THE SUB-DIVISION OF ALPHA-ADRENOCEPTORS IN VASCULAR SMOOTH MUSCLE

A thesis presented for the degree of

Doctor of Philosophy

by

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November 1985

Institute of Physiology

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DECLARATION

The work of this thesis was carried out by myself alone except for approximately 30 experiments on the blood gases of pithed rats which were carried out in collaboration with Mr. Tom Grant of the Department of Physiology, University of Glasgow.

PUBLICATIONS

1) Grant, T.L., McGrath, J.C. & O'Brien, J.W. (1984). The influence of blood gases on $alpha_1$- and $alpha_2$-adrenoceptor-mediated pressor responses in the pithed rat. British Journal of Pharmacology, 82, 216P.

2) Grant, T.L., O'Brien, J.W. & McGrath, J.C. (1984). The effects of nifedipine on pressor responses to alpha agonists and angiotensin II in the pithed rat. British Journal of Pharmacology, Proceedings from the 9th IUPHAR, London, 467P.

3) O'Brien, J.W. & McGrath, J.C. (1984). A comparison of Ca^{2+}-entry blockade on bolus and infusion responses to alpha-agonists in the pithed rat. Blood Vessels, 21, 4, 197.

4) O'Brien, J.W., Flavahan, N.A., Grant, T.L., McGrath, J.C. & Marshall, R.J. (1985). Influence of blood gases, Ca^{2+}-entry blockade and angiotensin converting enzyme inhibition on pressor responses to alpha-adrenoceptor agonists: evidence in vivo for subtypes of response independent of receptor subtype? Clinical Science, 68, suppl. 10, 99s-104s.

5) Grant, T.L., McGrath, J.C. & O'Brien, J.W. (1985). The influence of blood gases on alpha$_1$- and alpha$_2$-adrenoceptor-mediated pressor responses in the pithed rat. British Journal of Pharmacology, 86, 1, 69-77.

6) Daly, C.J., Eglen, R.M., McGrath, J.C., Miller, D.J., Muir, A.G.B. & O'Brien, J.W. (1985) Ca^{2+}-dependence of noradrenaline-induced contraction in rat aortic smooth muscle. British Journal of Pharmacology, 86, proc. suppl., 723P.

ACKNOWLEDGEMENTS

I would like to thank Dr. J.C. McGrath, Institute of Physiology, for his guidance and supervision in this work. I would also like to thank Professor O.F. Hutter of the Inststitute of Physiology for the opportunity to work in this department.

Thanks are also due to the staff of the Pharmacology department of Syntex Research for the enjoyable time spent there. I would like especially to thank Dr. R.L. Whiting (for his joint supervision of this project), Dr. R.M. Eglen (for the space in his laboratory?), Dr. A. Kilpatrick (for his help and advice with the HPLC system), Mrs. R. Clague (for the company and the crosswords) and finally Mr.B. Clarke (for the transportation - much appreciated).

I could not finish without thanking my colleagues in the laboratory for their entertaining presence. Especially Tom for enduring the past three years along with me and for his continual supply of chauvinistic comments.

Finally, a special thank- you to my husband Martin, for his support and encouragement during these difficult times.

1) The aim of this study was to examine the sub-division of alpha-adrenoceptors in vascular smooth muscle. These studies were carried out on the pithed rat and isolated rat aorta preparations.

2) Various parameters were investigated in an attempt to discover the physiological conditions which might be optimal for activation of the alpha-adrenoceptor subtypes. In the pithed rat, the influence of blood gases and the differential effects of calcium-entry blockade on the $alpha_1$- and $alpha_2$-adrenoceptor-mediated pressor responses were examined. The isolated rat aorta was used to study more closely the calcium dependency of the alpha receptor response, in vascular smooth muscle.

3) The influence of blood gases on the alpha-adrenoceptor subtype-mediated pressor response was studied in the pithed rat by varying the inspired gas mixture or the ventilation volume. Acidosis was found to favour the peak responses to the $alpha_2$-adrenoceptor agonist, xylazine, while alkalosis favoured the peak responses to the $alpha_1$-adrenoceptor agonist, phenylephrine. A combination of hypoxia and hypercapnia greatly depressed the $alpha_1$ response to phenylephrine whereas the $alpha_2$ response to xylazine remained relatively unaffected. When PaO_2 was varied in either acidotic or alkalotic conditions, the response to phenylephrine increased as PaO_2 increased.

4) To prevent hypoxia in air ventilated rats , large stroke volumes were required. This caused alkalosis and hence decreased responsiveness to xylazine. Consequently, air ventilated pithed rats gave poorer responses to xylazine than did those ventilated on 100% O_2. The results show that alpha$_1$- and alpha$_2$-adrenoceptor mediated pressor responses can be differentially affected by blood gases. The relative contribution of alpha$_1$- and alpha$_2$-adrenoceptors to vascular tone may be either under- or over-estimated depending on the arterial blood gases.

5) The pithed rat was also used to study the effect of the calcium-entry blocker, nifedipine, on the alpha$_1$- and alpha$_2$-adrenoceptor pressor responses. To do this, alpha$_1$ and alpha$_2$ agonists were either bolus injected or infused into the pithed rat's blood stream. It was found that nifedipine could inhibit the response initiated by both types of agonist, usually for both the bolus and infusion responses. Calcium-entry blockade was significantly greater on the more prolonged, secondary component of the pressor responses; whether produced by alpha$_1$ or by alpha$_2$ receptor activation. This demonstrates that calcium-entry occurs during the secondary component of the alpha response and can be initiated by either alpha$_1$- or alpha$_2$-adrenoceptor subtypes.

6) The shape of the response produced by infusing the alpha-agonists varied greatly. Most alpha$_1$ agonists, with the exception of SGD 101/75, did not achieve a plateau

during the 20 minute infusion time. The alpha$_2$ agonists, on the other hand, readily attained a plateau which was maintained throughout the remainder of the infusion. Phenylephrine and noradrenaline produced pressor responses which reached a maximum and then declined during the remainder of the infusion. These results indicate a difference in the mode of action by which the various alpha-agonists produce their infusion pressor responses.

7) The levels of noradrenaline in the rat arterial and venous plasma were measured during the infusion of noradrenaline, by an HPLC system. Arterial noradrenaline levels rose throughout the infusion whereas venous levels remained relatively unaffected.

8) The isolated rat aortic ring produced a biphasic, contractile response when stimulated by noradrenaline. The initial transient component (ITC) was relatively resistant to the removal of calcium from the external buffering saline and to calcium-entry blockade by nifedipine. It was therefore thought to be the result of mobilization of internal calcium. The slower secondary component (SSC) was reduced by the removal of calcium from the external buffering saline and by calcium-entry blockade, when carried out under reduced calcium conditions. It was considered to be the result of calcium-entry into the cell. The inorganic calcium-entry blocker, cadmium, and the intracellular calcium blocker, dantrolene sodium, were both found to reduce the ITC and the SSC equally well. These results

indicate that calcium-entry is involved in both components of the response to noradrenaline.

9) A buffering system was used, incorporating a mixture of EDTA and NTA, the calcium chelaters, to control the levels of calcium in the physiological saline bathing the tissues. This was done in order to demonstrate the differential effect on the ITC and SSC of gradually reintroducing calcium into a $[calcium]_{free}$ solution. The ITC remained relatively unaffected by these buffers but the SSC was gradually increased as the levels of calcium increased. This shows that it is the SSC which is more dependent on external calcium for its activation, than is the ITC.

10) Prazosin, the $alpha_1$ antagonist, was administered to the aorta before noradrenaline, and was found to inhibit the ITC in preference to the SSC. This may indicate that prazosin, being a high affinity antagonist, does not allow the short, sharp burst of agonist activity required to produce the ITC. As a result the ITC appears preferentially blocked.

INTRODUCTION

The Alpha Adrenergic Receptor

In most things the best place to begin is, naturally enough, at the beginning, however in scientific research, it is often difficult to tell where this is exactly. In the physiology of sympathetic receptors the "beginning" as such, came just after the turn of the century. In 1905, Langley described a "receptor substance" which he thought of as being located in the muscle rather than the nerve endings. He proposed that it existed in more than one form, this being the basis for physiological inhibition and excitation (Langley, 1905).

One year later, Dale produced his classical study on the effect of ergot alkaloids on adrenaline and sympathetic nerve stimulation in vascular and non-vascular smooth muscle. He found that excitatory or motor responses of the various organs were inhibited by the ergot alkaloids and in some cases became inhibitory responses after treatment (adrenaline reversal). On the other hand, if the tissue gave an inhibitory response to the stimuli, the addition of the ergot alkaloids produced no effect. Dale interpreted this to mean that there were two distinct types of "sympathetic myoneural junctions" which were activated by adrenaline and nerve stimulation and that only those blocked by ergot alkaloids were responsible for motor responses.

In 1904, Elliott was the first to suggest that adrenaline was liberated from the sympathetic nerve endings when they were stimulated and that the released adrenaline then acts

on the responsive cells (Elliott, 1904). Dale carried out another study on the actions of a large number of compounds related to adrenaline. He compared the potency of adrenaline and phenylethylamine derivatives in producing increases in blood pressure and responses in different muscular organs. He pointed out that noradrenaline more closely mimicked the effects of sympathetic stimulation, unfortunately this observation was overlooked for many years (Barger & Dale, 1910). This early work by Dale had in fact discovered two methods of differentiating types of receptors i.e. selective block by antagonists and comparing the relative potencies of agonists.

The first conclusive evidence that chemical transmission occurs was provided by Loewi in 1921. He set up two frog hearts in such a way that the Ringer solution flowed through the first heart on to the second. When he stimulated the sympathetic nerve to the first heart it beat faster and stronger and subsequently the second heart responded in the same way. This indicated that the nerve endings in the first heart were releasing a substance which Loewi named "Acceleranstoff" (Loewi, 1921). Following this work nerve transmission research concentrated on discovering the identity of the chemical involved in this process.

Cannon and Rosenbleuth (1933) called the sympathetic transmitter substance "sympathin" as its actions were not identical to those of adrenaline. They proposed that once released from the nerve endings the sympathin must combine

with one or other of two hypothetical substances within the innervated tissue. Whichever substance the sympathin combined with, determined the type of response produced. In effect, two sympathins were produced - sympathin E (excitatory) and sympathin I (inhibitory). They further expanded their theory by suggesting that the combined sympathin product could then circulate round the body and activate other tissues. This theory was proved wrong when in 1946 Von Euler established that noradrenaline is the chemical transmitter for all adrenergic nerves and there was no evidence that it was modified in any way before reaching the tissue. He kept their terminology, however, still believing that it was a difference in the transmitter which caused the different response in the effector tissues. He suggested that sympathin E was noradrenaline and that sympathin I was adrenaline.

It was two years later when Ahlquist (1948) carried out his classical study which enabled him to characterise the adrenergic receptor. He did this by studying the order of potency of a series of different adrenergic agonists in producing a response in various sympathetically innervated tissues. The sympathomimetic amines used were adrenaline, noradrenaline, alpha-methyl-adrenaline and isoprenaline. He found that there were two distinct order of potencies. Noradrenaline was much more potent in producing excitation of the smooth muscle in the various tissues tested (except for the gut, which it inhibited) than was isoprenaline. On the other hand, isoprenaline was the most potent drug at inhibiting smooth muscle and at stimulating the heart. He

concluded that there were two distinct types of adrenoceptors producing these responses. The receptor producing the first type of response he named "alpha" and the second type he named "beta".

This theory was further strengthened by the discovery of DCI, a dichloro-analogue of isoprenaline (Powell & Slater, 1958). This drug was found to be a selective inhibitor of those reponses which Ahlquist had classified as beta. Thus the terminology of alpha and beta receptors became accepted and lies at the basis of adrenergic neurotransmission research (for a review see Furchgott, 1972).

The concept of a single alpha and a single beta adrenoceptor-type survived until 1967 when Lands published work on the relative potencies of a series of sympathomimetic amines (beta-agonists) on both isolated tissues and whole animals. He and co-workers concluded that there were two different types of beta-receptors, which they termed $beta_1$ and $beta_2$ (Lands et al, 1967). However the sub-division of alpha receptors took much longer to discover. In fact, Furchgott in his review of the classification of adrenoceptors in 1972 concluded that there was no evidence to suggest other than a single type of alpha adrenoceptor.

The Pre-Synaptic Alpha$_2$ Receptor

The original observation of presynaptic receptors (although it wasn't appreciated as such at the time) was made by Brown and Gillespie (1956, 1957; Brown et al, 1958). The amount of noradrenaline found in the venous blood from the cat spleen or colon after adrenergic nerve stimulation, was greatly increased by dibenamine or phenoxybenzamine. They knew that these drugs blocked alpha receptors and so concluded that it was receptor blockade or some event subsequent to it that preserved the noradrenaline.

From that time however, the weight of evidence went against the post-junctional alpha receptor being involved in regulating the release of noradrenaline from the nerve terminal (for reviews see Gillespie, 1980; Langer, 1981). The idea that the concentration of noradrenaline in the synapse was regulated by neuronal and extra-neuronal uptake became so widely accepted that by 1966 both Brown and Gillespie had abandoned their theory of the involvement of alpha receptors.

The first suggestion of a feedback mechanism in which the release of transmitter was inversely related to the intensity of the tissue response came from experiments from prostaglandins. Hedqvist, (1969a,b) showed that when prostaglandins of the E series were infused into the cat spleen, the nerve-stimulated overflow of noradrenaline was reduced with no alteration to the uptake of infused noradrenaline. Prostaglandin E_2 could also antagonise the

effect of phenoxybenzamine in increasing noradrenaline overflow. The post-synaptic action of alpha-antagonists was still considered to be the method by which they increased overflow, and this inhibitory trans-synaptic signal was thought to be due to prostaglandin release (Haggendal, 1970; Farnebo and Hamberger, 1971).

A major step in discovering the pre-synaptic alpha receptor came about when researchers changed from looking at tissues with mainly alpha receptors to looking at the heart, with its predominantly beta receptors (Starke, 1971). It was found that alpha antagonists increased transmitter overflow without altering noradrenaline uptake or metabolism and without reducing the response (Starke, 1972, 1973; Farah & Langer, 1974). More research followed along these lines thus ending the theory of transsynaptic regulation as the mode of action of alpha antagonists (Kaumann, 1970; Langer et al., 1977). The new theory of presynaptic regulation was backed up by the findings of Werner and of Starke (Werner et al. 1972; Starke et al., 1972). Alpha agonists such as clonidine, phenylephrine, oxymetazoline and naphazoline all produced an inhibition of transmitter release after nerve stimulation of rat or rabbit heart.

As a result of these findings, it was suggested that there existed a pre-synaptic alpha receptor situated on the nerve varicosity membrane. Noradrenaline released from the nerve attached itself to the receptor thus reducing the amount of transmitter released on each subsequent nerve impulse (Starke, 1972; Langer 1971). In his review paper in

1974, Langer proposed that the pre- and post-synaptic alpha receptors were not identical. He suggested, "... the postsynaptic alpha-receptor that mediates the response of the effector organ should be referred to as $alpha_1$, while the presynaptic alpha-receptor that regulates transmitter release should be called $alpha_2$".

The Post-Synaptic Alpha$_2$ Receptor

Following the definition by Langer, Berthelsen and Pettinger (1977) suggested that the differentiation between alpha$_1$ and alpha$_2$ types of adrenergic receptor should be made on a functional rather than an anatomical basis. They found that the alpha-adrenergic receptor inhibiting the MSH-induced darkening of the frog skin behaved as though it was of the alpha$_2$ type but was located postsynaptically.

Now came studies on the relative potencies of various alpha agonists and antagonists at the pre- and post-junctional sites. Starke et al. (1975a) were the first to report that the drug yohimbine displayed a preferential blockade of the presynaptic alpha receptor as opposed to the postsynaptic receptor, in the rabbit main pulmonary artery. Other studies using agonists this time, showed that alpha agonists varied widely in their pre and postsynaptic potencies (Starke et al., 1975b,c). In the rabbit pulmonary artery, methoxamine was the most potent agonist tested at the postsynaptic receptor whereas oxymetazoline was the most potent presynaptically. They suggested this may be due to a structural difference in the two receptor sub-types.

Just as yohimbine had been found to be a selective antagonist at the alpha$_2$ receptor, the drug, prazosin, was then discovered to be selective for the alpha$_1$ receptor (Cambridge et al., 1977). Thus the classical pharmacological tools, of relative agonist and antagonist

potencies, were now available to identify and classify the sub-types of alpha adrenoceptors in the cardiovascular system and elsewhere.

The possibility of two populations of vascular post-junctional alpha adrenoceptors was demonstrated by Moulds and Jauernig in 1977. They discovered that prazosin acted as a competitive antagonist at alpha receptors but had selective effects on different vascular beds. The receptors on visceral beds, such as the uterine or splenic, were sensitive to prazosin whereas those on peripheral beds such as the palmar digital artery were resistant. Results supporting these findings were also made in whole animal preparations.

Bentley et al. (1977) showed that in pithed rats, prazosin was more potent in blocking the response to phenylephrine than to noradrenaline. In anaesthetised cats and pithed rats, Drew and Whiting (1979) found that prazosin blocked the blood pressure responses to phenylephrine better than those to exogenous noradrenaline or nerve stimulation. They suggested that two sub-types of post junctional alpha receptor existed - $alpha_1$ and one other.

Despite the evidence of an earlier paper (Drew, 1978), in which postsynaptic $alpha_2$ receptors had been found in the guinea-pig ileum, Drew still stated that the other postsynaptic adrenergic receptors found in the vascular system "...appear to differ from $alpha_2$-adrenoceptors".

It was Docherty and McGrath (Docherty et al., 1979; Docherty & McGrath, 1980) who finally stated that the two postsynaptic alpha adrenergic receptors were of the $alpha_1$ and $alpha_2$ sub-types. They used relatively selective agonists and antagonists for $alpha_1$ and $alpha_2$ receptors in the pithed rat. They found that xylazine's pressor response, in particular, was resistant to prazosin blockade whereas phenylephrine's was almost completely blocked. At the same time, Timmermans and co-workers (1979) presented additional information, in the pithed rat, that yohimbine had a greater inhibitory effect on the pressor response to guanfacine than phenylephrine. They also showed that prazosin only inhibited phenylephrine and not the guanfacine induced response. Since then, vascular postjunctional $alpha_2$-adrenoceptors have been clearly demonstrated in many animal in vivo preparations (for reviews see McGrath, 1982; Timmermans & van Zwieten, 1981 & 1982; see also McGrath, 1981 & 1983).

The classification of the postsynaptic $alpha_2$ receptor was much aided by the discovery of a more specific and potent antagonist in rauwolscine (Weitzell et al., 1979). This alloyohimbine derivative displayed the most selective competitive blockade of the presynaptic $alpha_2$ receptor when compared to other stereoisomers of yohimbine, in the pulmonary artery of the rabbit. Now, much research went into the discovery of new and more specific $alpha_2$ agonists and antagonists. Agonists such as BHT 933 and 920, UK14-304 and M7 were found to be specific for $alpha_2$ receptors in

the pithed rat whereas methoxamine, cirazoline and phenylephrine were specific for the $alpha_1$ receptors (Timmermans & van Zweiten, 1980a,b; van Meel et al., 1981; Drew, 1980). Prazosin and rauwolscine being in the process confirmed as the most selective $alpha_1$ and $alpha_2$ adrenoceptor antagonists, respectively.

Although the presence of $alpha_2$-receptors has been well documented in vivo, it has been much more difficult to show their existence in vitro. Although the results of Moulds and Jauernig (1977) have been taken as the first indication of the presence of vascular postsynaptic $alpha_2$-adrenoceptor, further in vitro investigations by Stevens and Moulds (1981 & 1982) concluded that the alpha receptors in human vessels are either different from other $alpha_1$ and $alpha_2$ adrenoceptors or are a mixed population of two or more types of receptors. The proposal of a heterogeneous population of $alpha_1$ and $alpha_2$ adrenoceptors was backed up in a paper by Flavahan and McGrath (1984).

One in vitro preparation which has been found to have postjunctional $alpha_2$-adrenoceptors is the canine saphenous vein (De Mey & Vanhoutte, 1981; Langer & Shepperson, 1981a,b). Other tissues tested by De Mey and Vanhoutte were the splenic and femoral arteries but they were found to be more sensitive to methoxamine than clonidine i.e. they possessed fewer $alpha_2$ receptors. This uneven distribution of alpha-adrenoceptor sub-types throughout the cardiovascular system has been documented in other papers (Vanhoutte, 1982; Langer, 1980). These results using

selective agonists have led to the proposal that the proportion of $alpha_1$ and $alpha_2$ adrenoceptors may vary between different vascular beds and between arteriolar and venous smooth muscle - the $alpha_2$ receptor being more prevalent on the venous side.

The differences in the effectiveness of various alpha-adrenoceptor antagonists at inhibiting the pressor responses to sympathetic stimulation and administered noradrenaline, in pithed rats, led Yamaguchi and Kopin (1980) to conclude that the pressor effects of the exogenous catecholamine are mediated predominantly by $alpha_2$-adrenoceptors, whereas the response to sympathetic stimulation is the result of activation of $alpha_1$-adrenoceptors in the region of the vascular neuro-effector junctions.

Langer suggested that the vascular postsynaptic $alpha_2$-adrenoceptor might be located at extrasynaptic sites which would be consistent with the previous observation (Langer, 1981). However, in the rabbit, as the stimulation frequency decreased, the major activation of smooth muscle changed from $alpha_1$-adrenoceptors to include an $alpha_2$-adrenoceptor component (McGrath et al., 1981). Supporting this, were the pressor effects of exogenous noradrenaline, which were mainly $alpha_1$ mediated at high doses but $alpha_2$ mediated at lower doses. It appeared therefore, that the $alpha_2$-adrenoceptor system on resistance vessels has a lower threshold for noradrenaline, but when higher doses of noradrenaline are used and both receptors activated, $alpha_1$

effects will dominate.

Another interesting observation which led away from the extrasynaptic theory was made by Hamilton and Reid (1981). In conscious rabbits, 6-OH-dopamine caused changes in pressor responsiveness to $alpha_1$-adrenoceptor agonists and to blockade of antagonists, but did not alter the responses to drugs acting on $alpha_2$ receptors. They suggested that the pressor response mediated via postsynaptic $alpha_1$ and $alpha_2$-adrenoceptors is controlled by different mechanisms, different sites of excitation-contraction coupling possibly being involved.

A clue to the possible difference between the mechanisms involved in alpha receptor sub-type activation, is the difference in time course between the two systems. The onset of the pressor response to $alpha_2$-adrenoceptor agonists is generally slower than those to $alpha_1$. This might be due to differences in solubility or longer diffusion times to an extrasynaptic receptor but Docherty and McGrath (1980b) showed that "mixed" agonists (those activating both $alpha_1$- and $alpha_2$-adrenoceptors) produce an initial rapid rise which is $alpha_1$ mediated and a slower secondary response which is mainly $alpha_2$ mediated. Another clue to the apparent difference in the mechanism of the two receptor subtypes, in producing a pressor response, is the difference in the involvement of calcium.

Calcium and the Alpha Adrenoceptor

It was Bohr (1963), who showed that the contraction of rabbit aortic strips in vitro to adrenaline comprised a rapid and a slow component. The rate limiting factor for the slow component appeared to be a process varying directly with the extra-cellular calcium concentration. Over the next 15 years or so, extensive research work on various smooth muscle preparations revealed that not only are calcium ions involved in excitation-contraction coupling but also, in the discharge of propagated action potentials. Drugs which are known to inhibit the slow inward current of calcium ions (which activate the contractile machinery), such as verapamil, D-600, nifedipine and diltiazem (Fleckenstein, 1977) have since been widely used to discover the difference in the involvement of calcium in the responses of the two adrenoceptor sub-types.

De Mey and Vanhoutte (1981) originally found that in dog saphenous veins, verapamil inhibited the contractions of methoxamine ($alpha_1$) more effectively than those of noradrenaline or clonidine ($alpha_2$). They concluded that "...contractions of vascular smooth muscle cells caused by $alpha_1$-adrenergic stimuli depend more on the influx of extracellular Ca^{2+} than $alpha_2$-adrenergic activation, which then must cause a relatively greater mobilisation of cellular stores of the activator ion". However, from then on, researchers found more evidence to support the opposite view.

