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**SOME FACTORS THAT AFFECT CALCIUM-  
SENSITIVITY IN THE RAT TAIL ARTERY**

A Thesis presented for the degree  
of Doctor of Philosophy

By

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## List of Abbreviations

Term	Abbreviation
Rat Tail artery	RTA
Calcium concentration-response curve	CCRC
Oxygen	O <sub>2</sub>
Oxygen tension	PO <sub>2</sub>
Noradrenaline	NA
Potassium chloride	KCl
5-hydroxytryptamine	5-HT
Bay K 8644	B.K
Palmitoyl carnitine	PC
Nifedipine	Nif.
Calcium	Ca <sup>2+</sup>
Extracellular calcium concentration	[Ca <sup>2+</sup> ] <sub>o</sub>
Free calcium concentration in buffer	[Ca <sup>2+</sup> ] <sub>free</sub>
Change in perfusion pressure	Δ P.P
Millimetres of mercury	mmHg
Molar	M
Buffer	≠
30% of 1st maximum response (index of calcium sensitivity)	EC <sub>30</sub>

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I would like to conclude by observing that this piece of academic exercise has only succeeded in scratching the surface of the great depth of information that awaits discovery for a better understanding of the subject towards the benefit of all physiological beings. In spite of all that this study has achieved, the words of Isaac Newton are most appropriate to consider at this final juncture - "I seem to have been only like a boy playing on the sea-shore ..... whilst the great ocean of truth lay undiscovered before me."

On the whole, the experience was great and very rewarding. This road I have passed; I will never pass it again.

## DECLARATION

The experimental work and other research which make up this thesis was carried out entirely by myself. No part of the material has previously been presented for any other degree. During the period of the study, some of the results have been published as detailed below under the List of Publications.

## LIST OF PUBLICATIONS

1. Fasehun, O.A., Jennett, S.M., McGrath, J.C. and Ugwu, A.C. (1985). What is a suitable O<sub>2</sub> tension for smooth muscle in vitro? Br. J. Pharmac., 86, 753P.
2. McGrath, J.C. and Ugwu, A.C. (1986). Effects of Bay K 8644 and nifedipine on isolated perfused rat tail artery under different oxygen tensions. Br.J. Pharmac., 88, 385P.
3. McGrath, J.C., Miller, D.J. and Ugwu, A.C. (1987). Desensitisation of responses to noradrenaline and KCl in the isolated perfused rat tail artery. Br.J. Pharmac., 90, 153P.
4. McGrath, J.C., Miller, D.J. and Ugwu, A.C. (1987). Factors affecting calcium-sensitivity and the restoration of desensitized responses in the isolated rat tail artery. J. Physiol., (In press).
5. Ugwu, A.C. (1987). Calcium-sensitivity and temperature effects on the desensitization of responses in different blood vessels isolated from the rat, rabbit and cat. J. Physiol., (In press).
6. Ugwu, A.C., McGrath, J.C. and Spedding, M. (1987). Comparisons of the effects of palmitoyl carnitine and Bay K 8644 on the desensitisation of the rat tail



## GENERAL SUMMARY

The calcium sensitivity of noradrenaline (NA) and potassium chloride (KCl)-induced contractions of vascular smooth muscle were investigated in calcium re-addition experiments under different oxygen tensions and temperatures in the isolated perfused rat tail artery (male Wistar, 300-350g). We started by establishing the most favourable conditions for responses of the smooth muscle in the preparation, and making an extensive analysis of the influence of experimental conditions on the contraction-response relationship to NA and in some cases, to KCl. This was carried out on both the proximal and distal portions of the vessel, applying NA both intraluminally and extraluminally. Both portions showed similar characteristics and intraluminal administration of NA was more effective than the extraluminal administration. But this difference in potency was abolished when cocaine was included in the saline during the comparisons of the routes of administration of NA.

Factors analysed (alone and in combination) included cocaine, propranolol, EDTA, extracellular and intracellular modification of pH, oxygen,  $[Ca^{2+}]_o$  (including the use of  $Ca^{2+}$  buffers). Following this the alpha-adrenoceptor population was analysed using selective antagonists (prazosin and rauwolscine) and  $Ca^{2+}$ -entry was manipulated using nifedipine (blocker) and Bay K 8644 (facilitator).

Both cocaine (4uM) (an uptake blocker) and propranolol (1uM) (a beta-adrenoceptor antagonist), individually and in combination, enhanced NA-induced responses. EDTA (23uM) stabilised NA responses by preventing NA oxidation without causing undue chelation of free calcium in the saline. There was no difference in the NA responses within the alkaline and acid range tested. This was the case whether we raised the extracellular pH with a higher concentration of sodium bicarbonate solution ( $\text{NaHCO}_3$ ) or the intracellular and extracellular pH by means of gassing the saline with a lower percentage of carbon dioxide ( $\text{CO}_2$ ). The rate at which a given gas mixture is bubbled into the saline did not significantly affect the pH of the solution and therefore the responses of the tissue.

The tissue apparently behaved differently in different oxygen tensions. Lowering the gassing oxygen in steps from 95% through 16% to 4%  $\text{O}_2$  decreased the responses with progressive rightward shift of the NA-induced concentration response curves.

Exposure to high  $\text{pO}_2$  tended to attenuate the initial rise in perfusion pressure to NA and increased the maximal maintained contraction. In addition, the pattern of interactions of the tail artery with various agonists and antagonists suggest that the  $\alpha$ -adrenoceptor population consists mainly of the  $\alpha_1$ -subtype, as

demonstrated with the selective  $\alpha_1$ -adrenoceptor antagonist, prazosin, and the selective  $\alpha_2$ -adrenoceptor antagonist, rauwolscine. In both the proximal and distal portions of the tissue, prazosin effectively blocked the responses even at a very low concentration of 1.0nM, whereas rauwolscine showed some blockade only at a high concentration of 1uM and above (probably due to its small  $\alpha_1$  antagonist properties).

The tissue contracted to NA in calcium-free saline but its responses increased with increase in the calcium concentration. Responses were more regularly reproducible in calcium-buffered saline than in unbuffered saline solution.

Construction of NA concentration-response curves in different calcium buffers (1uM to 5mM  $\text{Ca}^{2+}$ ), produced different levels of response with higher  $[\text{Ca}^{2+}]$  producing greater maxima and  $\text{pD}_2$  values. In the unbuffered saline, it was much more difficult to distinguish the effects of different concentrations of calcium on NA-induced responses. Only responses in zero calcium were prominently smaller. The indeterminate free  $\text{Ca}^{2+}$  level in nominally "zero" calcium saline might contribute to this effect. It was therefore more reliable to examine the sensitivity of the tissue to calcium in a calcium-buffered saline where the concentration of calcium can be more accurately

estimated.

Comparison of the sensitivity of the tissue to calcium by switching from buffered saline to the unbuffered or vice versa, using the same set of tissue could produce variable results, probably due to the desensitisation of the responses to calcium and/or the reduction of the free calcium concentration of the unbuffered saline by the residual buffers attached to the tissue or the organ bath.

Subsequent analysis of Ca sensitivity was thus carried out using Ca buffers. Compared with a first control curve, there was a small shift to the left of a 2nd curve with Bay K 8644 and a shift to the right with nifedipine. However, time controls showed a considerable rightward shift after the first curve, indicating that longitudinal studies were impracticable and thus that drug effects could be reliably estimated only by comparisons of 1st curves. A series of experiments was carried out before this was appreciated on the effects of Bay K 8644 and nifedipine in different  $O_2$  tensions. These results indicated qualitative trends but could only be properly understood after a more exhaustive study using first curves had been carried out. This applied to both NA-induced and KCl-induced contractions.

This "desensitisation" of the rat perfused tail

artery was particularly marked (relative to other preparations), and seemed to depend on  $[Ca^{2+}]_o$  ([free  $Ca^{2+}$ ]). A systematic study of some factors influencing desensitisation showed that an interval less than 3.5hr was insufficient for recovery; and when equilibrated for 5hr, the tissue did not lose sensitivity, showing that desensitisation is dependent on activation not time alone but is reversible. It was also found that Ca sensitivity could be retained by avoiding high levels of  $Ca^{2+}$  and/or pre-exposing the tissue to 0.3mM with CRCs taken only to 0.3mM  $Ca^{2+}$ , thus achieving both stability and reasonable level of sensitivity; doubling the glucose concentration after the 1st curve or using lower [NA] (0.3uM) than our standard NA of 3uM partially arrested desensitisation. Desensitisation occurred with activation by KCl, NA or 5-HT. Cross desensitisation between NA and 5-HT was not mutual. NA caused desensitisation to 5-HT but 5-HT did not cause desensitisation to NA. Thus NA responses in this tissue require very little  $[Ca^{2+}]_o$  but this is very easily missed if care is not taken to avoid desensitisation. As a corollary, it appears that the desensitisation of NA-induced responses in vascular smooth muscle at high "physiological saline" levels of  $[Ca^{2+}]$  is largely due to loss of sensitivity to  $[Ca^{2+}]_o$ . Comparison of the first and subsequent  $Ca^{2+}$  CRCs in 95%O<sub>2</sub> (and 16% and 4% O<sub>2</sub>), in an attempt to incorporate the first responses in an analysis of the properties of the tissues, showed that progressive

desensitisation occurred in both NA and KCl-induced contractions and irrespective of the presence of Bay K 8644, or nifedipine, although the 1st curve did show potentiation and inhibition respectively to these drugs. At this point we are left with a dilemma over the actual physiological  $[Ca^{2+}]_o$  sensitivity of this tissue and hence the likely quantitative effects in vivo of drugs which modify this, ie., should we accept the first curve as "normal" and regard the desensitised state as an experimental artefact, or vice versa?

Calcium sensitivity in perfused cutaneous arteries from 3 different species was examined at different temperatures to examine the general applicability of the desensitisation phenomenon which had been studied in detail in rat tail artery. This was repeated at 3 temperatures to find whether this further physiological variable was critical. The general order of sensitivity was  $24^{\circ}C \geq 37^{\circ}C > 42^{\circ}C$  and desensitisation occurred in the other species (rabbit ear artery, cat distal limb arteries to the paws), although to a smaller extent than in rat tail artery.

The effect on calcium sensitivity of the endogenous lipid metabolite, palmitoyl carnitine (PC), was tested at different oxygen tensions and found to resemble that of Bay K 8644, although palmitoyl carnitine additionally produced an increase in the height of responses.

This work has provided an experimental framework for the study of the  $\text{Ca}^{2+}$  sensitivity of vascular smooth muscle, suggests that "NA-induced contraction" requires lower levels of  $[\text{Ca}^{2+}]_o$  than had been thought previously; has clarified the effects of the  $\text{Ca}^{2+}$  facilitator Bay K 8644 against responses to NA and points to new recommendations for standard experimental conditions for studying such phenomena.

## GENERAL INTRODUCTION

This study was designed to investigate the mechanisms underlying the effects of blood gases on the calcium sensitivity of the rat tail artery. In addition, the study aimed at investigating the mechanisms behind the facilitatory action of the calcium channel activators and the blocking action of the calcium channel blockers on  $\alpha_1$ -adrenoceptor mediated excitation-contraction coupling in vascular smooth muscle and how this was affected by oxygen tension.

The main Ca-modulating drugs used were Bay K 8644 (a calcium entry facilitator) and Nifedipine (a calcium entry blocker). Their effects were determined against contractile responses obtained in different levels of calcium under hyperoxic (supramaximal); normoxic (physiological) and mildly hypoxic conditions.

In order to properly assess the effects of these drugs on calcium movement across the plasma membrane under various oxygen tensions, it was necessary to establish the optimum in vitro conditions which would suitably mimic in vivo conditions for the study of adrenergic excitation-contraction coupling in this blood vessel. Essentially, we consider that a high oxygen environment, such as is commonly employed in pharmacological studies, may give misleading results.

This was prompted by a study on the non-vascular smooth muscle of rat anococcygeus, in which responses to noradrenaline were not only reduced in size on moving to lower, i.e., more physiological, levels of oxygen, but the initial rate of contraction actually increased and the dependence of the contraction on nifedipine-sensitive  $\text{Ca}^{2+}$ -channels decreased (McGrath, 1982). This suggests that it might be fruitful to manipulate blood gases and calcium concentrations around the physiological levels rather than the artificial levels commonly employed. For example, more emphasis might be placed on 1.25mM calcium rather than 2.5mM and an oxygen tension of 120mmHg rather than 650mmHg. In order to cover the physiological range we also examined responses in mild hypoxia.

The initial hypothesis was that, if similar factors apply in the vascular system as in anococcygeus smooth muscles, then this has considerable importance for the in vitro study of the details of vascular regulatory processes and its extrapolation to in vivo conditions. For example, the interactions between calcium entry blockers and alpha-adrenoceptor antagonists, both employed in hypertension and heart failure could be misinterpreted.

We chose the isolated perfused rat tail artery to further investigate the phenomenon since its contractions to noradrenaline and potassium chloride

were known to be attenuated by nifedipine and facilitated by Bay K 8644 (Su et al., 1984) under the high oxygen tension customarily employed in pharmacological experiments.

Often isolated tissues are maintained in saline which is equilibrated at atmospheric pressure with a gas mixture of high oxygen content such as 100% or where carbon dioxide/bicarbonate buffering is employed, 95%. This produces partial pressures of oxygen of 300 to 650mmHg according to the efficiency of equilibration. Often the main objective is to avoid a hypoxic core within the tissue and it is assumed that a high oxygen tension will do no harm. However, this practice is not universal. Tyrode's solution is often bubbled with room air, which gives an oxygen partial pressure of approximately 150mmHg and provides viable conditions even for thick preparations such as the rabbit intestine. Those interested in the effects of hypoxia on smooth muscle routinely employ a bubbling mixture containing 16% oxygen for their normal oxygen tension (usually 120-100mmHg) and reduce this further to produce hypoxia (Detar and Bohr, 1968; Ebeigbe and Jennett, 1978).

Little attention has been paid to the difference between normoxia, eg., 16% oxygen (approx. 120mmHg) and hyperoxia, e.g. anything above atmospheric values and represented in this study by 90% - 95% oxygen (580 -

650 mmHg), or between normoxia and mild hypoxia, eg., 4% oxygen (approx. 30mmHg).

It has been noted that low oxygen tension appears to act in vascular smooth muscle as though it interferes with net influx or utilization of external calcium (Ebeigbe, Pickard and Jennett, 1980). However, little attention has been paid to the influence of different oxygen tensions on the action of drugs which modify calcium utilization during excitation-contraction coupling in vascular smooth muscle. Bay K 8644 and nifedipine are dihydropyridines with a similar structure except for the -CF- side-chain in Bay K 8644, which probably is responsible for its facilitatory role in calcium movement across the plasma membrane (see Fig. ii). Since the clearest demonstration of contraction of smooth muscle by Bay K 8644 is usually against potassium chloride-induced depolarization (Schramm and Towart, 1983), we used as activators both 100mM potassium chloride and a 3uM concentration of noradrenaline. We noted that most of the work done with nifedipine and Bay K 8644 (Mikkelsen, 1985; Cheung, 1985; Su et al., 1984; Schramm and Towart, 1983) had been carried out under the supramaximal levels of oxygen - usually 95% O<sub>2</sub>. It may be that the effects of the dihydropyridines or of the activators themselves may alter with oxygen tension and that phenomena seen in normoxic conditions or mild hypoxia may be more closely related to responses in vivo.

The rat tail artery functions in thermoregulation rather than for supplying oxygen to meet metabolic demand, so oxygen itself is perhaps less likely to fulfil a direct regulatory physiological role than in vessels supplying for example, skeletal muscle. We are therefore looking for the generalised influence of oxygen on coupling processes rather than to elucidate its role in autoregulation. There is a considerable literature available on this vessel using standard pharmacological methods which has uncovered some properties currently of interest. For example, it has been suggested that in this tissue  $\alpha_2$ -adrenoceptors are more prevalent in hypertensive rats (Hicks et al., 1984) and non-adrenergic neurotransmission, probably purinergic co-transmission (Bevan et al., 1985; Sneddon & Burnstock, 1984; Vidal et al., 1986), is present.

In order to justify our methods and to compare them with those employed previously, we started by making comparisons between the "classical" (non-physiological) conditions and what we consider to be more "physiological". In this justification of the methods we have also experimented with different methods for the analysis of the responses since this is considerably complicated by the time course of responses to agonists, which consist of combinations of phasic and tonic contractions, and by desensitization of tissues with time. In general, the proximal segment of the tail

artery of male rat (Wistar, 300-350g) was perfused at 2-3 ml/min with a pulsatile flow pump (Watson-Marlow, 6-roller pump with cassette pump head) and the perfusion pressure recorded. The peaks of the pulsatile pressure waves were measured for calculation of responses.

We made an initial analysis of the influence of experimental conditions and the effectiveness of various additives which are commonly employed in experiments of this type. Having established optimal conditions, the  $\text{Ca}^{2+}$ -sensitivity and its modulation by oxygen and  $\text{Ca}^{2+}$ -modulatory drugs was tackled by constructing concentration/response curves (CRCs) for calcium when the tissue was under the influence of a 3  $\mu\text{M}$  concentration of noradrenaline or high potassium chloride (100 mM, in low phosphate).

In the process of these experiments we observed that when we failed to pre-expose the tissue preparations to an activating agent prior to our usual experimental protocol, the first responses and /or the first concentration response curve (CRC) were highly exaggerated. Subsequent responses or CRCs were however desensitised and stabilised. This unusual response of the rat tail artery was unexpected from what had been included in earlier published papers and therefore aroused interest on two grounds. First, could it shed some light on the phenomenon of desensitisation and secondly, which level of sensitivity, initial or

"stabilised" reflects the in vivo responsiveness?

Desensitisation has been reported in several tissue preparations of several animals (Miranda, 1976), but it is apparent that the mechanism of desensitisation is not properly understood. However, it is known that the composition of the physiological saline solution in which the tissue is immersed may affect desensitisation. In the present study, therefore we tried to investigate some of the important factors that influence desensitisation with the aim of fully understanding the mechanisms of desensitisation of smooth muscle responses to calcium sensitivity under various physiological and pharmacological conditions. Since the phenomenon was so marked, this became an important element of the thesis.

## LITERATURE REVIEW

- (i) Properties of the rat tail artery
- (ii) Importance of perfusion techniques
- (iii) Drugs that enhance responses of smooth muscle responses
- (iv) Surface of administration of drugs
- (v) Oxygen tensions
- (vi) pH of the medium
- (vii) Calcium Buffers
- (viii) Calcium Channels
- (ix) Calcium channel facilitators
- (x) Calcium channel blockers
- (xi) Palmitoyl Carnitine
- (xii) Temperature effects

### The Rat Tail Artery

1. Anatomy of the rat tail:- Centrally located at the ventral surface of the tail of a rat is the rat tail artery (Fig. i/c). Its major physiological role is concerned with thermoregulation. The rat can use its tail for balance on the slack rope, especially for the black rat (*Rattus rattus*). The rat tail has over 210 rings of epidermal scales, between which are a few short bristles. The brown rat (*Rattus Norvegicus*) has a shorter tail than the combined length of the head and body. It is stout, and usually dark above and pale beneath. The entire ventral surface of the tail of a

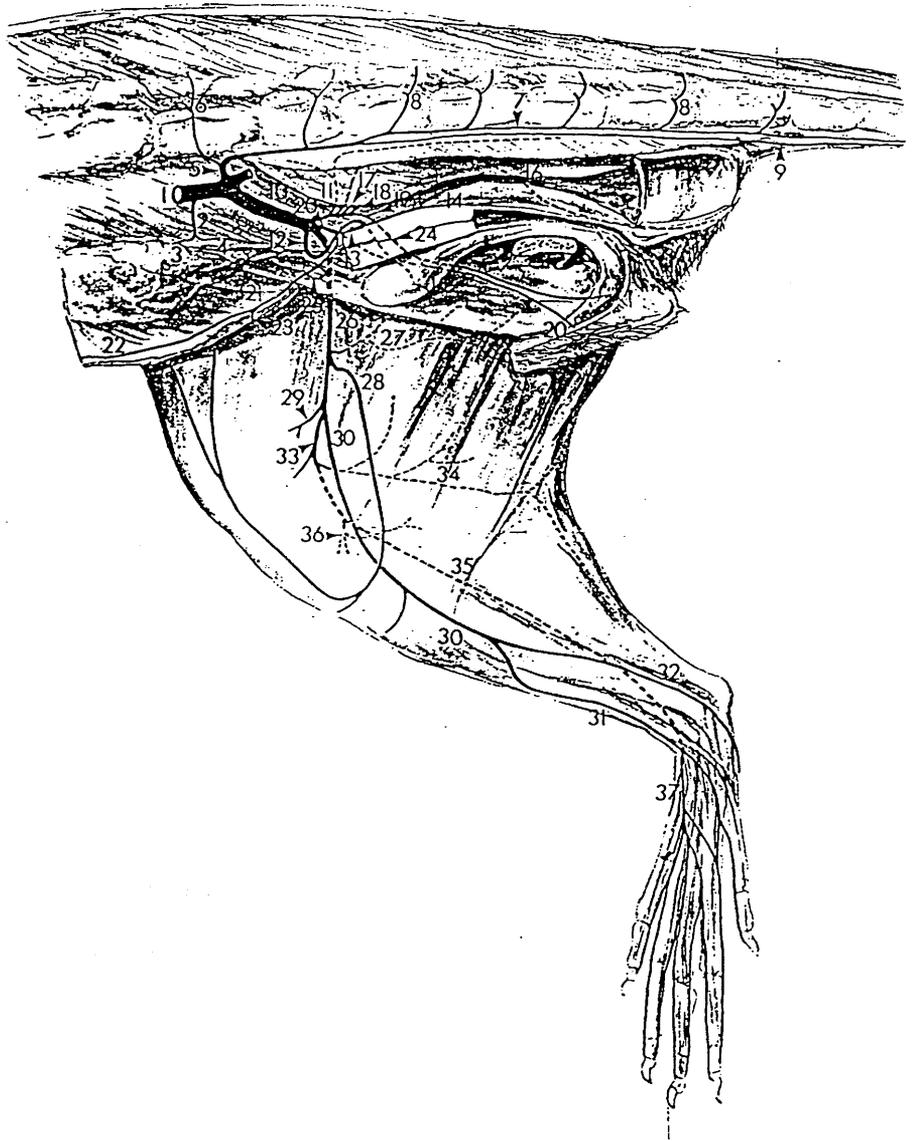


Figure (i/c). A diagram showing the rat tail artery in its natural position along with some other blood vessels. The rat tail artery, otherwise known as the caudal artery of the rat, is ventrally placed in the rat tail.

rat is covered by rows of scales which overlap like roof-tiles. Three short bristles project from under the edge of each scale. The surface is covered with orange-yellow, waxy grease for the Wistar albino laboratory rat (*Rattus norvegicus*).

2. Properties of the rat tail artery Differences in functional behavior occur among sections obtained from different vascular beds. The characteristics of the isolated rat tail artery make it a good model for the investigation of some properties of a resistance vessel. Some of its characteristics according to some studies by Henry and Lacuara (1971) on spiral strip include the following:

(i) Differences in contractile response with variations in the resting applied tension; below 500mg of applied tension, no mechanical activity could be registered and this activity was maximal for a resting tension of 1500mg.

(ii) About 75 min equilibration period is needed to obtain a uniform functional activity shown by uniform responses with 1.0uM adrenaline.

(iii) It showed greater sensitivity to adrenaline than to noradrenaline.

(iv) The threshold of the contractile response of the artery to KCl was with 60mM and the maximal effect was

with 120mM. In our own studies with this tissue, we would consider 100mM KCl as an adequate submaximal concentration for investigating the effects of drugs on calcium channel function.

(v) In the absence of external calcium the contractile response to adrenaline and KCl was lost, and was recovered upon readmission of calcium to the medium. The tension developed in the presence of KCl was more sensitive to changes in the calcium concentration in the medium.

(vi) Electrical field stimulation produced a phasic contraction which disappeared after exposure of the artery to 1.0ug/ml of phentolamine.

According to Henry and Lacuara (1971), the above 6 observations, in addition to the ability of the rat tail artery to react to the agonists employed, and besides its histological characteristics, indicate that this preparation may be successfully employed in physiological and pharmacological studies of the arterial smooth muscle.

Wade and Beilin (1970) observed that the baseline resistance of the rat tail artery remained stable for several hours and that maximal sensitivity occurred at 32° and 34°C. In addition, low concentrations of ( $\pm$ )-propranolol enhanced noradrenaline vasoconstriction.

They also found the rat tail artery to be a simple and relatively inexpensive adjunct to established methods of studying resistance vessel behaviour under varying experimental conditions. In some other studies, it was shown that electrical stimulation of this preparation activate solely the postganglionic nerve terminals and that tachyphylaxis to angiotensin, vasopressin and bradykinin preclude the use of this preparation for their assay (Nicholas, 1969). Hinke and Wilson (1962) had also shown that high  $[K^+]_o$  Krebs solutions alone constricted the vessel whereas low  $[Na^+]_o$  Krebs alone distended it.

While the  $\alpha$ -adrenoceptors population of the rat tail artery is predominantly  $\alpha_1$ -subtype (Medgett & Langer, 1984), the existence of  $\alpha_2$ -subtype was subject to controversy. However, there is evidence for a substantial population of  $\alpha_2$ -adrenoceptors in the distal segment of normotensive Sprague-Dawley rat tail artery (Medgett, 1985) and in spontaneously hypertensive (SHR) rat tail arteries (Medgett & Langer, 1984; Hicks et al. 1984). Cocaine (4 $\mu$ M) and propranolol (1 $\mu$ M) were found effective in blocking neuronal uptake and beta adrenoceptors, respectively (Medgett & Langer, 1984).

Neuropeptide Y, a polypeptide of 36 amino acids, caused a dose-dependent contraction of the rat tail artery and also increased the contraction caused by exogenous NA, 5-HT and KCl concentrations that gave

submaximal (but not maximal or near-maximal) contractions (Neild, 1987).

Rat tail artery may have purinergic co-transmission since - a, b-methylene ATP attenuates nerve-induced contraction (Medgett & Langer, 1985; Sneddon & Burnstock, 1984; Vidal, Hicks & Langer, 1986). The electrophysiology of rat tail artery, has also been studied by Cheung (1982).

#### Choice of isolated preparations and perfusion techniques

In our laboratory, Docherty and McGrath (1980a,b), Flavahan and McGrath (1982) and a host of other workers have devoted their time to studying the adrenergic responses in the whole animal under different experimental conditions. However, much of our practical knowledge of pharmacology and physiological chemistry and many of the theories of drug action have been attained from experiments conducted on isolated preparations. When we work with isolated preparations we can observe the direct actions of various factors on the organ of interest in a relatively controlled manner. These observations are reasonably unencumbered by the many compensatory mechanisms of the organism that often mask the drugs' direct actions. Yet, working at this level of isolated preparations, we remain within the realm of investigation that permits us to connect observations on the whole animal with the more intimate

action of drugs and individual molecules ( Tallarida and Jacob, 1979).

Nevertheless, the experimental necessity for adopting the in vitro method for the study of organs and tissues and the general advantages and disadvantages of this methodology are well known. For example, it has been suggested that the spiral strip preparation in studying the behaviour of vascular smooth muscle in vitro has the advantage of preserving the anatomical configuration of the muscle fibres. It also permits one to measure artery wall activity in hydrodynamic parameters. Thus, smooth muscle contractility is measured as a narrowing of the lumen and a reduction in flow at constant pressure (Hinke and Wilson, 1962). Experimentalists should therefore recognize the specific limitations of existing in vitro methods used to study blood vessels in particular, since the isolation of a tissue always imposes some restriction on the conclusions of the experimentalists. This is why there is the need to confirm the observations made in the in vitro preparations with those of the in vivo experiments. Where practicable, this method of confirmation of observations was tried in the present study.

A lot of workers examining blood vessels in vitro have adopted different styles in the setting up of their tissues in the organ bath. Helical strips of vascular

smooth muscles (VSM) have been used ( Ebeigbe, 1979; Ebeigbe, Gantzios & Webb, 1983; Aoki & Asano, 1986); rings of vascular smooth muscle ( Ahlquist, 1948; Hogestatt, et al. 1983); flat strips of vascular smooth muscle ( Ebeigbe, 1979; Weir & Weston, 1986).

However, an isolated blood vessel, unless perfused, is not subject to the consequences of the high, pulsatile, intravascular pressure that exists in vivo. These include the high circumferential mural tension which is unevenly distributed through the vessel wall thickness, and which causes a bulk flow of fluid from the intima towards the adventitial surface. Other routes of exchange between the circulation and the vessel wall, via the adventitial plexus and the vasa vasora, are also interrupted by isolation. In the tissue bath, movement of molecules into and out of the tunicae is governed mainly by diffusional forces. Because of these alterations with isolation, vessel wall thickness and diffusion characteristics may be critical for the exchange of materials, in particular oxygen and carbon dioxide, between the tissue and the bath solution.

In recognition of these facts, the perfusion technique was adopted in the in vitro experiments of the present study on the vascular smooth muscle of the tail artery.

It is also important to recognize, for in vitro experiments on VSM, the propagation of excitation which appears to take place over distances greater than several millimeters (Bevan and Ljung, 1974). Such propagation might to a great extent be precluded by cutting spiral (helical) strips and by using ring preparations of short length. Vessel ring and strip preparations also necessitate cutting the sympathetic terminal plexus. Some forms of propagated excitation in the vessel wall may be dependent on an intact terminal nerve plexus (Duling and Berne, 1970).

Lastly, repetitive electrical stimulation of postganglionic nerve elements supplying the isolated vessel causing a simultaneous activation of all neural elements, may result in a pattern of excitation different from that associated with the asynchronous activity that occurs in vivo. Asynchronous neuronal activity may be important in determining the pattern of contraction in sparsely innervated vessels (Bevan and Ljung, 1974). However, no nerve stimulation is involved in the present work.

#### Calcium and its role in muscle contraction

Filo, Bohr and Ruegg (1965) observed that an increase in the concentration of free  $\text{Ca}^{2+}$  (above approx.  $0.1\mu\text{M}$ ) in the cytoplasm of the smooth muscle cell triggers the interaction between the contractile proteins, actin and

myosin, which result in sliding of the filaments and shortening of the muscle. Blood vessel tension development induced through alpha-receptor activation may be due to  $\text{Ca}^{2+}$  mobilization from extracellular and intracellular sources. Some events have been proposed in connection with these processes. These are:

(a) an increase in  $\text{Ca}^{2+}$  entry from the extracellular space through  $\text{Ca}^{2+}$  channels in the smooth muscle plasmalemma (the  $\text{Ca}^{2+}$  entering the cell may be derived from extracellular free  $\text{Ca}^{2+}$ , or from extracellularly bound  $\text{Ca}^{2+}$ , e.g., on the external surface of the smooth muscle cells);

(b) a release of intracellularly bound  $\text{Ca}^{2+}$  into the myoplasm (this  $\text{Ca}^{2+}$  may come from several  $\text{Ca}^{2+}$  pools, e.g.  $\text{Ca}^{2+}$  bound on the internal surface of the plasmalemma or  $\text{Ca}^{2+}$  stored in intracellular organelles such as the sarcoplasmic reticulum).

Leijten, Cauvin, Lodge, Saida and van Breemen (1985) working on isolated rabbit aorta and rabbit mesenteric artery, provided evidence supporting the concept that alpha-adrenoceptor agonists mobilize  $\text{Ca}^{2+}$  from multiple sources for activation of the vascular smooth muscle cell. They proposed that, in a model of  $\text{Ca}^{2+}$  mobilization by noradrenaline in vascular smooth muscle, upon receptor occupation,  $\text{Ca}^{2+}$  from a labile small intracellular store on the inner plasmalemma is

released. This  $\text{Ca}^{2+}$ , they pointed out, does not function as activator  $\text{Ca}^{2+}$  but triggers  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum ( $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release). It had also been noted that at the same time  $\text{Ca}^{2+}$  from an extracellular bound store (on the external surface of the plasmalemma) is equally being dislodged and this enters the cell through receptor linked channels. These observations appear to suggest that these are the processes responsible for the early "phasic" component of the noradrenaline contraction.  $\text{Ca}^{2+}$  from the free extracellular  $\text{Ca}^{2+}$  pool also enters through receptor operated channels, supporting the maintained tension development (Leijten et al., 1985). It has been stated that the final common pathway in smooth muscle contractile activation by alpha-agonists is an increase in  $[\text{Ca}^{2+}]_i$ . This can be achieved either through the release of bound  $\text{Ca}^{2+}_i$  or by the entry of  $\text{Ca}^{2+}_o$  through different "channels", some of which may be blocked selectively by appropriate drugs (Loutzenhiser and van Breemen, 1981; Godfraind et al., 1982). According to McGrath (1985), it seems that different agonists might produce their responses by employing these different mechanisms to different extents. Some scope exists to vary  $[\text{Ca}^{2+}]_o$  and to use drugs which alter  $\text{Ca}^{2+}$  mobilisation in attempts to define any such differential activation by agonists and to see whether this correlates with anything else that we know about them, such as:

- (i) the subtype of receptor that we think they act through,
- (ii) measurements of "potency", defined in various ways, or
- (iii) their chemical structures.

Desensitisation of tissue responses:

When an effector is periodically stimulated and responses decline, two related definitions of the phenomenon have been given. (a) Tachyphylaxis which occurs when doses of a drug, given shortly after a previous equal dose of the same drug, produce an effect which is smaller for each subsequent dose (Ariens, 1964); and (b) desensitisation, which is the insensitivity of a tissue immediately after it has been treated with a large dose of agonist (Barlow, 1964). Different substances are known to cause desensitisation in different tissues. For example, substance P desensitised the intestinal muscle of the guinea-pig (Holzer & Petsche, 1983); cholinergic agonists, such as acetylcholine, acetylthiocholine and tetramethylammonium desensitised acetylcholine receptors of denervated rat soleus muscle (Anwyl & Narahashi, 1980); carbachol desensitised smooth muscle

cells in guinea taenia coli (Magaribuchi et al. 1973); and 5-hydroxytryptamine desensitised the guinea pig ileum (Gaddum, 1953). Reduction in the developed tension in ferret papillary muscles as a result of calcium overload had also been reported (Allen et al. 1985). It is therefore apparent that desensitisation of tissue responses is a generalised phenomenon and it could be triggered off in different tissues by different agents or factors. In this thesis, the term "desensitisation" will be used to describe the reduction in sensitivity to  $\text{Ca}^{2+}$  which occurs with sequential administration of the same concentration of activator.

#### Calcium Buffers:

The concept of metal-ion buffering is over 40 years old (Bjerrum, 1941; Rauflaub, 1960 ). In calcium buffering, a calcium chelator is required at some point, either to reduce free  $\text{Ca}^{2+}$  inside or outside the cell to less than  $1\text{nM}$  or to buffer the free  $\text{Ca}^{2+}$  in the range of  $0.1$  to  $10\mu\text{M}$ . The main aim of a calcium buffer is to minimise changes in free  $\text{Ca}^{2+}$  to less than  $0.1$  log unit. A buffer is essential to produce low  $\text{Ca}^{2+}$  levels when  $\text{Ca}^{2+}$  contamination is present, or when changes in total  $\text{Ca}^{2+}$  or  $\text{Ca}^{2+}$  distribution occur during the experiment.

Calculations of metal ion buffering are based on an equation similar in form to the Henderson-Hasselbach

equation for pH buffers.

$$ML = M \text{ (metal ion)} + L \text{ (ligand)}$$

$$pM = -\log_{10} \text{ (free metal ion concentration)}$$

$$= pK_d + \log_{10} ([L] / [ML]).$$

It has been reported that an ideal ligand is expected to satisfy six criteria as a  $Ca^{2+}$  buffer. These include the following:-

(a) The  $Ca^{2+}$  concentration should be adequately buffered over a range likely to be found inside the cell, ie. 0.1 to 10uM.

(b) The ligand should be specific for  $Ca^{2+}$  and not subject to interference from other ions likely to be present, ie.,  $Mg^{2+}$ ,  $Na^+$ ,  $K^+$  and  $Cl^-$ .

(c) The  $Ca^{2+}$  buffer should be insensitive to pH changes.

(d) The ligand should be non-toxic in the biological system.

(e) Unless it is being used as a calcium chelator, the equilibration should be fast enough to respond quickly to physiological changes.

It is known that a  $\text{Ca}^{2+}$  ligand buffers best when  $\text{pK}_{\text{app}}^{\text{Ca}} = \text{pCa}$ ; that is, the ligand is half-saturated with  $\text{Ca}^{2+}$ . However, it has been noted that any one of at least five problems can arise when using a  $\text{Ca}^{2+}$  buffer. These problems include :-

(1) how to calculate pCa

(2) the  $\text{K}_{\text{app}}^{\text{Ca}}$  may be sensitive to small changes in pH;

(3) the buffering capacity may not be adequate for the complete range of  $\text{Ca}^{2+}$  being studied.

(4) calculation of pCa is critically dependent on an accurate value for  $\text{K}_{\text{d}}$ ; since calculation of pCa may be affected by other cations or ligands.

(5) calculation of pCa may not respond fast enough to a change in  $\text{Ca}^{2+}$  concentration to buffer it adequately (Campbell, 1983).

EGTA and EDTA are good  $\text{Ca}^{2+}$  buffers over the range 0.3 to  $1\mu\text{M}$  and can be used up to  $3\mu\text{M}$  free  $\text{Ca}^{2+}$ . However over 90% of the ligand is known to be in the form of  $\text{LH}^{2-}_2$  at pH 7.4. At this pH a change of only 0.2 pH unit can cause a 2-3 fold change in the concentration of free  $\text{Ca}^{2+}$ . The pH of Ca/EGTA buffers must therefore be maintained within 0.02 pH if accurate data are to be obtained. This problem has led to the design of new high-affinity  $\text{Ca}^{2+}$  ligands which are much less sensitive to pH near the physiological pH range (Tsien, 1980). It

has also been stated that a further problem with EGTA is that addition of  $\text{Ca}^{2+}$  causes the release of  $\text{H}^+$  ions; and this can cause massive decreases in pH unless alkali is added (Campbell). However, another way of removing the problem of pH sensitivity is to ensure that more than 95% of the ligand is in the form LMg.

To calculate pCa accurately, it is necessary to know precisely the concentration ratio of total ligand to total calcium. Variations in ionic conditions often prevail resulting in several different values for the association constant of various ligands. The association constant can however be measured quite easily either by titration using pH as the end-point (Miller and Moisescu, 1976) or by using a  $\text{Ca}^{2+}$  electrode (Owen, 1976). It is worth noting that several pH buffers are available which do not bind  $\text{Ca}^{2+}$  significantly (Ashley and Moisescu, 1977) and that the binding of  $\text{Mg}^{2+}$  and  $\text{K}^+$  to EGTA can be taken into account by approximation methods (Ashley and Moisescu, 1977). In addition, the problem of activity coefficients can be ignored provided that the ionic conditions are the same as those for the measurement of the association constants (Campbell, 1983).

According to reports by Hellam and Podolsky (1969), the forward and backward rate constants for  $\text{Ca}^{2+}$  and EGTA at pH 7 are  $10^{6.3} \text{M}^{-1} \text{sec}^{-1}$  and  $0.4 \text{M}^{-1} \text{sec}^{-1}$ , respectively. Thus, EGTA may respond slowly in the cell

to rapid  $\text{Ca}^{2+}$  transients, but experiments with photoproteins in vitro show that free  $\text{Ca}^{2+}$  can be reduced to  $<1\mu\text{M}$  within a few milliseconds if sufficient EGTA is added (Tsien, 1980).

#### Use of NTA as an extracellular $\text{Ca}^{2+}$ buffer

For the experiments on smooth muscle into the effects of adrenaline, noradrenaline and various adrenoceptor agonists and antagonists, a range of extracellular  $[\text{Ca}^{2+}]$  needed to be established. This necessitated the use of suitable calcium buffers in the bathing media. "Suitable" means a buffer chosen to cover the range of  $[\text{Ca}^{2+}]$  likely to be encountered, combined with minimal interference with other ions or the properties of the preparations. With the desired conditions (notably pH and millimolar levels of free Mg), EGTA alone will only cover the lower range (from  $1\mu\text{M}$  to  $0.1\text{mM}$ ) of  $[\text{Ca}^{2+}]$  under study; the range was extended by the use of NTA (Nitrilo-triacetic acid) to cover the range (approximately  $10^{-5}$  to  $10^{-4}\text{M}$ ) between EGTA and the "self-buffering" by free  $\text{Ca}^{2+}$  which becomes effective at about millimolar  $[\text{Ca}^{2+}]$ .

NTA has been used in experiments on chemically "skinned" skeletal and cardiac muscle (see Miller and Smith, 1984 for references eg., Scharff, 1979), where it does not interfere with the contractile machinery. Apart from certain observations among some workers in the

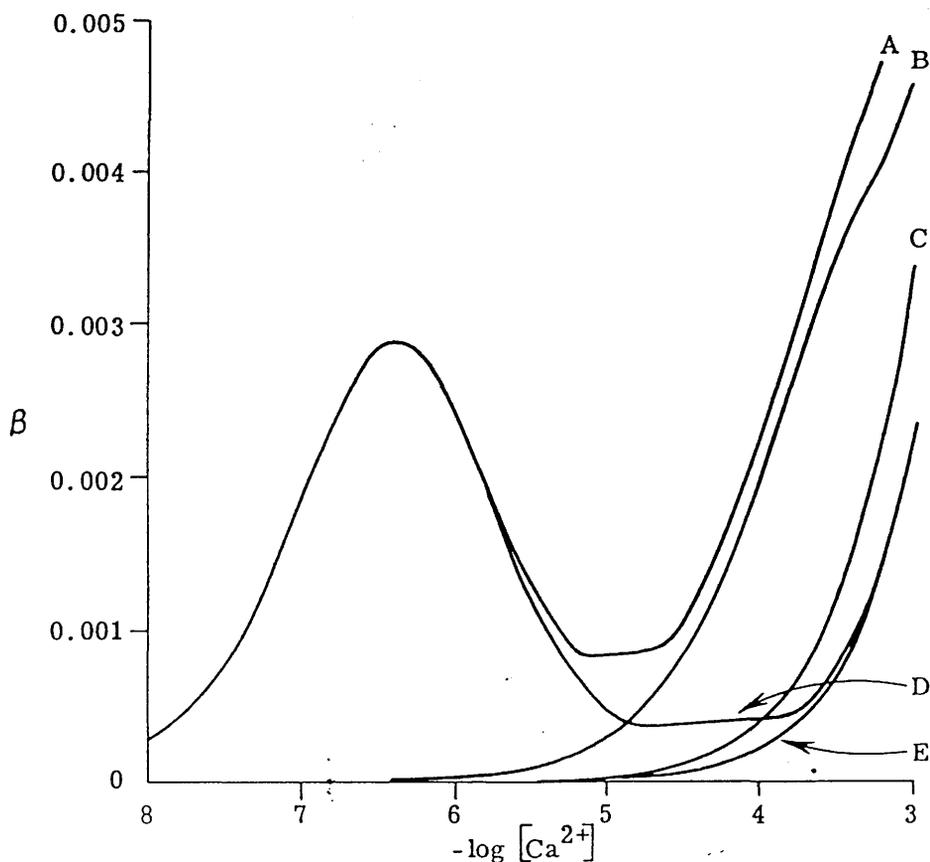


FIG. 5. Computer-derived plot of buffer value ( $\beta$ , *ordinate*), defined as  $\beta = \text{increment in total Ca (M)}/\text{decrement } (-\log [\text{Ca}^{2+}])$ , against  $-\log [\text{Ca}^{2+}]$ . Curves represent Ca buffering of following mixtures: EGTA, ATP, and NTA (A); NTA alone (B); ATP alone (C); EGTA alone (D); and unbuffered free Ca (E). Each buffer substance was present at 5 mM.  $[\text{Mg}]_{\text{total}}$  adjusted to keep  $[\text{Mg}^{2+}]$  at 2 mM. Ionic strength 0.1  $\text{pH}_a$  7.00, 20°C. Stability constants were taken from Ref. 12 or, failing that, from Ref. 20.

Figure (i/a). A graphic comparison of the calcium buffering capacities of NTA, ATP, EGTA and their combination as detailed in Miller and Smith (1984).

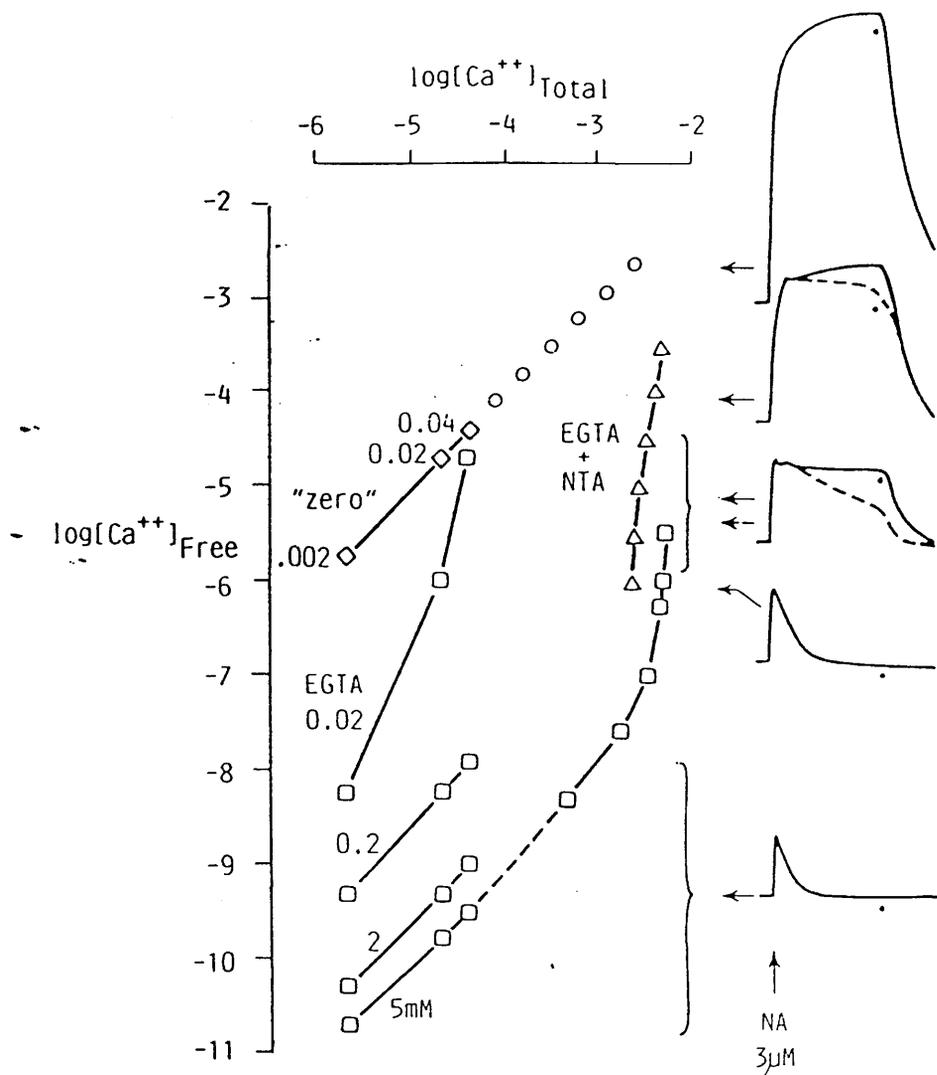
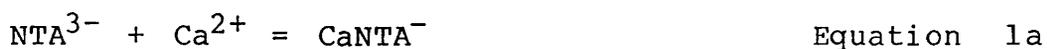


Figure (i/b). Calcium-dependent responses of the rat anococcygeus muscle in calcium buffers (NTA and EGTA) - a logarithmic relationship between the free and the total calcium concentrations and their influence on the time course of noradrenaline (NA) (3µM)-induced contractions. While EGTA allows the buffering of the lower range (1µM to 0.1mM) NTA extends this to even a lower range of approximately  $10^{-5}$  to  $10^{-4}$  M. A combination of the two provides a greater range of buffering capacity of the  $[Ca^{2+}]$  likely to be encountered in physiological and pharmacological experiments (see text and McGrath, Miller and Smith, 1984).

McGrath group (unpublished) which are loosely described as toxic (in the rat aorta and rat portal vein), there are no reports of deleterious effects of NTA on intact cells.

The hydrogen binding constants ( $-pK_a$ 's) and divalent ion binding constants ( $K_{Ca}$  &  $K_{Mg}$ ) are well suited to the present experimental conditions. The only complexity of any concern in the use of buffer is that two reactions with ions such as  $Ca^{2+}$  need to be considered:



$$K_{CaNTA} = [CaNTA^{-}] / [NTA^{3-}] \cdot [Ca^{2+}] \quad \text{Equation 1b}$$

or;



$$K_{Ca2NTA} = [Ca(NTA)_2^{4-}] / [NTA^{3-}]^2 \cdot [Ca^{2+}] \quad \text{Equation 2b}$$

The latter reaction only becomes significant when high levels of otherwise unbound NTA are present, since the concentration of the product,  $Ca(NTA)_2$ , is obviously proportional to  $[NTA^{3-}]^2$ . This is the case at low (relative to  $1/K_{CaNTA}$ ) concentrations of  $Ca^{2+}$ , such as in the range where the high range of EGTA-buffering overlaps with the lowest range of NTA-buffering.

Figure 5 {represented here in the thesis as Fig. i/a} of Miller & Smith (1984) (reproduced here with the permission of Dr. D.J. Miller) and (Fig i/b) from Dr. J.C. McGrath, show the effect of dual buffering of free  $\text{Ca}^{2+}$  by EGTA and NTA in equimolar mixture on Ca-buffer capacity over the pCa range 8 to 3.

### Calcium Channels

It has been suggested that extracellular  $\text{Ca}^{2+}$  ions enter the vascular smooth muscle cells in several ways, which include:

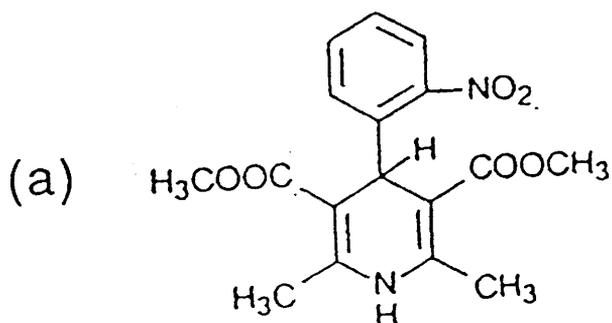
- (a) via specific membrane channels for  $\text{Ca}^{2+}$ ,
- (b) in exchange for  $\text{Na}^+$  by a hypothetical  $\text{Na}^+-\text{Ca}^{2+}$  exchange mechanism and
- (c) by a less well defined "passive leak" of the ion across the cell membrane, accounting for the  $\text{Ca}^{2+}$  uptake in the resting muscle (Bolton, 1979; van Breemen et al., 1979).

Bolton (1979) proposed the existence of two different types of membrane channels for  $\text{Ca}^{2+}$ , called potential- and receptor-operated channels (POCs and ROCs), in smooth muscle. By his definition, the POC is an ion channel population that opens when the potential across the membrane is reduced, eg. after exposure to  $\text{K}^+$ -rich

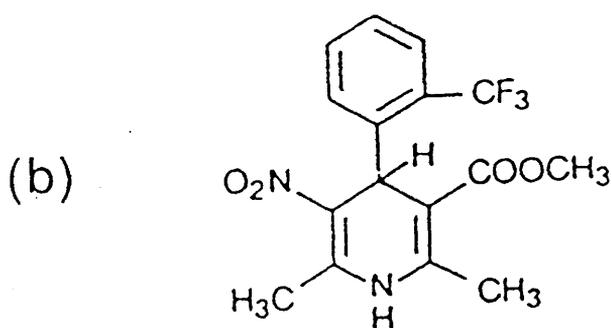
medium. This channel is considered to correspond to the "slow channel" in cardiac muscle. On the other hand, ROCs were suggested to be primarily controlled or operated by receptors for stimulant substances. The concept of two types of  $\text{Ca}^{2+}$  channel has proved useful in explaining the actions of various drugs on VSM. In addition,  $\text{Ca}^{2+}$  influx via ROCs, either alone or in combination with intracellular  $\text{Ca}^{2+}$  release, may underlie the mode of activation generally referred to as pharmacomechanical coupling. This phenomenon refers to the mode of activation in some VSM preparations (eg., the rabbit ear artery) in which certain agonists can induce contraction in the absence of membrane depolarization (Droogmans et al., 1977).

#### Calcium Channel Facilitators

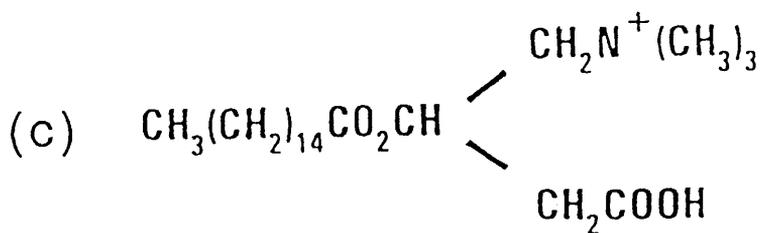
It is interesting to note that one of the most exciting recent developments in the study of calcium antagonists has been the discovery of compounds which activate both the voltage-operated channels and the adrenergic receptor-operated channels. Certain dihydropyridines like Bay K 8644 (Schramm et al., 1983a, 1983b); YC 170 (Takenaka and Maeno, 1982) and CGP 28392 (Erne et al., 1984) have been synthesized which, although apparently binding to the same site as antagonist dihydropyridines such as nifedipine, activate calcium channels. Very small structural changes are required to transform antagonistic activity into



Nifedipine



BAY K 8644



Palmitoyl Carnitine

Figure (ii). The chemical structures of calcium entry blocker, nifedipine and the calcium channel facilitators, Bay K 8644 and the endogeneously occurring palmitoyl carnitine. Note the close resemblance between nifedipine and Bay K 8644.

stimulatory activity (compare the chemical structures of Bay K 8644 and nifedipine shown in the diagrams, {Fig. i}). It has been shown that compounds such as Bay K 8644 exert opposite pharmacological effects to nifedipine, contracting smooth muscle and increasing myocardial force (Schramm et al., 1983). Such compounds can be very toxic as they increase calcium entry in heart and smooth muscle yet unlike agents working via cyclic AMP they do not stimulate relaxation processes (Spedding, 1985). In addition, they activate voltage-operated channels in both heart and smooth muscle and increased myocardial work is accompanied by coronary constriction. This is probably the reason why physiological stimulants of voltage-operated channels work by indirect mechanisms for there is no evidence yet for endogenous direct activation of voltage-operated channels. However, palmitoyl carnitine has recently been implicated in this respect by Spedding (1986) (Its chemical structure is shown in Fig. ii).

Therefore the discovery of structural analogues of the dihydropyridines (Schramm et al., 1983) that compete for common high affinity binding sites (Janis et al., 1984; Vaghy et al., 1984a,b), yet enhance rather than block  $\text{Ca}^{2+}$  current (eg., Bay K 8644, CGP 28 392) has led to speculation about basic mechanisms that regulate  $\text{Ca}^{2+}$ -channel gating (Hess et al., 1984). However, patch-clamp studies of isolated cardiac cells have demonstrated that enhancement of  $\text{Ca}^{2+}$ -channel current by

Bay K 8644 (Hess et al., 1984; Ochi et al., 1984) and CGP 28 392 (Kokubun and Reuter, 1984) results from a drug-induced prolongation of single channel open time and not from an increase in amplitude of unitary channel openings.

There is apparently a tendency to refer to many of the dihydropyridines as partial agonists. This is illustrated by the observation that the low concentrations of the " $\text{Ca}^{2+}$ -antagonists", nifedipine and nitrendipine, actually increase the contractility of cat papillary muscle (Strauer, 1974) and of guinea-pig isolated perfused hearts (Thomas et al., 1984). In addition, high concentrations of the " $\text{Ca}^{2+}$  agonists", eg. Bay K 8644, decrease contractility of isolated hearts (Thomas et al., 1984) and inhibit  $^{45}\text{Ca}^{2+}$  influx into cultured cells (Freedman and Miller, 1984).

