 35a, b, c and d: Effect of SRTX-b and ET-1 on concentration-response curves to NE and KCL in the anococcygeus :- a) 1 nM SRTX-b ; b) 1 nM ET-1 on NE concentration-response curves; c) 1 nM SRTX-b ; d) 1 nM ET-1 on KCl concentration response curves. Control curves ± S.E.M. are represented by open symbols, e.g. O–O and after application of ET-1 or SRTX-b by filled symbols, e.g. ●–●. (n = 4)

(a)


120
100
80
60
40
20
0
% MAXIMUM CONTROL
o-o NE
●-● NE + 1nM SRTX
−8.5
−8.0
−7.5
−7.0
−6.5
−6.0
−5.5
LOG [NE] (M)

(b)


140
120
100
80
60
40
20
0
% MAXIMUM CONTROL
o-o NE
●-● NE + 1nM ET−1
−8.5
−8.0
−7.5
−7.0
−6.5
−6.0
−5.5
LOG [NE] (M)


% MAXIMUM CONTROL
180
160
140
120
100
80
60
40
20
0
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
LOG [KCL] (M)
□-□ KCL
■-■ KCL + 1nM SRTX

(c)

(d)

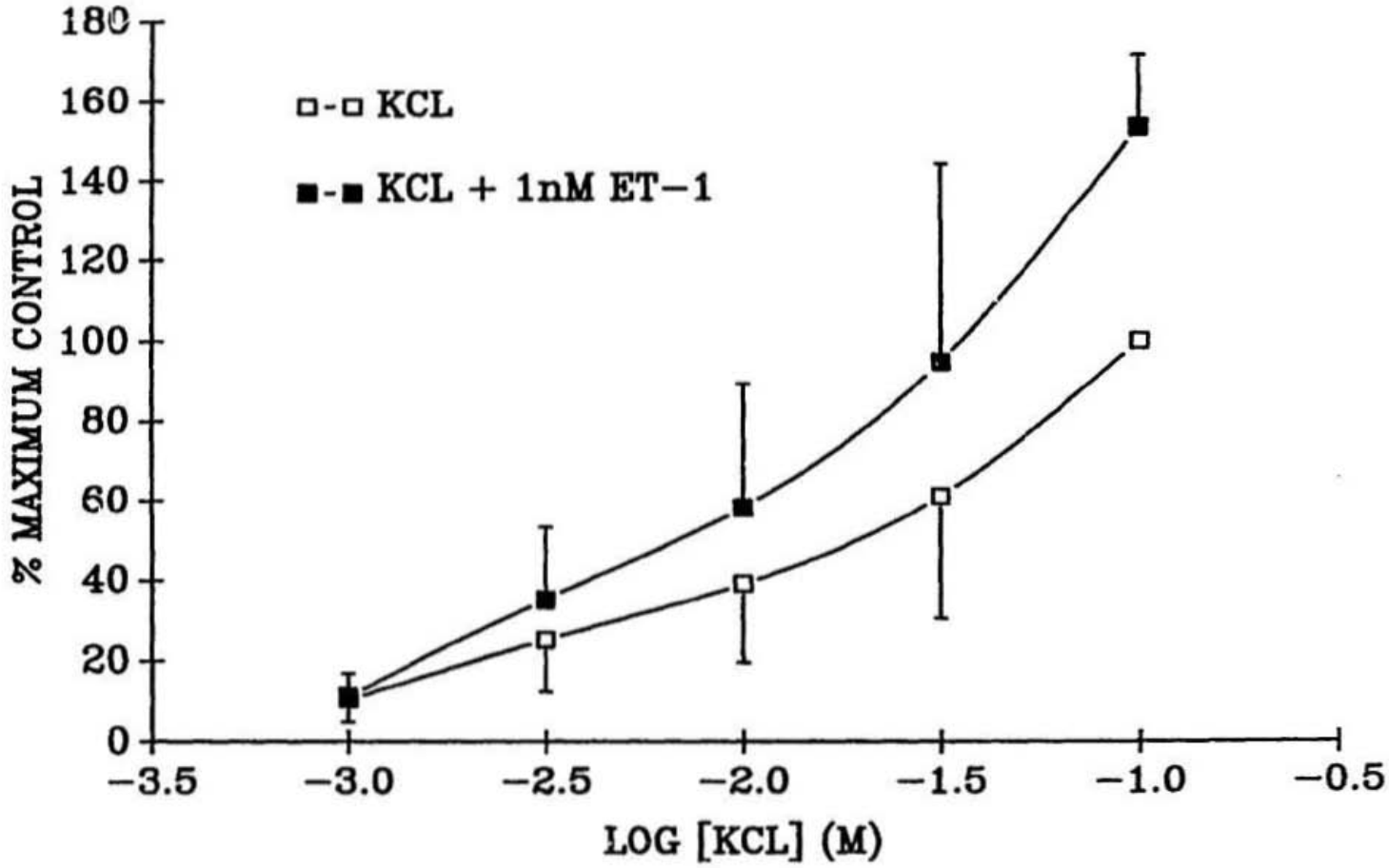
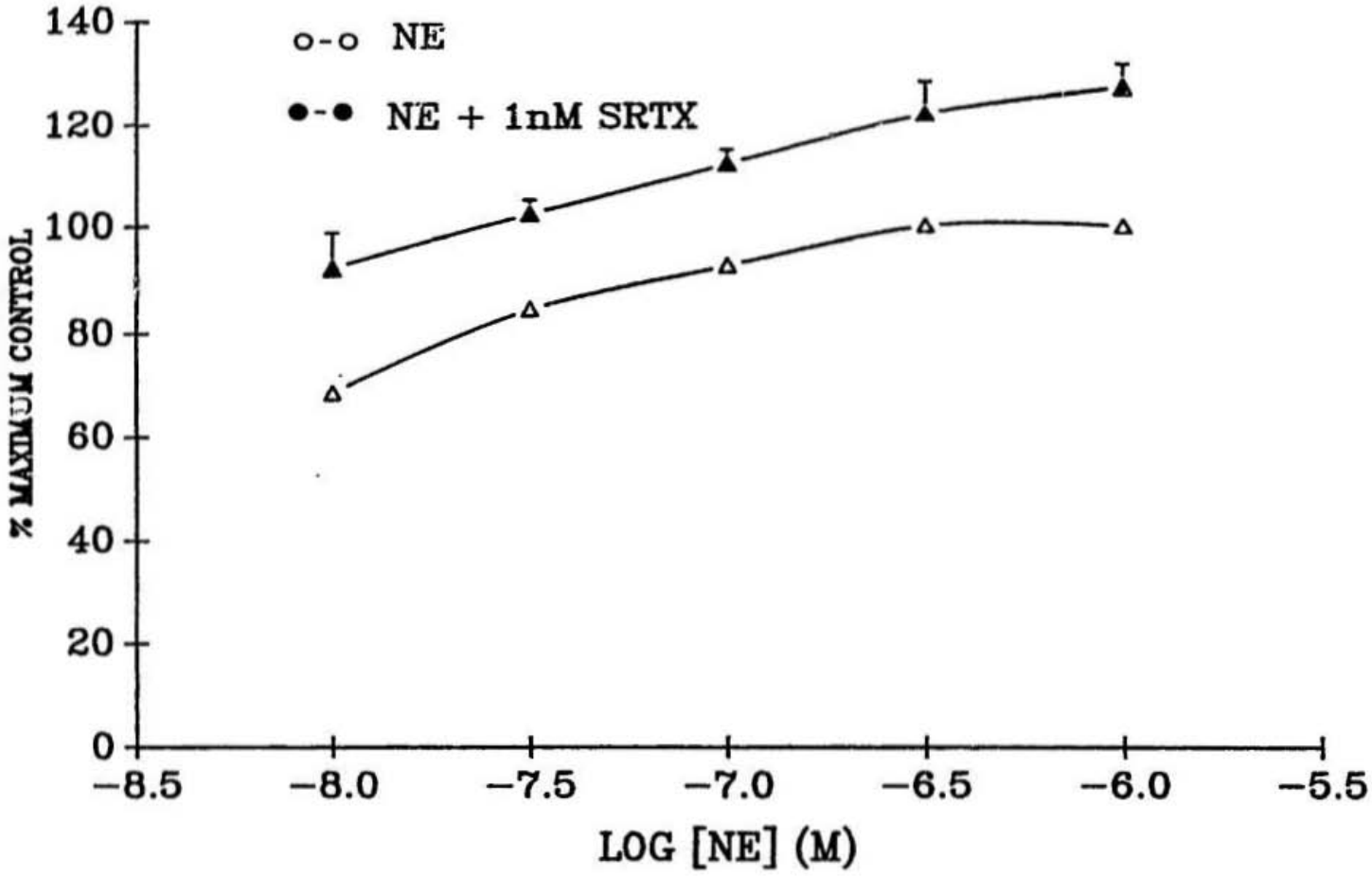

180
160
140
120
100
80
60
40
20
0
% MAXIMUM CONTROL
□-□ KCL
■-■ KCL + 1nM ET−1
−3.5
−3.0
−2.5
−2.0
−1.5
−1.0
−0.5
LOG [KCL] (M)

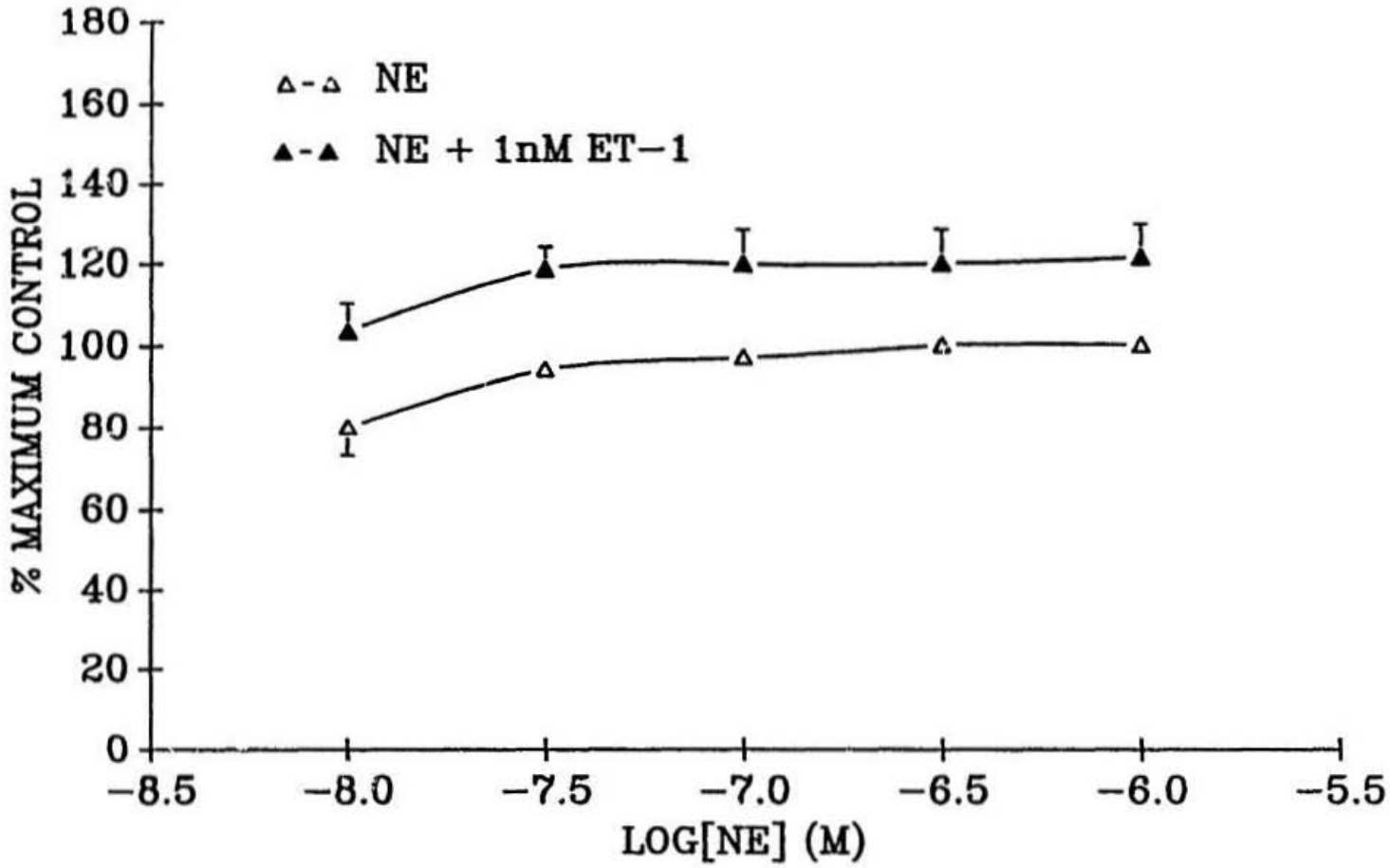
Figure 36a, b, c and d: Effect of SRTX-b and ET-1 on concentration-response curves to NE and KCL in the aorta :- a) 1 nM SRTX-b; b) 1 nM ET-1 on norepinephrine concentration-response curves; c) 1 nM SRTX-b; d) 1 nM ET-1 on KCl concentration-response curves .

Control curves ± S.E.M. are represented by open symbols, e.g. O-O and after application of ET-1 or SRTX-b by fil'ed symbols, e.g. ●-● . (n = 4)

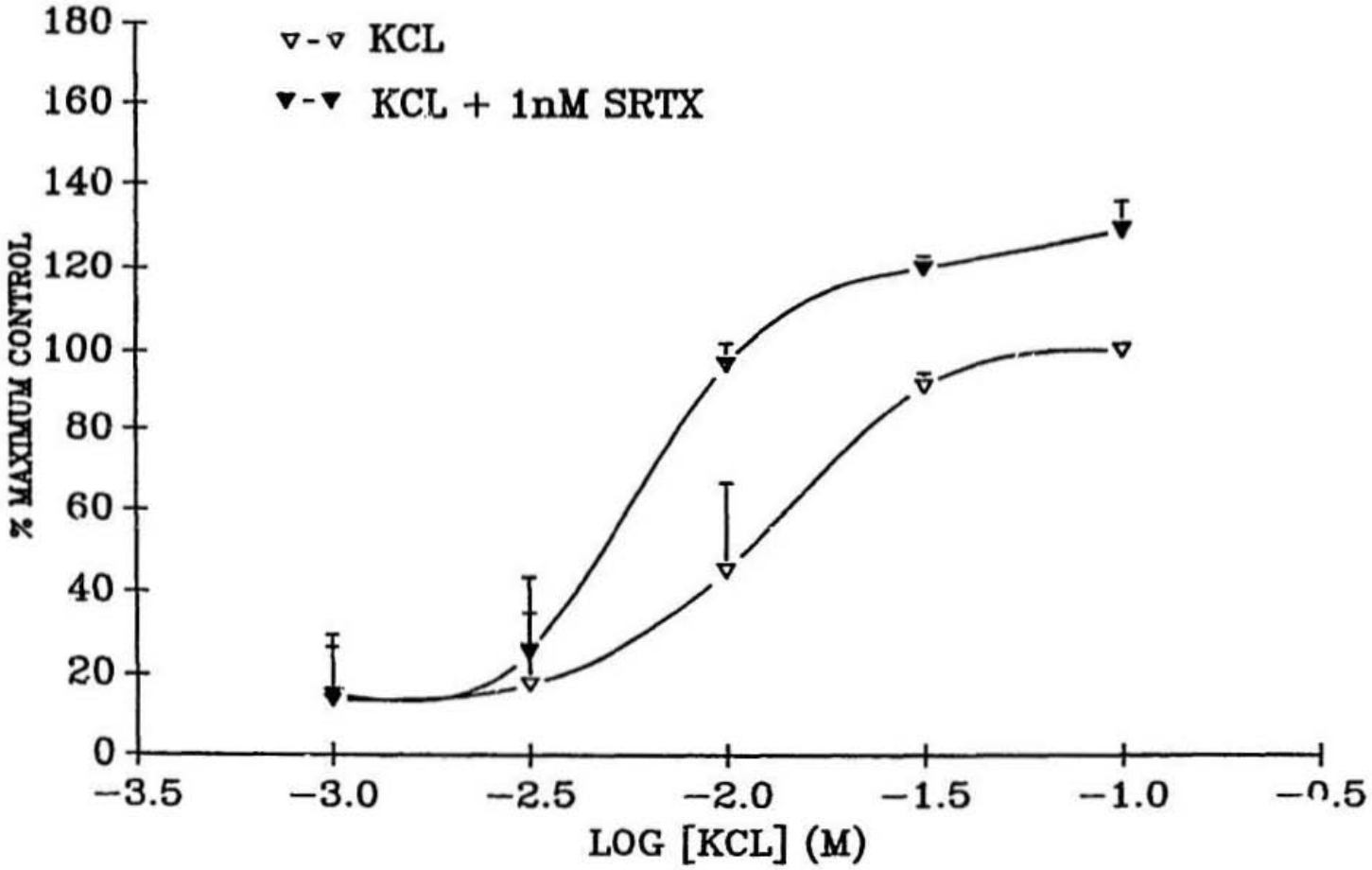
(a)


o-o NE
●-● NE + 1nM SRTX
% MAXIMUM CONTROL
140
120
100
80
60
40
20
0
−8.5
−8.0
−7.5
−7.0
−6.5
−6.0
−5.5
LOG [NE] (M)