Van Meel et al. (1981b) showed that in pithed rats the stimulation of alpha$_2$ receptors by BHT 920 was inhibited by the calcium entry blockers verapamil, nifedipine and D-600, whereas alpha$_1$ stimulation was virtually unaffected. This was backed up by the finding that diltiazem and verapamil inhibit M7 and noradrenaline more than phenylephrine (Langer & Shepperson, 1981). Other evidence supporting the Ca^{2+}-entry theory for alpha$_2$-adrenoceptors came from van Zweiten et al., 1982; Cavero and Lefevre-Borg, 1981. So, the conclusion from all this evidence was that vascular smooth muscle contraction in vivo initiated by post-synaptic alpha$_2$-adrenoceptor activation is induced by an influx of extracellular calcium, whereas a transmembrane influx of calcium ions is probably not required directly in linking alpha$_1$-adrenoceptor activation to vasoconstriction.

However this theory did not agree with biochemical evidence at the time (Fain & Garcia-Sainz, 1980). In this review, alpha$_1$-adrenoceptors were said to mediate their effects via a turnover of phosphatidylinositol and a release of intracellular Ca^{2+} ions as well as a calcium inward current through calcium gates. Alpha$_2$-adrenoceptors on the other hand, mediated their effects via a non-specific decrease of adenylate cyclase through a mechanism independent of calcium.

At the present time, there is still much controversy about the involvement of calcium in the response to alpha-adrenoceptor activation. Most of the evidence points towards the van Meel theory, of calcium entry being

primarily involved in the $alpha_2$-adrenoceptor response and has been shown in both in vivo and in vitro preps (Langer & Shepperson, 1982; van Meel et al., 1983a,b; Timmermans et al., 1983; Saeed et al., 1983 and Cavero et al., 1983).

Recently there has been some evidence that the calcium involvement is much more complicated than previously supposed. In pithed rats and cats, Timmermans et al. (1983), examined the effects of the calcium entry blocker, nifedipine, on the vasopressor effects of the selective $alpha_1$-adrenoceptor agonist Sgd 101/75. They found that Sgd 101/75 was indeed effectively antagonised. Therefore in view of the current thinking on calcium-entry blockade and $alpha_1$-adrenoceptors, they concluded that this agonist was different from other $alpha_1$-agonists and their results may be indicative of a further subdivision of vascular postjunctional $alpha_1$-adrenoceptors.

This idea was nothing new as evidence of subtypes of the $alpha_1$ receptor had been postulated before (Coates & Weetman, 1982; Ruffolo et al., 1982; McGrath, 1982) in various in vitro preparations and Hirst and Neild (1980a,b; 1981) had even put forward the theory that there is a third postjunctional alpha-adrenoceptor, the gamma receptor.

However, the complicated story of calcium involvement in the alpha- adrenoceptor response continued to be less clear cut with the findings of Flavahan and McGrath (1982). They showed that the pressor response to amidephrine, the

selective $alpha_1$-agonist, consisted of two components: an early rapid peak which was resistant to nifedipine and a second, slower component which was nifedipine-sensitive. Both components were antagonised by prazosin but not by rauwolscine and so were said to be $alpha_1$-adrenoceptor mediated (see also, Vizi & Ludvig, 1983; Mathy et al., 1983). So, this was evidence of another $alpha_1$-adrenoceptor response which appeared to be mediated at least partly, by Ca^{2+} influx.

The previous two observations were both conducted in vivo, however, Janssens and Verhaege (1984) recently proposed that in the dog saphenous veins, $alpha_1$- and $alpha_2$-adrenoceptors cause both influx of Ca^{2+} ions and release intracellular Ca^{2+}. They also concluded that the source of the intracellular calcium for the two receptor sub-types is the same and that the mechanism of influx is likely to be the same too. Alabaster (1984) also concluded that both $alpha_1$- and $alpha_2$-adrenoceptor activation, by phenylethanolamine derivatives in particular, are equally dependent on extracellular calcium influx. So, the story of calcium involvement still remains very complex.

In the future, ligand-binding techniques will possibly purify and characterize exactly what receptors are present in vascular smooth muscle. At the present time, the results of binding experiments support the concept of $alpha_1$ and $alpha_2$ subtypes (Wood et al., 1979; U'Prichard & Snyder, 1979). However, there have been observations in favour of the existence of at least two conformational states of the

$alpha_2$-adrenoceptor, which are differentiated by high or low affinity for agonists (U'Prichard et al., 1979; Daiguji et al., 1981). The danger in these studies is to classify too many unnecessary receptor subtypes, as there are still examples of ligand-binding sites, which are waiting to be ascribed a function.

Another new and interesting development in the characterisation of the two receptor subtypes is the involvement of EDRF (endothelium-derived relaxing factor) as a modulator of contractile agonist effects (Cocks & Angus, 1983). Egleme et al. (1984) showed that the endothelium depresses contractions of the rat aorta induced by alpha-adrenoceptor agonists. There is evidence to suggest that this EDRF causes a greater depression of $alpha_2$ mediated responses than $alpha_1$ and that the EDRF controls both calcium entry and calcium release evoked by alpha-adrenoceptor agonists in vascular smooth muscle (Godfraind et al, 1985).

If the best place to begin is the beginning, then the best place to end must be the end. Unfortunately, as yet, there is no final conclusion to the story of the $alpha_2$-adrenoceptor. As much as we presume to understand about its location, structure and mode of action, as much again remains elusive, at this time. As we learn more of: the importance of calcium; why there is a difference in activation time course between the $alpha_1$- and $alpha_2$-adrenoceptor sub-types; why the $alpha_2$ receptor is so

difficult to demonstrate in "in vitro" preparations and what is the exact involvement of EDRF, we may eventually reveal the functional relevance of the alpha$_2$-adrenoceptor. What follows is one very small step in that giant leap for mankind.

The Subdivision Of Alpha-Adrenoceptors In Vascular Smooth Muscle

Purpose Of The Present Investigation

The present study was designed to investigate various factors which appear to affect the two alpha-adrenoceptors sub-types in different ways. The first series of experiments was a continuation and expansion of the work done on the influence of blood gases on the $alpha_1$- and $alpha_2$-adrenoceptor mediated pressor responses in the pithed rat, by Flavahan and McGrath (1982). In section one, the blood gases of pithed rats were altered by means of varying the ventilation rate, stroke volume or the actual composition of the inspired gases. The effect of this was investigated on the pressor responses to phenylephrine, chosen as a typical $alpha_1$-adrenoceptor agonist, and xylazine, chosen as a typical $alpha_2$-adrenoceptor agonist. This was done in an attempt to discover the optimal conditions for eliciting a selective response from the different adrenoceptor sub-types. It was also hoped that the various blood gas conditions would reveal something of the relative physiological importance of the two receptor sub-types under normal and pathological conditions.

The second section looked at another factor influencing the two alpha-adrenoceptor sub-types i.e. calcium. In his review paper, McGrath (1983) suggested that as most researchers measure the peak pressor response of agonists they inject into the pithed rat, they may miss an $alpha_2$-adrenoceptor-mediated effect:- $alpha_2$ adrenoceptors, when

activated, appear to have a slower onset of action.

The high concentrations of mixed agonists, produced when they are bolus injected, show an early dominance of the $alpha_1$-adrenoceptor effect. Any $alpha_2$-adrenoceptor effect will only become evident later on, perhaps when the drug reaches a lower "equilibrium" concentration. In order to test this theory, with respect to calcium involvement, $alpha_1$-, $alpha_2$- and mixed-adrenoceptor agonists were both bolus injected and infused into the pithed rat and the time course of the pressor response measured. To see if the involvement of calcium varied with the time course of the response, nifedipine, the calcium-entry blocker, was used against both methods of drug administration.

As a further guide to the idea that low, prolonged concentrations of alpha agonists will preferentially activate $alpha_2$-adrenoceptors, the levels of noradrenaline present in the plasma of the pithed rat during infusion of the drug were measured using an HPLC system.

In the third section, an in vitro system, the rat aortic ring, was used as a simpler model than the whole animal preparation, to carry out a more detailed study on the involvement of calcium in the alpha-adrenoceptor response. Smooth muscle contraction to noradrenaline has two components which are dependant to different extents on external calcium levels (van Breemen, 1977). So firstly, inorganic and organic calcium-entry blockers were used to

examine what effect they had against the contractile response to noradrenaline.

Free calcium levels in the physiological saline bathing the isolated tissues can be accurately calculated in a buffered system incorporating known levels of $(Ca^{2+})_{total}$, EGTA and NTA (Miller & Smith, 1984). The object of the last study was to examine the effects of removing calcium from the physiological saline and reintroducing it to the aorta in known aliquots. This was done in order to examine what happened to the two components of the adrenergic response when their extracellular source of calcium was limited.

METHODS

1) IN VIVO EXPERIMENTS

The Pithed Rat

Male Wistar rats (245-265g) were placed in a restraining box and pithed under halothane anaesthesia (4% halothane in a mixture of 1 volume of oxygen to 2 volumes of nitrous oxide, reducing to 2.5% halothane to maintain anaesthesia) by the method of Gillespie, MacLaren & Pollock (1970). Following tracheal cannulation, a steel tube was inserted into the orbit and foramen magnum to the spinal column, stopping at the sixth cervical vertebra. A thin steel rod was then pushed down this outer tube, down the spinal column to the lower lumbar region thus completing the pithing.

Carotid arterial pressure was monitored (Elcomatic EM 752) via a heparinised cannula (200 units per ml saline) and the heart rate was extracted from the pressure transducer by an instantaneous rate meter (Lectromed 4522). Heart rate and blood pressure were displayed on a UV Oscillograph (SE Labs 6150). The right jugular vein was cannulated for drug injections and infusions (McLennan DS 201 digital syringe). Injections were administered in a fixed volume of 1 ml/kg followed by a similar volume of 0.9% saline and the agonists infused in a volume of 1.5 ml/kg/hr. The temperature of the rat was maintained at 37^{o}C using a tungsten lamp and the rectal temperature was monitored with a thermometer.

The rats were ventilated using a Harvard Rodent Respirator at a rate of 60 strokes per minute with one of the following gas mixtures;

(i) air

(ii) 100% O_2

(iii) 30% O_2 with varying CO_2 levels (0-4%), or

(iv) varying O_2 levels with 0 or 4% CO_2.

The gas mixtures were made up in Douglas bags via rotameters and checked using a gas analyser (Morgan Analytical 801 A). The stroke volume was varied in rats ventilated with air or 100% O_2 (1.8-4.0 ml/stroke) thus altering acid-base balance. Stroke volume was fixed at 3.5ml in rats ventilated by methods (iii) and (iv) : the remainder of the gas was N_2. Rats used in the infusion experiments were ventilated at 2.0ml/stroke (60 strokes/min) with 100% O_2 to produce well oxygenated, slightly acidic blood gases, thus optimising any prolonged response (see results section one). The rats were allowed to stabilise for 20-30 mins before the commencement of the experiment.

Carotid arterial blood samples (0.6ml) were taken for blood gas analysis (IL 213 analyser) both before the experiment and after. In later experiments, a new analyser the IL 1302 needed only 90ul samples. pH and $PaCO_2$ values were within the ranges 7.73-7.10 and 10-66 mmHg, respectively. PaO_2 varied between 50 and 470 mmHg. Pressor responses were assessed as changes in diastolic arterial blood pressure (mmHg) and changes in heart rate as beats/min. McGrath & McKenzie (1975) showed that in pithed

rats the diastolic blood pressure is little affected by cardiac effects and can therefore, provide a good estimate of vascular reactivity.

The results were analysed, in the infusion experiments, by comparing the area under the graph (a.u.g. in mmHg.min) between control and nifedipine-blocked experiments. The areas were calculated from the graphs of mean data. This was done using a digitising tablet linked to an Apple IIe computer (see Moss, 1981). For the bolus experiments, the area was measured from t=0 mins, (when the drug was injected) to the time taken for the response to return to baseline. Some drug responses did not return to the baseline so their responses were measured until they returned to a constant value above the baseline. For the infusion responses the area was measured from t=0 mins, (when the infusion began) to the end of the infusion at t=20 mins.

Nifedipine was used throughout this study as a calcium-entry blocker because it has been shown to inhibit the slow inward current of calcium ions (Fleckenstein, 1977). It selectively blocks noradrenaline-dependent calcium influx, leaving efflux unaltered and produces a concentration dependent reduction of noradrenaline-induced contraction (Godfraind, 1981). It has little $alpha_1$-adrenoceptor blocking activity which is negligible at the 0.3mg/kg dose used in this study (Flavahan, 1983) and its affinity for $alpha_2$ receptors is also negligible (about 450 times less

than yohimbine), (van Meel et al., 1981). If injected intra-arterially it causes only a slight, transient decrease in the diastolic blood pressure of 10.3 +/- 1 mmHg which returns to baseline values after approximately 3.7 +/- 0.2 mins, (n=14). Other calcium-entry blockers may have had additional effects apart from the one desired. For example, verapamil can interfere directly with alpha-adrenoceptors (van Meel et al., 1981; Jim et al., 1981) or with cellular mobilization of calcium (Thorens & Haeusler, 1979).

2) PLASMA NORADRENALINE ANALYSIS

a)Sample Preparation

2ml blood samples were taken from the pithed rats which were infused with either 0.9% saline (control) or lug/kg/min noradrenaline (each infused for 20 mins). The samples were taken from either the carotid artery or the jugular vein and placed immediately into heparinised tubes, rotamixed for a couple of minutes and placed on ice to await centrifugation.

The blood was taken either:

1) 30 mins after pithing i.e. just before infusion began,
2) 5 mins into the infusion i.e. where the maximum blood pressure response to NA infusion is seen,
3) at 20 mins when the infusion was finished.

When enough samples had been collected they were spun at 4000g in a Gallenkamp Angle Head Centrifuge for 20 mins and the plasma was decanted into 1ml EDTA tubes and rotamixed before storing overnight at -12^{o}C.

The plasma was thawed out and 1ml placed into 10ml conical tubes with 20mg of alumina and 5ml of Tris-EDTA buffer. 250ul of 2ng/ml (control expts.) or 20ng/ml (NA expts.) of 3,4-dihydroxybenzylamine hydrobromide (DHBA) were added to each tube as an internal standard. The tubes were capped and rotamixed for 1/2 hr after which the eluate was aspirated off and the alumina washed 4 times with ice cold distilled water. The NA was then eluted with 250ul of 0.1M perchloric acid added to the tubes which were then rotamixed for a further 1/2 hr (adapted from Eriksson & Persson, (1982)).

b) HPLC Techniques

The technique of high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) was employed to assay the levels of plasma NA. The components of this system are,

1) a microparticulate, high resolution chromatographic column (Altex Ultrasphere I.P. reverse phase, 4.5mmID x 25cm),

2) a pump capable of the high pressures necessary to pass fluid through such a column (Altex pump 110 A),

3) and after amine separation, a detector able to detect and quantitatively measure the NA (ESA 5100 A).

In addition this system included, an automatic sampler (Kontron MSI 660) for automatic injections of 100ul onto the column, in-line filters, a solvent reservoir and a pen recorder (Servoscribe RE 541). Eluant from the solvent

reservoir is filtered , pressurised and pumped through the column. The mixture of solutes injected into the system is separated into components on travelling down the column. The individual solutes are measured as they pass through the detector, the resultant voltage signal once amplified is recorded as peaks on the chart recorder. Peak heights are proportional to quantity as verified by assay of standards.

c) Ion-Pair Chromatography

In ion-pair chromatography the mobile phase is made acidic to ensure complete ionisation of the catecholamine (CA) molecules. The ion-pairing agent (sodium octylsulphonate) enters into an equilibrium complex with the ionised catecholamine molecule. This "ion-pair" then behave as a non-ionic species with some nonpolar (lipophilic) characteristics. The most recent theory is that the ion-pair reagent primarily attaches itself to the stationary phase (the column microparticles) by hydrophobic attraction, partitioning into it in a dynamic exchange process. The initial reverse-phase mode of the column has basically been changed to an ion-exchange mode. Ionised CAs then partition onto the newly formed stationary phase by conventional ion-exchange processes. The actual mechanism by which the column separates CAs is probably a mixture of this theory and an earlier one which proposed that the ion-pair is formed in the mobile phase prior to partition into the stationary phase (CAs only interacting with the stationary phase when in an ion-pair form).

d) Solvent Pretreatment

The buffer used in these experiments was a 90% Phosphate / 10% Methanol mixture taken to pH3 with phosphoric acid. This is a buffer employed in ion-pairing chromatography, the reagent being sodium octylsulphonate. Before the methanol is added, the mobile phase, as it is known, is filtered and degassed using a high vacuum pump and Millipore solvent Clarification kit with 0.45um aqueous filters. This prevents contamination of the pump and column by microparticulate debris and reduces the possibility of air bubbles forming which can disrupt solvent flow through the column and cause severe baseline noise.

Solutions Used In HPLC Analysis

i) Tris-EDTA Buffer (pH 8.6)

12g Tris Base
2g EDTA
100ml HPLC-grade water
Taken to pH 8.6 with HCl

ii) Phosphate Buffer (pH 3.0)

13.6g Potassium dihydrogen orthophosphate 0.1M
0.037g EDTA 0.1mM
0.057g Sodium octylsulphonate 0.26mM
100ml HPLC-grade Methanol
Diluted to 1 litre with HPLC-grade water
Taken to pH 3 with conc. orthophosphoric acid

e) Data Analysis

The amount of plasma noradrenaline present in the two types of experiments (saline or NA infused) were calculated by constructing a standard curve to NA i.e. known amounts of NA were added to the conical tubes and put through the whole extraction process along with the internal standard DHBA (both dissolved in 0.1M Perchloric acid). These samples were then injected onto the column immediately before the plasma. The height of the NA peak was measured and expressed as a ratio over the DHBA peak (this allows for any fluctuation in % recovery between extraction processes) and this was graphed against initial NA concentration. Then, when the plasma sample peaks were also expressed as a ratio over their DHBA peaks, a value could be read off the standard curve for the actual concentration of NA in 1ml of plasma.

3) IN VITRO EXPERIMENTS

Male Sprague-Dawley rats (300-400g) were stunned and killed by cervical dislocation. The aorta was removed and sectioned into rings of 3-4mm. These were set up in 0.5ml flow-through organ baths perfused with Tris buffer, 16 mls/min, (Watson Marlow pump, 502s). The buffer in the baths was maintained at pH 7.4, 37^{o}C and gassed with 100% O_2. The high flow rate was neccessary to ensure that the Tris solution measured 37^{o}C when it reached the baths. The temperature of the perfusing solution in the water bath (Grant Instruments) was kept below 40^{o}C to minimise the oxidation of the noradrenaline when it was added, and all

the connecting tubes were lagged with aluminium foil. The aortic rings were hooked on to fixed pins in the baths and looped over the transducer pins (AME AE875) which were then racked back to produce a tension in the tissues of 1-1.5g. These were then left to equilibrate for 1/2-1hr (adapted from Downing et al, (1983)).

The drugs were added to the bottles of perfusate. Noradrenaline (NA) was used as the agonist in these in vitro experiments primarily because it is the endogenous transmitter. It is a potent activator of the $alpha_1$-adrenoceptors present on the isolated, thoracic rat aorta and it is well documented that it produces both the fast and slow components of contraction which are thought to be dependent upon intracellular and extracellular calcium respectively (Godfraind & Kaba, 1972; Godfraind et al., 1982; Heaslip & Rahwan, 1982).

NA is not a selective $alpha_1$ agonist and can produce both pre- and post-synaptic $alpha_2$-adrenoceptor effects and can stimulate beta receptors. However, from the evidence available at this time it is accepted that in rat aorta $alpha_1$-adrenoceptors are the predominant or exclusive sub-type present (Rufflo, 1984), therefore NA's lack of specificity becomes less important. NA has less beta activity than the more "$alpha_1$selective" agonist phenylephrine (see discussion). Phenylephrine is also slightly less potent than NA on the rat aorta (Digges & Summers, 1983; Godfraind et al, 1982) although it does

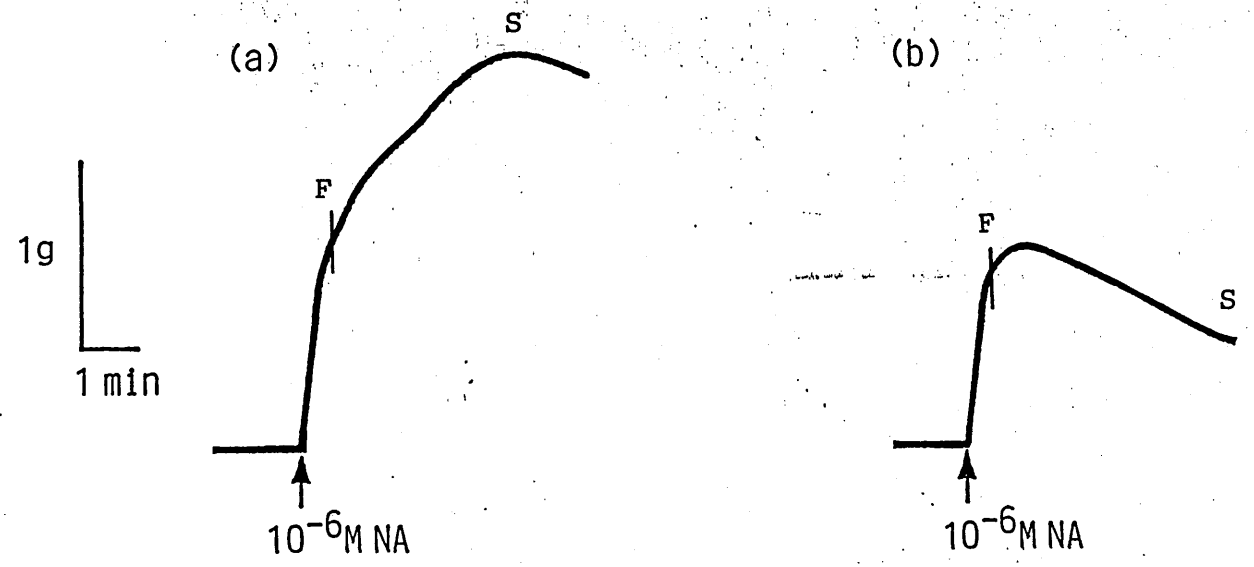
produce the fast and slow phases of the response similar to NA, and its fast response can also be antagonised by prazosin, the selective alpha$_1$ antagonist, (Wilson et al., 1983).

The dose of 10^{-6}M NA was chosen as it is slightly sub-maximal (see fig 21) (Downing et al., 1983) but still produces a good fast and slow component and has been used successfully in other studies (Godfraind et al., 1982).

The NA was generally exposed to the tissue for 4 mins to elicit a response i.e. the tubes feeding the organ baths were swapped to the bottle containing a Tris/NA mixture for 4 mins and then returned to normal Tris buffer for 1/2hr before the next NA "pulse". At the start of every experiment the tissues were pulsed 4 times with NA to obtain a reproducible response. All normal calcium experiments ($[Ca^{2+}]_{free}$=2mM) were carried out with 30uM EDTA present in the Tris to prevent the breakdown of noradrenaline.

The contraction of the rat aorta to NA is bi-phasic; an initial, transient contraction (ITC) and a secondary, slower contraction (SSC), fig 1a. For the purposes of this study, the abrupt change in slope of the contraction was used to differentiate the components of the response to noradrenaline. For the majority of the experiments this inflexion point was easy to identify as the concentration of noradrenaline used (10^{-6}M), although sub-maximal, ensured that a biphasic response was always produced. In experiments where the division between the ITC and the SSC

FIG 1 Representative traces illustrating the fast (F) and slow (S) components of the biphasic contraction of the rat isolated thoracic aorts to 10^{-6}M noradrenaline: (a) in 2mM Ca^{2+} saline and (b) in Ca^{2+}-free saline.


(a)
S
F
1g
1 min
10^{-6}M NA
(b)
F
S
10^{-6}M NA

was not so easy to determine e.g. the prazosin-blocked aortas, the ITC was measured as the response 30 secs after the initiation of the contraction. This time course was chosen because the ITC for 10^{-6}M noradrenaline was always completed within the first 30 secs of the contraction. The SSC was measured as the maximum response elicited during the exposure to the agonist. Contractions were plotted as % of maximum, since the maximum contractions elicited by noradrenaline on the aorta varied greatly between animals; i.e. Range in i) 2mM Ca^{2+} solutions (n = 2 to 9):
ITC= 0.68 ±0.02g to 1.44 ±0.22g; SSC= 0.81 ±0.09g to 2.25g
ii) Calcium-free solutions (n = 2 to 9):
ITC= 0.69 ±0.02g to 1.28g; SSC= 0.68 ±0.04g to 0.87 ±0.07g

To study the effects of antagonists on the initial fast contraction, Ca^{2+} was excluded from the Tris buffer. This $[Ca^{2+}]_{free}$-solution isolated (to some extent) the ITC, fig 1b. The $[Ca^{2+}]_{free}$-solution was created by excluding all calcium salts from the saline and chelating remaining calcium contamination with 1mM EGTA. This was later found to be insufficient so 5mM EGTA was used instead. The tissues were perfused with this $[Ca^{2+}]_{free}$ saline for 4 mins before the agonist was administered. The tissues were exposed to the agonist for 4 minutes and were then returned to normal Tris for 1/2hr before the next $[Ca^{2+}]_{free}$ exposure. When an antagonist effect was being examined the antagonist was present at all times, i.e. before during and after pulsing with NA.