It has been reported that the modulation of  $\text{Ca}^{2+}$  current by Bay K 8644 is voltage-dependent. In voltage-clamped cardiac Purkinje fibres, Bay K 8644 (0.2 to 2.0  $\mu\text{M}$ ) acts as a  $\text{Ca}^{2+}$ -channel agonist when clamp pulses are applied from holding potentials negative to approximately -50 mV, but rapidly reverses to an antagonist at more depolarised holding potentials (Sanguinetti and Kass, 1984b). The agonist effect of Bay K 8644 on  $\text{Ca}^{2+}$  current can also be reversed by rapid pulsing (Sanguinetti & Kass, 1985). In effect therefore, Bay K 8644 can act as either an agonist or antagonist at

a given concentration depending upon cellular membrane potential. In some experiments involving the study of the effects CGP 28 392 on slow response action potentials recorded from  $K^+$ -depolarized papillary muscles, including protocols designed to enhance any possible  $Ca^{2+}$  antagonists, Kamp et al., (1985) came up with results which indicate that CGP 28 392 is a partial  $Ca^{2+}$ -channel agonist and suggest that its effects on  $Ca^{2+}$  current are voltage dependent. CGP 28 392 increased  $V_{max}$  more than two fold at low rates of stimulation (1 or 12 pulses  $min^{-1}$ ), but had no significant effect on  $V_{max}$  during rapid pulsing (200 pulses  $min^{-1}$ ). Further, the enhancement of  $V_{max}$  by CGP 28 392 is dependent upon extracellular  $[K^+]$ , while increasing extracellular  $[K^+]$  from 22mM to 27mM suppresses the frequency-dependent agonist effects and increases the antagonism effects on  $V_{max}$ .

### Calcium Channel Blockers

Dihydropyridine calcium ( $Ca^{2+}$ ) antagonists have been shown to block the movement of  $Ca^{2+}$  through voltage-sensitive channels of a diverse group of cell types, including smooth muscle (Karaki & Weiss, 1984), cardiac muscle (Lee & Tsien, 1983) and several cultured cell lines (Freedman & Miller, 1984). Calcium ( $Ca^{2+}$ ) current block by the dihydropyridines is modulated by voltage. This is supported by the finding that block by nitrendipine and nisoldipine is enhanced at depolarized

potentials (Sanguinetti & Kass, 1984a).

It has been shown that nifedipine, a  $\text{Ca}^{2+}$  entry blocker, can selectively block the "non-adrenergic" contraction leaving the adrenergic component of nerve-induced contraction in rat vas deferens (French & Scott, 1981). Nifedipine does not, however, act by interfering with the excitatory junctional potentials (ejp's) but it does prevent the occurrence of the muscle action potential (Blakely et al., 1981). Since the adrenergic ( $\alpha_1$ -mediated) contraction is not modified, it can be inferred that the  $\alpha_1$ -induced contraction does not require an action potential but utilizes, perhaps, a more direct excitation-contraction coupling process. If this is the case it could suggest a different type of  $\alpha$ -adrenoceptor compared with those involved in depolarising the cell membrane. This might also explain why the vas deferens has such a dense adrenergic innervation, since the "adrenergic" response would not be transmitted between cells (McGrath, 1982).

Many of the studies carried out on the membrane effects of the dihydropyridine, nifedipine, have been obtained from electrophysiological studies on the heart. Nifedipine effectively reduced the slow inward current of  $\text{Ca}^{2+}$  in the mammalian ventricular myocardium (Bayer & Ehara, 1978). It was suggested that nifedipine inhibits rather selectively the slow  $\text{Ca}^{2+}$  channels without affecting the "fast  $\text{Na}^+$  channels", since this drug did

not influence the upstroke velocity of the action potential in the myocardial cell (Rodenkirchen et al., 1977). Voltage clamp studies revealed that nifedipine did not alter the rates of activation and inactivation of the slow channels, but rather reduced the number of channels available for calcium transport (Kohlhardt & Fleckenstein, 1977; Bayer & Ehara, 1978).

In comparison with its mechanism of action on the heart, the relaxing effect of nifedipine on VSM has been attributed to inhibition of stimulation-induced  $\text{Ca}^{2+}$  entry (Fleckenstein, 1977; Bolton, 1979; Cauvin et al., 1983; Andersson & Hogestatt, 1984). It is known that the concentration-response relationships for the inhibitory effects of nifedipine on  $\text{K}^{+}$ -induced contraction and  $^{45}\text{Ca}^{2+}$  influx are almost identical in the rat aorta and superior mesenteric artery, suggesting that this drug attenuated contraction by blocking  $\text{Ca}^{2+}$  translocation through the cell membrane (Godfraind, 1983). In addition, resting  $\text{Ca}^{2+}$  uptake (passive leak) in smooth muscle does not appear to be appreciably influenced by nifedipine (Godfraind, 1983).

It has been reported that  $^3\text{H}$ -Nifedipine binds with high affinity to cardiac membranes (Holck et al., 1982) and that the unlabelled drug potently inhibits the binding of the tritiated nifedipine analogues nitrendipine and nimodipine to cardiac, smooth muscle and brain membranes (Belleman et al., 1982; Ehlert et

al., 1982). In contrast to verapamil, nifedipine in reasonable concentrations ( $< 1 \mu\text{M}$ ) does not interact with  $\alpha$ -adrenoceptors (van Meel et al. 1981; Larsson et al. 1984). Diltiazem keeps an intermediate position between the cardiovascular effects of nifedipine and verapamil (Fleckenstein and Fleckenstein, 1984). Nifedipine, a lipophilic drug, would be expected to enter the smooth muscle cell but there is no consensus on its intracellular effects (Walus et al. 1981; Spedding, 1983). Generally therefore,  $\text{Ca}^{2+}$  entry blockade apparently is the main mechanism behind the relaxing effect of nifedipine on vascular smooth muscles although other effects may contribute.

### Palmitoyl Carnitine

It has been reported that inhibition of fatty acid oxidation by ischaemia causes marked accumulation of acyl carnitine derivatives in the cytosol. These amphiphilic derivatives accumulate in cell membranes where they have detergent properties and affect channel gating (Mir & Spedding, 1986). Palmitoyl carnitine is a lipid metabolite that accumulates in the heart following ischaemia and has been suggested to be responsible for some aspects of ischaemic cell damage (Neely & Feuvray, 1981). It is known to increase  $\text{Ca}^{2+}$  current in the heart by a surface charge effect (Inoue & Pappano, 1983). In addition, it resembles Bay K 8644 as a direct activator of calcium channels in smooth muscle (Mir & Spedding,

1986; Ugwu, et al. 1987). It resembles Bay K 8644 in  $K^+$ -depolarized smooth muscle and, like Bay K 8644, has selective interactions with the different types of calcium antagonists (Spedding and Mir, 1987).

#### Temperature effects on blood vessels

Smooth muscle tone and sensitivity in several vascular beds are known to change with temperature. Smith (1952) showed that arteries from swine and dogs constricted when cooled to between  $4^{\circ}$  and  $6^{\circ}C$  and that the arteries were most sensitive to adrenaline at  $17^{\circ}C$ . Glover, Strangeways & Wallace (1968) noted an increase in tone when femoral and ear arteries of the rabbit were cooled from  $37^{\circ}C$  to  $3^{\circ}C$ . The ear artery was most sensitive to noradrenaline at  $24^{\circ}C$ , whereas the sensitivity of the femoral artery decreased progressively below  $37^{\circ}$ . It has also been observed that the maximal sensitivity in dose response curves to noradrenaline measured in whole tails of anaesthetized intact normal rats was seen between  $32^{\circ}$  and  $34^{\circ}C$  (Wade & Beilin, 1970). At  $37^{\circ}C$ , they found that decreased sensitivity was usually observed but there was no change in baseline resistance. In addition sensitivity was reduced at  $40^{\circ}$  and  $42^{\circ}C$  and after a period at  $42^{\circ}C$  normal sensitivity at  $34^{\circ}C$  did not always return. It was reported that temperatures below  $30^{\circ}C$  were associated with an increase in baseline resistance and a decreased pressor response to noradrenaline.

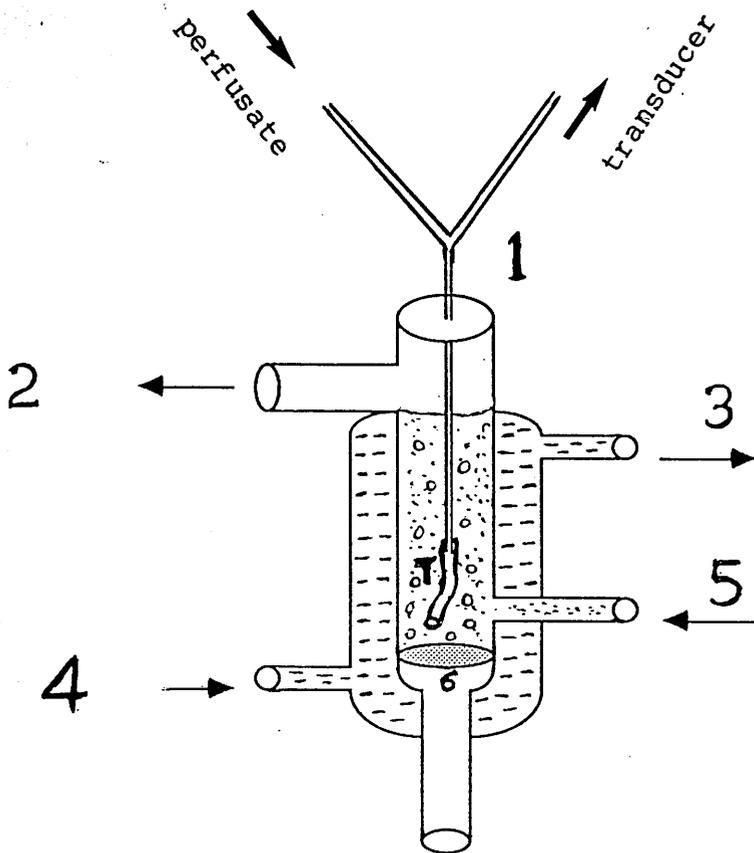
## GENERAL METHODS

### (i) Preparation of the tissue for recording perfusion pressure.

1-2cm lengths of the proximal or distal segment of the rat tail artery were prepared for recording the perfusion pressure in vitro. Male Wistar rats (300 to 350gm) were killed by a blow on the head and exsanguination. The ventral tail artery was rapidly removed and placed in aerated calcium-buffered physiological saline solution (CB/PSS). The vascular smooth muscle was cannulated at the proximal end and subsequently mounted in a 5ml jacketed organ bath. It was perfused with, and bathed in, saline of similar composition. The saline solution was maintained at a temperature of 37°C and at a pH of 7.2 to 7.4.

#### Buffered saline:-

The calcium-buffered physiological saline solution had the following composition (millimolar unless otherwise specified):- EGTA (ethylene glycol bis-(B-aminoethyl ether) N,N,N'-tetraacetic acid), 2.5 (i.e ;  $0.9\text{g l}^{-1}$ ); NTA (nitrilo-triacetic acid, i.e. N,N-bis(carboxymethyl)glycine, free acid); 2.5 (i.e;  $0.475\text{g l}^{-1}$ ); NaCl, 111.5; NaOH, 7.5; NaHCO<sub>3</sub>, 24.8; KH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub> .7H<sub>2</sub>O, 1.2; KCl, 4.7; CaCl<sub>2</sub> was



- T Tissue
- 1 Cannula to pressure transducer.
- 2 Bath overflow
- 3 Water (37°C) Outlet
- 4 Water (37°C) Inlet
- 5 Saline solution
- 6 Glass sinter:

Figure (iii). Diagrammatic representation of arrangement of 5ml organ bath used for this study. The cannulated blood vessel was suspended in the physiological saline bubbled with the appropriate gas mixture from the glass sinter and kept at constant temperature with circulating water from the water bath. Perfusion pressure was monitored by Devices recorder via the cannula and pressure transducer.

varied from 4.69 (for calcium buffer 1, i.e; // 1) to 2.35 ( for calcium buffer 6, i.e; // 6 ); a molar concentration of glucose i.e.  $2\text{gl}^{-1}$ ; cocaine,  $4\mu\text{M}$ ; propranolol,  $1\mu\text{M}$ ; and EDTA (ethylenediaminetetra-acetic acid disodium salt),  $23\mu\text{M}$ . Another saline solution used in some experiments (examining the  $\text{Ca}^{2+}$  dependence of the contraction induced by depolarisation) had high potassium chloride, low phosphate (which allowed the use of  $[\text{Ca}^{2+}]_o = \text{or} > 5\text{mM}$  without precipitation), and had identical composition to the one described above with the following exceptions:- NaCl, 24; KCl, 100;  $\text{KH}_2\text{PO}_4$ , 0.1.

#### Unbuffered saline:-

The calcium-unbuffered saline solution was made up of the following composition (in millimolar concentration):- NaCl, 119;  $\text{NaHCO}_3$ , 24.8;  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.2; KCl, 4.7;  $\text{CaCl}_2$ , 2.5; glucose,  $2\text{gl}^{-1}$ ; cocaine,  $4\mu\text{M}$ ; propranolol,  $1\mu\text{M}$ ; and EDTA (ethylene-diaminetetra-acetic acid disodium salt),  $23\mu\text{M}$ .

#### Gassing the saline with different gas mixtures:-

The saline was bubbled with gas mixtures of different oxygen tensions. These were :- 615mmHg (95% $\text{O}_2$  and 5% $\text{CO}_2$ ); 120mmHg (16% $\text{O}_2$ ; 5% $\text{CO}_2$  & 79% $\text{N}_2$ ); and 32mmHg (4% $\text{O}_2$ , 5% $\text{CO}_2$  & 91% $\text{N}_2$ ). In a few experiments, 64%, 32%, 8% and zero  $\text{O}_2$  were used. In addition,

different carbon dioxide and bicarbonate levels were used as detailed in the appropriate sections.

Except for the pressurised bottled gas mixture (95%O<sub>2</sub> : 5%CO<sub>2</sub> from BOC) which we tested for its composition and bubbled directly into the saline solution, the gas mixtures were made up in Douglas bags using the rotameters from an anaesthetic trolley. The physiological saline was gassed with these mixtures by a small aquarium pump.

(ii) Determination of perfusion pressure.

The preparations were tested for leakage and those which were satisfactory were set up for perfusion. The artery segments were mounted vertically with the cannulated proximal end of each tissue uppermost. The preparations were placed in a jacketed organ bath containing 5ml of the physiological saline solution (Fig. iii). The free distal end which was lowermost in the organ bath opened into the solution. The lumen of the artery segment was perfused by directing the physiological saline solution from a reservoir (kept in the water-bath) at a constant rate of 2-3ml/min with a pulsatile flow pump (Watson-Marlow peristaltic cassette pump, 501U with 501M multi-channel pumphead - maintained at 14% of its maximum flow) and the perfusion pressure was recorded. This rate of flow was shown in preliminary experiments to be adequate for recording the optimal

vasoconstrictor responses to noradrenaline (NA) or to high concentration of potassium chloride (KCl). The vasoconstrictor responses were measured as an increase in perfusion pressure at constant flow, using an Elcomatic EM751 pressure transducer and Devices recorder. These increases in perfusion pressure were determined as the difference between the resting perfusion pressure and the peak response. The perfusate passing through the artery segment via the cannula mixed freely with the identical physiological saline solution in the organ bath that bathed the adventitial surface of the artery segment. The peaks of the pulsatile pressure waves were measured for calculation of responses.

### (iii) General Experimental Protocol

A standard stabilisation period of 90 to 120mins in activator-free buffer 6 solution was allowed before any responses were obtained. The usual protocol involved changing the perfusing solution by briefly stopping perfusion and switching it to a new solution containing the required concentration of  $\text{Ca}^{2+}$ , NA or KCl or any other drug whose effect on the tissue was being tested. The bathing solution was then replaced within one minute of restarting the perfusion. This allowed for the time taken for the perfusate to move from the reservoir in the water-bath to the tissue in the organ bath. Consequently both surfaces of the tissues were always exposed to an identical medium during the experiments.

Standard exposure of the tissues to NA or other drug or KCl was for 5mins during which time the maximum response to that concentration of the activator was obtained.

Concentration/response curves (CRCs) to agonists like noradrenaline (NA) and 5-Hydroxytryptamine (5-HT), or other types of activating agents such as potassium chloride (KCl) or concentration-response relationships to adrenoceptor antagonists such as prazosin (for  $\alpha_1$ ) and rauwolscine (for  $\alpha_2$ ) were constructed by starting in the lowest concentration of the drug. This was performed using different procedures - cumulative (e.g. in desensitisation experiments) and non-cumulative (e.g. NA curves with adrenoceptor antagonists). Similarly, the concentration-response curves for the sensitivity of the tissue to calcium ( $\text{Ca}^{2+}$ ) in the presence or absence of the calcium channel activators ( Bay K 8644, palmitoyl carnitine) or to the calcium entry blocker (Nifedipine) were constructed each time by starting with the lowest concentration of calcium.

Concentration-response curves to calcium (CCRCs), for example, were constructed by starting in the lowest [ $\text{Ca}^{2+}$ ] (buffer 6, for calcium-buffered experiments (Table 1) and zero (i.e. apart from contamination level) or 0.039mM (1/64 of 2.5mM {normal} calcium) for calcium-unbuffered experiments (Table 2), adding the activator (NA or KCl) and then changing stepwise to higher [ $\text{Ca}^{2+}$ ] at 5min intervals to a maximum of 5mM (or

Table 1. A comparison of the quantitative values of the free and the total calcium concentrations in the calcium buffered saline in different buffers (1 to 6) and in 0.5 to 4 times the normal calcium concentration of 2.5mM.

Buffer name	$[Ca^{2+}]_{free}$ (M)	$[Ca^{2+}]_{total}$ (mM)
6	$1 \times 10^{-6}$	2.3470
5	$3 \times 10^{-6}$	2.4720
4	$1 \times 10^{-5}$	2.6899
3	$3 \times 10^{-5}$	3.1214
2	$1 \times 10^{-4}$	3.9009
1	$3 \times 10^{-4}$	4.685
0.5 $Ca^{2+}$	$1.25 \times 10^{-3}$	[Buff.1] + 1.57
1 $Ca^{2+}$	$2.50 \times 10^{-3}$	[Buff.1] + 2.82
2 $Ca^{2+}$	$5.00 \times 10^{-3}$	[Buff.1] + 5.32
4 $Ca^{2+}$	$10.00 \times 10^{-3}$	[Buff.1] + 10.32

Table 2. A comparison of the quantitative values of the total external calcium (mM) present in the unbuffered calcium saline for zero calcium to 4 times the normal calcium concentration of 2.5mM.

Multiples of normal $[Ca^{2+}]$	$[Ca^{2+}]_{ext.}$ (mM)	-log Molar values
Zero	$1.00 \times 10^{-2}$	4.70
1/64	$3.90 \times 10^{-2}$	4.41
1/32	$7.80 \times 10^{-2}$	4.11
1/16	$1.56 \times 10^{-1}$	3.81
1/8	$3.125 \times 10^{-1}$	3.51
1/4	$6.25 \times 10^{-1}$	3.20
1/2	1.25	2.90
1.0	2.50	2.60
2.0	5.00	2.30
4.0	10.00	2.00

sometimes to 10mM with KCl). The concentration of NA (3uM) or KCl (100mM) was kept constant while changing  $[Ca^{2+}]$ . At the end of the construction of the CCRC, the perfusion was stopped and the bathing and perfusing solutions were replaced by activator-free buffer 6 or unbuffered 0.039mM  $Ca^{2+}$  saline solution and allowed to re-equilibrate (to baseline pressure) before constructing another CCRC.

Preliminary experiments showed that washing and "resting" in buffer 6 or 0.039mM  $Ca^{2+}$  for calcium CRCs minimised desensitisation i.e. it was worse if  $[Ca^{2+}]$  was higher. For the calcium buffered experiments, the stepwise increase in  $[Ca^{2+}]_o$  started from buffer 6 ( $2.29 \times 10^{-6}$  M) through a series of buffers referred to as buffers 5 to 1 which took the  $[Ca^{2+}]_o$  to 300uM. Further increments in  $[Ca^{2+}]$  were obtained by further addition of  $CaCl_2$  to buffer 1, up to a total  $[Ca^{2+}]_{free}$  of 5.32mM or at times to 10.32mM (in buffer 1). Details of the  $[Ca^{2+}]$  in the various buffers are outlined in Table 1. Similarly, for the calcium unbuffered experiments, the  $[Ca^{2+}]_o$  was increased in stepwise manner from 0.039mM to 5mM or at times to 10mM, each time doubling the earlier concentration. Intervals of 15min were allowed between curves in the initial protocol but this was varied in other experiments. A total time of 45minutes was taken to complete the construction of each control curve, giving a routine cycle of 1 curve per hour.

In a series of experiments illustrating the decline in sensitivity to NA illustrated by the NA concentration-response curve, EGTA and NTA were included (2.5mM each) so that any toxic effect would be constant throughout, and total  $\text{CaCl}_2$  was adjusted to keep  $[\text{Ca}^{2+}]_{\text{free}}$  at 2.5mM.

In the earlier experiments, preliminary studies were carried out to test the difference between (a) perfusing and superfusing the tissue in an air-filled organ bath and (b) perfusing and superfusing tissue in an organ bath containing identical solutions and gaseous mixtures as the perfusate in the reservoir. Our results showed that there was no significant difference in the results obtained by both methods. However, we settled for method (b) after assessing the NA responsiveness caused by surface of entry. It is also simpler to perform and keeps tissue viable for longer experiments. In addition, it prevents partial drying out of tissue encountered with method (a).

In some experiments, as in Section Three, longitudinal studies were carried out on the same tissue during the investigation of the influence of different oxygen tensions on the responsiveness of the tissue to NA and KCl in the presence and absence of Bay K 8644 and nifedipine. This method was found to be unsuitable for testing the calcium-sensitivity of the rat tail artery. This is because of desensitisation after the first

concentration-response curve. A non-longitudinal method was therefore adopted for most of the studies.

### Drugs and chemicals

The following substances were used:-

Bay K 8644,	Bayer
Nifedipine	Bayer
Palmitoyl Carnitine	Sigma
(-)-Noradrenaline bitartrate salt	Sigma
E.D.T.A.	B.D.H.
E.G.T.A.(ethylene glycolbis-{B-aminoethyl ether} N,N,N'-tetraacetic acid)	Sigma
N.T.A.(nitrilo-triacetic acid, i.e., N,N-bis {carboxymethyl}glycine, free acid)	Sigma
Cocaine HCl	McCarthy's
DL-Propranolol HCl	I.C.I.
Prazosin Hydrochloride	Pfizer
Rauwolscine Hydrochloride	Roth
5-Hydroxytryptamine creatinine sulphate	Sigma
(-)-Phenylephrine HCl	Sigma
Xylazine Hydrochloride	Sigma
Angiotensin II	Ciba
Amidephrine Mesylate	Mead Johnson
POCA	Syntex

POCA, a carnitine acyltransferase inhibitor, is

chemically known as Sodium 2-[5-(4-chlorophenyl)-penty]-oxirane-2-carboxylate.

All drugs were obtained as pure powders. Stock solutions of Bay K 8644 and nifedipine were dissolved in alcohol then diluted with water (one part of alcohol in nine parts of distilled water v/v) and shielded from direct light because of their photosensitivity. All other drugs were dissolved in distilled water and diluted in the appropriate physiological saline. Dissolution of the drugs was enhanced with an ultrasonic vibrator (Kerry / Ultrasons Annemasse).

Dissolution of palmitoyl carnitine was performed with minimal shaking of the solution. This was to reduce frothing. An ultrasonicator was therefore not used in dissolving palmitoyl carnitine. Thus, in all our experiments involving palmitoyl carnitine frothing was absent. This was also because of the small concentration (0.1 $\mu$ M) which we used. With higher concentrations frothing can handicap experimentation.

#### Calculation of Results and Statistical Analysis.

All results have been expressed, or represented on graphs, as the mean  $\pm$  S.E.M. Statistical analysis was performed using Student's t test for paired or unpaired data, as appropriate and the 0.05 level of probability was regarded as significant.

Most of the experiments were "whole day" experiments lasting for at least 9 hours each time. In the desensitisation experiments in particular, the first curve characteristically shows increased sensitivity to calcium with the maximum occurring at a sub-physiological calcium level of  $0.3\mu\text{M}$ , although we tested up to  $5\text{mM}$ . However, sensitivity and the maximum response decreased steadily with subsequent curves.

This raised the problem of quantification of Calcium-Sensitivity, since there was no steady maximum to correct to and the maximum also varies with time. To avoid this problem, we expressed sensitivity in each curve for each individual tissue as the calcium concentration which allows a response of 30% of the first maximum.

Thus this  $\text{EC}_{30}$ , as we have called it, is steadily increasing (or moving to the right on the graph). This value was further expressed as the negative logarithm, so that the rightward shift will represent a falling index of sensitivity. Most graphs on calcium sensitivity experiments were therefore drawn to show the  $-\log \text{EC}_{30}$  values against Time in mins in order to see what happens to consecutive curves in a series.

For experiments on the agonist/antagonist interactions on adrenoceptors, agonist dose ratio values were determined from the concentration producing 50% of

the maximum response in the absence and presence of each concentration of the antagonist. For each preparation a good number (>6) of unpaired control experiments (with no antagonist added) were performed to obtain a statistically valid estimate of any change in sensitivity, which was then used to compensate for changes during the experiments with antagonists as suggested by Furchgott (1972). Generally, if a blockade is competitive, a plot of the logarithm of (concentration-ratio - 1) against the negative logarithm of the molar concentration of antagonist will yield a straight line with a slope of unity and intercept along the abscissa of  $pA_2$ , which equals the negative logarithm of the dissociation constant ( $-\log K_B$ ) under equilibrium conditions (Arunlakshana and Schild, 1959). Antagonism was considered competitive if the 95% confidence limits for the Schild plot, drawn by linear regression, overlaps unity.

## SECTION I

# ESTABLISHMENT OF OPTIMAL CONDITIONS FOR INVESTIGATING THE EFFECTS OF CALCIUM AND OXYGEN IN THE ISOLATED PERFUSED RAT TAIL ARTERY

### SUMMARY

1. The general properties of the isolated perfused rat tail artery and its responses under the influence of different drugs and conditions were investigated. Proximal and distal segments were investigated separately but their results were basically similar.
2. Cocaine, which is a neuronal noradrenaline uptake blocker, tended to enhance the responses of the rat tail artery to noradrenaline.
3. Propranolol, a beta-blocker, also tended to increase the responses of the rat tail artery to noradrenaline.
4. In the presence of EDTA (23uM), the responses of the rat tail artery to noradrenaline were better maintained than when EDTA was omitted.
5. The difference in the intraluminal and extraluminal administration of noradrenaline to the isolated perfused rat tail artery was eliminated by the inclusion of cocaine in the saline.
6. The responses of the tissues tended to be greater in 95%O<sub>2</sub> than in 16%O<sub>2</sub> at a constant 5%CO<sub>2</sub>. In general,

the lower the oxygen tension, there was a tendency for the responses to be lower.

7. Carbon dioxide tension, over the range produced by bubbling with 2%-8% CO<sub>2</sub> made little difference to responses of the tissue to noradrenaline.
8. Similarly, altering the pH by varying bicarbonate concentration had little effect.
9. Prazosin, an alpha-1 adrenoceptor antagonist, (0.1µM) was more potent in antagonising the responses of the tissue than was rauwolscine, an alpha-2 adrenoceptor antagonist. Rauwolscine antagonised the responses only at high concentrations (>1µM) which probably block α<sub>1</sub>. The responses of the proximal and distal segments were basically similar, suggesting a homogeneous population of α<sub>1</sub>-adrenoceptors.

## INTRODUCTION

The rat tail artery is considered to be a model of a peripheral resistance vessel with a good population of alpha adrenergic receptors (Medgett et al, 1984). It meets most of the requirements of a characteristic peripheral resistance vascular smooth muscle (Wade & Beilin, 1978). It has been extensively used to test the effects of different drugs and often it is activated with exogenous noradrenaline (NA), field stimulation or potassium (KCl).

The results of the detailed study by Henry and

Laucuara (1971) in the cut strips of the rat tail artery showed that 500-1500mg tension was the range for contractile activity; that 75min equilibration period was needed for uniform results; that it was more sensitive to adrenaline than to noradrenaline and it developed tachyphylaxis to angiotensin II. They also found that the range for contractile response to KCl was 60-120mM and that these responses were dependent on external calcium.

In a separate study, Hinke and Wilson (1962) found that in the intact tail artery segment, identical responses were produced with 0.1ug/ml adrenaline and 0.5ug/ml noradrenaline, 0.4ug/ml angiotensin II, and 2.5mU/ml Pitressin in normal Krebs solution. In addition, high  $[K^+]_o$  Krebs solution alone constricted the artery while low  $[Na^+]_o$  distended (relaxed) the arterial segment. Also high  $[K^+]_o$  solutions potentiated the response to all four vasopressor agents. In the light of these earlier experiments, the rat tail artery has been constituted into a model for various experimental investigations. Its electrophysiological properties have been investigated by Cheung (1984), and its various adrenoceptor subtypes have also been studied (Medgett and Langer, 1984,1986; Hicks et al. 1985). The purinergic receptor at which ATP acts as a co-transmitter with noradrenaline has also been investigated (Sneddon and Burnstock, 1984; Hicks et al. 1985; Vidal et al. 1986).

In this section we examined the effects of various factors and conditions that affect the responses of such a resistance vessel with a view to establishing suitable conditions for a subsequent study of the influence of different levels of oxygen and calcium.

### Experimental protocol

The preparation of the rat tail artery for recording perfusion pressure, the determination of perfusion pressure, the drugs and chemicals used and also the statistical analysis were carried out as detailed in the section on General Methods.

For the experimental protocol, a standard stabilisation period of 90 to 120mins in activator-free solution (unbuffered saline) was allowed before any responses were obtained. In this part of the work, most investigations were conducted on noradrenaline concentration-response curves (NA CRCs). Some were

however carried out on potassium chloride (KCl)-induced contractions. Before obtaining the NA CRC, the tissue was exposed ("primed") for 5min with 3 $\mu$ M NA in order to stabilise the responses. In this way we were able to obtain reproducible curves as shown in Fig. 1.1a. It should be noted, however, that this results in a degree of desensitisation compared with that initially present, as will be shown and discussed in Section 2.

To construct a noradrenaline CRC (after priming the tissue), the usual protocol involved changing the perfusing solution by briefly stopping perfusion and switching it to a new solution containing the required concentration of NA. The bathing solution was then replaced within one minute of restarting the perfusion. This allowed for the time taken for the perfusate to move from the reservoir in the water-bath to the tissue in the organ bath. Consequently both surfaces of the tissues were always exposed to an identical medium during the experiments. Standard exposure of the tissues to NA was for 5min (approx.) during which time the maximum response to that concentration of the activator was obtained. Concentration/response curves to NA were constructed by starting in the lowest [NA]. The activator (NA) was cumulatively added in increasing steps from 30nM to 30 $\mu$ M and each time 5min was allowed for the tissue response to attain its maximum and 10min washout for recovery. The concentration of Ca<sup>2+</sup> (2.5mM) was kept constant while changing the

concentrations of NA. In other words, after obtaining the maximum response for each NA concentration the perfusion was stopped and the bathing and perfusing solutions were replaced by activator-free saline and the tissue response was allowed to re-equilibrate before obtaining the response for the next NA concentration. Intervals of 10min were allowed between NA concentrations while 15min intervals were allowed between curves. A total time of 95minutes was taken to complete the construction of each control curve, giving a routine cycle of 1 curve every 110min.

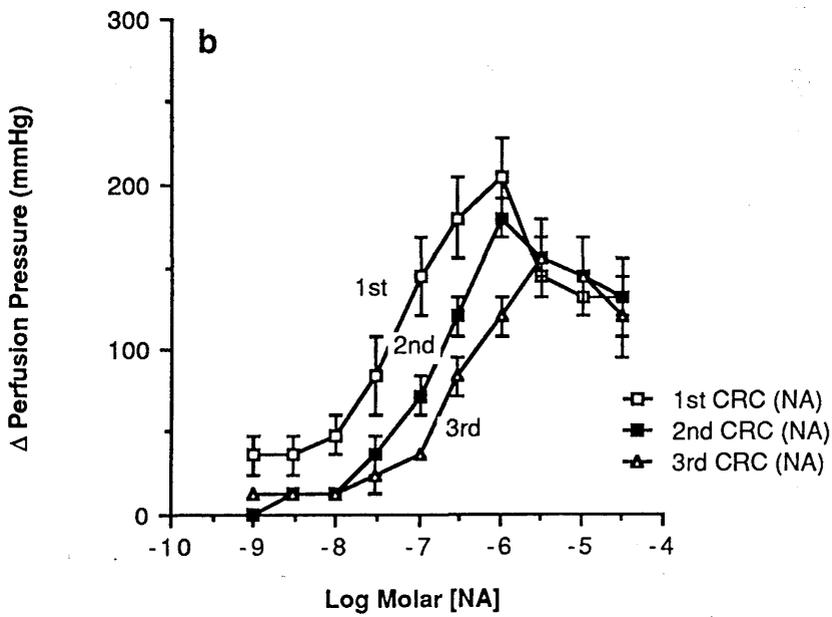
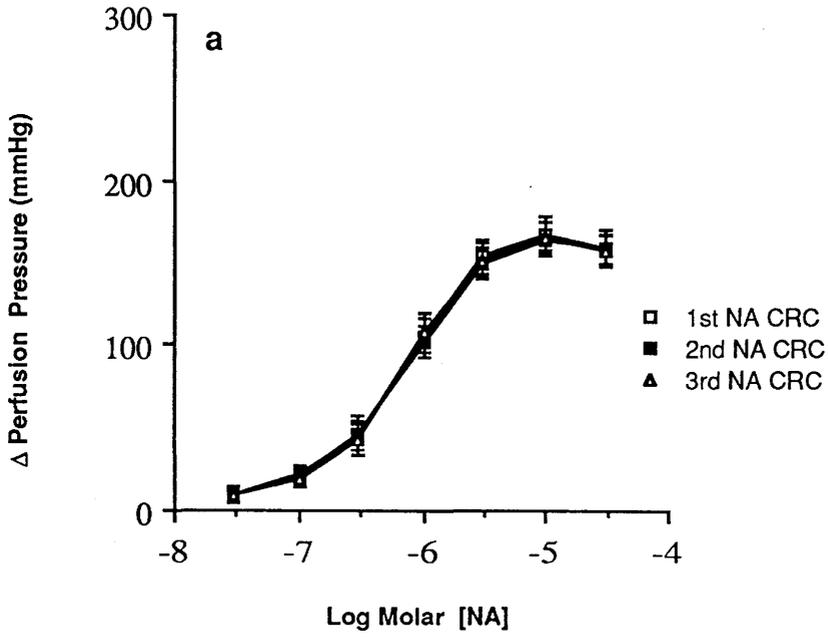
In a series of experiments investigating the effects of cocaine (4 $\mu$ M), propranolol (1 $\mu$ M), EDTA (23 $\mu$ M), the NA control curve was obtained before including the drug in the saline in the subsequent curve. For the experiments on the surface of administration of NA to the tissue, each surface was tested individually. In this case the vessel was set up so that the perfusate ran out of the bath and did not mix with the bathing solution around the vessel. Experiments on different oxygen tensions involved varying the percentage of oxygen (95, 16 and 4%) in the gassing mixture. The influence of these gas mixtures was tested in turn during the construction of a complete NA CRC. In the experiments on the adrenoceptor antagonists, a NA control CRC was obtained first before constructing the subsequent curves in the presence of the antagonists (prazosin and rauwolscine).

## RESULTS

### (i) Responses of the tail artery to noradrenaline

Noradrenaline, in normal calcium (2.5mM), cocaine (4uM), propranolol (1uM) and EDTA (23uM) produced concentration-dependent contractions resulting in the typical sigmoid shaped concentration-response curve (Fig. 1. 1). The tissue preparations were pre-exposed in 3uM NA for 5min in order to stabilise the responses (sensitise, stabilise or prime the tissue) before constructing the concentration response curves to NA. This makes the responses to successive curves reproducible, making it possible to test the effects of other drugs. When primed three times before constructing the NA CRCs, the responses became highly reproducible but tended to be smaller in magnitude. The sensitivity/reproducibility of NA-induced responses was quite similar to that of Medgett and Langer (1984 & 1986) on the isolated perfused rat tail artery of normotensive rats. In this part of the work, the tissue was primed only once. The mean  $pd_2$  or  $-\log EC_{50}$  for NA was  $6.40 \pm 0.05$  (n=32; first curves). The absolute perfusion pressure change from the mean basal value of 34mmHg was  $+180 \pm 24$ mmHg for the NA maximum.

Figure 1. 1. (a) Consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the proximal segment of the isolated perfused rat tail artery (Male Wistar) recorded by perfusion pressure at 95%O<sub>2</sub>. Tissue was exposed to 3uM NA for 5min ("priming") before obtaining the 1st (1st NA Con.) and subsequent non-cumulative CRCs. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. After the priming concentration, reproducible curves were obtained. Points shown indicate mean ± s.e.m. (n=32). (b) This figure is the same as Fig. 2. 3. and it shows comparable curves without priming.



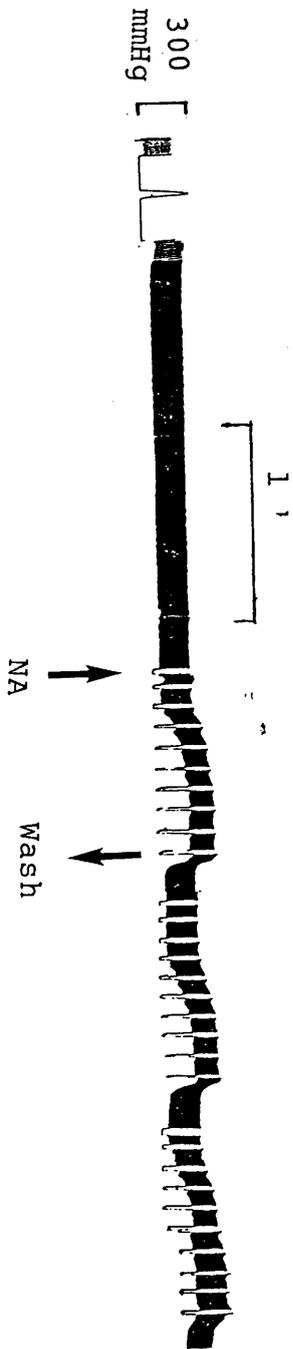


Figure 1.1c. This is an example of the perfusion pressure responses to increasing concentrations of noradrenaline (NA) (30nM to 30uM) in the rat tail artery in 95% O<sub>2</sub>. Concentration response curves (CRCs) to NA were constructed in the presence of 2.5mM Ca<sup>2+</sup>. Here NA was added cumulatively after obtaining priming responses.

(ii) Proximal and distal segments compared

The proximal segment of the rat tail artery produced responses to noradrenaline which were similar to those of the distal segment in the presence of cocaine (4uM), propranolol (1uM) and EDTA (23uM) (Fig. 1. 5-20). However, there were some cases in which the distal portion appeared to have some exaggerated responses over those of the proximal, e.g. when the intra and extraluminal routes of administration of NA were compared with or without cocaine: otherwise they were basically the same (Fig. 1. 8). It has been suggested by Medgett and Langer, (1984) that a small population of  $\alpha_2$ -adrenoceptor might exist in the distal segment which could, therefore, produce some extra response additional to that of the predominantly  $\alpha_1$ -adrenoceptors population of the proximal segment. This was tested later in a part of the study using prazosin and rauwolscine. We had no evidence from our experiments to support the suggestion by Medgett & Langer (1984) that  $\alpha_2$ -adrenoceptors exist in the rat tail artery (see Fig. 1. 15-20).

(iii) Effects of Cocaine, Propranolol and their combination

The presence of cocaine, an adenergic uptake<sub>1</sub> blocker (4uM), tended to enhance the responses of the rail tail artery to NA (Fig. 1. 3), shifting the curve

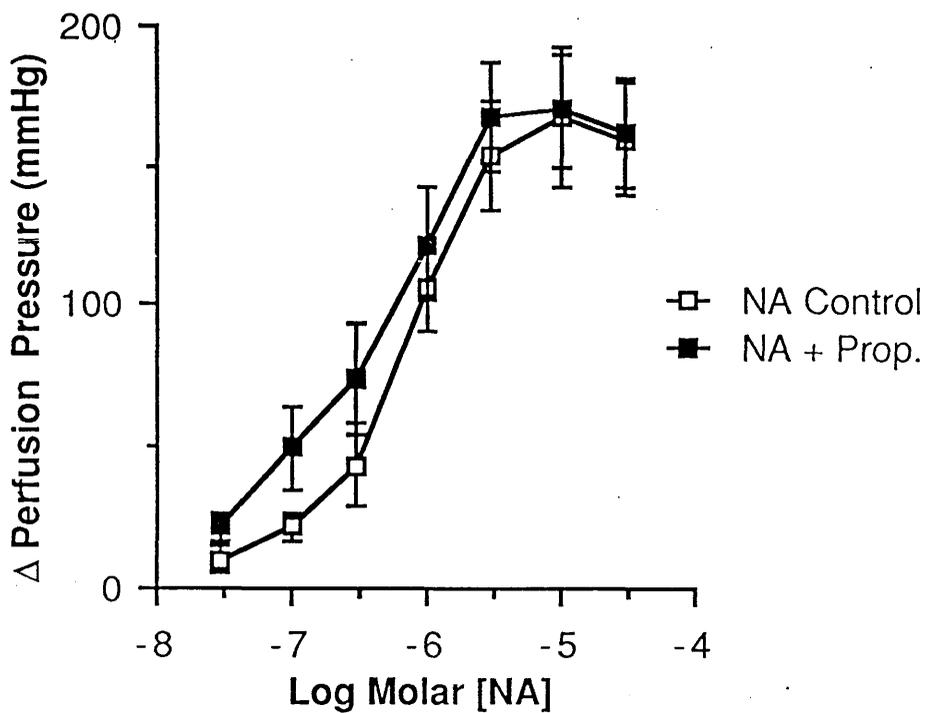


Figure 1. 2. Consecutive log concentration-response curves (CRC) to noradrenaline (NA) in absence (NA Control) and then in the presence of propranolol (NA + Prop.) (1 $\mu$ M). The proximal portion of the tissue was pre-exposed to 3 $\mu$ M NA for 5min ("priming") before obtaining the non-cumulative CRCs at 95%O<sub>2</sub>. Concentration range tested was 30nM-30 $\mu$ M NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean  $\pm$  s.e.m. (n=12). The responses tended to be greater in the presence of propranolol but the difference between these and the controls was not statistically significant.

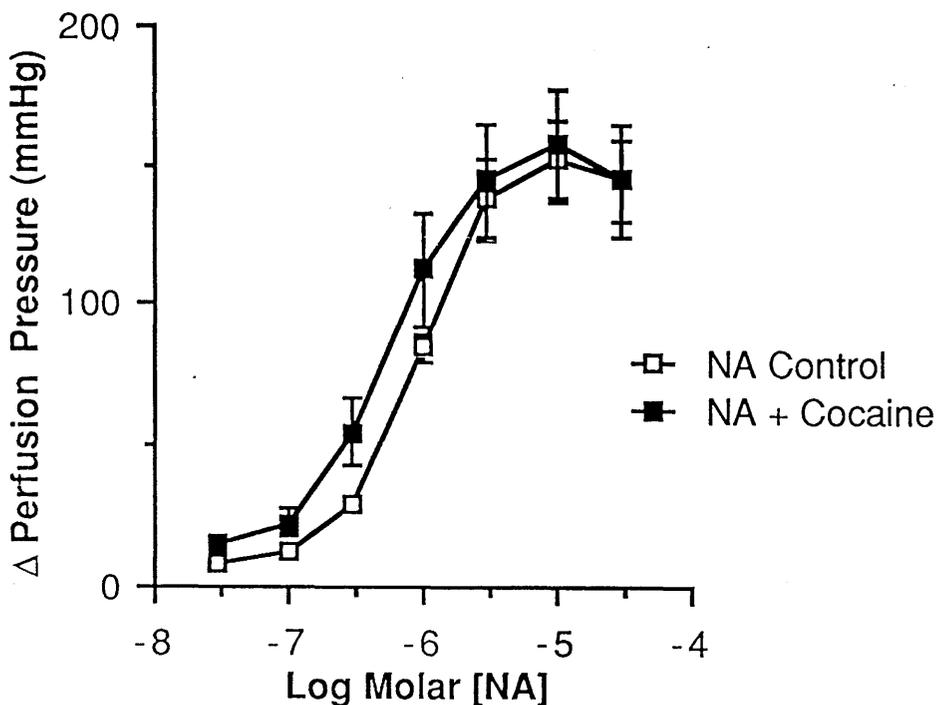


Figure 1. 3. Consecutive log concentration-response curves (CRC) to noradrenaline (NA) in absence (NA Control) and then in the presence of cocaine (NA + Cocaine) (4 $\mu$ M). Tissue (proximal segment) was pre-exposed to 3 $\mu$ M NA for 5min ("priming") before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg) at 95%O<sub>2</sub>. Concentration range tested was 30nM-30 $\mu$ M NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean  $\pm$  s.e.m. (n=12). There was a tendency for the responses to be greater in the presence of cocaine but the difference between these and the controls was not statistically significant.

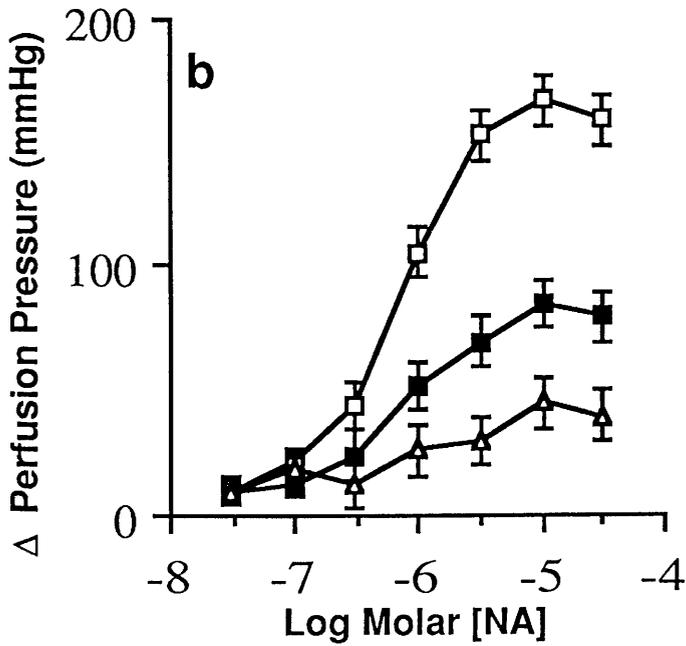
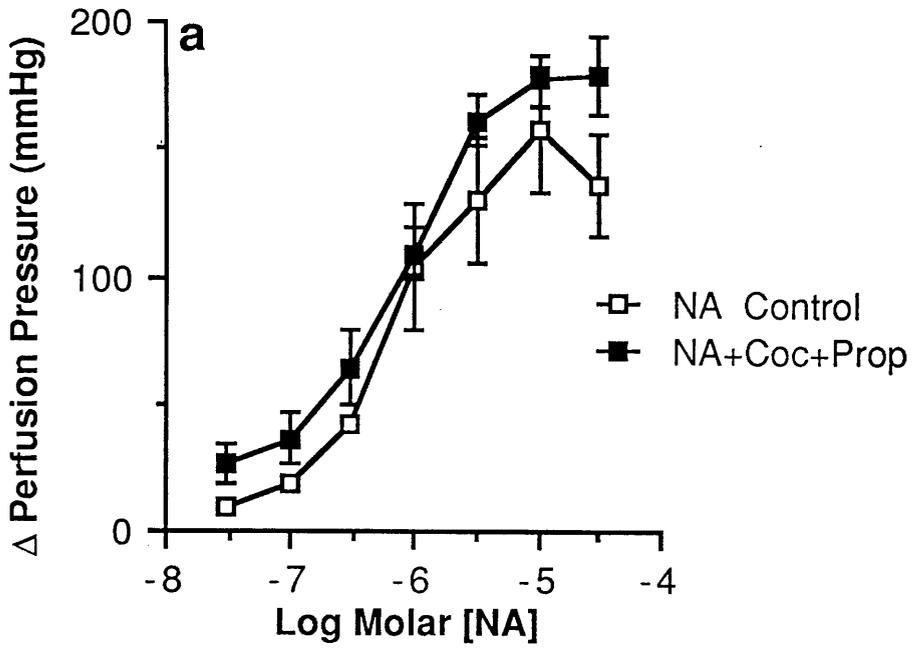
to the left of the control and therefore giving a slightly higher mean  $-\log EC_{50}$  value. However, these differences from the control were not statistically significant. There was also no significant change in the maximum value of the responses when compared with the control. A similar tendency towards potentiation of responses to noradrenaline was observed with propranolol, a beta-adrenergic blocker (1uM), present in the saline (Fig. 1. 2). A combination of both drugs in the saline in the presence of EDTA (23uM) similarly showed a tendency towards enhancing the tail artery responses to noradrenaline with some slight increase in both the sensitivity and the maximum value (Fig. 1. 4a). Despite these rather small effects both drugs were subsequently routinely employed in all our experiments except those involving action by KCl.

(iv) Effect of EDTA

EDTA (23uM) in the presence of cocaine (4uM) and propranolol (1uM) prevented the fast deterioration of NA responses. In the absence of EDTA the responses to NA rapidly waned (Fig. 1. 4b). Both the sensitivity of the tissue to NA and the mean maximum value significantly declined. EDTA is known to prevent the oxidation and rapid breakdown of NA in solution by chelating heavy metal ions which catalyse the oxidation. Although the inclusion of this drug could be disadvantageous to experiments in which the sensitivity of a tissue to

Figure 1. 4. (a) Consecutive log concentration-response curves (CRC) to noradrenaline (NA) in absence ( ) and then in the presence of cocaine (4uM) and propranolol (1uM) ( ). The proximal segment of the rat tail artery preparations were pre-exposed to 3uM NA for 5min ("priming") before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg) at 95%O<sub>2</sub>. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=10-12). The responses tended to be greater in the presence of cocaine and propranolol but the difference between these and the controls was not statistically significant.

(b) This shows the responses of the tissue in absence ( , ) and in the presence of 23uM EDTA ( ). Points shown indicate mean ± s.e.m. (n=8-10). Responses in the absence of EDTA were significantly smaller than those in the presence of EDTA (0.01<p<0.05).



calcium is being studied because of its chelation of calcium, provided that the concentration of calcium is much greater than that of EDTA, no problems arise. 23uM EDTA was therefore routinely used in all our subsequent experiments that involved NA.

(v) Effects of route of administration of drugs

Our results showed that in general, comparing exposure to NA of both surfaces with each individually, the responses seemed better when the drug was administered intraluminally and worse when it came through the extraluminal (for both the proximal and distal portions of the vessel) (Fig. 1. 5 & 6). When the NA was administered through both surfaces of the vessel (as control), and then administered through either of the surfaces only, the curve lies on the left of the control for the intraluminal administration (Fig. 1. 5) and on the right of the control for the extraluminal administration ( Fig. 1. 6). Both the sensitivity and the mean maximum value of the tissue responses to NA were apparently enhanced by intraluminal and attenuated by extraluminal administration of NA to the vessel. However this occurred only in the absence of cocaine.

In the presence of cocaine (4uM), there was no difference between the intraluminal and extraluminal administration of NA (Fig. 1. 8c) or between the intraluminal surface and both surfaces together (Fig. 1.

Figure 1. 5. Effects of surface of administration on response to NA in isolated perfused rat tail artery. Consecutive log concentration-response curves (CRCs) to noradrenaline (NA) in the absence of cocaine and propranolol were constructed first for intraluminal administration of NA ( ) and then for NA administration through both surfaces ( ). Tissue was pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg) at 95%O<sub>2</sub>. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=10-12). There was a tendency for the maximum responses to be greater by intraluminal application in the proximal segment (a) but not in the distal (b). The difference in responses between these methods of drug administration was not statistically significant.

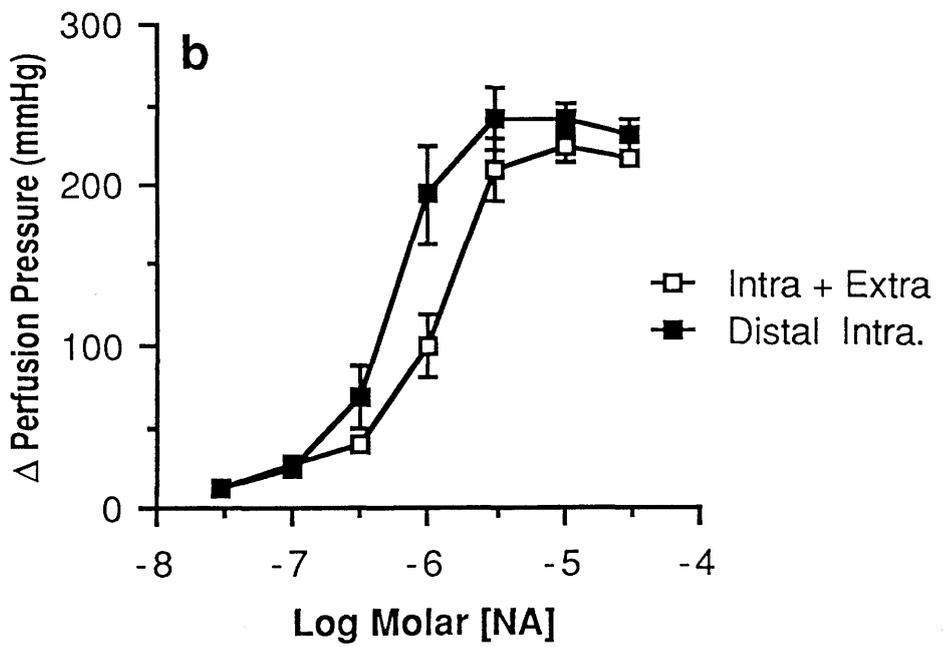
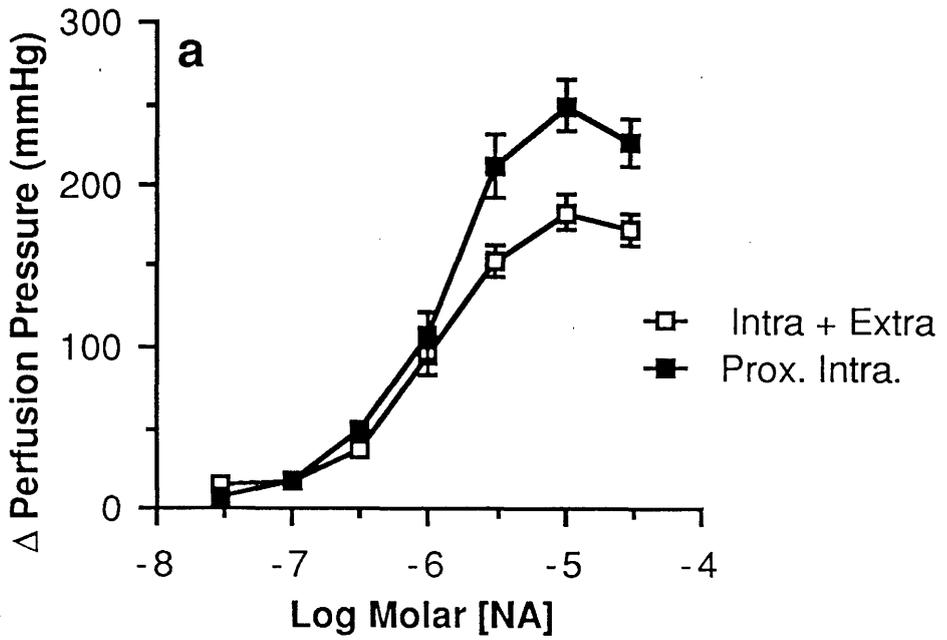


Figure 1. 6. Effects of surface of administration on response to NA in proximal segment of the isolated perfused rat tail artery. Consecutive log concentration-response curves (CRCs) to noradrenaline (NA) in the absence of cocaine and propranolol were constructed first for extraluminal administration of NA ( ) and then for NA administration through both surfaces ( ). Tissue was pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg) at 95%O<sub>2</sub>. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=12). In the proximal segment (a), responses to drug application through both surfaces were significantly greater than with application through the extraluminal surface only and these were at 4 concentrations of NA only (asterisked) (p<0.05). There was no such difference in the distal segment where both methods of drug administration gave similar responses (b).