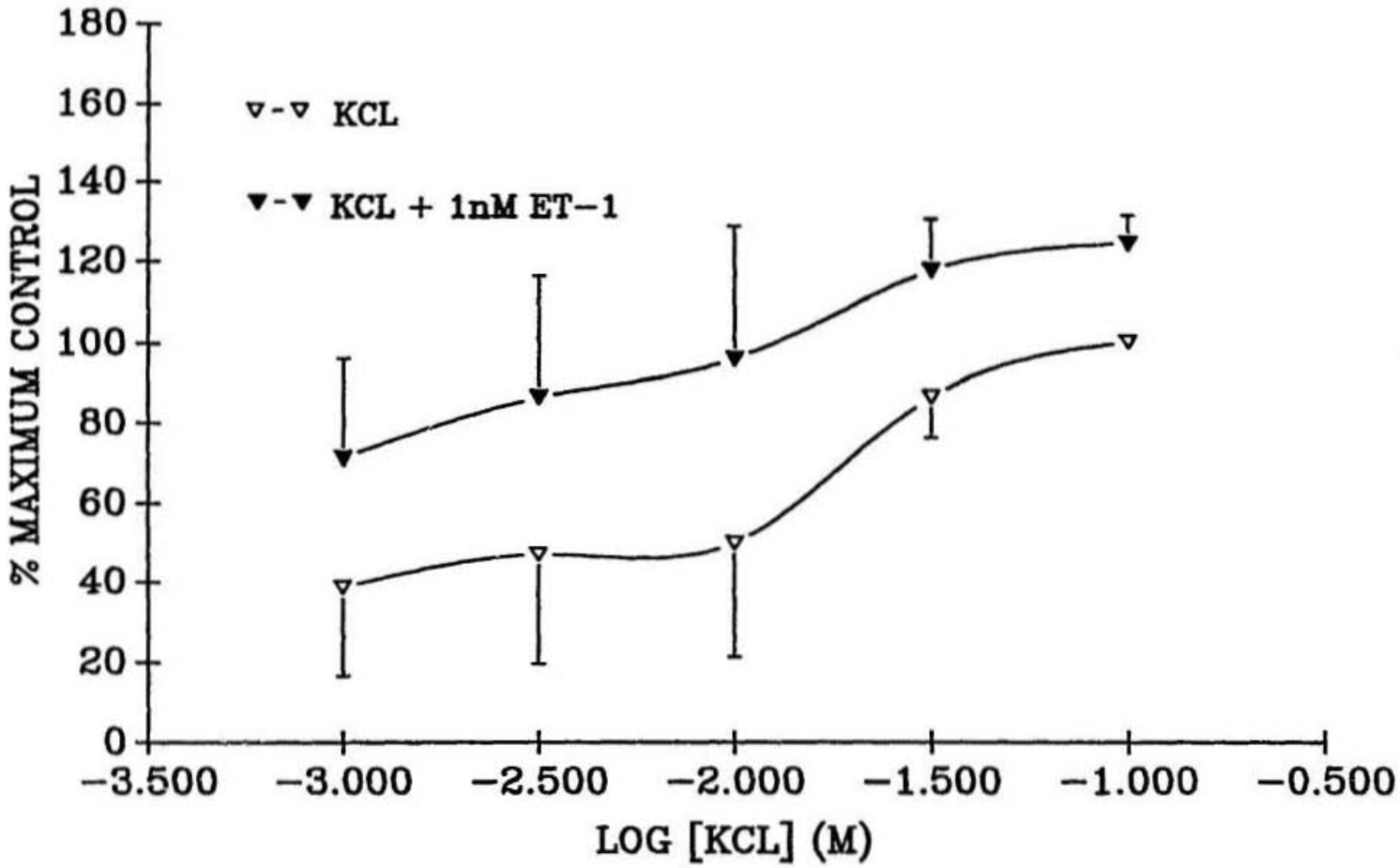
(b)


180
160
140
120
100
80
60
40
20
0
% MAXIMUM CONTROL
△-△ NE
▲-▲ NE + 1nM ET−1
−8.5
−8.0
−7.5
−7.0
−6.5
−6.0
−5.5
LOG[NE] (M)

(c)


▽-▽ KCL
▼-▼ KCL + 1nM SRTX
% MAXIMUM CONTROL
180
160
140
120
100
80
60
40
20
0
−3.5
−3.0
−2.5
−2.0
−1.5
−1.0
−0.5
LOG [KCL] (M)

(d)


180
160
140
120
100
80
60
40
20
0
% MAXIMUM CONTROL
▽-▽ KCL
▼-▼ KCL + 1nM ET-1
-3.500
-3.000
-2.500
-2.000
-1.500
-1.000
-0.500
LOG [KCL] (M)

CHAPTER 4

DISCUSSION

4.1. General considerations.

This study focused on the similarities and differences between the mode of actions of ET-1, SRTX-b and NE on vascular and non-vascular smooth muscle. The rationale for choosing to compare the responses of both vascular and non-vascular smooth muscle preparations to endothelin and sarafotoxin versus NE reflects the widespread biological effects of these peptides on both vascular and non-vascular smooth muscle, as well as their endocrine effects on the adrenal glands, kidney and neurohypophysis. A comparison with the effects of NE was also considered logical since the effects of this amine on smooth muscle have been extensively studied.

For such a comparison to be valid it was considered important to minimize the possible variables to a controllable number. Cognisance was taken of criticism of isolated preparations being in an artificial medium and perhaps reflecting a non-physiological situation. However, the choice of an isolated preparation avoids the effects of the peptides on neuronal, endocrine and cardiovascular variables. Criteria earlier described in the methods section were used to select the experimental tissues. Consequently, the final choice of aorta and anococcygeus muscle was not only the result of pharmacological screening but also of the published properties of these tissues and the fact that both the control and experimental tissues were obtainable from the same animal.

Furthermore, the choice and emphasis on studying the role and sources of calcium utilized in mediating the effects of ET-1 and SRTX-b reflects a major

interest in the role of a cation that is known to carry external signals to the cell interior with a resultant biologic response. Moreover, calcium has been implicated in altered smooth muscle reactivity in hypertension (Fitzpatrick and Szentivanyi, 1980; Kwan, 1985; Dominiczak and Bohr, 1990), and the finding of high endothelin contents in bronchial exudates of asthmatics (Nomora *et al.*, 1989) suggests that the relationship between calcium, endothelin and smooth muscle dysfunction is an important issue.

The involvement of several amplifier enzymes such as phospholipase C, phospholipase A_2, phospholipase D, adenylate cyclase and guanylate cyclase in the signal transduction process made it imperative to study the contribution or impact of the products of these enzymes in the absence of confounding variables of nerves, circulating hormones and their metabolites.

It has been stated that ET-1 and SRTX-b share the same membrane receptor and cellular mode of action (Kloog and Sokolovsky, 1989) but, since there are only 66% identical amino acid sequences between ET-1 and SRTX-b (Hirata *et al.*, 1988a; Watanabe *et al.*, 1989; Yanagisawa and Masaki, 1989a), this conclusion may be questioned. Studies based upon differences in recovery time following contractions to ET-1 versus SRTX-b with recovery in either Ca^{2+}-containing or Ca^{2+}-free Krebs suggest a role for Ca^{2+} in the binding and dissociation of ET-1 and SRTX-b to their receptor(s). It is speculated that these differences in apparent dissociation constants may reflect the already noted differences in the amino acid composition of ET-1 versus SRTX-b and resultant differences in receptor binding properties.

Recently, in an endothelin conference review, Webb (1991) noted that the IUPHAR Committee on Receptor Nomenclature and Drug Classification recommended that the ET-1 'selective' receptor described by Arai *et al.*(1990)

and which may be the vascular smooth muscle receptor, be named the ET_A receptor, and the nonselective receptor described by Sakurai, be named the ET_B receptor. The endothelin receptor cloned by Arai has 427 amino acid residues (48.5 kDa) and that by Sakurai *et al.* (1990) consists of 415 amino acid residues (46.9 kDa). They contrast remarkably with two receptors earlier isolated in cultured rat mesangial cells (Sugiura *et al.*, 1989) with Mw. of 58,000 and 34,000. It is unclear whether the differences in molecular weight of the receptors is a tissue phenomenon or the result of techniques applied in the isolation process.

4.2. Role of extracellular calcium (Ca^{2+}).

The first description of the importance of Ca^{2+} in the fluid bathing tissues was made by Ringer (1883) and by implication an intracellular messenger involved in contraction. A series of observations then showed that calcium regulates not only contraction but also many other cellular processes such as secretion of hormones (Kojima *et al.*, 1985), neurotransmitters (Israel *et al.*, 1979; Knight and Baker, 1982),and glycogen metabolism in the liver (Cohen, 1979; Garcia-Sainz and Hernandez-Sotomayor, 1985). Some of the responses mediated by Ca^{2+} are brief (and often repetitive), but others are quite prolonged. Th. *a* diversity of calcium messenger patterns has come to be recognized as organizational diversity of calcium messenger systems characteristic of cell type, tissue or organ (Rasmussen, 1986). Thus, there is a striking difference in the manner by which Ca^{2+} serves to couple stimulus to contraction in skeletal muscle as compared to smooth muscle or cardiac muscle. The ultrastructural arrangement which allows for differential source or storage of Ca^{2+} has been related to phasic and tonic responses in the three types of muscles mentioned. In skeletal muscle, a distinct source of Ca^{2+}

involved in the contraction and relaxation is the sarcoplasmic reticulum (Ebashi *et al.*, 1978). Depolarization of the plasma membrane, linked to the sarcoplasmic reticulum via the T system (a functional complex), induces the release of Ca^{2+} leading to contraction; relaxation is achieved by repolarization and reaccumulation of Ca^{2+} by the sarcoplasmic reticulum (see review by Rasmussen, 1986). In cardiac muscle, Ca^{2+} cycles across both the plasma membrane and the sarcoplasmic reticulum membrane. Depolarization of the plasma-membrane leads to an influx of Ca^{2+} through voltage dependent channels, which results in the release of Ca^{2+} from the sarcoplasmic reticulum via a calcium-induced calcium release process (Chapman, 1979). The resulting rise in intracellular calcium initiates a contractile response, and relaxation is achieved by a reversal of the Ca^{2+} cycle.

The situation in smooth muscle is quite different as, notably in vascular smooth muscle, smooth muscle has the capability to maintain a contractile response for hours with or without depolarization (Bolton, 1979; Hashimoto *et al.*, 1986). The sarcoplasmic reticulum of smooth muscle has been shown to be an intracellular organelle of variable size but in vascular smooth muscle it can be as extensive as in mammalian cardiac muscle and can accumulate Ca^{2+} and other divalent cations, e.g.Sr^{2+}(Devine *et al.*, 1972). It is noteworthy that Devine *et al.* (1972) demonstrated that there is a general correlation among different smooth muscles between the importance of extracellular Ca^{2+} in receptor-mediated contractile responses and the relative volume of sarcoplasmic reticulum in the muscle (see review, Minneman, 1988). For example, rabbit mesenteric vein had only 2.2% sarcoplasmic reticulum and depended completely on extracellular Ca^{2+} for agonist-induced contractions, whereas strips of main pulmonary artery had 5.1% sarcoplasmic reticulum and

retained a significant contractile response to agonist in the absence of extracellular Ca^{2+} (Devine *et al.*, 1972). Possibly the size of the storage pool for releasable calcium influences the relative contribution which extracellular and intracellular Ca^{2+} make to agonist-mediated responses in smooth muscle, although this does not necessarily explain how receptor activation is linked to both sources of calcium (Minneman review, 1988). In many muscles, the different phases of contraction have a different dependence on extracellular Ca^{2+} (Bevan *et al.*, 1982). However there is no clear correlation between phasic and tonic contractions and the importance of Ca^{2+} influx (see review, Minneman 1988). In rabbit aorta and ear artery, rapid phasic contractions caused by NE are not dependent on the presence of extracellular Ca^{2+} (Deth and van Breemen, 1977), whereas in rat mesenteric arteries or resistance vessels, NE-induced rapid phasic contractions are abolished in a Ca^{2+}-free medium (Godfraind and Miller, 1983). In rat anococcygeus muscle, slow tonic contractions to NE are less sensitive to inhibition by Ca^{2+} entry blockers than are phasic contractions (Oriowo, 1984), while the reverse holds true in rabbit aorta (Deth and van Breemen, 1977).

The results in Table 3 show the relative contribution of Ca^{2+} to the contractile process in rat aorta and anococcygeus muscle for ET-1, SRTX-b and NE respectively to be 17±3%, 15±1.7% and 21±2.3% of maximal contraction in the aorta and 5±.3%, 5±.7% and 6±1.08% of maximal contraction in the anococcygeus muscle in normal Krebs solution. A noteworthy characteristic of these contractions was that the peptides ET-1 and SRTX-b, whether in Ca^{2+}-free or Ca^{2+}-containing medium, were able to initiate tonic contractions in both the aorta and anococcygeus, whereas only phasic contractions to NE were seen in both the aorta and anococcygeus when Ca^{2+}-free Krebs was used (Figure

8). These results are consistent with those of Wallnöfer *et al.* (1989) from mesenteric resistance vessels (MRV). The failure to tone in a Ca^{2+}-free medium after a transient contraction induced by NE in rabbit inferior vena cava and rabbit aorta has also been noted by Khalil and van Breemen (1990) and Miasiro and Paiva (1990) respectively. The finding of a small sustained contraction induced by the peptides in Ca^{2+}-free medium is consistent with that of other reports of the actions of ET-1(Huang *et al.* 1990; Miasiro and Paiva, 1990). Most comparative studies of ET-1 and SRTX-b allude to their similarities of action and their responses in Ca^{2+}-free are also quite similar. However, the SUMMARY of cell signalling processes presented in this thesis(given overleaf) does show a variety of difference amongst the agonists under examination. The non-maintenance of tone for NE-induced contraction in a Ca^{2+}-free medium, in contrast to the peptides, suggests that extracellular Ca^{2+} has a major role to play in maintaining tone with NE.