A major part of this study was to examine the effect, on both the fast and slow components of NA's contraction, of removing and reintroducing Ca^{2+} into the buffered saline. This was done at first by adding increasing concentrations of Ca^{2+} to the $[Ca^{2+}]_{free}$ solution (with and without EGTA present) and pulsing for 4 mins prior to NA, as described above. This proved unsuccessful as there was insufficient buffering of the Ca^{2+} when it reached higher concentrations. The contractile response was not increased gradually as the calcium concentration increased.

Therefore a buffering system was used incorporating known levels of $[Ca^{2+}]_{total}$, EGTA and nitrilotriacetic acid (NTA) (Miller & Smith, 1984) in the Tris saline. Thus the level of $[Ca^{2+}]_{free}$ in the solution could be accurately calculated. Once the solutions had been corrected for pH, this system proved highly successful and could be used in two ways to study the aortic responses:

i) these buffers were pulsed for 4 mins before NA was added, thus allowing both fast and slow responses to occur and then the tissues were returned to normal Ca^{2+} for 1/2hr as described above or,

ii) the tissues were exposed to Buffer 6, the lowest Ca^{2+} buffer, for 10 mins before NA was added and then the buffers were sequentially perfused (containing increasing levels of Ca^{2+}) with NA present at all times, to view the maximum slow contraction elicited.

The concentration of $[Ca^{2+}]_{free}$ in the solutions was calculated using the method described by Miller & Smith

(1984) which takes the form of a programme written for the "Texas" calculator, written by Godfrey Smith. The log $K_{app}Ca^{2+}$ (the apparent affinity constant for the binding of calcium ions to EGTA) is first calculated with respect to:

i) purity (Sigma is c. 96.45%),

ii) ionic strength,

iii) temperature.

This is then entered into the programme along with the level of calcium added (allowing for the calcium contamination, typically 20 uM) and the pCa (-log concentration of calcium, M) is then calculated, (see also Smith & Miller, 1985).

Solutions Used In IN VITRO Experiments

i) Tris Buffer : pH 7.4 37^{o}C

Sodium chloride 146.5mM	Trizma hydrochloride 7mM
Potassium chloride 6.0mM	Trizma base 3mM
Magnesium chloride 1.0mM	Glucose 5.5mM
Calcium chloride 2.0mM	Gassed with 100% O_2

ii) Calcium Buffers (1 litre)

Tris buffer 987.8mls

EGTA 0.95g (2.5mM)

NTA 0.475g (2.5mM)

1M Sodium hydroxide 7.5mls (to dissolve the EGTA/NTA mix)

Then the following amounts of calcium are added to produce the levels of $[Ca^{2+}]_{free}$ stated below:

		$[Ca^{2+}]_{free}$
Buffer 1. Calcium chloride	4.69mls	$3x10^{-4}$mM
Buffer 2. B.1) 668.8mls + B.6)	331.20mls	$1x10^{-4}$mM
Buffer 3. " 331.2mls "	668.80mls	$3x10^{-5}$mM
Buffer 4. " 146.6mls "	853.40mls	$1x10^{-5}$mM
Buffer 5. " 53.4mls "	946.50mls	$3x10^{-6}$mM
Buffer 6. Calcium chloride	2.35mls	$1x10^{-6}$mM

4) STATISTICAL ANALYSIS

Statistical comparisons were made using Student's t-test for paired or unpaired data. Linear correlations were assesed using linear regression analysis. The difference between two means were considered statistically significant if $p < 0.05$. pD_2 values were determined from the relevant dose/response curves. In the "in vivo" experiments, the response to phenylephrine in alkalotic conditions (see fig 4) the data suggested a quadratic relationship. Thus, the curve of best fit was employed, choosing the model:

$$y=a_0+a_1x+a_2x^2+e$$

where, y=response

x=gas tension

An estimate of the coefficients a_0,a_1 and a_2 and the constant e was made using the regression analysis routine in MINITAB (Ryan et al, 1976). This procedure chooses the coefficients to minimise the mean of the squares of the difference between the measured values and those predicted

byficients to minimise the mean of the squares of the difference between the measured values and those predicted by the equation. For this case the predicted equation was:

$$y=25.9 + 0.545x - 0.0013x^2$$

This fit (and the linear relations indicated in the other regression analyses) is being used in a purely descriptive manner and not to make statistical inferences about the underlying processes.

5) DRUGS and COMPOUNDS

(-) amidephrine hydrochloride (Mead Johnstone)

azepexole/BHT 933 (Thomae)

cadmium chloride (BDH)

cirazoline hydrochloride (Synthelabo)

dantrolene sodium (Norwich-Eaton Pharmaceuticals)

3,4-dihydroxybenzylamine hydrobromide (Sigma)

EDTA (Sigma)

EGTA (Sigma)

NTA (Sigma)

methoxamine hydrochloride (Burroughs Wellcome)

M7 / 2-N-dimethylamino-5,6,dihydroxy-1,2,3,4-tetrahydronaphthalene (supplied by Syntex)

nifedipine (Bayer)

noradrenaline bitartrate (Koch-Light)

oxymetazoline hydrochloride (Merck)

phenylephrine hydrochloride (Sigma)

prazosin hydrochloride (Pfizer)

(+/-) propranolol hydrochloride (Sigma)

rauwolscine base (Inverni Della Beffa)

siegfried 101/75 (Siegfried Zofingen)

sodium octylsulphonate (Fisons)

xylazine hydrochloride (Bayer)

Drugs were dissolved in 0.9% saline except for the following:

nifedipine - pithed rat: 10 mg in 1ml of cremophor heated to approx. 75^{o}C and diluted in distilled water to give desired concentration.

rat aorta: 10 mg in 1ml of alcohol and diluted to desired conc. in distilled water.

prazosin - distilled water

rauwolscine - a w/w solution of ascorbic acid in distilled water.

RESULTS

THE INFLUENCE OF BLOOD GASES ON ALPHA$_1$ AND ALPHA$_2$ ADRENOCEPTOR-MEDIATED PRESSOR RESPONSES IN THE PITHED RAT

In a preliminary study of oxygen-ventilated rats (McGrath et al, 1982), it was found that altering the artificial ventilation volume, and hence varying the blood gases, changed the susceptibility of adrenaline's pressor response to alpha-blockers. In respiratory acidosis the alpha$_2$ antagonist rauwolscine was more potent than in alkalosis, while the opposite was found for the alpha$_1$ antagonist, prazosin. This suggested that the responses mediated through the alpha receptor sub-types might be optimal under different blood-gas conditions. This section further investigates this phenomenon using the agonists phenylephrine (alpha$_1$) and xylazine (alpha$_2$) as responses to these might be expected to be affected differentially by alteration of the ventilation.

1.1) General Effects of Varying Ventilation

The effects of manipulating the ventilation on the arterial blood gases and resting heart rate of the pithed rat are summarised below.

1.1.a) Arterial pH and $PaCO_2$

As shown in figure 2 (a & b), increasing the ventilation stroke volume (1.8-3.5ml for O_2; 2.5-4.0ml for air) increased the arterial pH and decreased the $PaCO_2$. In air ventilated rats, if the stroke volume was reduced below 2.5ml, the rats became so hypoxic that the heart stopped.

In O_2-ventilated rats, extremely low pH and high $PaCO_2$ levels could be achieved by reducing the stroke volume below 2.5ml. The rats remained viable because they were still hyperoxic.

1.1.b) PaO_2

As shown in figure 2 (c), increasing the stroke volume (2.5-4.0ml) modestly increased the PaO_2 in air-ventilated rats. Approximately physiological PaO_2 could be obtained only if the rats were ventilated at a stroke volume of 4.0ml. However, as shown in figure 2 (a & b), this would produce a high pH and low $PaCO_2$ levels. In contrast, O_2-ventilated rats were always hyperoxic, even at very low stroke volumes. Increasing the stroke volume produced marked increases in PaO_2. At the highest stroke volume studied, PaO_2 values in excess of 300mmHg were obtained.

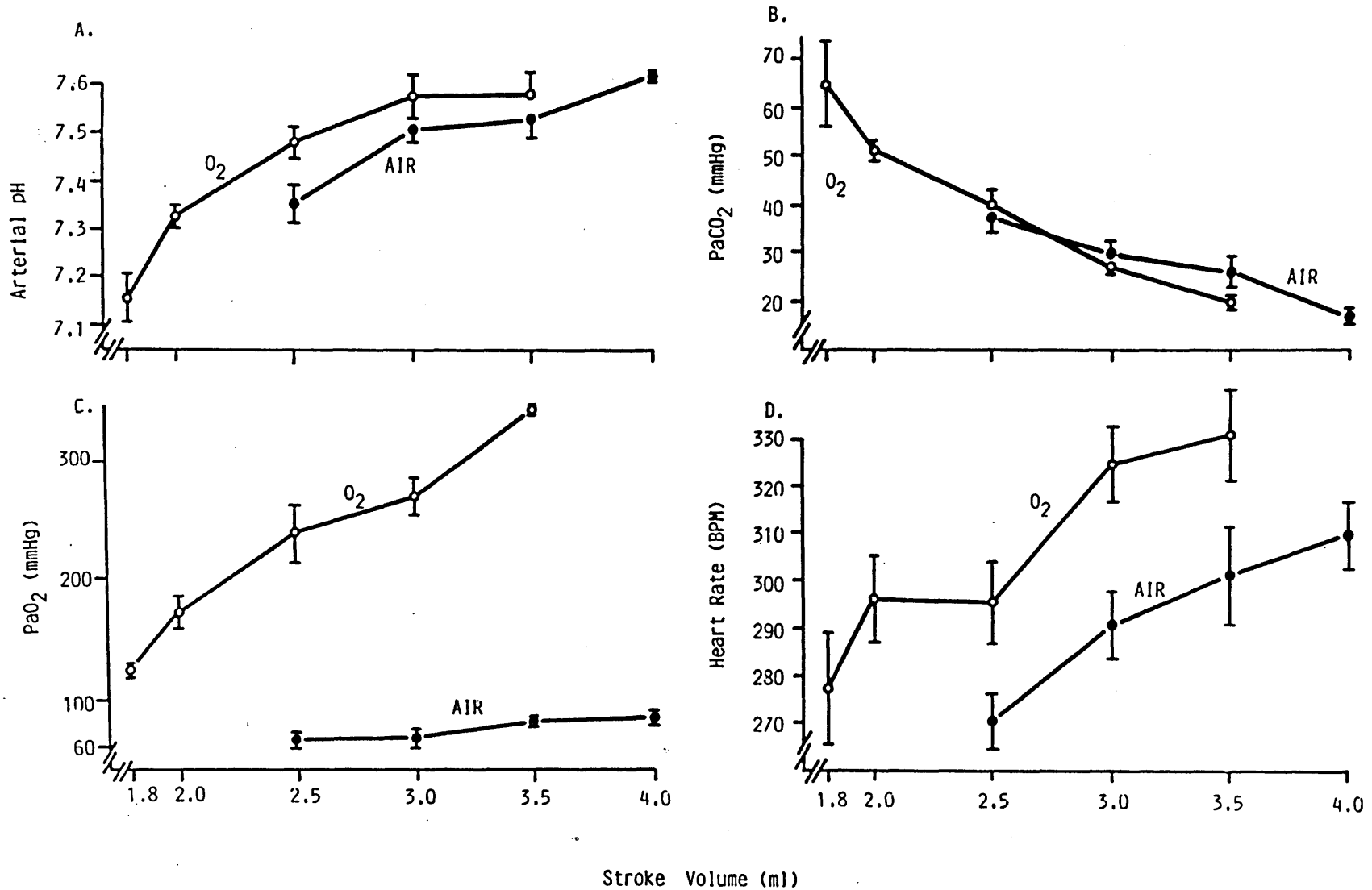
1.1.c) Heart rate

In both air and O_2-ventilated rats, the basal heart rate increased with increasing stroke volume. Over the directly comparable range of stroke volumes (i.e. 2.5-3.5ml). O_2-ventilated rats always had higher basal heart rates than air-ventilated rats (see figure 2d).

1.2) Phenylephrine

In the pithed rat, pressor responses to phenylephrine were biphasic, consisting of a rapid initial increase which reached a peak 10-12 secs after injection,

FIG. 2 A summary of the effects of varying the ventilatory stroke volume on (a) arterial pH, (b) $PaCO_2$, (c) PaO_2 and (d) heart rate in the pithed rat. Rats were ventilated either with air, closed symbols (n=21), or with 100% O_2, open symbols, (n=16).


A.
Arterial pH
7.6
7.5
7.4
7.3
7.2
7.1
O_2
AIR
B.
$PaCO_2$ (mmHg)
70
60
50
40
30
20
O_2
AIR
C.
PaO_2 (mmHg)
300
200
100
60
O_2
AIR
D.
Heart Rate (BPM)
330
320
310
300
290
280
270
O_2
AIR
1.8
2.0
2.5
3.0
3.5
4.0
Stroke Volume (ml)

followed by a slower secondary response with a peak at 22-30 secs (fig 3(a)). Baseline was re-established at between 1.5 and 9 min according to the dose and the blood gas status (see below). Responses were measured at the early peak, at 30 secs and at 2 min. In the dose range tested (0.1 - 30.0 ug/kg), all components of the response were blocked by prazosin (1 mg/kg), (Flavahan & McGrath, 1981) and are thus considered to be mediated by $alpha_1$-adrenoceptors.

1.2.a) Air

At every dose of phenylephrine studied, the responses decreased with increasing $PaCO_2$ over the range 19-42 mmHg (fig 4a, i-iii), (further increases in $PaCO_2$ were not possible since reducing stroke volume made the rats so hypoxic that the heart stopped). This relationship was similar whether the early peak or the 30 sec response was measured. At doses falling in the steepest part of the dose/response curve (e.g. 3 ug/kg), the correlation was clearer for the peak than for 30 sec (R:peak= 0.79, $0.001<P<0.01$; R:30sec= 0.51, not. sig., where R = regression coefficient). Therefore fig 4a shows the peak responses. No significant correlation was found when the response was plotted against PaO_2. The responses were short-lived on air compared to those obtained when respiring with O_2 or a "normoxic" mixture (see below). Responses at 2 min were detectable only with 10 and 30 ug/kg and hypocapnic conditions.

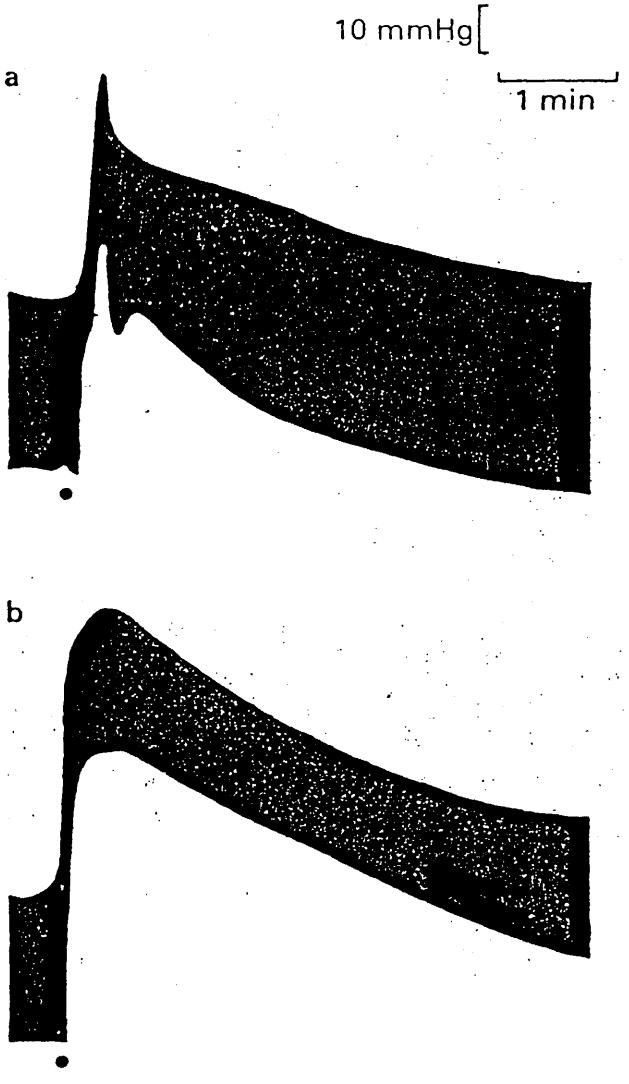
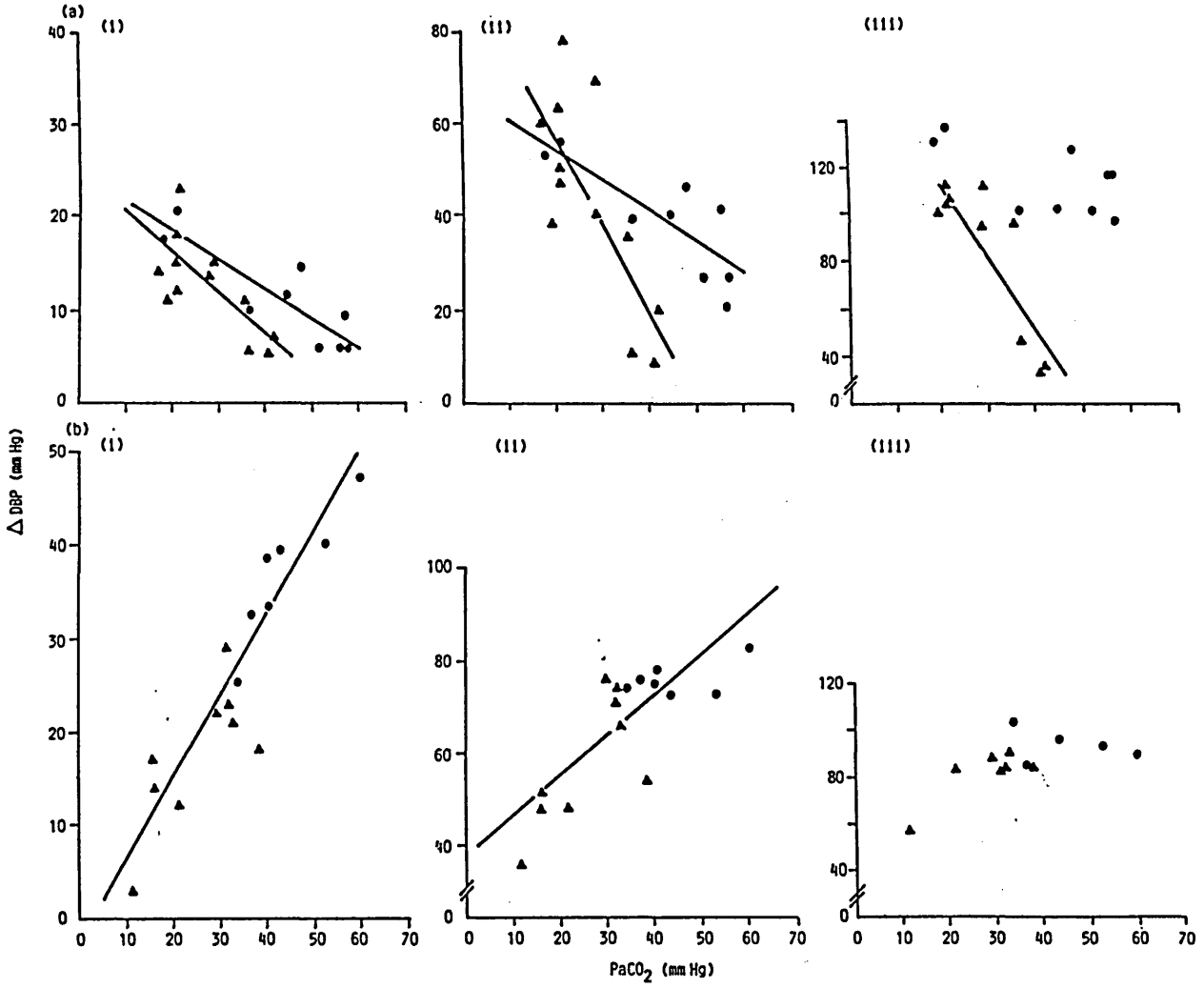


Figure 3 Shows typical pressor responses to (a) phenylephrine ($3\,\mu g\,kg^{-1}$) and (b) xylazine ($0.5\,mg\,kg^{-1}$) in the pithed rat.

FIG. 4 The effects of $PaCO_2$ on the peak change in diastolic arterial blood pressure (/\DBP mmHg) to (a) phenylephrine (i) 0.3 ug/kg (ii) 3.0 ug/kg (iii) 30 ug/kg and (b) xylazine (i) 0.05 mg/kg (ii) 0.5 mg/kg (iii) 5.0 mg/kg. Rats were respired on air (triangles) or 100% O_2 (circles) and ventilatory stroke volume varied. Regression lines are shown in (a) for air (n=10-12) and O_2 (n=9) groups ; in (b) for all points combined (air, n=7-9; O_2, n=5-7). For phenylephrine (30 ug/kg) on O_2 and for the combined data on xylazine (5mg/kg) the correlation is not significant since the responses are maximal.


(a)
(I)
(II)
(III)
(b)
(I)
(II)
(III)
Δ DBP (mm Hg)
PaCO2 (mm Hg)

1.2.b) O_2

The preparation was viable over a wider range of $PaCO_2$ levels than could be obtained in air-ventilated rats (18-57 mmHg). The responses decreased with increasing $PaCO_2$ but not as markedly as with air ventilation. Peak responses are shown in fig 4a since the correlation was better (R:peak= 0.79, $0.001<P<0.01$; R:30sec= 0.67, $0.01<P<0.05$ at 3 ug/kg). With the highest dose of phenylephrine (30 ug/kg), peak responses did not decline with increasing $PaCO_2$ but responses measured at 30 sec did. This may indicate that the "peak" is maximal and therefore is insensitive to change whereas the declining phase, measured at 30 sec, remains sensitive. No significant correlation was found between PaO_2 and the response. The response persisted beyond 2 min at doses of 3 ug/kg or greater. This prolonged part of the response declined with increasing $PaCO_2$.

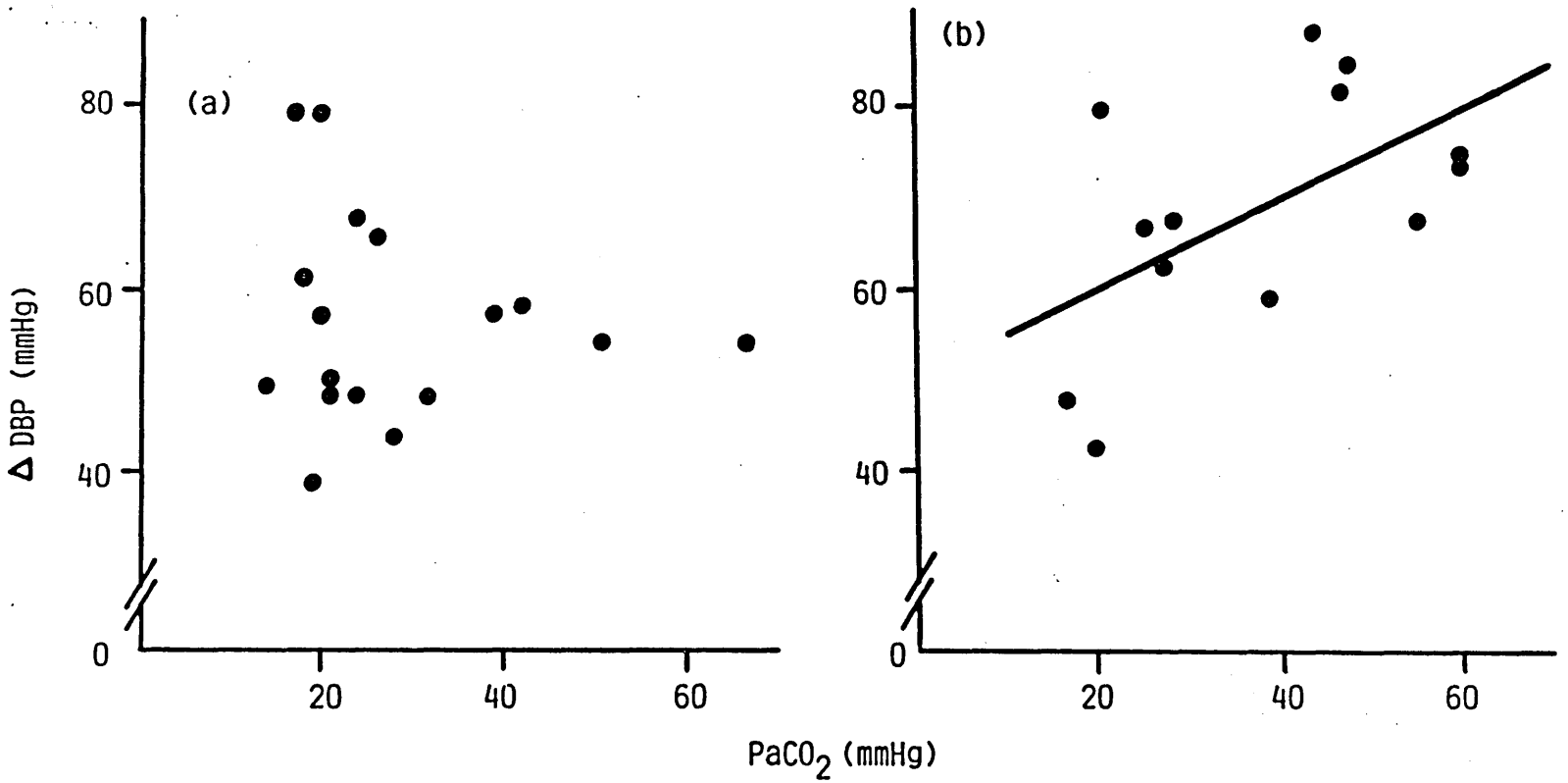
1.2.c) "Normoxic"

Rats ventilated with a gas mixture containing 30% O_2 and a varying amount of CO_2 (0-4%), had responses to phenylephrine of similar magnitude to those in O_2-ventilated rats (fig 5a) but there was no significant correlation between the response and $PaCO_2$.