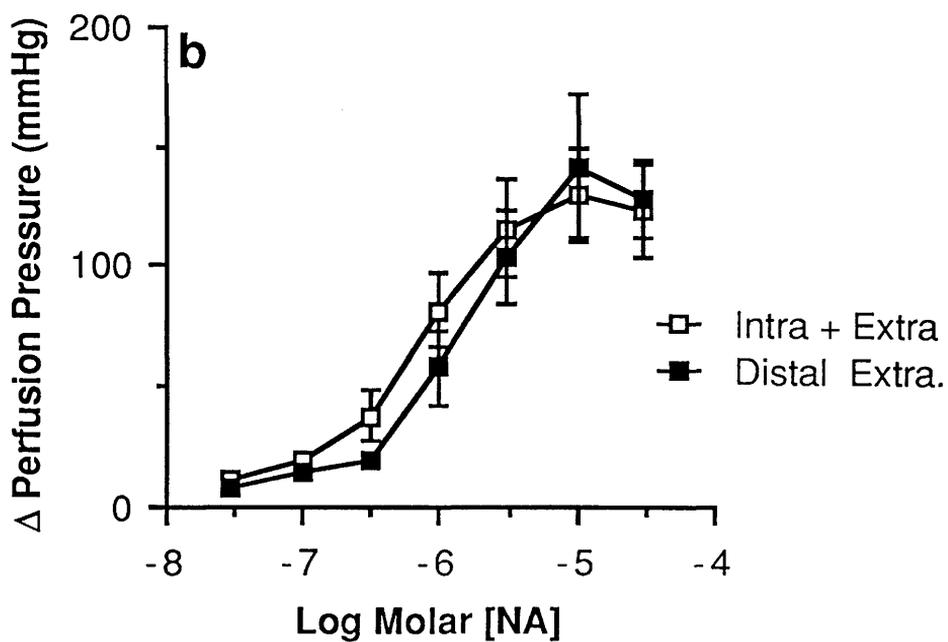
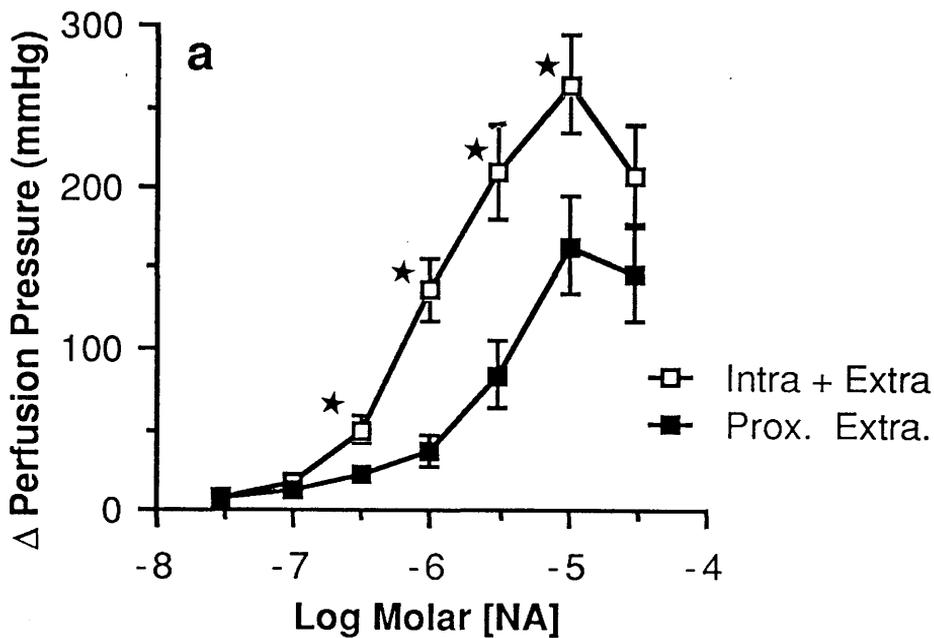


Figure 1. 7. Effects of surface of administration on responses to NA in isolated perfused rat tail artery with cocaine present. Consecutive log concentration-response curves (CRCs) to noradrenaline (NA) in the presence of cocaine (4uM) were constructed first for intraluminal administration of NA ( ) and then for NA administration through both surfaces ( ). Tissue was pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg) at 95%O<sub>2</sub>. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=12). In both the proximal (a) and distal (b) segments, there was no significant difference between responses obtained with administration through both surfaces together and administration through the intraluminal surface only.

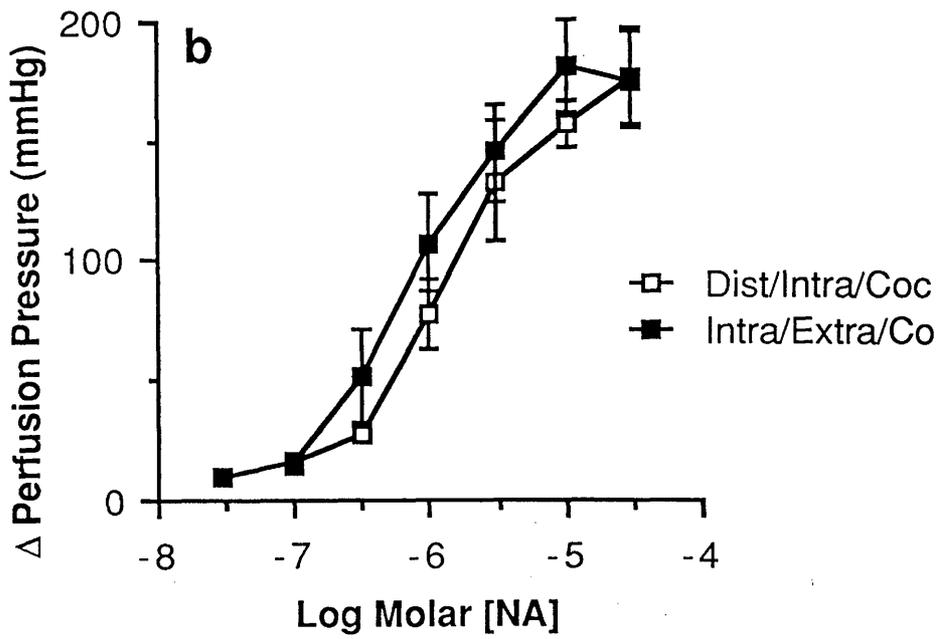
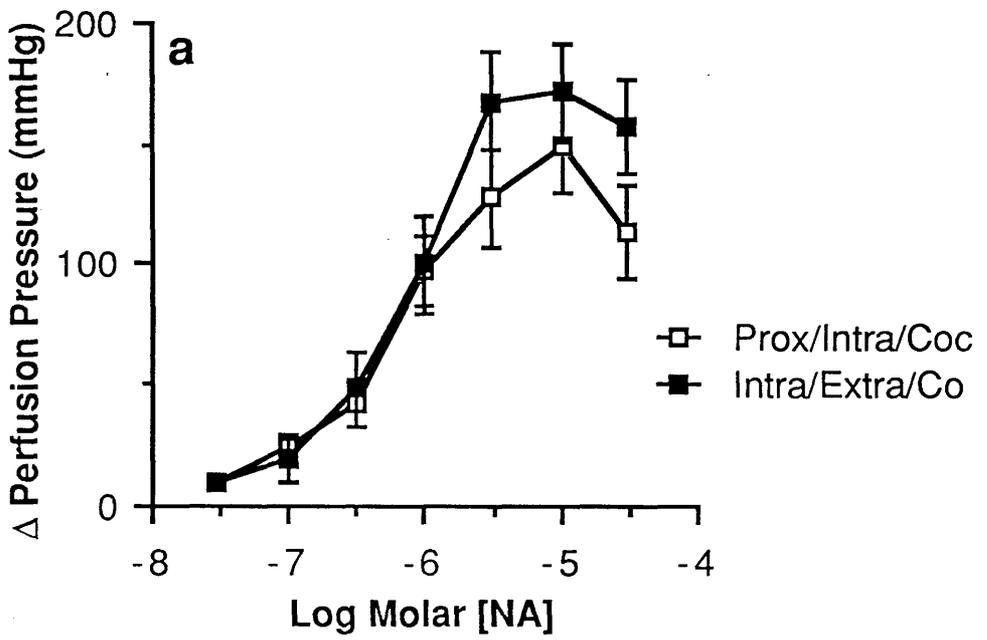
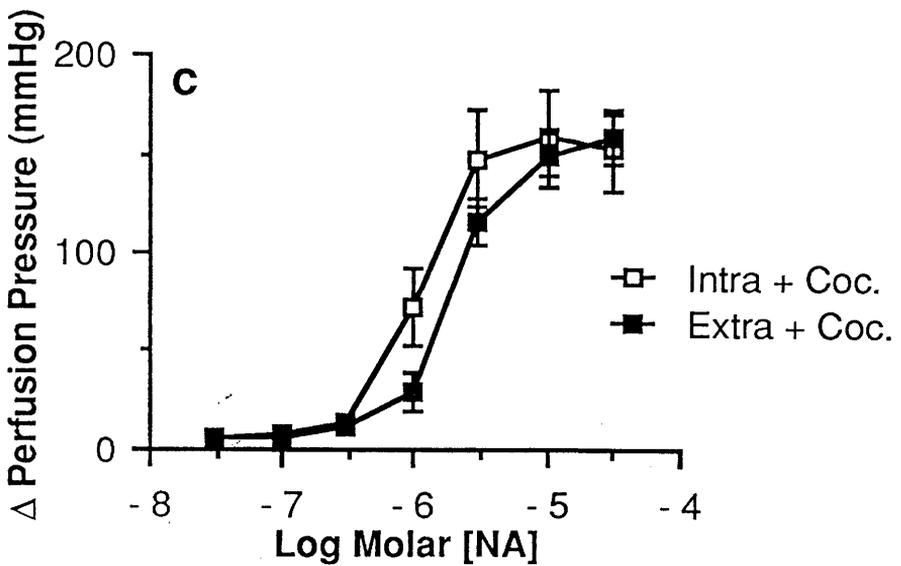
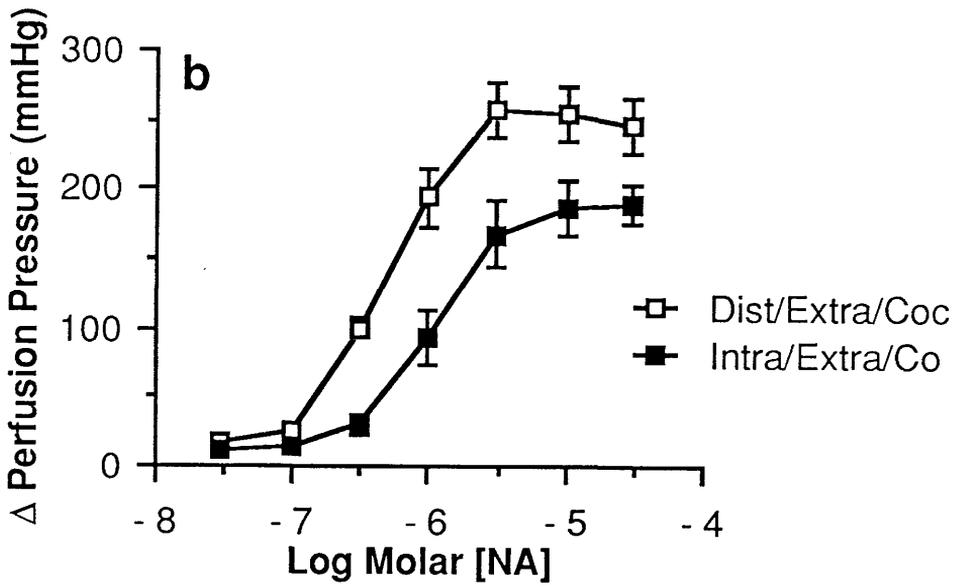
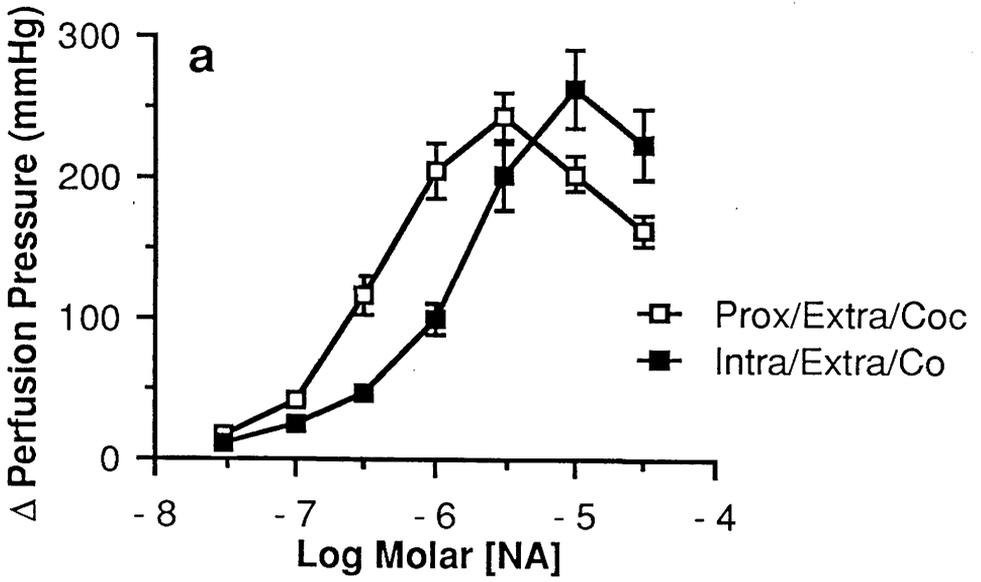


Figure 1. 8. Effects of surface of administration on response to NA in isolated perfused rat tail artery presence of cocaine. Consecutive log concentration-response curves (CRCs) to noradrenaline (NA) in the presence of cocaine (4uM) were constructed first for extraluminal administration of NA ( ) and then for NA administration through both surfaces ( ). Tissue was pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg) at 95%O<sub>2</sub>. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=10-12). In the proximal segment (a) there was no significant difference between response to the combined methods of drug administration in the presence of cocaine and administration through the extraluminal surface only except at two concentration levels (0.3 and 1uM NA). In the distal (b) segment, the intraluminal administration in the presence of cocaine gave significantly higher responses than the administration through both surfaces except at the 2 lowest NA concentrations. Cocaine abolishes the difference between the intraluminal and extraluminal administrations of NA in the rat tail artery. Only responses in the proximal segment are shown (c).



7). Thus, cocaine enhanced the responses better through the extraluminal surface than through both surfaces together (Fig. 1. 8a&b). Cocaine therefore abolished the difference between the two routes of surface of entry of NA.

(vi) Effects of oxygen tensions

Over the range tested, oxygen tensions had little effect on responses to NA. At a constant carbon dioxide tension of 32mmHg (5%CO<sub>2</sub>) high oxygen tension of 580-650mmHg (95%O<sub>2</sub>) and 92%O<sub>2</sub>, produced responses that were not significantly different from those with physiological oxygen tension of 100-120mmHg (16%O<sub>2</sub>) (Fig. 1. 9a&b). This was the case for both proximal and distal segments of the vessel although there was a tendency for lower responses at 16% in some experiments, e.g. in Fig. 1. 9c). This is complicated by the tendency for the 2nd curve to be smaller, but reversing the order of gassing suggested that there was no substantial difference between 95% and 16% O<sub>2</sub> versus NA CRCs (Fig. 1. 9c). However, the time courses of responses were affected by O<sub>2</sub> tensions. At 16%O<sub>2</sub>, contraction was biphasic with most NA concentrations: an early rapid but transient component was followed by a slower, maintained component, normally of smaller size. At 95%O<sub>2</sub>, the response was more monotonic (Fig. 1. 9d&e). In the rat tail artery, as in the anococcygeus and the portal vein, this change to a

Figure 1. 9. Effects of O<sub>2</sub> on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the presence of cocaine (4uM) and propranolol (1uM): in 16%O<sub>2</sub> (16% Oxygen), then in 92%O<sub>2</sub> (92% Oxygen) and finally in 95%O<sub>2</sub> (95%O<sub>2</sub>-Contr.). Tissue preparations were pre-exposed to 3uM NA for 5min ("priming") before obtaining the non-cumulative CRCs recorded by perfusion pressure (mmHg). Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=10-12). In the proximal segment (a) the responses the responses in the different oxygen levels were not statistically significantly different. In the distal segment (b), responses tended to be smaller in 16% but there were no significant differences .

In another set of experiments (c), the responses were first tested in 95% O<sub>2</sub> and 5% CO<sub>2</sub> (95%O<sub>2</sub>/5%CO<sub>2</sub>), then in 16% O<sub>2</sub> and 5% CO<sub>2</sub> twice (16%O<sub>2</sub> - 1st; 16%O<sub>2</sub>/5%CO<sub>2</sub>) before testing again in 95% O<sub>2</sub> (95%O<sub>2</sub> - 2nd). Here the responses in the 2nd 95%O<sub>2</sub> were less than those in the first 95% at 2 NA concentrations (asterisked) but similar to the responses in 16% O<sub>2</sub>.

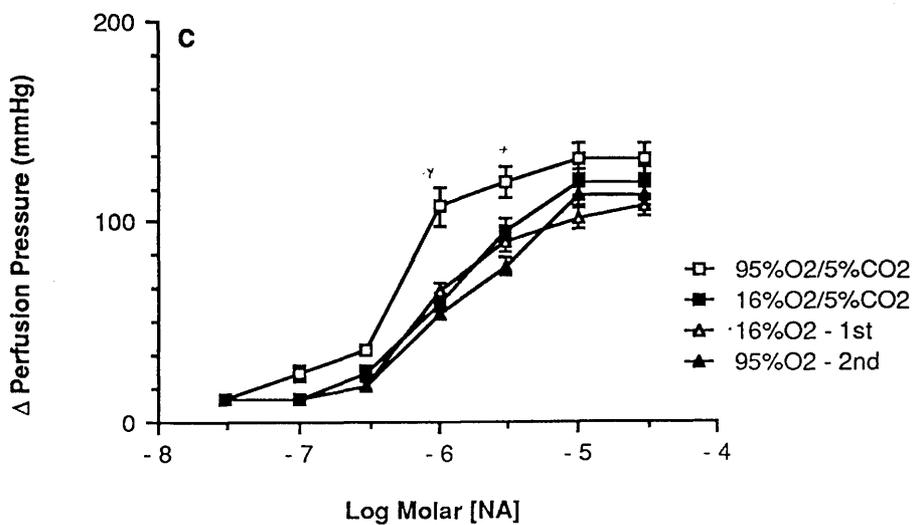
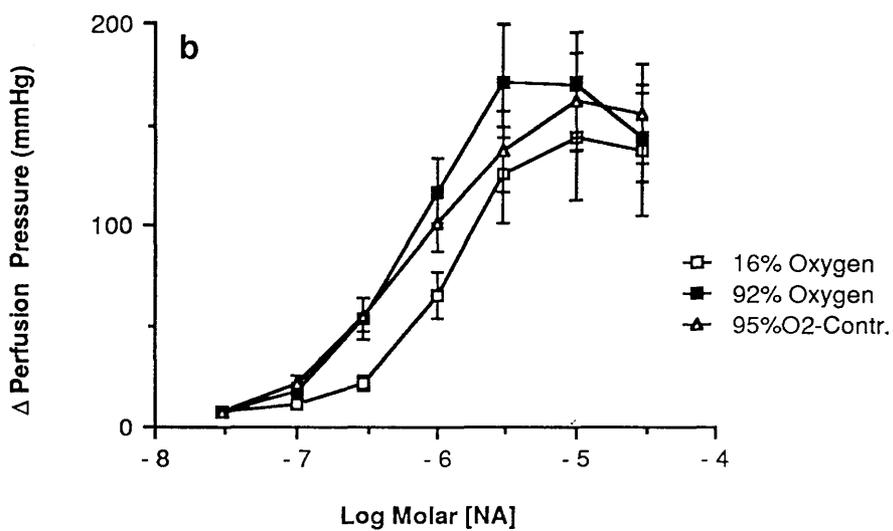
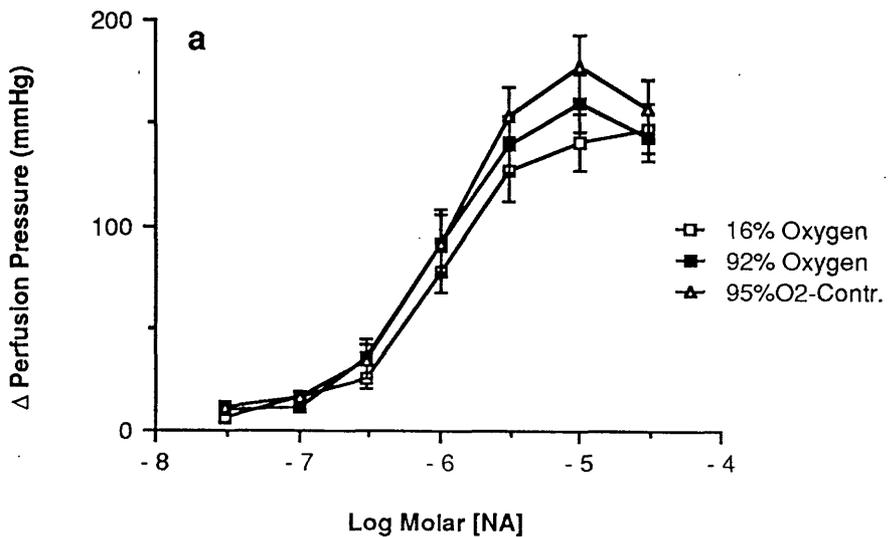


Figure 1.9 (i-iii). A comparison of the influence of 16% and 95%O<sub>2</sub> on noradrenaline-induced concentration response curves in absolute values (mmHg) (ii) and when the results are expressed as percent of the control maximum (iii). 95% tended to attenuate the initial rise in perfusion pressure to NA and increased the maximal maintained contraction (i).

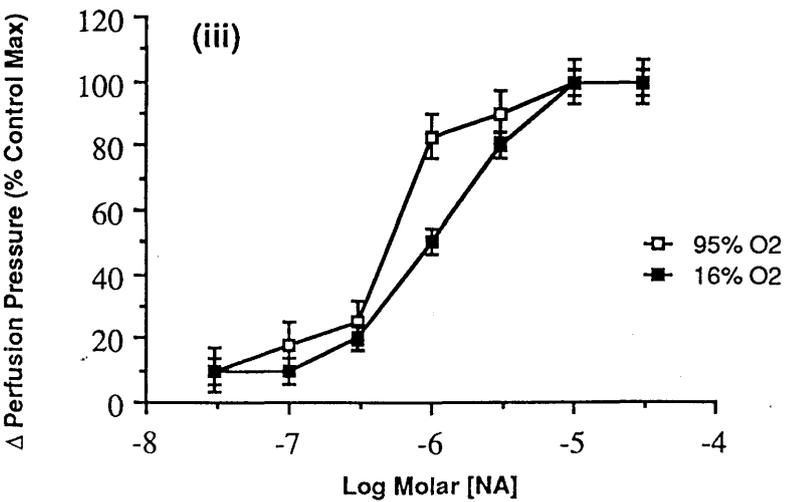
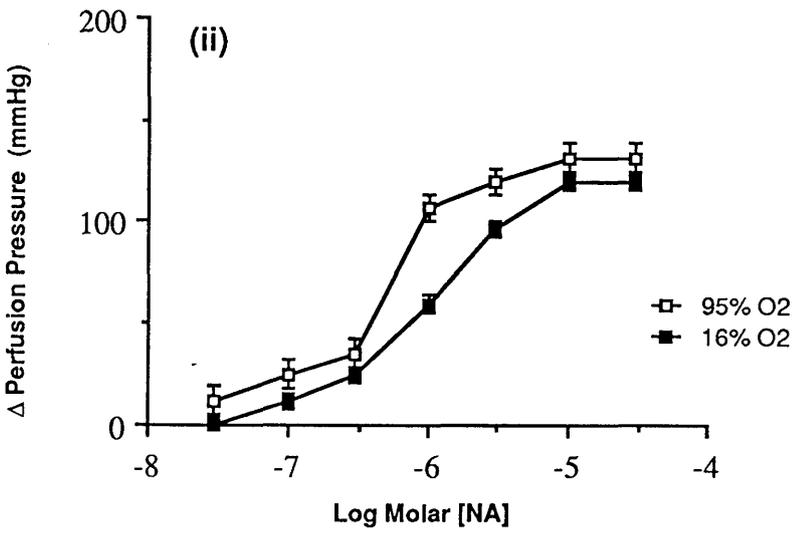
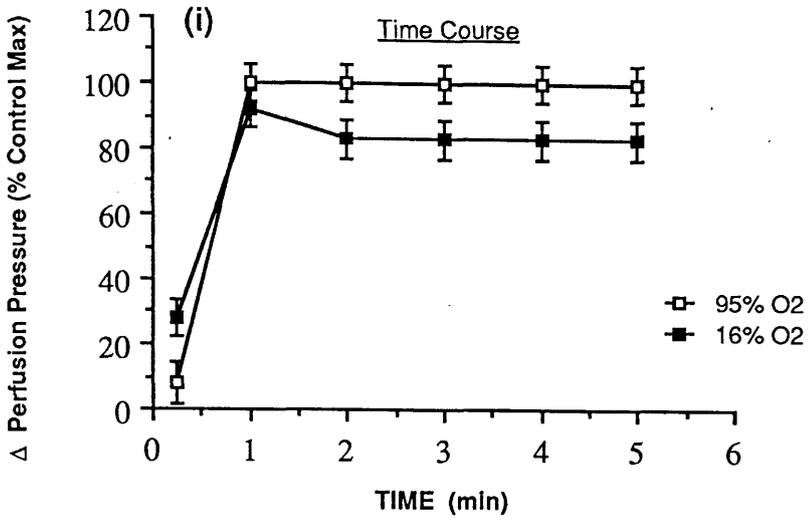
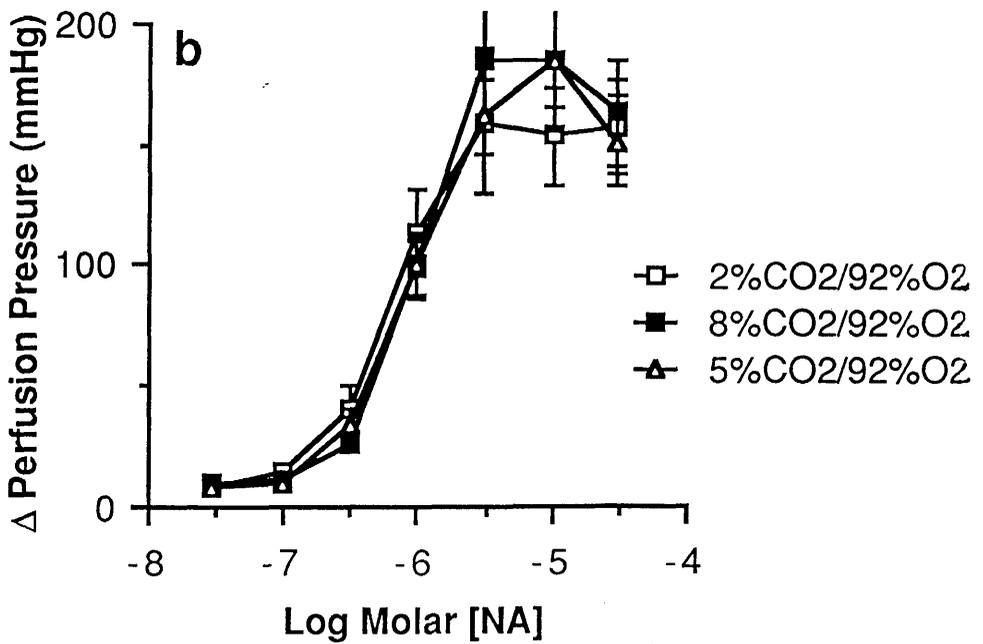
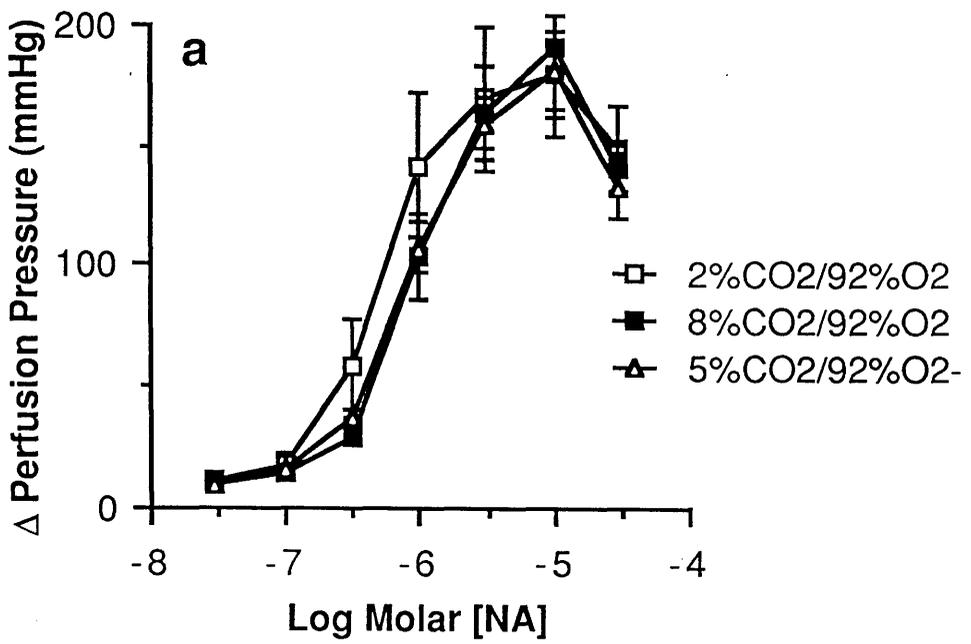


Figure 1. 10. Effects of CO<sub>2</sub> on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the presence of cocaine (4uM) and propranolol (1uM): first in 2% CO<sub>2</sub> (2% CO<sub>2</sub>/92%O<sub>2</sub>), then in 8%CO<sub>2</sub> (8%CO<sub>2</sub>/92%O<sub>2</sub>) and then in 5%CO<sub>2</sub> (5%CO<sub>2</sub>/92%O<sub>2</sub>-Con) at a constant 92%O<sub>2</sub>. Tissue preparations were pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=10-12). In both the proximal (a) and the distal (b) segments there was no significant difference in the responses over the range of carbon dioxide levels tested.



monotonic response in hyperoxia resulted both from a diminution in the rate of rise of the first component and an increase in the height of the second component (although the latter was not significant in the present series of experiments when the maximum was measured.

When mild hypoxia (4%O<sub>2</sub>) was employed (in calcium experiments), the curve was slightly shifted to the right of the 16%O<sub>2</sub> curve but this was not statistically significant. 4%O<sub>2</sub> therefore has the tendency to produce not only a lower sensitivity to calcium but also a lower mean maximum when compared with 16%O<sub>2</sub> or 95%O<sub>2</sub> (Fig. 3. 1a).

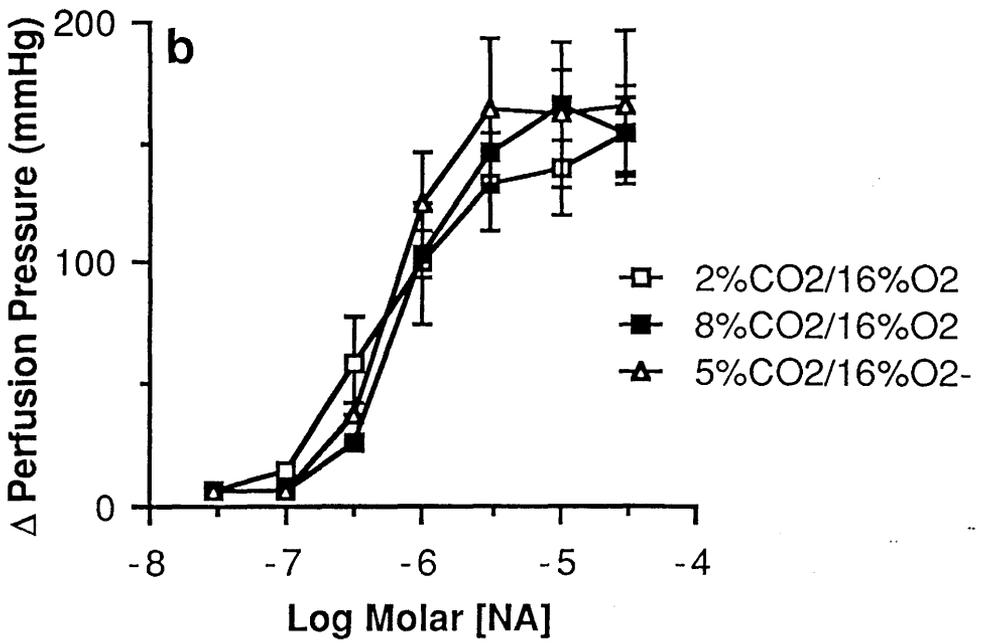
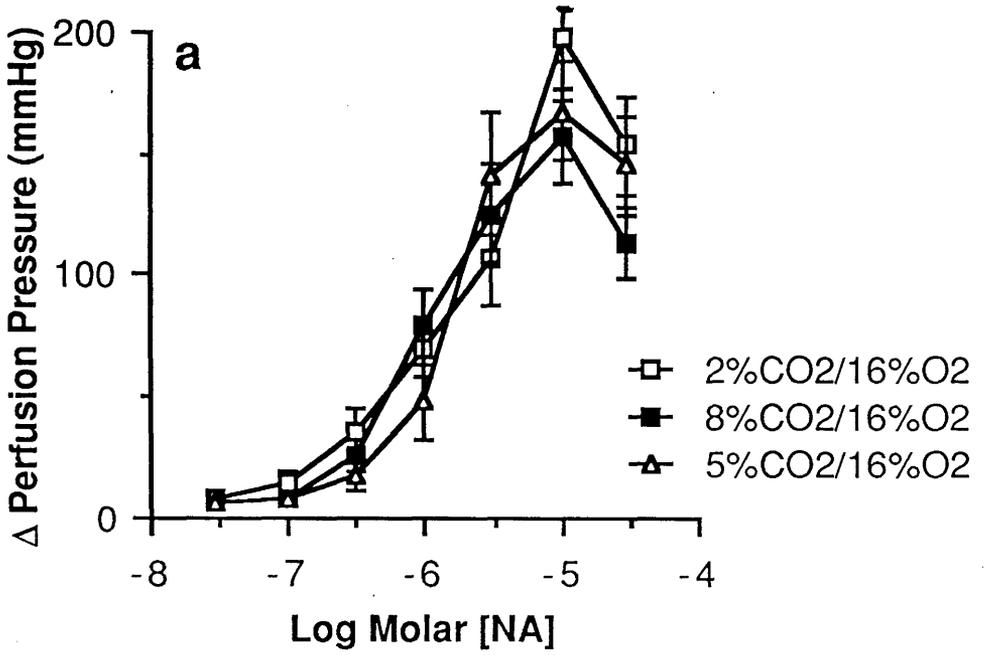
(vii) Effects of pH of the saline

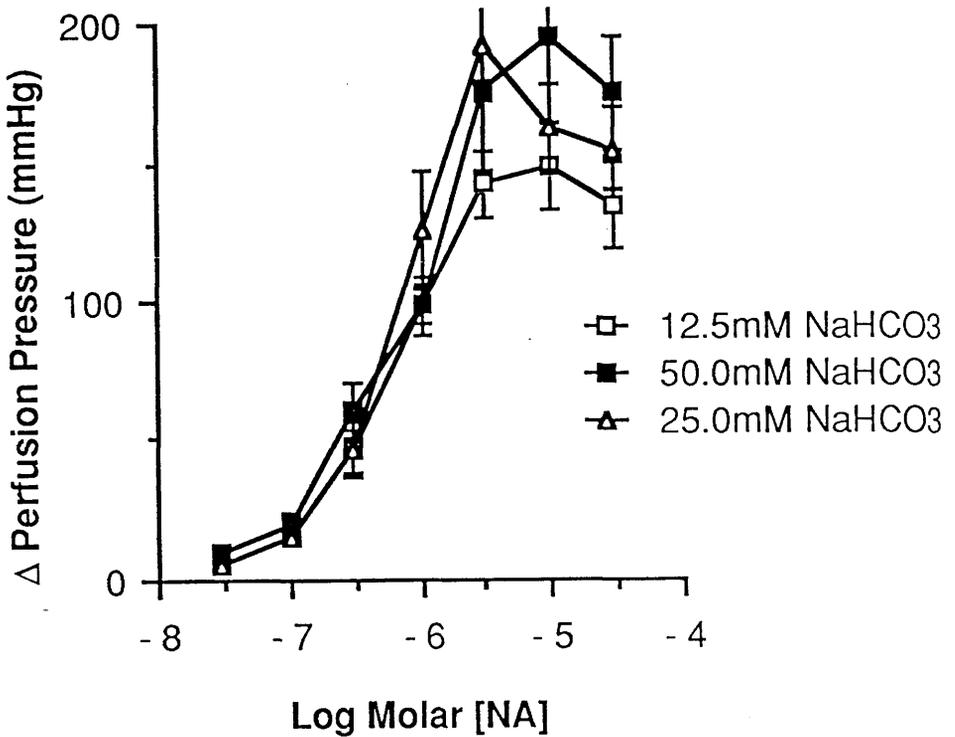
When the pH of the physiological saline is altered by varying the CO<sub>2</sub> in the gas mixture, both intracellular and extracellular pH should change: this was first examined at a constant oxygen tension of 580mmHg (92%O<sub>2</sub>). Lowering the carbon dioxide tension from 5% to 2% slightly raised the pH of the saline from 7.35 to 7.50 (see Table 3). This resulted in a slight but non-significant increase in the sensitivity of the tissue to NA in both the proximal and distal portions of the vessel (Fig. 1. 10). The sensitivity and the mean maximum were not significantly altered when the pH was lowered to 7.20 by raising CO<sub>2</sub> from 5% to 8%.

Table 3. A comparison of the changes in the pH of the saline with changes in the carbon dioxide and oxygen tensions.

pH	pCO <sub>2</sub> (mmHg)	pO <sub>2</sub> (mmHg)
7.6	23.4	428
7.5	28.0	470
7.4	30.5	566
7.3	42.0	660
7.2	50.0	721
7.1	51.0	755

Figure 1. 11. Effects of CO<sub>2</sub> on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the presence of cocaine (4uM) and propranolol (1uM): first in 2% CO<sub>2</sub> (2% CO<sub>2</sub>/16%O<sub>2</sub>), then in 8%CO<sub>2</sub> (8%CO<sub>2</sub>/16%O<sub>2</sub>) and then in 5%CO<sub>2</sub> (5%CO<sub>2</sub>/16%O<sub>2</sub>-Con) at a constant 16%O<sub>2</sub>. Tissue preparations were pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs. Concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Points shown indicate mean ± s.e.m. (n=10-12). In both the proximal (a) and the distal (b) segments there was no significant difference in the responses over the range of carbon dioxide levels tested.





This was repeated with the oxygen tension kept constant at the physiological level of 100-120mmHg (16%O<sub>2</sub>). The effects of changing pH were similar to the above (580mmHg: (95%O<sub>2</sub>) (Fig. 1. 11).

When the pH of the saline was changed by varying the concentration of sodium bicarbonate in the saline, in an attempt to change only the extracellular pH, no significant effects on NA sensitivity were found over the range studied (HCO<sub>3</sub><sup>-</sup> 12.5mM to 50mM: pH 7.10 to 7.65) (Fig. 1. 12).

(viii) Characterisation of the adrenergic receptors and the effects of  $\alpha$ -adrenoceptor antagonists

The characterisation of the adrenergic receptors in the rat tail artery was carried out by examining the effects of selective  $\alpha$ -adrenoceptor antagonists on the tissue. Prazosin (0.1nM to 0.1 $\mu$ M) was used to test for  $\alpha_1$ -adrenoceptors and rauwolscine (1nM to 1 $\mu$ M) was employed to test for  $\alpha_2$ -adrenoceptors.

Prazosin effectively inhibited the NA-induced contractions in both the proximal and distal portions of the rat tail artery and a significant effect was observed at concentrations as low as 0.1nM prazosin in each segment (Fig. 1. 13a&b). Increasing the concentrations of prazosin further shifted the curve to the right. Construction of a Schild Plot for prazosin

Figure 1. 13 a&b. Effects of prazosin on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the (a) proximal and (b) distal segments of the rat tail artery: control ( ) in the presence of increasing concentrations of prazosin (0.1, 1 and 10nM). Cocaine (4uM), propranolol (1uM) and EDTA (23uM) were present in the saline. Tissue preparations were pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs at 95%O<sub>2</sub>. NA concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Responses were calculated as % of maximum response of the control. Points shown indicate mean ± s.e.m. (n=10-12). Prazosin effectively inhibited the NA-induced responses in both the proximal and distal segments.

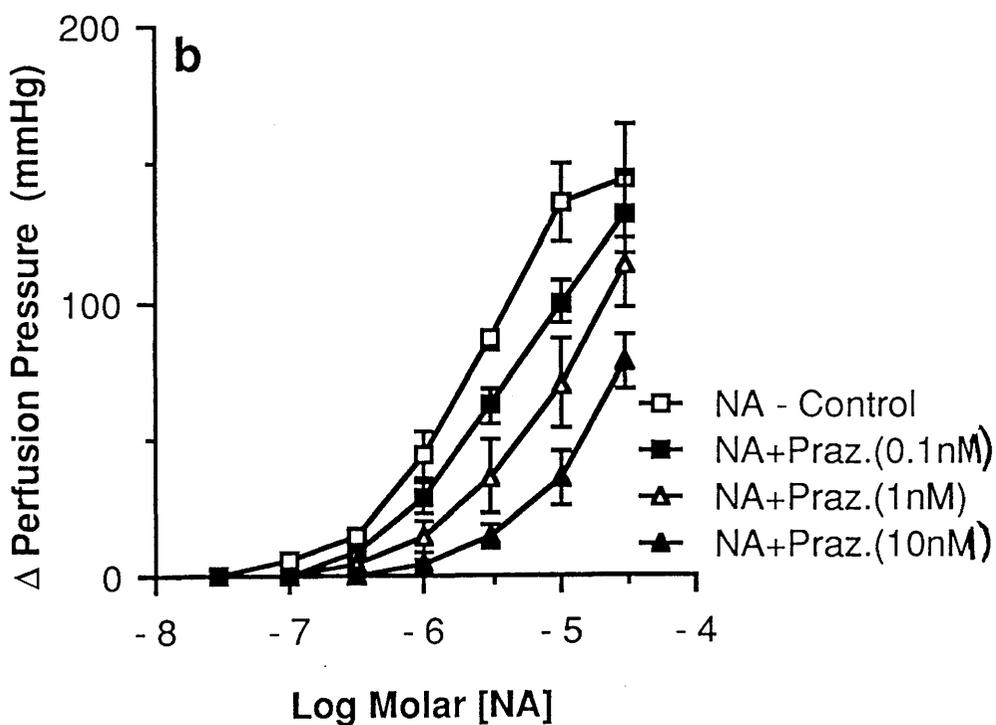
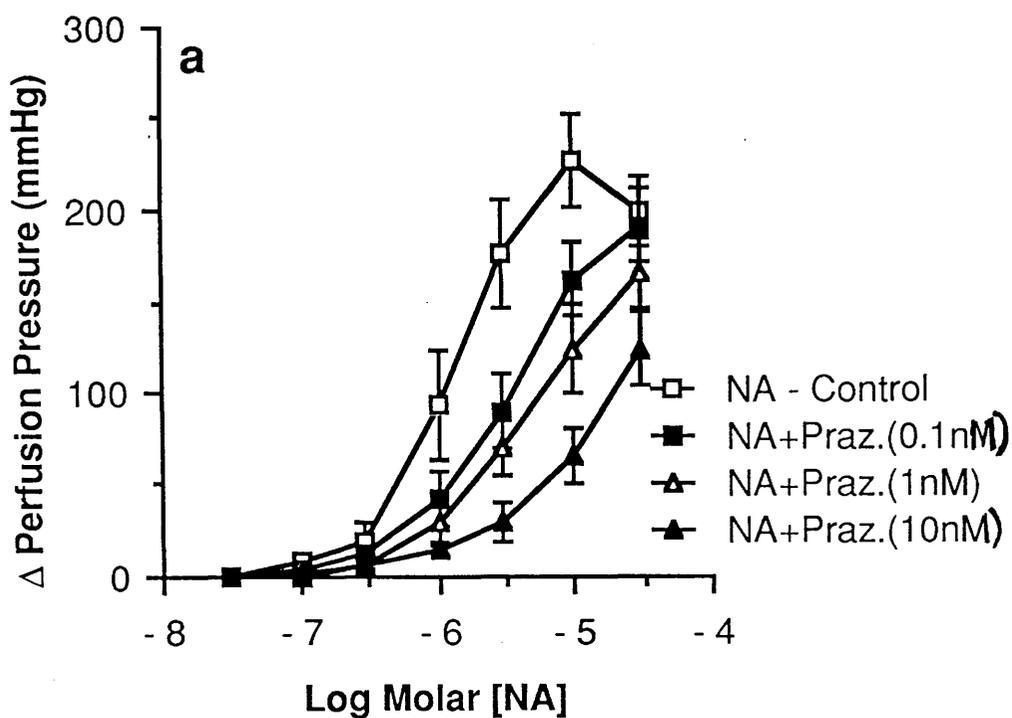


Figure 1.13 c&d. Schild plot for the interaction of prazosin and NA was constructed for each concentration of prazosin. The  $pA_2$  value and slope were obtained by linear regression by using the individual data point for concentration-ratio (CR) (calculated at the  $EC_{50}$ ) and the individual agonist concentrations from each concentration of antagonist. The intercept of the linear regression line with the line,  $\log(CR-1)=0$ , gave an estimate of the  $pA_2$ . The points shown represent the mean  $\pm$  s.e.m. at each concentration and the line is based on the mean  $pA_2$  and mean slope from the group of experiments (see Table 4 for the values of the  $pA_2$  and the slope).

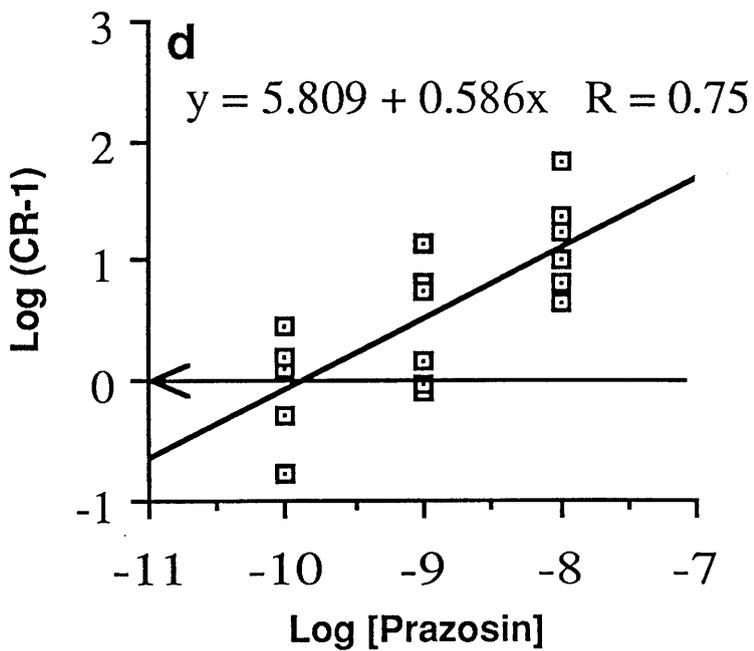
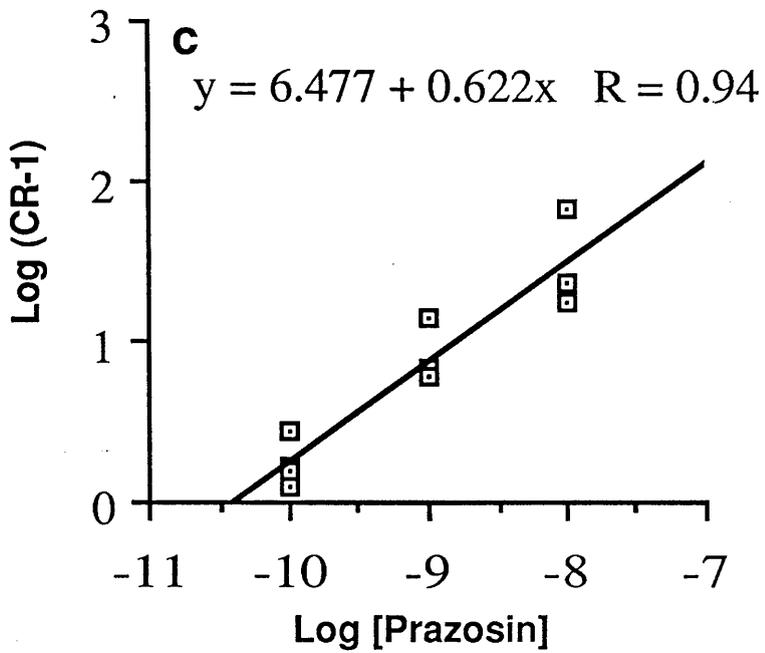


Figure 1.14 a&b. Effects of rauwolscine on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the (a) proximal and (b) distal segments of the rat tail artery: control ( ) in the presence of increasing concentrations of rauwolscine (1nM, 10nM, 0.1uM, 1uM). Cocaine (4uM), propranolol (1uM) and EDTA (23uM) were present in the saline. Tissue preparations were pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs at 95%O<sub>2</sub>. NA concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Responses were calculated as % of maximum response of the control. Points shown indicate mean ± s.e.m. (n=10-12). Rauwolscine was virtually ineffective in inhibiting the NA-induced responses.

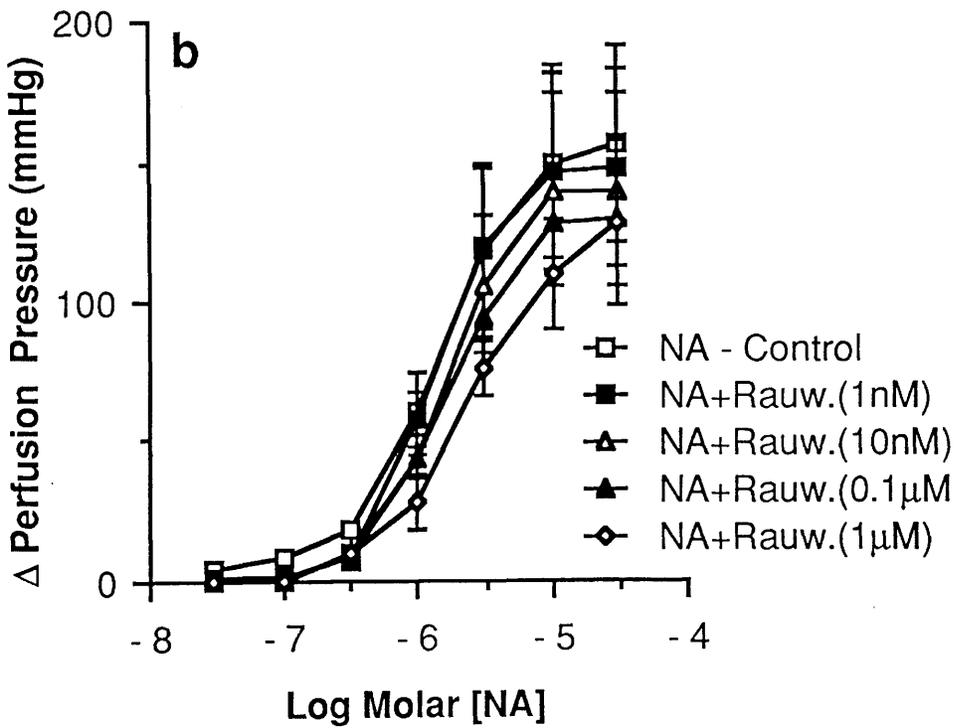
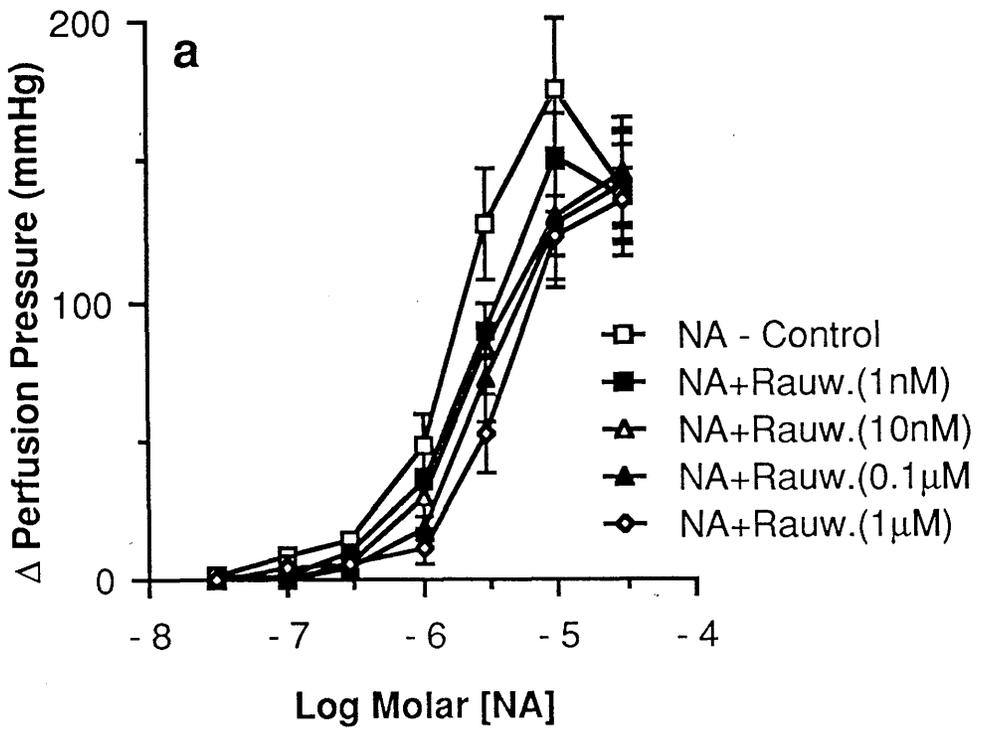


Figure 1.14 c&d. Schild plot for the interaction of prazosin and NA was constructed for each concentration of prazosin. The  $pA_2$  value and slope were obtained by linear regression by using the individual data point for concentration-ratio (CR) (calculated at the  $EC_{50}$ ) and the individual agonist concentrations from each concentration of antagonist. The intercept of the linear regression line with the line,  $\log(CR-1)=0$ , gave an estimate of the  $pA_2$ . The points shown represent the mean  $\pm$  s.e.m. at each concentration and the line is based on the mean  $pA_2$  and mean slope from the group of experiments (see Table 4 for the values of the  $pA_2$  and the slope).