There is clearly a role for extracellular calcium in the contractile response to ET-1, SRTX-b and NE as evidenced by a loss of >80% of the contractile response in Ca^{2+}-free medium, however, this role differs for each agonist. For example, whereas NE is capable of initiating a phasic contractile response in the absence of extracellular Ca^{2+}, only tonic responses to ET-1 and SRTX-b were observed (Figure 8); true phasic response to ET-1 and SRTX-b, in both rat aorta and anococcygeus, require extracellular Ca^{2+}. The converse was true for the initiation and maintenance of tonic responses.

The route of entry of extracellular Ca^{2+} into the cell has received considerable attention. These routes, often referred to as channels, can be subdivided on the basis of their selective permeability to particular ions,

SUMMARY

	AORTA	ANOCOCCYGEUS
Role of extracellular Ca^{2+}	**Amplification of contraction**	**Amplification of contraction**
	ET-1 ≡ SRTX-b ≡ NE (Normal Krebs)	ET-1 ≡ SRTX-b ≡ NE (Normal Krebs)
	Maintenance of tone	**Maintenance of tone**
	ET-1 ≡ SRTX-b (independent of Ca_e)	ET-1 ≡ SRTX-b (independent of Ca_e)
	NE (dependent on Ca_e)	NE (dependent on Ca_e)
Nifedipine-sensitive processes	SRTX-b ≡ NE (insensitive)	SRTX-b ≡ NE (insensitive)
	ET-1 (sensitive component)	ET-1 (sensitive component)
Ryanodine-sensitive processes	no significant inhibition	SRTX-b > ET-1 > NE
Nifedipine/Ryanodine sensitive processes	ET-1 ≡ NE (significant inhibition)	SRTX-b (no significant inhibition)
	SRTX-b (no significant inhibition)	ET-1 ≡ NE (significant inhibition)
Indomethacin-sensitive processes	ET-1 ≤ SRTX-b ≡ NE (no significant inhibition)	ET ≥ SRTX-b > NE (no significant inhibition)
NDGA-sensitive processes	SRTX-b ≡ NE (no significant inhibition); ET-1 = significant inhibition)	ET-1 ≤ NE (significant inhibition); SRTX = no effect
Cross-desensitization	SRTX and ET-1 do not cross-	SRTX desensitizes NE response

and also by the stimulus which primarily controls channel permeability (see review by Hinneman, 1988). Within each class of channels which are permeable to a particular ion and opened by a particular stimulus, there are also subclasses. So far three distinct types, namely, "L", "N" and "T" type, of Ca^{2+} channels have been identified in different cell types (Sturek and Hermsmeyer, 1986; Friedman *et al.*, 1986; Bean, 1985). These channels can be distinguished on the basis of the strength of depolarization required for channel activation, the time course of inactivation, and the sensitivity to dihydropyridine Ca^{2+}-channel agonists and antagonists. "L-type" channels require relatively strong depolarizations for activation, inactivate slowly, and are modulated by dihydropyridine Ca^{2+}-channel antagonists. "T"-type" channels are activated by smaller depolarization, inactivate rapidly, and are insensitive to dihydropyridines. "N-type" channels also require a fairly strong depolarization for activation, inactivate with an intermediate time course, and are also insensitive to dihydropyridines (Nowycky *et al.*, 1985, Fox *et al.*, 1987). Besides the three subclasses of voltage operated channels, suggestions of receptor-operated Ca^{2+} channels have been made (Bolton, 1979; van Breemen *et al.*, 1979). However, there has been only one report of single channel recording that supports the existence of receptor operated Ca^{2+} channels in smooth muscle (Benham and Tsien, 1987). Other second messenger operated channels have been recorded in neutrophils (von Tscharner *et al.*, 1986), lymphocytes (Kuno and Gardner, 1987) and rat brain (Fraser and Sarnacki, 1990).

Voltage-operated Ca^{2+} channels are known to be quite heterogeneous in nature, with the existence of multiple types of Ca^{2+} currents in neuronal and nonneuronal cells determined using voltage clamp methods (Sala and

Matteson,1990; Bean, 1989; see review by Shearman *et al.*, 1989). N-type channels have been reported to exist only in neuronal cells (Tsien *et al.*, 1988; Miller, 1987) leaving "L" and "T" type channels whose distribution in terms of relative density in smooth muscle is not known. Claims for the existence and non-existence of 'T'-channels in smooth muscle are emerging (Ganitkevich and Isenberg, 1990; Matsuda *et al.*, 1990) and the situation is unclear. As the L-type channel is the only type of Ca^{2+}-channel so far identified that is sensitive to dihydropyridines (DHPs), it is also referred to as the "DHP-receptor" (see review by Shearman, 1989).

Thus far, attempts have been made to highlight events at the level of receptor and amplifier enzymes. The effects of these enzymes on Ca^{2+}-channel modulation, and the elaboration of other "second messengers" like IP_3 and DAG from membrane catabolism, have been compositely assesed by interference with enzymes or products of their pathways.These processes are given in the SUMMARY (page 184) and schematically in Figure 37.

This study has avoided receptor binding studies. Findings by other investigators (Hirata *et al.*,1989; Ambar *et al.*,1989) have not shown any differences in binding parameters between ET-1 and SRTX-b, although such studies have demonstrated the existence of receptor subtypes. Furthermore, the demonstration of low cross-reactivity between antibodies raised against ET-1 and SRTX-b clearly suggest immune system recognition and by the same token, receptor recognition (Fleminger *et al.* ,1989).

The failure of binding and biochemical studies to distinguish between ET-1 and SRTX-b receptors does not necessarily lead to the conclusion that these peptides interact with an identical population of receptor.

The role of DHP-sensitive Ca^{2+}-channels in the maintenance of contractile

responses to ET-1, SRTX-b and NE was explored using the channel antagonist nifedipine. In the aorta, nifedipine (10 μM) significantly reduced the maximum responses to ET-1, but not NE and SRTX-b, and also reduced the sensitivity of the response to these agonists in the aorta (Figure 9a, b and c). In the anococcygeus, 10 μM nifedipine significantly reduced the maximal response to ET-1, but responses to NE and SRTX-b were not significantly reduced (Figure 10a, b and c). However, it is noteworthy that the decrease in the maximum responses to ET-1, SRTX-b and NE was more than that observed for the aorta. The resistance of NE induced contractions in the anococcygeus to nifedipine is consistent with the finding of Oriowo (1984) but the divergence in the sensitivity of responses to ET-1 in aorta and SRTX-b in anococcygeus were surprising for peptides that are said to act on the same receptor and have the same mode of action (Kloog and Sokolovsky, 1989). Despite the clear dependence on extracellular Ca^{2+}, the inability of nifedipine treatment alone to completely inhibit the responses in both preparations, indicates that cellular mechanisms other than voltage-operated calcium channels must also play a significant role in the contractile processes activated by the two peptides and NE. It should also be noted that the concentration of nifedipine, 10 μM, that was used in these studies is high and the inhibitory effects noted against ET-1 may reflect some non-specific actions of this substance that are not yet documented. Recently, Inoue *et al.*(1990) demonstrated that ET-1 augments unitary Ca^{2+}-channel currents in the guinea pig portal vein and they were able to characterize two types of unitary Ba^{2+} currents with conductances of 22 pS and 12 pS. Nifedipine was said to inhibit both types of unitary channel current, although the sensitivity of 22 pS Ca^{2+} channel was 20-fold higher than the 12

pS Ca^{2+}-channel. Although Inoue *et al.* (1990) did not specifically examine the effects of ω-conotoxin on the 12 pS current generated by ET-1, other studies (Lawson and Chatelain, 1989; Topouzis *et al.*, 1989) suggest that the 12 pS current is an N-type channel. Thus, based upon slope conductance analysis, the two types of current that ET-1 activates in the guinea pig portal vein could represent those produced by "L" and "N"-type Ca^{2+}-channels.

Resink *et al.* (1990) have demonstrated the internalization of ET-1 receptors by cultured human vascular smooth muscle cells and have therefore suggested that this process may be relevant to the characteristically persistent contractile effects of this peptide on the vasculature. In view of its persistent contractile effects, it is likely that the SRTX-b receptor undergoes a similar process, however, this has not yet been demonstrated.

Iino *et al.* (1988) have demonstrated the existence of two classes of calcium stores in guinea pig portal vein, pulmonary artery and taenia coli - one with both "Ca^{2+}-induced Ca^{2+} release" (CICR) and "IP_3-induced Ca^{2+} release" (IICR) mechanisms ($\underline{S}\alpha$) and the other only with the IICR mechanisms ($\underline{S}\beta$). These authors demonstrated that after ryanodine treatment, the Ca^{2+} store ($\underline{S}\alpha$) lost its capacity to hold calcium and went further to suggest that the Ca^{2+} released from $\underline{S}\alpha$ produces the initial phase of contracture. By far the most interesting question raised by Iino *et al.* (1988) is: "If the second messenger for the agonist is indeed IP_3 as has been suggested in many other cells (Berridge and Irvine, 1984), then why do agonists not release Ca^{2+} from $\underline{S}\beta$ in the absence of extracellular Ca^{2+} ?" Iino *et al.* (1988) suggested a number of possibilities - one is that following agonist action the released IP_3 may not reach the $\underline{S}\beta$ site, alternatively IP_3 may not be used as the second messenger or finally, that IICR from $\underline{S}\beta$ requires extracellular Ca^{2+}. Iino *et*

al. (1988) argued that since the IICR has been shown to be dependent on a pCa^{2+} ($-\log[Ca^{2+}]$) of approximately 7 (Iino, 1987), it is possible that IP_3 releases Ca^{2+} from Sβ only when there is sufficient Ca^{2+} influx to keep the Ca^{2+} concentration in the vicinity of the store higher than a certain critical level. They finally submitted that there could certainly be other possibilities which needed further study and clarification. The suggestion by Iino *et al.* (1988) that ryanodine seems to hold the Ca^{2+} channels of the Sα stores fixed in an open configuration has been supported by the finding of Hisayama and Takayanagi (1988). In the presence of ryanodine, even if stored Ca^{2+} was depleted by caffeine, the rate of rise of the extracellular Ca^{2+}-induced contraction remained at a higher level.

My data with anococcygeus muscle are consistent with the finding of Hisayama and Takayanagi (1988) concerning the effects of ryanodine, but the conclusion from my data is that the Ca^{2+} mobilizing mechanisms, and ability to sustain them, are likely to be agonist dependent. In this regard, the Rasmussen model for Ca^{2+} cycling across the membrane and the association of protein kinase C with the plasma membrane in the presence of diacylglycerol sustaining the cellular response may only be valid in the presence of extracellular Ca^{2+}. The inability of NE to maintain a sustained contraction in the absence of extracellular Ca^{2+} questions this model, in that α-adrenoceptor activation is thought to involve the production of diacylglycerol and inositol trisphosphate(see review by Minneman, 1988). Furthermore, the ability of ET-1 and SRTX-b to maintain sustained tone in the absence of extracellular Ca^{2+} indicates that there is more to sustained cellular response than membrane association of diacylglycerol and Ca^{2+} mobilization by IP_3. A crucial question is if NE, as for ET-1 and SRTX-b,

produces IP_3 and DAG on receptor activation, why is the response to NE phasic-like and those to ET-1 and SRTX-b tonic-like in a Ca^{2+}-free Krebs? No definite answer can be provided at this time.

Further experiments were conducted to compare and evaluate the role of extracellular versus intracellular Ca^{2+} in the contractile response to ET-1, SRTX-b and NE. In these experiments, control cumulative Ca^{2+} concentration response curves were constructed in the presence of an appropriate agonist, tissues were then washed and incubated in 10 μM ryanodine for one hour following which the Ca^{2+} concentration response curves were repeated. In this study, the same concentrations of the agonists (i.e. 10 nM ET-1,10 nM SRTX-b and 1μM NE) were used as in other phases of this project. After washing and recovery to baseline, the tissues were again subjected to ryanodine treatment in the presence of 10 μM nifedipine in light-proof chambers and Ca^{2+} concentration response curves were constructed in the presence of the appropriate agonist. Responses to ET-1 in the anococcygeus muscles were significantly enhanced by ryanodine treatment alone (Figures 15a, b and c). Treatment with nifedipine significantly reduced the responses to ET-1 and NE but significantly less so for SRTX-b (table 4) . It is apparent that the extracellular Ca^{2+} requirements for ET-1 and NE induced contractions partly utilize nifedipine-sensitive voltage operated channels. There is, however, a component of each contraction which is nifedipine-insensitive. The response to SRTX-b following depletion of the intracellular ryanodine-sensitive store and nifedpine blockade was not significantly reduced, suggesting that Ca^{2+} entry may depend upon non-selective cation channels (Owen *et al.*, 1986; Patridge and Swandulla, 1988; Oortigiesen *et al.*, 1990).

Additional experiments to explore the preliminary finding that ryanodine

and NE depleted the same store(s) of intracellular calcium were conducted. Figure 17a demonstrates that repeated stimulation with NE in anococcygeus could only deplete the tissues of calcium to a certain basal level beyond which further depletion seemed impossible, even for an extended stimulation of 2-3 hours. Figure 18a, b and c demonstrate calcium response curves of the three agonists under examination following depletion of the NE sensitive Ca^{2+} stores with repeated NE exposure. When compared to depletion of cellular Ca^{2+} stores with ryanodine (Figures 15a, b and c), the responses were similar in the anococcygeus except that nifedipine did not significantly reduce the NE response as was the case following ryanodine treatment. These data confirm the postulate of Iino *et al.* (1988) that Sα stores are sensitive to ryanodine and IP_3 generated by agonist. The only discrepancy was the insignificant reduction of NE response by nifedipine following NE induced-depletion of cellular Ca^{2+} stores. Furthermore, the data indicate that ryanodine, unlike NE, holds intracellular Ca^{2+}-channels in an open configuration such that the intracellular stores lose their capacity to reaccumulate Ca^{2+} following stimulation, whereas NE depletes the cellular stores of Ca^{2+} but leaves them with the ability to reaccumulate and maintain a functional capacity. The availability of Ca^{2+} stores that are sensitive only to IP_3 explains the capacity of NE to evoke a contraction after treatment with ryanodine. An interesting development is the isolation of the IP_3 receptor (Chadwick *et al.*, 1990) and the inability to isolate Ca^{2+}-induced Ca^{2+} release channels from the sarcoplasmic reticulum of smooth muscle. It was concluded that, although there is an IP_3 receptor in smooth muscle, there may not be a distinct ryanodine receptor (Chadwick et *al.*, 1990). It is most probable that this store varies from tissue to tissue since Patel and Triggle (1986) were able

to deplete the rat tail artery of Ca^{2+} by repeated exposure to NE.

Further attempts to address the question of which intracellular calcium pools were sensitive to ryanodine and/or NE involved experiments in which tissues were repeatedly stimulated with NE in a Ca^{2+}-free Krebs and then incubated in ryanodine for one hour. The results from anococcygeus (Figures 20a, b and c) maintained a pattern similar to those with ryanodine alone which were earlier described (Figures 15a, b and c).