1.2.d) Varying O_2 under acidotic and alkalotic conditions

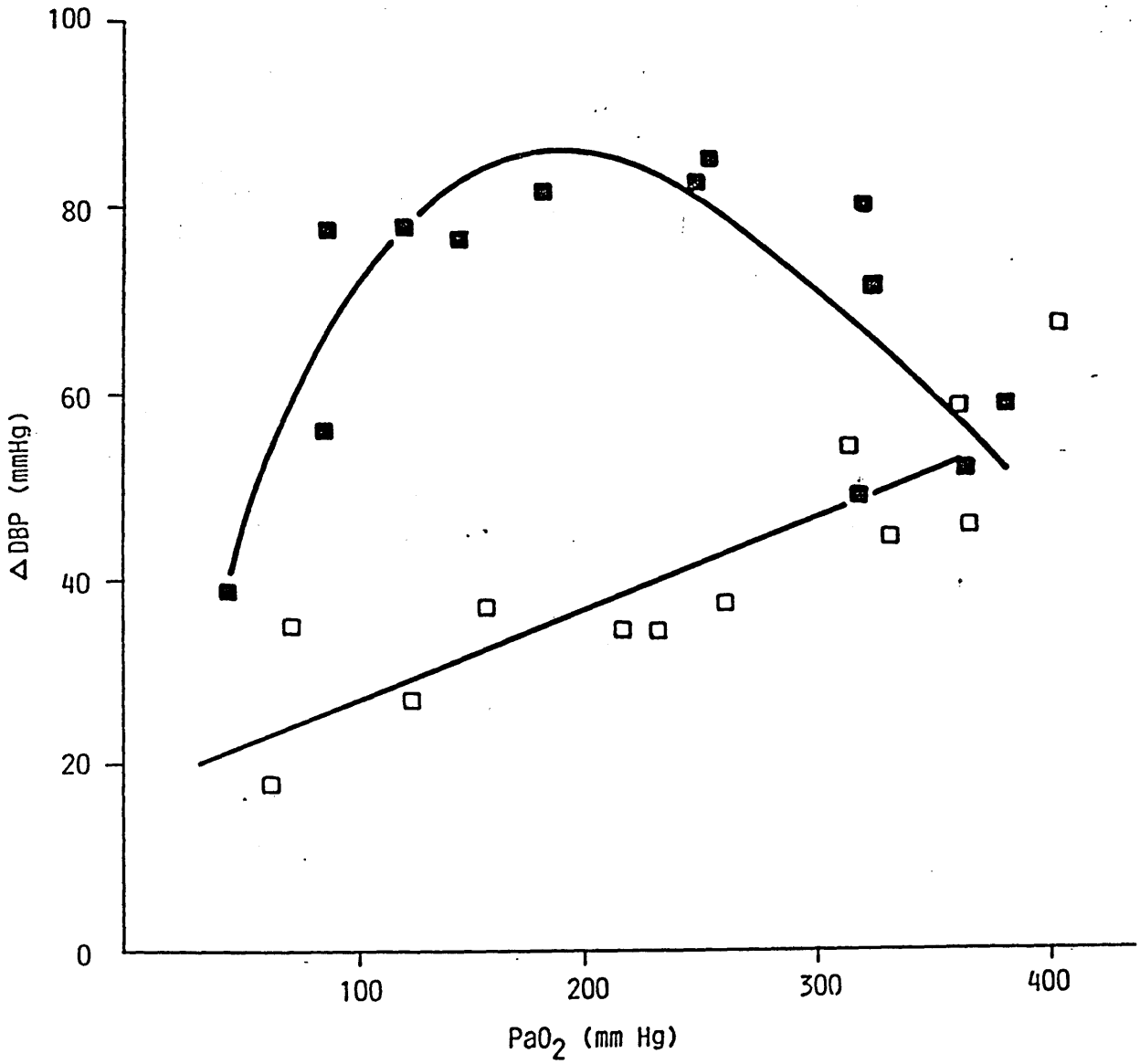
The O_2 content of the inspired gas was varied (15-100%) at constant CO_2 levels of either 0% CO_2 (alkalotic conditions) or 4% CO_2 (acidotic conditions). Under both alkalotic

FIG. 5 The effects of varying the $PaCO_2$ on peak diastolic blood pressure responses to (a) phenylephrine (3.0 ug/kg, n=17) and (b) xylazine (0.5 mg/kg, n=13). Rats were ventilated at a constant stroke volume with 30% O_2 to produce normal PaO_2 levels. The % of inspired CO_2 was varied in order to vary the $PaCO_2$.


(a)
(b)
80
60
40
0
20
40
60
Δ DBP (mmHg)
PaCO2 (mmHg)

conditions ($PaCO_2$= 19.0 +/- 0.9mmHg; pH= 7.53 +/- 0.02, n=13) and acidotic conditions ($PaCO_2$= 52.0 +/- 1.1mmHg; pH= 7.26 +/- 0.01, n=12) responses increased with increasing PaO_2 (fig 6). However in alkalotic rats, responses reached an optimum at a PaO_2 of around 200mmHg. Any further increase in PaO_2 produced a reduction in response to phenylephrine.

FIG. 6 The effects of PaO_2 on the peak diastolic blood pressure response to phenylephrine (3.0 ug/kg). Rats were ventilated at 3.5 ml/st with either 0% CO_2 (closed symbols), producing alkalosis, or 4% CO_2 (open symbols), producing acidosis. The inspired % of O_2 was varied at both levels of CO_2. The top line is a line of best fit (13 experiments, quadratic relationship - see methods) using the regression analysis routine in MINITAB (Ryan et al., 1976). The bottom line was obtained from regression analysis (12 experiments).


100
80
60
40
20
0
ΔDBP (mmHg)
100
200
300
400
PaO2 (mm Hg)

1.3) Xylazine

Pressor responses to xylazine were biphasic but less markedly so than were those to phenylephrine. The pressure rose sharply for 12 to 15 sec and then continued to rise more slowly to a peak at 30 sec or more (fig 3b). Baseline was regained between 3-13 min depending on dose. At high doses (>0.5mg/kg) the response remained at a plateau for several minutes before declining. Responses were measured at 30 sec, at the peak (between 30 sec and 5 min according to dose) and at 5 min. At high doses of xylazine, the initial peak is sensitive to prazosin and therefore contains an $alpha_1$ component. The remainder of the response is resistant to prazosin but susceptible to the $alpha_2$ antagonist rauwolscine (Flavahan & McGrath, 1981a & b). However in the dose range employed in this study (0.01-10mg/kg), the pressor response to xylazine is not significantly affected by prazosin (Grant & McGrath, unpublished observations).

1.3.a) Air

At each dose of xylazine the response increased with increasing $PaCO_2$. This relationship was similar whether the response was measured at 30 sec or at the peak but did not hold at 5 mins. Fig 4b shows peak responses (R:peak= 0.71, R:30sec= 0.80; $0.001<P<0.01$ for both, and R:5min= 0.28, not significant, for 0.1 mg/kg xylazine). There was no correlation with PaO_2.

1.3.b) O_2

Ventilation with O_2 allowed continuation of the $PaCO_2$/response relationship into the hypercapnic range. At low doses of xylazine (0.01-0.05mg/kg) responses continued to increase with increasing $PaCO_2$ over the range studied (34-60 mmHg). At higher doses (>0.5mg/kg) responses reached a plateau level at $PaCO_2$= 35 mmHg. The correlation was similar at each of the three time intervals after injection (e.g. R:peak= 0.84; R:30sec= 0.82; R:5min= 0.96; $0.001<P<0.01$ in all cases for 0.1mg/kg xylazine) see fig 4b. There was no significant correlation of responses with PaO_2.

1.3.c) Comparison of air and oxygen

On air ventilation, since the responses varied with $PaCO_2$, responses to low doses of xylazine were widely scattered. Nevertheless, if the "air" and "O_2" groups are compared, the responses on "air" are significantly smaller at the lower doses ($P<0.001$ for 0.05mg/kg; $0.001<P<0.01$ for 0.5mg/kg; $0.01<P<0.05$ for 5mg/kg). This was not the case for any dose of phenylephrine ($0.05<P$ for 0.3, 3.0 and 30 ug/kg).

1.3.d) "Normoxic"

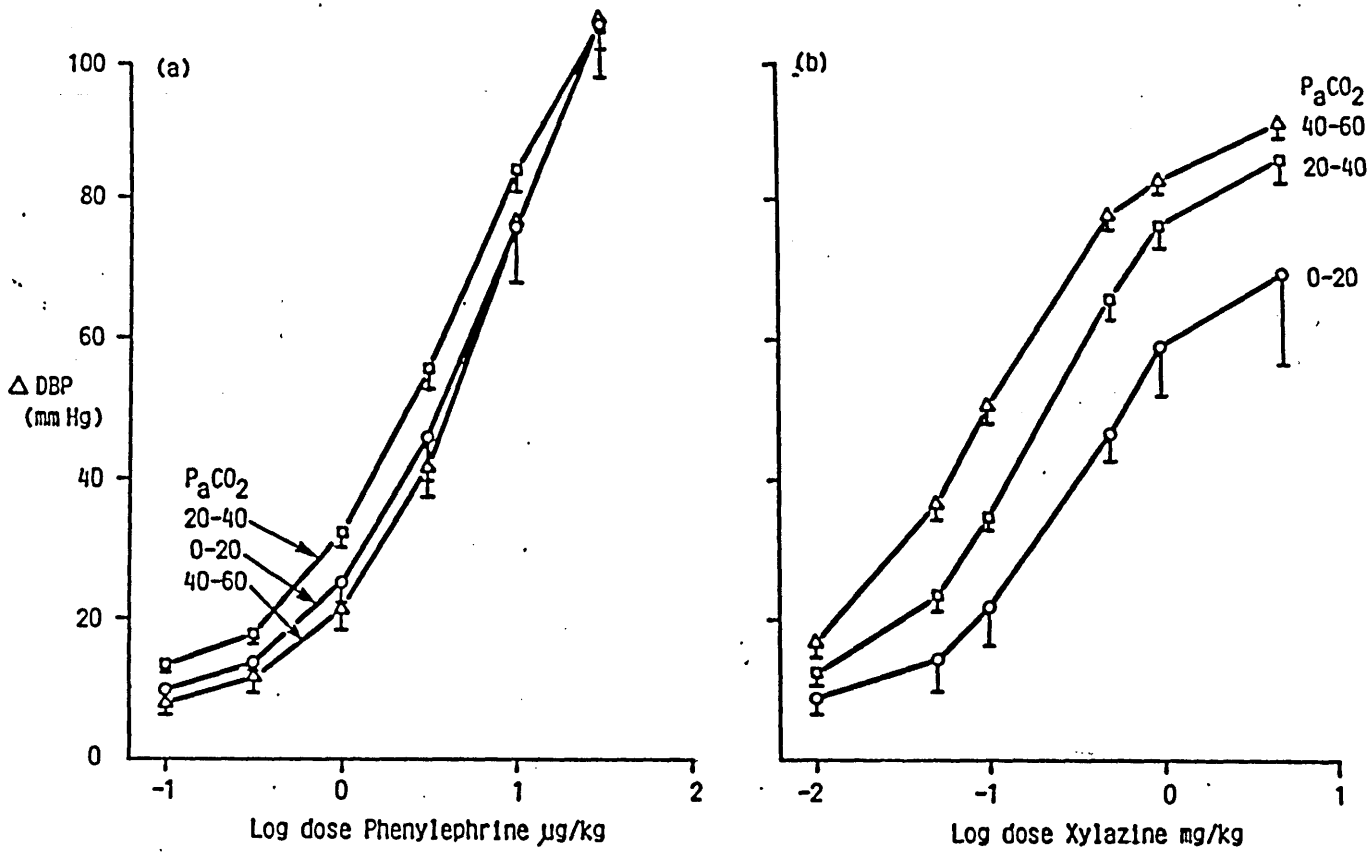
When PaO_2 was held constant at approximately physiological levels (PaO_2= 100 mmHg) and the $PaCO_2$ was varied (0-4%), the responses followed the same relationship as when the ventilation was varied on air or 100% O_2. Fig 5b shows the peak responses for 0.5mg/kg xylazine (R= 0.62, $0.01<P<0.05$

That is, responses increased with increasing $PaCO_2$.

1.4.a) Dose/response curves and blood gases.

A comparison of dose/response curves for phenylephrine and xylazine at different levels of $PaCO_2$ (fig 7) shows that xylazine's response will be relatively underestimated and phenylephrine's response overestimated under the type of alkalotic conditions likely to be found in "over ventilated" air-ventilated, pithed rats. Conversely, when 100% O_2 is employed, rats can remain viable even at PaO_2=100 mmHg with an "under-ventilation" which induces severe acidosis; in these conditions the relative effect of xylazine might be overestimated. "Physiological" blood gases could be mimicked by ventilating the rats with 40% O_2, 60%N_2 at 2.5ml/stroke and 60 strokes/min. These would seem to be the optimal conditions for studying physiological adrenergic mechanisms in the pithed rat.

FIG 7 The effects of varying the $PaCO_2$ on the log dose/response curve to a) phenylephrine and b) xylazine in the pithed rat. Experiments were separated according to $PaCO_2$ in the ranges i) 0-20 mmHg, ii) 20-40 mmHg and iii) 40-60 mmHg. Table c) shows the effect of increasing $PaCO_2$ on the relative potencies of the two agonists.



(c) Summary of ED_{50} mmHg values with increasing P_aCO_2

ED_{50} mmHg (µg/Kg)	P_aCO_2 mmHg 0-20	P_aCO_2 mmHg 20-40	P_aCO_2 mmHg 40-60
Xylazine	603	219	96
Phenylephrine	3.8	2.5	4.2
Potency Ratio $\frac{Phe}{Xyl}$	159	88	23

A COMPARISON OF Ca^{2+}-ENTRY BLOCKADE ON BOLUS AND INFUSION RESPONSES TO ALPHA-AGONISTS IN THE PITHED RAT.

The previous results show that $alpha_1$- and $alpha_2$-adrenoceptor mediated pressor responses can be differentially modulated by alterations in the acid-base balance. Responses to phenylephrine are decreased with $PaCO_2$ and increase with PaO_2 whereas those to xylazine are increased with $PaCO_2$ and remain unaffected by PaO_2. Phenylephrine boluses produce a rapid, transient response whereas xylazine boluses produce a slower response with a more dominant second phase. This pattern of $alpha_1$-type and $alpha_2$-type responses does not hold true for all alpha-adrenoceptor agonists. Amidephrine produces a persistent $alpha_1$-mediated pressor response, which is potentiated by respiratory acidosis. Similar to the second phase of xylazine's response, this prolonged $alpha_1$-response is susceptible to calcium entry blockers, (Flavahan & McGrath, 1982).

This provided support for the proposal that it is the excitation-contraction coupling rather than the receptor which is the critical element influenced by blood gases. It emphasised the need to consider the components within the time course of the response in pithed rats, which never represents an equilibrium but rather shows a series of linked events reflecting the rise and fall of the blood concentration of the drug (Docherty & McGrath, 1980).

Therefore, the study described in this section was

carried out in order to examine more closely the time course of the response to a bolus of alpha-agonist. Infusions of the drugs were also studied as they may be more indicative of the equilibrium response. The effect of calcium entry blockade on these two types of response was used as a means of examining one of the receptor-activated processes involved i.e. opening of Ca^{2+} channels.

2.1) General

The rats in this study were respired to keep their blood gases well oxygenated but slightly acidic. Respiring them on 100% O_2, 60 strokes/min and 2.0ml/stroke had previously been discovered to prolong the time-course of alpha-agonists injected (see previous section). This prolonged part of the response was thought to involve Ca^{2+}-entry and therefore should be susceptible to the calcium antagonist, nifedipine. Doses of the agonists were chosen to give a blood pressure response of approx. 60mmHg (so falling on the steeply rising section of the dose/response curve). For typical examples of the pressor responses to infusions of the alpha-agonists see fig 8. This proved difficult to estimate, but for most drugs there appeared to be almost a one to one correlation between the concentration of agonist needed to give a certain response from a bolus injection and that needed to give the same ultimate response from an infusion, fig 9. Nifedipine was injected intra-arterially (to avoid cardiac depression), in the fixed dose of 0.3mg/kg, 15-20 mins before the agonist was administered. It produced a transient depressor response of 10.3 ±1 mmHg which returned

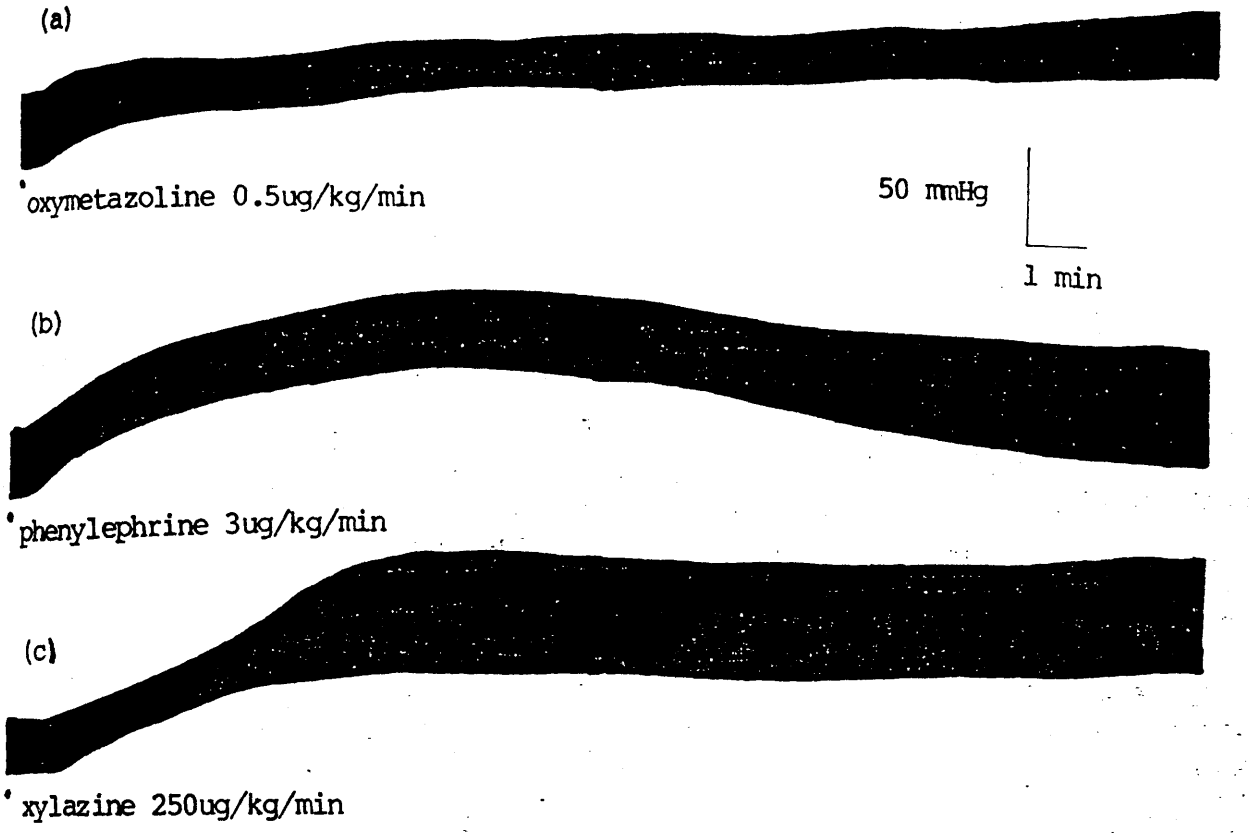
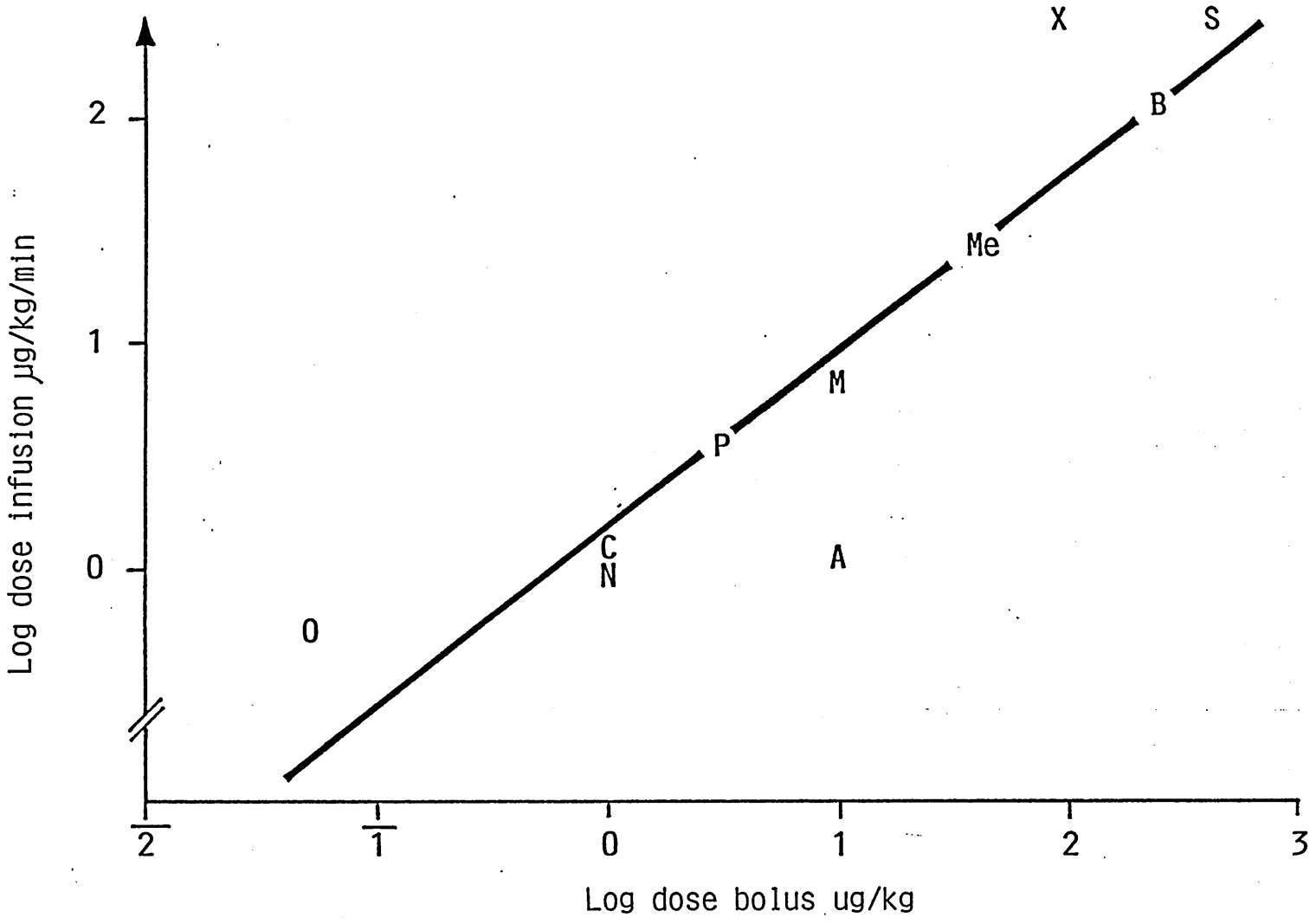


FIG. 8 Shows typical pressor responses to (a) oxymetazoline; (b) phenylephrine and (c) xylazine in the pithed rat.