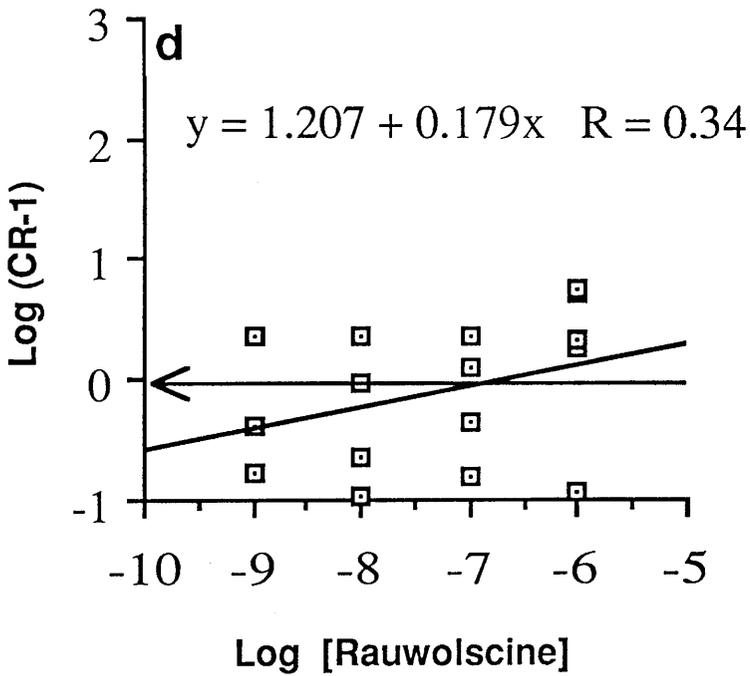
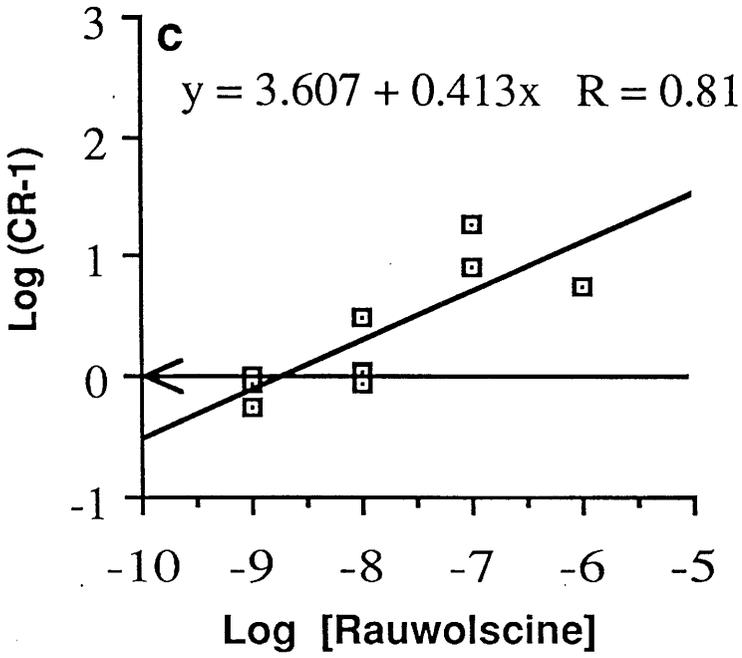
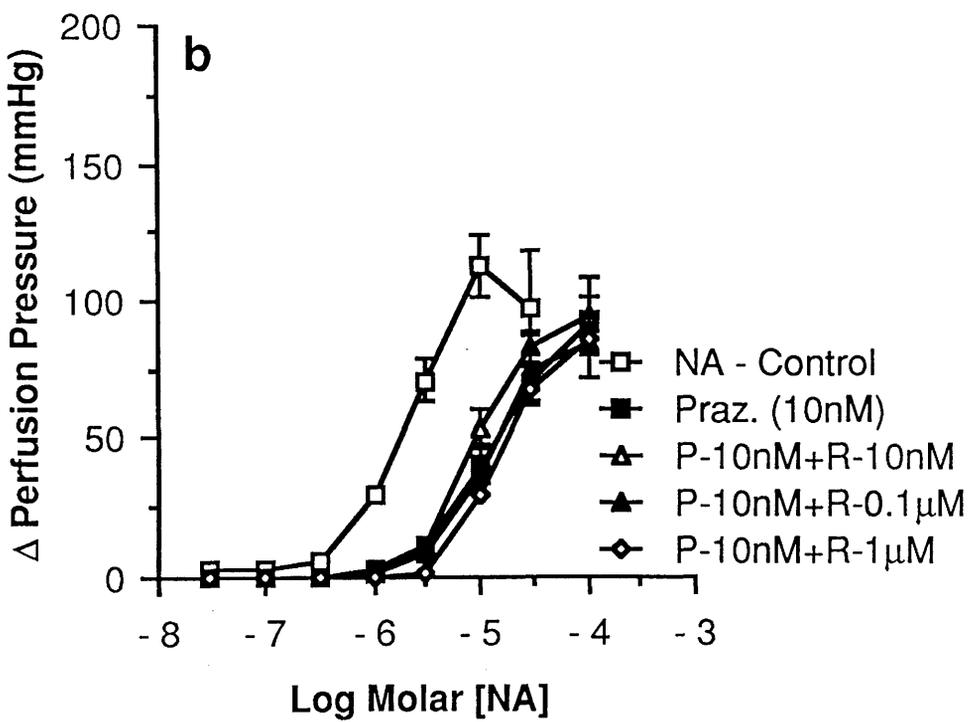
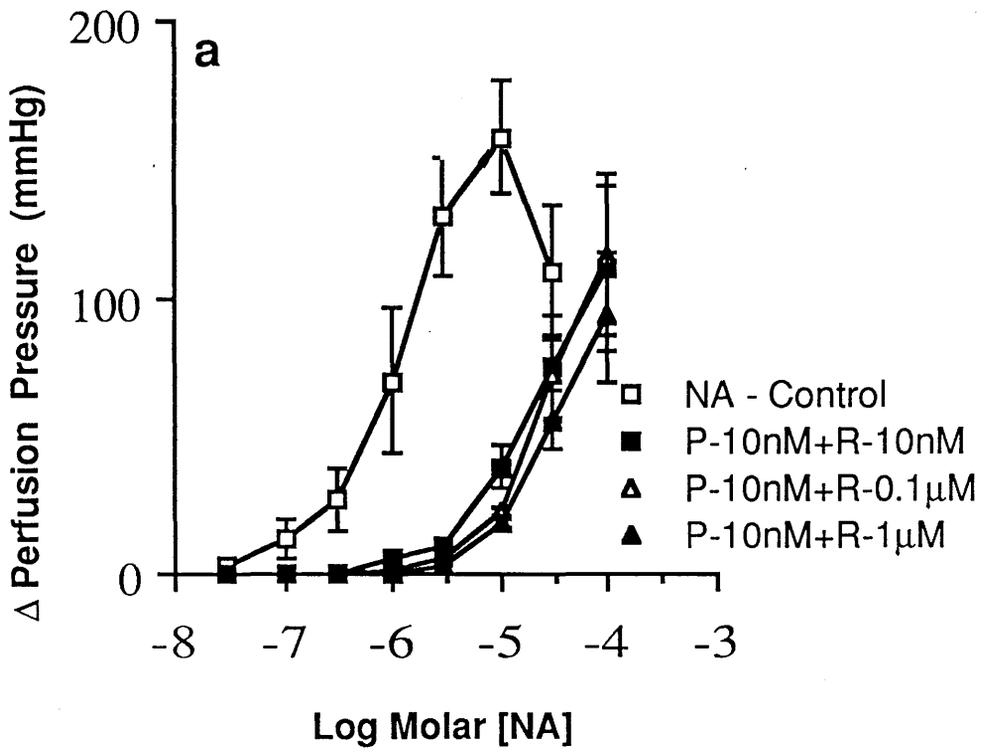


Figure 1.15 a&b. Effects of rauwolscine in the presence of 10nM prazosin on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the (a) proximal and (b) distal segments of the rat tail artery: control ( ) in the presence of increasing concentrations of rauwolscine (10nM, 0.1uM and 1uM). Cocaine (4uM), propranolol (1uM) and EDTA (23uM) were present in the saline. Tissue preparations were pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs at 95%O<sub>2</sub>. NA concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Responses were calculated as % of maximum response of the control. Points shown indicate mean ± s.e.m. (n=10-12). Rauwolscine was virtually ineffective in inhibiting NA-induced responses after the effect of prazosin (10nM) only. (In the proximal segment, the effect of prazosin alone was mistakenly missed out).



gave a regression line with slope of 0.6 and a  $pA_2$  value approximately 11 (Fig. 1. 13c&d). This indicates the presence of a good population of  $\alpha_1$ -adrenoceptors (see Table 4).

In both the proximal and distal segments of the rat tail artery, rauwolscine was virtually ineffective in inhibiting the NA-induced contractions, except at the considerably higher concentration of  $1\mu M$  (Fig. 1. 14a&b). The regression line of the Schild plot for rauwolscine (Fig. 1. 14c&d) had calculated  $pA_2$  values and slopes for the proximal and distal segments as shown in Table 4.  $\alpha_2$ -adrenoceptors were apparently absent in this tissue.

A combination of these two antagonists in the proximal and distal segments produced effects basically similar to those of prazosin alone (Fig. 1. 15 & 16).

When prazosin was kept constant at  $10nM$  and the concentration of rauwolscine was gradually increased from  $10nM$  to  $1\mu M$  (Fig. 1.15), rauwolscine was virtually ineffective. The  $pA_2$  values were however smaller than with prazosin as calculated from the Schild plot (Fig. 1.15c&d) (Table 4).

If on the other hand, the concentration of rauwolscine was kept constant at a fairly high concentration of  $1\mu M$ , and the concentrations of prazosin

Figure 1.15 c&d. Schild plot for the interaction of rauwolscine and NA was constructed for each concentration of rauwolscine. The  $pA_2$  value and slope were obtained by linear regression by using the individual data point for concentration-ratio (CR) (calculated at the  $EC_{50}$ ) and the individual agonist concentrations from each concentration of antagonist. The intercept of the linear regression line with the line,  $\log(CR-1)=0$ , gave an estimate of the  $pA_2$ . The points shown represent the mean  $\pm$  s.e.m. at each concentration and the line is based on the mean  $pA_2$  and mean slope from the group of experiments (see Table 4 for the values of the  $pA_2$  and the slope).

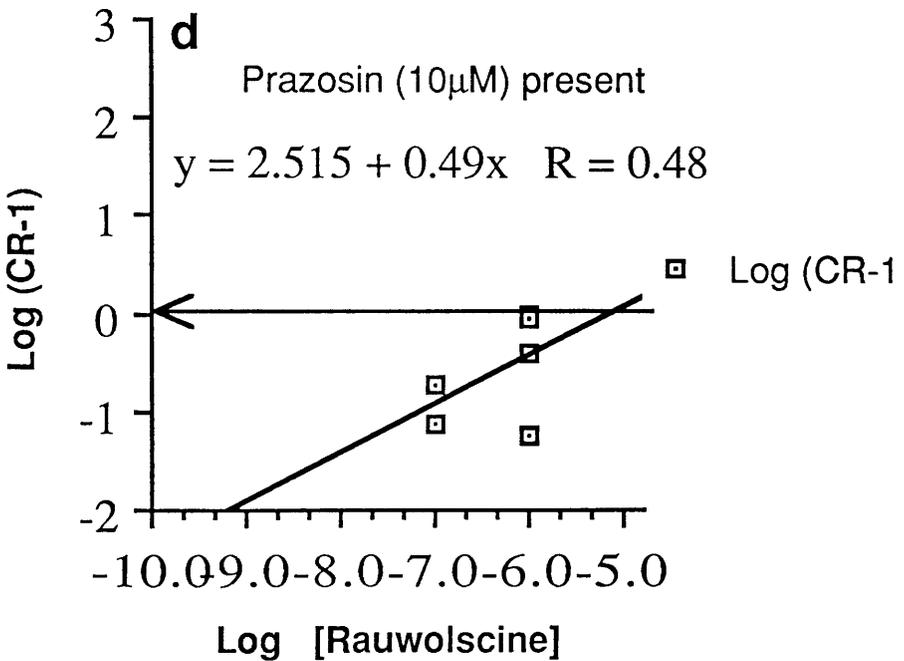
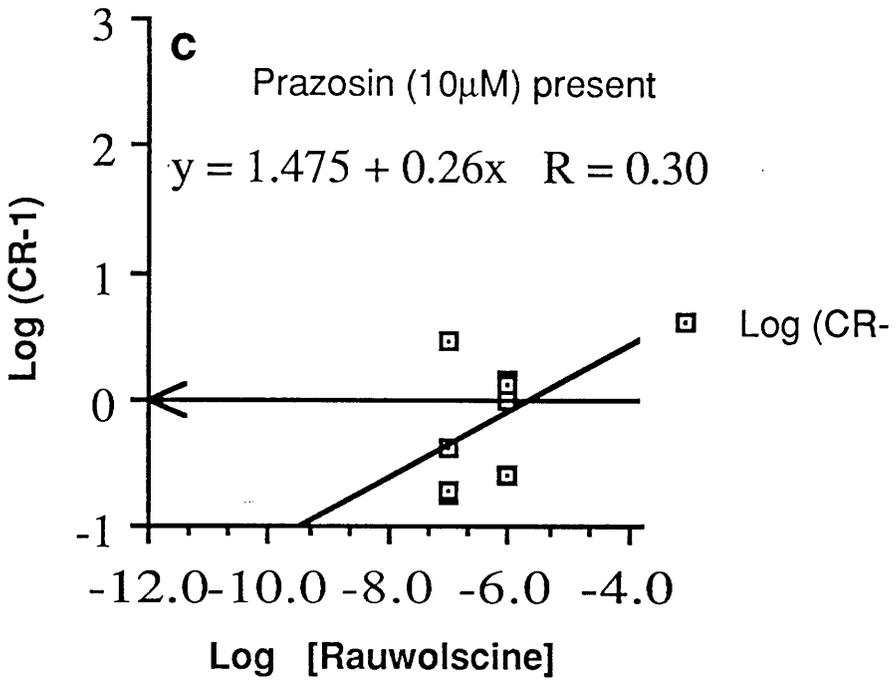


Figure 1.16 a&b. - Effects of prazosin in the presence of rauwolscine (1uM) on consecutive log concentration-response curves (CRC) to noradrenaline (NA) in the (a) proximal and (b) distal segments of the rat tail artery: control ( ) in the presence of increasing concentrations of prazosin (0.1nM, 1nM, 10nM and 0.1u). Cocaine (4uM), propranolol (1uM) and EDTA (23uM) were present in the saline. Tissue preparations were pre-exposed to 3uM NA for 5min before obtaining the non-cumulative CRCs at 95%O<sub>2</sub>. NA concentration range tested was 30nM-30uM NA in 2.5mM [Ca<sup>2+</sup>]. Responses were calculated as % of maximum response of the control. Points shown indicate mean ± s.e.m. (n=10-12). The high concentration of rauwolscine (1uM) alone inhibited the NA-induced responses. Increasing concentrations of prazosin further inhibited these responses.

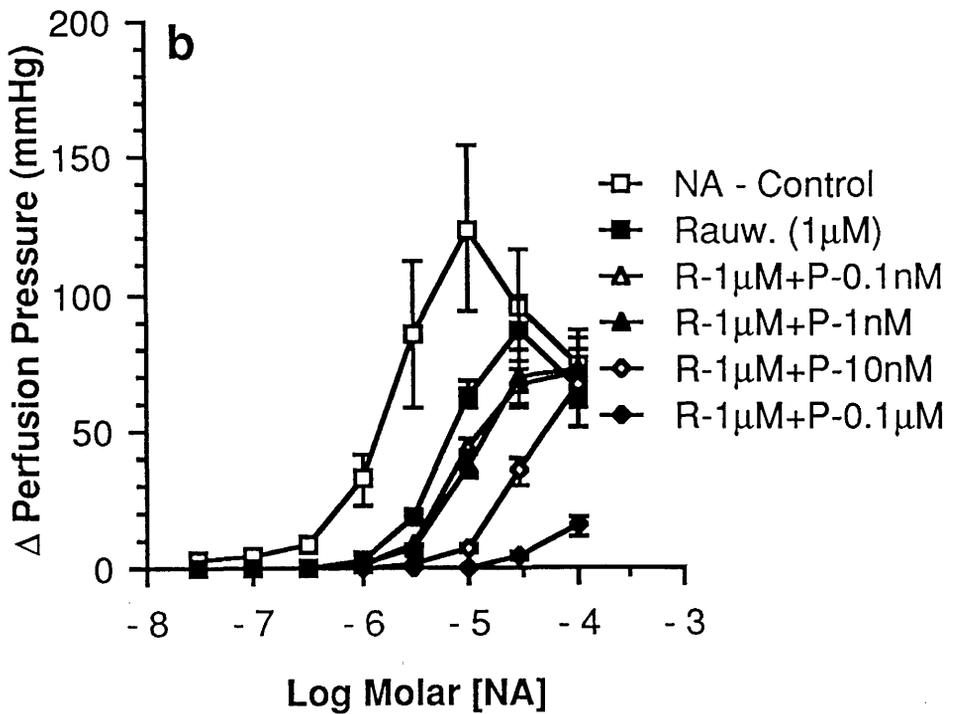
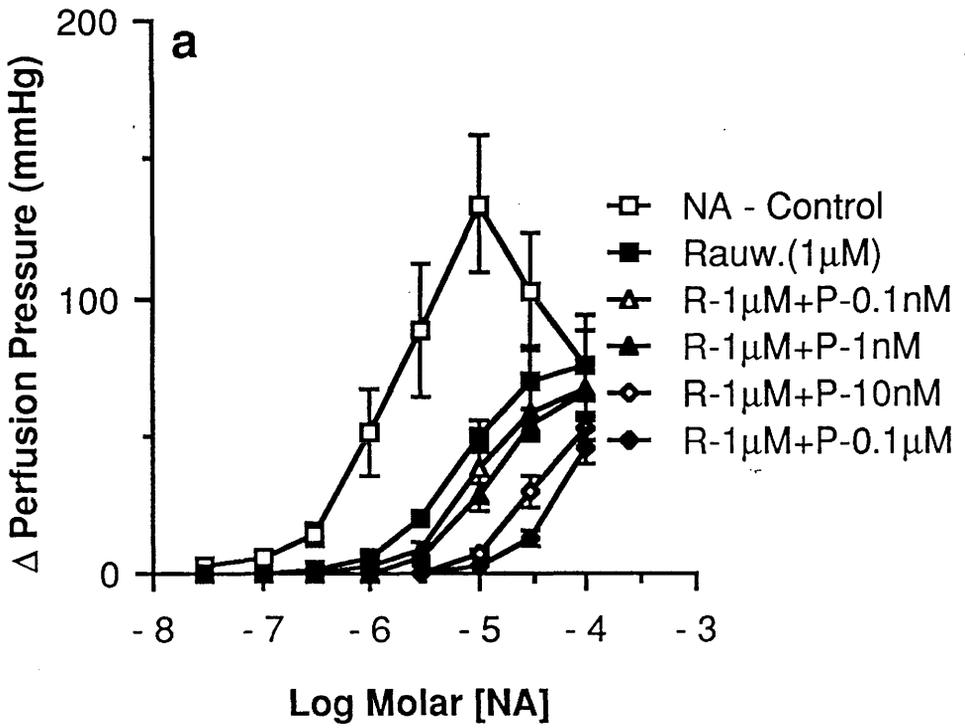
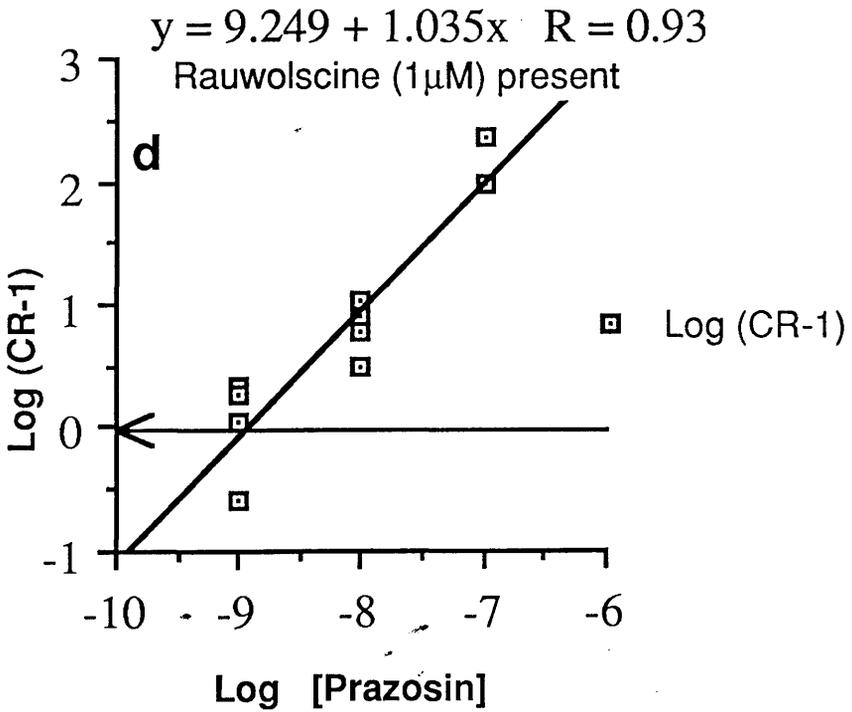
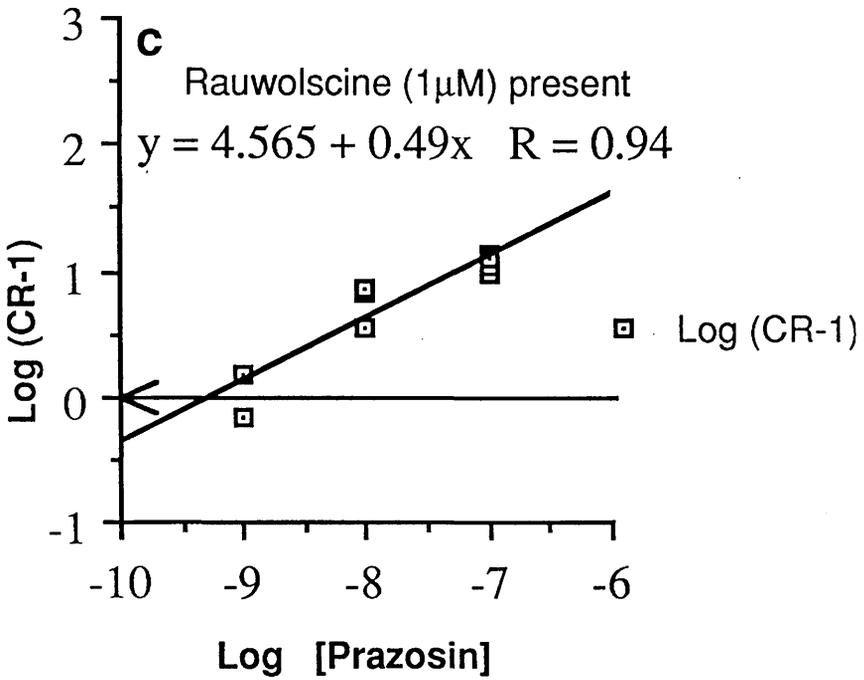


Figure 1.16 c&d. Schild plot for the interaction of prazosin and NA was constructed for each concentration of prazosin. The  $pA_2$  value and slope were obtained by linear regression by using the individual data point for concentration-ratio (CR) (calculated at the  $EC_{50}$ ) and the individual agonist concentrations from each concentration of antagonist. The intercept of the linear regression line with the line,  $\log(CR-1)=0$ , gave an estimate of the  $pA_2$ . The points shown represent the mean  $\pm$  s.e.m. at each concentration and the line is based on the mean  $pA_2$  and mean slope from the group of experiments (see Table 4 for the values of the  $pA_2$  and the slope).



gradually increased from 0.1nM to 0.1uM (Fig. 1.16), then the responses which had already been inhibited by the high concentration of rauwolscine, were further reduced as the concentrations of prazosin increased. These results suggest that the inhibition caused by rauwolscine was probably due to its  $\alpha_1$ -adrenoceptor effects at high concentrations. No antagonism was observed at lower concentrations of rauwolscine. This is consistent with  $\alpha_2$ -adrenoceptors being virtually absent in this tissue. A Schild plot from the responses showed a slope value close to unity.

In each case the degree of antagonism was calculated by expressing the contractile responses in the presence of antagonists as a percentage of the control maximum (i.e. in the absence of the antagonists). Neither of the two antagonists influenced the basal perfusion pressure of the rat tail artery.

Table 4. Parameters of the Schild plot for the interaction of antagonists with the receptors of noradrenaline (alpha-adrenoceptors) in the isolated perfused rat tail artery in 95% O<sub>2</sub> in 2.5mM Ca<sup>2+</sup>.

Antagonist	pA <sub>2</sub>	Slope	n	P/D	Relevant figure
Prazosin	10.41 (10.39-10.41)	0.622 (0.62-0.63)	12	P	13 c
	9.42 (9.41-9.43)	0.586 (0.57-0.59)	10	D	13 d
Rauwol-scine	8.72 (8.70-8.74)	0.41 (0.40-0.42)	12	P	14 c
	6.75 (6.58-6.92)	0.18 (0.17-0.19)	10	D	14 d
Rauwol-scine + (Prazosin)	5.67 (5.57-5.77)	0.26 (0.24-0.28)	10	P	15 c
	5.13 (5.07-5.19)	0.49 (0.47-0.51)	12	D	15 d
Prazosin + (Rauwol-scine)	9.31 (9.29-9.33)	0.49 (0.48-0.50)	12	P	16 c
	8.93 (8.88-8.95)	1.03 (1.02-1.04)	10	D	16 d

Values are the mean (95% confidence limits) of n estimates. n stands for number of observations and P and D represent the proximal and distal segments of the artery, respectively.

## DISCUSSION

Responsiveness to NA was examined in both the proximal and distal portions of the vessel and by applying NA both intraluminally and extraluminally. Both portions showed similar characteristics and the intraluminal administration of NA was in general more potent than the extracellular administration. The inclusion of cocaine in the saline abolished the difference between the two surfaces of entry of NA. This confirmed similar observations made in the rat tail artery by Venning and de la Lande (1984). However, administration to both surfaces simultaneously gave consistent responses which were not greatly different from those to either surface alone. So subsequent experiments used this method for all drug addition since it also simplified establishment of equilibrium not only for agonists but also for test drugs.

Both cocaine (4 $\mu$ M) (an uptake<sub>1</sub> blocker) and propranolol (1 $\mu$ M) (a beta-adrenoceptor blocker) individually and in combination, enhanced NA-induced responses to a small degree. EDTA (23 $\mu$ M) stabilised NA responses by preventing NA oxidation without causing undue chelation of free calcium in the saline. The responses were little influenced by pH. This was the case whether we changed the extracellular pH by varying sodium bicarbonate (NaHCO<sub>3</sub>) or by means of gassing the saline with a varying percentage of carbon

dioxide (CO<sub>2</sub>).

The tissue's responses were fairly typical of the basic responses obtained with a variety of other vascular resistance vessels when they are activated by agonists with regard to NA's potency and time course of contraction. On the other hand, responses to NA of the tail artery were little influenced by modification of intracellular or extracellular pH and variations of partial pressures of oxygen and carbon dioxide in the "normal" range. This is, perhaps, not surprising in a vessel serving temperature control rather than metabolic demand.

The pattern of interactions of the tail artery with various agonists and antagonists suggests that the  $\alpha$ -adrenoceptor population consists mainly of the  $\alpha_1$ -subtype. This was demonstrated with the selective  $\alpha_1$ -adrenoceptor antagonist, prazosin, and the selective  $\alpha_2$ -adrenoceptor antagonist, rauwolscine. In both the proximal and distal portions of the tissue, prazosin effectively blocked the responses even at a very low concentration of 1.0nM, whereas rauwolscine showed some blockade only at a high concentration of 1 $\mu$ M and above (probably due to its  $\alpha_1$  antagonist properties). There was no evidence of the small  $\alpha_2$ -adrenoceptor population in the distal portion suggested by Medgett and Langer (1984, 1986).

Medgett & Langer (1984, 1986) reported that alpha-1 adrenoceptors are the main sub-type found in the proximal segment. A mixture of alpha-1 and alpha-2 adrenoceptor sub-types exists in the distal segment of the normotensive Sprague-Dawley rat tail artery (Medgett, 1985) and in spontaneously hypertensive (SHR) rat tail arteries (Medgett & Langer, 1984; Hicks et al. 1984). However, Medgett and Langer (1984) expressed doubts as to whether alpha-2 adrenoceptor sub-type exists in the Wistar strain of rats, which was the strain of rats we used in the present study. We have no evidence of the existence even of a very small population of alpha<sub>2</sub>-adrenoceptors which might have explained the slight differences in the sensitivities of the two segments to NA.

Little attention has been paid to the difference between normoxia, e.g. 16% oxygen, and hyperoxia, e.g. greater than 90% oxygen; or between normoxia and mild hypoxia, e.g. 4% oxygen (approx. 30mmHg). McGrath, (1982) had shown that reduction in oxygen tension from the supramaximal level 95% to the physiological level of 16%, attenuated the alpha-adrenoceptor mediated noradrenaline-induced contractions in the rat anococcygeus smooth muscle. The time courses of contractions in the two oxygen tensions were also found to be different. In the light of this observation, we decided to examine the effects of lowering the oxygen tensions on our tissue preparation. For the first set of the experiments, we used three different oxygen tensions of 95%, 92% & 16% with a constant carbon dioxide level of 5%, and the rest made up from nitrogen.

92%O<sub>2</sub> was chosen so that in future experiments where changes in the carbon dioxide tension would be carried out this could be varied up to 8%.

We observed that there were reductions in the

responses of the tail artery to noradrenaline throughout the dose-response curve as the oxygen tension was lowered from 95% to 16%. Both the curve in general and the EC<sub>50</sub> values were shifted to the right going from 95%O<sub>2</sub> to 16%. The maximum response was also reduced as the oxygen tension dropped from 95% to 16%. This situation was common to both the proximal and distal segments of artery. On the whole, when gassing was started with 95%O<sub>2</sub> rather than 16%, then the dose-response curve in 16%O<sub>2</sub> was regularly shifted to the right when compared with that of 95%O<sub>2</sub> in noradrenaline-induced contractions and this was true whether it was the proximal or the distal segment of the isolated perfused rat tail artery that was being considered.

In the comparisons between the 95% and 16% oxygen tensions, we also observed differences in their time courses. The choice of 95%O<sub>2</sub> in favour of 16%O<sub>2</sub> was originally with the objective of avoiding a hypoxic core within the tissue since it is assumed that a high PO<sub>2</sub> will do no harm. Our own observations showed that the size of the maximum contraction at equilibrium was smaller at 16% than at 95% in each preparation. The most marked effect was a change in the time course of contraction.

The general pattern was similar. At 16%, contraction was biphasic with most NA concentrations: an early rapid but transient component was followed by a slower,

maintained component, normally of smaller size. At 95%, the response was more monotonic. There were two elements to this:

(i) A diminution in the rate of rise of the first component. In anococcygeus attenuation of the rate of rise was clear (McGrath, 1982) and this was true to a lesser extent, in the tail artery, In portal vein, in which the initial rise is very rapid and spontaneous activity is present, this could not be discerned (Fasehun, Jennett, McGrath and Ugwu, 1985; Fasehun, Jennett and McGrath, 1986).

(ii) An increase in the height of the second component, which masked the first phase. This occurred in all preparations.

On the basis of these observations, there are parallels with responses obtained in vivo in the pithed rat: with ventilation on oxygen, alpha-1-mediated pressor responses were enhanced compared with those under normoxia at constant pH & pCO<sub>2</sub> (Grant, et al, 1984). It is concluded that exposure to high pO<sub>2</sub> :  
attenuates the initial rise in tension to NA, and  
increases the maximal maintained contraction.

This suggest that high pO<sub>2</sub> alters the response beyond the receptor and thus may provide false information on excitation-contraction coupling. With thin preparations, it is suggested that physiological pO<sub>2</sub> is appropriate

(Fasehun, Jennett, McGrath & Ugwu, 1985).

These observations were similar in both the proximal and distal segments of the rat tail artery.

After assessing differences in responsiveness to NA we settled for method (b) in subsequent studies since it is simpler to perform and keeps the tissues viable for longer experiments. Partial drying out produced problems with method (a).

Neuronal uptake of noradrenaline into the sympathetic nerve endings is known to be blocked quite effectively by such drugs as cocaine, desipramine and other related drugs (Iversen, 1960). Many workers have employed cocaine in their studies and have reported that cocaine blocked the uptake mechanism in their preparations (e.g. McGrath et al, 1982; Medgett & Langer, 1984). Cocaine has advantages over the rest of these neuronal uptake blockers including its specificity of action on the neuronal uptake mechanisms. Desipramine is known, for example, to have other actions which can interfere with its neuronal uptake blocking action including blockade  $\alpha$ -adrenoceptors (Bowman and Rand, 1980). We thus decided to use cocaine (4 $\mu$ M) routinely to block neuronal uptake of NA. In our experiments, cocaine (4 $\mu$ M) potentiated the noradrenaline-induced contractions of both the proximal and distal segments of the rat tail artery. In a few preliminary experiments desipramine (4 $\mu$ M) showed similar results as cocaine confirming the mechanism of cocaine's action.

Beta-adrenoceptor activity in this tissue with NA as agonist has been reported and is blocked by propranolol (Medgett & Langer, 1984). Since the effect of beta-adrenoceptor activity in the vascular smooth muscle is

muscular relaxation, which will interfere with the contraction mediated by alpha adrenoceptors, we decided to block any beta-adrenoceptor activity with propranolol ( 1uM ). Our results showed that propranolol slightly enhanced the rat tail responses to noradrenaline-induced contractions in some tissues and thus justifying its use.

There was a tendency for a combination of both cocaine and propranolol to enhance the responses of the tissue to noradrenaline. Thus both drugs were routinely employed in NA-induced contractions.

EDTA prevents the oxidation of noradrenaline in solution by chelating the heavy metal ions which catalyse the reaction and so, workers like McGrath (1978-1986) and Medgett & Langer (1983- 1985), have consistently employed it in their studies involving noradrenaline in solution. Because most of our experiments would be carried at the physiological mammalian temperature of 37°C which is close to the optimum oxidation temperature of 42°C for noradrenaline, we tested the effect of EDTA on the responses of our tissue to noradrenaline. Our results showed that in the presence of EDTA the contractile responses of the isolated perfused rat tail artery to low concentrations of noradrenaline were increased. Our preliminary tests showed that a suitable concentration of EDTA for allowing maintained responses to NA

without significantly interfering with free calcium levels in solution is 23uM. This is the same concentration of EDTA regularly employed by McGrath and co-workers in other preparations, the odd choice of 23uM arising historically from a g/litre quantity. In all our studies involving noradrenaline 23uM EDTA was used.

In order to justify our methods and to compare them with those employed previously, we started by making comparisons between the "classical" (non-physiological) conditions and what we consider to be more "physiological". In this justification of the methods we have also experimented with different methods for the analysis of the responses. This method of approach later proved advantageous especially with our later discovery of the highly marked desensitisation to calcium sensitivity of the responses of the rat tail artery.

In conclusion, we have succeeded in establishing the conditions for the optimisation of responses of the isolated perfused rat tail artery for reasonable studies of the sensitivity of the tissue to calcium and oxygen. These include the need to employ cocaine, propranolol and EDTA routinely in all experiments involving NA responses; the establishment of the insignificant effects of surface of administration of NA in presence of cocaine, pH of the saline, and the normoxic and

hyperoxic conditions of oxygen tensions; and finally the antagonism of  $\alpha_1$ -adrenoceptor mediated contractions by prazosin but not rauwolscine. All these apply equally to both the proximal and the distal segments of the rat tail artery. The responses of the tissue to calcium and oxygen in relation to the factors were examined in the next section.

## SECTION II

### CALCIUM-SENSITIVITY OF SMOOTH MUSCLE CONTRACTION IN THE ISOLATED PERFUSED RAT TAIL ARTERY USING CALCIUM BUFFERS - SOME ASPECTS OF DESENSITISATION AND THE EFFECTS OF BAY K 8644 AND NIFEDIPINE

#### SUMMARY

1. Desensitisation and the effects of Bay K 8644 and nifedipine on the calcium-sensitivity of smooth muscle contraction were studied in the isolated perfused rat tail artery, employing the activators noradrenaline (NA) (3 $\mu$ M) and potassium chloride (KCl) (100mM). Experiments were conducted in Ca<sup>2+</sup>-buffered saline. Activators were added when [Ca<sup>2+</sup>]<sub>free</sub> was low (1 $\mu$ M) and then [Ca<sup>2+</sup>]<sub>free</sub> was increased stepwise to give a Ca<sup>2+</sup>-concentration/response curve (CRC).
2. There was a progressive rightward shift of the CRCs with time when a series of curves was constructed. Desensitisation was not a function of time elapsed before first testing. The higher the calcium concentration to which the tissue was exposed during activation, the greater was desensitisation. The progressive loss in sensitivity could be attenuated by restricting the range of free calcium used for CRCs to between 1 $\mu$ M

and 300uM  $\text{Ca}^{2+}$ . Desensitisation could be reduced also by increasing the interval between CRCs. Results were similar whether activation was by NA or high KCl.

3. When the tissues were pre-exposed to NA (3uM) ("priming") before constructing CRCs, desensitisation was produced more quickly and thus sensitivity became more "stable". However, the  $[\text{Ca}^{2+}]$  during priming and the maximum  $[\text{Ca}^{2+}]$  in a CRC determined the stable level, high  $[\text{Ca}^{2+}]$  reducing sensitivity. Priming and maximum at 300uM  $\text{Ca}^{2+}$  was optimal for avoiding progressive desensitisation.

4. Thus the rat tail artery exhibits higher sensitivity to  $\text{Ca}^{2+}$  on initial contact with activators. The results suggest that desensitisation at some stage in excitation-contraction coupling, possibly by  $\text{Ca}^{2+}$  overload, occurs when high extracellular  $[\text{Ca}^{2+}]$  (2.5 or 5mM) is present during activation by NA. This can be prevented by avoiding high  $[\text{Ca}^{2+}]$  or through allowing recovery by leaving longer intervals between CRCs, thus allowing prolonged reproducibility of high sensitivity to  $\text{Ca}^{2+}$  which, otherwise, is lost.

5. Bay K 8644 increased the sensitivity to  $\text{Ca}^{2+}$  but

did not alter the rate of desensitisation. Desensitisation complicated demonstration of potentiation by Bay K 8644 in the same tissue. However, potentiation between consecutive CRCs could be shown after the 3rd or 4th control. This was the case for responses activated by either NA or KCl.

6. Nifedipine decreased the sensitivity to  $Ca^{2+}$  at the first CRC but thereafter CRC's were not significantly different from their controls until the 6th test in which sensitivity was actually higher. Inhibition could be seen between consecutive curves when nifedipine was given after drug-free control responses, and this reduced sensitivity below that produced by the normal course of desensitisation.

## INTRODUCTION

The progressive fall in responsiveness of vascular smooth muscle, such as in the rat tail artery, with time or with consecutive periods of activation by agonists, has apparently been recognized by many workers (Medgett and Langer, 1984, 1986; Spedding, 1985; Su et al., 1984). However, lack of information on the possible causes of the initial decline between the first and the subsequent responses, has led to the neglect of the first

concentration - response curve; the second or even the third being taken as the control curve for the analysis of test drugs by these workers (see Su et al., 1984; Medgett and Langer, 1984,1986; Aoki & Asano, 1986).

The reason for discarding the first curve is that a significant difference usually exists between the first and the subsequent curves, thereby creating problems for analysis of the data. The basis of this difference is unclear and it is as reasonable to assume that the initial state is as likely to reflect the physiological properties of the tissue as does the subsequent desensitised condition: both cannot. We have therefore made a study of the initial and subsequent sensitivity of the smooth muscle of rat tail artery with particular reference to the influence of extracellular  $[Ca^{2+}]_{free}$ . Strategies for avoiding desensitisation are examined and the effects are studied of drugs influencing  $Ca^{2+}$  channel function.

## METHODS

### Experimental protocol

The materials and methods used in this section of the work were the same as detailed under the General Methods section. Thus the tissue was prepared for recording perfusion pressure with constant flow. The drugs and chemicals used along with the method of statistical analysis were also the same as in the section on General Methods. The detailed experimental protocol for this section is given below.

In a series of experiments illustrating the decline in sensitivity to NA illustrated by NA concentration-response curve, EGTA and NTA were included (2.5mM each) so that any toxic effect would be constant throughout, and total  $\text{CaCl}_2$  was adjusted to keep  $[\text{Ca}^{2+}]_{\text{free}}$  at 2.5mM.

In all the experiments involving NA-induced contractions, cocaine (4uM), propranolol (1uM) and EDTA (23uM) were always present.

## Drugs and chemicals

The following substances were used:- Bay K 8644, nifedipine (both from Bayer), (-)-noradrenaline bitartrate salt (Sigma), E.D.T.A. (B.D.H.), E.G.T.A.(ethylene glycol bis-{B-aminoethyl ether} N,N,N'-tetraacetic acid)(Sigma), N.T.A.(nitrilotriacetic acid, i.e., N,N-bis {carboxymethyl}glycine, free acid)(Sigma), Cocaine HCl (McCarthy's), DL-Propranolol HCl (I.C.I.).

Stock solutions of Bay K 8644 and nifedipine were dissolved in alcohol then diluted with water (one part of alcohol in nine parts of distilled water) and shielded from direct light because of their photosensitivity. All other drugs were dissolved in distilled water, v/v and diluted in the appropriate physiological saline.

Statistics

Results were expressed as the mean  $\pm$  S.E.M. Statistical analysis was performed using Student's t test for paired or unpaired data, as appropriate and the 0.05 level of probability was regarded as significant.

## RESULTS.

### A. Responses to NA in varying $[Ca^{2+}]$ and responses to $Ca^{2+}$ in varying [NA]

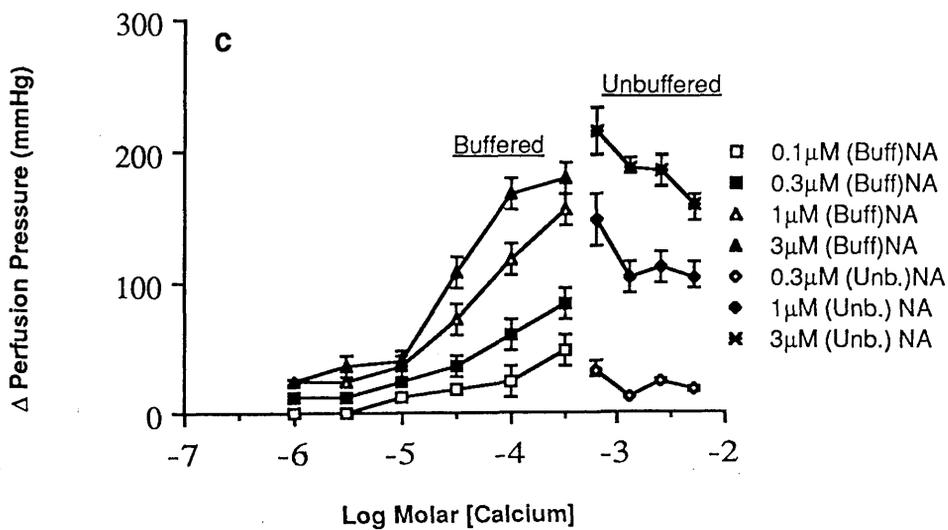
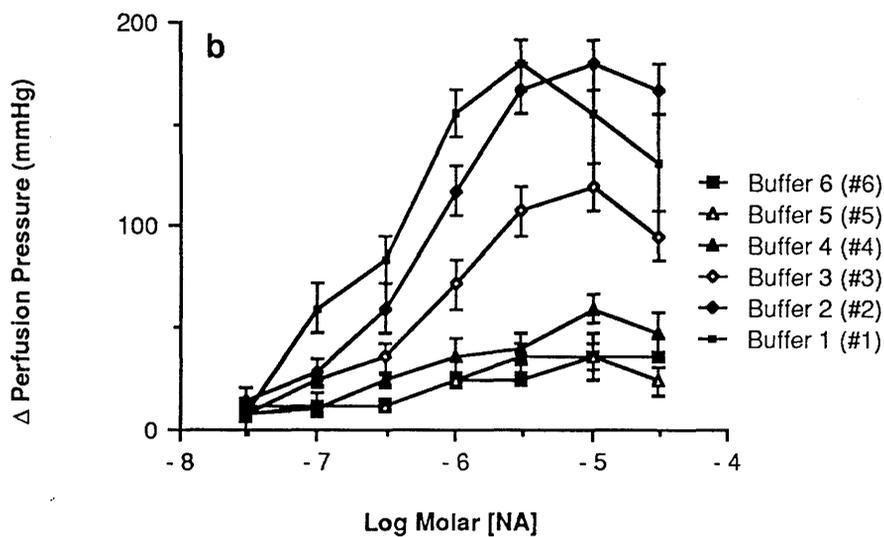
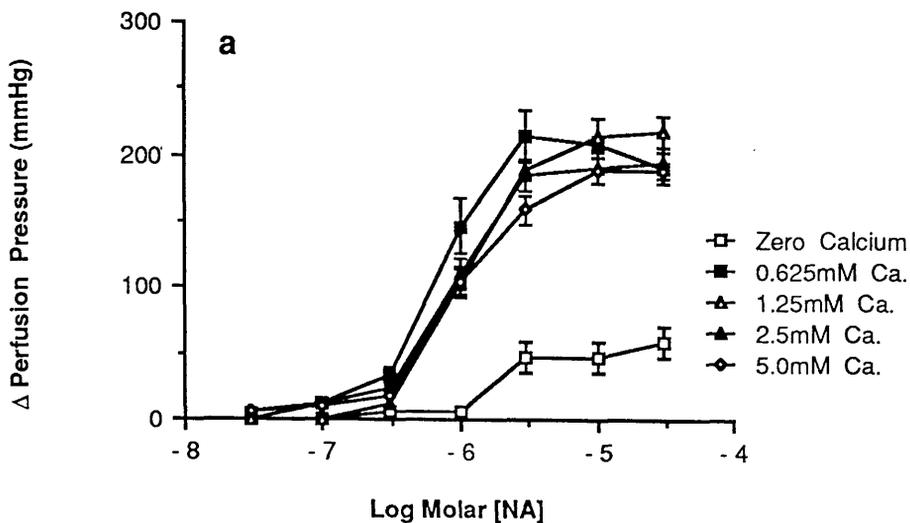
In calcium-unbuffered saline, NA (3 $\mu$ M) induced small but maintained contractions in "nominally" zero  $[Ca^{2+}]$ . Increasing the  $[Ca^{2+}]$  led to increased responses throughout the NA CRC but it was difficult to distinguish between the responses over the range of  $[Ca^{2+}]$  tested, i.e. from 0.625 to 5.0mM  $Ca^{2+}$  (Fig. 2. 1a).

In the calcium-buffered saline, in which a range of lower concentrations of  $[Ca^{2+}]$  could be employed, responses to NA increased as the free external calcium in the saline ( $[Ca^{2+}]_{free}$ ) was increased from 1 $\mu$ M (buffer 6) to 0.3mM (buffer 1) (Fig. 2. 1b) (see Table 1 for the concentrations of free calcium concentrations in the buffers).

Similarly, when Ca CRCs were constructed in increasing NA concentrations, (0.1, 1.0 and 3 $\mu$ M), the responses increased with increasing  $[Ca^{2+}]$  (Fig. 2. 1c). The responses in NA 3 $\mu$ M and 1 $\mu$ M did, however, deviate from the rule. Although, sensitivity to calcium and maximum response were greater than in the lower [NA] the maximum was not maintained after 0.3mM even though we tested up to 5mM  $Ca^{2+}$ .

Figure 2. 1. Influence of  $[Ca^{2+}]$  on NA CRC. Consecutive log concentration-response curves to NA were obtained at different calcium concentrations in (a) unbuffered calcium and (b) buffered calcium saline in 95%O<sub>2</sub>. The vessel was exposed to 3uM NA before constructing the non-cumulative NA CRCs beginning in each case with the lowest calcium concentration and then with increasing concentrations of calcium up to 5.0mM. In the unbuffered saline (a), in the nominally "zero Ca<sup>2+</sup>", were significantly lower than in the other Ca<sup>2+</sup> concentrations but not different from each other between the concentrations. In the buffered saline, increasing the free calcium concentration in the saline (from buffer 6 to 1) increased the responses and the tissue sensitivity to calcium. In each case, the vessel contracted in nominally "zero calcium". Asterisks indicate that CCRC responses in zero Ca<sup>2+</sup> are significantly smaller than curves with higher  $[Ca^{2+}]$  ( $p < 0.05$ ).

Activating the tissue with increasing NA concentrations (0.1, 0.3, 1, and 3uM) increased responses of the CCRCs in buffered saline but decreased them in the unbuffered saline (c). (d) This shows the plot of  $pD_2$  for NA versus ( $[Ca^{2+}]$ ) for (a) and (b) together.



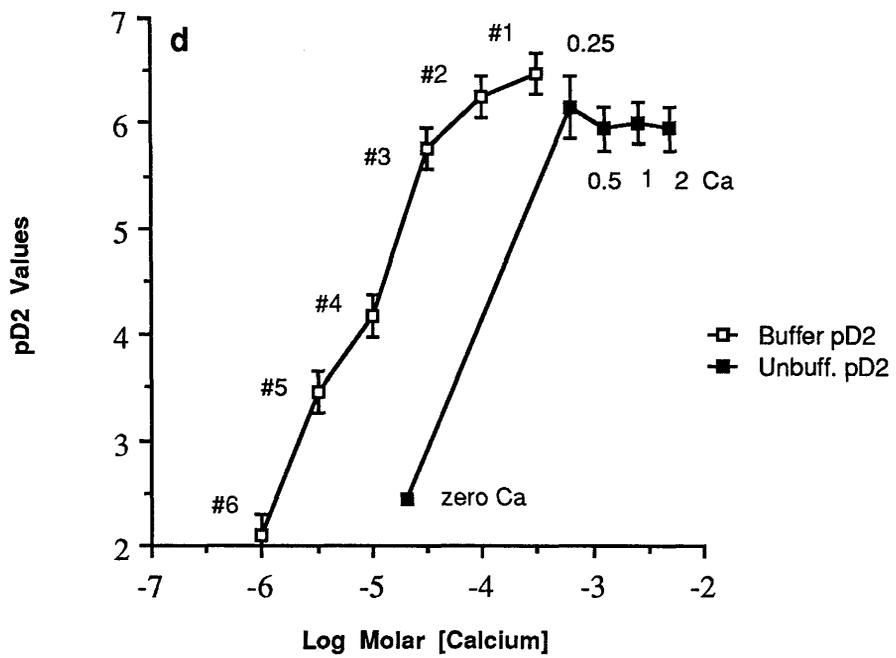
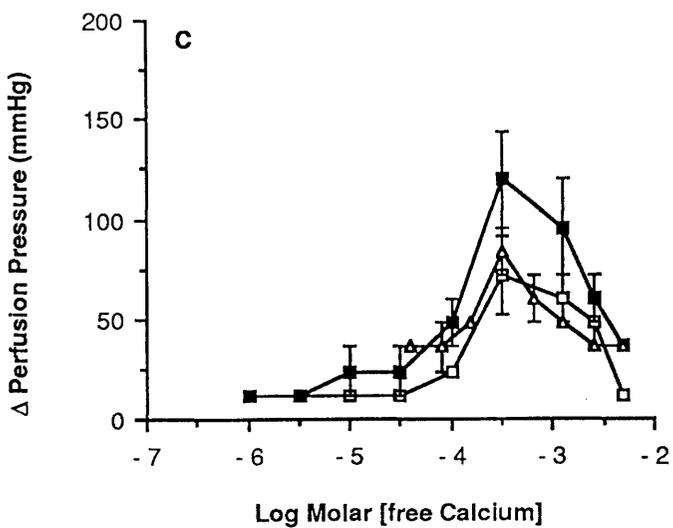
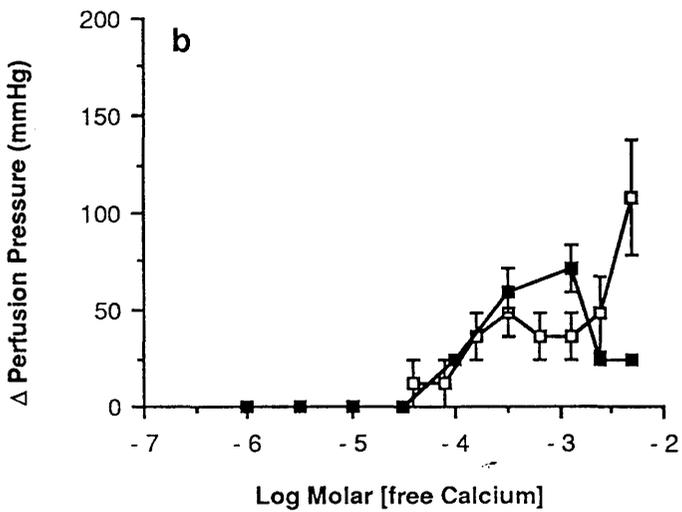
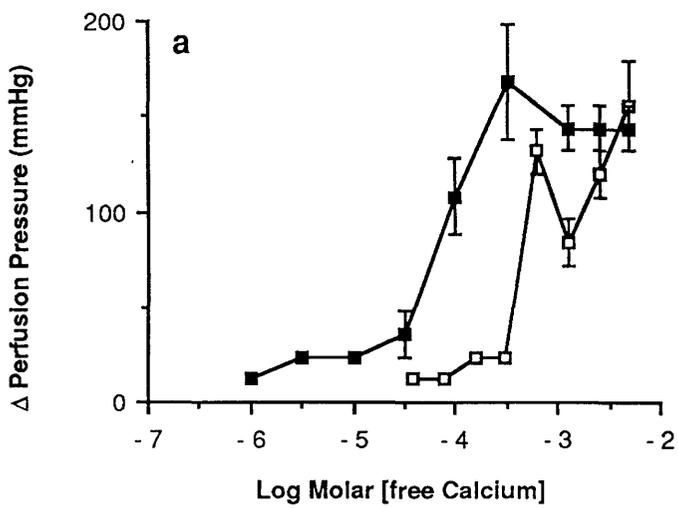


fig. 2.1d

Figure 2. 2. Comparison of buffered and unbuffered Ca CRC's in the same tissue. Consecutive log concentration-response curves to external Ca in the isolated perfused rat tail artery at different NA concentrations in unbuffered (□) and buffered (■) calcium saline in 95%O<sub>2</sub> using (a) 3uM NA, (b) 0.3uM NA and (c) 1uM NA. The vessel was exposed to 3uM NA before constructing the non-cumulative CCRCs. In the unbuffered saline, the responses to increasing calcium concentrations produced less of a regular pattern than with the buffered saline. Activation of the vessel with 1uM NA for Ca CRC in buffered saline and then switching to the unbuffered saline for the subsequent Ca CRC tended to alter its level of response to calcium (c).



The calcium-sensitivity of the rat tail artery in calcium-buffered saline in unpaired tissues was not significantly higher than that in the calcium-unbuffered saline when the tissues were activated in each case by the same concentration of NA (Fig. 2. 2a&b). Switching the tissue from buffered saline to unbuffered saline or vice versa in paired tissues (Fig. 2. 2c), reduced responses in the calcium-unbuffered saline and this could be due to desensitisation of the responses.

B. Responses to NA in  $[Ca^{2+}]_{free} = 2.5mM$

Even at the lowest NA concentrations ( $[NA] = 0.10$  to  $30nM$ ) tested to activate the rat tail artery in  $[Ca^{2+}]_{free} = 2.5mM$ , there was always an initial "supersensitive" response which was not consistently reproducible: increasing the  $[NA]$  produced lower responses until the usual sigmoid shaped NA concentration-response curve was reached (Fig. 2. 3a). This phenomenon may be related to the initial "supersensitive" response in low  $[Ca^{2+}]_{free}$  when the tissue is activated by NA (Fig. 2. 4a). When 3 consecutive cumulative CRCs to NA were constructed with 15min gaps, they moved progressively to the right and the maximum decreased. The 1st curve was bell-shaped but subsequent curves became more sigmoid, losing the top of the bell (Fig. 2. 3a).

Figure 2. 3. (a) Three consecutive cumulative concentration-response curves to NA in the isolated perfused rat tail artery in  $2.5\text{mM Ca}^{2+}$  in calcium buffered saline in  $95\%O_2$ . The curves moved rightwards and the maximum was reduced as the series progressed.