The possibility that the effectiveness of ryanodine could be enhanced by the simultaneous presence of an agonist which effectively holds the ryanodine-sensitive release channel in an open state, thus enhancing Ca^{2+} depletion, was explored. In order to address this possibility, experiments were designed in which ryanodine alone, ryanodine and caffeine, and ryanodine and agonist, respectively, were used to deplete the tissue of cellular Ca^{2+}. In the anococcygeus (Figures 22a, b and c) Ca^{2+} concentration- response curves in the presence of 10 nM SRTX-b and 10 nM SRTX-b plus ryanodine showed no significant differences when evaluated by ANOVA. The data presented are consistent with those of Iino *et al.* (1988) who concluded that ryanodine acts on the calcium induced calcium release (CICR) channels only when they are open, and that ryanodine does not have any appreciable effect on the IP_3-induced calcium release (IICR) channels even if the drug is applied when these channels are open. From my data concerning interactions between caffeine and ryanodine I conclude that caffeine and ryanodine likely act on the same calcium pool(s). Support for this hypothesis is provided by the fact that the effects of ryanodine with and without caffeine were not significantly different from each other. Figure 23a, b and c illustrate the responses obtained in anococcygeus with the use of ET-1. Evaluation of the

three independent variables by ANOVA showed that the observed difference was not significant thus confirming earlier findings with SRTX-b concerning depletable Ca^{2+} pools.

Further elucidation of the routes of entry of extracellular calcium had become necessary as blockade with nifedipine had been shown to be only partially effective and then only when used at a high concentration. To explore the roles of Ca^{2+} entry via dihydropyridine modulated Ca^{2+}-channels in contractions induced by ET-1, SRTX-b and NE a known L-type calcium channel opener Bay K 8644 was used with depolarizing (15 mM K^+) Krebs buffer. Figure 26a, b and c show the responses in anococcygeus to Bay K 8644 without and with 1 nM ET-1 and SRTX-b and 100 nM NE (subcontractile concentrations). Contractile response to the three agonists was noted but since the response of the anococcygeus to Bay K 8644 (Figure 27) did not demonstrate strict concentration dependence, these differences were not statiscally evaluated for significance. This suggest the involvement of non-selective cation channels and perhaps, "N" and "T"-type channels in the contractile process of the smooth muscle as proposed by several authors (D'Orleans-Juste *et al.*, 1989; Van Renterghem *et al.*, 1989; Patridge and Swandulla, 1988; Inoue *et al.*, 1990).

In further experiments conducted to compare and evaluate the role of extracellular versus intracellular Ca^{2+} in the contractile response to ET-1, SRTX-b and NE in the aorta (Figures 16a, b and c), a combination of ryanodine and nifedipine produced a marked and significant inhibition (ANOVA) of the contractile response to ET-1 and NE, but not SRTX-b (see also Table 4). This suggests that an intracellular source of Ca^{2+}, which is maintained by extracellular Ca^{2+} entering via a nifedipine-sensitive route plays a

significant role in determining the contractile response to ET-1 whereas nifedipine-insensitive routes are utilized by and SRTX-b.

Experiments to expand on the preliminary finding that ryanodine and NE depleted the same stores of intracellular calcium in the aorta (Figure 17b) demonstrate that repeated stimulation with NE in a Ca^{2+}-free Krebs could only deplete the tissues of calcium to a certain basal level beyond which further depletion seemed impossible. Figures 19a, b and c demonstrate responses to ET-1, SRTX-b and NE from the aorta following depletion of NE-sensitive Ca^{2+} stores with repeated NE exposures . When compared to depletion of cellular Ca^{2+} stores with ryanodine, it can be seen that the results were similar (Figure 16a, b and c). Sequential depletion in aorta of NE-sensitive Ca^{2+} stores with repeated NE exposures followed by incubation in ryanodine (Figures 21a, b and c) were not different from Figures 19a, b and c or Figure 16a, b and c thus confirming our earlier speculation that ryanodine and NE acted on the same depletable Ca^{2+} pool.

In a parallel study with the aorta, designed to determine whether ryanodine was more effective in the presence of an agonist, ryanodine alone, ryanodine and caffeine, and ryanodine plus either the agonist SRTX-b or ET-1 were compared (Figures 24a-c and 25a-c respectively). No significant differences were observed (ANOVA). Nifedipine, even at 10 μM, proved to be only partially effective at inhibiting the responses to SRTX-b, ET-1 and NE in the aorta and thus, additional experiments were designed to elucidate the role and routes of extracellular Ca^{2+} utilization. Figures 28a, b and c demonstrate enhancement of responses to ET-1, SRTX-b and NE over the control responses induced by 10 μM Bay K 8644 in a depolarizing (15 mM K^+) Krebs alone. In contrast to anococcygeus, the responses of aorta to Bay K 8644

demonstrated a strict concentration dependence (Figure 27b). No explanation for the "flapping" responses of anococcygeus to Bay K 8644 when contrasted with the sustained tone and concentration-dependent responses exhibited by the aorta to Bay K 86344 (Figure 27a and b) can be offered, although electrophysiological studies may provide an insight.

4.4 Role of arachidonic acid metabolites.

There is a direct association between phosphatidylinositol turnover, diacylglycerol production, and arachidonic acid release (see review by Rasmussen, 1986). Arachidonic acid could be generated by one of three mechanisms: from phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol with further metabolism to phosphatidic acid and arachidonic acid or from phosphatidylcholine to lysophosphatidylcholine and arachidonic acid. The metabolism of phosphatidic acid to arachidonic acid from these pathways is catalyzed by phospholipase A_2 (see review by Exton, 1988). An alternate pathway is the hydrolysis of phosphatidylcholine by phospholipase C and D to arachidonic acid (Irving and Exton, 1987). The view that agonist increases diacylglycerol levels solely via the stimulation of phosphatidylinositol bisphosphate (PIP_2) breakdown and resultant DAG leading to phosphatidic acid is not strictly supported by experimental data. Emergent biochemical data (Irving and Exton, 1987; Bocckino *et al.*, 1985) have revealed that fatty acid composition of DAG and/or PA produced greatly exceeds the decrease in mass of inositol phospholipids. Thus, agonists may induce the breakdown of another phospholipid, phosphatidylcholine, and support for this hypothesis is provided by the agonist induced increases in phosphocholine and choline seen in hepatocytes, 3T3 fibroblasts and smooth

muscle cells (see review by Exton, 1988).

There seems to be a prevalent notion (see Rasmussen, 1986) that once produced, arachidonic acid is rapidly metabolized largely via the cyclooxygenase pathway to endoperoxides, prostaglandins and thromboxane A_2 with negligible contribution of the lipoxygenase pathway. Data from recent studies with endothelin (Rapoport *et al.*, 1990) seem to agree with this notion. However, it is well established that some lipoxgenase products (Vanhoutte *et al.*, 1985) do affect smooth muscle function, and a role for lipoxygenase metabolites in mediating the effects of ET-1 has been described by Resink *et al.* (1989). Furthermore, the work of Reynolds *et al.* (1989) has demonstrated that phorbol ester treatment dissociated endothelin-stimulated phosphoinositide hydrolysis and arachidonic acid release in vascular smooth muscle cells. This later study suggests parallel or synergistic mechanisms for initiating the contraction of smooth muscle.

In view of the possible role of arachidonic acid metabolites in agonist induced contraction, the effects of ET-1, SRTX-b and NE were explored by constructing calcium concentration response curves in the presence of these agonists and in the absence or presence of the cyclooxygenase inhibitor, indomethacin (10 μM),(Figure 29a, b and c for anococcygeus). The responses to the three agonists in presence of indomethacin were not significantly different from each other as analysed by ANOVA. Thus, at least in the anococcygeus, products of cyclooxygenase are of negligible consequence in the contractile process (see Table 4). The results were similar for the aorta (Figures 30a, b and c; Table 4). These findings are in contrast to Rapoport *et al.* (1990) who noted a significant inhibition by indomethacin of ET-1 induced responses in the aorta. Further examination of the effects of the

three agonists in the anococcygeus in the absence and presence of 10μM nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, revealed that responses to ET-1 and NE were significantly reduced when compared to controls by "t"-test analysis (Figure 31a-c,and Table 4). In the aorta (Figure 32a, b and c), responses to SRTX and NE when compared to controls were not significantly reduced by NDGA, whereas that to ET-1 was significantly reduced when examined by the "t"-test respectively. The inability of (ANOVA) to detect a significant difference may reflect the low number of experimental replications. The results with ET-1 were consistent with the findings of Resink *et al.* (1989) who observed almost complete inhibition of [^{3}H]-arachidonic acid release by NDGA, suggesting that such extracellular labelled materials represented eicosanoid metabolites derived from lipoxygenase activity. Resink *et al.* (1989) also found that endothelin-induced arachidonic acid release was relatively insensitive to both indomethacin and meclofenamate (cyclooxygenase inhibitor), thus confirming our finding of lack of effect of indomethacin on responses to ET-1, SRTX-b and NE. The findings of Resink *et al.* (1989) also suggest that it is phospholipase A_2 that mediates the generation of the lipoxygenase product following endothelin stimulation as they report that neomycin, a putative phospholipase C inhibitor (Slivka and Insel, 1987), did not inhibit arachidonic acid release. Resink *et al.* (1989) concluded that the lack of inhibition by neomycin indicates that activation of phospholipase A_2 by endothelin may occur through a pathway that is independently parallel with, rather than secondarily sequential to, the phospholipase C pathway. Such independent pathways have been proposed for α_1-adrenergic receptors (Slivka and Insel, 1988; Burch *et al.*, 1986). The data from my experiments do not support this hypothesis, at least in the aorta,

since SRTX-b was not significantly inhibited by NDGA. However, in the anococcygeus, the NE response was significantly inhibited by NDGA. This further points to the diversity of pathways utilized by the different agonists in different tissues. Reynolds *et al.* (1989) have shown the dose-response relationship of endothelin to [^{3}H]-inositol phosphate formation and [^{3}H]-arachidonic acid release in rabbit renal artery vascular smooth muscle cells and have alluded to the fact that since the EC_{50} values for both responses were very similar, 0.2-0.4 nM, both responses were, most likely, mediated by a single population of endothelin receptors. Reynolds *et al.* (1989) noted that, in the FRTL5 thyroid cells, pertussis toxin completely inhibited α_1-adrenergic receptor mediated PLA_2 activation but did not affect α_1-adrenergic receptor-mediated phospholipase C activation (Burch *et al.*, 1986). These data indicate that α_1-adrenergic receptors of these cells were coupled to PLC and PLA_2 by different G proteins. Reynolds *et al.* (1989) concluded that the coupling of α_1-adrenergic receptors to phospholipase C (PLC) and phospholipase A_2 (PLA_2) activation occurred in parallel and was independently regulated in those cells.

The question of non-specific actions of NDGA (Rimele and Vanhoutte, 1983) was addressed by designing experiments which compared the inhibition of contractile response produced by NDGA on agonist cumulative concentration response curves, e.g. ET-1, NE compared to inhibition by NDGA of calcium concentration response curve in the presence of 50 mM K^+. Figure 33a and b showed that in the anococcygeus preparation, where significant inhibition of the maximal response to NE by NDGA was achieved, no significant inhibition by NDGA of K^+ induced contractions were noted. Figures 34a and b show that in the aorta, no significant reduction of contractile response to either NE or

K^+ by NDGA was achieved. A comparison of figures 33a and b to figures 34a and b leaves some possibility for equivocation and as such the specificity of NDGA may be questionable. Nonetheless, the more recent work by Scriabine, Pan and Vanhoutte (1990) has shown that the NDGA action was specific for lipoxygenase.

4.5. Agonist cross-desensitization.

In the course of tissue screening, it was noted that ET-1, SRTX-b or NE, when cumulatively administered to reach bath concentrations of 10^{-7} M or 0.1 μM for ET-1 or SRTX-b, and 10 μM for NE, often resulted in desensitization to subsequent stimulation. It was reasoned that this might be desensitization or tissue toxicity and since the experimental protocol demanded repeated administration of a particular agonist, this question had to be addressed. As earlier pointed out in the Materials and Methods section, it was observed that if the concentration of 10 nM for ET-1 and SRTX-b or 1 μM for NE was not exceeded, desensitization on subsequent stimulation was not apparent and reproducible concentration response curves to ET-1, SRTX-b and NE could be obtained for up to 4 hours (Table 1). In the study by Miasiro and Paiva (1990), ET-1 pretreatment in rabbit aortic rings and cultured rat aorta smooth muscle cells did not affect responses to angiotensin II and NE, thus demonstrating homologous desensitization to ET-1. However, the concentration used was 100 nM (0.1 μM) which, as demonstrated in this study, is sufficient to induce myogenic activity in anococcygeus or subsequent non-response in the aorta. At the concentrations used in my study, which were approximately EC_{50} values for these agonists, desensitization was not observed and thus our findings are not consistent with those of Miasiro and Paiva (1990). A good discourse of the phenomena of desensitization (Triggle, 1980) does not seem

to relate desensitization to dose or concentration, which the author defines as diminished response during, or subsequent to, the initial action of a drug (variously described as tachyphylaxis, tolerance, refractoriness or subsensitivity). Recent developments in this area have clearly indicated that conditions which decrease or increase agonist-receptor interaction, either chronically or acutely, result in opposing alterations in effector sensitivity (Triggle, 1980). Given the agonist-receptor internalization of ET-1 as noted by Resink *et al.* (1990), it could be argued that internaliation of ligand-receptor complex leads to loss of receptor, thereby providing the basis for desensitization. However, Auguet *et al.* (1990) had demonstrated that even at concentrations as low as 30 nM, ET-1 but not SRTX-b induces cross desensitization of rat aorta to SRTX-b or ET-1, respectively. In a second set of experiments, Auguet *et al.* (1990) using 0.1 μM of ET-1 or SRTX-b demonstrated that pretreatment with SRTX-b did not affect subsequent responses to ET-1 or SRTX-b whereas pretreatment with ET-1 significantly reduced responses to ET-1 and SRTX-b. Auguet *et al.* (1990) concluded that ET-1 and SRTX-b may activate a common receptor since ET-1 induces desensitization to both ET-1 and SRTX-b and went on to postulate that SRTX-b, unlike ET-1 (Hirata *et al.*, 1988a), may be more readily dissociable from its binding site and hence does not normally induce desensitization. Furthermore, Auguet *et al.* (1990) suggest that the long lasting effect of the peptides of the ET/SRTX family is more likely to be due to their post-receptor events rather than to the nature of binding of the drug to the receptor (irreversible or not), since the rate of washout was the same with the contraction induced by ET-1 and that induced by SRTX-b. My data, as determined by loss of myogenic activity in anococcygeus (Figure 6 and Table

3), show differing dissociation characteristics of ET-1 and SRTX-b. The argument that this activity may be a post-receptor event may be conceded on the basis of the work of Van Renterghem *et al.* (1989) and Sakata *et al.* (1989) who related ET-1-induced "spiking" activity to Ca^{2+}- activated K^+ channels and cytosolic Ca^{2+} spikes to mechanical response of tonus respectively.

However, my data (Figure 35a and b) demonstrate the phenomenon of cross-desensitization between SRTX-b and NE in rat anococcygeus and the absence of cross-desensitization between ET-1 and NE. This contrasts markedly with the data presented in Figure 35c and d which were designed as controls for the NE response by substituting cumulative concentration response curves of NE with KCl. In this subset, enhancement of the maximal response to KCL was noted with both ET-1 and SRTX-b. Statistical evaluation of responses by ANOVA showed significant differences for the effects of SRTX-b on the NE response and the SRTX-b effect on the KCl concentration response curves. This contrasts with the ET-1 effect on NE and KCl concentration response curves where comparisons in the anococcygeus were not significant as assessed by ANOVA. In the aorta (Figures 36a, b, c and d), there was no desensitization, as assesed by ANOVA analysis to NE or KCl after pretreatment with either ET-1 or SRTX-b.