FIG. 9 The correlation of the log bolus concentration (ug/kg) vs the log infusion concentration (ug/kg/min) of the alpha agonists, used to produce a pressor response of approximately 60 mmHg, in the pithed rat. The line was obtained by regression analysis. Key: oxymetazoline (O); noradrenaline (N); cirazoline (C); amidephrine (A); phenylephrine (P); M7 (M); methoxamine (Me); BHT 933 (B); xylazine (X); SGD 101/75 (S). n=3-6.


X
S
B
Me
M
P
C
N
A
O
2
1
0
Log dose infusion µg/kg/min
2
1
0
1
2
3
Log dose bolus ug/kg

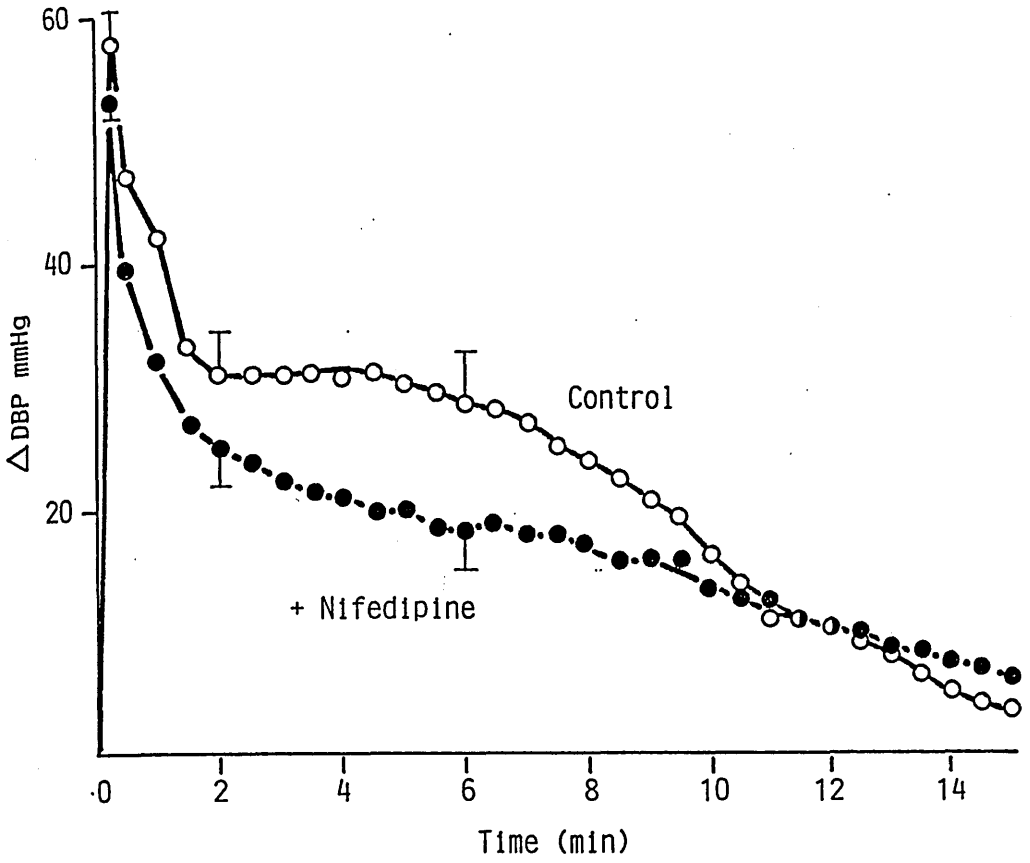
to baseline after 3.7 ±0.2 mins (n=14). For the following figures in this section, only representative s.e.m.'s (standard error of the mean) have been shown per line, to aid the clarity of the figures. Commonly these are at the peak and declining phase of the bolus responses and at the beginning, rising phase and plateau (if there is one) of the infusion response. The alpha-agonists were chosen to give as varied a type of response as possible: 1) "typical" short lasting $alpha_1$-agonists, phenylephrine, cirazoline and methoxamine, 2) long-lasting $alpha_1$-agonists amidephrine and SGD 101/75, 3) "typical" long-lasting $alpha_2$-agonists xylazine and azepexole and 4) a rapid $alpha_2$-agonist, M7.

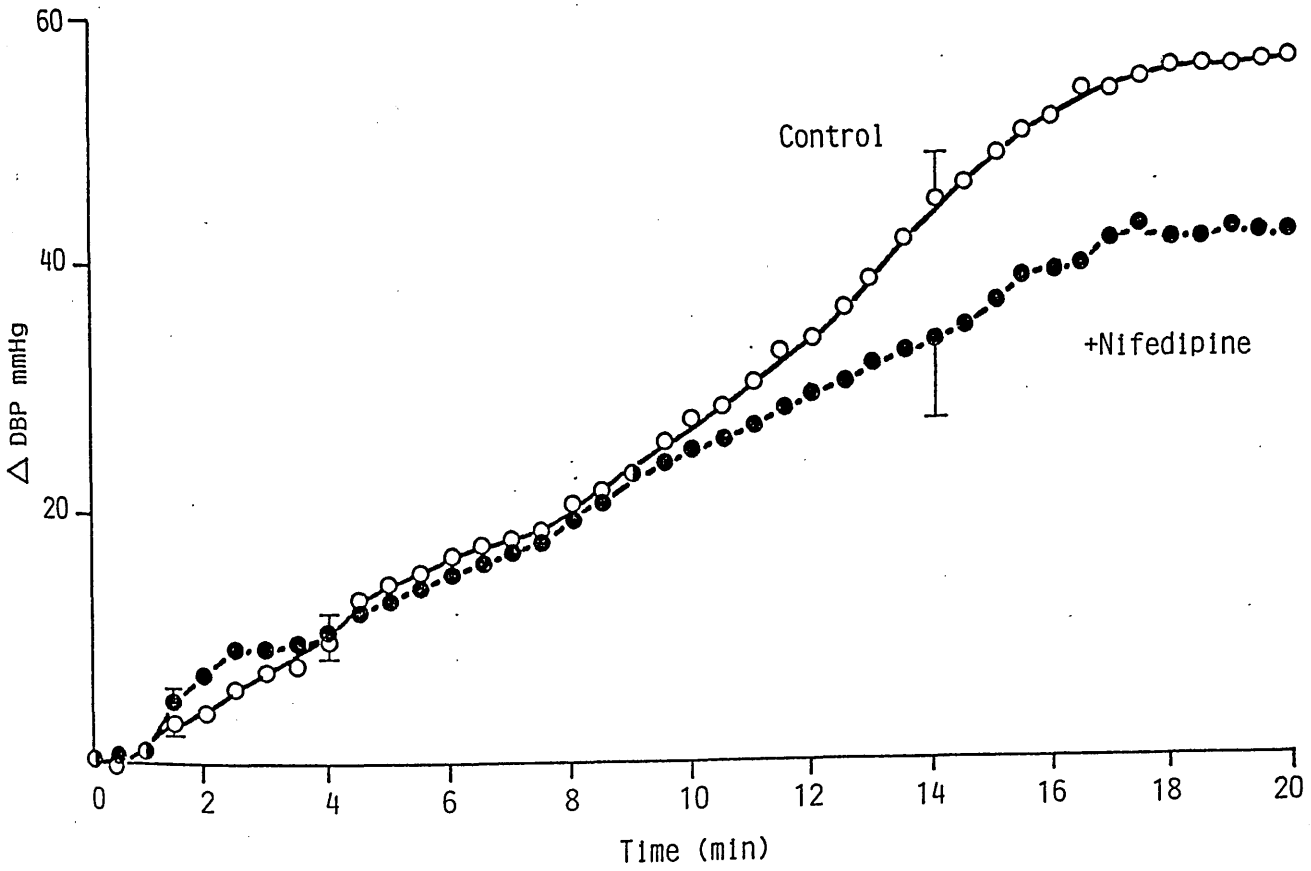
2.2) Vasoconstrictor Responses to $Alpha_1$-Adrenoceptor Agonists

2.2.a.) Amidephrine

BOLUS: Expanding from Flavahan's work (1982), bolus injections of amidephrine (10ug/kg) were given to rats respired as described above. They produced a rapid blood pressor response of 58 +/- 3mmHg ($\bar{x}$ +/- the standard error of the mean, s.e.m., n=3) lasting for over 15 mins, fig 10a. Nifedipine (0.3 mg/kg) was then employed to block the response. It had no effect on the maximum response (54 +/- 1mmHg, not significant, n=3). However the secondary phase of the response was significantly blocked ($0.01<P<0.05$; t=5 mins). The time taken for the response to decay to 50% of its maximum ($t_{1/2}$) was 6 +/- 1 mins for the control and 2 +/- 0.6 mins for the nifedipine blocked rats ($0.01<P<0.05$). When the area under the graph was measured the inhibition

FIG 10 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of amidephrine (10 ug/kg), and B) an infusion of amidephrine (1 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure (/\DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=3-4.


60
40
20
Δ DBP mmHg
Control
+ Nifedipine
0
2
4
6
8
10
12
14
Time (min)


60
40
20
Δ DBP mmHg
Control
+Nifedipine
0
2
4
6
8
10
12
14
16
18
20
Time (min)

was 26%.

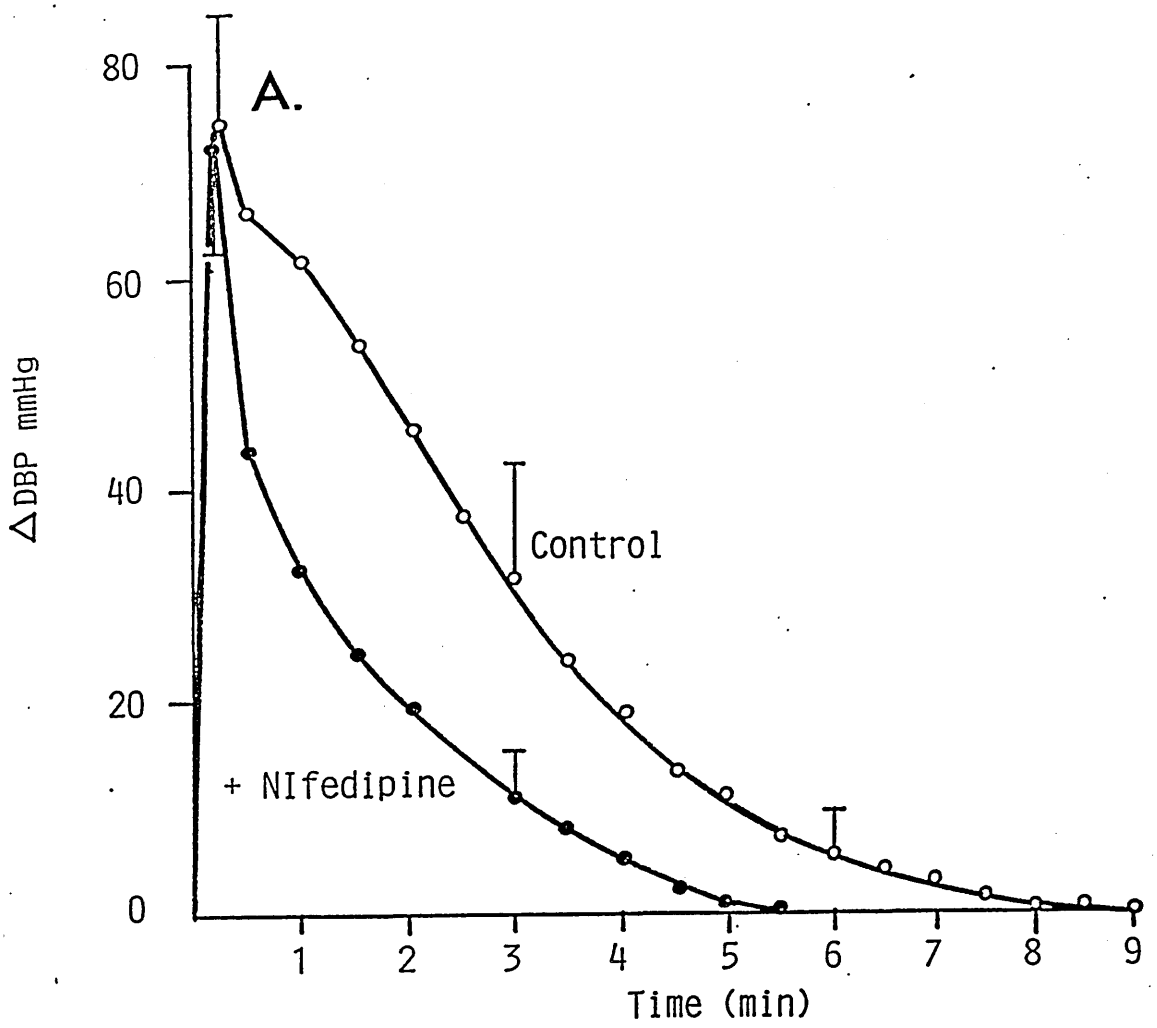
INFUSION: A dose of lug/kg/min amidephrine was found to produce an eventual maximum of 56 +/-8 mmHg (n=4) under these conditions, fig 10b. The infusion response was of slow onset and did not achieve a plateau within the 20 mins infusion time. Nifedipine was found to reduce the maximum to 42 +/- 8 mmHg ($0.01<P<0.05$, n=4). The area under the graph revealed only a 19% inhibition.

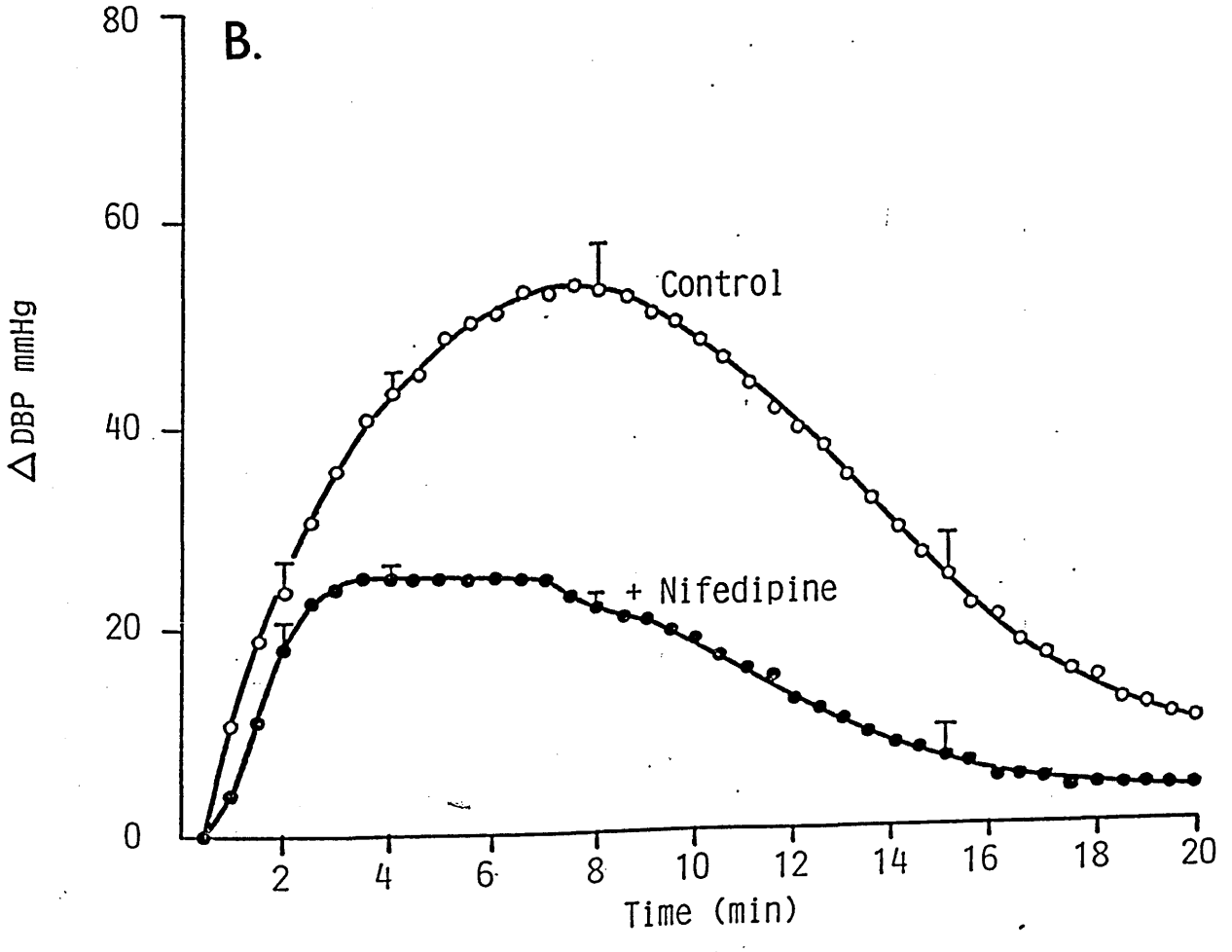
2.2.b) Phenylephrine

BOLUS: In the pithed rat, phenylephrine (3ug/kg) evoked a rapid, short-lived pressor response of 75 +/- 14mmHg (n=5) in the presence of propranolol (1 mg/kg to block any cardiac effects), fig 11a. At this point in the study (summer), myself and co-workers were experiencing variable results with phenylephrine and tried various ways of eliminating the possible reasons. None were found to be successful and so the large s.e.m. stands. However, one theory considered to be a possibility, is that the pollen level may affect the rats' responses in isolated tissues or pithed rat preparations (McGrath, unpublished observations).

The phenylephrine response under these ventilation conditions was more prolonged than that found when the rat was respired to produce "normal" blood gases:- 9.0 +/- 0.1 mins as compared to 6.6 +/- 0.4 mins. The responses were measured every 30 seconds (and at the first 15 secs) and the mean responses were plotted along with their s.e.m.s.

FIG 11 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of phenylephrine (3 ug/kg), and B) an infusion of phenylephrine (3 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure (/\DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=5 for each group.


A.
80
60
40
20
0
Δ DBP mmHg
Control
+ NIfedipine
1
2
3
4
5
6
7
8
9
Time (min)


B.
80
60
40
20
0
Δ DBP mmHg
Control
+ Nifedipine
2
4
6
8
10
12
14
16
18
20
Time (min)

Nifedipine (0.3 mg/kg) was then tested against phenylephrine and did indeed block at least part of the response. The initial, rapid phase of the response remained virtually unaffected (72 +/- 14 mmHg, not significant(n.s.), n=5). However the secondary phase was significantly blocked ($0.001<P<0.01$; t=2 mins). The time taken for the maximum response to decay by 50% ($t_{1/2}$) was 2.5 +/- 0.5 mins for the control and 1.0 +/- 0.2 mins for the nifedipine blocked rats ($0.001<P<0.01$). When the area under the graph was measured the control response was 955 mmHg.min and the nifedipine-blocked response was 435 mmHg.min so there was an average inhibition of 54%.

INFUSION: Various concentrations were tried in an attempt to achieve the same maximum response as for the bolus injection. The closest was a dose of 3 ug/kg/min phenylephrine (in the presence of 1 mg/kg of propranolol) which gave a maximum blood pressor response of 54 +/- 4.7 mmHg (n=5), fig 11b. The infusion response was of rapid onset, the maximum response being achieved on average after 6.6 +/- 0.8 mins but was maintained only for a couple of minutes before the response waned to approx. 20% of max. This phenomenon will be discussed in the next section but because of the waning of the response separate rats were used for the nifedipine-blocked experiments.

Nifedipine was found to reduce the max. response to 25 +/- 1.1 mmHg ($p< 0.001$) and to reduce the time to max. to 4.0 +/- 0.4 mins. When the area under the graph was measured (control= 3218 mmHg.min : blocked= 1332 mmHg.min)

the infusion-induced response was 59% inhibited. The infusion in the presence of nifedipine maintained its maximum for longer than the control, appearing to reach a plateau for about 4 mins, but it still displayed the same tailing off of the response to approx. 20% of its max.

INFUSION TACHYPHYLAXIS?: In an effort to explain why the infusions of phenylephrine were unable to maintain their maxima, various parameters were investigated. Firstly the infusion pump was checked and found to be working properly! Secondly, the responses to several other infusion concentrations were examined. 6,8,10 and 17 ug/kg/min phenylephrine were all unable to sustain their responses over the 20 mins of infusion to a greater or lesser extent. This effect was not due to breakdown of the drug in the syringe as it was bio-assayed in other animals and found to be fully potent. As this did not appear to be a dose related effect, the possibility of tachyphylaxis was next examined.

A 3 ug/kg/min infusion was administered to the rat and when the response started to wane (reduced to 20 +/- 5 mmHg) the infusion rate was increased x2. This increased the response to 50 +/- 9 mmHg which eventually decreased to 20% of its max. after 13 mins (n=3). As increasing the infusion rate could still increase the response, bolus injections of various agonists (both $alpha_1$, $alpha_2$ and non-adrenergic vasopressors) were next tried to see if they could elicit a response after an infusion. The agonists

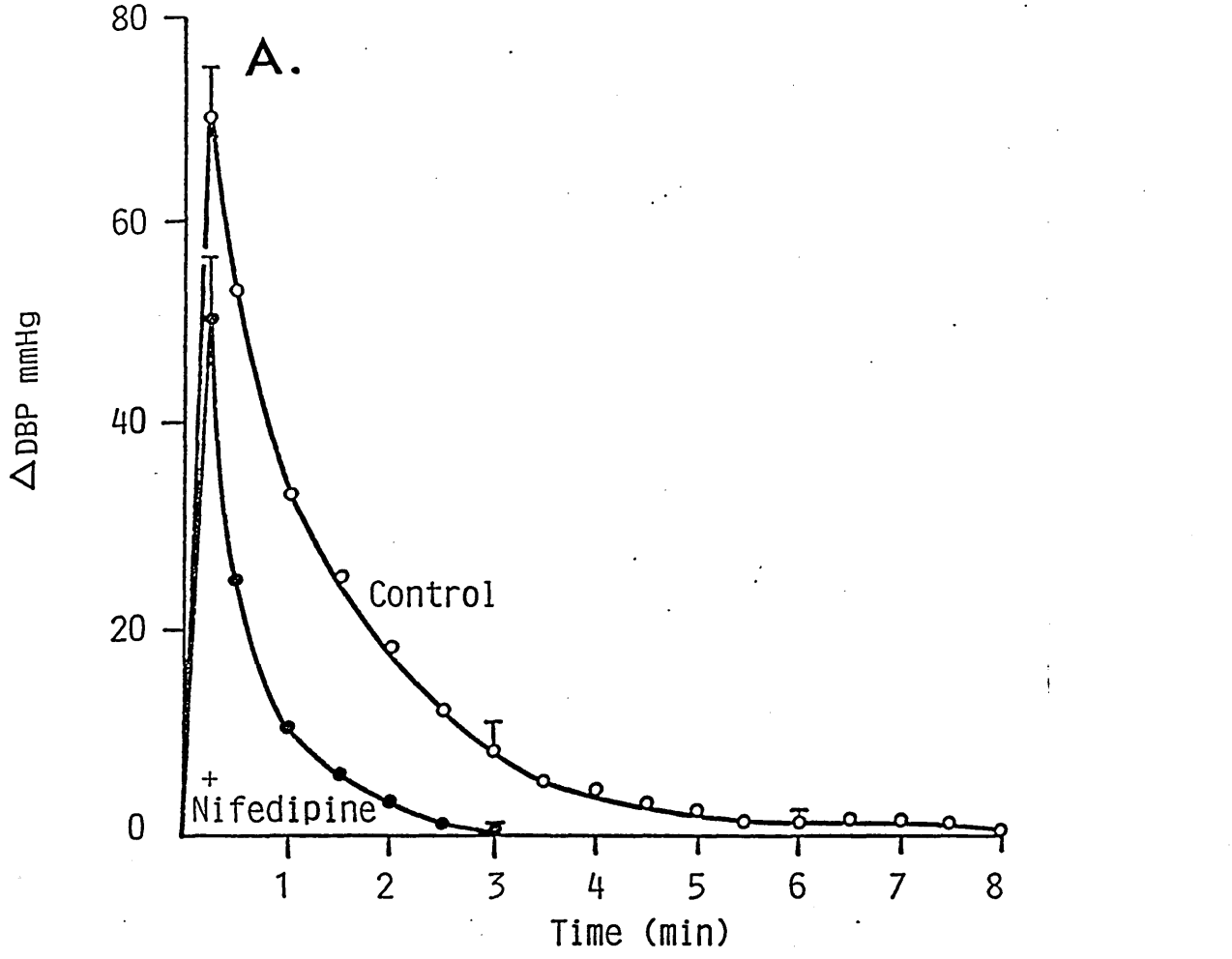
listed below all gave normal maximum responses but the duration of the responses were reduced by the following percentages: phenylephrine (3ug/kg) 70% +/-2, n=2; cirazoline (lug/kg) 84%, n=1; methoxamine (40ug/kg) 50% +/-6, n=3; M7 (10ug/kg) 72%, n=1 and angiotensin II (0.4ug/kg) 30% +/-3, n=3.