(b) When the tissue was exposed for sustained period to NA in  $2.5\text{mM Ca}^{2+}$  for 30-45min with 15min recovery periods, it was clear that by the 3rd exposure the responses had diminished but thereafter remained constant. When this pattern was compared with responses in  $2.5\text{mM Ca}^{2+}$  obtained in CCRCs (see Fig. 2. 4), the trend was about the same. They had been "stabilised" by the "priming" effect of the early activation periods. This is also illustrated in the histograms with the mean of  $140 \pm 20\text{mmHg}$  for the 1st maximum (c).

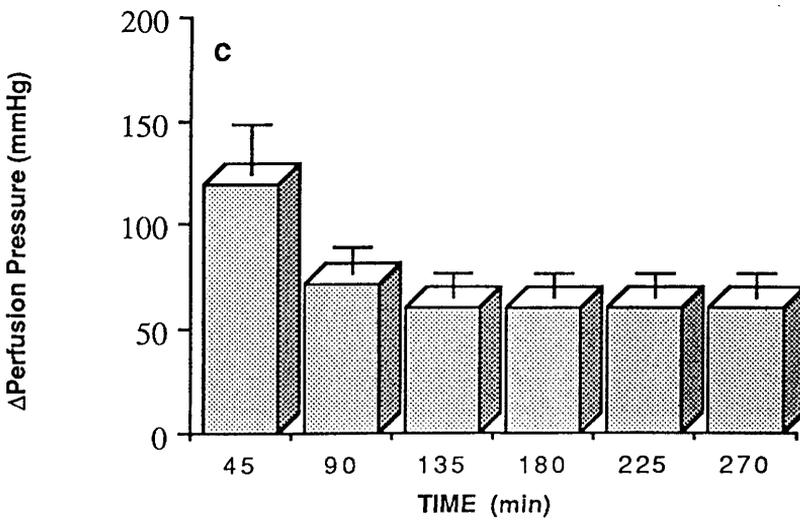
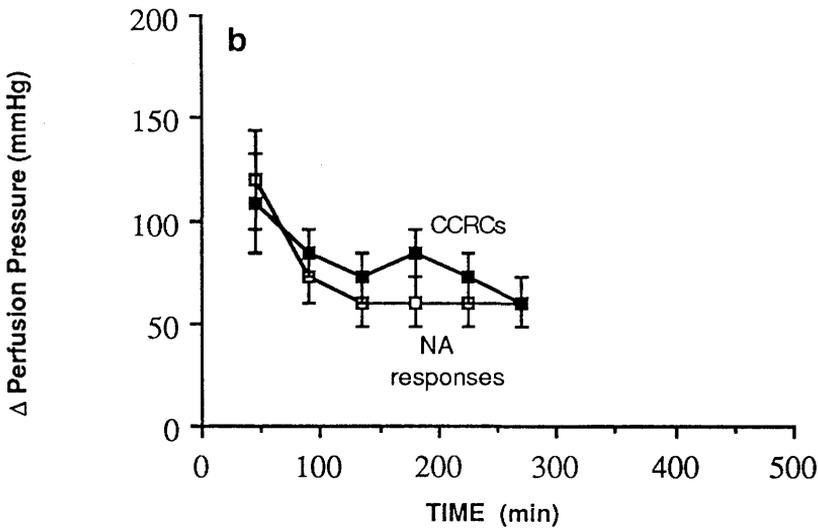
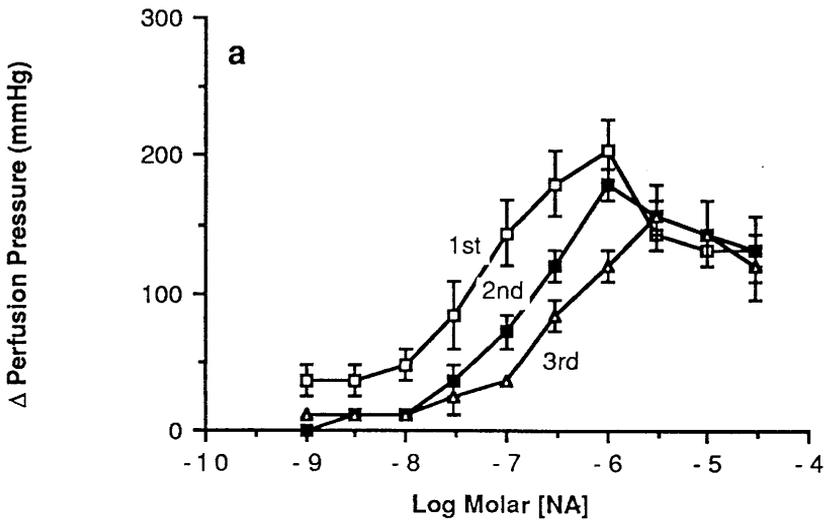
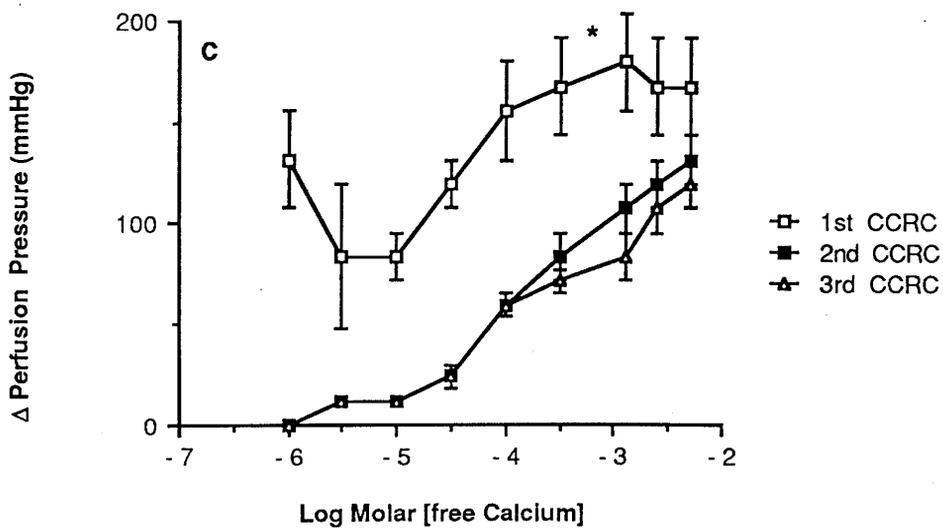
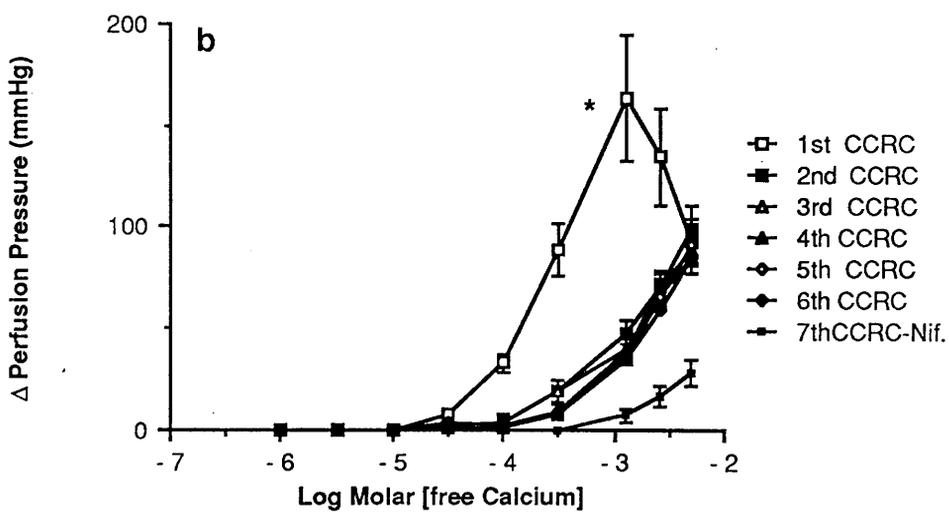
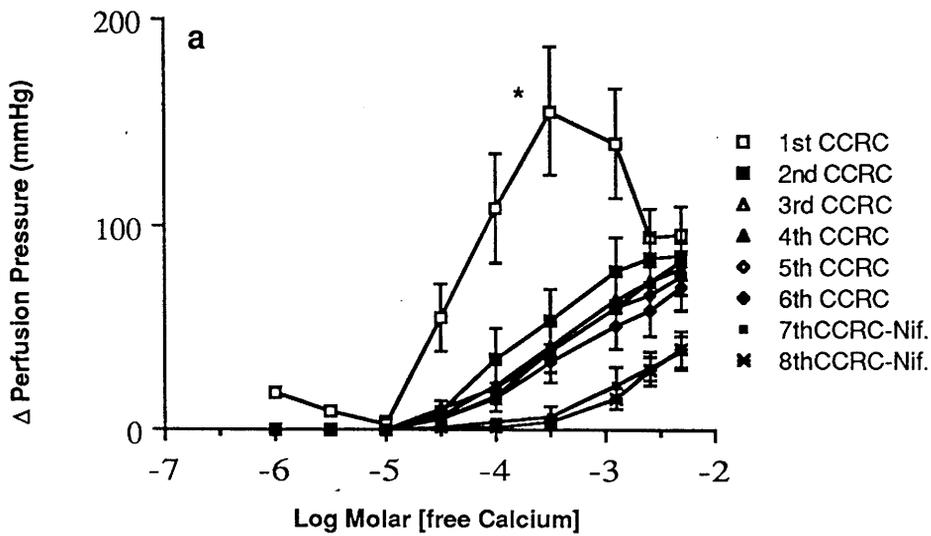


Figure 2. 4. Ca sensitivity of responses to NA and KCl. (a) Eight consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA-induced (3 $\mu\text{M}$ ) contractions in the isolated perfused rat tail artery in calcium buffered saline in 95% $\text{O}_2$ , the last two (7th & 8th) in the presence of nifedipine (0.1 $\mu\text{M}$ ).

(b) Seven consecutive cumulative  $\text{Ca}^{2+}$  CRCs for KCl (100mM)-induced contractions in the isolated perfused rat tail artery in calcium buffered saline in 95% $\text{O}_2$ , the last one in the presence of nifedipine.

(c) Three consecutive cumulative  $\text{Ca}^{2+}$  CRCs for 5-HT (3 $\mu\text{M}$ )-induced contractions in the isolated perfused rat tail artery in calcium buffered saline in 95% $\text{O}_2$ ..

Pressor responses in mmHg were plotted against concentration of free calcium. For NA, KCl and 5-HT-induced contractions, the 1st curve showed high sensitivity to  $\text{Ca}^{2+}$  with the maximum at the sub-physiological  $[\text{Ca}^{2+}]$  of 0.3mM for NA and 1mM for KCl and 5-HT. The subsequent curves moved rightwards and the sensitivity and maximum response steadily declined with with time. Points shown represent mean  $\pm$  s.e.m. Apart from the responses at the highest  $[\text{Ca}^{2+}]$ , the subsequent CCRCs were significantly lower their corresponding controls ( $0.01 < p < 0.05$ ).



Since sensitivity to  $[Ca^{2+}]$  was to be assessed (in subsequent experiments) by cumulative CRCs lasting 45min each, a total of six consecutive 45min long exposures to NA 3uM were produced in 2.5mM  $Ca^{2+}$ . Responses declined after the 1st and stabilised at a lower level after the 3rd (Fig. 2. 3b). When this was compared with NA responses at 2.5mM  $[Ca^{2+}]$  in Ca CRCs (superimposed NA responses in Fig. 2. 3b), the magnitudes of the pressor responses were similar. Having established that responses show desensitisation at a constant high level, the progress of  $Ca^{2+}$  sensitivity was then determined.

C.  $Ca^{2+}$  sensitivity of contractile responses to NA and  $K^+$

Arteries were exposed to a concentration of 3uM NA (Fig. 2. 4a) or 100mM  $K^+$  (Fig. 2. 4b) in the presence of a low concentration of free calcium ions in solution ( $[Ca^{2+}]_{free} = 1uM$ ; buffer 6). Cumulative increases of  $[Ca^{2+}]_{free}$  up to 5.0mM (twice the calcium concentration commonly employed), elicited concentration-dependent contractions. Eight such  $Ca^{2+}$  concentration response curves (CCRC) were obtained at 45 minutes intervals. In such a series, the sensitivity of the tissues to  $[Ca^{2+}]_{free}$  steadily declined. A similar observation was made with 3uM 5-HT as for 3uM NA, with sensitivity declining with subsequent curves (Fig. 2. 4c).

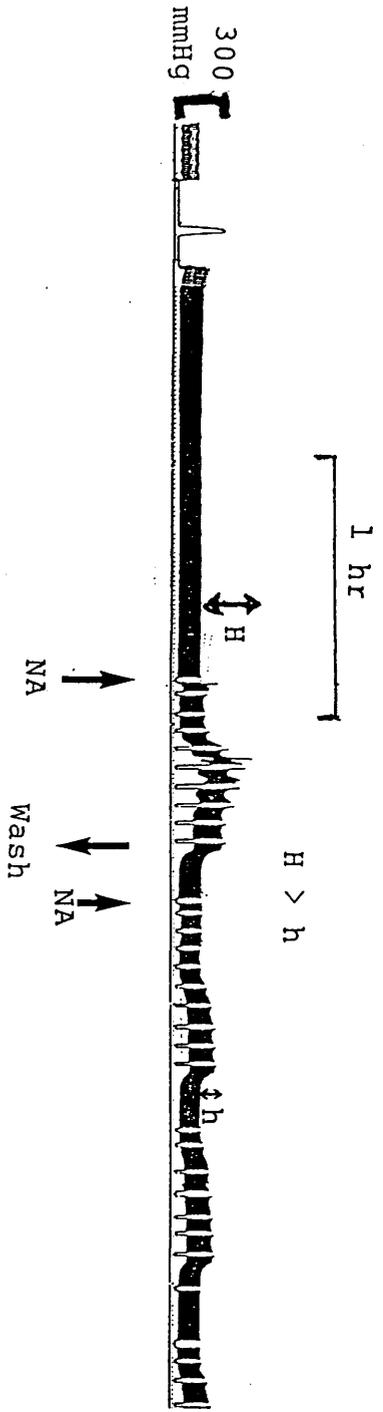


Figure 2.4 a-a. This original tracing shows the 1st and the 2nd (and 3rd) concentration response curves (CRCs) to calcium in the rat tail artery in 95%O<sub>2</sub>. NA was present during the cumulative addition of calcium. Desensitisation of the responses occurred after the CRC.

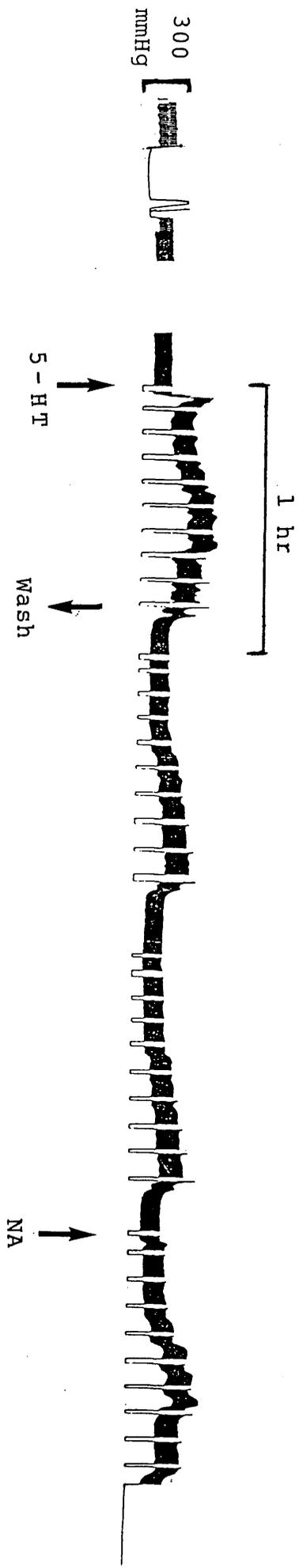


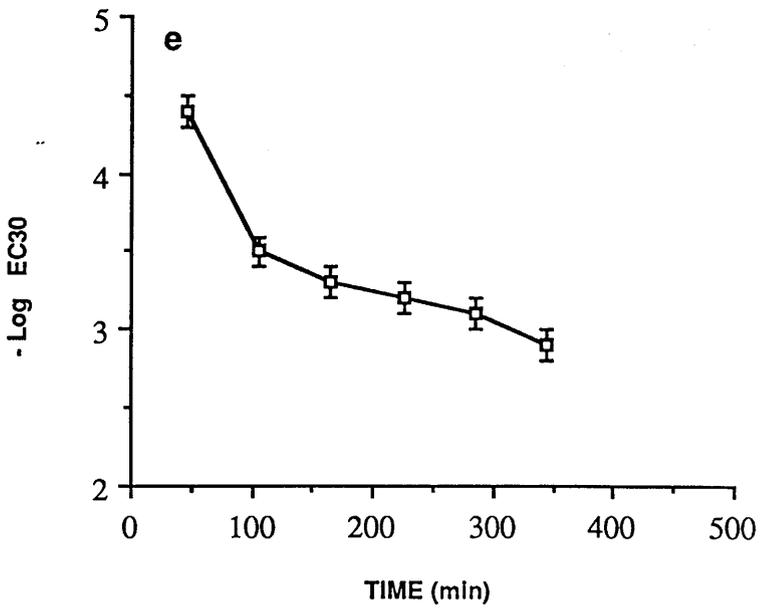
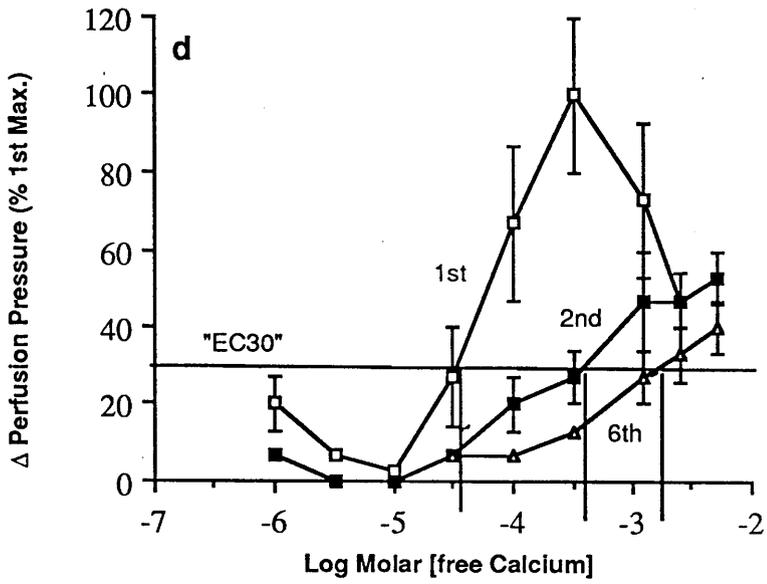
Figure 2.4 c-c. A tracing of a representative recording of a cumulative concentration-response curve to 5-HT (3 $\mu$ M) in a rat tail artery. Note the high but variable response with low concentrations of calcium. Desensitisation of responses occurred after the 1st CCRC. Activation of the tissue at the 4th determination with NA instead of 5-HT almost restored the responses to the original level. Also see Fig. 2.13. NA responses were not desensitised after 5-HT-induced contractions.

The absolute sizes of the maximum responses obtained within the 1st CCRC to NA (3uM) or  $K^+$  (100mM) were similar (Figs. 2. 4a&b). With either activator, the maximum response, the  $-\log(EC_{30})$  and the  $-\log(EC_{50})$  of the first curves were statistically significantly greater than in the second or subsequent curves. The same situation applied to 5-HT (Fig. 2.4c). The first CCRC lay further to the left than any subsequent CCRC. For NA there was a progressive shift which decreased after the 1st CCRC. However, for KCl-induced responses the second and the subsequent curves were not statistically significantly different from each other in any of the above parameters. They were almost indistinguishable from each other.

(i) Noradrenaline

For NA (3uM), the first CCRC lay further left than the subsequent curves (by  $1.13 \pm 0.20$  log units at the level of  $-\log EC_{30}$  values) compared with the 2nd. This represents the distance between the mean of the individual pairs of  $EC_{30s}$ . This first CCRC was "bell-shaped". It peaked at  $[Ca^{2+}]_{free} = 0.3uM$  to  $1.25mM$  with 30% (i.e. approximately 50mmHg) of its maximum attained by 30uM (i.e.,  $EC_{30} = \text{approx. } 30uM$ ). The second and subsequent CCRCs showed a more sigmoid correlation of response with  $\log [Ca^{2+}]$  and responses had not attained a true maximum even at 5mM. The second curve had reached

Figure 2.4 d & e. Illustration of the response level ( $EC_{30}$ ) shown for assessment of  $Ca^{2+}$  sensitivity. Consecutive cumulative concentration-response curves (CCRCs) for NA-induced (3 $\mu$ M) contractions in calcium buffered saline in 95% $O_2$  showing the 1st, 2nd and 6th. Pressor responses in % of 1st maximum were plotted against concentration of free calcium. Sensitivity and maximum response steadily declined with with time. The  $EC_{30}$  values (30% of the 1st maximum) were chosen to represent an index of calcium sensitivity (e). These values steadily declined in a series of control curves when plotted against time from initial set of tissues (d). When the calcium sensitivity of KCl-induced (100mM) contractions were expressed as a percentage of the maximum within each curve, desensitisation still occurred. Points shown represent  $mean \pm s.e.m.$  (n=12). Both the sensitivity and the maximum response of the subsequent CCRCs were significantly lower than the control (1st CCRC) ( $0.01 < p < 0.05$ ).



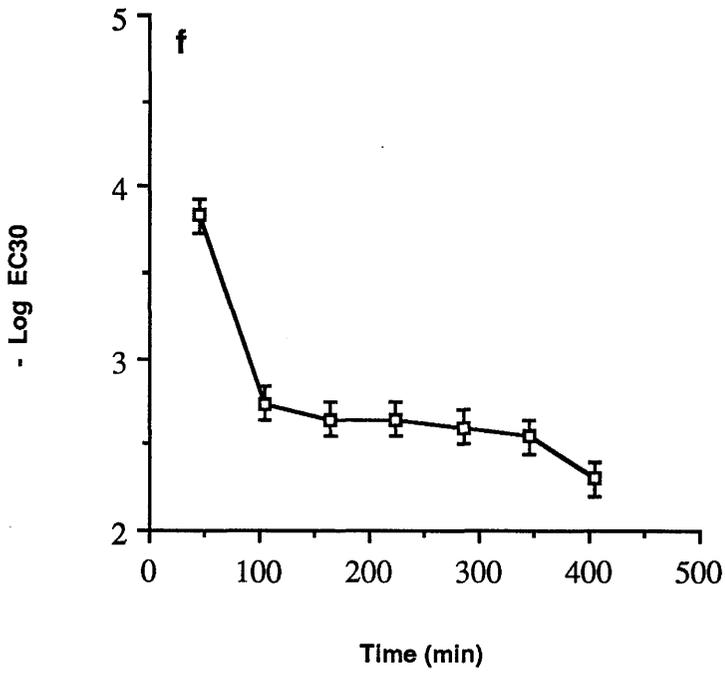


Fig. 2.12

50mmHg which is approximately 50% of its "maximum" by 300uM; and the third CCRC by about 600uM with the rightward shift decreasing thereafter. For example, the 6th CCRC attained 50mmHg by  $[Ca^{2+}] = 1mM$  (Fig. 2. 4a).

The rightward shift (decline) in the sensitivity of tissue to calcium (represented by the  $EC_{30}$  values, i.e. at 30% of control maximum) (Fig 2.4d) showed a steady decline with subsequent curves. This rightward shift of the  $EC_{30}$  values, expressed as the  $-\log EC_{30}$  values, was used as an index of the fall in sensitivity (Fig. 2. 4d).

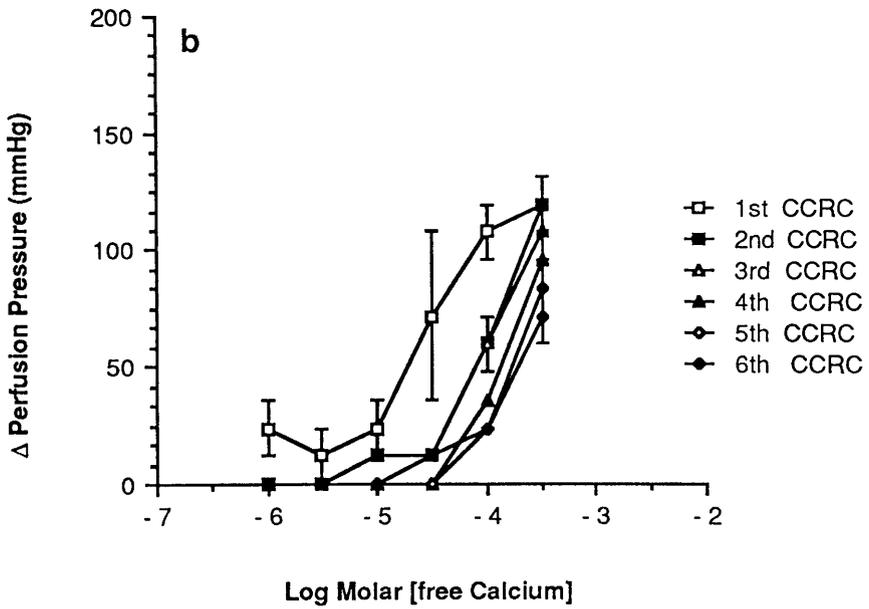
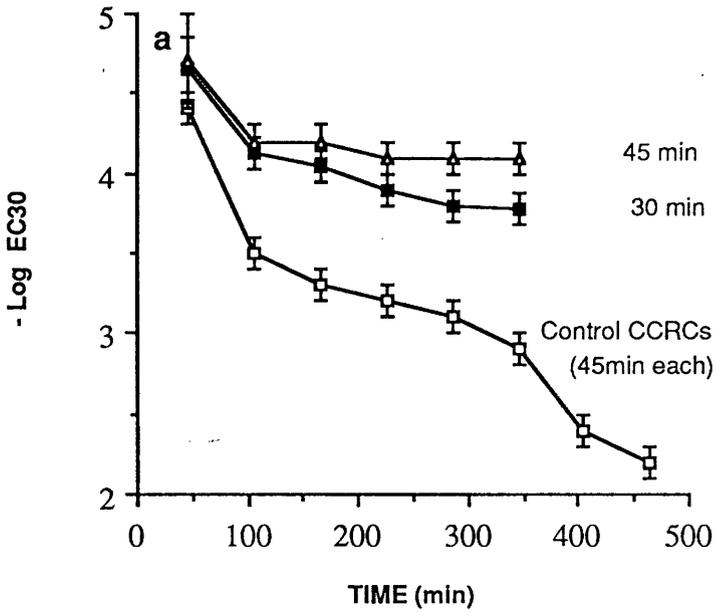
(ii) Potassium Chloride

High potassium chloride (KCl, 100mM), also produced a "bell-shaped" 1st curve which was not repeated in the 2nd or subsequent curves and was, in this respect, similar to NA. However, the  $[free Ca^{2+}]$  required for any given response (at 2.5mM  $Ca^{2+}$ ) with KCl was approximately 10 times higher than with NA. This means that all curves lay further to the right (including the declining leg of the "bell-shaped" first curve (Fig. 2. 4b). One obvious implication of this result is that at the physiological free calcium concentration of 1.25mM, an increase in  $[free Ca^{2+}]$  in the saline causes a steep fall in response of the first curves whereas it produces a steep rise in the subsequent curves. In addition, the

[free  $\text{Ca}^{2+}$ ] for a 50mmHg response changes from just above 100uM in the first curve to 1.25mM in the second curve - a rightward log shift of  $0.91 \pm 0.15$  at the level of  $\text{EC}_{30}$  values.

The bell-shaped first curve makes correction of responses for inter-tissue variability difficult and presents problems in how to compare  $\text{Ca}^{2+}$  sensitivity at different times, even in a single tissue, when the "shape" and maximum are changing. We have expressed all pressor responses as a percentage of the 1st maximum to the particular activator, whether this was obtained in a CCRC or during priming. These 1st maxima were not significantly different between series with the exception of those in the presence of nifedipine. Thus we have compensated only for tissue variability in the height of responses.  $\text{Ca}^{2+}$  sensitivity was expressed as the concentration producing 30% of this 1st maximum ( $\text{EC}_{30}$ ) (interpolated as  $-\log \text{EC}_{30}$ ). Thus, when the maximum changes, our  $-\log \text{EC}_{30}$  values no longer represent a true  $-\log \text{EC}_{30}$  for that curve, but a slightly lower value, exaggerating the extent of desensitisation. However even expressed as a percentage of the maximum within each curve, desensitisation still occurs (Fig. 2. 4f).

Figure 2. 5. Effect of limiting maximum  $[Ca^{2+}]$  on desensitisation. Consecutive cumulative  $Ca^{2+}$  concentration-response curves (CCRCs) for NA-induced (3 $\mu$ M) contractions. in calcium buffered saline in 95% $O_2$ . -Log  $EC_{30}$  values (a) (from absolute curves {b}) were plotted against time (min). Limiting  $[Ca^{2+}]$  to 0.3 $\mu$ M in CCRCs, (30 min) by which point the tissue had already achieved its maximum, reduced desensitisation when compared with the controls (45min). This was also the case even when an extra 15mins was allowed to compensate for the longer activation time when constructing CCRCs up to 5mM (a). Points shown represent mean  $\pm$  s.e.m. (n=6-8). Apart from the 1st determination, the sensitivity for each CCRC was significantly than the corresponding controls ( $0.01 < p < 0.05$ ).



D. Effects of the range of  $[Ca^{2+}]_{free}$  used to construct the CRC

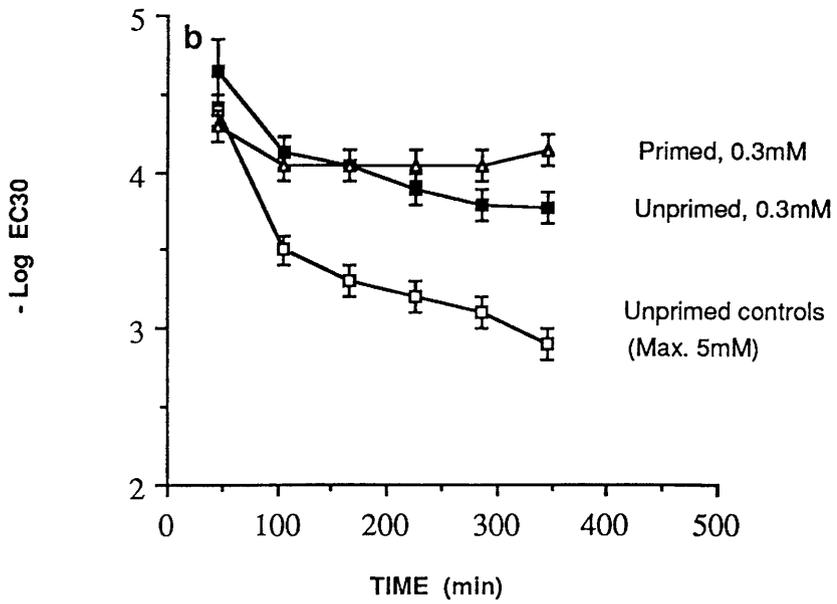
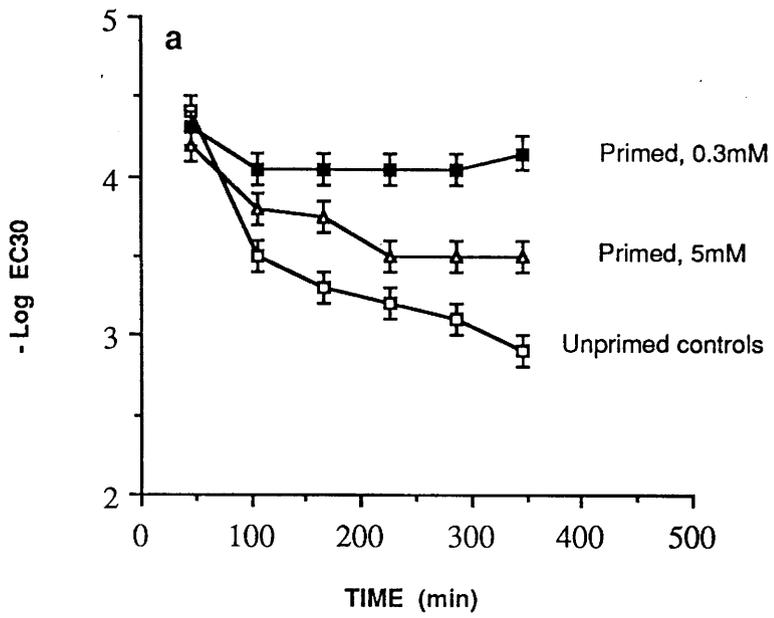
The highest concentration of  $[Ca^{2+}]$ , to which the NA-activated tissue was exposed, affected  $Ca^{2+}$ -sensitivity. At the first determination of  $Ca^{2+}$ -sensitivity, the maximum contraction usually occurred by 300uM  $Ca^{2+}$ . When the tissue was exposed to a maximum of 300uM  $Ca^{2+}$  (buffer 1), desensitisation of subsequent CRC's was less than when  $[Ca^{2+}]_0$  was taken up to 5mM (Fig. 2. 5). Another factor enters these later experiments since the tissues are exposed to NA for a shorter time (30min), which might have accounted for less desensitisation. However, desensitisation was still reduced even when the tissues were left contracted in 300uM  $Ca^{2+}$  for longer so that the total time for constructing each CCRC was 45min, as for the controls with maximum 5mM  $Ca^{2+}$ .

E. Effects of a "Priming" contraction to NA in different levels of  $[free Ca^{2+}]$  before constructing CCRCs

(i) "Priming" in Buffer 1:

If the tissues were exposed for 5min to NA (3uM) in Buffer 1 ("primed") before constructing the first CCRC (up to 5mM  $Ca^{2+}$ ), desensitisation was partially arrested (Fig. 2.6a&c). Therefore either priming in 300uM  $Ca^{2+}$  or taking CRCs to a 'maximum'  $[Ca^{2+}]$  of 300uM reduced

Figure 2. 6. Effect of priming tissue in  $0.3\mu\text{M Ca}^{2+}$ . Consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA-induced ( $3\mu\text{M}$ ) contractions, constructed in calcium buffered saline in  $95\% \text{O}_2$ .  $-\text{Log EC}_{30}$  values (from absolute curves {c & d}) were plotted against time (min). The tissue was primed with  $3\mu\text{M NA}$  in  $0.3\text{mM Ca}^{2+}$  and CCRCs constructed to  $0.3\text{mM Ca}^{2+}$  only (data from {c}) and in another set, to  $5\text{mM}$  (data from {d}). Priming or priming and limiting  $[\text{Ca}^{2+}]$  to  $0.3\mu\text{M}$  enhanced stability. For primed tissues, the sensitivities of the 1st and 6th CCRCs were similar. Points shown represent  $\text{mean} \pm \text{s.e.m.}$  ( $n=6-8$ ). Apart from the 1st determination, the sensitivity for each CCRC was significantly greater than the corresponding controls ( $0.01 < p < 0.05$ ).



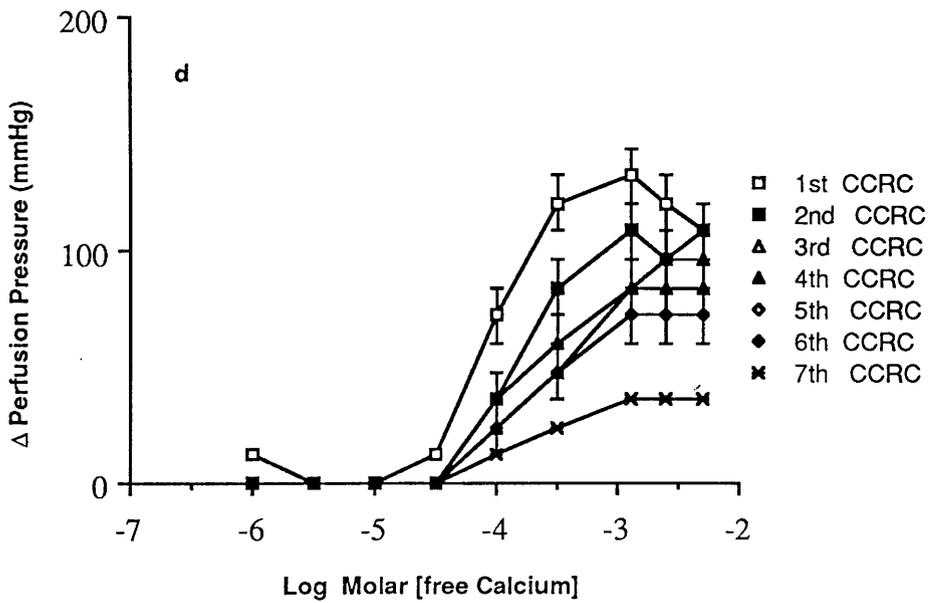
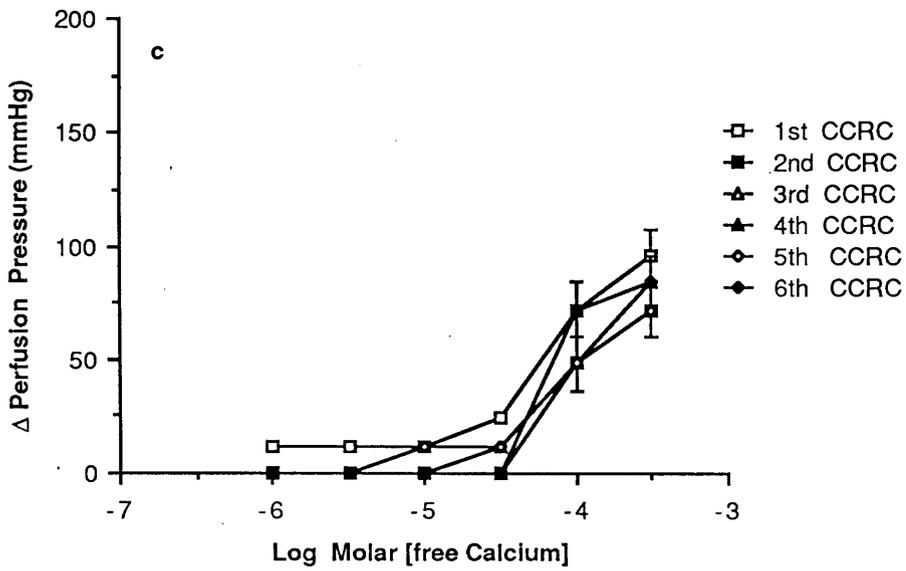


Figure 2. 7. Effect of priming tissue in 2.5mM  $\text{Ca}^{2+}$ . Consecutive cumulative concentration-response curves (CCRCs) for NA-induced (3uM) contractions, constructed in calcium buffered saline in 95% $\text{O}_2$ . -Log  $\text{EC}_{30}$  values (from absolute curves {b}) were plotted against time (min) (a). The tissues were primed with 3uM NA in 2.5mM  $\text{Ca}^{2+}$  and CCRCs constructed to 5mM  $\text{Ca}^{2+}$  only (b). Priming in 2.5mM [ $\text{Ca}^{2+}$ ] and constructing CCRCs to 5mM led to loss of sensitivity especially with the first two CCRCs. Points shown represent mean  $\pm$  s.e.m. (n=6-8). The sensitivity for the 1st 2 determinations were significantly lower and thereafter significantly greater than the corresponding controls (0.01 <p<0.05).

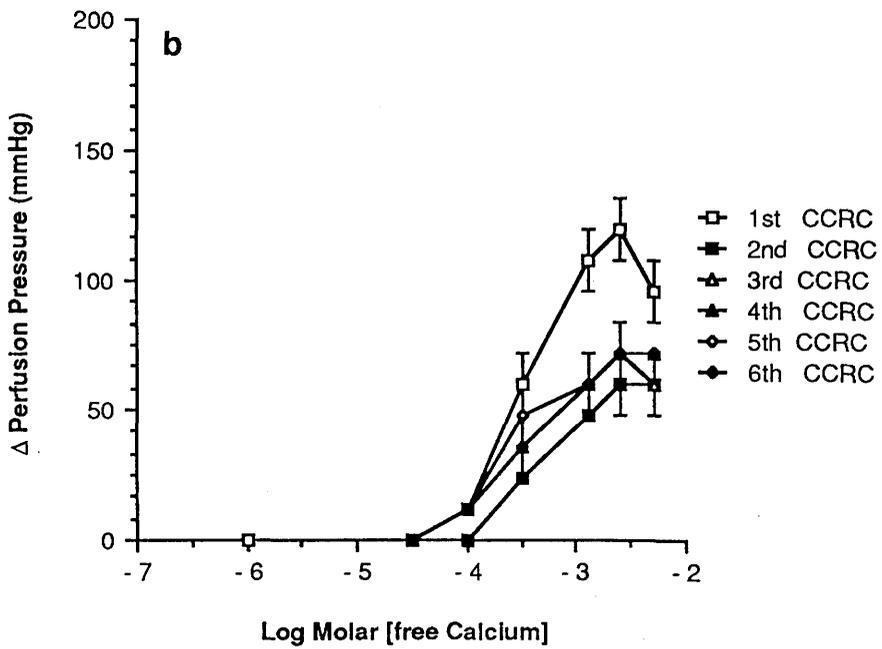
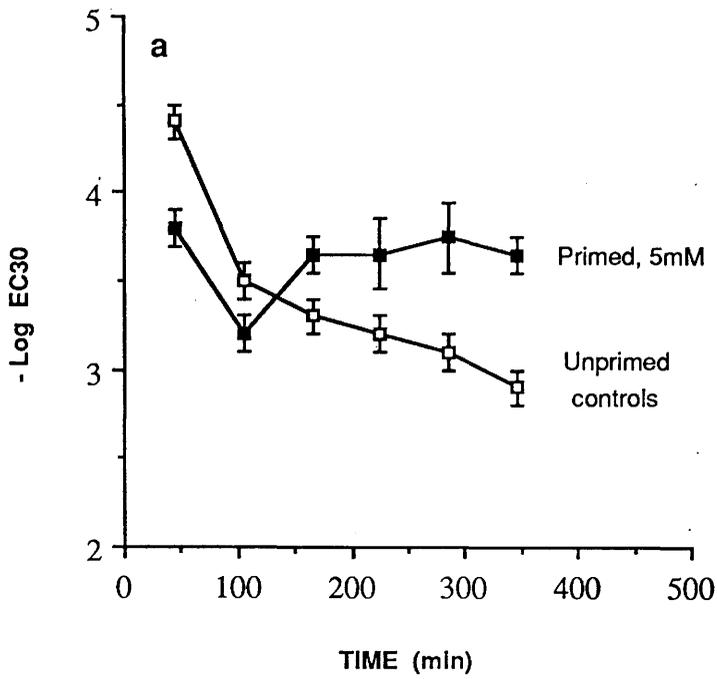
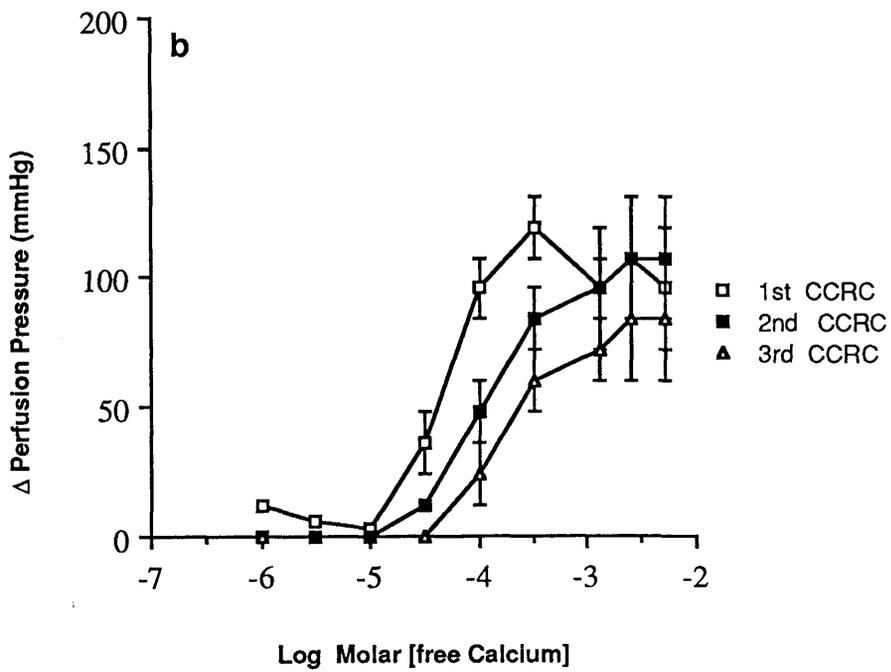
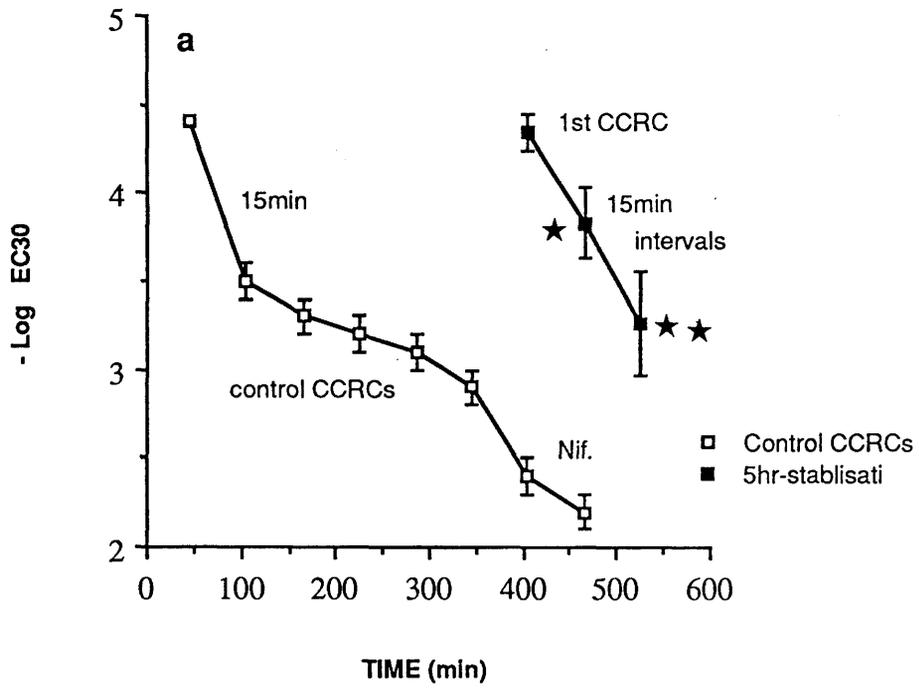


Figure 2. 8. Effect of 5hr stabilisation period. Consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA-induced (3 $\mu\text{M}$ ) contractions were constructed in calcium buffered saline in 95% $\text{O}_2$ . -Log  $\text{EC}_{30}$  values (from absolute curves {b}) were plotted against time (min). 5hr instead of the usual 1.5 hr stabilisation period did not affect the tissue sensitivity but desensitisation occurred with subsequent 15min intervals between CCRCs. Points shown represent mean  $\pm$  s.e.m. (n=6-8). Asterisks indicate significant loss in sensitivity compared with the 1st CCRC after a 5hr stabilisation period (\*\* p<0.01; \* p<0.05).



desensitisation. Combining the two was even more effective: the  $EC_{30}$  for the 6th CRC was not significantly different from the first (Fig. 2. 6b). Of course, the 1st was slightly less sensitive than without priming so that the stable level was actually slightly desensitised. If on the other hand CCRCs were taken to a 'maximum' of 5mM after priming in 300uM (Fig. 2. 6a&d), desensitisation occurred which was half way between that in 300um 'maximum' and that in the unprimed controls.

(ii) "Priming" in 2.5mM  $Ca^{2+}$ :

Priming in 2.5mM  $Ca^{2+}$  accelerated desensitisation for the 1st two curves although there was some recovery thereafter. This shifted the first curve to the position of the usual second curve. The second curve was shifted to the position of the usual sixth curve (Fig. 2. 7). Thus while this procedure produced a degree of stability (analogous to Fig. 2. 3), it did so by making the tissues insensitive to  $Ca^{2+}$  by approximately one log unit.

F. Influence of initial incubation period

An initial stabilisation period of 5 hours allowed the same sensitivity as the usual 1.5 hour period (Fig. 2. 8). This indicates that high sensitivity remains if the tissue is not activated. Short intervals between subsequent CCRCs produced similar desensitisation after

Figure 2. 9. Effect of increasing time intervals after 3rd CCRC. Consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA-induced (3 $\mu\text{M}$ ) contractions were constructed in calcium buffered saline in 95% $\text{O}_2$ . (a)  $-\text{Log EC}_{30}$  values (data from {c}) were plotted against time (min). After the 3rd CCRC, 2hr instead of 15min intervals between CCRCs partially restored sensitivity by the 5th CCRC when compared with the controls.

With 1hr intervals between CCRCs, desensitisation still occurred but more slowly (b). Points shown represent  $\text{mean} \pm \text{s.e.m.}$  ( $n=6-8$ ). Apart from the 1st CCRC (in {b}), the sensitivity for each CCRC was significantly greater than in the corresponding controls ( $0.01 < p < 0.05$ ).

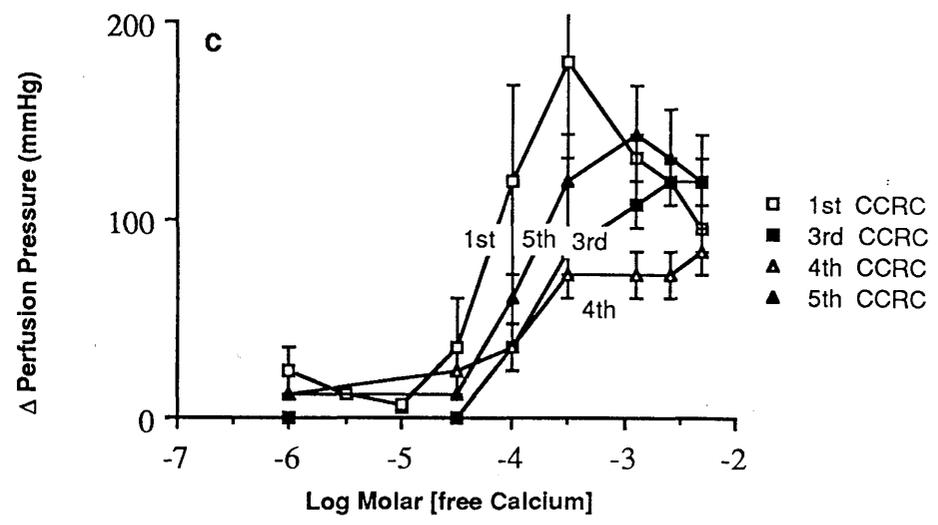
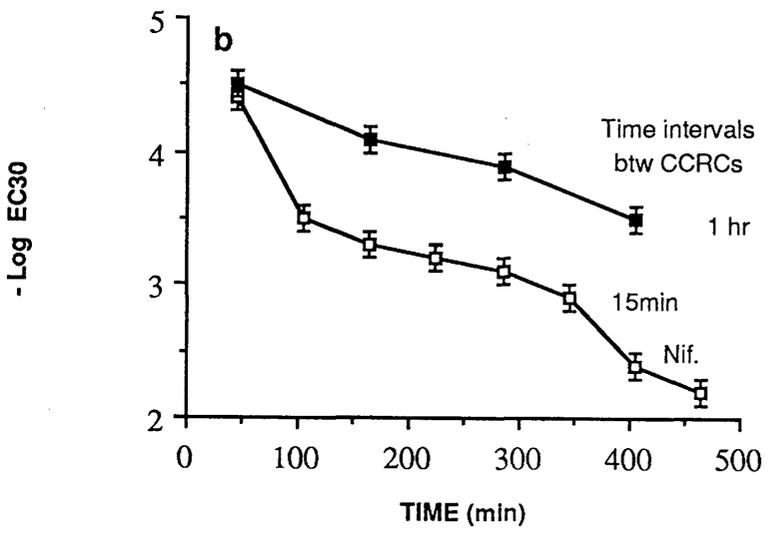
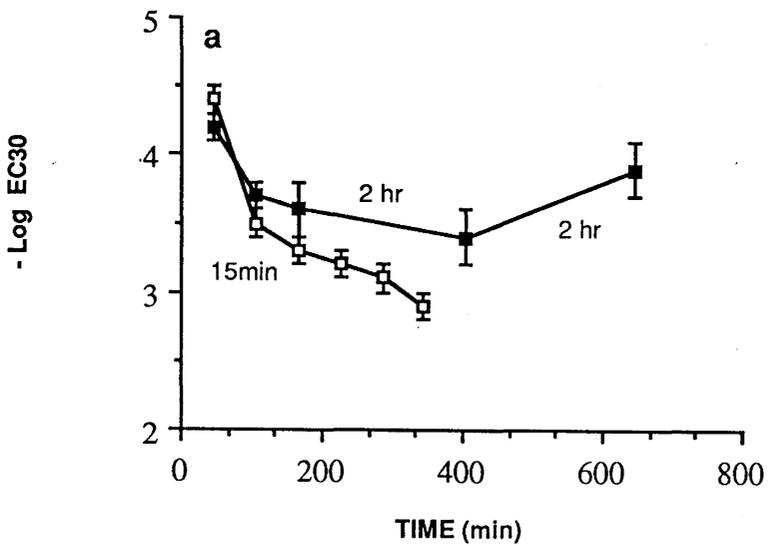
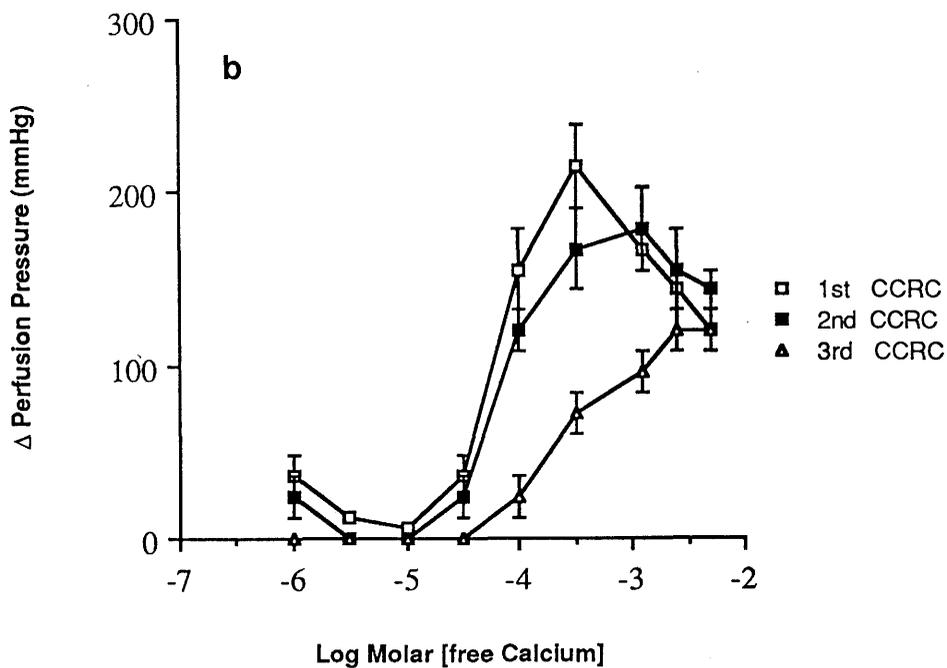
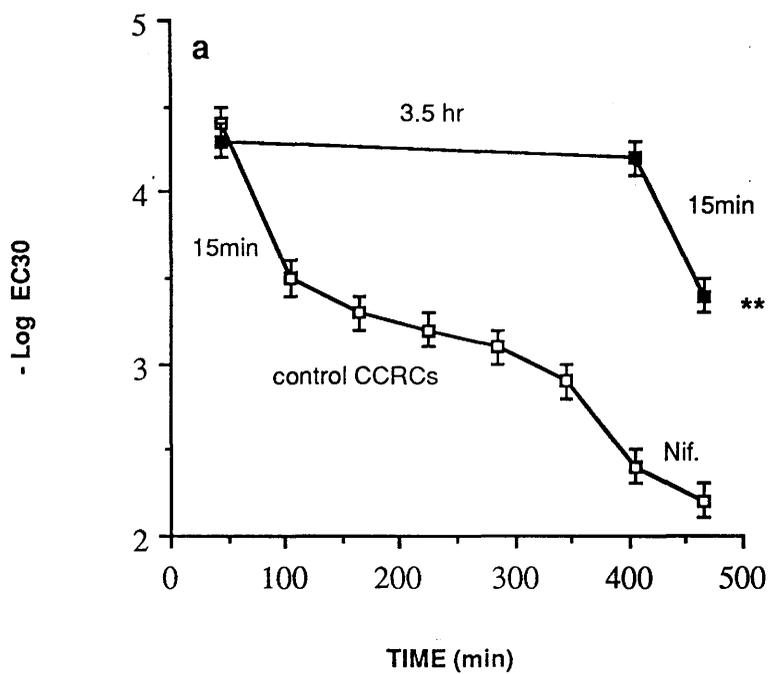


Figure 2.10. Effect of 3.5hr interval between 1st and 2nd CCRCs. Consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA-induced (3 $\mu\text{M}$ ) contractions were constructed in calcium buffered saline in 95% $\text{O}_2$ . -Log  $\text{EC}_{30}$  values (from absolute curves {b}) were plotted against time (min). 3.5hr instead of 15min interval between the 1st and 2nd CCRC completely restored tissue sensitivity but a subsequent 15min interval allowed desensitisation. Points shown represent mean  $\pm$  s.e.m. (n=6-8). The sensitivity of the 3rd CCRC (asterisk) after a 3.5hr interval between the 1st and 2nd CCRCs was significantly lower than the previous CCRCs (\*\* p<0.01).