Conclusions

The most obvious conclusion that can be reached from the present study is that ET-1 and SRTX-b interact with a heterogeneous population of receptors. If ET-1 and SRTX-b do interact with a homogeneous receptor population, then signal transduction pathways are differentially

activated(see also Galron *et al.*1990). However,my data does suggest that the affinity of ET-1 and SRTX-b binding to their receptor(s) in the anococcygeus is increased in the presence of extracellular Ca^{2+} and this is reflected by the time interval needed for disappearance of induced myogenic activity in the anococcygeus muscle in Ca^{2+}-containing and Ca^{2+}-free Krebs buffer. A comparison of this interval in either Krebs solution shows that SRTX-b dissociates faster from its receptor than does ET-1. In contrast ,the effects of NE can be terminated in less than 2 minutes. ET-1 has been shown to be internalized by endocytosis along with its receptor (Resink *et al.*, 1990) and this may explain the basis of the persistent contractile activity of the peptides.

In some respects, the resistance of the effects of SRTX-b to inhibition by nifedipine in the anococcygeus bears strong similarities to the resistance of NE to nifedipine. Moreover, the demonstration of cross-desensitization of NE effects by SRTX-b in the anococcygeus suggest that these agonists may share a common post-receptor mechanism coupled via a common G-protein.The significant reduction in the aorta of ET-1, but not NE and SRTX-b, responses by nifedipine points to the divergent utilization of Ca^{2+} channels by the two peptides in different tissues. The validity of this suggestion is not diminished by the finding of no significant differences (ANOVA) between the three agonist following nifedipine treatment because other protocols (nifedipine and ryanodine) have demonstrated significant differences between the peptides (ANOVA), Table 4. The electrophysiologic characteristics of small depolarizations (-7 mV) for ET-1 in contrast to -20 mV for NE (Wallnöfer *et al.*, 1989), and the suggestion of the role of "N"-type channels (Inoue *et al.*, 1990) for ET-1 along with the sensitivity of response to

dihydropyridines, suggest that ET-1 may be opening "L" and "N"-type calcium channels whereas SRTX-b may be opening "L" and "T"-type calcium channels depending on the preponderance of channels in the tissues. "N"-type channels, however, have not previously been associated in smooth muscle.

The inability, after ryanodine treatment, of nifedipine to further inhibit SRTX-b responses in the aorta is in contrast to the near complete inhibition of ET-1, after ryanodine treatment, thus lending additional support to the divergent use of calcium pools by the three agonists. This leads to the conclusion that the processes affecting Ca^{2+} mobilization are an intrinsic property of an agonist and the availability of sensitive Ca^{2+} stores in a particular tissue determines the effectiveness of the agonist.

Since ET-1, SRTX-b and NE have been shown to produce DAG and IP_3 as universal second messengers the inability of NE to sustain tone in the absence of extracellular Ca^{2+} suggests that ET-1 and SRTX-b must be producing something in addition to DAG and IP_3 which could not be determined by my protocols. However, the demonstration by Price *et al.* (1989) that oncogenic ras could stimulate phosphatidylcholine hydrolysis, diacylglycerol release and arachidonic acid production without altering inositol phosphate levels in Swiss-3T3 cells is consistent with the findings of Hirata *et al.* (1988a) concerning the actions of ET-1 in vascular smooth muscle cells. Furthermore, the demonstration that ET-1 is a potent mitogen which stimulates c-myc and c-fos proto-oncogene expression (see review by Simonson and Dunn, 1990b) leaves room for speculation that oncogenic ras can be stimulated by ET-1 and SRTX-b. Oncogenically activated ras proteins contain single amino acid substitutions and are generally unresponsive to GTPase activating protein, a cellular protein that stimulates GTP hydrolysis by normal $p21^{ras}$ (Trahey and McCormick,

1987) in an "active" (GTP-bound) conformation. Price *et al.* (1989) have suggested that ras rapidly activates a number of cellular signalling systems, leading to a sustained increase in diacylglycerol levels. In contrast, Sunako *et al.* (1990) propose a phospholipase C system generating IP_3 and DAG as an explanation for ET-1 induced changes in diacylglycerol during sustained tone. Sunako *et al.*(1990) do concede that there could be other mechanisms of DAG production. The suggestion by Price *et al.* (1989) fits the Rasmussen (1986) model of sustained cellular response and would, perhaps, explain the basis of ET-1 and SRTX-b sustained tone in the absence of extracellular Ca^{2+}. Additional studies are required to address this matter.

By far the most important conclusion derived from the ryanodine studies is that IP_3 may not be the intracellular Ca^{2+} mobilizer for contractions induced by ET-1 or SRTX-b whereas those induced by NE have a significant IP_3 dependency. Based on the characteristics of Sα and Sß intracellular Ca^{2+} stores (Iino *et al.*, 1988), and the emergent "L" and "N"-like Ca^{2+} channel utilization by ET-1 and "L" and "T" type Ca^{2+} channel utilization by SRTX-b (Inoue *et al.*, 1990; Lawson and Chatelain, 1989; Topouzis *et al.*, 1989), it can be postulated that the primary electrophysiologic event in the action of these peptides involves the opening of "N"-like or "T"-type Ca^{2+} channels with a resultant Ca^{2+} induced Ca^{2+} release from Sα stores initiating contraction. Maintenance of tone is brought about by activation of protein kinase C with sustained levels of diacylglycerol not involving the inositol phosphate pathway. NE can be postulated to act via the activation of "L" and "T" type Ca^{2+} channels leading to Ca^{2+}-induced Ca^{2+} release to initiate contraction. However,there is a dependence on extracellular Ca^{2+} to sustain activity of diacylglycerol and protein kinase C to maintain tone. The sharp spike of

contraction induced by NE in a Ca^{2+}-free medium is likely due to IP_3-mediated mobilization of Ca^{2+} from Sß stores. The non-maintenance of tone is due to the reaccumulating property of Sß stores thereby reducing intracellular Ca^{2+} to a level below which the diacylglycerol and protein kinase C activity cannot be maintained. The absence of the characteristic spiked contraction for the two peptides is evidence that the Sß store is not stimulated by ET-1 and SRTX-b. This also explains the the ability of NE, in the absence of extracellular Ca^{2+}, to mobilize intracellular Ca^{2+} from Sα and Sß as opposed to the peptide use of Sα stores predominantly. In a Ca^{2+}-free medium, NE leads to the production of IP_3 which mobilizes Sß Ca^{2+} to release Ca^{2+} from Sα stores to generate the phasic contraction. The rapid reaccumulation characteristics of Sß stores lowers Ca^{2+} below a critical level for contraction while IP_3 is metabolized. I further speculate that the small slowly rising tone of peptide induced contraction may be due to activation of oncogenic ras with production of diacylglycerol which, in association with protein kinase C, can function at low intracellular Ca^{2+} levels. Thus, large contractions may be dependent on Ca^{2+}-induced Ca^{2+} release fostered by the entry of extracellular Ca^{2+} through "L", "T", "N" or, perhaps, non-specific cation channels.

The effects of NDGA have been clearly demonstrated to be specific for lipoxygenase products which could be generated from arachidonic acid derived from either the phosphatidylinositol or the phosphatidylcholine pathway.

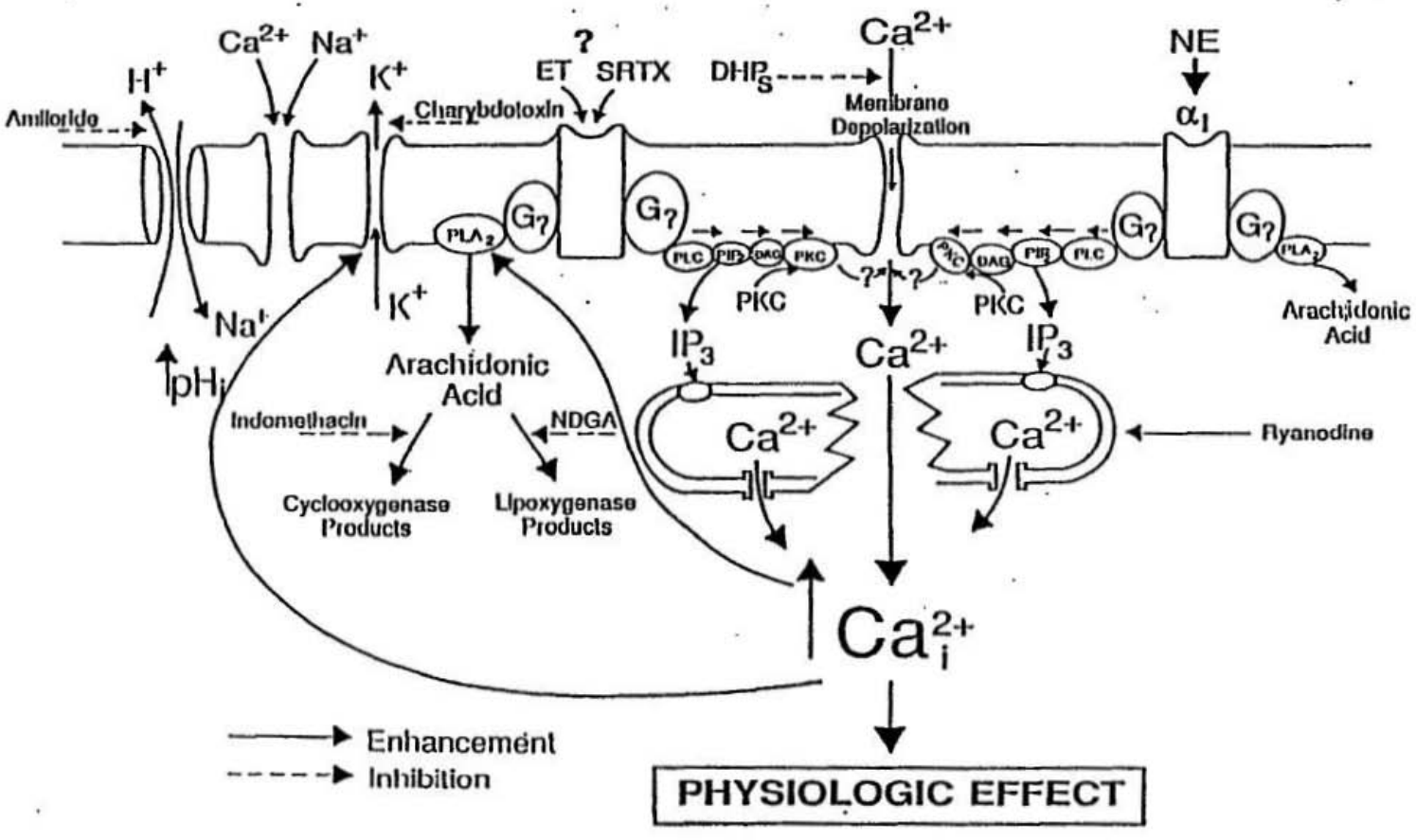
Further electrophysiologic characterization of the Ca^{2+} channel types in smooth muscle, as well as the effect of proto-oncogenes induced by ET-1 and SRTX-b, will be needed to completely characterize the physiologic effects of these peptides. The differences in the cell signalling processes in vascular

versus non-vascular smooth muscle demonstrated in this study suggest that SRTX-b and ET-1 may not act on a homogeneous receptor population. Other evidence in the literature is also indicative of receptor subtypes for these related peptides (Webb, 1991).

In conclusion, my studies indicate that ET-1 initiates contraction in the rat thoracic aorta and anococcygeus by depolarizing the plasma membrane with a resultant influx of Ca^{2+} through voltage-operated channels and non-specific cation channels. The incoming Ca^{2+} leads to calcium induced calcium release from the Sα stores. Intracellular calcium, having risen above the critical concentration activates phospholipase A_2 to produce 5-lipoxygenase products to sustain the contraction. In the latter tonic phase, activation of ras oncogene produces sustained levels of diacylglycerol which, in association with protein kinase C, maintains tone for long periods even in the face of a diminshing calcium concentration. The major membrane lipid catabolized is most probably phosphatidylcholine rather than phosphatidylinositol(see review by Exton, 1988).

A summary of the signalling processes that are involved is represented in Figure 37.

Figure 37: Schematic summary of cell signalling processes in smooth muscle activated by ET, SRTX, NE. DHP, dihydropyridine; PLC, phospholipase C; DAG, diacylglycerol; PLA_2, phospholipase A_2; PKC, protein kinase C, G?, G-protein.


Ca2+
Na+
H+
K+
?
ET
SRTX
DHPs
Ca2+
NE
Amiloride
Charybdotoxin
Membrane Depolarization
α1
Gq
PLA2
PLC
PIP2
DAG
PKC
PKC
PKC
IP3
IP3
Arachidonic Acid
Arachidonic Acid
Na+
pHi
K+
Ca2+
Indomethacin
NDGA
Ryanodine
Ca2+
Ca2+
Cyclooxygenase Products
Lipoxygenase Products
Cai2+
Enhancement
Inhibition
PHYSIOLOGIC EFFECT

REFERENCES

Ambar, I., Kloog, Y., Schvartz, I., Hazum, E. and Sokolovsky, M. (1989) Competitive interaction between endothelin and sarafotoxin: binding and phosphoinositide hydrolysis in rat atria and brain. *Biochem. Biophys. Res. Commun.* 158, 195-201.

Ando, K., Hirata, Y., Shichiri, M., Emori, T. and Marumo, F. (1989) Presence of immunoreactive endothelin in human plasma. *FEBS Lett.* 245, 164-166.

Ando, J., Komatsuda, T. and Kamiya, A. (1988) Cytoplasmic calcium response to fluid shear stress in cultured vascualr endothelial cells. *In Vitro* 24, 871-877.

Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S.(1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 348, 730-732.

Auch-Schwelk, W., Katuszic, Z. and Vanhoutte, P.M. (1989) Contractions to oxygen derived free radicals are augmented in the aorta of the spontaneously hypertensive rat. *Hypertension* 13, 859-864.

Auguet, M., Delaflotte, S., Guillon, J.M., Rouberg, P., Chabrier, P.E., Braquet, P. (1990) Endothelin-1, but not sarafotoxin S6b, induces cross-desensitization of rat aorta contraction. *Eur. J. Pharmacol.* 187, 293-294.

Auguet, M., Delaflotte, S., Chabrier, P.E., Pirotzky, E., Clostre, F. and Braquet, P. (1988) Endothelin and Ca^{2+} agonist Bay K 8644: different vasoconstrictive properties. *Biochem. Biophys. Res. Commun.* 156, 186-192.

Badr, K.F., Murray, J.J., Breyer, M.D., Takahasi, K., Inagami, T. and Harris, R.C. (1989) Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney. *J. Clin. Invest.* 83, 336-342.

Bdolah, A., Wollberg, Z., Fleminger, G. and Kochva, E. (1989) SRTX-d, a new native peptide of the endothelin/sarafotoxin family. *FEBS Lett.* 256, 1-3.

Bean, B.P. (1985) Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity and pharmacology. *J. Gen. Physiol.* , 1-30.

Bean, B.P. (1989) Classes of calcium channels in vertebrate cells. *Annu. Rev. Physiol.* 51, 367-385.

Benham, C.D. and Tsien, R.W. (1987) A novel receptor-operated Ca^{2+}-channel activated by ATP in smooth muscle. *Nature (Lond.)* 328, 275-278.