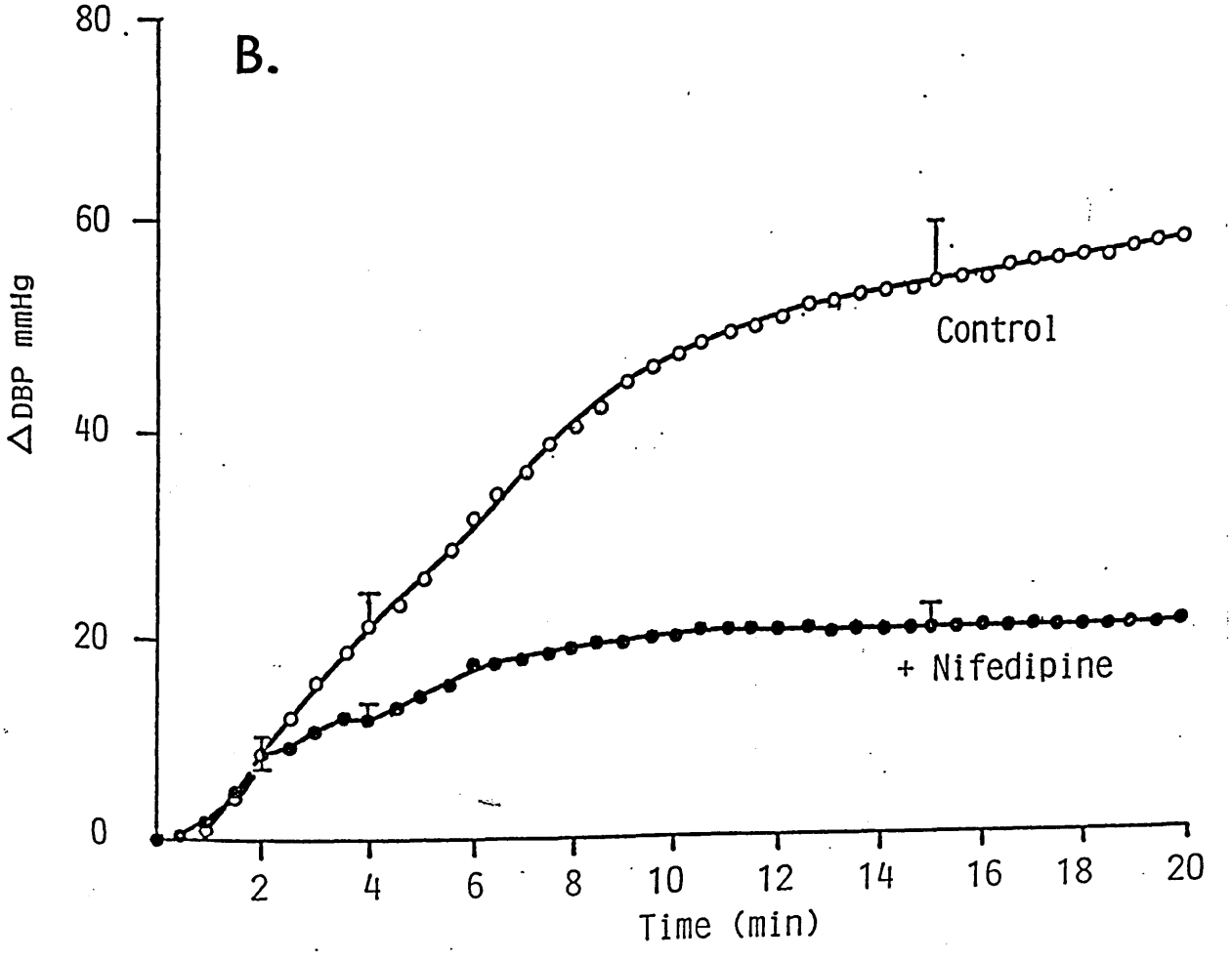
2.2.c) Cirazoline

BOLUS: Like phenylephrine, cirazoline (lug/kg) evoked a rapid, short-lived pressor response of 70 +/- 5 mmHg, $t_{1/2}$=1.1 +/-0.1 mins, (n=6), fig 12a. The response was measured every 30 secs (and the first 15 secs) and plotted as above. Nifedipine (3ug/kg) was injected to block the response and the time course was plotted on the same graph. Both the maximum response and the time course appeared blocked (max. response = 50 +/- 7 mmHg; $0.05<P<0.1$, $t_{1/2}$ = 0.4 +/- 0.1 mins; $P<0.001$, n=6) and when the area under the graph was measured the inhibition was calculated as 68%.

INFUSION: The infusion concentration found to give the closest match to the maximum pressor response elicited by the bolus injection was that of lug/kg/min, which gave a maximum response of 57.4 +/- 6.6 mmHg (n=6), fig 12b. The infusion response to cirazoline was unlike that of phenylephrine. It was of slow onset and did not achieve a plateau within the 20 minutes of administration. In a first series of experiments nifedipine was injected into the rat when the first infusion response had returned to baseline and the infusion was repeated. However, very little

FIG 12 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of cirazoline (1 ug/kg), and B) an infusion of cirazoline (1 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure (/\DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=6 for each group.


A.
80
60
40
20
0
ΔDBP mmHg
Control
+
Nifedipine
1
2
3
4
5
6
7
8
Time (min)


B.
80
60
40
20
0
ΔDBP mmHg
Control
+ Nifedipine
2
4
6
8
10
12
14
16
18
20
Time (min)

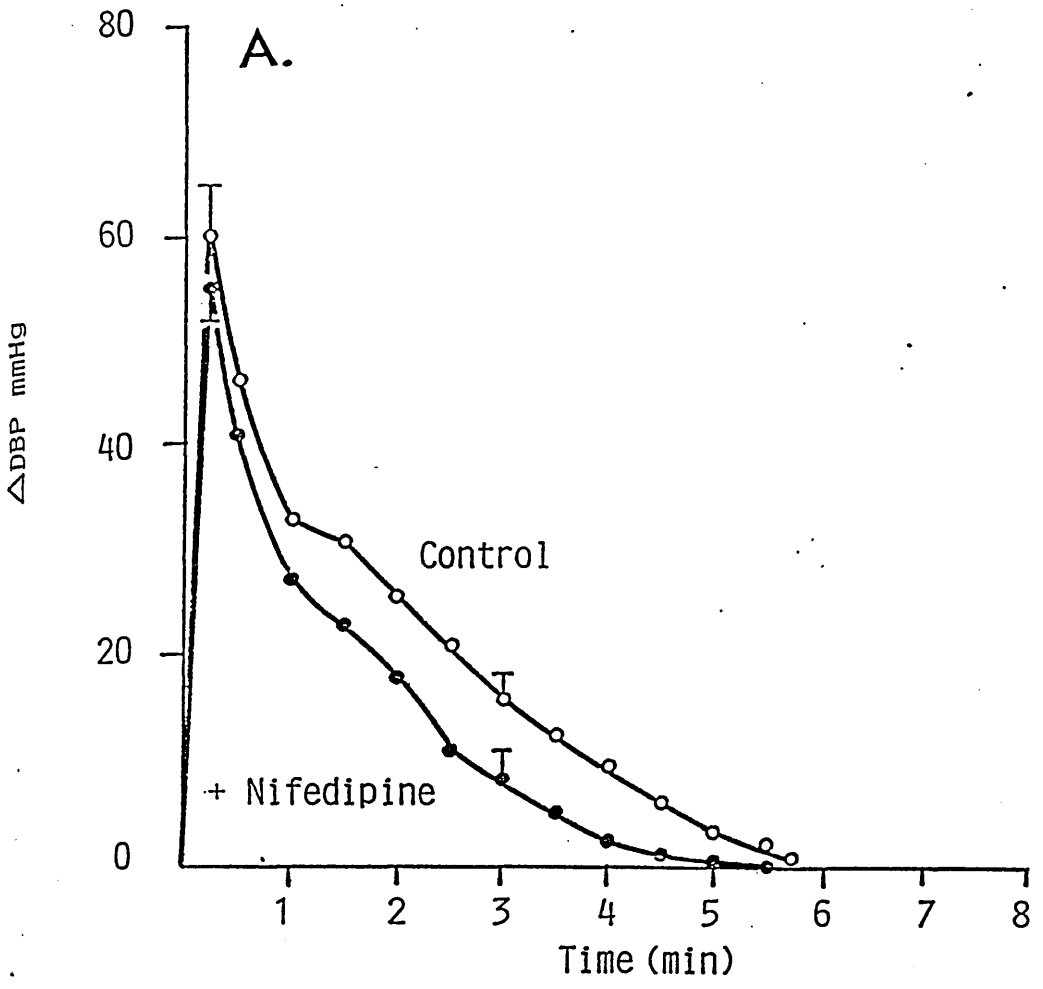
blockade of the maximum response was seen (15.1% +/- 2.6; n=3).

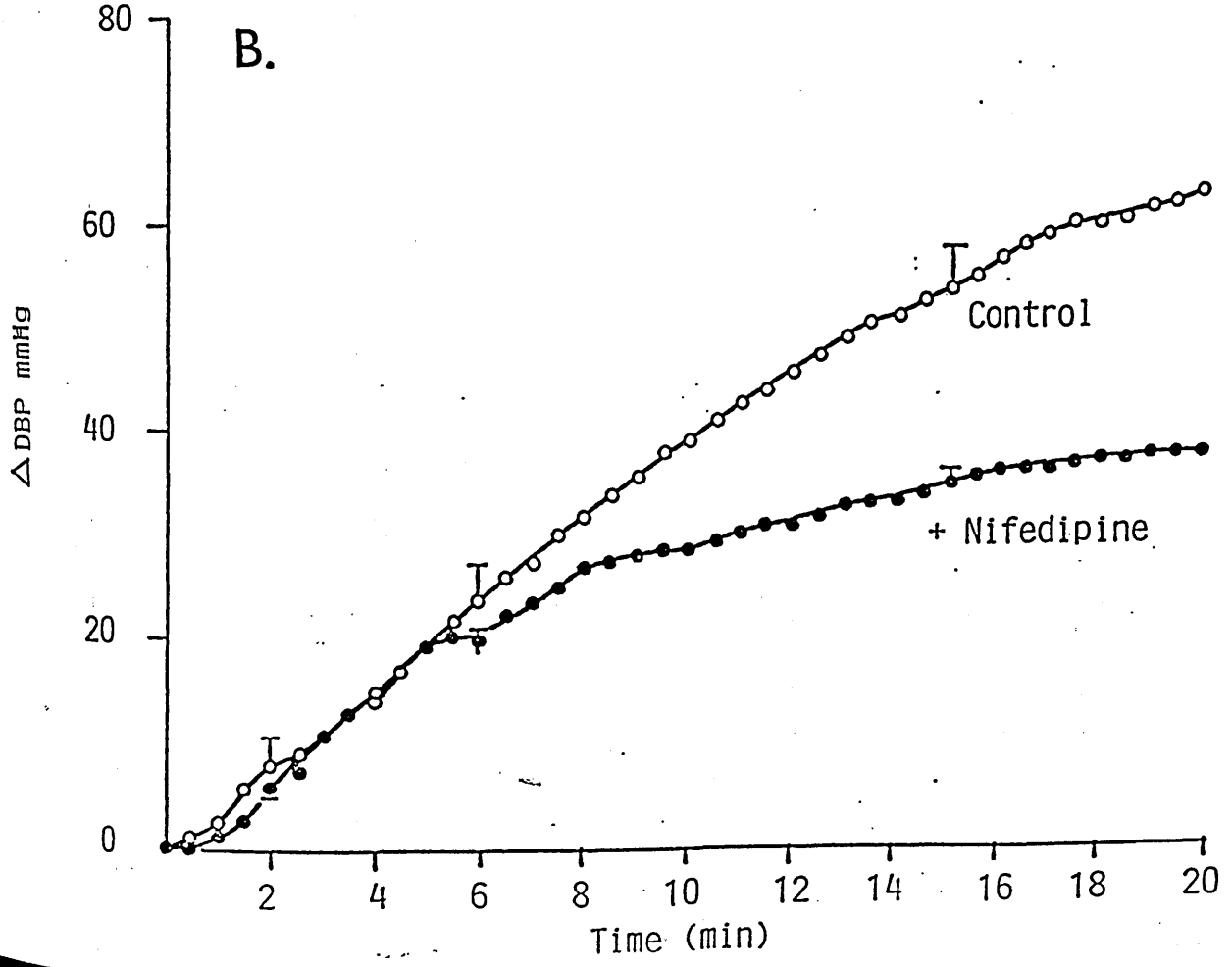
To test if this was due to repeat infusions being potentiated, two infusions were given to the same rat with 30 mins between them (the usual time taken for the first response to wane and nifedipine to be administered, in the previous experiments) but without any antagonist. It was found that on the second administration the maximum B.P. response was potentiated by 24% +/- 6 (n=3). Therefore, all future experiments involving infusions were carried out in separate rats for the control and antagonist. Nifedipine-blocked rats now gave a maximum response of 20.3 +/- 2.2 mmHg ($0.01>p>0.001$; n=6), the infusions reaching a plateau around 10 mins and maintaining it for the duration. Compared with controls, the area under the graph showed an inhibition of 58%.
(N.B.:- because of the waning of the response in the phenyl ephrine infusion experiments, these had been carried out in separate rats for the control and antagonist.)

2.2.d) Methoxamine

BOLUS: Methoxamine was another $alpha_1$ agonist displaying the typical rapid, short-lived response, fig 13a. A dose of 40ug/kg produced a maximum response of 60 +/- 5 mmHg (n=4), lasting for 5.8 mins, ($t_{1/2}$=1.8 +/-0.5 mins) . When nifedipine was injected before methoxamine, neither the max. response nor the time course appeared to be much affected (max. response = 55 +/- 3 mmHg; n.sig., $t_{1/2}$ = 1.1 +/- 0.1

FIG 13 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of methoxamine (40 ug/kg), and B) an infusion of methoxamine (25 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure ($\angle\backslash$DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=4 for each group.


A.
80
60
40
20
0
△DBP mmHg
Control
+ Nifedipine
1
2
3
4
5
6
7
8
Time (min)


B.
80
60
40
20
0
△DBP mmHg
Control
+ Nifedipine
2
4
6
8
10
12
14
16
18
20
Time (min)

mins; n.sig., n=4). The area under the graph revealed that only 31% of the response was blocked.

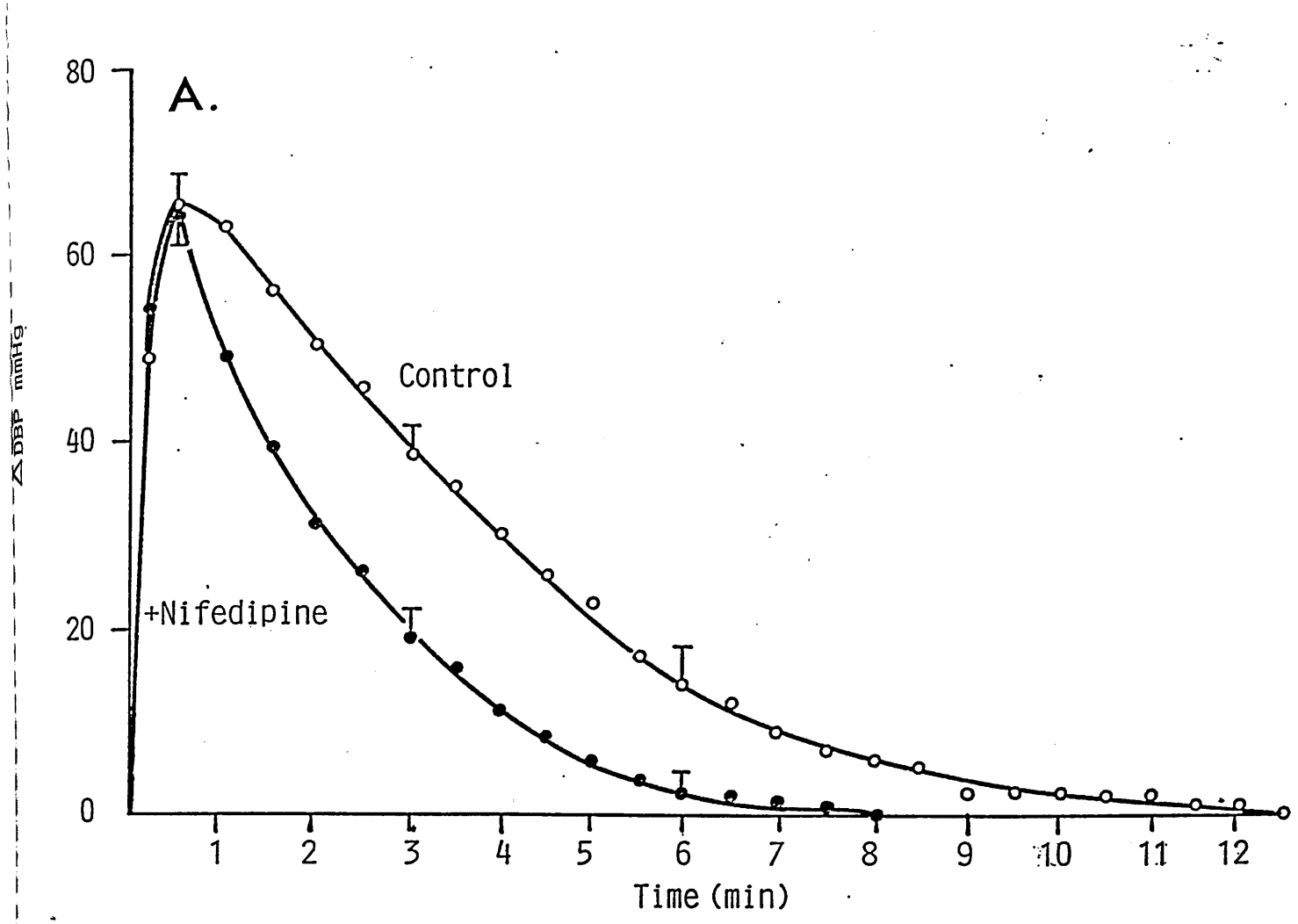
INFUSION: An infusion of 25ug/kg/min of methoxamine was found to give a maximum pressor response of 63 +/- 5 mmHg (n=4), fig 13b. The infusion was like cirazoline's in pattern i.e. slow onset and not reaching a plateau within the 20 mins. When nifedipine was used it did not appear to have much affect within the first 6 mins but eventually reduced the maximum to 37 +/- 1 mmHg ($p<0.001$; n=4) after 20 mins. The area under the graph revealed an inhibition of only 30%.

2.2.e) Oxymetazoline

BOLUS: This drug, over which some controversy exists as to its $alpha_1/alpha_2$ status, produced a rapid, fairly prolonged response in the pithed rat, fig 14a. A dose of 50ng/kg produced a max. pressor response of 64 +/- 3 mmHg (n=4) lasting for 12 mins +/- 2 ($t_{1/2}$=5.0 +/-0.5 mins). After nifedipine, the maximum response was unaffected (max. response = 64 +/- 4 mmHg; n.sig.; n=4) but the time course of the response (as measured every 30 secs) was reduced ($t_{1/2}$=2.3 +/- 0.6 mins, $0.01<P<0.05$). A 45% inhibition was measured using the area under the graph (a.u.g.).

INFUSION: The infusion response to 0.5ug/kg/min of oxymetazoline was like that of the previous two drugs, fig 14b. There was a slightly more rapid onset, but the response never reached a plateau (max. response = 64 +/- 3

FIG 14 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of oxymetazoline (50 ng/kg), and B) an infusion of oxymetazoline (0.5 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure ($\angle\!\!\setminus$DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=4-6.


A.
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Δ DBP mmHg
Control
+Nifedipine
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7
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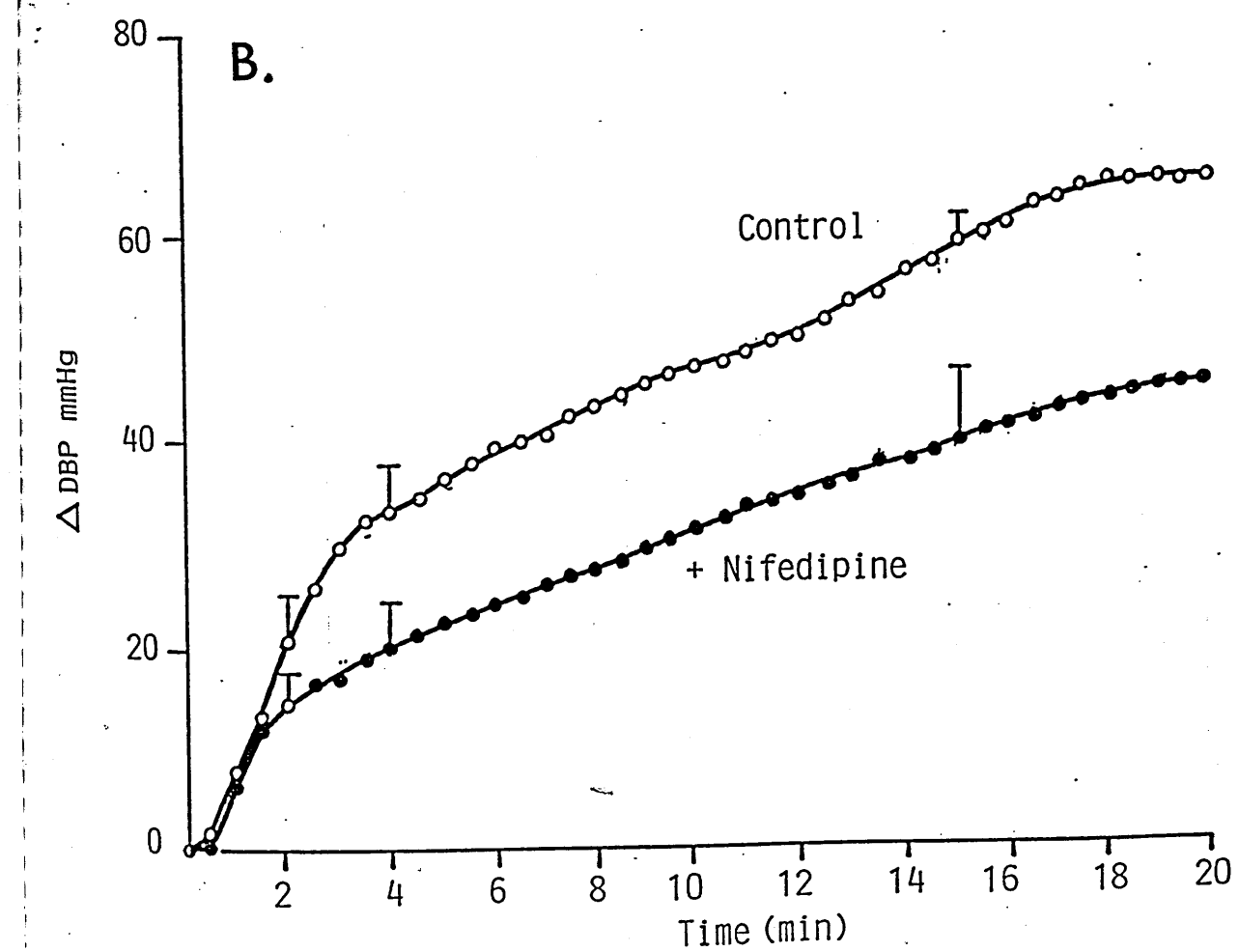
11
12
Time (min)


B.
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Δ DBP mmHg
Control
+ Nifedipine
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Time (min)

mmHg; n=6). Nifedipine did not affect the response for the first two minutes but eventually reduced the response to 44 +/- 7 mmHg (0.05>p>0.01; n=6), a 34% inhibition (a.u.g.).