5 hours as after 1.5 hours. Thus the deterioration in sensitivity is a function of activation and not a general deterioration with time.

G. Influence of length of interval between CCRCs.

In the experiments described above, the protocol allowed only a short rest period of 15min between exposures to NA. Increasing the gap between CCRCs reduced desensitisation. Increasing the interval to 2 hours after 3 CCRCs at 15min gaps partially arrested deterioration and allowed some restoration of sensitivity after a 2nd 2 hour interval (Fig. 2. 9a&c). This suggested that 2 hr is insufficient to allow full recovery to initial sensitivity, but indicates that some recovery towards initial levels is possible.

With 1 hour gaps between CCRCs desensitisation still occurred, though more slowly (Fig. 2. 9b).

However, virtually no desensitisation occurred when 3.5 hours was allowed between the 1st and 2nd CCRCs: it did occur when a subsequent short gap of 15mins was allowed before the 3rd CCRC (Fig. 2. 10). This shows that the tissue recovers its original high sensitivity after 3.5 hours.

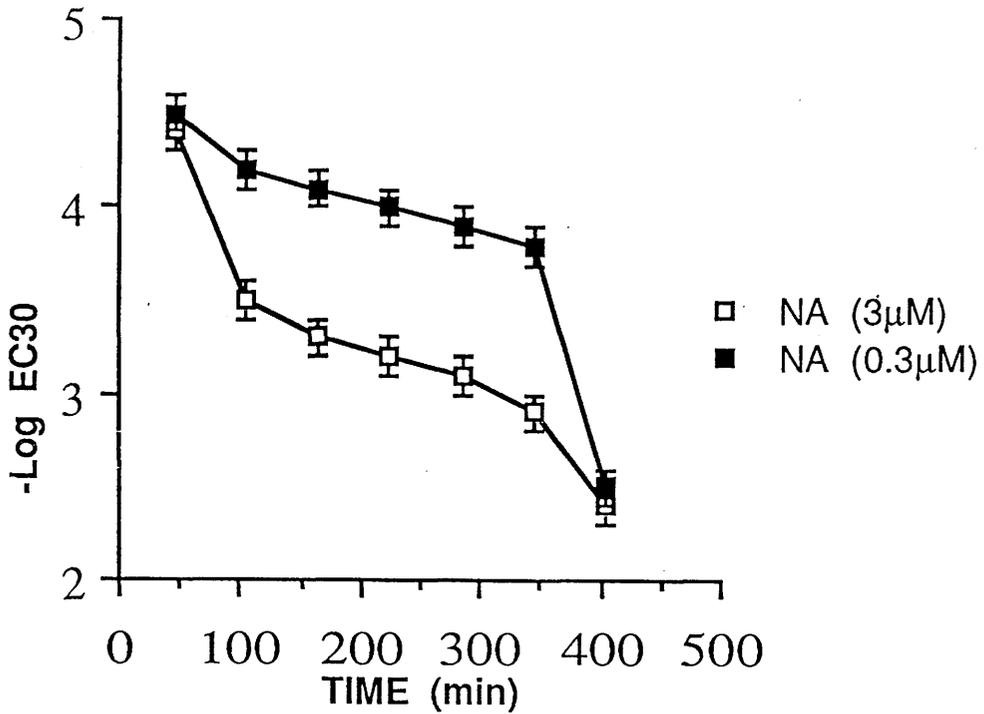


Figure 2. 11. Effect of tissue activation with lower [NA] (0.3µM). Consecutive cumulative  $Ca^{2+}$  concentration-response curves (CCRCs) for NA-induced (0.3µM) contractions were constructed in calcium buffered saline in 95% $O_2$ . -Log  $EC_{30}$  values were plotted against time (min). When tissue was activated with 0.3µM instead of the usual 3µM NA, desensitisation also occurred but at a slower rate than in the controls (3µM). Thus the level of sensitivity remained higher with 0.3µM NA after the first curve. In each case the 7th (final) curve was obtained in the presence of nifedipine (0.1µM). Points shown represent mean  $\pm$  s.e.m. (n=6-12). Apart from the 1st and the 7th (nifedipine effect) CCRCs, the sensitivity for each CCRC was significantly greater than the corresponding controls ( $p < 0.01$ ).

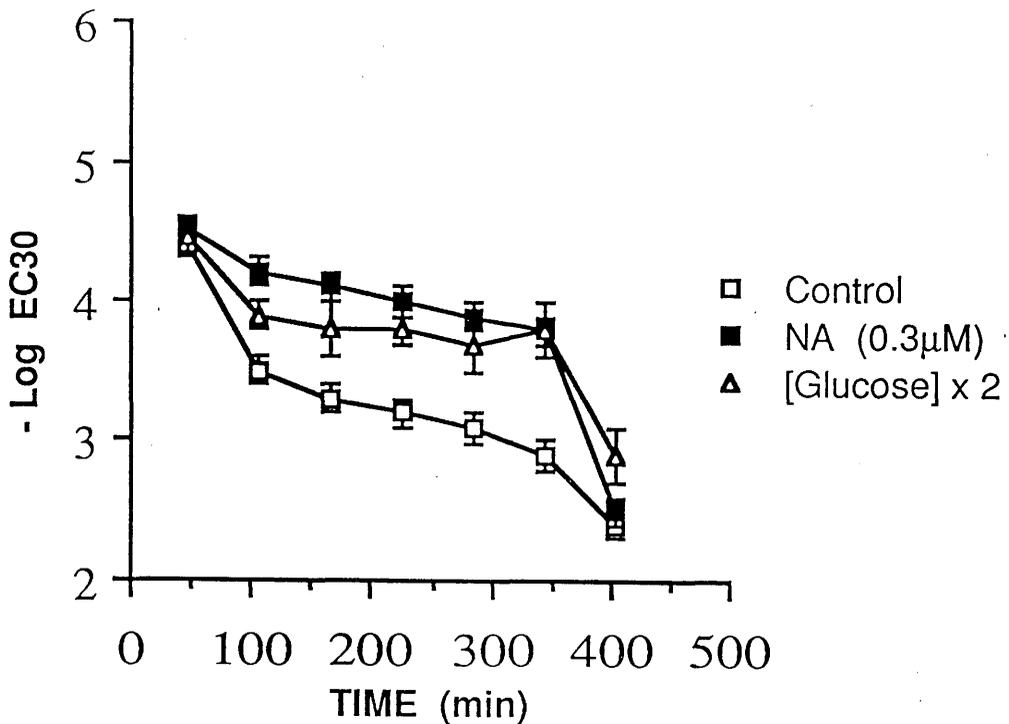
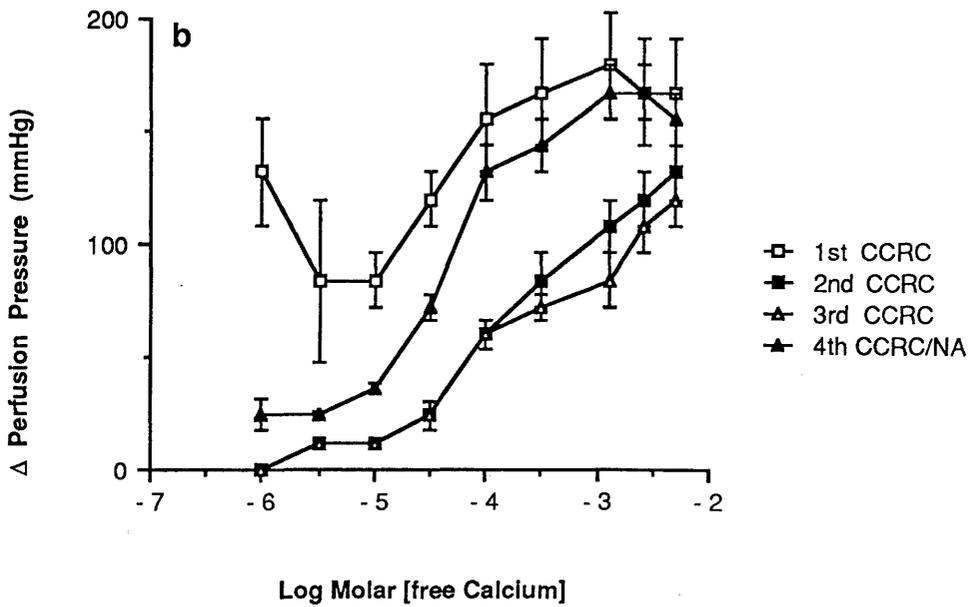
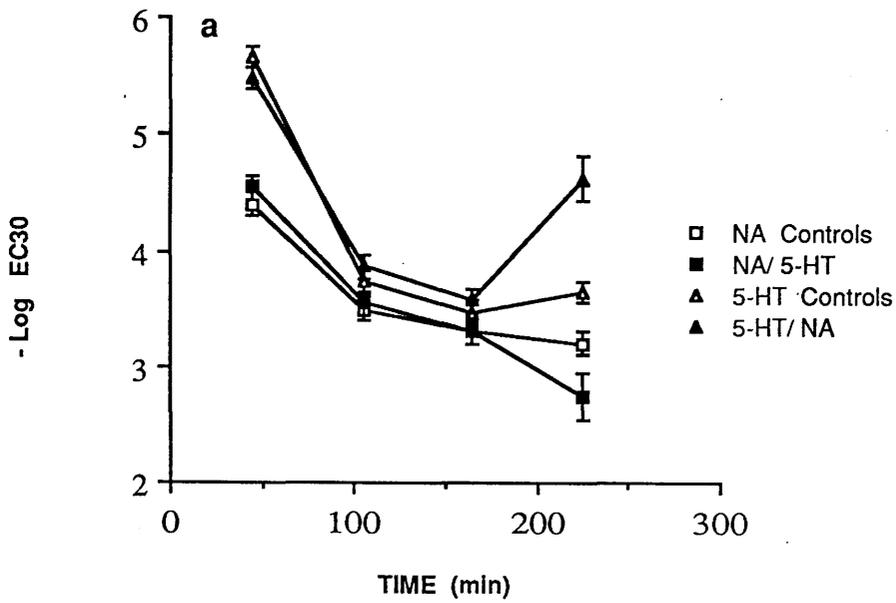


Figure 2. 12. Effect of doubling the glucose concentration in the saline. Consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA-induced ( $3\mu\text{M}$ ) contractions were constructed in calcium buffered saline in  $95\% \text{O}_2$ .  $-\text{Log EC}_{30}$  values were plotted against time (min). When the concentration of the glucose in the saline was doubled, desensitisation occurred at a slower rate and the level of sensitivity to calcium was similar to tissue responses activated by  $0.3\mu\text{M}$  NA in normal glucose concentration. Points shown represent mean  $\pm$  s.e.m. ( $n=6-8$ ). Apart from the 1st and the 7th (nifedipine effect) CCRCs, the sensitivity for each CCRC was significantly greater than the corresponding controls ( $0.01 < p < 0.05$ ).

Figure 2. 13. Cross-desensitisation between NA and 5-HT. Consecutive cumulative  $\text{Ca}^{2+}$  concentration-response curves (CCRCs) for NA (3 $\mu\text{M}$ ) and 5-HT-induced (3 $\mu\text{M}$ ) contractions were constructed in calcium buffered saline in 95%  $\text{O}_2$ . -Log  $\text{EC}_{30}$  values were plotted against time (min). While 5-HT responses were desensitised after NA-induced contractions, the NA responses were not desensitised after 5-HT-induced contractions. Points shown represent mean  $\pm$  s.e.m. (n=6-8). Sensitivity of 5-HT at the determination was significantly greater than the corresponding NA 1st CCRC. Sensitivity of NA after three 5-HT determinations was significantly greater than either NA or 5-HT at the 4th determination ( $p < 0.01$ ).



#### H. Effect of Activation in Low [NA]

When the tissue was activated with a lower [NA] (0.3uM) rather than the 3uM regularly used in this study for constructing CCRCs, desensitisation still occurred but this was significantly lower than that with 3uM NA (Fig. 2. 11).

#### I. Effect of [Double Glucose]

When the concentration of glucose in normal saline (2mM) was doubled and CCRCs constructed under 3uM NA activation, desensitisation was significantly reduced when compared with the controls, giving similar level of calcium-sensitivity as in CCRCs where normal [glucose] was used but the tissue was activated with 0.3uM NA (Fig. 2.12).

#### J. Effect of Activation by 5-HT

Desensitisation of the tissue responses to calcium at the first determination with 5-HT (3uM) was similar to that with NA (3uM) (Fig. 2. 13). However, with 5-HT, calcium sensitivity was significantly higher at very low calcium concentration than was the case with NA (Fig. 2.13b). Their sensitivities were similar in subsequent curves. 5-HT (3uM) given after three NA consecutive CCRCs, was desensitised by NA. However, NA (3uM) given after three 5-HT consecutive CCRCs, did not show

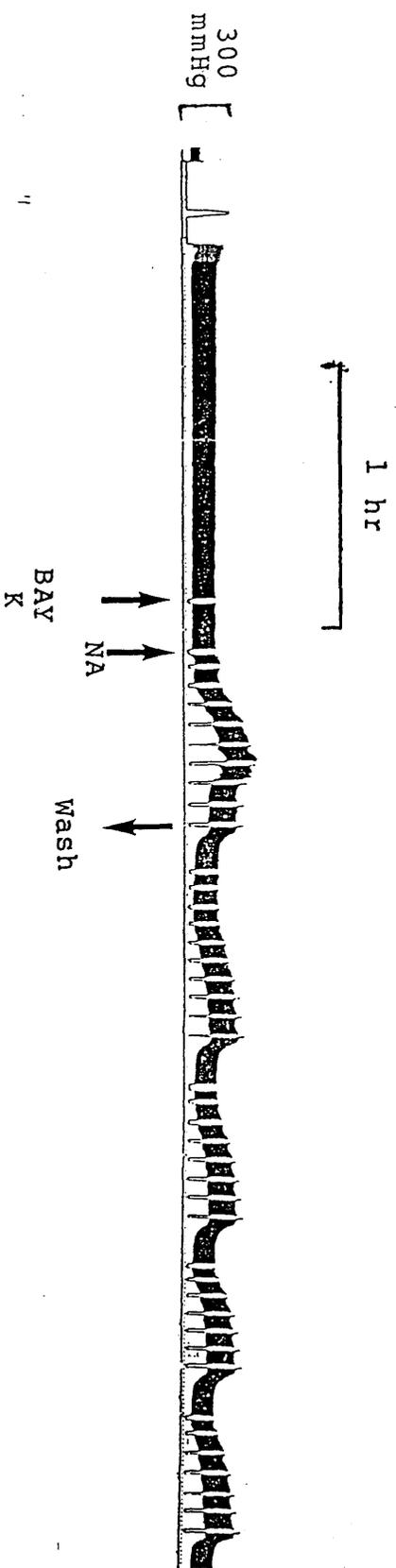
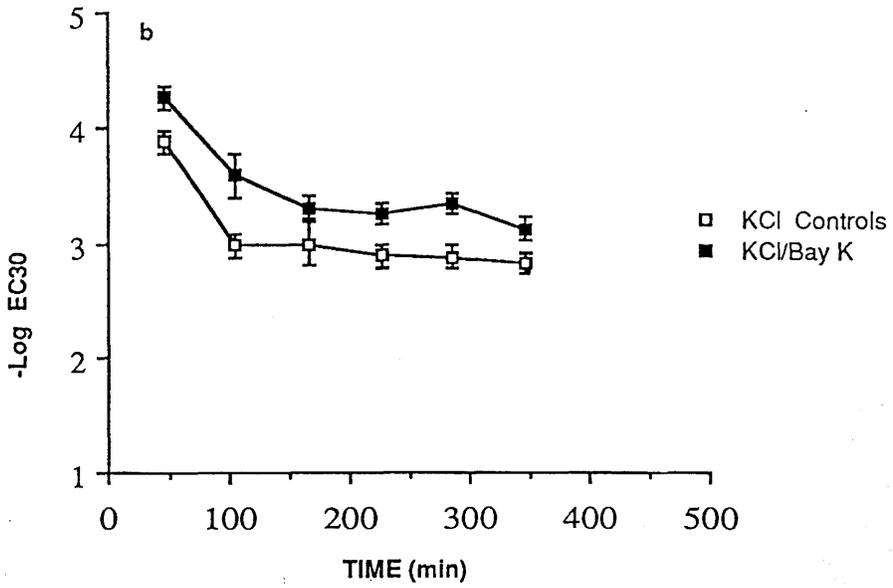
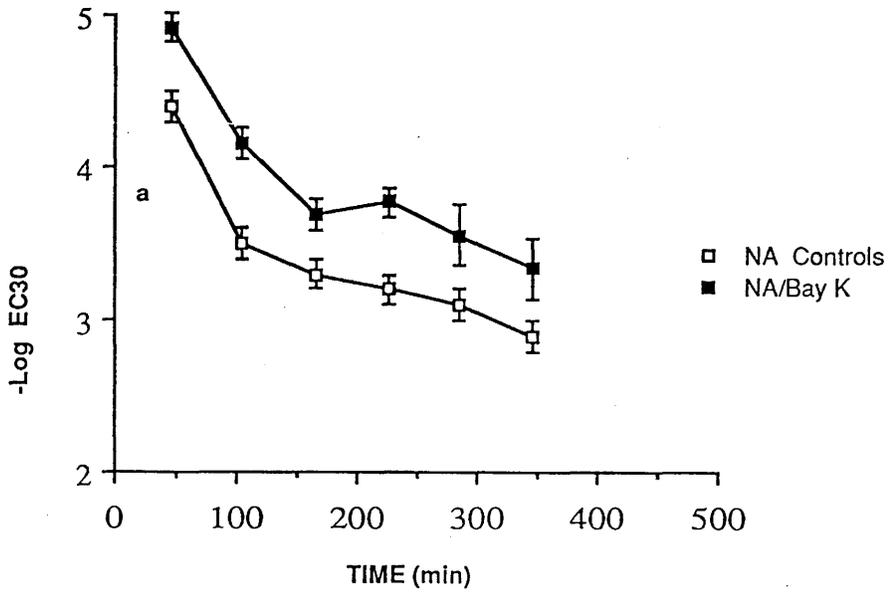


Figure 2.14. A tracing of the cumulative calcium concentration response curves (CCRCs) in the presence of Bay K 8644 (0.1μM). Desensitisation still occurred but was decreased than in the controls. In the absence of an activator, e.g. NA, Bay K 8644 had no effect and did not alter the baseline pressure at 37°C in 95%O<sub>2</sub>

Figure 2. 15. Effect of Bay K 8644 (0.1 $\mu$ M) on (a) NA and (b) KCl-induced contractions in buffered saline. Consecutive cumulative concentration-response curves (CCRCs) of the isolated perfused rat tail artery were constructed in the presence of Bay K 8644 in calcium buffered saline in 95%O<sub>2</sub>. -Log EC<sub>30</sub> values (from absolute curves {c} for NA) were plotted against time (min). In each case, Bay K 8644 potentiated the responses but did not arrest desensitisation. Points shown represent mean  $\pm$  s.e.m. (n=6-8). The sensitivity for each CCRC was significantly greater than the corresponding controls in (a) but not (b) (p<0.05).



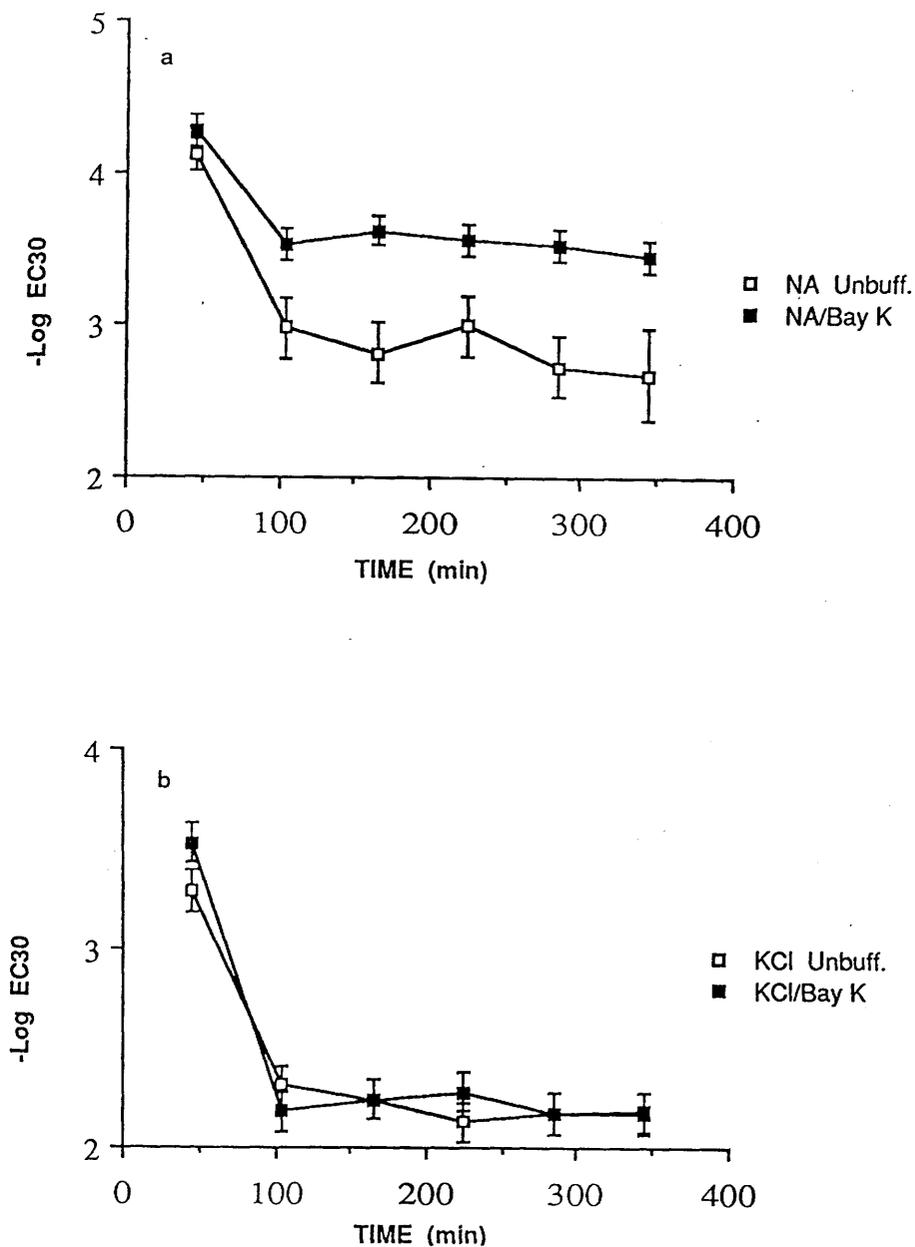


Figure 2.16. Effect of Bay K 8644 (0.1 $\mu$ M) on (a) NA and (b) KCl-induced contractions in unbuffered saline. Consecutive cumulative concentration-response curves (CCRCs) of the isolated perfused rat tail artery were constructed in the presence of Bay K 8644 in calcium unbuffered saline in 95%O<sub>2</sub>.  $-\text{Log EC}_{30}$  values were plotted against time (min). In each case, Bay K 8644 potentiated the responses but did not arrest desensitisation. Points shown represent mean  $\pm$  s.e.m. (n=8-16). Apart from the 1st determination, the sensitivity for (a) but not (b) was significantly greater than the corresponding controls (p<0.05).

desensitisation by 5-HT (Fig. 2. 13).

K.        Effects of Bay K 8644 and Nifedipine on the  
          CCRC and on desensitisation

Bay K 8644

Bay K 8644 (0.1 $\mu$ M) on its own, unlike the activators NA or KCl, did not produce any change in the baseline tension of the artery (as measured by the perfusion pressure) during the cumulative or non-cumulative addition of calcium in the saline (n = 12), when it was added at any point within the range of  $[Ca^{2+}]_{free}$  used. However, Bay K 8644 (0.1 $\mu$ M) potentiated the first CCRC with NA or KCl (Fig. 2. 14).

Bay K 8644 increased the sensitivity to  $Ca^{2+}$  but desensitisation still occurred. However, the  $EC_{30}$  in each CCRC indicated greater sensitivity than in the equivalent Bay K 8644-free control (Fig. 2.15). The use of  $EC_{30}$  based on initial maximum was considered valid since the mean value for this initial maximum was not significantly altered by Bay K 8644 (0.1 $\mu$ M). The calcium threshold value for a pressor response was also less when Bay K 8644 was present in the saline. In general, for a given CCRC (1st, 2nd etc) the effect of Bay K 8644 was a parallel displacement to the left, whether the responses were NA or KCl-induced contractions in calcium-buffered saline (Fig. 2. 15a&b)

or in calcium-unbuffered saline (Fig. 2. 16a&b).

### Nifedipine

Nifedipine (0.1 $\mu$ M), attenuated the calcium-dependent contractions of the rat tail artery, without altering the baseline. Nifedipine decreased sensitivity to  $Ca^{2+}$  at the first CCRC compared with untreated controls but thereafter CRC's were not significantly different from their time controls until the 6th test in which sensitivity was actually significantly higher (Fig. 2. 18). This contrasts with experiments in which nifedipine was absent for the first 3 or 6 CRC's but when it was subsequently added it then reduced  $Ca^{2+}$ -sensitivity at the next test (Fig. 2. 18).

Comparison of  $Ca^{2+}$  sensitivity by employing the  $EC_{30}$  based on the initial maximum is not so straightforward with nifedipine as with the other groups of experiments since it made the 1st maximum significantly smaller. This has the effect of overestimating sensitivity to  $[Ca^{2+}]$  after nifedipine when comparing with other situations. This can be taken into account by

- (i) calculating  $EC_{30}$  using the actual maximum in each individual curve, or
- (ii) estimating the  $[Ca^{2+}]$  to produce a fixed absolute response.

In either case, compared with time controls, nifedipine (0.1 $\mu$ M) led to a significant decrease ( $p < 0.05$ ) in the

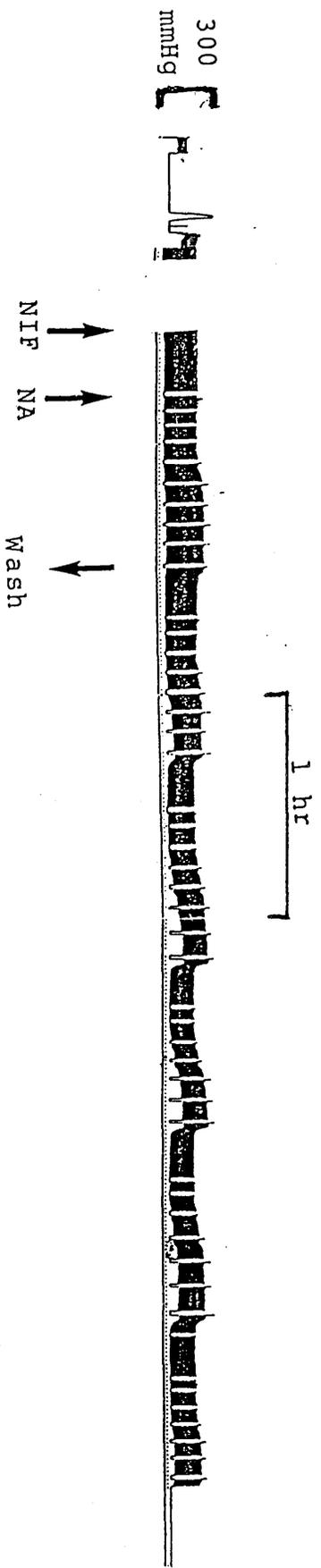
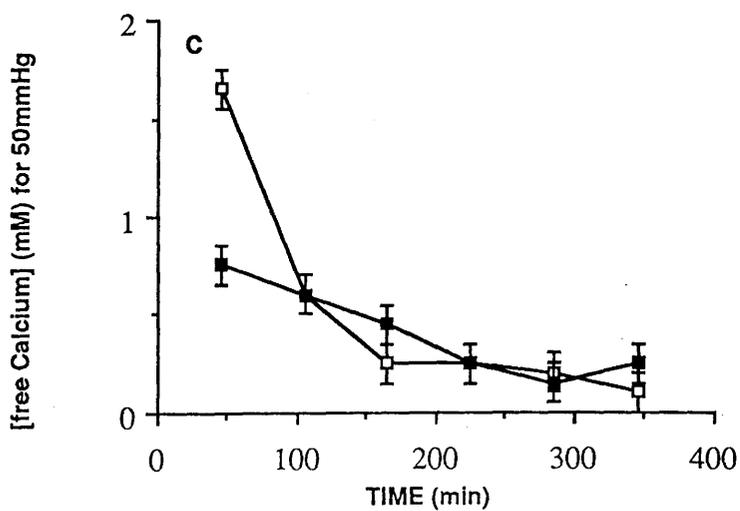
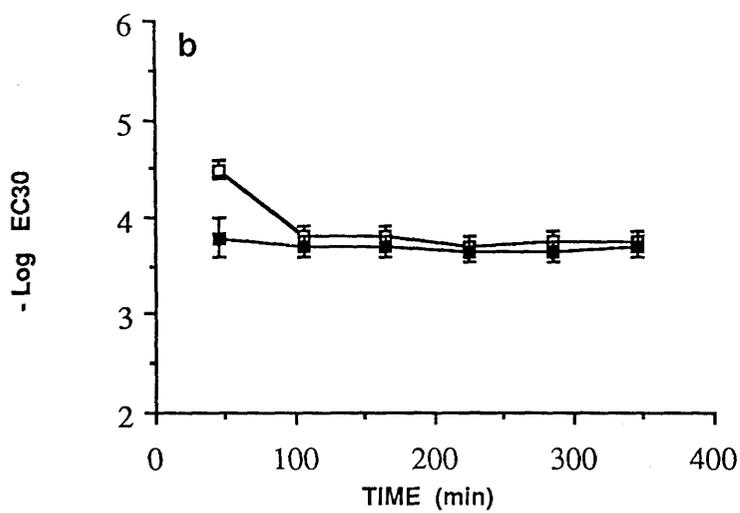
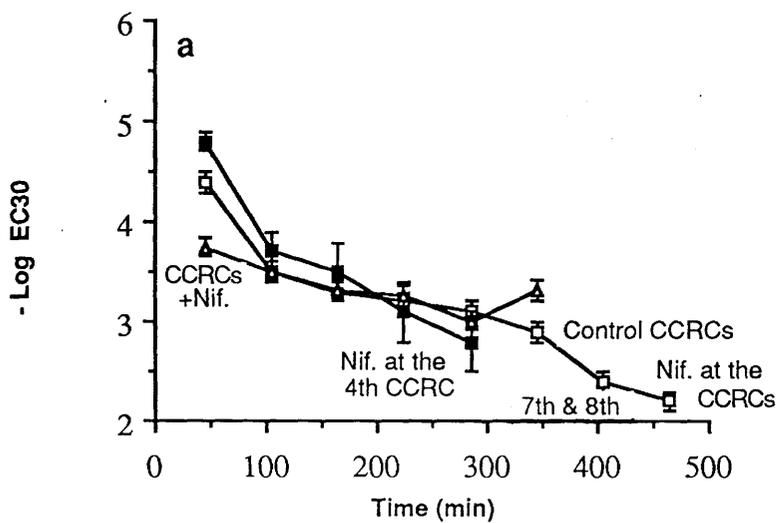


Figure 2.17. A tracing of the cumulative calcium concentration response curves in the presence of nifedipine (0.1μM). The 1st CCRC was depressed by nifedipine. Thereafter, there was no further depression of the responses. In the absence of an activator, e.g. NA, nifedipine had no effect and did not alter the baseline pressure in 95% O<sub>2</sub> at 37°C.

Figure 2. 18. Effect of continuous presence of Nifedipine (0.1 $\mu$ M) and its presence at only the 4th, 7th and 8th CCRCs. Consecutive cumulative concentration-response curves (CCRCs) for NA-induced contractions in the isolated perfused rat tail artery were constructed in the continuous presence of nifedipine ( ) and at only the 4th and 5th ( ) and finally only at the 7th and 8th CCRCs for each set of tissues (see {a}). Desensitisation occurred in the 1st CCRC and tissue stabilised at the control level if nifedipine was present throughout. Introducing nifedipine at the 4th, 5th, 7th or 8th CCRC led to steady decline in tissue sensitivity, especially at the 7th CCRC ( ). Points shown represent mean  $\pm$  s.e.m. (n=16-24). In the presence of nifedipine, only the sensitivity at the 1st determination was significantly lower than the corresponding CCRC (p<0.05).

When sensitivity was expressed as percent of own maximum for each tissue in the presence of nifedipine ( ) (b), only the 1st determination showed a significantly lower sensitivity than the corresponding controls ( ) (p<0.05).





## DISCUSSION

Clearly the sensitivity of rat tail artery to noradrenaline or to  $[Ca^{2+}]_{free}$  (activated by NA or high  $K^+$ ) starts at a high value and declines as the experiment progresses. The instability with which this property endows the tissue can be avoided by an initial desensitisation procedure which leaves a stable but, by definition, less sensitive preparation. It is not unequivocally clear whether (i) the initial high sensitivity represents the "physiological" state of the smooth muscle and the subsequent decline represents pathological changes, or (ii) the initial state is one of pathological supersensitivity induced by removing the tissue from its usual *in vivo* environment to one of artificial saline and it subsequently reverts to a normal physiological state. If the first hypothesis is correct then experiments carried out in the desensitised phase may not accurately reflect events *in vivo*. The same applies to the "supersensitive" phase in the second hypothesis. It is of some importance to distinguish between these since the two states represent an order of magnitude difference in sensitivity to external calcium ( $[Ca^{2+}]_o$ ) the more sensitive of which brings it very close to the type of calcium intracellular ( $[Ca^{2+}]_i$ ) values expected during activation as observed in some studies by Campbell, (1983), Spedding and and Mir (1987), Schramm and Towart (1983c). Even if the initial "supersensitive" phase is pathological it should be of

interest to ascertain just what are the factors which bring this about. If it is "normal" then interest focusses on what is lost or what "antagonistic" factor accumulates when it becomes less sensitive. A further consequence is that this loss of sensitivity to  $\text{Ca}^{2+}$  may, in part, underlie the loss of sensitivity to NA found in consecutive CCRCs (Fig. 2. 3): the initial sensitive state places contraction within the physiological range for NA whereas the desensitised curve lies outwith this.

To further elucidate the mechanism underlying desensitisation, we attempted to establish some factors which influence it. We have concentrated on the sensitivity to  $[\text{Ca}^{2+}]_0$  since this shows up desensitisation even when the response in  $2.5\text{mM Ca}^{2+}$  is little altered. However, it does seem that these changes in  $\text{Ca}^{2+}$ -sensitivity underlie changes in sensitivity in NA concentration/response curves (see below).

First, it was clear that desensitisation followed activation of the tissue but that recovery occurred with time. Our main protocol, with only 15min gap between the end of one CCRC and the start of the next, allowed insufficient recovery so that desensitisation became cumulative. However, although 1 hr intervals reduced the rate of desensitisation, it was necessary to allow a very long gap of 3.5 hrs for complete recovery. It seems unlikely that such gaps would be employed by most

workers so we assume that this type of desensitisation will commonly be present. Long intervals between CCRCs attenuated desensitisation and also allowed some recovery of already desensitised tissues. It has been noted, in the intestinal muscle of the guinea-pig, that the degree of desensitisation and the time needed for recovery are directly related to concentration of substance P and contact time (Holzer and Petsche, 1983).

Secondly, the highest calcium concentration to which the tissue was exposed affected  $\text{Ca}^{2+}$ -sensitivity. The higher the  $[\text{Ca}^{2+}]_o$ , the greater the desensitisation. This suggests that Ca overload may be responsible for the deterioration of responses in this smooth muscle as it does in cardiac muscle (Allen, et al, 1985). It has been proposed that an internal binding site for calcium on the cell membrane regulates the rate of desensitisation (Nastuk & Parsons, 1970; DeBassio et al., 1976). An accumulation of excess free intracellular calcium, in "Ca overload", may contribute to desensitisation of this tissue via such an internal binding site. This seems to be the case also in guinea-pig ileum where excess calcium accelerates desensitisation (Magaribuchi, Ito & Kuriyama, 1973). The different degrees of desensitisation produced by priming in different concentrations of  $\text{Ca}^{2+}$  show that priming can produce some stability of subsequent responses but that the remaining level of sensitivity will depend on  $[\text{Ca}^{2+}]$  both during priming and in the

subsequent tests. Therefore priming accelerates desensitisation but leaves the tissue relatively insensitive. This stabilisation may explain why many workers initially activate their preparations several times until the responses are reproducible (see Su et al., 1984; Aoki & Asano, 1986). Clearly, avoidance of high  $[Ca^{2+}]$  during priming as well as during subsequent parts of the protocol leads to stable preparations which are significantly less desensitised.

It is interesting to note that priming in 300uM  $Ca^{2+}$  (buffer 1) leads to less desensitisation. One of the probable explanations is that on 1st activation, Ca channels open "more" than on any subsequent occasion. Therefore, if  $[Ca^{2+}]_o$  is high on 1st activation, this leads to Ca overload; but if  $[Ca^{2+}]_o$  is not high the 1st time the tissue is activated, then Ca overload does not occur.

Second activation produces "less" channel opening. Therefore, if priming is the 1st activation of the tissue, then the 1st curve is equivalent to the 2nd activation and therefore produces less damage.

It was observed that a smaller concentration of NA (0.3uM), which is one-tenth of the concentration in normal use in this study, similarly produced desensitisation of the first curve but the subsequent curves stabilised at a higher level of sensitivity than

with 3uM NA (Fig.2. 11). This suggests that extracellular  $[Ca^{2+}]$  is not the only factor in desensitisation. A further requirement is a sufficiently high stimulus from the activator, presumably by an increased opening of  $Ca^{2+}$  channels (either more channels or longer open-time).

The partial arrest of desensitisation by doubling the glucose concentration in the saline suggests that desensitisation is partly a function of metabolic processes. The decline in responses following  $Ca^{2+}$ -overload could be linked to the inability of the cell to continuously cope with the maintenance of Ca ion efflux at the same rate as influx. Usually increased  $Ca^{2+}$  influx into the cell would be expected to cause increased responses within a limit, but this influx must ultimately be balanced by rate of efflux of the ions from the cell. Initially, during the first CCRC, the cells can maintain this equilibrium. Even as this first curve proceeds, particularly into high  $[Ca^{2+}]$  levels, the increase in response alters, as "overload" occurs. By the subsequent curves, cell "fatigue" has become evident as the cell strives to restore equilibrium, having partially depleted its energy reserve. At this point the increase in the cell's substrate needs in the form of glucose, restored part of this lost energy and the cell was therefore able to cope with the Ca ion influx/efflux system. Consequently, desensitisation was partially arrested.

It would normally be expected that 5-HT would be desensitised after NA probably because 5-HT would normally act on both the 5-HT receptors and the  $\alpha_1$ -adrenoceptors. This is more so when it is observed that the  $Ca^{2+}$  EC<sub>30</sub> of 5-HT was also high as with NA; the 1st curve of 5-HT was bell-shaped but not the 2nd as with NA; 5-HT maximum response was similar to that of NA; and based on some preliminary studies with 5-HT antagonist, cyproheptadine (0.1 $\mu$ M) 5-HT responses were inhibited showing the presence of 5-HT receptors in the rat tail artery. Medgett and Langer (1986) working with the rat tail artery have shown that receptors of 5-HT exist in this tissue.

It would normally be expected that 5-HT would be desensitised after NA probably because of a greater population of  $\alpha$ -adrenoceptors in this tissue than the 5-HT receptors. However, it was interesting to observe that NA was not desensitised after 5-HT responses. The reason for this is not clear but it could be associated with some sort of augmentation of 5-HT responses by NA.

Bay K 8644 increased  $Ca^{2+}$ -sensitivity but did not alter desensitisation. This suggests that Bay K 8644 sets up a new equilibrium position for tissue responses by allowing further opening of  $Ca^{2+}$  channels by an activating stimulus but at the same time it allows the

accumulation of  $\text{Ca}^{2+}$ , perhaps even enhancing it. This would explain the combination of desensitisation within each preparation coupled with potentiation of responses relative to time controls.

Nifedipine decreased  $\text{Ca}^{2+}$ -sensitivity and to some extent partly arrested desensitisation. Interpretation of the effects of nifedipine is not straightforward. Clearly, nifedipine prevented the characteristic 1st response if given before any activation had occurred. Subsequent responses, however, were no different from time controls (expressed as  $-\log \text{EC}_{30}$ ). This suggests that the initial high sensitivity is produced by a high functional effectiveness of dihydropyridine-sensitive channels and that desensitisation in large part consists of the loss of their function. This correlates with the results with Bay K 8644 presumably by a functional antagonism at the dihydropyridine-sensitive channels.

On its own, the observation that the second CCRC in the presence of nifedipine is similar to its time control might suggest that after the 1st curve, i.e., in desensitised tissue, the main part, if not all, of the response involves  $\text{Ca}^{2+}$  influx through dihydropyridine-insensitive channels. However, the acute effect of nifedipine, given after partial desensitisation, suggests that in tissues which have not been exposed to nifedipine, some dihydropyridine-sensitive channels remain functional. Therefore acute

nifedipine leaves tissues less sensitive than when it is present throughout. To explain this we suggest that when nifedipine is present from the beginning, it may actually attenuate desensitisation by limiting  $\text{Ca}^{2+}$  influx so that the dihydropyridine-insensitive channels (or other elements in the excitation-contraction process which are involved in desensitisation) remain more effective than in the absence of nifedipine. This would be consistent with the concept of Ca overload and its prevention by nifedipine. For practical purposes, the important point is that dihydropyridine-sensitive channels are significantly involved in the response to NA but this element is partly lost after desensitisation.

In conclusion, we have established several factors which influence desensitisation in the rat tail artery. Our results are consistent with a desensitisation of  $\text{Ca}^{2+}$  channels by  $\text{Ca}^{2+}$  overload when high  $\text{Ca}^{2+}$  (2.5 or 5mM) is present during, or subsequent to, activation by NA thus causing reduced sensitivity in subsequent responses. This can be reversed spontaneously by leaving long intervals between tests or can be avoided by restricting  $\text{Ca}^{2+}$  to  $\leq 300\mu\text{M}$  during activation. There is also some evidence that desensitisation can be partly arrested by blockade of  $\text{Ca}^{2+}$  channels with nifedipine. Some stabilisation of sensitivity can be achieved by a "priming" contraction in  $\text{Ca}^{2+}$  (300 $\mu\text{M}$  or 2.5mM) but this does not necessarily clarify further experimentation.

Most importantly, it does seem that the initial high sensitivity to  $Ca^{2+}$  is a state which is "normal" for this tissue in vitro unless it is exposed to the vigorous insult of a prolonged contraction by a non-physiological concentration of NA at a high  $[Ca^{2+}]$ .

## SECTION III

### EFFECTS OF OXYGEN ON CALCIUM CONCENTRATION-RESPONSE CURVES AND THE INFLUENCE OF BAY K 8644, PALMITOYL CARNITINE AND NIFEDIPINE ON THESE RESPONSES

#### SUMMARY

1. The effects of different oxygen tensions on calcium concentration-response curves (CCRCs) of the isolated perfused rat tail artery and the influence of Bay K 8644, palmitoyl carnitine and nifedipine on these responses were studied. The responses were induced by noradrenaline (NA) (3 $\mu$ M) or and potassium chloride (KCl) (100mM) in Ca<sup>2+</sup>-buffered saline. Under control conditions, oxygen tensions between 4% and 95% did not significantly alter the responses of the rat tail artery.
2. Irrespective of the conditions used, there was a significant rightward shift of the subsequent CCRCs when compared with the first, i.e. desensitisation of the 2nd and subsequent CCRCs.
3. Because of desensitisation the interpretation of the effects of Bay K 8644 and nifedipine and the influence on the this of O<sub>2</sub> depended on the experimental protocol. When the effects of the various agents were tested sequentially in the same

tissue (first protocol), the effects of desensitisation prevented clear interpretation. Once a more satisfactory protocol had been devised, however, the data could be satisfactorily explained.

4. However, comparisons were made between the first curves in different tissues, subjected to different conditions (second protocol), the effects of each agent became apparent. By repeating CCRCs in each set of conditions, the desensitisation under different conditions also could be studied. This second protocol allowed demonstration of the facilitatory influence of Bay K 8644 and the inhibitory effect of nifedipine on the corresponding control CCRCs.
  
5. In the second protocol, the effect of the desensitisation of the first curve was specifically noted. This results from the rightward shift of the 2nd and subsequent curves as the oxygen tension is reduced.
  
6. The effects of palmitoyl carnitine (0.1 $\mu$ M) on the Ca pEC<sub>30</sub> (NA as activator) resembled those of Bay K 8644 (0.1 $\mu$ M): increasing it by 0.5 to 1.0 log units for each curve in the series when compared with the time controls. Palmitoyl carnitine also increases the maximum response. The effects of palmitoyl carnitine is clearer at 4%O<sub>2</sub> than at 16% or 95%O<sub>2</sub>.

7. Nifedipine added at the 7th CRC produced a significant rightward shift of the curve and reduction in the maximum, showing that the nifedipine-sensitive channels are still active even after the tissue has lost some sensitivity over six CRCs.
  
8. In conclusion, the results showed that in the rat tail artery, the action in life of drugs which influence  $Ca^{2+}$  channels may be fruitfully explored under physiological oxygen tension. While the effect of Bay K 8644 appears to be specific for facilitation of  $Ca^{2+}$  function, palmitoyl carnitine appears to have in addition, some further effect on the tissue response.

## INTRODUCTION

The modulatory effects of different levels of oxygen in altering the responses of smooth muscles to different activating agents have been extensively studied by various groups of workers and they agree that reduction of the  $PO_2$  in the fluid bathing smooth muscle preparations may have some effect on their responsiveness to drugs or to nerve stimulation in different organs (Garry, 1928; Day & Vane, 1963; Smith & Vane, 1966; Detar & Bohr, 1968; Ebeigbe & Jennett,

1978; McGrath, 1982, 1984, 1985; Chang & Detar, 1980; De Mey & Vanhoutte, 1981; Grant et al., 1984; McGrath & O'Brien, 1987). It is possible that high oxygen tension alters the response beyond the receptor and thus may provide false information on excitation-contraction coupling.

An earlier observation on the rat anococcygeus smooth muscle (McGrath, 1982) suggested that noradrenaline's susceptibility to the calcium entry blocker (CEB) nifedipine was less at low  $pO_2$ , and Su et al, (1984) reported that contraction of the rat tail artery by noradrenaline and potassium chloride are attenuated by nifedipine and facilitated by Bay K 8644 under the high oxygen tension customarily employed in pharmacological experiments. Similarly, it has been suggested that low oxygen tension may cause reduced net influx or utilization of external calcium in vascular smooth muscle (Ebeigbe, Pickard & Jennett, 1980). It therefore seemed possible that the oxygen level could influence the effects on calcium channels of Bay K 8644 and nifedipine.

Since the reported effects of Bay K 8644 and nifedipine on smooth muscle contractions have always been carried out under high oxygen tension (see Schramm et al., 1985; Artalejo & Garcia, 1986; Donoso, 1986), we decided to investigate their influence under different oxygen tensions on the isolated perfused rat tail

artery, using calcium-buffered saline.

Two sets of experiments were carried out. In each set, calcium-buffered and unbuffered physiological salines were used. The first set which involved a longitudinal protocol with drugs and  $O_2$  tensions changed in each tissue, was carried out before the effects of desensitisation on the calcium sensitivity of the isolated perfused rat tail artery were evident to us and the experimental design did not take into account the loss of sensitivity in the course of the experiment. It was the unexpected results from this set of experiments that led to the discovery of the characteristic and highly marked desensitisation of responses which was documented in Section 2.

In the second set of experiments, therefore, the main emphasis was put on the first response in each tissue and comparisons were made between tissues. The opportunity was also taken to observe desensitisation under the varying conditions. In the second set of experiments, the effects of oxygen tension per se was studied as well as the effects of Bay K 8644 and palmitoyl carnitine at different oxygen tensions. In the first set we had switched from one set of conditions to another and had therefore been unable to fully separate the effects of the agent under study from the effects of desensitisation.

## Experimental Protocol

The methods of preparation of the tissue for recording perfusion pressure, and the determination of the perfusion pressure, the drugs and chemicals and the statistical analysis were the same as in the section on General Methods.

The general experimental protocol was similar to that in Section Two, except in a few areas mentioned below. In this section of our study, we utilised both calcium-buffered and calcium-unbuffered salines. In the experiments with calcium buffering, CCRCs were constructed by starting in nominally zero calcium unbuffered saline in which  $[Ca^{++}]_{free}$  was cumulatively increased from about 40uM to 5mM. Responses were triggered by 3uM [NA] or 100mM [KCl]. In other experiments with calcium-buffered physiological saline,  $[Ca^{++}]_{free}$  was buffered with EGTA (2.5mM) and NTA (2.5mM) and again contractions were induced by NA (3uM) or KCl (100mM).  $[Ca^{++}]_{free}$  in the saline was varied between 1uM and 5mM.

The experimental protocols in the two sets of experiments were basically the same with a few exceptions.

In the first set of experiments, a CCRC was

constructed gassing the tissue in 95%O<sub>2</sub>, followed by one in 16%O<sub>2</sub> and then one in 4%O<sub>2</sub> (in the same tissue). This was repeated in the presence of Bay K 8644 (0.1μM) and then again in nifedipine (0.1μM) making 9 CCRCs in all.

In the second set of experiments, a different set of tissues was used to test responsiveness in each set of conditions. Inter-tissue comparisons were made between the first CCRCs. By repeating the CCRC's, desensitisation could be followed under the various conditions. In this way the effects of Bay K 8644, palmitoyl carnitine and nifedipine were tested: this was repeated at different O<sub>2</sub> tensions.

## RESULTS.

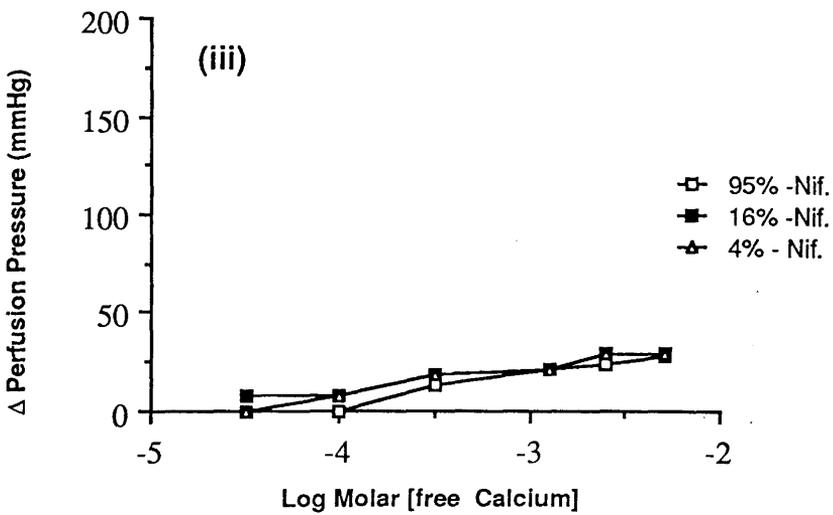
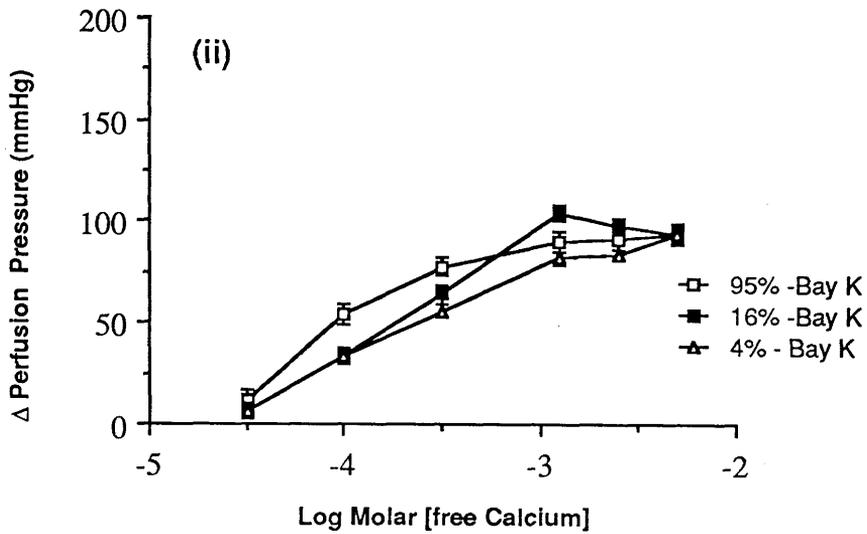
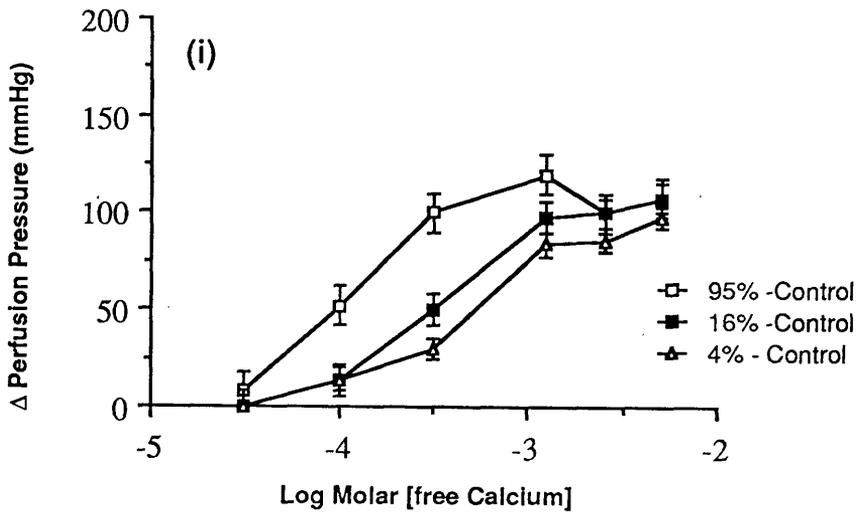
### First Method

#### Variation of $O_2$ , Bay K and nifedipine longitudinally in each tissue

When the oxygen tension was varied sequentially from 95% to 16 % and then 4% for the time controls, it was observed that plot of  $-\log EC_{30}$  values against time (min.) clearly shows that the second CCRC was desensitised. This process continued as the experiment proceeded from 16% to 4%  $O_2$ , though at a slower rate (Fig. 3.1a & b). This was basically the same pattern of results obtained for the control curves for desensitisation at 95% $O_2$  throughout as detailed in Section 2. The situation was the same whether the tissue responses were induced by 3 $\mu$ M NA or 100mM KCl.