Berridge, M.J. (1985) The molecular basis of communication within the cell. *Sci. Amer.* 53, 142-152.

Berridge, M.J. and Irvine, R.F. (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312, 315-321.

Besch, H.R. (1985) Effects of ryanodine on cardiac subcellular membrane fractions. *Fed. Proc.* 44, 2960-2963.

Bevan, J.A., Bevan, R.D., Hwa, J.J., Owen, M.P., Tayo, F.M. and Winquist, R.J. (1982) Calcium extrinsic and intrinsic (myogenic) vascular tone. In: *Calcium Modultors*, Godfraind, T., Albertini, A. and Paloette, R, Eds., pp. 125-132. Elsevier, Amsterdam.

Bieger, D. and Triggle, C. (1985) Pharmacological properties of mechanical responses of the rat oesophageal muscularis mucosae to vagal and field stimulation. *Br. J. Pharmacol.* 84, 93-106.

Boarder, M.R. and Mariott, D.B. (1989) Characterization of endothelin-1 stimulation of catecholamine release from adrenal chromaffin cells. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5), S223-S224.

Bocckino, S.B, Blackmore, P.F. and Exton, J.H. (1985) Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin, epinephrine and angiotensin II. *J. Biol. Chem.* 260, 14201-14207.

Bolton, T.B. (1979) Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.* 59, 606-718.

Borges, R., Von Grafenstein, H. and Knight, D.E. (1989) Tissue selectivity of endothelin. *Eur. J. Pharmacol.* 165, 223-230.

Brain, S.D. (1989) The direct observation of arteriolar constriction induced by endothelin in vivo. *Eur. J. Pharmacol.* 160, 401-403.

Brody, M.J., Haywood, J.R. and Touw, K.B. (1980) Neural mechanisms in hypertension. *Ann. Rev. Physiol.* 42, 441-453.

Burch, R.M., Luini, A. and Axelrod, J. (1986) Phospholipase A_2 and phospholipase C are activated by distinct GTP binding proteins in response to α_1-adrenergic stimultion in FRTL5 thyroid cells. *Proc. Natl. Acad. Sci. U.S.A.* 83, 7201-7205.

Carafoli, E. and Penniston, J.I. (1985) The calcium signal. *Sci. Amer.* 261, 66-73.

Cassel, D. and Selinger, Z. (1976) Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. *Biochem. Biophys. Acta 452, 538-551.*

Cernacek, P. and Stewart, D.J. (1989) Immunoreactive endothelin in human plasma: marked elevations in patients in cardiogenic shock. *Biochem. Biophys. Res. Commun.* 161, 562-567.

Chadwick, C., Saito, A. and Fleischer, S. (1990) Isolation and characterization of inositol trisphosphate receptor from smooth muscle.

Proc. Natl. Acad. Sci. U.S.A. 87, 2132-2136.

Chapman, R.A. (1979) Excitation-contraction coupling in cardiac muscle. *Prog. Biophys. Mol. Biol.* 35, 1-52.

Chen, G. and Suzuki, H. (1990) Calcium dependency of the endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery. *J. Physiol.* 421, 521-534.

Cherry, P.D., Furchgott, R.F., Zawadzki, J.V. and Jothianandan, D. (1982) The role of endothelial cells in the relaxation of isolated arteries by bradykinin. *Proc. Natl. Acad. Sci. U.S.A.* 79, 2105-2110.

Cocks, T.M. and Angus, J.A. (1983) Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature* 305, 627-630.

Cohen, P. (1979) The hormonal control of glycogen metabolism in mammalian muscle by multivalent phosphorylation. *Biochem. Soc. Trans.* 7, 459-480.

Cozza, E.N., Gomez-Sanchez, C.E., Foecking, M.F. and Chiou, S. (1989) Endothelin binding to cultured calf adrenal zona glomerulosa cells and stimulation of aldosterone secretion. *J. Clin. Invest.* 84, 1032-1035.

Creed, K.E. and Gillespie, J.S. (1973) The effect of excitatory or inhibitory nerve stimulation on the membrane potential of the rat anococcygeus muscle. *J. Physiol.* 237, 47-48.

Creed, K.E., Gillespie, J.S. and Muir, J.C. (1975) The electrical basis of exictation and inhibiiton in the rat anococcygeus muscle. *J. Physiol.* 245: 33-47.

Criscione, L., Thomann, H., Rodriguez, C., Eglème, C. and Chiesi, M. (1989) Blockade of endothelin-induced contractions by dichlorobenzamil: mechanisms of action. *Biochem. Biophys. Res. Commun.* 163, 247-254.

D'Orleans-Juste, P., DeNucci, G. and Vane, J.R. (1989) Endothelin-1 contracts isolated vessels independently of dihydropyridine-sensitive Ca^{2+} channel activation. *Eur. J. Pharmacol.* 165, 289-295.

DeLean, A., Stadel, J.M. and Lefkowitz, R.J. (1980) A ternary complex model explains the agonist-specific binding properties of adenylate cyclase-coupled ß-adrenergic receptor. *J. Biol. Chem.* 255, 7108-7117.

DeMey, J.G. and Vanhoutte, P.M. (1982) Heterogenous behaviour of the canine arterial and venous wall: Importance of the endothelium. *Circ. Res.* 51, 439-447.

DeMey, J.G. and Vanhoutte, P.M. (1983) Anoxia and endothelium-dependent reactivity of the canine femoral artery. *J. Physiol. (Lond.)* 335. 65-74.

deNucci, G., Thomas, R., D'Orleans-Juste, P., Antunes, E., Walder, C., Warner, T.D. and Vane, J.R. (1988) Pressor effects of circulating endothelin are

limited by its removal in pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxation factor. *Proc. Natl. Acad. Sci. U.S.A.* 85, 9797-9800.

Deth, R. and van Breemen, C. (1977) Relative contributions of Ca^{2+} influx and cellular Ca^{2+} release during drug induced activation of rabbit aorta. *Pfluggers Arch. Eur. J. Physiol.* 348, 13-23.

Devine, C.E., Somlyo, A.V. and Somlyo, A.P. (1972) Sarcoplasmic reticulum and excitation contraction coupling in mammalian smooth muscles. *J. Cell Biol.* 52, 690-718.

Dominiczak, A.F. and Bohr, D.F. (1990) Cell membrane abnormalities and the regulation of intracellular calcium concentration in hypertension. *Clin. Sci.* 79, 415-423.

Ebashi, S., Mikawa, T., Hirata, M. and Nonomura, Y. (1978) The regulatory role of calcium in muscle. *Ann. N.Y. Acad. Sci.* 307, 451-461.

Eglen, R.M., Michel, A.D., Sharif, N.A., Swank, S.R. and Whiting, R.L. (1989) The pharmacological properties of the peptide, endothelin. *Br. J. Pharmacol.* 97, 1297-1307.

Emori, T., Hirata, Y., Ohata, K., Shichiri, M. and Marumo, F. (1989) Secretory mechanisms of immunoreactive endothelin in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.* 160, 93-100.

Exton, J.H. (1988) Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme. *FASEB J.* 2, 2670-2676.

Feletou, M. and Vanhoutte, P.M. (1988) Endothelium-dependent hyperpolarization of canine coronary artery smooth muscle. *Br. J. Pharmacol.* 93, 515-524.

Fitzpatrick, D.F. and Szentivanyi, A. (1980) The relationship between increased myogenic tone and hyper-responsiveness in vascular smooth muscle of spontaneously hypertensive rats. *Clin. Exp. Hypertens.* 2, 1023-1037.

Fleming, W.W., Westfall, D.P., De La lande, I.S. and Jehlett, L.B. (1972) Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmacol. Exp. Ther.* 181, 339-345.

Fleminger, G., Bousso-Mittler, D., Bdolah, A., Kloog, Y. and Sokolovsky, M. (1989) Immunological and structural characterization of sarafotoxin/endothelin family of peptides. *Biochem. Biophys. Res. Commun.* 162, 1317-1323.

Folkow, B. (1990) "Structural factor" in primary and secondary hypertension. *Hypertension* 16, 89-101.

Forstermann, U., Trogisch, G. and Busse, R. (1984) Species-dependent differences in the value of endothelium-derived vascular relaxing factor.

Eur. J. Pharmacol. 106, 639-643.

Fox, A.P., Nowycky, M.C. and Tsien, R.W. (1987) Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol.* 394, 149-172.

Fraser, C.L. and Sarnacki, P. (1990) Inositol 1,4,5-trisphosphate may regulate rat brain Ca^{2++} by inhibiting membrane bound Na^{+}-Ca^{2++} exchanger. *J. Clin. Invest.* 86, 2169-2173.

Freissmuth, M., Casey, P.J. and Gilman, A.G. (1989) G-proteins control diverse pathways of transmembrane signaling. *FASEB J.* 3:2125-2131.

Friedman, M.E., Suarez-Kurtz, G., Kaczorowski, G.J., Katz, G.M. and Reuben, J.P. (1986) Two calcium currents in a smooth muscle cell line. *Am. J. Physiol.* 250, H699-H703.

Fu uda, Y., Hirata, Y., Yoshimi, H., Takatsugu, K., Kobayashi, Y., Yanagisawa, M. and Masaki, T. (1988) Endothelin is a potent secretagogue for atrial natriuretic peptide in cultured rat atrial myocyte. *Biochem. Biophys. Res. Commun.* 155, 167-172.

Furchgott, R.F., Zawadzki, J.V. and Cherry, P.D. (1981) Role of endothelium in vasodilator response to acetylcholine. In: *Vasodilation*, Vanhoutte, P. and Lensen, I., Eds., pp. 49-66, Raven, New York.

Furchgott, R.F. and Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature (Lond.)* 288, 373-376.

Furchgott, R.F. (1988) Endothelium-dependent relaxation in systemic arteries. In: *Relaxing and Contracting Factors; Biological and Clinical Research*, Paul M. Vanhoutte, Ed., pp. 1-26, Clifton, NJ: Humana Press Inc.

Furchgott, R.F. (1983) Role of the endothelium in responses of vascular smooth muscle. *Circ. Res.* 53, 557-573.

Galron, R., Kloog, Y., Bdolah, A. and Sokolovsky, M. (1989) Functional endothelin/sarafotoxin receptors in rat heart myocytes: structure-activity relationships and receptor subtypes. *Biochem. Biophys. Res. Commun.* 163, 936-943.

Galron, R., Kloog, Y., Bdolah, A. and Sokolovsky, M. (1990) Different pathways of endothelial sarafotoxin-stimulated phosphoinositide hydrolysis in myocytes. *Eur. J. Pharmacol.* 188, 85-88.

Ganitkevich, V.Y. and Isenberg, G. (1990) Contribution of two types of calcium channels to membrane conductance of single myocytes from guinea-pig coronary artery. *J. Physiol. (Lond.)* 426, 19-42.

Garcia-Sainz, J.A. and Hernandez-Sotomayor, S.M.T. (1985) Adrenergic regulation of gluconeogenesis: possible involvement of two mechanism of

signal transduction in α_1-adrenergic action. *Proc. Natl. Acad. Sci. U.S.A.* 82, 6727-6730.

Gillespie, M.N., Owasoyo, J.O., McMurtry, I.F. and O'Brien, R.F. (1986) Sustained coronary vasoconstriction provoked by a peptidergic substance released from endothelial cells in culture. *J. Pharmacol. Exp. Ther.* 236, 339-343.

Gillespie, J.S. (1972) The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.* 45, 404-416.

Godfraind, T. and Miller, R.C. (1983) Specificity of action of Ca^{2+} entry blockers: comparison of their actions in the rat arteries and in human coronary arteries. *Circ. Res.* 52 (Suppl. 1) I81-I91.

Goetz, K.L., Wang, B.C., Madwed, J.B., Zhu, J.L. and Leadley, R.J. Jr. (1988) Cardiovascular, renal and endocrine responses to intravenous endothelin in conscious dogs. *Am. J. Physiol.* 255, R1064-R1068.

Goetz, K., Wang, B.C., Leadley, R. (Jr.), Zhu, J.L., Madwed, J. and Bie, P. (1989) Endothelin and sarafotoxin produced dissimilar effects on renal blood flow, but both block the antidiuretic effects of vasopressin. *Proc. Soc. Exp. Biol. Med.* 191, 425-427.

Golby, F.S. and Beilin, L.S. (1972) Relationship between arterial pressure and the permeability of arterioles to carbon particles in acute hypertension in the rat. *Cardiovasc. Res.* 6, 384-390.

Goldberg, M.T. and Triggle, C.R. (1977) An analysis of the action of lanthanum on aortic tissue from normotensive and spontaneously hypertensive rats. *Can. J. Physiol. Pharmacol.* 55, 1084-1090.

Goto, K., Kasuya, K., Matsui, N., Takuwa, Y., Kurihara, H., Ishikawa, T., Kimura, S., Yanagisawa, M. and Masaki, T. (1989) Endothelin activates the dihydropyridine-sensitive voltage-dependent Ca^{2+} channel in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.* 86, 3915-3918.

Goyal, R.K. (1984) The anococcygeus muscle; a promising smooth muscle preparation for the autonomic research. *Ind. J. Pharmacol.* 16, 68-78.

Graur, D., Bdolah, A., Wollberg, Z., Kochva, E. (1988/89) Homology between snake venom sarafotoxin and mammalian endothelin. *Israel J. Zool.* 35, 171-175.

Griffith, T.M., Edwards, D.H., Lewis, M.J., Newby, A.C. and Henderson, A.H. (1984) The nature of endothelium-derived vascular relaxant factor. *Nature* 308, 645-648.

Gustafson, T.L., EPISTAT : a statistical package designed for analysis of small data sets. Round Rock, TX 78664, U.S.A.

Hashimoto, T., Hirta, M., Itoh, T., Kanmura, Y. and Kuriyama, H. (1986) Inositol 1,4,5-trisphosphate activates pharmacomechanical coupling in smooth muscle of rabbit mesenteric artery. *J. Physiol.* 370, 605-618.

Hay, D.W.P. (1990) Mechanism of endothelin-induced contraction in guinea pig trachea: comparison with rat aorta. *Br. J. Pharmacol.* 100, 383-392.

Hickey, K.A., Rubanyi, G.M., Paul, R.J. and Highsmith, R.F. (1985) Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am. J. Physiol.* 248, C550-C556.

Hirata, Y., Yoshimi, H., Takaichi, S., Yanagisawa, M. and Masaki, T. (1988b) Binding and receptor down regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett.* 239, 13-17.

Hirata, Y., Yoshimi, H., Muramo, F., Watanabe, T.X., Kumagaye, S., Nakajima, K., Kimura, T. and Sakakibara, S. (1989) Interaction of synthetic sarafotoxin with rat vascular endothelin receptors. *Biochem. Biophys. Res. Commun.* 162, 441-447.

Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagai, S., Nakajima, K. and Sakakibara, S. (1988a) Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 154, 868-875.

Hisayama, T. and Takayanagi, I. (1988) Ryanodine: its possible mechanism of action in caffeine-sensitive calcium store of smooth muscle. *Pflugers Arch.* 412, 376-381.

Huang, X-N., Yisayama, T., Takayanagi, I. (1990) Endothelin-induced contraction of rat aorta: contributions made by Ca^{2+} influx and activation of contractile apparatus associated with no change in cytoplasmic Ca^{2+} level. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 341, 80-87.

Huttner, I., Boutet, M. and More, R. (1973) Studies on protein passage through arterial endothelium. I. Regional differences in permeability to fine structural protein tracers in arterial endothelium of normotensive rat. *Lab. Invest.* 28, 678-685.