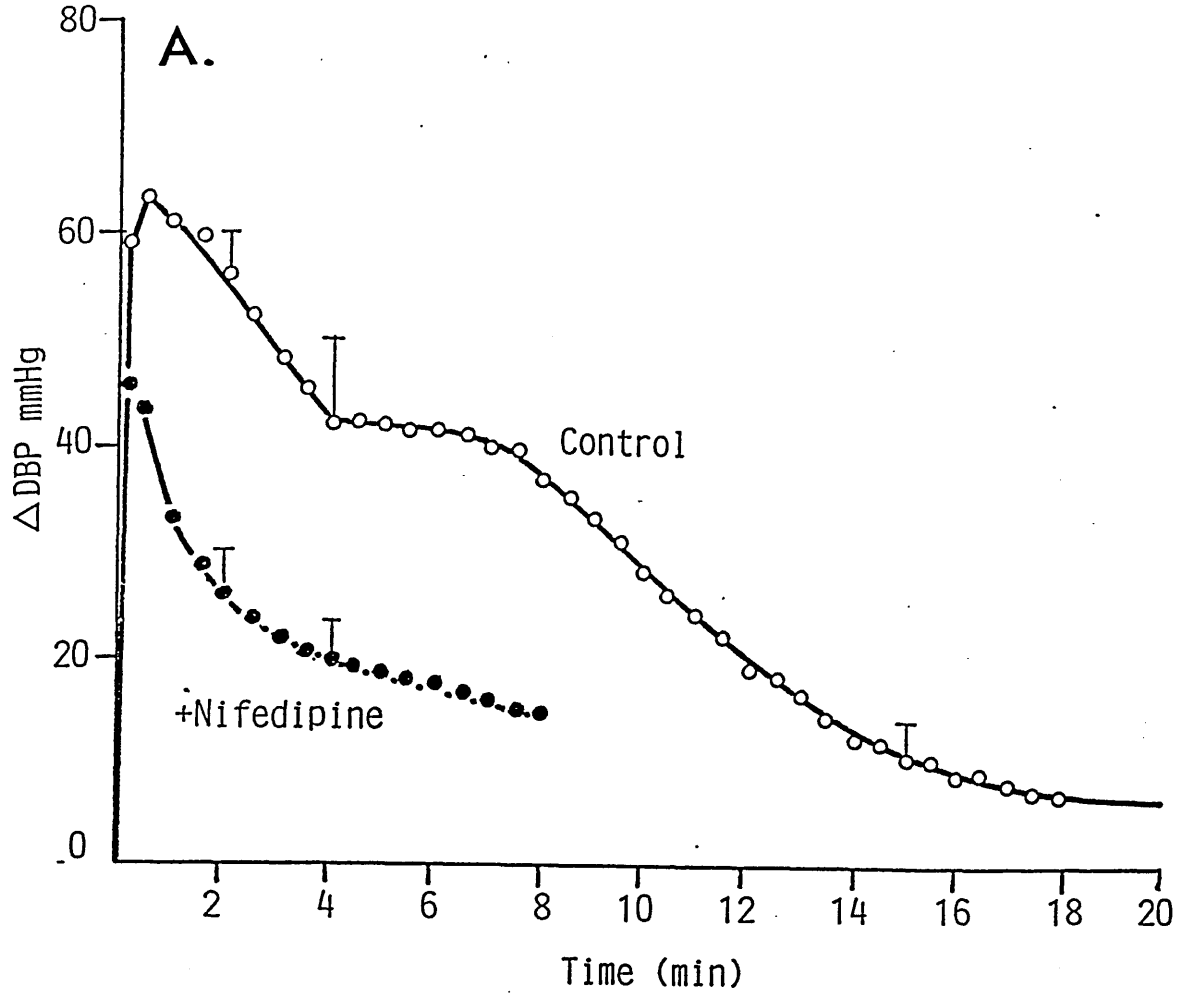
2.2.f) SGD 101/75

BOLUS: A 0.5mg/kg bolus injection of SGD 101/75 elicited a rapid, prolonged pressor response of 63 +/- 6 mmHg (max. response), lasting for 18 mins, $t_{1/2}$=10.1 +/- 0.9 mins, (n=5). Fig 15a shows that nifedipine reduced the maximum response by 29% to 45 +/- 7 mmHg (0.001<P<0.01; n=5) and the time course to $t_{1/2}$=3 +/- 0.8 mins (P<0.001). The nifedipine line is not continued for the bolus because unfortunately the recordings were terminated at this point, when the blood pressure no longer appeared to be falling. For the purposes of measuring the area under the graph, the line was extrapolated up to 20 mins to match the control curve. By this method the area under the graph showed a 55% inhibition. The control response to SGD 101/75 was more distinctly biphasic than the other alpha$_1$-agonists examined and it was this second phase which was blocked more i.e. at t=3 mins, the response was blocked by 57%, from 37 +/- 4 mmHg to 16 +/- 2 mmHg, 0.001<P<0.01.

INFUSION: The infusion response to 0.25mg/kg/min of SGD 101/75 was of rapid onset and reached a plateau, after about 8 mins, of 58 +/- 7 mmHg (n=5), fig 15b. The plateau was better maintained than that to phenylephrine but still had a tendency to fall off, slightly, towards the end of the infusion. When nifedipine was injected first, a plateau was not reached until 17 mins and the maximum pressor response

was reduced to 45 +/- 7 mmHg ($0.01<P<0.05$; n=7). The total inhibition was calculated as only 22% (a.u.g.).

FIG 15 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of SGD 101/75 (0.5 mg/kg), and B) an infusion of SGD 101/75 (0.25 mg/kg/min) in the pithed rat. The changes in diastolic blood pressure (/\DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=5-7.


A.
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Δ DBP mmHg
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+Nifedipine
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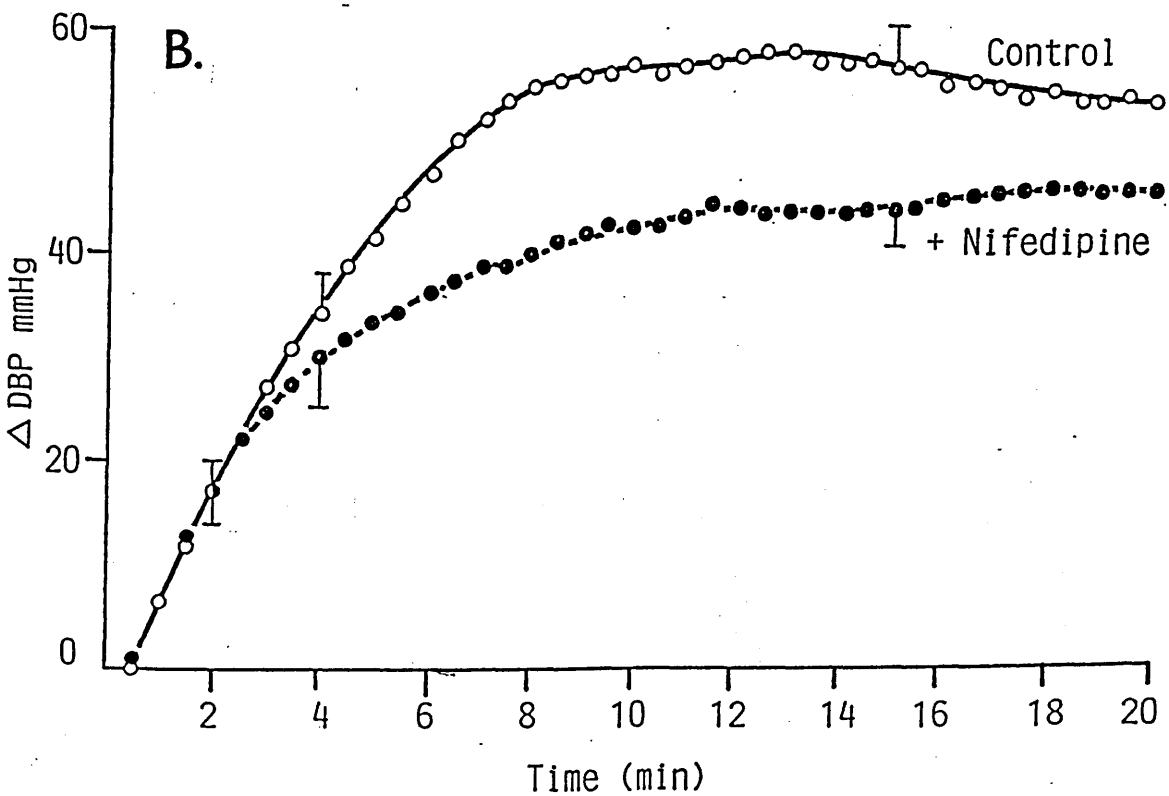
Time (min)


B.
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Δ DBP mmHg
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+ Nifedipine
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20
Time (min)

2.3) Vasoconstrictor Responses to Alpha$_2$-Adrenoceptor Agonists

2.3.a) Xylazine

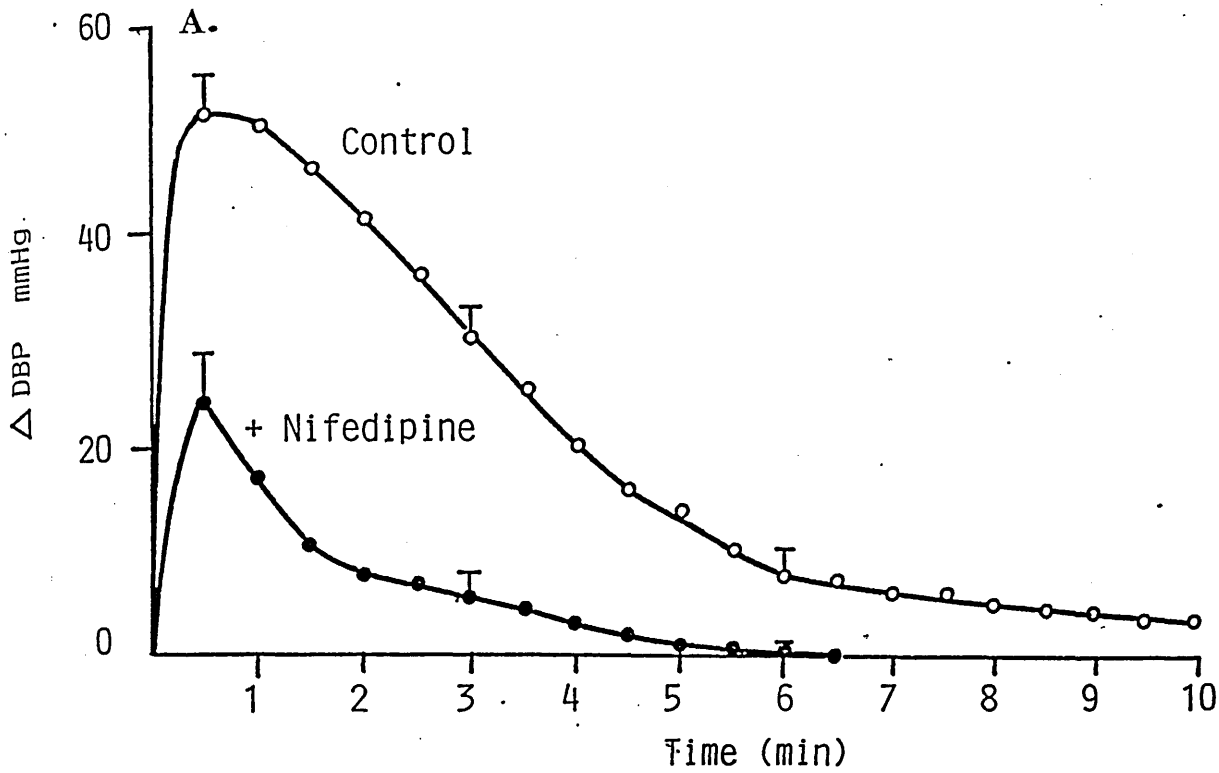
BOLUS: Xylazine was chosen as a typical alpha$_2$ agonist as it produces a slow, prolonged pressor response in the pithed rat, fig 16a. A dose of 0.1mg/kg elicited a maximum change in diastolic blood pressure of 51 +/- 4 mmHg, lasting for 10 mins, $t_{1/2}$=3.7 +/- 0.2 mins (n=5). Nifedipine caused a 77% inhibition of the total response (a.u.g.), reducing the maximum peak height to 24 +/- 5 mmHg ($0.01>p>0.001$) and reducing $t_{1/2}$ to 1.5 +/- 0.1, $P<0.001$ (n=5).

INFUSION: The infusion response to xylazine was different to any seen so far as it produced a rapid and maintained increase in blood pressure, fig 16b. The concentration of 0.25mg/kg/min of xylazine produced a rapid response which achieved a maximum of 71 +/- 3 mmHg in about 8 mins and maintained it for the duration (n=5). This response was blocked by nifedipine which reduced the maximum response to 31 +/- 2 mmHg ($p<0.001$) and caused an inhibition of 56% (a.u.g.).

2.3.b) M7

BOLUS: M7 (10ug/kg) produced a slow, prolonged pressor response of 57 +/- 2 mmHg (max.) lasting for 10 mins, $t_{1/2}$=4.2 +/- 0.5 mins (n=6), fig 17a. The maximum peak height was reduced to 40 +/- 4 mmHg ($0.01>p>0.001$, n=6) i.e. 30% inhibition by nifedipine and the time course was reduced

FIG 16 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of xylazine (0.1 mg/kg), and B) an infusion of xylazine (0.25 mg/kg/min) in the pithed rat. The changes in diastolic blood pressure (ΔDBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=5 for each group.


A.
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Δ DBP mmHg
Control
+ Nifedipine
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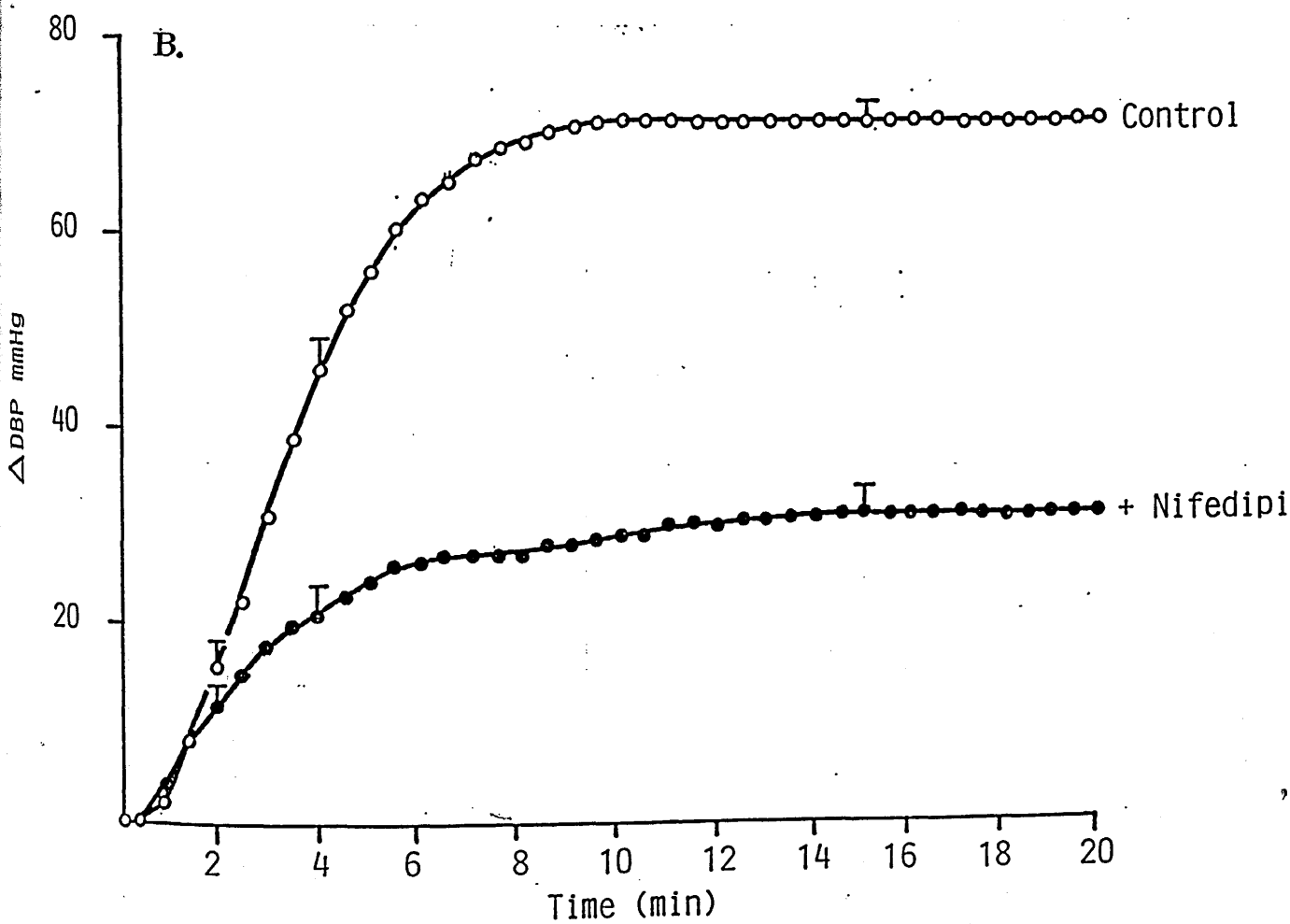
Time (min)


B.
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20
Δ DBP mmHg
Control
+ Nifedipi
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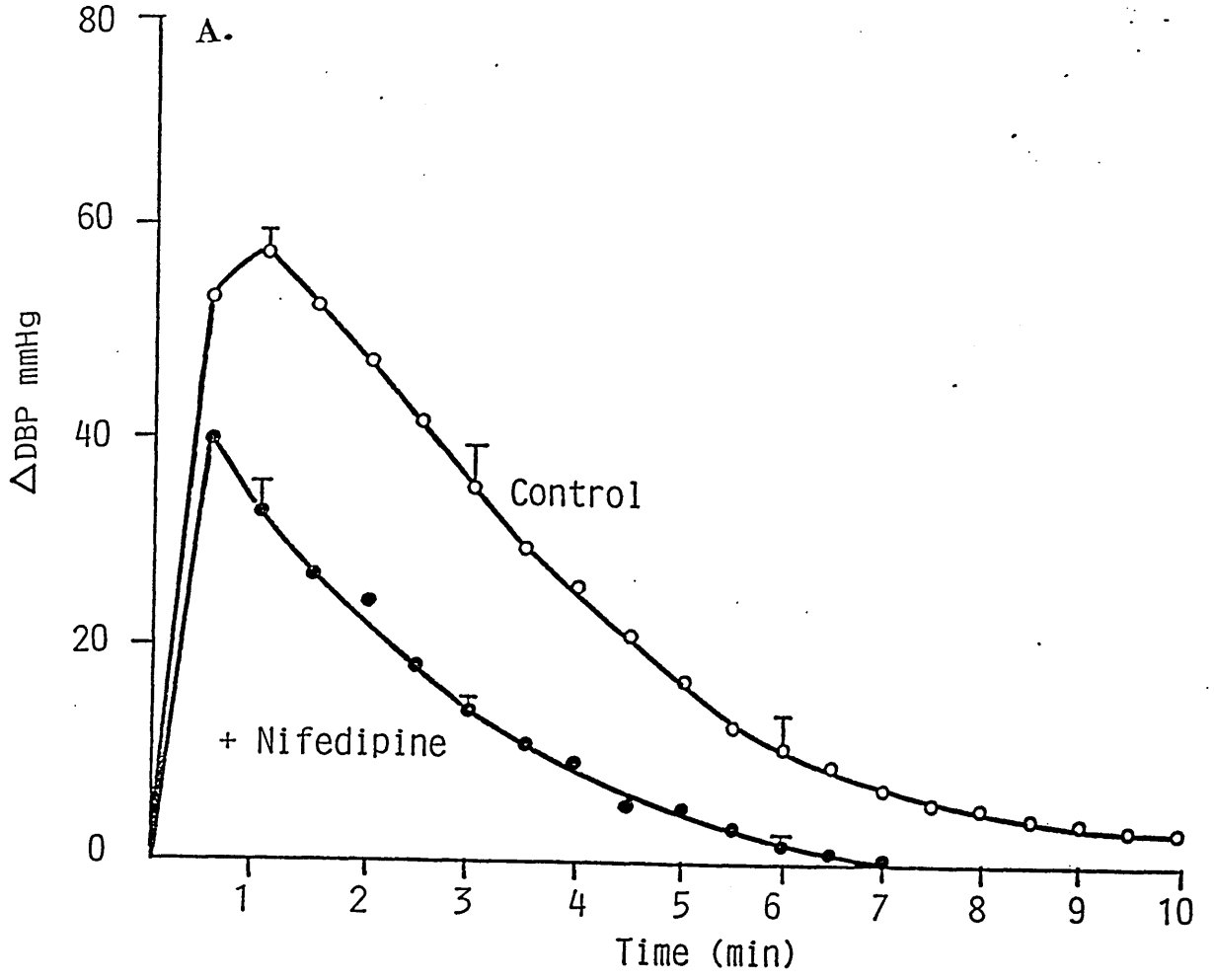
18
20
Time (min)

FIG 17 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of M7 (10 ug/kg), and B) an infusion of M7 (6 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure (/\DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=5-6.


A.
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ΔDBP mmHg
Control
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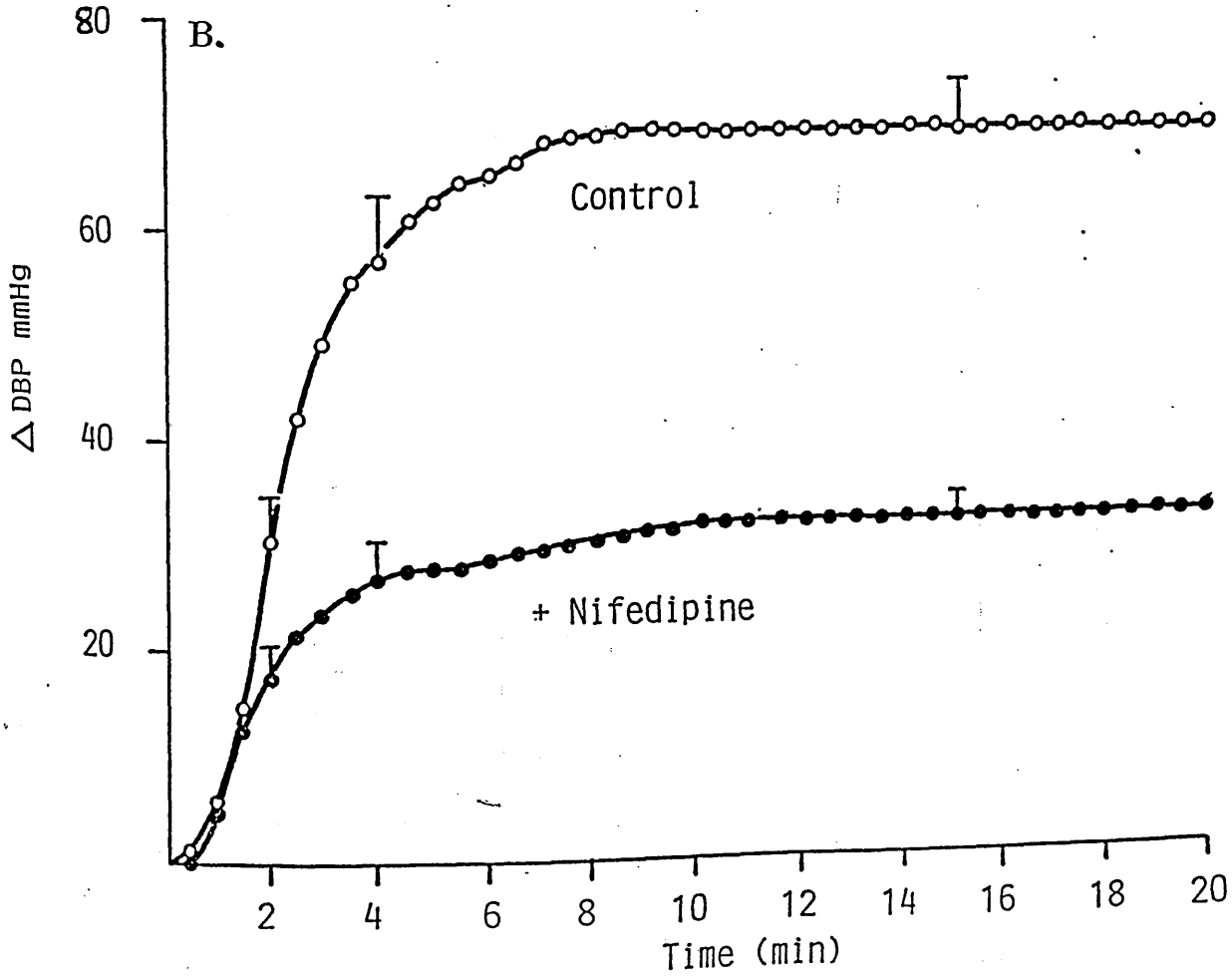
Time (min)


B.
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Δ DBP mmHg
Control
+ Nifedipine
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Time (min)

to 7 mins, i.e. also a 30% inhibition. The total inhibition was measured as 57% (a.u.g.) and $t_{1/2}$ was reduced to 2.5 +/- 0.3 mins ($0.01<P<0.05$). D-600 was another calcium-entry blocker which was used to try to block the M7 response, this time with more success. A dose of 0.3mg/kg caused a 63% +/- 8 reduction of the maximum response and the duration of the response was reduced by 47% +/-6 (n=3).