The introduction of Bay K 8644 into the saline before the second sequence of variation of oxygen tension (95%, 16% then 4%), temporarily interrupted the course of desensitisation, increasing the level of sensitivity for NA-induced contractions (Fig. 3.2a & b). For KCl-induced responses, only the first curve (for 95% $O_2$ ) was at a higher level of sensitivity than the controls when Bay K 8644 was present in the saline. Variation of oxygen tensions did not alter the continuous fall in the sensitivity of the tissue with time.

Figure 3. 1a. First protocol , NA (3uM): Sequential  $\text{Ca}^{2+}$  CRCs - 3 gas tensions in controls, repeated with Bay K 8644 then nifedipine. (a) Controls - 1st to 3rd curves; order 95%, 16% then 4%  $\text{O}_2$ . Effect of sequential variation of oxygen tensions during the tests for the influence of (b) 4th to 6th curves, as (a) but with Bay K 8644 (0.1uM) and (c) 7th to 9th curves, as (a) but with nifedipine (0.1uM) (in calcium buffered saline). Pressor responses were plotted as % of control (drug-free) maximum i.e. in 95% $\text{O}_2$ . Subsequent curves tended to move rightwards with time and with decreasing oxygen tension.



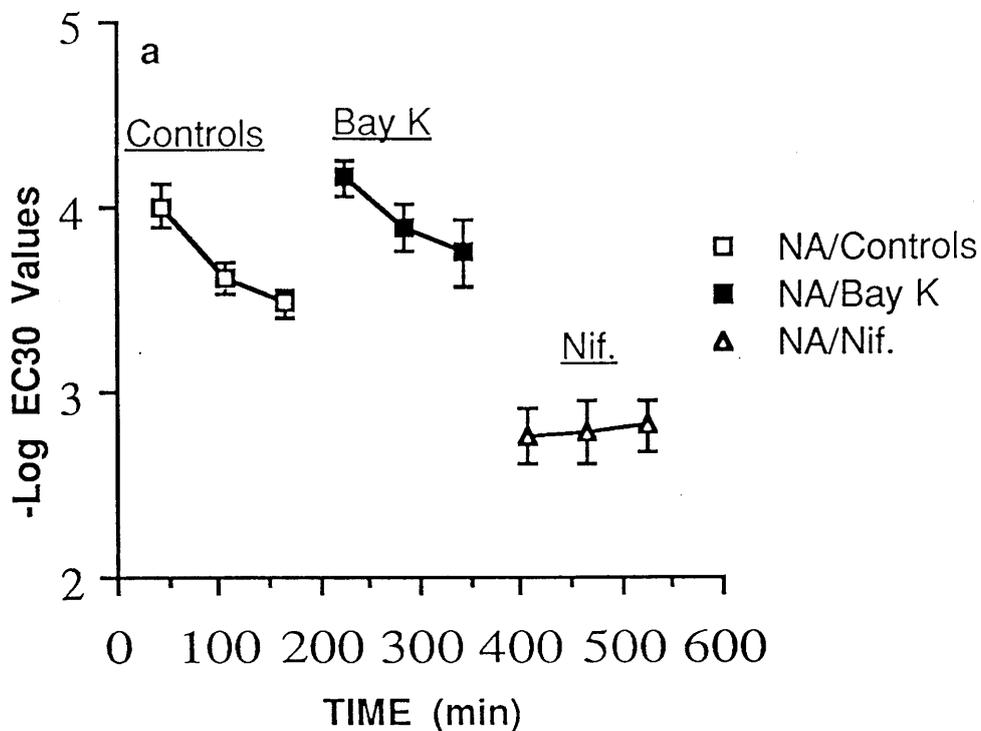
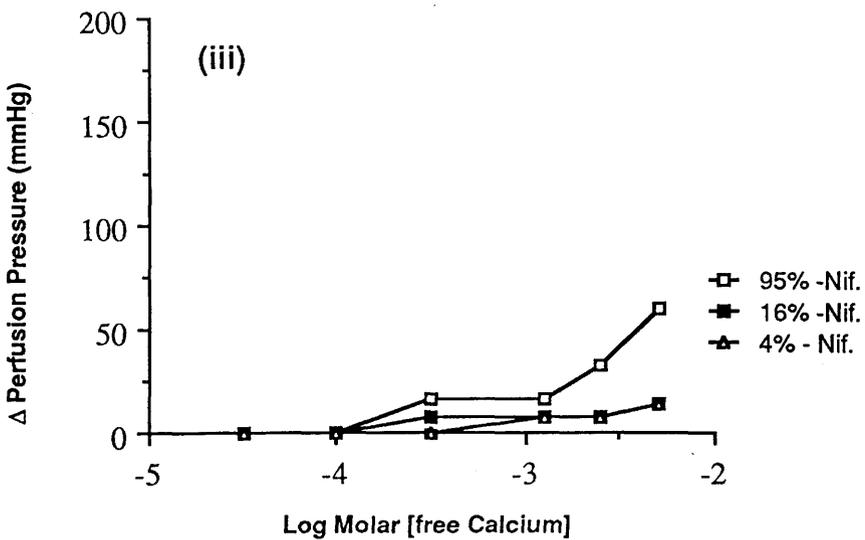
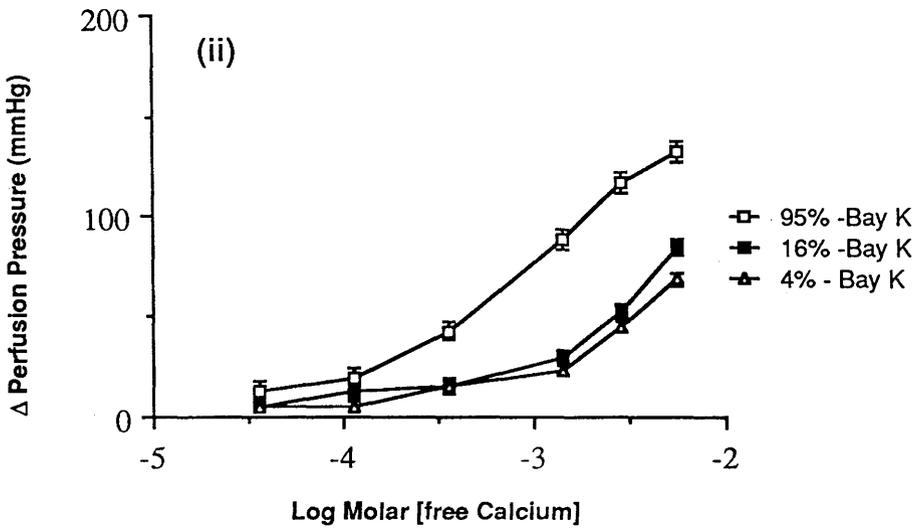
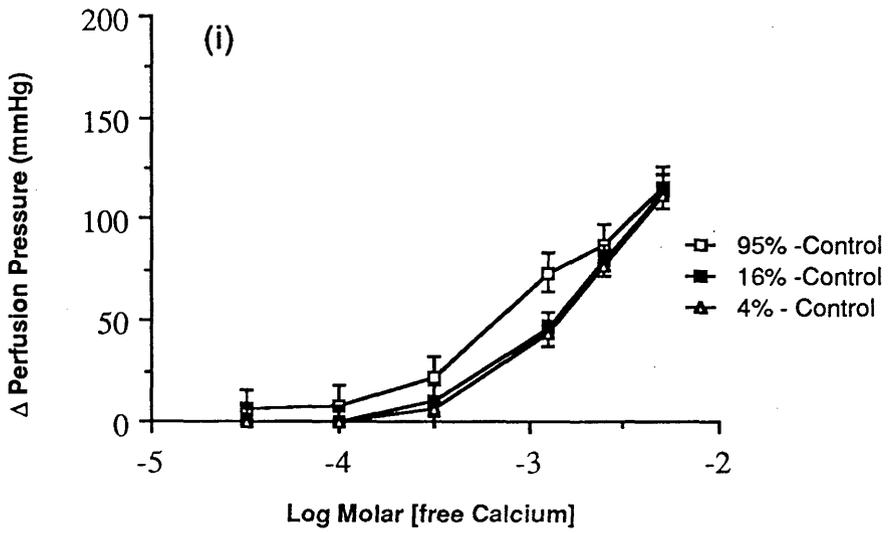


Figure 3. 1b. When the  $-\log EC_{30}$  values were plotted against time, it was clear that desensitisation occurred in the tissue with time, Bay K 8644 (0.1 $\mu$ M) tended to facilitate responses and nifedipine (0.1 $\mu$ M) attenuated them. It was still possible to conclude that within each group of 3 curves, sensitivity declined and thus to correlate this with falling  $pO_2$ . However, reference to Fig. 3.5 shows this to be incorrect.

Figure 3. 2a. First protocol, KCl (100mM): Sequential  $\text{Ca}^{2+}$  CRCs - 3 gas tensions in controls, repeated with Bay K 8644 then nifedipine. (a) Controls - 1st to 3rd curves; order 95%, 16% then 4%  $\text{O}_2$ . Effect of sequential variation of oxygen tensions during the tests for the influence of (b) 4th to 6th curves, as (a) but with Bay K 8644 (0.1uM) and (c) 7th to 9th curves, as (a) but with nifedipine (0.1uM) (in calcium buffered saline). Pressor responses were plotted as % of control (drug-free) maximum i.e. in 95% $\text{O}_2$ . Subsequent curves tended to move rightwards with time and with decreasing oxygen tension.



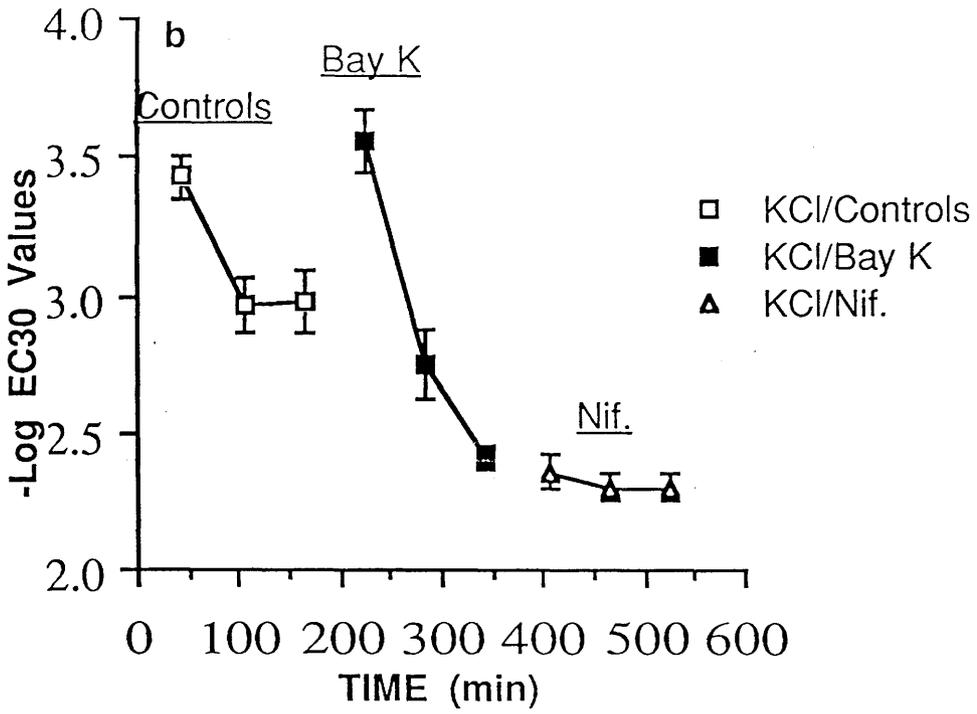


Figure 3. 2b. When the  $-\log EC_{30}$  values were plotted against time, it was clear that desensitisation occurred in the tissue with time, Bay K 8644 tended to facilitate responses and nifedipine attenuated them. It was still possible to conclude that within each group of 3 curves, sensitivity declined and thus to correlate this with falling  $pO_2$ . However, reference to Fig. 3.6 shows this to be incorrect.

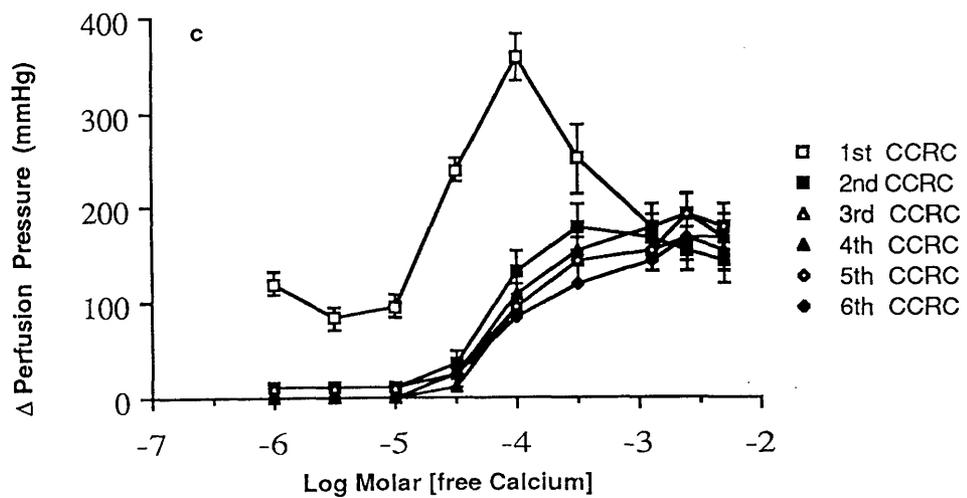
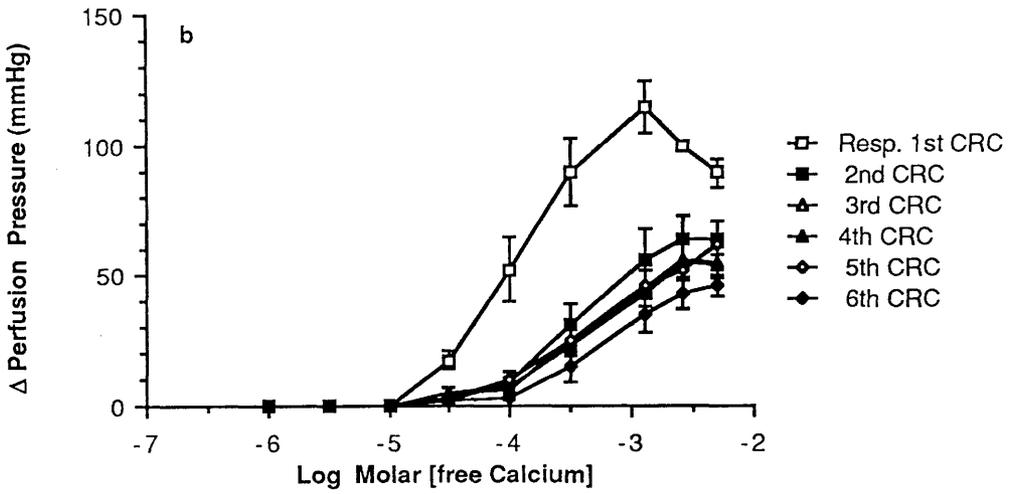
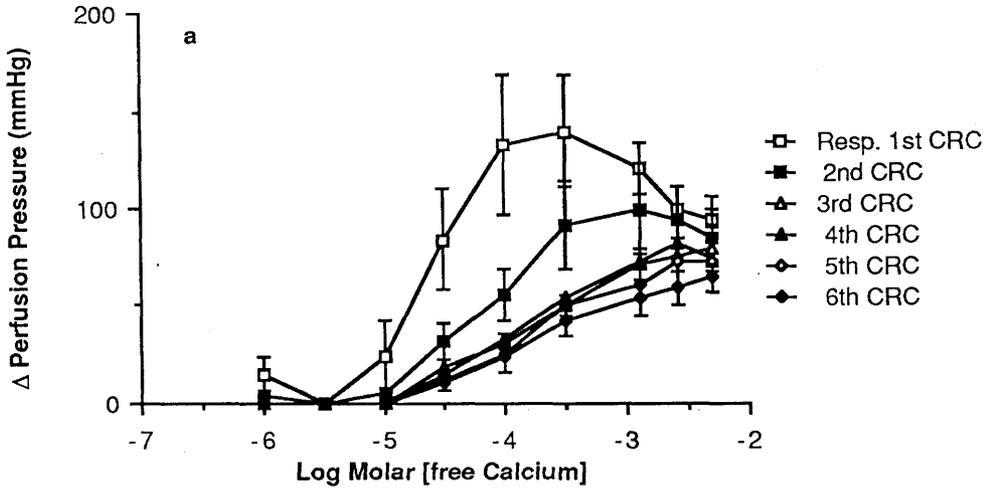
The presence of nifedipine during the third sequence resulted in a reduction of sensitivity to lower than control levels. For the NA-induced responses, there was a tendency for lower oxygen tensions (4% and 16% O<sub>2</sub>) to have a slightly higher level of sensitivity than at a higher oxygen tension (95%O<sub>2</sub>), but the difference was not significant (Fig. 3.1). In contractions induced by KCl, variations of the oxygen tensions had virtually no effect on the influence of nifedipine on the level of sensitivity (Fig. 3.2).

### Second Method

#### Variation of oxygen tension, Bay K 8644, palmitoyl carnitine and nifedipine on separate tissues and analysis of desensitisation

When Bay K 8644 was present during NA or KCl-induced contractions (Figs. 3. 3 a&b), the first CCRC showed significantly greater sensitivity than the subsequent curves, which progressively moved to the right with time. In the presence of palmitoyl carnitine (0.1uM) during NA-induced contractions, the pattern of responses was similar to that with Bay K 8644 (Fig. 3. 3c). Plotting the pressor responses in the presence of either Bay K 8644 or palmitoyl carnitine in 95%O<sub>2</sub> as % of its own maximum (Fig. 3. 4) clearly shows that both Bay K 8644 and palmitoyl carnitine facilitated the responses to

Figure 3. 3. Second protocol: Putative facilitation present throughout. Effect of (a) Bay K 8644 (0.1uM) and (c) palmitoyl carnitine (P.C) (0.1uM) on NA (3uM) induced responses and (b) of Bay K 8644 on KCl (100mM)-induced responses in 95%O<sub>2</sub>. Consecutive concentration-response curves to external calcium (CCRCs) were constructed in calcium buffered saline. Pressor responses were plotted against log free calcium concentration ( $[Ca^{2+}]_o$ ). The sensitivity and maximum response were significantly lower for the subsequent CCRCs than for the controls (1st curve) ( $0.01 < p < 0.05$ ).



similar levels.

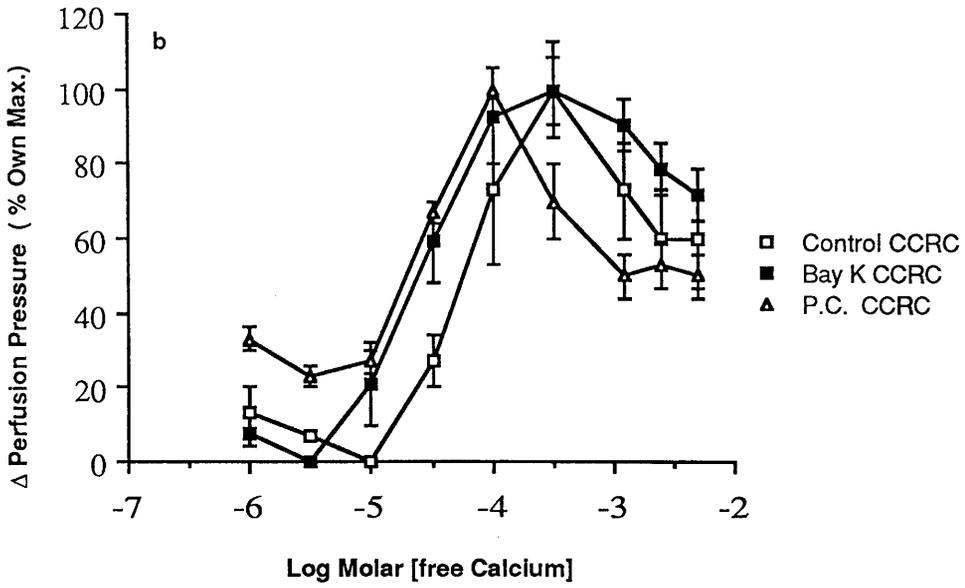
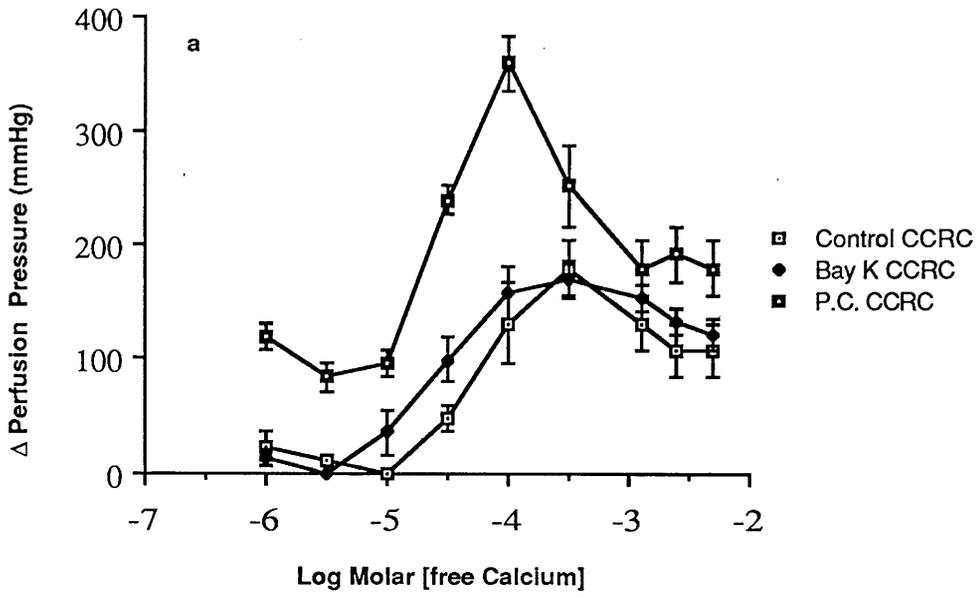
In 95%O<sub>2</sub> Bay K 8644 potentiated the responses induced by NA (Fig. 3. 5a) or by KCl (Fig. 3. 6) throughout the concentration range. A similar result was obtained with palmitoyl carnitine potentiating the NA-induced responses in 95%O<sub>2</sub> (Fig. 3. 7a).

In 16% or 4% O<sub>2</sub> however, there was clear potentiation only at the 1st CCRC except in KCl- induced contractions (Fig. 3.6). For subsequent curves, Bay K 8644 and palmitoyl carnitine (0.1μM each) in 16% O<sub>2</sub> showed the tendency of potentiating responses induced by NA but the difference from the controls was non-significant. In 4% O<sub>2</sub>, fewer CCRCs were facilitated by Bay K 8644 and palmitoyl carnitine than at 95% O<sub>2</sub>. This was illustrated by the plot of the -log EC<sub>30</sub> values against time (Figs. 3. 5 b&c; 3. 7 b&c).

A focus on the 1st curve showed that Bay K 8644 significantly facilitated responses of the 1st curve at 95%, 16% and 4% O<sub>2</sub> in NA-induced contractions (p<0.05) (Fig. 3.8). Palmitoyl carnitine significantly facilitated responses of the 1st curve only at 95% and 4% but not at 16% in NA-induced contractions (p<0.05) (Fig. 3.8).

When the absolute values of the maxima for the 1st CCRC with Bay K 8644 or palmitoyl carnitine were

Figure 3. 4. Comparison of the effects of Bay K 8644 (0.1uM) and (c) palmitoyl carnitine (P.C) (0.1uM) on NA-induced responses (2nd protocol) in 95%O<sub>2</sub>. Only first curves in calcium buffered saline from each experiment are shown. (a) Pressor responses (mmHg) were plotted against log molar free calcium concentration ( $[Ca^{2+}]_o$ ). (b) Pressor responses as % of "its own" maximum within each curve were plotted against log molar free calcium concentration. In each case it was evident that both Bay K 8644 and palmitoyl carnitine facilitated NA-induced responses in the rat tail artery. P.C in addition significantly increased the maximum response (a) ( $p < 0.01$ ).



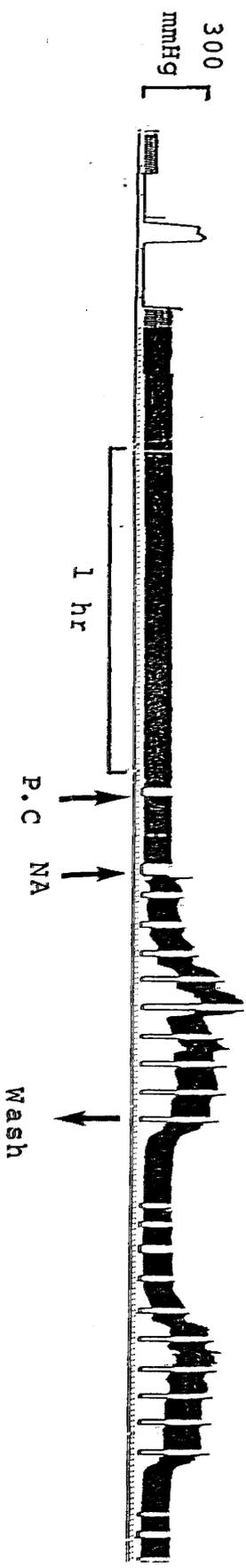


Figure 3.4 c. A tracing of the cumulative calcium concentration response curves in the presence of palmitoyl carnitine (0.1 $\mu$ M). Desensitisation here was as marked as in the controls. In the absence of an activator, e.g. NA, palmitoyl carnitine had no effect and did not alter the baseline pressure in 95% O<sub>2</sub> at 37°C.

Figure 3. 5. Effect of Bay K 8644 (0.1 $\mu$ M) on responses induced by 3 $\mu$ M NA in (a) 95%O<sub>2</sub>; (b) 16%O<sub>2</sub> and (c) 4%O<sub>2</sub>. Consecutive concentration-response curves to external calcium (CCRCs) were constructed in calcium buffered saline. -Log EC<sub>30</sub> values of the pressor responses were plotted against time (min).

In 95%O<sub>2</sub>, Bay K 8644 significantly potentiated the responses for all the 6 CCRCs when compared with the corresponding controls ( $p < 0.05$ ). In 16%, only the 1st EC<sub>30s</sub> was significantly affected by Bay K 8644 than in 95% and 4% O<sub>2</sub> although the first curve showed clear facilitation. This difference can be attributed to the high control values at 16% control, perhaps, left less scope for facilitation.

Nifedipine (0.1 $\mu$ M) introduced at the 7th CCRC in each control, attenuated the already desensitised and stabilised responses leaving them at similar levels of calcium sensitivity.

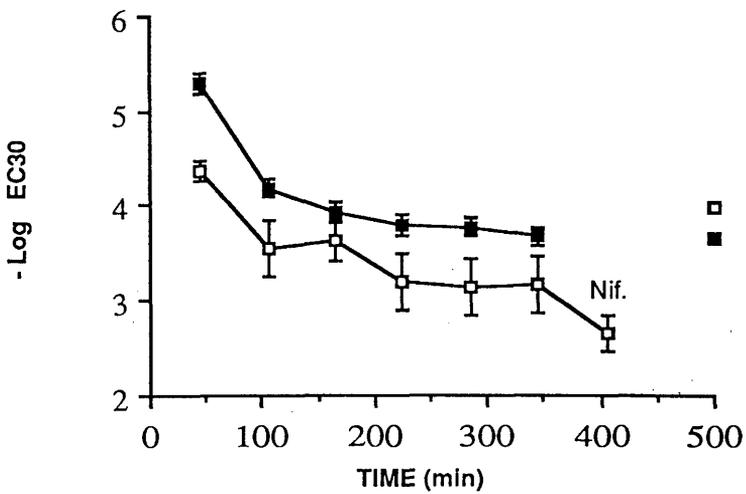
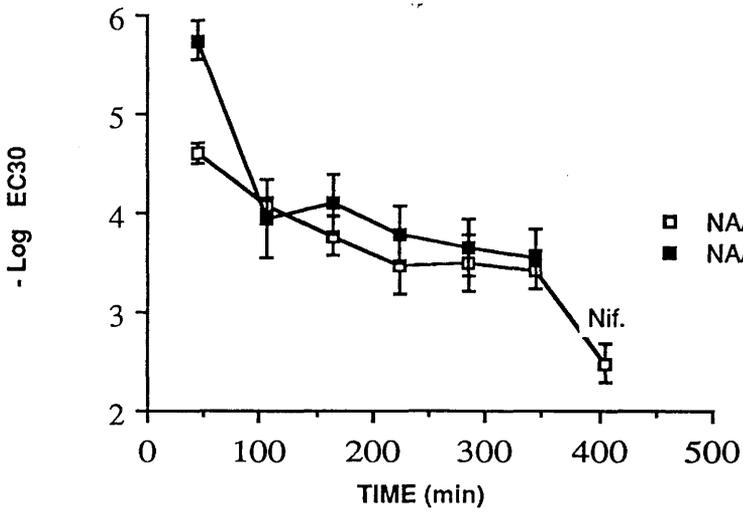
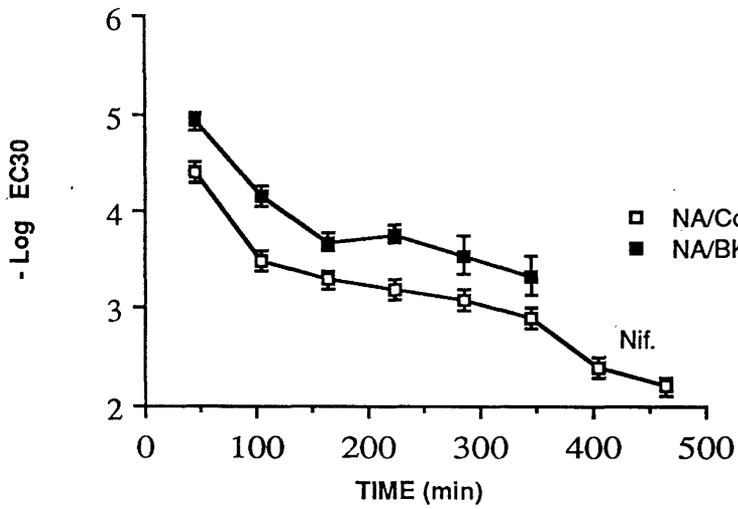
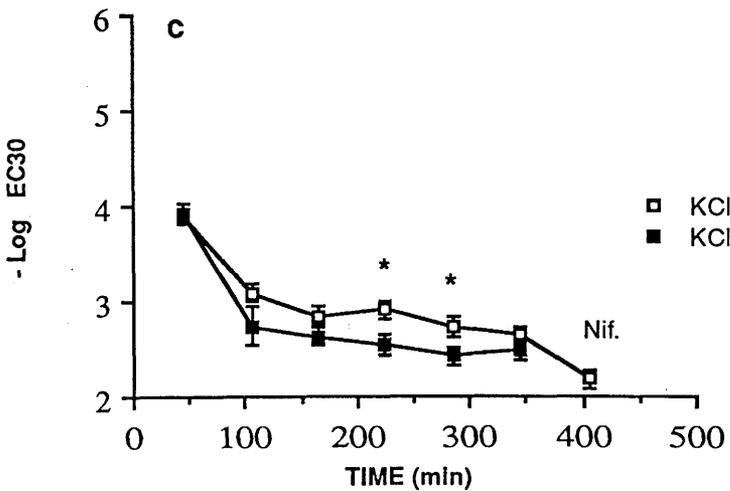
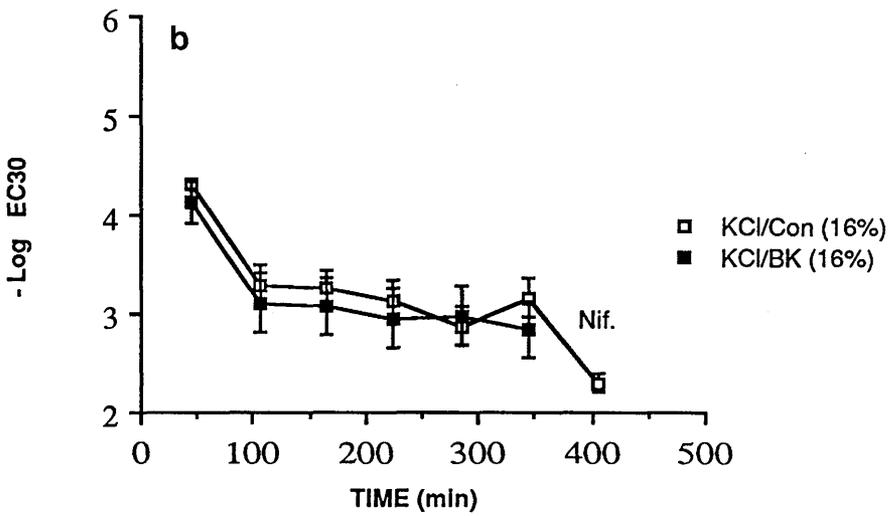
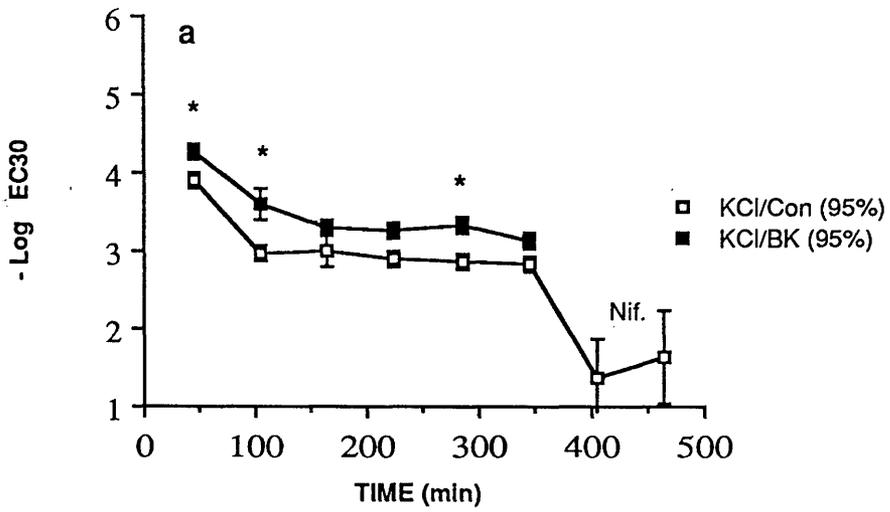


Figure 3. 6. Effect of Bay K 8644 (0.1 $\mu$ M) on responses induced by 100mM KCl in (a) 95%O<sub>2</sub>; (b) 16%O<sub>2</sub> and (c) 4%O<sub>2</sub>. Consecutive concentration-response curves to external calcium (CCRCs) were constructed in calcium buffered saline. -Log EC<sub>30</sub> values of the pressor responses were plotted against time (min).

In 95%O<sub>2</sub>, Bay K 8644 potentiated ( $p < 0.05$ ) the responses only at the CCRCs asterisked when compared with the corresponding controls. In 16% responses were little affected while in 4% they had the tendency of being attenuated rather than being potentiated. However the difference from the control responses was significant at the 4th and 5th CCRCs. .

Nifedipine (0.1 $\mu$ M) introduced at the 7th CCRC in the oxygen level tested, attenuated the already desensitised and stabilised responses to similar level of calcium sensitivity. Asterisks indicate sensitivity which is significantly greater than the corresponding controls ( $p < 0.05$ ).



compared with the controls in 95%, 16% and 4% O<sub>2</sub>, the responses were consistently higher in palmitoyl carnitine than with Bay K 8644 when compared with the controls at each oxygen level (Fig. 3.9). This was particularly so at 95%O<sub>2</sub>. Here the difference between the values for palmitoyl carnitine and Bay K 8644 or controls was highly significant ( $p < 0.001$ ) (Fig. 3.9).

For KCl-induced contractions at 4% and 16% O<sub>2</sub>, the absolute maximum values tended to be higher with Bay K 8644 than in the controls but this did not apply in 95%O<sub>2</sub> where the maximum response in Bay K 8644 appeared to smaller (Fig. 3.9).

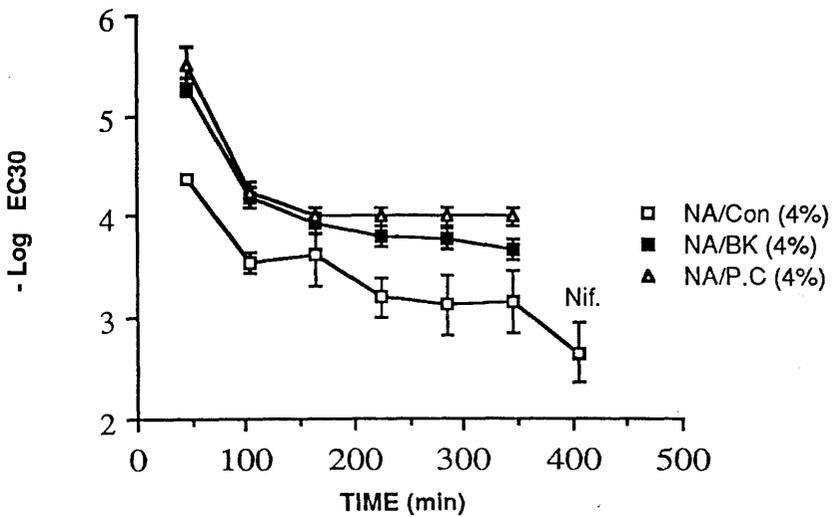
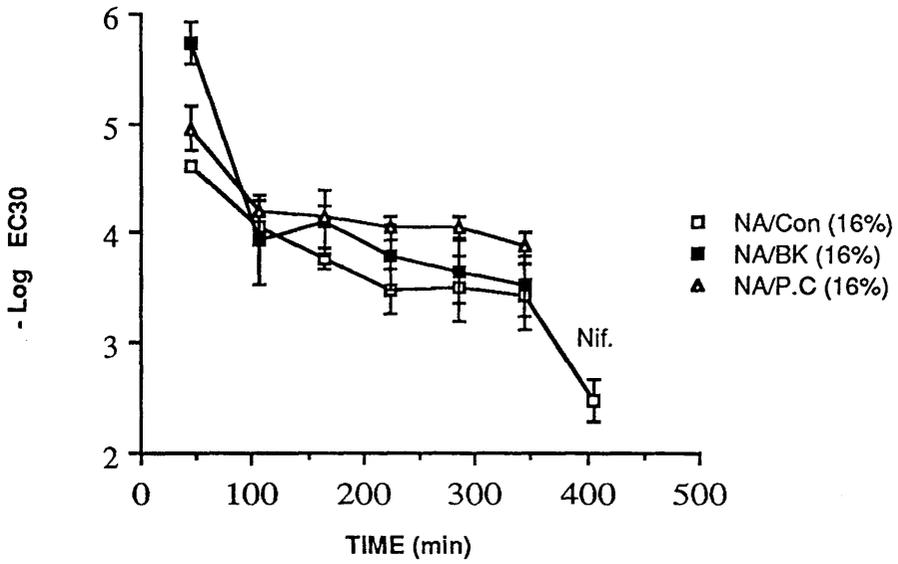
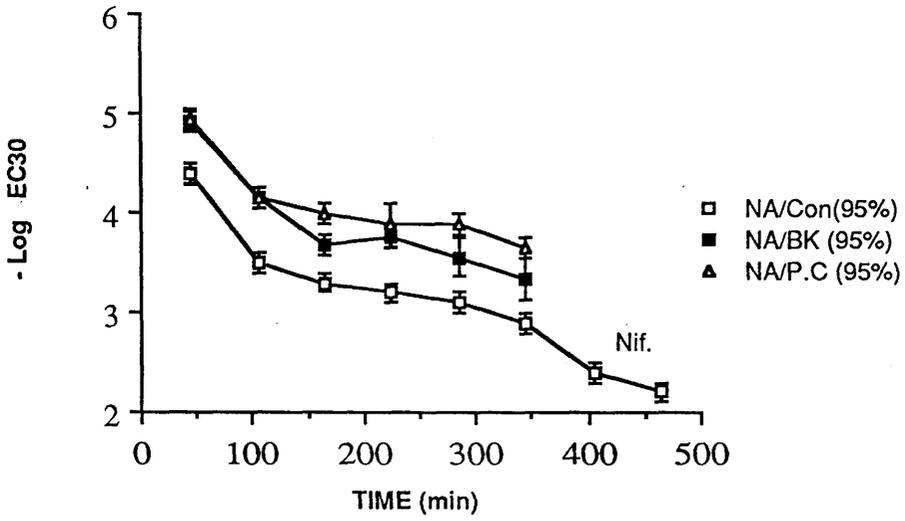
In most of the experiments, as a matter of routine, nifedipine was introduced before the 7th (and 8th) CCRC. In each case nifedipine attenuated the responses of the tissue in 95%, 16% and 4% O<sub>2</sub> (Fig. 3.5; Fig. 3.6; and Fig. 3.7).

In one series of experiments nifedipine was present for the six CCRCs in 95%O<sub>2</sub>. The 1st CCRC (as illustrated by the  $-\log EC_{30}$  values) was significantly depressed ( $p < 0.05$ ) but the subsequent CCRCs were similar to the controls (Fig. 2.18). Because this was the only occasion in which the 1st CCRC and the absolute maximum were significantly reduced more than than the controls, we decided to analyse it in different ways: as percent of the 1st maximum of the controls; as percent

Figure 3. 7. Effect of Palmitoyl carnitine (0.1 $\mu$ M) and Bay K 8644 (0.1 $\mu$ M) on responses induced by 3 $\mu$ M NA in (a) 95%O<sub>2</sub>; (b) 16%O<sub>2</sub> and (c) 4%O<sub>2</sub>. Consecutive concentration-response curves to external calcium (CCRCs) were constructed in calcium buffered saline. -Log EC<sub>30</sub> values of the pressor responses were plotted against time (min).

In 95%O<sub>2</sub>, both palmitoyl carnitine and Bay K 8644 significantly potentiated ( $p < 0.05$ ) the responses for all the 6 CCRCs when compared with the corresponding time controls. In 16% and 4% O<sub>2</sub> fewer EC<sub>30</sub>s were significantly affected by palmitoyl carnitine and Bay K 8644, otherwise the responses in palmitoyl carnitine were essentially the same in 95%, 16% and 4% O<sub>2</sub> giving a smaller shift after the 1st curve. Responses in palmitoyl carnitine were of greater magnitude and were better maintained than in controls (or in Bay K 8644) (see fig. 3.9).

Nifedipine (0.1 $\mu$ M) introduced at the 7th CCRC in the oxygen level tested, attenuated the already desensitised and stabilised responses to similar level of calcium sensitivity.



of maximum within each curve and comparing the  $[Ca^{2+}]$  that will produce fixed pressor response of 50mmHg in the presence and absence of nifedipine. In each case the first CCRC in the presence of nifedipine was significantly ( $p < 0.05$ ) reduced but the subsequent curves were similar to the controls (Fig. 2.18). The introduction of nifedipine during a series of CCRCs whether at the beginning of the 1st CCRC (Fig. 2.18) or the 4th CCRC (Fig. 2.18) or at the 7th CCRC (Fig. 3.5; Fig. 3.6; Fig. 3.7), always significantly reduced sensitivity when compared with the time controls (or the CCRC before the introduction of nifedipine).

Potentialiation of NA-induced responses by Bay K 8644 was greatest with 16%  $O_2$  in the 1st CCRC and least in the subsequent curves. With 4% (except at the 3rd CCRC) and 95%  $O_2$ , the potentialiation by Bay K 8644 was uniform throughout the six consecutive CCRCs for NA (Fig. 3.5). For KCl-induced contractions, the results were different. Here Bay K 8644 potentialiated the responses in 95%  $O_2$  but not 16%  $O_2$  or 4% (significant at the 4th and 5th CCRCs (Figs. 3.6). This was essentially the same observations made with the first protocol (see Fig. 3.2b). This means that in general, whether the effects of various oxygen tensions were tested sequentially on the same tissue or on the first curve of each tissue, Bay K 8644 potentialiated KCl-induced responses only in 95%  $O_2$  but attenuated them in 16% and 4%  $O_2$  only in the 1st series.

Figure 3. 8. Comparison of the effects of Bay K 8644 (0.1 $\mu$ M) and palmitoyl carnitine (0.1 $\mu$ M) on the 1st CCRC of NA-induced responses in 95%, 16% and 4% O<sub>2</sub>. Consecutive concentration-response curves to external calcium (CCRCs) were constructed in calcium buffered saline. (a) -Log EC<sub>30</sub> values for the pressor responses were plotted against oxygen levels (95%, 16% and 4%). When compared with the time controls, both Bay K 8644 and palmitoyl carnitine significantly facilitated the responses to about the same extent at 95% and 4% (0.1 < p < 0.05) but Bay K was more effective than palmitoyl carnitine at 16%. Essentially the effects were similar in the three oxygen tensions.

(b) In responses induced by KCl (100mM) in the 1st CCRC, Bay K 8644 potentiated responses in 95%O<sub>2</sub> (p < 0.05) while in 16% and 4% O<sub>2</sub>, the responses tended to be greater in the presence of Bay K 8644 than in the time controls but the difference was not significant. The effects of palmitoyl carnitine were not tested on KCl-induced contractions.

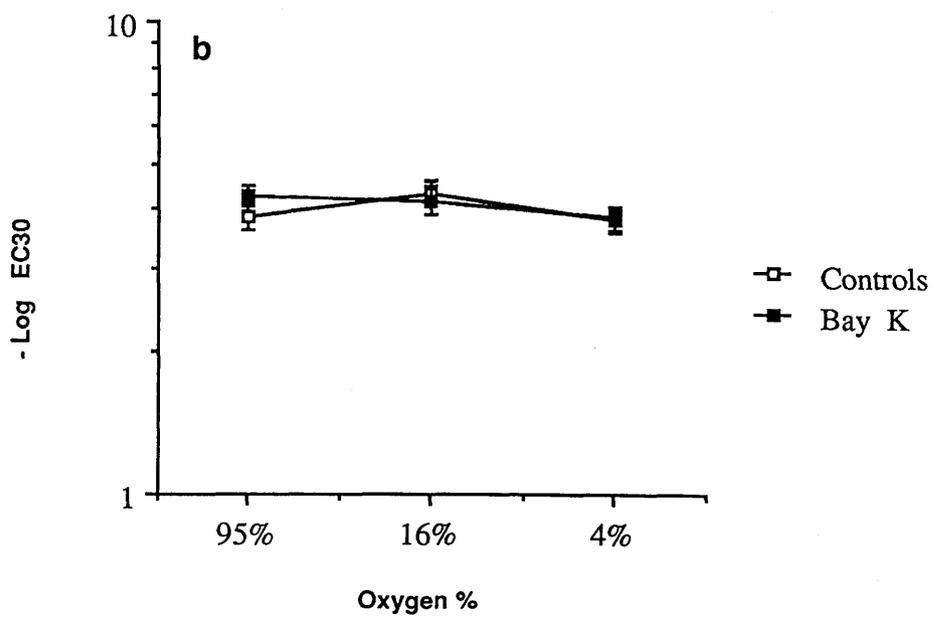
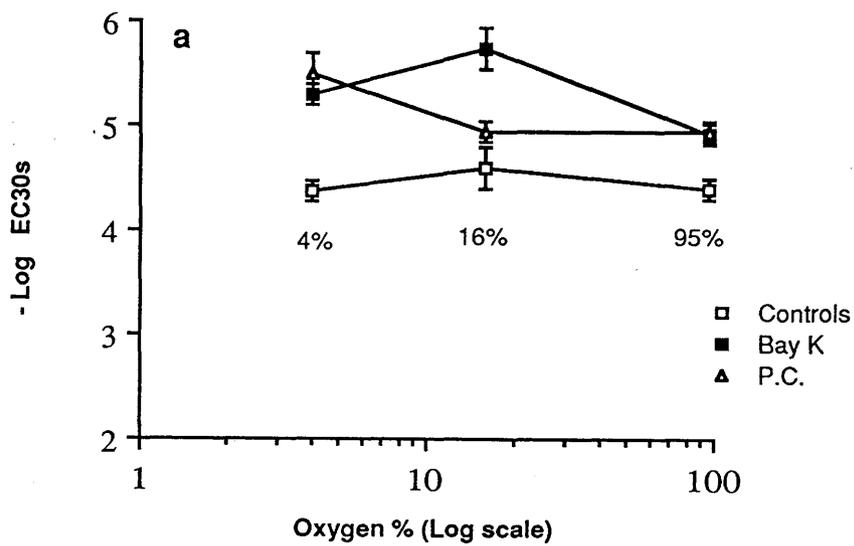
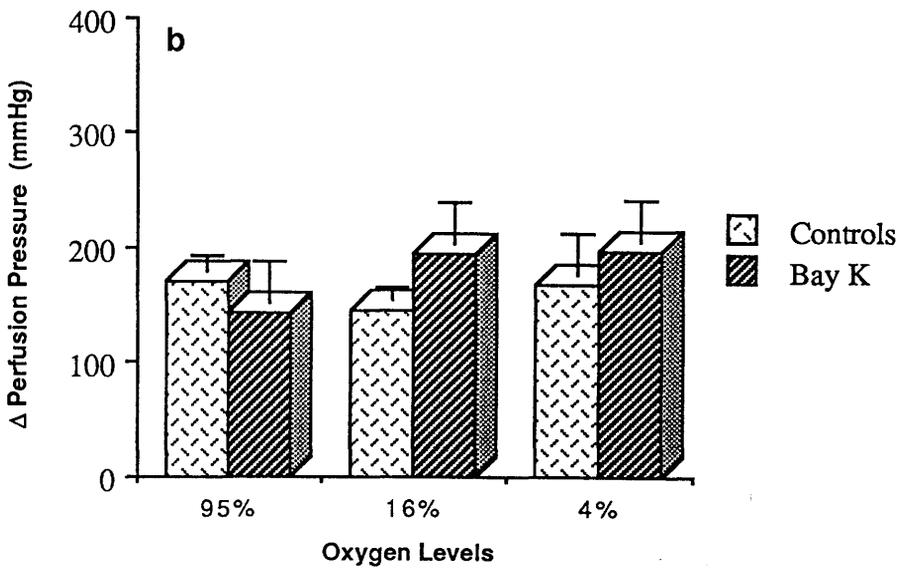
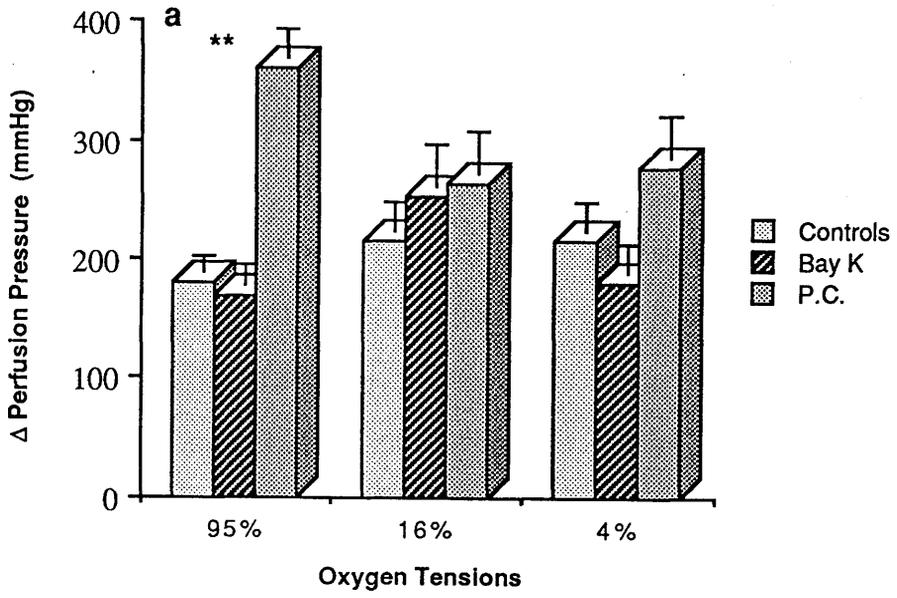


Figure 3. 9. Comparison of the effects of Bay K 8644 (0.1 $\mu$ M) and palmitoyl carnitine (0.1 $\mu$ M) on the maximum responses of the 1st CCRC of NA-induced responses in 95%, 16% and 4% O<sub>2</sub>. Consecutive concentration-response curves to external calcium (CCRCs) were constructed in calcium buffered saline. (a) Pressor responses were plotted against oxygen levels (95%, 16% and 4%). When compared with the controls, responses in palmitoyl carnitine was of greater magnitude and were better maintained. The maximum response of P.C in 95% O<sub>2</sub> was significantly greater than the controls (0.01 < p < 0.05). The maximum responses for NA- and KCl-induced contractions in the presence of Bay K 8644 were similar to the controls for the different oxygen tensions tested.



Palmitoyl carnitine (0.1uM), on the other hand facilitated the NA-induced responses to approximately the same extent across the range of oxygen tensions tested. Both the size of responses and the level of sensitivity to calcium were increased when compared with the controls.

Nifedipine (0.1uM) attenuated the responses after the 6th curve in each of the three oxygen tensions in spite of the already lowered sensitivity of the tissue responses to calcium. This was the case for both NA and KCl.

Generally,  $Ca^{2+}$  sensitivity of the NA-induced (3uM) responses was greater than for KCl (100mM) for both time controls and in the presence of Bay K 8644 (Figs. 17-21). Responses were essentially of the same maximum magnitude and degree of desensitisation in both the calcium buffered and unbuffered salines and in the presence or absence of Bay K 8644 (Fig. 3.1a; 3.2a).

## DISCUSSION

From the results of the present study, it appears that over the range tested oxygen tension had no substantial effect on the  $\text{Ca}^{2+}$  sensitivity of the tail artery response to NA or KCl and neither did it influence the effects upon these of Bay K 8644, palmitoyl carnitine or nifedipine. With calcium-buffered saline the results were more consistent than with unbuffered saline and it was possible to more accurately quantify the free calcium concentration in the saline than in the unbuffered saline.

When  $\text{Ca}^{2+}$  channels open in response to an appropriate change in membrane potential, they allow  $\text{Ca}^{2+}$  ions to move down their electrochemical gradient into the cytoplasm. This inflow of  $\text{Ca}^{2+}$  not only transfers depolarizing charge into excitable cells but also carries a specific message to be decoded by  $\text{Ca}^{2+}$  receptor proteins. The signal leads to the initiation of contraction in heart and smooth muscle cells, transmitter release from nerve cell synaptic terminals, hormone secretion by gland cells, and other important cellular responses (Hagiwara & Byerly, 1981, 1983; Reuter, 1983; Tsien, 1983). In linking membrane potential changes to a messenger substance,  $\text{Ca}^{2+}$  channels perform a function that is vital and possibly unique (Tsien et al, 1983, Hille, 1984). Fox et al (1986), suggested that  $\text{Ca}^{2+}$  channels in a variety of

cell types share the ability to express different modes of gating behaviour; shifts between these gating modes can be an important mechanism in modulatory effects of neurochemicals and drugs.

Bay K 8644 mainly increases the mean open time of the calcium channel (Hess et al, 1984; Kokubun & Reuter, 1984; Ochi et al, 1984), resulting in an increased probability of degree of openness in the voltage ranges normally covered by such experiments. Different models have been put forward to explain the opposite actions of different 1,4-dihydropyridines. The most popular was introduced by Fox et al (1986), who has proposed three so-called "gating modes" to explain these effects. The modes are characterized by their different single channel calcium current behaviour. Mode 0 is characterized by tracings without calcium currents, mode 1 by tracings with short openings, and mode 2 by long-lasting openings. In general, dihydropyridines are proposed to bind preferentially to one mode, stabilising the channel in this mode. Under this interpretation, a calcium antagonist is a compound that stabilises the calcium channel in mode 0, whereas a calcium agonist does so with mode 2. Mode 2 might therefore predominate when Bay K 8644 facilitates responses in the rat tail artery.