Hwang, K.S. and van Breemen, C. (1987) Ryanodine modulation of ^{45}Ca efflux and tension in rabbit aortic smooth muscle. *Pflügers Arch.* 408, 343-350.

Ignarro, L.J., Byrns, R., Buga, G.M., Wood, K.S. and Chaudhuri, G. (1987) Pharmacological evidence that endothelium derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study endothelium-dependent and nitric oxide-elicited vascular smooth muscle relaxation. *J. Pharmacol. Exp. Ther.* 244, 181-189.

Iino, M., Kobayashi, T. and Endo, M. (1988) Use of ryanodine for functional removal of the calcium store in smooth muscle cells of guinea pig. *Biochem. Biophys. Res. Commun.* 152, 417-422.

Iino, M. (1987) Calcium dependent inositol trisphosphate induced calcium release in the guinea pig taenia caeci. *Biochem. Biophys. Res. Commun.* 142, 47-52.

Inoue, Y., Olke, M., Nakao, K., Kitamura, K. and Kuriyama, H. (1990)

Endothelin augments unitary calcium currents on the smooth muscle cell membrane of guinea-pig portal vein. *J. Physiol.* 423, 171-191.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) The human endothelin family: Three structurally and pharmacologically distinct isopepties predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.* 86 2863-2867.

Irving, H.R. and Exton, J.H. (1987) Phosphatidylcholine breakdown in rat liver plasma membranes. *J. Biol. Chem.* 262, 3440-3443.

Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988b) Positive chronotropic action of endothelin, a novel endothelium-derived vasoconstrictor peptide. *Pflugers Arch.* 413, 108-110.

Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K. and Masaki, T. (1988a) Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea pig atria. *Am. J. Physiol.* 255, H970-H973.

Israel, M., Dunant, Y. and Manaranche, R. (1979) The present status of the vesicular hypothesis. *Prog. Neurobiol.* 13, 237-275.

Itoh, Y., Yanagisawa, M., Ohkubo, S., Kimura, C., Kosaka, T., Inoue, A., Ishida, N., Mitsui, Y., Onda, H., Fujino, M. and Masaki, T. (1988) Cloning and sequence anlaysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett.* 231, 440-440.

Janis, R.A. and Triggle, D.J. (1973) Effect of diazoxide on aortic reactivity to calcium in spontaneously hypertensive rats. *Can. J. Physiol. Pharmacol.* 51, 621-626.

Jenden, D.J. and Fairhurst, A.S. (1969) The pharmacology of ryanodine. *Pharmacol. Rev.* 21, 1-25.

Jones, C.R., Hiley, C.R., Pelton, J.T. and Mohr, M. (1989) Autoradiographic visualization of the binding sites for [^{125}I]endothelin rat and human brain. *Neurosci. Lett.* 97, 276-279.

Kamm, K.E. and Stull, J.T. (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Ann. Rev. Pharmacol. Toxicol.* 25, 593-620.

Katusic, Z.S., Shepherd, J.T. and Vanhoutte, P.M. (1984) Vasopressin causes endothelium-dependent relaxation of the canine basilar artery. *Circ. Res.* 55, 575-579.

Katusic, Z.S., Shepherd, J.T., Vanhoutte, P.M. (1987) Endothelium-dependent contractions to stretch in canine basilar arteries. *Am. J. Physiol.* 252, H671-H673.

Khalil, R.A. and van Breemen, C. (1990) Intracellular free calcium

concentration/force relationship in rabbit inferior vena cava activated by norepinepehrine and high K^+. *Pflugers Arch. Eur. J. Physiol.* 416, 727-734.

Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1988) Structure activity relationship of endothelin: importance of the C-terminal moiety. *Biochem. Biophys. Res. Commun.* 156, 1182-1186.

Kloog, Y., Bousso-Mittler, D., Bdolah, A. and Sokolovsky, M. (1989) Three apparent receptor subtypes for endothelin/sarafotoxin family. *FEBS Lett.* 253, 199-202.

Kloog, Y., Ambar, I., Sokolovsy, M., Kochva, E., Wollberg, Z. and Bdolah, Z. (1988) Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain. *Science* 242, 268-270.

Kloog, Y. and Sokolovsky, M. (1989) Similarities in mode and sites of action of sarafotoxins and endothelins. *Trends in Pharmacol. Sci.* 10, 47-49.

Knight, D.E. and Baker, P.F. (1982) Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J. Membr. Biol.* 68, 107-140.

Kochva, E., Viljoen, C.C. and Botes, D.P. (1982) A new type of toxin in the venom of snakes of the genus Atractaspis (Atractaspideinae). *Toxicon* 20, 581-592.

Kojima, I., Kojima, K. and Rasmussen, H. (1985) Intracellular calcium and adenosine 3',5'-cyclic monophosphate as mediators of potassium-induced aldosterone secretion. *Biochem. J.* 228, 69-76.

Kokesi, C., Imai, M., Hirata, Y., Yanagisawa, M. and Masaki, T. (1989) Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? *Am. J. Physiol.* 256, R858-R866.

Komuro, I., Kurihara, H., Siugiyama, T., Takaku,F. and Yazaki, Y. (1988) Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett.* 238, 249-252.

Kuno, M. and Gardner P. (1987) Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature (Lond.)* 326, 301-304.

Kurihara, H., Yoshizumi, M., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., Hamaoki, M., Kato, H. and Yazaki, Y. (1989) Transforming growth factor-ß stimulates the expression of endothelin mRNA by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 159, 1435-1440.

Kwan, C.Y. (1985) Calcium-handling defects and smooth muscle pathophysiology, in *Calcium and Contractility*, Smooth Muscle, Grover, A.K. and Daniel, E.E., Eds., Humana Press, Clifton, NJ 299-325.

Lakatta, E.G., Capograssi, M.C., Kort, A.A. and Stern, M.D. (1985)

Spontaneous myocardial calcium oscillations: overview with emphasis on ryanodine and caffeine. *Fed. Proc.* 44, 2977-2983.

Lawson, K. and Chatelain, P. (1989) Contractions of rat aorta to endothelin are sensitive to nickel and cadmium ions but not to nicardipine or *ω*-conotoxin. *Br. J. Phamacol.* 98, 840p.

Leijten, P.A.A. and van Breemen, C. (1984) The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. *J. Physiol.* 357, 327-339.

Lin, W.W., Lee, C.Y. and Chuang, D.-M. (1989) Cross desensitization of endothelin and sarafotoxin-induced phosphoinositide turnover. *Eur. J. Pharmacol.* 166, 581-582.

Loeb, A.L. and Peach, M.J. (1989) Endothelial and smooth muscle interactions in hypertension. In: *Blood Vessel Changes in Hypertension: Structure and Function*, R.M.K.W. Lee, Ed., Vol. I, 25-44.

Long, C.J., Shikano, K. and Berkowitz, B.A. (1987) Anion exchange resins discriminate between nitric oxide and EDRF. *Eur. J. Pharmacol.* 142, 317-318.

Lund-Johansen, P. (1977) Hemodynamic alteration in hypertension-spontaneous changes and effects of drug therapy. A review. *Acta Med. Scand.* (603) Suppl., 1-14.

Lüscher T.F. and Vanhoutte, P.M. (1986) Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension* 8, 344-348.

Lüscher, T.F. (1990) The endothelium: target and promoter of hypertension. *Hypertension* 15, 482-485.

MacCumber, M.W., Ross, C.A. and Snyder, S.H. (1990) Endothelin in brain: receptors, mitogenesis and biosynthesis in glial cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 2359-2363.

MacCumber, M.W., Ross, C.A., Glaser, B.M. and Snyder, S.H. (1989) Endothelin: visualization of mRNAs by in situ hybridization provides evidence for local action. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7285-7289.

Maggi, C.A., Giulani, S., Patacchini, R., Santicioli, P., Turini, D., Barbanti, G. and Meli, A. (1989) The C-terminal hexapeptide, endothelin-(16-21), discriminate between different endothelin receptors. *Eur. J. Pharmacol.* 166, 121-122.

Marshall, J.J. and Kontos, H.A. (1990) Endothelium-derived relaxing factors: a perspective from in vivo data. *Hypertension* 16, 371-386.

Martin, W., Villani, G.M. Jothianandan, D. and Furchgott, R.F. (1984) Selective blockade of endothelium-dependent and glycerol trinitrate-induced

relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.* 232, 708-716.

Matsuda, J.J., Volk, K.A. and Shibata, E.F. (1990) Calcium currents in isolated rabbit coronary arterial smooth muscle myocytes. *J. Physiol. (Lond.)* 427, 657-680.

Mellander, S. and Johansson, B. (1968) Control of resistance, exchange, and capacitance functions in peripheral circulation. *Pharmacological Reviews* 20, 117-196.

Miasiro, N. and Paiva, A.C.M. (1990) Homologous desensitization of the effects of endothelin on rabbit aortic rings and on cultured rat aorta smooth muscle cells. *Eur. J. Pharmacol.* 179, 151-158.

Miller, V.M., Aarhus, L.L. and Vanhoutte, P.M. (1986) Modulation of endothelium-dependent responses by chronic alterations of blood flow. *Am. J. Physiol.* 251, H520-H527.

Miller, R.J. (1987) Multiple calcium channels and neuronal function. *Science* 235, 46-52.

Miller, V.M. and Vanhoutte, P.M. (1985) Endothelial α_2-adrenoceptors in canine pulmonary and systemic blood vessels. *Eur. J. Pharmacol.* 118, 123-129.

Miller, V.M. and Vanhoutte, P.M. (1989) Is nitric oxide the only endothelium-derived relaxing factor in canine femoral veins? *Am. J. Physiol.* 257, H1910-H1916.

Minneman, K.P. (1988) α-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. *Pharmacol. Rev.* 40, 87-119.

Morgan, J.P. and Morgan, K.G. (1984) Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol. (Lond.)* 351, 155-167.

Mulvany, M.J. (1989) Contractile properties of resistance vessels related to cellular function. In *Blood Vessel Changes in Hypertension: Structure and Function*, R.M.K.W. Lee, Ed. pp. 1-24. Boca Raton, Florida: CRC Press Inc.

Myers, P.R., Guerra, R. Jr., Bates, J.N. and Harrison, D.G. (1989) Studies on the properties of endothelium-derived relaxing factor (EDRF), nitric oxide, and nitrosolthiols: Similarities beween EDRF and S-nitroso-L-cysteine (CysNO). *J. Vasc. Med. Biol.* 1: 106.

Nakajima, K., Kumagaye, S., Nishio, H., Kuroda, H., Watanabe, T.X., Kobayashi, Y., Tamaoki, H., Kimura, T. and Sakakibara, S. (1989) Synthesis of endothelin-1 analogues, endothelin-3 and sarafotoxin S6b: structure-activity relationships. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5), S8-S12.

Nayler, W.G. (1990) Endothelin: isoforms, binding sites and possible implications

in pathology. *Trends in Pharmcol. Sci. 11, 96-99.*

Nomora, A., Uchida, Y., Kameyama, M., Saotome, M., Oki, K. and Hasegawa, S.(1989) Endothelin and bronchial asthama. *Lancet* ii, 747-748.

Nowycky, M.C., Fox, A.P. and Tsien, R.W. (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature (Lond.)* 316, 440-443.

O'Brien, R.F. and McMurtry, I.F. (1984) Endothelial cell supernates contract bovine pulmonary artery rings. *Am. Rev. Respir.* 126, A337 (abstr).

Ohlstein, E.H., Horohonich, S. and Hay, D.W.P. (1989) Cellular mechanisms of endothelin in rabbit aorta. *J. Pharmacol. Exp. Ther.* 50, 548-555.

Oortgiesen, M., van Kleef, R.G.D.M. and Vijverberg, H.P.M. (1990) Novel type of ion channel activated by Pb^{2+}, Cd^{2+} and AL^{st} in cultured mouse neuroblastoma cells. *J. Membr. Biol.* 113, 261-268.

Oriowo, M.A. (1984) Effect of calcium entry blockers and divalent cations on noradrenaline induced contractions of the rat anococcygeus muscle. *Arch. Int. Pharmacodyn. Ther.* 271, 45-52.

Owen, D.G., Segal, M. and Baker, J.L. (1986) Voltge-clamp analysis of a Ca^{2+} and voltage-dependent chloride conductance in culture mouse spinal neurons. *J. Neurophysiol.* 55, 1115-1135.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524-526.

Patel, A.I. and Triggle, C.R. (1986) Pharmacological characterisation of calcium channels in vascular smooth muscle from hypertensive and normotensive animals. Unpublished M.Sc. Thesis, Memorial University of Newfoundland.

Patridge, L.D. and Swandulla, D. (1988) Calcium activated non-specific cation channels. *Trends Neurosci.* 11, 69-72.

Peach, M.J., Loeb, A.L., Singer, H.A. and Saye, J. (1985) Endothelium-derived vascular relaxing factor. *Hypertension* 7 (Suppl. I) I-94-I-100.

Pernow, J., Hemsén, A. and Lundberg, J.M. (1989) Tissue specific distribution, clearance and vascular effects of endothelin in the pig. *Biochem. Biophys. Res. Commun.* 161, 647-653.

Price, B.D., Morris, J.D.H., Marshall, C.J. and Hall, A. (1989) Stimulation of phosphatidylcholine hydrolysis, diacylglycerol release, and arachidonic acid production by oncogenic ras is a consequence of protein kinase C activation. *J. Biol. Chem.* 264, 16638-16643.

Pohl, U., Busse, R. and Bassenge, E. (1987) Endothelial cells as oxygen sensors in *Mechanisms of Vasodilation* IV (Vanhoutte, P. M., ed.) Raven, New York.

Rand, M.J. and Varma, B. (1970) The effects of cholinomimetic drugs on responses to sympathetic nerve stimulation and noradrenaline in the rabbit ear artery. *Br. J. Pharmacol.* 38, 758-770.

Rapoport, R.M., Stauderman, K.A. and Highsmith, R.F. (1990) Effects of EDCF and endothelin on phosphatidylinositol hydrolysis and contraction in rat aorta. *Am. J. Physiol.* 258, C122-C131.

Rasmussen, H. (1989) The cycling of calcium as an intracellular messenger. *Sci. Amer.* 261, 66-73.

Rasmussen, H. (1986) Calcium messenger system. *New Engl. J. Med.* 314, 1094-1101 and 1164-1170.

Rees, D.D., Palmer, R.M.J. and Moncada, S. (1989) Role of endothelium derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.* 86, 3375-3378.

Resink, T.J., Scott-Burden, T., Boulanger, C., Weber, E. and Bühler, F. (1990) Internalization of endothelin by cultured human vascular smooth muscle cells: characterization and physiological significance. *Mol. Pharmacol.* 38, 244-252.

Resink, T.J., Scott-Burden, T. and Bühler, F.R. (1989) Activation of phospholipase A_2 by endothelin in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 158, 279-286.

Reynolds, E.E., Mok, L.L.S. and Kurokawa, S. (1989) Phorbol esters dissociates endothelin-stimulated phosphoinositide hydrolysis and arachidonic acid release in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 160, 868-873.

Rimele, T.J. and Vanhoutte, P.M. (1983) Effects of inhibitors of arachidonic acid metabolism and calcium entry on responses to acetylacholine, potassium and norepinephrine in the isolated canine saphenous vein. *J. Pharmacol. Exp. Ther.* 225, 720-728.

Ringer, S. (1883) A third contribution regarding the influence of the inorganic constituents of the blood on the ventricles contraction. *J. Physiol.* 4, 222-225.

Rodman, D.M., McMurtry, I.F., Peach, J.L. and O'Brien, R.F. (1989) Comparative pharmacology of rat and porcine endothelin in rat pulmonary artery. *Eur. J. Pharmacol.* 165, 297-300.

Ru Hu, J., Von Harsdorf, R. and Lang, R.E. (1988) Endothelin has potent inotropic effect in rat atria. *Eur. J. Pharmacol.* 158, 275-278.

Rubanyi, G.M., McKinney, M. and Vanhoutte, P.M. (1987) Biphasic release of endothelium-derived relaxing factor(s) by acetylcholine from perfused canine femoral arteries. Characterization of muscarinic receptors. *J. Pharmacol. Exp. Ther.* 240, 802-808.

Rubanyi, G.M. and Vanhoutte, P.M. (1987) Nature of endothelium-derived relaxing factor: are there two relaxing mediators? *Circ. Res.* 34, 317-326.

Rubanyi, G.M., Wilcox, D.E. and Greenberg, S. (1989) Studies on endothelium derived relaxing factor (EDRF) released from canine femoral arteries by acetylcholine (ACh) and its identity as nitric oxide (NO)(abstract). *J. Vasc. Med. Biol. 1, 111.*

Rubanyi, G.M., Lorenz, R.R. and Vanhoutte, P.M. (1985) Bioassay of endothelium-derived relaxing factor. *Am. J. Physiol.* 215, H1077-H1080.

Rubanyi, G.M. and Vanhoutte, P.M. (1985) Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. *J. Physiol. (Lond.)* 364, 45-56.

Saida, K., Mitsui, Y. and Ishida, N. (1989) A novel peptide, vasoactive intestinal contractor of a new (endothelin) peptide family. *J. Biol. Chem.* 264, 14613-14616.

Sakata, K., Ozaki, H., Kwon, S.-C. and Karaki, H. (1989) Effects of endothelin on the mechanical activity and cytosolic calcium levels in various types of smooth muscle. *Br. J. Pharmacol.* 98, 483-492.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348, 732-735.

Sala, S. and Matteson, D.R. (1990) Single channel recordings of two types of calcium channels in rat pancreatic beta-cells. *Biophys. J.* 58, 567-571.

Sato, K., Ozaki, H. and Karaki, H. (1988) Multiple effects of caffeine on contraction and cytosolic free Ca^{2+} levels in vascular smooth muscle of rat aorta. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 338, 443-448.

Schofield, P.R. and Abbott, A. (1989) Molecular pharmacology and drug action: structural information casts light on ligand binding. *Trends in Pharmacol. Sci.* 10, 207-212.

Scriabine, A., Pan, M. and Vanhoutte, P.M. (1990) Effect of lipoxygenase inhibitors on Ca^{2+}-induced constriction of the rabbit ear artery. *Gen. Pharmacol.* 21, 235-239.

Sharma, R.V. and Bhalla, R.C. (1989) Regulation of cytosolic free Ca^{2+} concentration in vascular smooth muscle cells by A- and C-kinases. *Hypertension* 13, 845-850.

Shearman, M.S., Sekiguchi, K. and Nishizuka, Y. (1989) Modulation of ion channel activity: a key function of protein kinase C enzyme family. *Pharmacol. Rev.* 41, 211-237.

Shiba, R., Yanagisawa, M., Miyauchi, T., Ishii, Y., Kimura, S., Uchiyama, Y.,

Masaki, T. and Goto, K. (1989) Elimination of intravenously injected ET-1 from the circulation of the rat. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5) S98-S101.

Shibata, S., Kuchii, M. and Taniguchi, T. (1975) Calcium flux and binding in the aortic smooth muscle from spontaneously hypertensive rats. *Blood Vessels* 12, 279-289.

Shichiri, M., Hirata, Y., Emori, T., Ohia, K., Nakajima, T., Sato, K., Sato, A. and Marumo, F. (1989) Secretion of endothelin and related peptides from renal epithelial cell lines. *FEBS Lett.* 256, 203-206.

Shimokawa, H., Aarhus, L.L. and Vanhoutte, P.M. (1987) Porcine coronary arteries with regenerated endothelium have a reduced endothelium-dependent responsiveness to aggregating platelets and seretonin. *Circ. Res.* 61, 256-270.

Shinmi, O., Kimura, S., Sawamura, T., Sugita, Y., Yoshizawa, T., Uchiyama, Y., Yanagisawa, M., Goto, T., Masaki, T. and Kananzawa, I. (1989) Endothelin-3 is a novel neuropeptide: isolation and sequence determination of endothelin-1 and endothelin-3 in porcine brain. *Biochem. Biophys. Res. Commun.* 164, 587-593.

Silver, P.J. and Stull, J.T. (1982) Regulation of myosin light chain and phosphorylase phosphorylation in tracheal smooth muscle. *J. Biol. Chem.* 257, 6145-6150.

Simonson, M.S., Wann, S., Mene, P., Dubyak, G.R., Kester, M., Nakazato, Y., Sedor, J.R. and Dunn, M.J. (1989) Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-fos expression and mitogenesis in rat mesangial cells. *J. Clin. Invest.* 83, 708-712.

Simonson, M.S. and Dunn, M.J. (1990a) Endothelin: Pathways of transmembrane signalling. *Hypertension* 15 (Suppl. I), I5-I12.

Simonson, M.S. and Dunn, M.J. (1990b) Cellular signaling by peptides of the endothelin gene family. FASEB J. 4, 2989-3000.

Singer, H.A., Saye, J.H. and Peach, M.J. (1984) Effects of cytochrome P-450 inhibitors on endothelium dependent relaxation of rabbit aorta. *Blood Vessels* 21, 223-230.

Slivka, S.R. and Insel, P.A. (1987) α_1-Adrenergic receptor mediated phosphoinositide hydrolysis and prostaglandin E_2 formation in Madin-Darby canine kidey cells. *J. Biol. Chem.* 262, 4200-4207.

Slivka, S.R. and Insel, P.A. (1988) Phorbol ester and neomycin dissociate bradykinin receptor-mediated arachidonic acid release and polyphosphoinositide hydrolysis in Madin-Darby canine kidney cells. *J. Biol. Chem.* 263, 14640-14647.

Sokolovsky, M., Galron, R., Kloog, Y., Bdolah, A., Indig, F.E., Blumberg, S. and

Fleminger, G. (1990) Endothelins are more sensitive than sarafotoxins to neutral endopeptidase: possible physiologic significance. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4702-4706.

Steinsland, O.S., Furchgott, R.F. and Kirpekar, S.M. (1973) Inhibition of adrenergic neurotransmission by parasympathetics in the rabbit ear artery. *J. Pharmacol. Exp. Ther.* 184, 346-356.

Sturek, M. and Hermsmeyer, K. (1986) Calcium and sodium channels in spontaneously contracting vascular smooth muscle cells. *Science* 233, 475-478.

Sugiura, M., Snajdar, R.M., Schwartzberg, M., Badr, K.F. and Inagami, T. (1989) Identification of two types of specific endothelin receptors in rat mesangial cell. *Biochem. Biophys. Res. Commun.* 163, 1396-1401.

Sunako, M., Kawahara, Y., Hirata, K., Tsuda, T., Yokoyama, M., Fukuzaki, H. and Takai, Y. (1990) Mass analysis of 1,2-diacylglycerol in cultured rabbit vascular smooth muscle cells; comparisons of stimulation by angiotensin II and endothelin. *Hypertension* 15, 84-88.

Sutko, J.L., Ito, K. and Kenyon, J.L. (1985) Ryanodine: a modifier of sarcoplasmic reticulum calcium release in striated muscle. *Fed. Proc.* 44, 2984-2988.

Sutter, M.C. and Ljung, B. (1977) Contractility, muscle mass and agonist sensitivity of isolated portal veins from normo- and hypertensive rats. *Acta Physiol. Scand.* 99, 484-495.

Suzuki, N., Matsumoto, H., Kitada, C., Yanagisawa, M., Miyauchi, T., Masaki, T. and Fujino, M. (1989) Immunoreactive endothelin-1 in plasma detected by sandwich-type enzyme immunoassay. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5) S151-S152.

Tabrizchi, R. and Triggle, C.R. (1990) Comparison between the vasoactive actions of endothelin and arginine vasopressin in pithed rats after pretreatment with Bay K 8644, nifedipine or pertussis toxin. *J. Pharmacol. Exp. Ther.* 253, 272-276.

Tabuchi, Y., Nakamura, M., Rakugi, H., Nagano, M., Mikami, H. and Ogihara, T. (1989) Endothelin inhibits presynaptic adrenergic neurotransmission in rat mesenteric artery. *Biochem. Biophys. Res. Commun.* 161, 803-808.

Takasaki, C., Tamiya, N., Bdolah, A., Wollberg, Z. and Kochva, E. (1988) Sarafotoxins S6: several isotoxins from Atractaspis engaddensis (burrowing asp) venom that affect the heart. *Toxicon* 26, 543-548.

Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. (1987) Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* 328, 313-318.

Taylor, S.G., Southerton, J.S., Weston, A.H. and Baker, J.R.J. (1988) Endothelium-dependent effects of acetylcholine in rat aorta: a comparison with sodium nitroprusside and cromakalim. *Br. J. Pharmacol.* 94, 853-863.

Topouzis, S., Pelton, T.J. and Miller, R.C. (1989) Contractile effects of endothelin and [$Ala^{3,11}$]endothelin in rat isolated aorta. *Br. J. Pharmacol.* 96, 101P.

Trahey, M. and McCormick, K. (1987) A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* 238, 542-545.

Triggle, D.J. (1980) Desensitization. *Trends in Pharmacol. Sci.* 1, 395-399.

Triggle, D.J. (1984) Cellular calcium metabolism: activation and antagonism. *J. Asthma* 21, 375-385.

Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R. and Fox, A.P. (1988) Multiple type of neuronal calcium channels and their selective modulation. *Trends Neurosci.* 11, 431-438.

Uchida, Y., Ninomiya, H., Saotome, M., Nomura, A., Ohtsuka, M., Yanagisawa, M., Goto, K., Naskai, T. and Hasegawa, S. (1988) Endothelin, a novel vasoconstricotr peptide, as a potent bronchoconstrictor. *Eur. J. Pharmacol.* 154, 227-228.

van Breemen, C., Aaronson, P., Loutzenhiser, R. and Meisheria, K. (1979) Sodium-calcium interaction in mammalian smooth muscle. *Pharmacol. Rev.* 30, 167-208.

Vanhoutte, P.M. (1974) Inhibition by acetylcholine of adrenergic neurotransmission in vascular smooth muscle. *Circ. Res.* 34, 317-326.

Vanhoutte, P.M. and Katusic, Z.S. (1988) Endothelium-derived contracting factor: Endothelin and/or superoxide anion? *Trends in Pharmacol. Sci.* 9:229-230.

Vanhoutte, P.M., Rimele, T.J. and Flavahan, N.A. (1985) Lipoxygenase and calcium entry in vascular smooth muscle. *J. Cardiovasc. Pharmacol.* 7, S47-S52.

Vanhoutte, P.M. Rubanyi, G.M., Miller, V.M. and Houston, D.S. (1986) Modulation of vascular smooth msucle contraction by endothelium. *Ann. Rev. Physiol.* 48, 307-320.

Vanhoutte, P.M. and Miller, V.M. (1989) α_2-Adrenoceptors and endothelium-derived relaxing factor. *Am. J. Med.* 87 (3C), 1S-5S.

Vanhoutte, P.M. (1989) Endothelium and control of vascular function: state of the art lecture. *Hypertension* 13, 658-667.

Vanhoutte, P.M., Auch-Schwelk, W., Boulanger, C., Janssen, P.A., Katusic, Z.S., Komori, K., Miller, V.M., Schini, V.B. and Vidal, M. (1989) Does endothelin-1 mediate endothelium-dependent contractions during anoxia? *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5) 124-128.

Van Renterghem, C., Vigne, P., Barhanin, J., Schmid-Alliana, A., Frelin, C. and Zdunski, M. (1989) Molecular mechanism of endothelin-1 action on aortic cells. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5), S186-S187.

von Tscharner, V., Prod'hom, B., Baggiolini, M. and Reuter, H. (1986) Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature (Lond.)* 324, 369-372.

Wallnöfer, A., Weir, S., Rüegg, U. and Cauvin, C. (1989) The mechanism of action of endothelin-1 as compared with other agonists in vascular smooth muscle. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5) S23-S33.

Watanabe, H., Miyazaki, H., Kondoh, M., Masuda, Y., Kimura, S., Yanagisawa, M., Masaki, T. and Murakami, K. (1989) Two distinct types of endothelin receptors are present on chick cardiac membranes. *Biochem. Biophys. Res. Commun.* 161, 1252-1259.

Webb, D.J. (1991) Endothelin receptors cloned, endothelin converting enzyme characterized and pathophysiological roles for endothelin proposed. *Trends in Pharamcol. Sci.* 12, 43-46.

Wei, E.P. and Kontos, H.A. (1990) H_2O_2 and endothelium-dependent cerebral arteriolar dilation: Implications for the identity of endothelium-derived relaxing factor generated by acetylcholine. *Hypertension* 16, 162-169.

Weiser, E., Wollberg, Z., Kochva, E. and Lee, S.Y. (1984) Cardiotoxic effects of the venom of the burrowing asp, Atractaspis engaddensis (Atractaspididae, Ophidia). *Toxicon* 22, 767-774.

Wileman, T., Harding, C., Stahl, P. (1985) Receptor mediated endocytosis. *Biochem. J.* 232, 1-14.

Wollberg, Z., Shabo-Shina, R., Intrator, N., Bdolah, A., Kochva, E., Shavit,G., Oron, Y., Vinde, B.A. and Gitters, S. (1988) A novel cardiotoxic polypeptide from the venom of Atractaspis engaddensis (Burrowing asp): Cardiac effects in mice and isolated rat and human heart preparations. *Toxicon* 26, 525-534.

Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S., Nakajima, K., Watanabe, T.X., Sakakibara, S., Goto, K. and Masaki, T. (1988b) Primary structure, synthesis and biological activity of rat endothelin and endothelium-derived vasoconstrictor peptide. *Proc. Natl. Acad. Sci. U.S.A.* 85, 6964-6967.

Yanagisawa, M., Kurihara, H., Kimura, S., Tombe, Y., Kobayashi, M., Mitsui, Y., Yazak, Y., Goto, K. and Masaki, T. (1988a) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond.)* 332, 411-415.

Yanagisawa, M. and Masaki, T. (1989b) Endothelin, a novel endothelium-derived peptide. *Biochem. Pharmacol.* 38, 1877-1883.

Yanagisawa, M. and Masaki, T. (1989a) Molecular biology and biochemistry of the endothelins. *Trends in Pharmacol. Sci.* 10, 374-378.

Yanagisawa, M., Inoue, A., Takuwa, Y., Mitsui, Y., Kobayashi, M. and Masaki, T. (1989) The human preproendothelin-1 gene: possible regulation by endothelial phosphoinositide turnover signaling. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5), S13-S17.

Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S.J., Kimura, S., Uchiyama, Y., Polak, J.M., Masaki, T. and Kanazawa, I. (1990) Endothelin: a novel peptide in the posterior pituitary system. *Science* 247, 462-464.

Yoshizumi, M., Kurihara, H., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., Yazaki, Y. (1989) Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. *Biochem. Biophys. Res. Commun.* 161, 859-864.

Zeidel, M.L., Brady, H.R., Kone, B.C., Gullans, S.R. and Brenner, B.M. (1989) Endothelin, a peptide inhibitor of Na^+-K^+-ATPase in intact renal tubular cells. *Am. J. Physiol.* 257, C1101-1107.