It is now widely accepted that the dihydropyridine-receptor is an integral subunit of the calcium channel

(Schramm et al, 1986). The chemical similarity between calcium agonist and antagonist dihydropyridines and their pharmacological profile suggest that these compounds also bind to the dihydropyridine (DHP) receptor. Calcium agonists are exemplified by Bay K 8644 and CGPP 392. These compounds increase cardiac contractility and divalent cation influx even though they are very similar in structure to nifedipine and other DHP  $\text{Ca}^{2+}$  antagonists. These new nifedipine analogues have vasoconstrictor and positive inotropic properties: Bay K 8644 (Schramm et al, 1983) and CGP 28392 (Erne et al, 1984). Important differences in molecular conformation may help to explain why such similar compounds produce seemingly opposite effects (Fox et al, 1986).

It was observed that in the first series Bay K 8644 did not potentiate the responses in 95% $\text{O}_2$  throughout the concentration-response curve. This applies to both NA and KCl responses. The results resemble the findings of Schramm et al. (1985) in which they observed in KCl-depolarized tissue gassed with 95%  $\text{O}_2$ , that Bay K 8644 failed to potentiate the responses throughout the concentration-response curve and that Bay K 8644 actually attenuated the responses as the  $[\text{Ca}^{2+}]$  increased beyond 1.25mM. One explanation for this could be that calcium overload occurs and is exacerbated at high  $\text{Ca}^{2+}$ . In the present study responses in high  $\text{Ca}^{2+}$  were not actually smaller than in controls but

responses were sharply reduced compared with lower  $\text{Ca}^{2+}$  levels. This would be consistent with potentiation of  $\text{Ca}^{2+}$  entry throughout the  $[\text{Ca}^{2+}]$  range with enhanced overload at high  $[\text{Ca}^{2+}]$ .

Sanguinetti and Kass (1984), showed that the effects of Bay K 8644 are voltage dependent and that it normally behaves like a calcium agonist, but it has calcium antagonist effects under partly depolarized conditions. Another possible explanation has been offered for the antagonist action of Bay K 8644 (Schramm et al, 1986). They suggested that all dihydropyridines shift the open probability curve of the single calcium channel in a dose-dependent manner to more negative potentials, which is probably due to a prolongation of the mean open time. Increasing the probability of the channel being open at a given membrane potential enhances the probability that the calcium channel will inactivate. The main difference between calcium agonists and calcium antagonists lie in the efficacy of shifting the open probability curve to the left. The stronger this shift, the more channels inactivated, and the greater is the calcium antagonist action of a compound. This would explain why calcium modulating dihydropyridines generally have a dose range in which they show calcium agonist behaviour, and another one in which they act as calcium antagonists. This might well be the case with the rat tail artery preparation but it does not help to explain the present results unless there is a difference in membrane

potential caused by increasing  $[Ca^{2+}]$  above 1mM, which seems unlikely.

The  $Ca^{2+}$  sensitivity of NA-induced contraction was clearly potentiated by Bay K 8644 at each of the  $O_2$  tensions tested (see first CCRCs in fig. 3.5). This suggests that the  $Ca^{2+}$  channels and their modulatory structure are fully functional even at the lowest  $O_2$  tensions of 4% which produces mildly hypoxic conditions ( $PO_2 = 32-36$ mmHg). The only change from this pattern which was observed was the similarity of the  $pEC_{30S}$  in controls and Bay K 8644 in 16% $O_2$ , after the first CCRC. This seemed to be due to a reduced desensitisation in controls. It does not affect the present interpretation, and we have not pursued it further, but clearly, had we chosen 16% $O_2$  for testing Bay K 8644, and had not relied upon the first curve, we might have gained the erroneous impression that Bay K 8644 was ineffective versus NA.

In contrast, and perhaps surprisingly, Bay K 8644 had less marked effects versus KCl-induced contraction. Only at 95% $O_2$  was a clear facilitation found at the first CCRC. At 4% $O_2$  the Bay K 8644-treated tissues were actually less sensitive to  $Ca^{2+}$  after the first curve. It is possible that the potential-operated  $Ca^{2+}$  channels, and indeed the membrane potential itself might be affected by the oxygen tension in such a way that the above mentioned antagonist effect of Bay K 8644 becomes

apparent. If there is such an effect, and we would not stress this too strongly on the present evidence, then it is specific to channels activated by KCl rather than NA (see above).

This interpretation is based on results from the second protocol (comparison of first curves). The data from the first protocol (longitudinal study) does, however, fit well with this. Figure 3.1b shows the clear facilitation by Bay K 8644 of responses to NA at all O<sub>2</sub> levels. Figure 3.2b shows in contrast, that while there is clear evidence at 95%O<sub>2</sub> of facilitation of KCl-induced responses, there was little sign of this at 16% or 4%O<sub>2</sub>.

Palmitoyl carnitine (P.C) was tested using only the second method and NA. With P.C, the Ca<sup>2+</sup> sensitivity of the tissue was potentiated: it increased the -log EC<sub>30</sub> by 0.5 to 1.0 log units for each curve in the series. This was essentially similar at 95%, 16% and 4% O<sub>2</sub> except that at 16% the controls tended to be more sensitive while sensitivity in palmitoyl carnitine was the same as at 95%O<sub>2</sub> giving a smaller shift. However, P.C responses were of greater magnitude and were better maintained than in controls or in Bay K 8644. Thus although P.C did not prevent the relative loss of sensitivity to extracellular Ca<sup>2+</sup> which produces desensitisation, even after desensitisation, responses in P.C were at least as large as in non-

desensitised controls. This suggests that P.C exerts other intracellular effects on  $\text{Ca}^{2+}$  mobilisation or efficiency of contraction which can offset the effects of  $\text{Ca}^{2+}$ -overload and produce greater facilitation than would  $\text{Ca}^{2+}$ -channel facilitation alone.

In general, both palmitoyl carnitine (P.C) and Bay K 8644 increase sensitivity to  $\text{Ca}^{2+}$  judged by the  $\text{pEC}_{30}$ ; P.C also increases maximum in CCRC but not at physiological  $[\text{Ca}^{2+}]$ ; all these are clear at 4%  $\text{O}_2$  which is mildly hypoxic but not really ischemic; and P.C therefore is a potential endogenous facilitator of  $\text{Ca}^{2+}$  sensitivity in vascular smooth muscle, possibly in ischemia.

From these results, it can be said that P.C can facilitate pressor responses to NA even in mildly hypoxic conditions and that this is partly attributable to facilitation of  $\text{Ca}^{2+}$  channel function. In addition, P.C and Bay K 8644 increase the sensitivity to  $\text{Ca}^{2+}$  in a similar manner. P.C also increases the maximum but does not retain increased response at physiological  $[\text{Ca}^{2+}]$ . All these effects are clear at 4%  $\text{O}_2$ , a mild hypoxic condition, which is not really what we call proper ischaemia (a condition during which P.C is produced and accumulates).

Our results also showed that the nifedipine-sensitive channels are quite active even after the 6th or 7th

consecutive CCRCs for NA or KCl and using both protocols. This might suggest that the dihydropyridine receptor site are always open for binding and occupation by either nifedipine or Bay K 8644 since any of these two dihydropyridines can respectively produce blockade or facilitation of tissue responses to calcium sensitivity even after the 6th consecutive CCRCs when the tissue sensitivity is already low but fairly stabilised.

The adoption of the two protocols has provided evidence for the involvement of desensitisation of the calcium sensitivity of the rat tail artery. In addition, these experiments provide guidance on some important factors in the design of an experiment to measure sensitivity of smooth muscle to calcium. They show that NA and KCl- induced responses in the rat tail artery are very sensitive to calcium under different oxygen tensions but this can easily be missed if care is not taken to avoid desensitisation. This is better achieved in experiments with calcium-buffered physiological saline than with the calcium-unbuffered saline in which results are less consistent, especially with the 1st protocol. The indeterminate nominally zero calcium level might account for this.

Construction of noradrenaline concentration-response curves in different calcium buffers (1 to 6), produced increasing responses as higher concentrations of free calcium in buffered saline were employed. In the

unbuffered saline, with the range of  $[Ca^{2+}]$  employed it was difficult to distinguish the effects of different concentrations of calcium in both the proximal and distal segments of the vessel. Only responses in zero calcium were prominently smaller. This illustrates again the high  $[Ca^{2+}]_o$  sensitivity of the preparation. From comparison of responses in buffered/unbuffered saline, an estimate of the free  $Ca^{2+}$  level in the "zero Ca" could be made. This is approximately 30uM in the tail artery which is similar and comparable to estimates using the same method in rat anococcygeus (10uM) (McGrath, Miller & Smith, 1984) and in rat portal vein (30uM) (Fasehun, et al, unpublished). The high  $Ca^{2+}$  sensitivity of the preparation clearly makes it impossible to estimate  $Ca^{2+}$  sensitivity accurately without buffering  $Ca^{2+}$ .

An attempt was made to assess any gross effects of the buffers by comparing the sensitivity of the tissue to calcium with and without buffers on the same tissue by switching from buffered saline to the unbuffered and vice versa. This produced variable results, probably because of the reduction of the free calcium concentration of the unbuffered saline by the residual buffers attached to the tissue or to the organ bath (Fig. 2.2).

In subsequent experiments buffered  $Ca^{2+}$  was employed, occasionally reproducing experiments without buffers as a check.

## SECTION IV

# EFFECTS OF TEMPERATURE ON CALCIUM CONCENTRATION-RESPONSE CURVES AND ON DESENSITISATION - COMPARISON WITH OTHER SPECIES

### SUMMARY

1. The effects of temperature on the calcium concentration-response curves and on the desensitisation of the isolated perfused rat tail artery were studied. For comparison, these effects were also studied in thermoregulatory vessels from other species (rabbit and cat): the isolated perfused rabbit central ear artery and the cat distal limb arteries (supplying the paws). The  $\text{Ca}^{2+}$  sensitivity of and the pressor responses to noradrenaline (NA) (and potassium chloride {KCl} in the rat tail only) in  $\text{Ca}^{2+}$ -buffered saline was non-sequentially studied at  $24^{\circ}$ ,  $37^{\circ}$  and  $42^{\circ}\text{C}$ .
2. In each tissue, a single temperature was employed and 6-8 consecutive CCRCs were constructed. In each tissue, sensitivity to  $[\text{Ca}^{2+}]_o$  was similar at  $37^{\circ}\text{C}$  and  $24^{\circ}\text{C}$  and less at  $42^{\circ}\text{C}$  at the first determination, but desensitisation was less marked at  $42^{\circ}\text{C}$  so that the difference declined or, in the case of the rat tail artery, the sensitivity

actually became relatively greater at 42°C than at 24°C or 37°C.

3. Bay K 8644 (0.1μM), which was tested at 37°C, produced consistent potentiation on the rat tail artery but not on the rabbit central ear artery or the cat distal hind limb arteries. Some potentiation occurred in the rabbit ear artery but only on the first concentration-response curve (CRC).
4. Nifedipine (0.1μM) produced an inhibitory effect on Ca<sup>2+</sup> sensitivity on all tissues, at all temperatures, after the sixth curve, even though sensitivity had already declined.
5. In conclusion, the results showed that in the rat tail artery, the rabbit ear artery, and the distal limb artery to the paws of a cat, temperature changes or drugs affecting Ca<sup>2+</sup> channels can be radically different between those perceived when a fresh tissue is first tested and when the same tissue has been previously subjected to activation.

## INTRODUCTION

It has long been recognized that changes in temperature have considerable effects on the tone and sensitivity of vascular smooth muscle. It was shown that arteries from swine and dogs constricted when cooled to between 4° and 6°C and that the arteries were most sensitive to adrenaline at 17°C (Smith, 1952). An increase in tone had also been observed when femoral and ear arteries of the rabbit were cooled from 37° to 3°C (Glover et al. 1968). The ear artery was most sensitive to NA at 24°C, whereas the sensitivity of the femoral artery decreased progressively below 37°C. Wade and Beilin (1970) found that the vascular resistance of the isolated rat tail preparation increased at temperatures below 30°C, and sensitivity to noradrenaline was maximal between 32° and 34°C. They maintained that 34°C was closer to the true tail temperature of rats maintained at an environmental temperature of 22°C. Keatinge (1964) described a decrease in the smooth muscle resting membrane potential in isolated aortic strips as the temperature was reduced. Moderate cooling of canine cutaneous veins was reported to increase the contractile responses evoked by NA or adrenergic nerve stimulation (Vanhoutte et al, 1970a,b) and receptor reserve theory had been involved in the explanation of this phenomenon (Flavahan et al, 1985). There is therefore considerable evidence for radical changes in vascular smooth muscle responses by

temperature changes. However, the influence of desensitisation and sensitivity to calcium and Bay K 8644 and/or nifedipine under these conditions are not known. The present study aimed at investigating the effects of temperature on calcium concentration-response curves and on desensitisation and comparisons of these with those in other species.

In this study, special attention has been paid to desensitisation processes, which we have now fully established to be operating quite significantly in the isolated perfused rat tail artery. In general, vascular smooth muscle preparations are pre-exposed several times to an activating agent or to field stimulation until the responses are reproducible (see Aoki & Asano, 1986). In such studies the first concentration-response curve (CRC) or the first few responses are generally disregarded and are, therefore, never used for the analysis of data. Since desensitisation appears to occur in most vascular smooth muscle preparations, e.g. in the rat tail artery (McGrath, Miller & Ugwu, 1987; Ugwu, 1987) and guinea-pig ileum (Gaddum, 1953; Holzer & Petsche, 1983), and seems to depend on  $[Ca^{2+}]_o$  ([free  $Ca^{2+}$ ]), we have compared the first with the subsequent ' $Ca^{2+}$  CRCs' to assess the value of the 1st responses in an analysis of the properties of various thermoregulatory blood vessels at different temperatures and in the presence or absence of Bay K 8644 or nifedipine.

## Experimental protocol

The methods used in the present section of this study for the preparation of the tissue for recording and for the determination of perfusion pressures were the same as with our earlier studies on desensitisation in Section Two as detailed under General Methods section. The parts of the experimental protocol which were specific to this section are however summarised below.

Lengths (1-2cm) of the proximal segment of rat tail artery (male Wistar, 300-350gm); central ear artery of New Zealand rabbit (2-3kg) and the arteries leading to the paws of the fore- and hindlimbs of cats (2-4kg), were perfused at 2-3ml/min with a Krebs' bicarbonate saline ( $[Ca^{2+}]$  2.5mM,  $PO_2$  580-650mmHg, pH 7.2-7.3 at  $37^\circ C$ ), and immersed in a similar medium. Perfusion pressure indicated vasoconstrictor responses. CRCs for  $Ca^{2+}$  were constructed by reducing  $[Ca^{2+}]_o$  to 1uM then activating with noradrenaline (NA) (3uM) with or without Bay K 8644 (0.1uM) or nifedipine (0.1uM) at  $24^\circ C$ ,  $37^\circ C$  or  $42^\circ C$ .  $[Ca^{2+}]_o$  was increased in steps from 1uM to 5mM using  $Ca^{2+}$  buffers (NTA {nitrilotriacetic acid} and EGTA {2.5mM of each}). At least 6 curves were determined in each tissue, separated by 15min "rest" in 1uM  $[Ca^{2+}]_o$ . This led to a steady decline in sensitivity to  $[Ca^{2+}]_o$  shown by a rise in the pEC30 (-log concentration of  $Ca^{2+}$  producing 30% of maximum response in the 1st curve).

## RESULTS

### Effects of 24°C

At the first determination at 24°C, sensitivity to  $[Ca^{2+}]_o$  was similar for all the three types of tissues. In the subsequent determinations, up to the 4th CCRCs, the level of sensitivity to calcium was the same in both the cat and the rabbit arteries while that of the rat tail artery was significantly lower (Fig. 4.1, 4.8). This suggests that the tail artery was more sensitive to desensitisation at 24°C than either the cat vessel or the rabbit ear artery. By the 6th CCRC however, the level of sensitivity in the cat artery had significantly dropped below those of either rat or rabbit arteries (Fig. 4.8a). In general, desensitisation was observed least in the rabbit ear artery during a prolonged experimental protocol when compared with its occurrence in the rat or cat arteries.

### Effects of 37°C

At 37°C, the calcium-sensitivity of the three types of arteries were the same at the first determination. For the subsequent curves, up to the 6th, the level of sensitivity in the rat tail artery significantly decreased when compared with those of the cat or rabbit

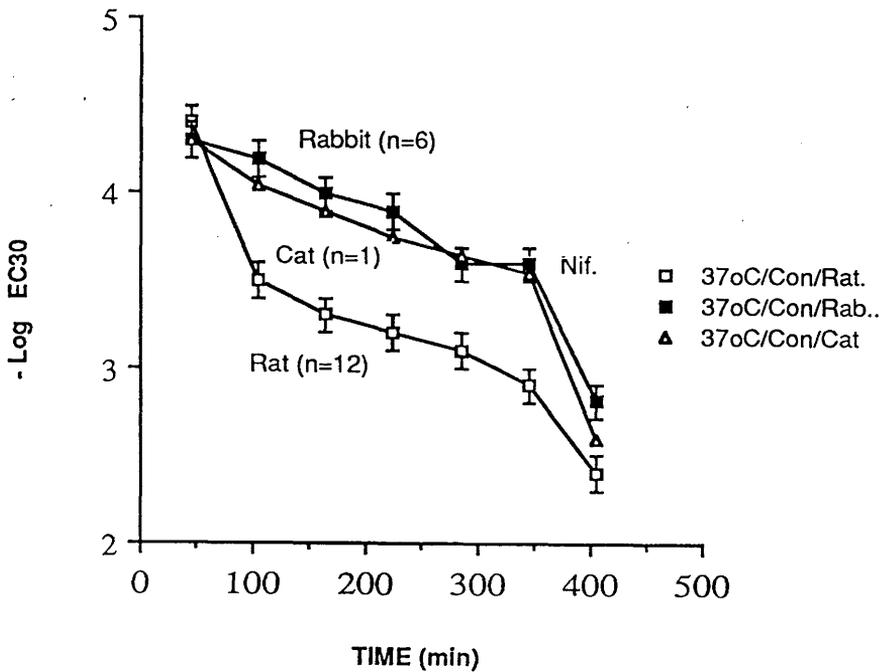


Figure 4. 1. Comparison of the control values for  $\text{Ca}^{2+}$ -sensitivity ( $-\log \text{EC}_{30}$ ) against time (min) for consecutive noradrenaline (NA)-induced contractions in rat, rabbit and cat perfused arteries at  $37^{\circ}\text{C}$ . Nifedipine ( $0.1\mu\text{M}$ ) was present for the 7th CRC. Points shown are mean  $\pm$  S.E.M. ( $n=12, 6$  &  $1$  respectively). Apart from the 1st and 7th (nifedipine effect) CCRCs the sensitivity was significantly greater for the rabbit and cat than for the rat ( $0.01 < p < 0.05$ ).

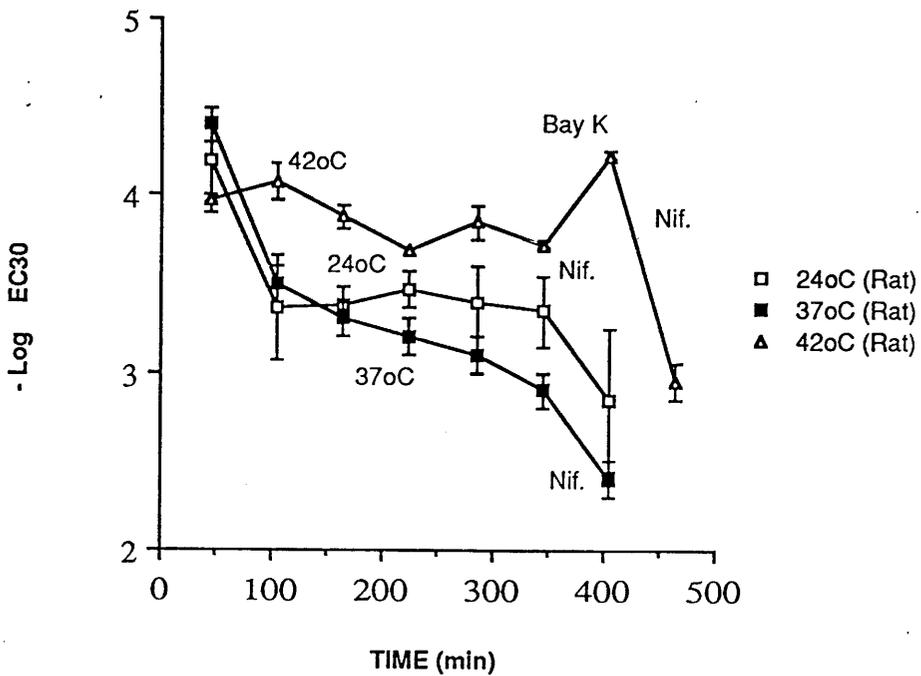


Figure 4. 2. Comparison of the calcium-sensitivity NA-induced contractions of the isolated perfused rat tail artery, at 24, 37 and 42°C, when calcium was cumulatively re-added. Points shown are mean  $\pm$  S.E.M. (n=12). Apart from the 1st determination the sensitivity at 42°C was significantly greater than at 24°C or 37°C ( $p < 0.05$ ).

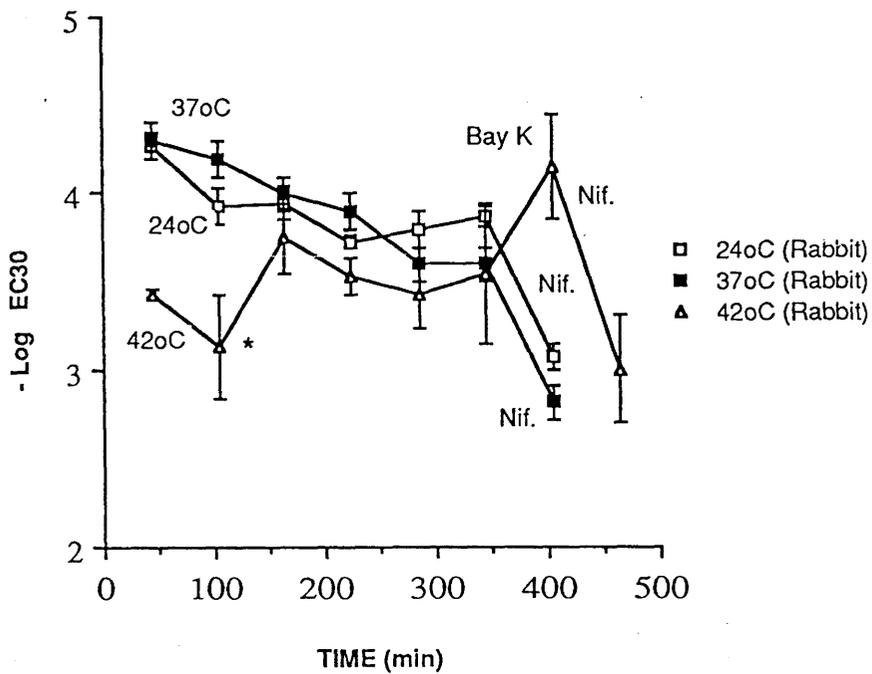


Figure 4. 3. Comparison of the calcium-sensitivity NA-induced contractions of the isolated perfused rabbit ear artery, at 24, 37 and 42°C. Calcium was cumulatively re-added. Points shown are mean  $\pm$  S.E.M. (n=6). The sensitivity at the temperatures were similar except for the 1st two CCRCs at 42°C which were significantly smaller than at 24°C and 37°C (p<0.0%).

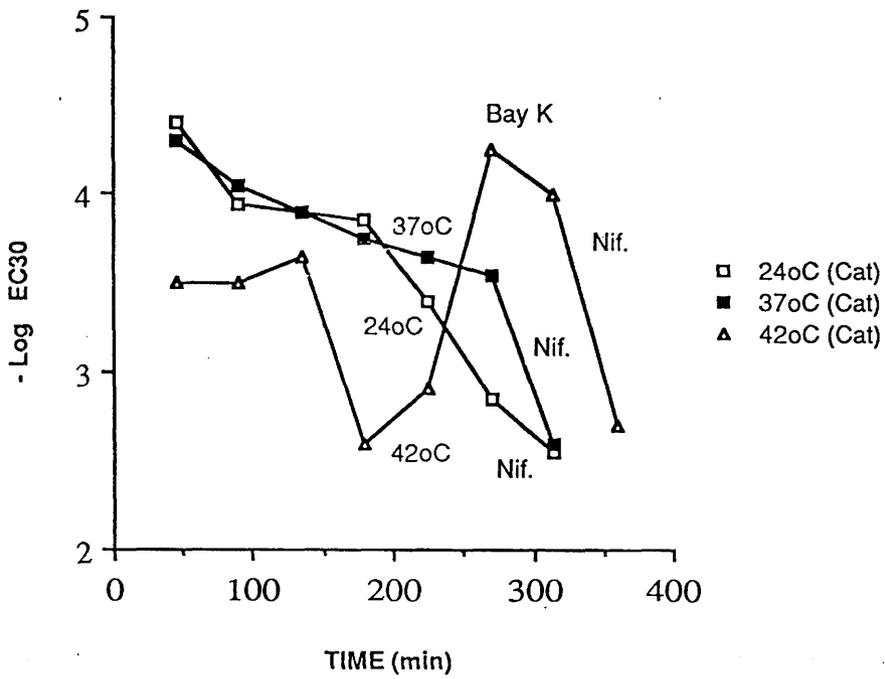


Figure 4. 4. Comparison of the calcium-sensitivity of NA-induced contractions of the isolated perfused distal hindlimb artery which leads to the paws of the cat, at 24, 37 and 42°C. Calcium was cumulatively re-added during experiment. Points shown are mean  $\pm$  S.E.M. (n=1).

(Fig. 4.8). Desensitisation was therefore a more significant feature of the rat tail artery at 37°C than of the cat or rabbit arteries (Fig. 4.4a). The extent of desensitisation in the cat and rabbit arteries was the same throughout the prolonged experimental protocol. There was, therefore, no substantial difference between 24° and 37°C (Fig. 4.8).

### Effects of 42°C

At 42°C, the sensitivity to calcium of the three tissues started off at a lower level but showed less desensitisation. At the first and second determinations, the levels of sensitivity of the cat and rabbit arteries were both lower than those of the rat tail artery (Fig. 4.8). By the 3rd determination, their sensitivities were similar. By the 4th and 5th determinations, the sensitivity in the cat artery dropped below those of the rat and rabbit arteries. At the 6th determination however, the sensitivity was completely restored (Fig. 4.8).

### Effects of Bay K 8644 at 37°C

#### (i) Rat Tail Artery

Bay K 8644 (0.1µM) which was tested at 37°C, produced consistent potentiation in the rat tail

Figure 4.4b. A comparison of the original representative tracings of the calcium concentration response curves in the (i) rat tail artery, (ii) rabbit ear artery and (iii) the distal hindlimb artery of the cat that leads to the paws at 37°C in 95% O<sub>2</sub>. Desensitisation was more pronounced in the rat than in either the rabbit or the cat.

1 hr



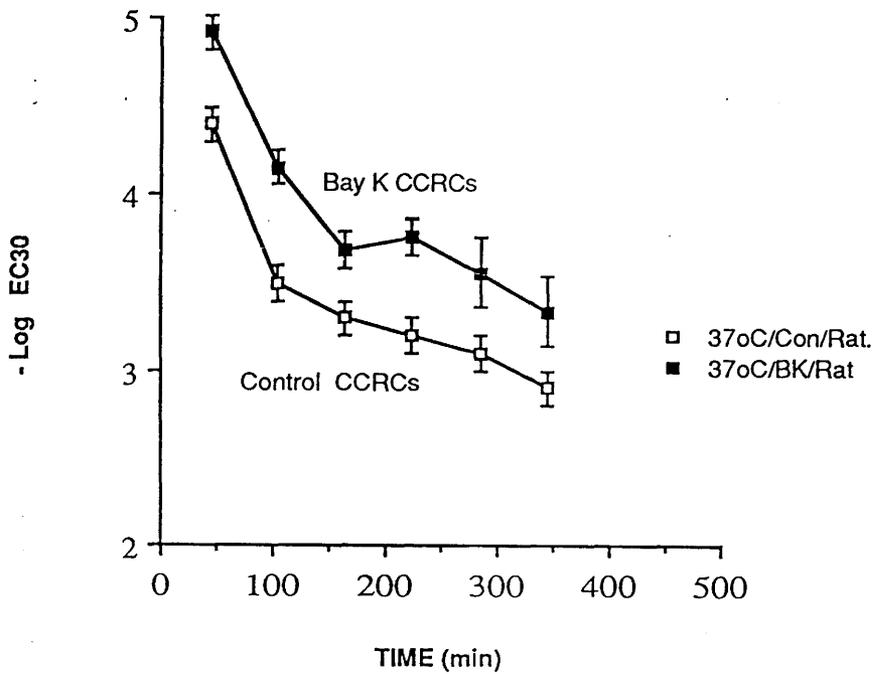


Figure 4. 5. Influence of Bay K 8644 (0.1 $\mu$ M) on the calcium-sensitivity of NA-induced contractions of the isolated perfused rat tail artery, at 37°C, when calcium was cumulatively re-added. Points shown are mean  $\pm$  S.E.M. (n=12). The sensitivity for each CCRC was significantly greater than the corresponding controls (p<0.05).

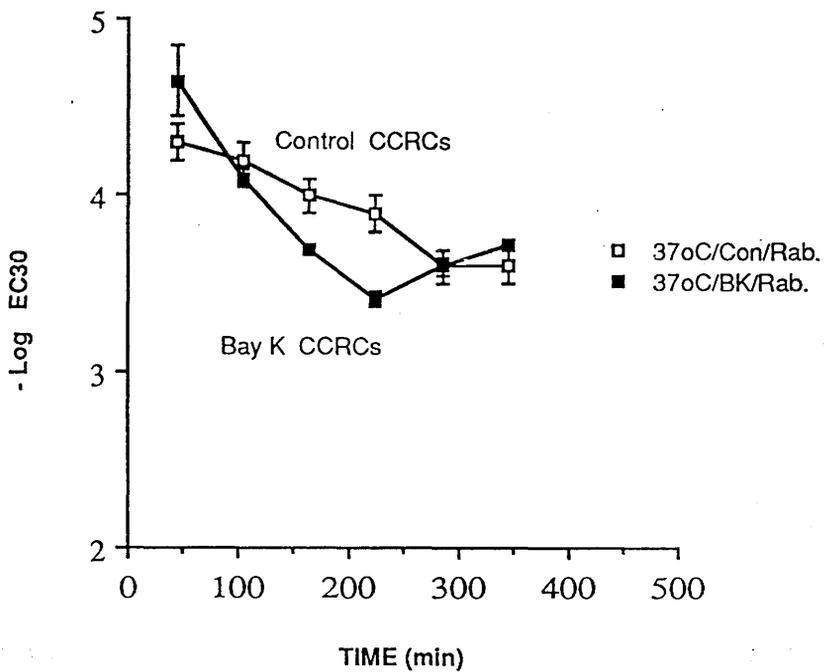


Figure 4.6. Influence of Bay K 8644 (0.1 $\mu$ M) on the calcium-sensitivity of NA-induced contraction of the isolated perfused rabbit ear artery, at 37°C. Calcium was cumulatively re-added during the experiments. Points shown are mean  $\pm$  S.E.M. (n=6). Only the sensitivity at the 3rd CCRC was significantly lower than the corresponding controls (p<0.05).

artery but not on the rabbit central ear artery or the cat distal hind limb arteries (Figs. 4. 4-6). From the 1st to the 6th CCRCs in the rat tail artery, Bay K 8644 facilitated the responses when compared with the corresponding controls. However it failed to arrest the desensitisation process.

(ii) Rabbit Ear Artery

At the 1st determination, Bay K 8644 (0.1 $\mu$ M) showed a tendency ( $p > 0.05$ ) to facilitate the responses, with the responses in presence of Bay K 8644 apparently greater than the controls although the difference was not statistically significant ( $p > 0.05$ ) (Fig. 4.6). At the 5th CCRC, the sensitivity of the rabbit ear artery to calcium at 37°C was significantly attenuated by Bay K 8644 ( $p < 0.05$ ) (Fig. 4.6). The results might suggest that the rabbit ear responses at 37°C were already maximally contracted, so that further opening of calcium channels by Bay K 8644 did not increase the responses. It seemed that the continuous and prolonged presence of Bay K in the saline facilitated calcium entry through the channels rather resulted in calcium overload with a consequent decrease in responses and tissue sensitivity to calcium by the 5th CCRC.

(iii) Cat Distal Limb Artery

Bay K 8644 (0.1 $\mu$ M) did not alter the responses of

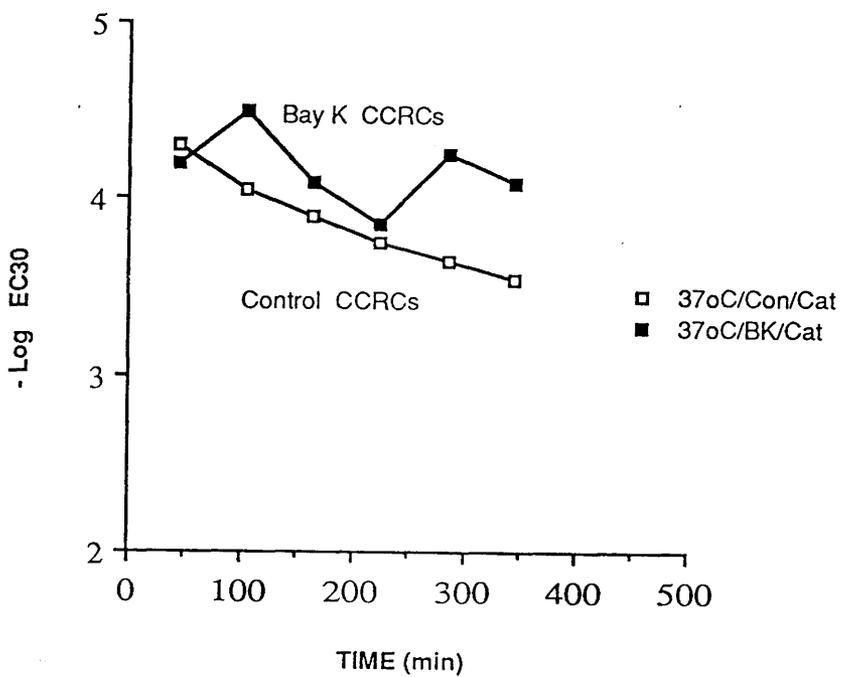


Figure 4.7. Influence of Bay K 8644 (0.1 $\mu$ M) on the calcium-sensitivity of NA-induced contractions of the isolated perfused distal hindlimb artery which leads to the paws of the cat, at 37°C, when calcium was cumulatively re-added. Points shown are mean  $\pm$  S.E.M. (n=1).

the cat arteries at the first determination of sensitivity to calcium (Fig. 4.7). However, at the subsequent determinations, Bay K 8644 clearly potentiated the responses of the tissue especially for the 2nd, 5th and 6th CCRCs. It appeared as if the tissue was already fully facilitated at the first determination so that the presence of Bay K 8644 at that stage had no effect on the responses.

#### Effects of Nifedipine at 37°C

Nifedipine (0.1µM) produced an inhibitory effect on Ca<sup>2+</sup> sensitivity on all the three tissues, at all temperatures, after the sixth curve, even though sensitivity had already declined (p <0.05). (Fig. 4.1-4; Fig. 4.8).

Nifedipine was tested at all seven CCRCs only in the rat tail artery and only then at 37°C as reported in Section 2 of the work. In these conditions, nifedipine decreased the sensitivity to Ca<sup>2+</sup> at the first CRC but thereafter CRC's were not significantly different from their controls until the 6th test in which sensitivity was actually higher. Inhibition could be seen between consecutive curves when nifedipine was given to a control tissue, and this reduced sensitivity below that produced by the normal course of desensitisation.

Figure 4. 8. Comparison of the control values of  $\text{Ca}^{2+}$ -sensitivity ( $-\log \text{EC}_{30}$ ) against time (min) for consecutive noradrenaline (NA)-induced contractions in the rat, rabbit and cat perfused arteries at (a)  $24^{\circ}\text{C}$ , (b)  $37^{\circ}\text{C}$  and (c)  $42^{\circ}\text{C}$ . Nifedipine ( $0.1\mu\text{M}$ ) was present for the 7th CRC. Points shown are mean  $\pm$  S.E.M. ( $n=12, 6$  &  $1$  respectively). Sensitivity was significantly greater for the rabbit and cat than for the rat at  $37^{\circ}$  and  $24^{\circ}\text{C}$ . At  $42^{\circ}\text{C}$  sensitivity was similar at the 3 temperatures apart from the 1st two determinations where that of the the rabbit was significantly lower than that of the rat ( $p<0.05$ ).

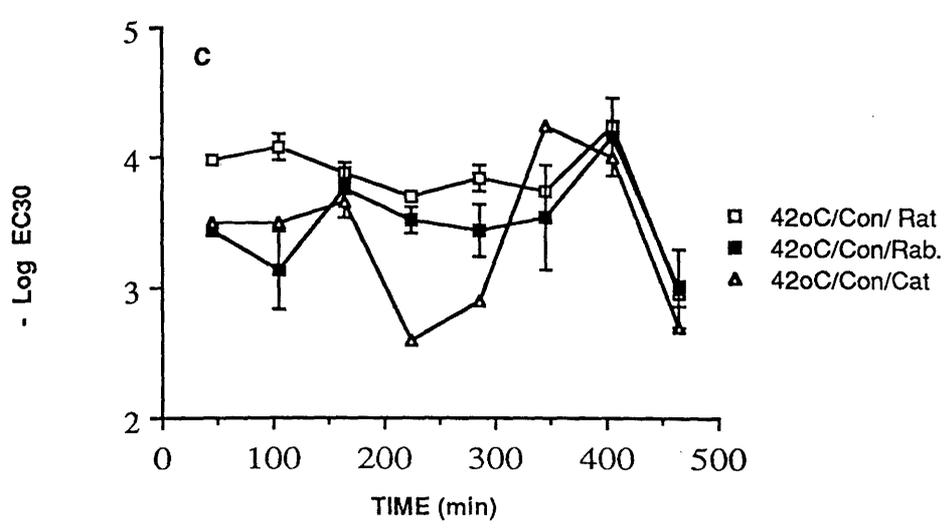
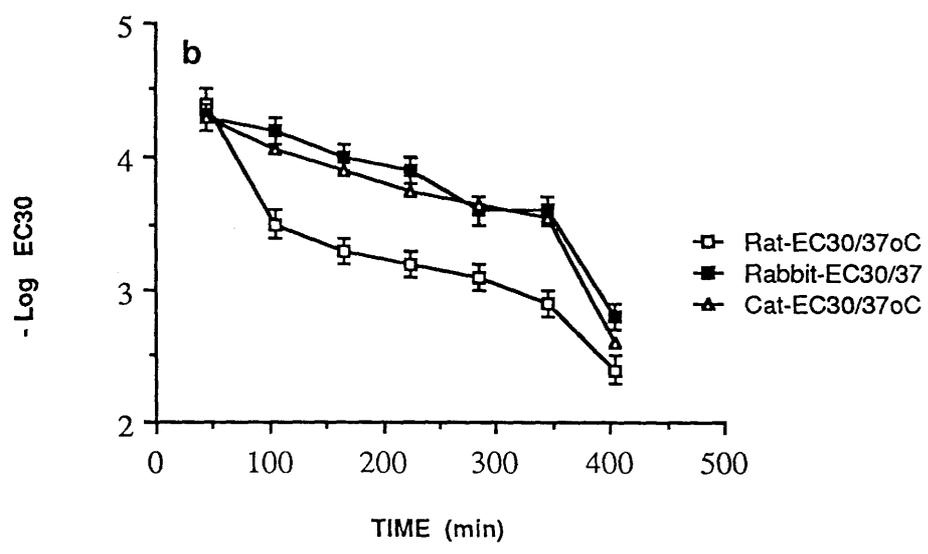
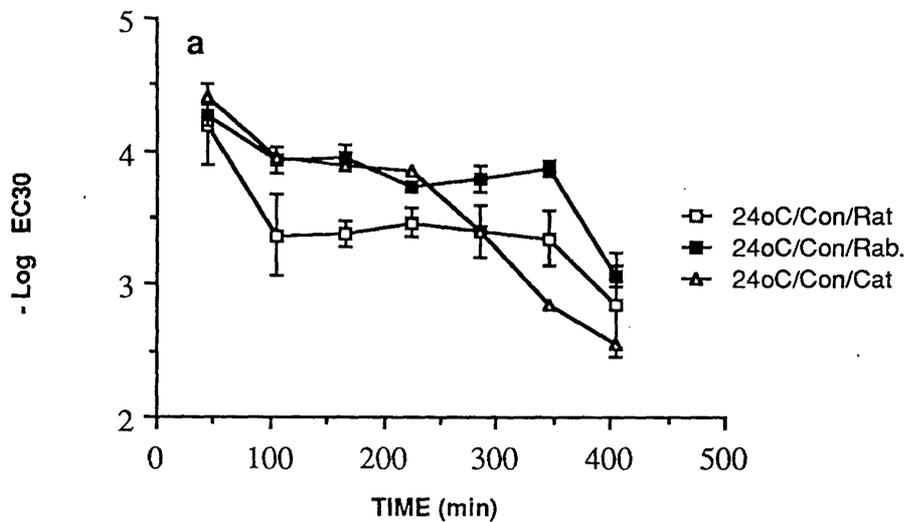
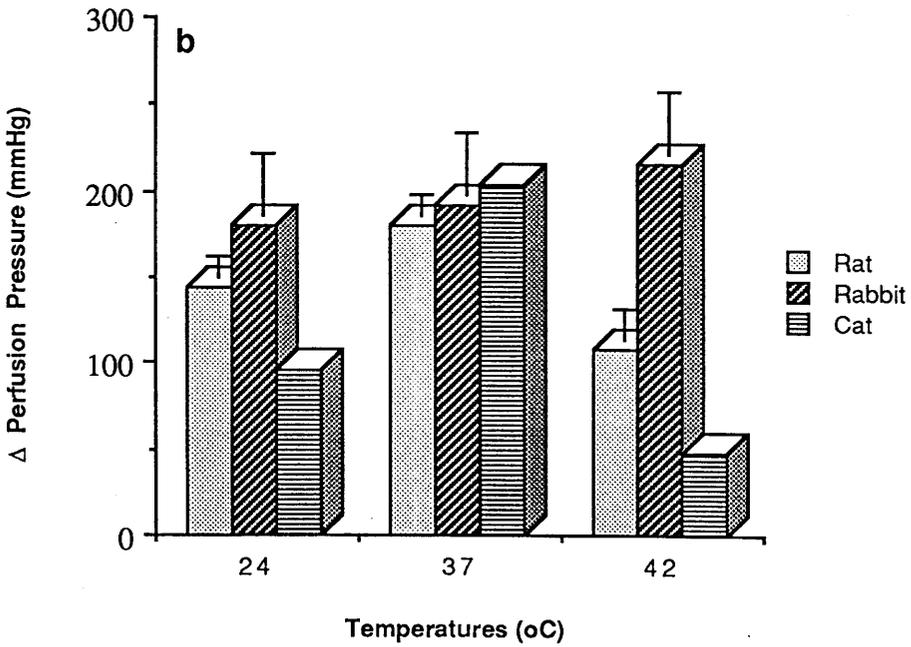
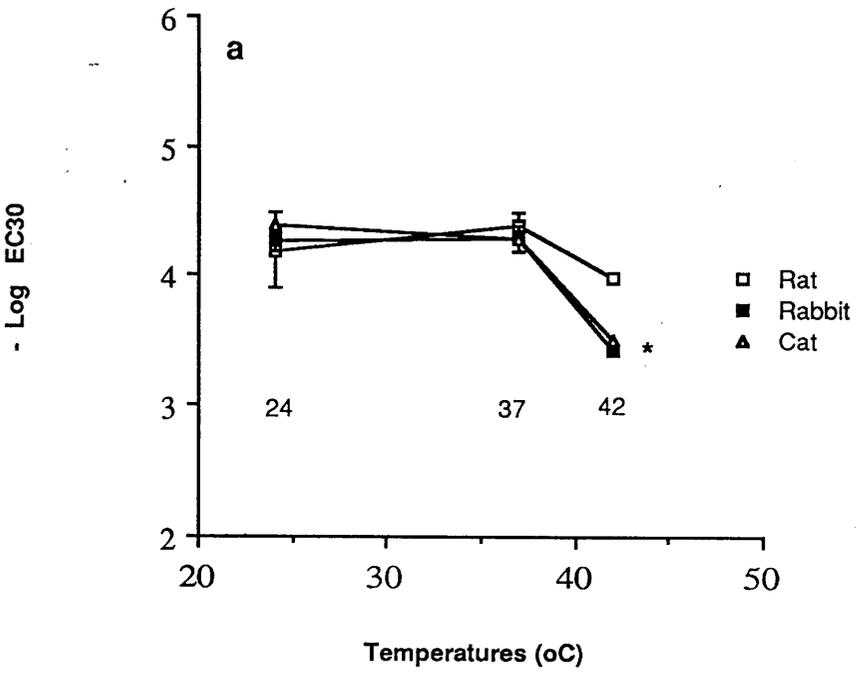


Figure 4. 9a. Comparison of the calcium-sensitivities ( $-\log EC_{30s}$ ) of the first CCRCs of the rat, rabbit and cat at  $24^{\circ}$ ,  $37^{\circ}$  and  $42^{\circ}$  C. Their values were similar at  $24^{\circ}$  and  $37^{\circ}$  C. At  $42^{\circ}$  C the sensitivity of the rabbit (or cat) was significantly lower than that of the rat ( $p < 0.05$ ).

(b) Comparison of the maximum responses (mmHg) of the first CCRCs of the rat, rabbit and cat at  $24^{\circ}$ ,  $37^{\circ}$  and  $42^{\circ}$  C. Their values were similar at  $24^{\circ}$  and  $37^{\circ}$  C. Apart from that of the rabbit, the maximum response was significantly lower at  $42^{\circ}$  C than at  $24^{\circ}$  or  $37^{\circ}$  C ( $p < 0.05$ ).



## DISCUSSION

The rat tail artery, the central ear artery of a rabbit and the distal artery supplying the paws of a cat share the physiological role of thermoregulatory functions. Their contractile activities and reactions to drugs and other factors are therefore likely to be subject to the influence of temperature.

In the rat tail artery Wade and Beilin (1970) suggested that a decrease in the smooth muscle resting membrane potential with reduction in temperature may be related to the increased sensitivity to noradrenaline which they observed at 32° to 34° C, compared with temperatures higher than this range since a moderate reduction of the resting membrane potential might be expected to reduce the further depolarisation necessary to reach threshold levels. The reduced sensitivity to noradrenaline, delayed relaxation and increased resting tone at still lower temperatures, as recorded in their studies, may be due to inhibition of energy dependent processes concerned with transmembrane fluxes of ions and smooth muscle contraction. Their suggestion was based on an earlier study by Keatinge (1964) which showed that in isolated rat aortic strips, the smooth muscle resting membrane potential decreased as the temperature was reduced.

In the present study, the desensitisation of the

responses in the isolated perfused rat tail artery (loss of  $\text{Ca}^{2+}$  sensitivity) after the 1st CCRC was more marked at  $24^{\circ}$  and  $37^{\circ}\text{C}$  and less pronounced at  $42^{\circ}\text{C}$ . However, sensitivity started off lower at  $42^{\circ}\text{C}$ . Taken together this may suggest that the mechanisms for the binding or extrusion of  $\text{Ca}^{2+}$  may be particularly effective at increased temperature.

The rabbit ear artery is known to be most sensitive to noradrenaline at  $24^{\circ}\text{C}$  (Glover et al. 1968). Our observations on  $\text{Ca}^{2+}$  with the single concentration of NA tested confirm that there is no loss of sensitivity on moving from  $37^{\circ}\text{C}$  to  $24^{\circ}\text{C}$  but that sensitivity did reduce on moving to  $42^{\circ}\text{C}$ . Desensitisation was less marked than the rat tail artery under similar conditions.

The distal artery to the paws of a cat showed a pattern quite similar to sensitivity of the rabbit ear artery to increasing  $[\text{Ca}^{2+}]_o$  in the saline, especially at  $37^{\circ}\text{C}$ . At  $42^{\circ}\text{C}$ , the pattern was also similar up to the 4th CCRC whereafter it became less stable. However, for the cat there was only a single observation.

The deterioration of NA-induced contractions of the 1st and subsequent CCRCs in the cat was not as marked as in the rat tail artery and this could partly be related to species differences. A similar result was observed in the cat spleen strips where it was observed that the maximum response or the gradient obtained with the

second exposure gave only a small shift to the right of the dose response curve at the level of the ED<sub>50</sub> (Summers and Tilman, 1979). Their results suggested that the two dose-response curves to noradrenaline can be obtained in the same preparation. Our results show that this is not possible with the rat tail artery responses to NA unless it is primed.

The different tissues showed that even in the desensitised state (after the 6th CCRC), nifedipine was still effective indicating that the dihydropyridine-sensitive channels were still open (at 37°C). In the rat tail artery where the continuous presence of nifedipine was tested at 37°C, our results showed the nifedipine-sensitive channels were blocked after the 1st CCRC, so that the presence of nifedipine in the subsequent CCRCs did not alter the course of desensitisation processes of the control curves.

In general, the results showed that in the rat tail artery, the rabbit ear artery, and the distal limb artery to the paws of a cat, temperature changes or drugs affecting Ca<sup>+</sup> channels can be radically different between those perceived when a fresh tissue is first tested and when the same tissue has been subjected to activation.

## GENERAL DISCUSSION

Based on the evidence of our results, the hypothesis originally set out about oxygen's effects on smooth muscle, did not entirely apply to the isolated perfused rat tail artery. The initial hypothesis was based on results from rat anococcygeus muscle. Noradrenaline's susceptibility to the calcium entry blocker, nifedipine was less at low oxygen tension (McGrath, 1982): if similar factors apply in the vascular system then this would have considerable importance for the *in vitro* study of the details of vascular regulatory processes and its extrapolation to *in vivo* conditions. Tail artery was not, in fact, altered in exactly the same way as anococcygeus. In general, the reduction of oxygen tension tended to produce smaller responses (resulting in slightly lower  $pD_2$  and maximum), but these differences were not statistically significant. More critically, at 16%  $O_2$ , which had been used in anococcygeus, rat tail artery was susceptible to inhibition by nifedipine and to potentiation by Bay K 8644. Furthermore, this picture did not change even at 4%  $O_2$ .

However, while investigating the factors and conditions that influence the physiological and pharmacological responses of the rat tail artery, we found several phenomena which deserved further examination. For example there was marked

desensitisation in the preparation with the protocol which we followed, so we decided to investigate the phenomenon of desensitisation itself, in an attempt to explain the considerable attenuation of the sensitivity to external calcium which we had found. This eventually led us to propose more suitable experimental protocols for the investigation of the effects of drugs that involve calcium-sensitivity in smooth muscle.

It is a common observation that the responses to NA of isolated vascular smooth muscle change as the experiment proceeds. In order to obtain reproducible data, experimenters often attempt to stabilise responses by some procedure: usually this is exposure to a high concentration of NA and is called something like sensitisation, desensitisation or priming. This always begs the question of whether the original response or the stabilised response represents the "physiological state".

We came across this problem when attempting to study the role of external calcium. It quickly became clear that consecutive responses to NA, KCl and 5-HT diminished. Desensitisation by some other agents on some other tissues have also been reported. For instance, substance P in intestinal muscle of the guinea-pig (Holzer & Petsche, 1983); carbachol in the guinea-pig taenia coli (Magaribuchi et al. 1973); acetylcholine in rat soleus muscle (Anwyl & Narahashi,

1980).

In the present study, when we looked at the  $\text{Ca}^{2+}$  requirement for contractions, the diminution of consecutive responses to NA (or KCl or 5-HT) was even worse than would be expected in sensitised or primed tissue preparations. We thus decided to look first at the extent of desensitisation to NA in the usual sort of  $[\text{Ca}^{2+}]$  employed in this type of pharmacological experiment and then to look in more detail at how sensitivity to external  $[\text{Ca}]$  changed with time. We have provided evidence that desensitisation is dependent on activation by calcium, not just time. In addition, we can partly retain calcium sensitivity by avoiding high levels of calcium. By stabilising the tissue, its sensitivity to calcium is lost although by keeping the calcium low in priming and in constructing the curve, both stability and a reasonable level of sensitivity is achieved. Loss of metabolic energy by the smooth muscles also appear to contribute to phenomenon of desensitisation as shown by the partial arrest of desensitisation by increasing the glucose concentration after the first curve.

On the other hand, receptor activation per se in this tissue does not appear to be responsible for the observed desensitisation since it is common to both receptor mediated responses by NA and 5-HT and also to voltage mediated responses by KCl. In addition, low

concentrations of NA also produced desensitisation in rat tail artery although to a lesser extent than a higher concentration. Exposure of the tissue to high calcium and the maintenance of calcium overload in the cells for a prolonged period of time seem to be the main factors responsible for the highly marked desensitisation in the rat tail artery. The presence of calcium channel facilitators did not substantially alter calcium overload or the subsequent desensitisation. Thus desensitisation was still observed in the presence of Bay K 8644.

This may be explained since the facilitator does not actually increase the response at high  $[Ca^{2+}]$  where, presumably  $[Ca^{2+}]$  entry is already fully facilitated. This would suggest that it does not perhaps increase  $Ca^{2+}$  entry at this time and consequently does not produce any greater  $Ca^{2+}$  overload than in controls. On the other hand, it was interesting that nifedipine seemed to be able to partly arrest desensitisation. Despite its acute blockade of responses, as shown on the first curve, it appeared to slow desensitisation thereafter. This may have been due to a reduction of  $Ca^{2+}$  entry which prevented  $Ca^{2+}$  overload. This left responses as large as time controls despite the acute inhibition of  $Ca^{2+}$  entry by nifedipine. The ability of a "priming" activation in low  $[Ca^{2+}]$  was also of great interest. Taking all of the results together, it would seem that the effect of the first activation of the

tissue is quite different from subsequent ones, producing circumstances which lead to Ca overload if a high external  $\text{Ca}^{2+}$  exists. If the tissue is protected from high external  $\text{Ca}^{2+}$  during the first activation, then sensitivity remains high. A second or subsequent activation is less capable of producing the damage which leads to desensitisation. An explanation for this could be that the first massive insult of activation by NA or KCl causes far more  $\text{Ca}^{2+}$  influx, presumably due to opening of more  $\text{Ca}^{2+}$  channels or opening them for longer, but that either this never happens again due to some depletion within the coupling process or the induction of more effective processes for dealing with intracellular  $\text{Ca}^{2+}$ . Consequently if overload is not initiated on this first occasion, it never occurs again to the same extent (unless the tissue recovers this property over a long period).

Variations in the oxygen tension or the temperature of the tissue hardly influenced the trend to desensitisation except that the high temperature of  $42^{\circ}\text{C}$  seemed to slow it. In some ways this was reminiscent of the effect of nifedipine. Perhaps in both cases the prevention of overload by the relatively low sensitivity to  $\text{Ca}^{2+}$  in the first CCRC conferred protection as noted above.

In general, desensitisation is more pronounced in the rat tail artery than in other similar vessels we have

studied such as the rabbit ear artery and the distal hind limb artery leading to the paws of a cat.

These results support the observations made by various workers that desensitisation occurs in most smooth muscles (Miranda, 1976; Magaribuchi et al. 1973; Allen et al. 1985). In each case however, different agents have been responsible for desensitisation. High  $[Ca^{2+}]$  and the length of exposure of the tissue to high  $[Ca^{2+}]$  are responsible for desensitisation in the rat tail artery. The failure to recognize these in experiments with this tissue may drastically affect the trend of results and consequently their interpretation. This is particularly the case when longitudinal studies are carried out on the same tissue.

The present study has therefore provided guidance on how to design an experiment to measure sensitivity of smooth muscle to calcium, and made two further important points. First, noradrenaline responses in this kind of vascular smooth muscle are very sensitive to calcium but this is easily missed if care is not taken to avoid desensitisation. Secondly, desensitisation of noradrernaline, potassium chloride and 5-HT responses in vascular smooth muscle is in large part due to loss of sensitivity to calcium.

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