The responses obtained to bolus injections of M7 were not as steeply rising as had been expected from the earlier work of Drew (1980). In an attempt to investigate why, the blood gases of the rats were altered by increasing the stroke volume from 2.0ml to 3.5ml. Increasing the stroke volume changed the blood gases by increasing the PaO_2 and decreasing the $PaCO_2$. The hypothesis was that, rapid responses, such as $alpha_1$-type responses, are increased in higher PaO_2. However it was found that the maximum response was reduced by 20% +/-3 ($0.01<P<0.05$, n=4) but the time course was unaffected.

Finally to investigate quantitatively, the proportion of the M7 response produced by the two alpha-receptor sub-types, rauwolscine (an alpha $_2$-antagonist) and prazosin (an $alpha_1$-antagonist) were employed. When rauwolscine (0.2mg/kg) was injected first (before the prazosin) it caused a 50% +/-13 inhibition of the maximum and a 52% +/-7 reduction in the duration of the response. Prazosin, however, reduced the maximum by only 28% +/-8 and the time course by 49% +/-1 when it was injected first.

If rauwolscine was injected after prazosin, it blocked the M7 maximum pressor response a further 37% +/-8 but increased the time course by 21% +/-9. Prazosin on the other hand if injected after rauwolscine, increased the maximum response by 16% +/-6 but had no further effect on the duration of the response. As rauwolscine was the more potent antagonist of the M7 response, M7 appears to be relatively selective for the $alpha_2$-adrenoceptor.

INFUSION: Like xylazine, M7 produced a response which rapidly attained a maximum and remained at a plateau for the rest of the infusion time, fig 17b. 6ug/kg/min of M7 gave a maximum pressor response of 69 +/- 5 mmHg (n=5). This was inhibited 52% (a.u.g.) by nifedipine, the maximum response being reduced to 32 +/- 3 ($p<0.001$, n=5).

2.3.c) Azepexole

BOLUS: Azepexole (BHT-933, 250ug/kg) produced a slow, prolonged pressor response, like the other $alpha_2$-agonists assayed, fig 18a. A maximum of 55 +/- 4 mmHg was reduced to 39 +/- 7 mmHg ($0.05>p>0.01$, n=4) by nifedipine and the time course was reduced from 9.5 mins to 4.5 mins. The $t_{1/2}$ value was reduced from 3.8 +/- 0.1 mins to 1.8 +/- 0.4 mins, $0.001<P<0.01$. The nifedipine block was calculated as a 66% total inhibition (a.u.g.).

FIG 18 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of azepexole (250 ug/kg), and B) an infusion of azepexole (0.1 mg/kg/min) in the pithed rat. The changes in diastolic blood pressure ($\angle\!\!\setminus$DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=4-5.

INFUSION: An infusion of this drug (0.1mg/kg) gave a rapid, maintained plateau of 47 +/- 2 mmHg (n=5), fig 18b. This was inhibited by 50% in total (a.u.g.) and the maximum was reduced to 24 +/- 3 mmHg by nifedipine ($p<0.001$, n=5).

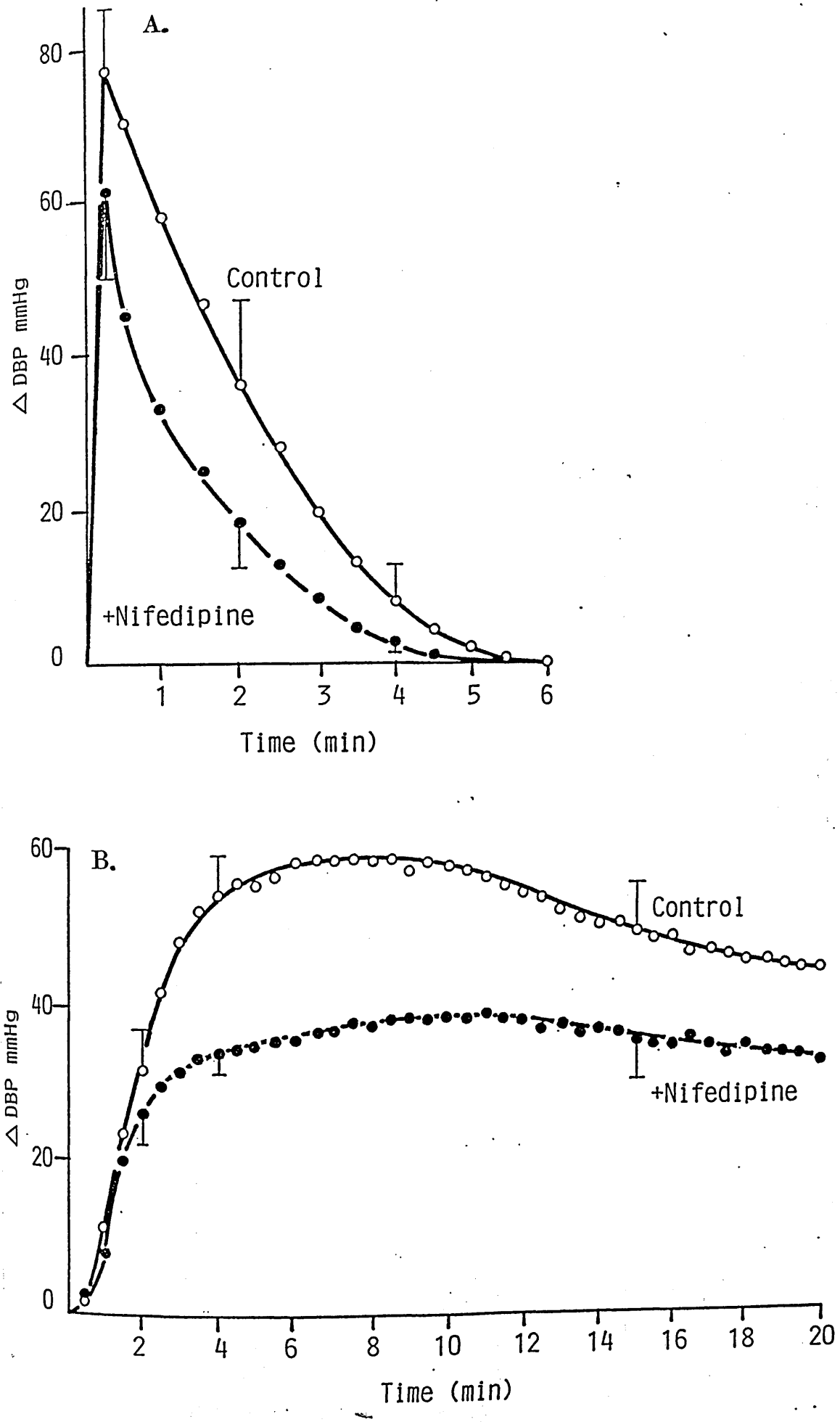
2.4) Vasoconstrictor Responses to Noradrenaline

ALPHA$_1$ OR ALPHA$_2$?: The bolus response to noradrenaline is susceptible to blockade by alpha$_1$ and alpha$_2$ antagonists. When prazosin (1mg/kg), an alpha$_1$ blocker, was injected 5 mins before NA (1ug/kg) the maximum response was reduced from 76 +/- 8 mmHg (n=6) by 21%. The time course was also reduced by 25%. If rauwolscine (0.2mg/kg), an alpha$_2$ blocker, was subsequently injected, a further block of only 13% was seen on the maximum but the time course was reduced by another 22%.

However, if rauwolscine was injected first, it reduced NA's maximum by only 5% but reduced the time course by 33%. When prazosin was subsequently injected, the maximum response was reduced by a further 58% but the time course only reduced another 12%. Under these conditions therefore, prazosin inhibits the maximum response preferentially while, rauwolscine inhibits the time course. All experiments with NA were carried out with 1mg/kg of propranolol present to inhibit any cardiac effects.

BOLUS: When nifedipine was administered there was little effect on the maximum but the overall response (assessed as area under the graph) was reduced, fig 19a. The maximum was

FIG 19 Effect of nifedipine (0.3 mg/kg) on the time course of the pressor response to A) a bolus injection of noradrenaline (1 ug/kg), and B) an infusion of noradrenaline (1 ug/kg/min) in the pithed rat. The changes in diastolic blood pressure (/\DBP, mmHg) produced by the agonist have been plotted against time (mins). Open symbols represent control response; filled symbols represent responses after nifedipine. Vertical bars represent SEM, n=5-6.


A.
80
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△ DBP mmHg
Control
+Nifedipine
1
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3
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5
6
Time (min)
B.
60
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20
0
△ DBP mmHg
Control
+Nifedipine
2
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14
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Time (min)

reduced to 61.3 +/- 12 mmHg ($0.01<P<0.05$, n=5) and $t_{1/2}$ was reduced from 1.9 +/- 0.4 mins to 1.1 +/- 0.2 mins, $0.01<P<0.05$. The total block was 46% (a.u.g.).

INFUSION: The infusion response to NA showed characteristics of both phenylephrine and xylazine, fig 19b. The concentration of lug/kg/min produced an infusion which rapidly attained a maximum of 58 +/- 5 mmHg but only maintained it for about 4 mins before waning to 42 +/- 6 mmHg after 20 mins (n=6). Nifedipine reduced the maximum to 38 +/- 4 mmHg ($0.001<P<0.01$, n=5), with an overall inhibition of 31% (a.u.g.).

INFUSION TACHYPHYLAXIS?: To investigate this phenomenon of the infusion response waning, NA was kept infusing for up to 1 hour and the response slowly decreased throughout. If, when the response was waning, the infusion rate was increased by a factor of two, the blood pressure increased and if the infusion was stopped and the response allowed to return to baseline a second infusion response could be obtained.

At the time when the response was decreasing, both prazosin and rauwolscine were injected to see what proportion of the response left was $alpha_1$ or $alpha_2$-receptor mediated. Rauwolscine (1mg/kg) was found to have only a transient effect, reducing the response of 62 +/- 7 mmHg to 11 +/- 2 mmHg below the baseline ($0.001<P<0.01$; n=3) but allowing recovery of the response to 42 +/- 13 mmHg after 10 mins. Whereas prazosin (1mg/kg) on the other hand

completely blocked the response, taking it from 61 +/- 4mmHg to 11 +/- 4 mmHg below the baseline ($0.001<P<0.01$; n=3) with no recovery of the response up to 1hr after the antagonist was injected. It appeared as if the response to NA towards the end of the infusion was due to the activation of mainly $alpha_1$ receptors as only prazosin had a lasting inhibitory effect.

If this was so, then rauwolscine given before the infusion of NA should produce a "phenylephrine-type" infusion response. A greater concentration of NA (2 ug/kg/min) had to be infused after 1 mg/kg of rauwolscine was injected to produce a maximum response of 61 +/- 2 mmHg (n=2). The response waned by 38%, which was greater than the 28% reduction when no $alpha_2$-blocker was present, but not as large as the 80% reduction of the response to phenylephrine. The levels of NA present during the infusion then became of interest and this was further investigated using HPLC methods.

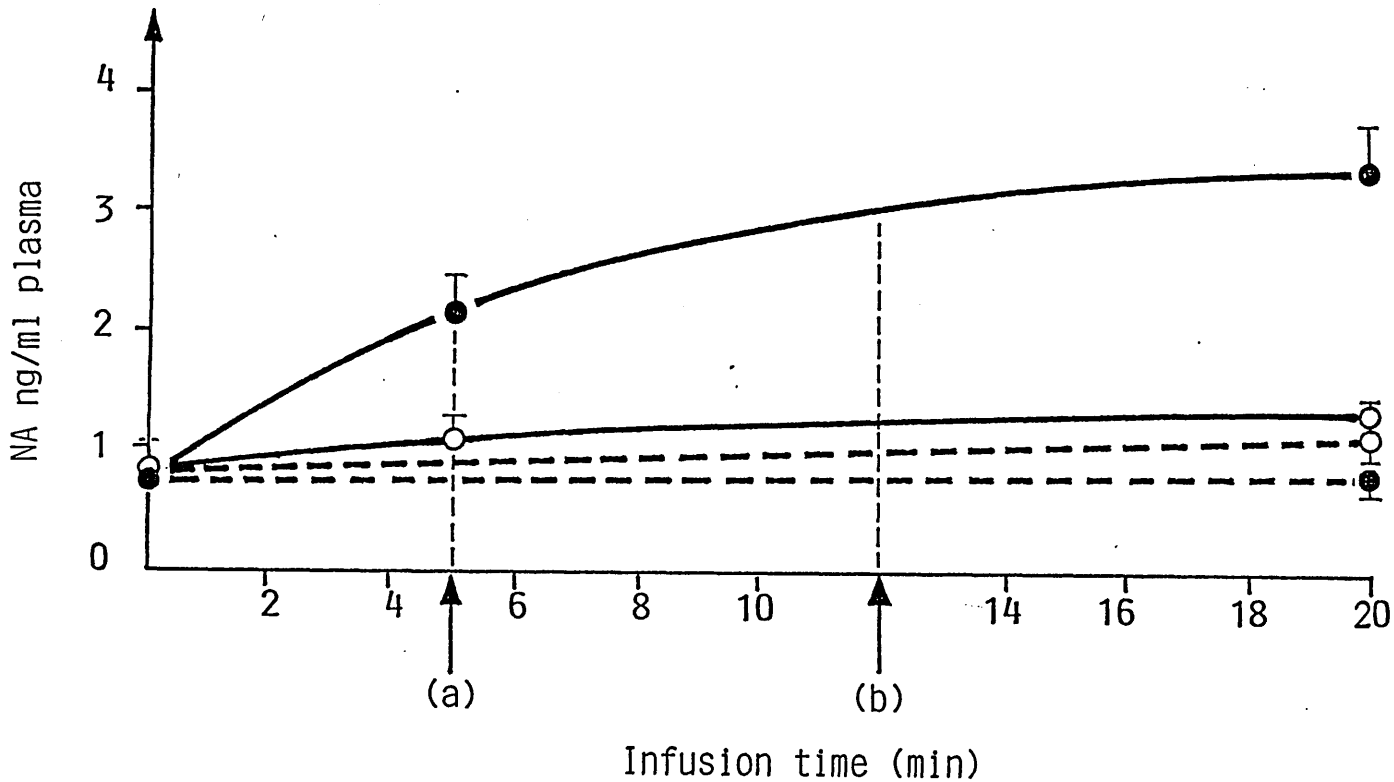
2.5) Measurement Of Noradrenaline Levels In The Plasma Of The Pithed Rat

In an attempt to find out what were the levels of NA present in the blood stream of the pithed rat, arterial or venous blood samples were taken from rats which had been infused with NA as above, fig 20. The 2.0ml samples were taken before the infusion (after the stabilization period), during the infusion (t=5 mins, when NA was exerting its maximum pressor effect) and at the end of the 20 min infusion; both arterial and venous samples were taken (n=4 for each sample). As a control, other rats were infused with 0.9% saline (the NA vehicle) at the same rate for 20 mins and blood samples taken at the same time intervals.

Due to the size of the samples needed, at first, different rats were used for the arterial and venous samples and only two samples were taken per rat. This was later found to be unnecessary as, on analysis, there was no significant difference between the levels of NA in the plasma if the samples were all taken from the one rat. The levels of noradrenaline found in rats' plasma when samples were taken **only after** the infusion of noradrenaline, were found to be 3.0 +/- 0.1 ng/ml for arterial blood and 1.3 +/- 0.1 ng/ml for venous blood (n=2). The levels found in the plasma after the infusion, when samples had previously been taken before the infusion, were 3.5 +/- 0.5 ng/ml for arterial blood and 1.3 +/- 0.1 ng/ml for venous blood (n=3, N.S.)

After the blood had gone through the catecholamine

FIG 20 Concentration of noradrenaline (ng/ml plasma) before, during and after the infusion of 1 ug/kg/min of noradrenaline in the pithed rat. The points are connected with the lines of best fit. Broken lines connect control levels (saline infused); solid lines connect noradrenaline infused levels. Closed symbols represent arterial plasma levels; open symbols represent venous plasma levels. (a) indicates the time at which the maximum pressor response for that particular concentration of noradrenaline is produced; (b) indicates the time from which the pressor response begins to decline. Vertical bars represent SEM, (n=4 for each sample).


NA ng/ml plasma
4
3
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18
20
(a)
(b)
Infusion time (min)

extraction process, the levels of NA were measured using ion-pair reverse phase chromatography. Table 1 shows the levels of NA found in the rats' plasma. It is clear from the results that the level of NA in arterial plasma was still at a maximum when the blood pressure response was on the decline. Surprisingly, almost no increase in the level of venous NA was found during or after the infusion.

TABLE 1 Summarises the levels of noradrenaline (ng/ml plasma) found in the arterial and venous blood of the pithed rat. Samples were tested in both control (saline infused) and noradrenaline infused (1 ug/kg/min) animals before, during and after the infusions. Data is expressed as mean values +/- SEM, (n=6).

	Concentration of noradrenaline ng/ml plasma			
	Control (saline infused)		Noradrenaline infused (1µg/kg/min)	
	Arterial blood	Venous blood	Arterial blood	Venous blood
Before infusion	0.76 ± 0.04	0.80 ± 0.20	0.76 ± 0.04	0.80 ± 0.20
During infusion	-	-	2.20 ± 0.30	1.10 ± 0.20
After infusion	0.79 ± 0.17	1.14 ± 0.20	3.40 ± 0.40	1.30 ± 0.05

Ca^{2+}-DEPENDENCE OF NORADRENALINE-INDUCED CONTRACTION IN RAT AORTIC SMOOTH MUSCLE

The previous studies in this thesis, on the importance of calcium to the alpha-adrenoceptor response, had been carried out on the whole animal preparation, the pithed rat. This section examines the effects of Ca^{2+} on an in vitro preparation from the rat, the aorta. This tissue was chosen because its contractile response to concentrations of noradrenaline of 10 nM and above is biphasic (Godfraind & Kaba, 1972). The initial fast component was believed to be due to the release of intracellular Ca^{2+} and the second, slow component to be due to the influx of extracellular calcium (Godfraind & Kaba, 1972; Van Breemen, 1977). In this one tissue preparation, therefore, two phases of contraction to one receptor sub-type (Ruffolo, 1985; Downing et al., 1983; Digges & Summers, 1983) could be produced and examined in comparison to the two phases of the alpha pressor response already looked at in section 2.

The first phase, thought to be relying more on internal calcium mobilisation, could be compared with the first phase of an $alpha_1$ response in the pithed rat where all previous evidence had pointed to that response being due to mobilisation of intracellular Ca^{2+} (Langer & Shepperson, 1982; van Meel et al., 1983a, b; Timmermans et al., 1983, Saeed et al., 1983; Cavero et al., 1983). The second phase of the aortic response, thought to be due to calcium entry, can be likened to the second phase of the alpha response in the pithed rat, which can be produced by either $alpha_1$ or

alpha$_2$ agonists. It was believed that by studying the effects of calcium and calcium-entry blockade on this simpler model more information could be gained on the hypothesis that the involvement of calcium varied with the time course of the response rather than with receptor subtype involved.

3.1) General

Smooth muscle response to noradrenaline has two components: an initial rapid, transient contraction (ITC) and a secondary, slower contraction (SSC). The concentration of noradrenaline used to study these components was 10^{-6}M which was chosen as it is sub-maximal, see fig 21, and has been used on the rat aorta previously (Godfraind et al, 1982). A couple of preliminary experiments were carried out, to ensure reproducibility of the results.

First, it was checked that repeat "pulses" (see Methods) of NA produced similar responses and that there was no evidence of potentiation or tachyphylaxis, fig 22. After the first two pulses there was no significant difference in the response produced (either the ITC or the SSC) for up to 9 pulses examined. In all cases, the tissues were given 4 pulses of NA (i.e. 4 separate, 4 minute perfusions of NA through the tissues, with 1/2hr recovery in normal Tris saline between each concentration of agonist) for 2 hours before the experiment began, to ensure minimal changes in sensitivity to subsequent concentrations of agonist.

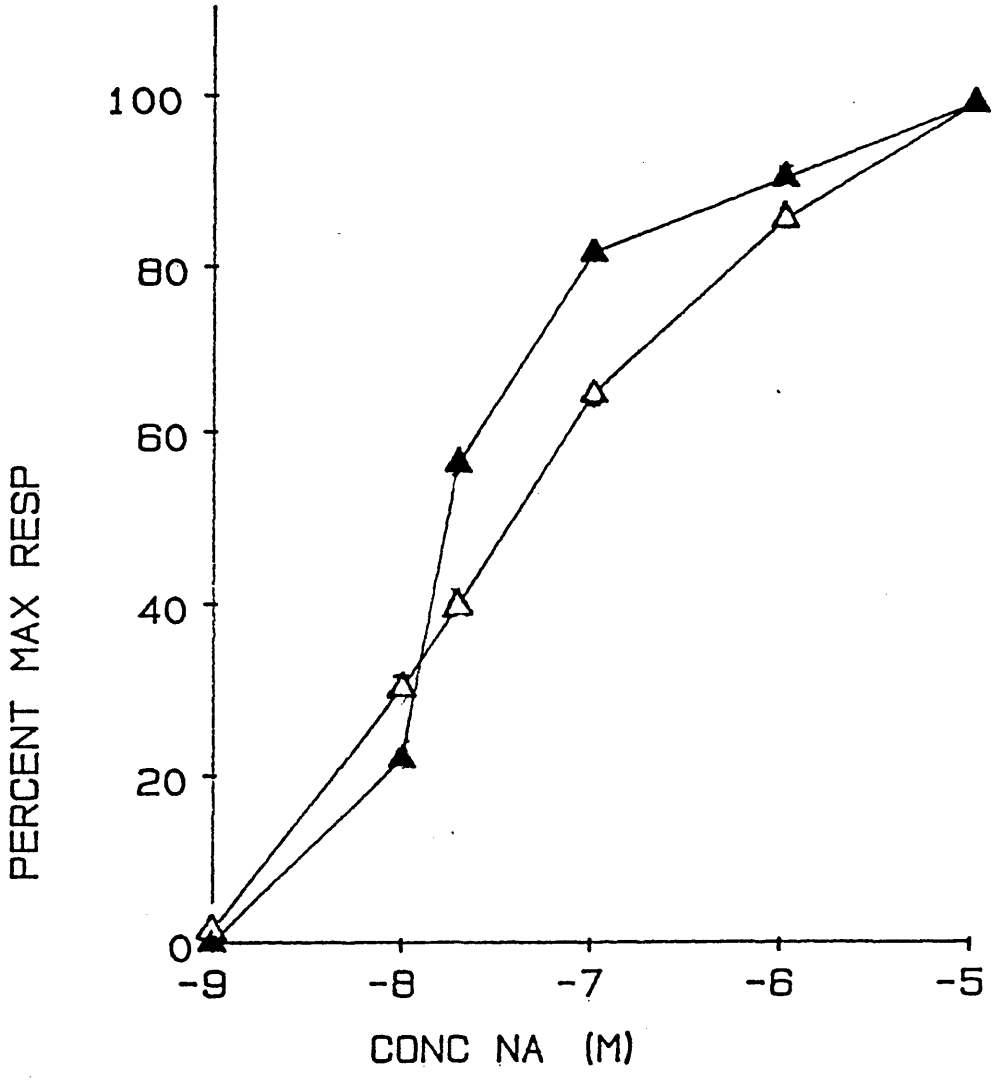


FIG 21 Dose response curve for noradrenaline on the isolated rat aorta. Responses are expressed as a percentage of the maximum response elicited by noradrenaline, in mg of isometric tension. Open symbols represent the slow component of the response; closed symbols respresent the fast component of the response. 100%: Fast = 1.1g; Slow = 2.0g (n=2).

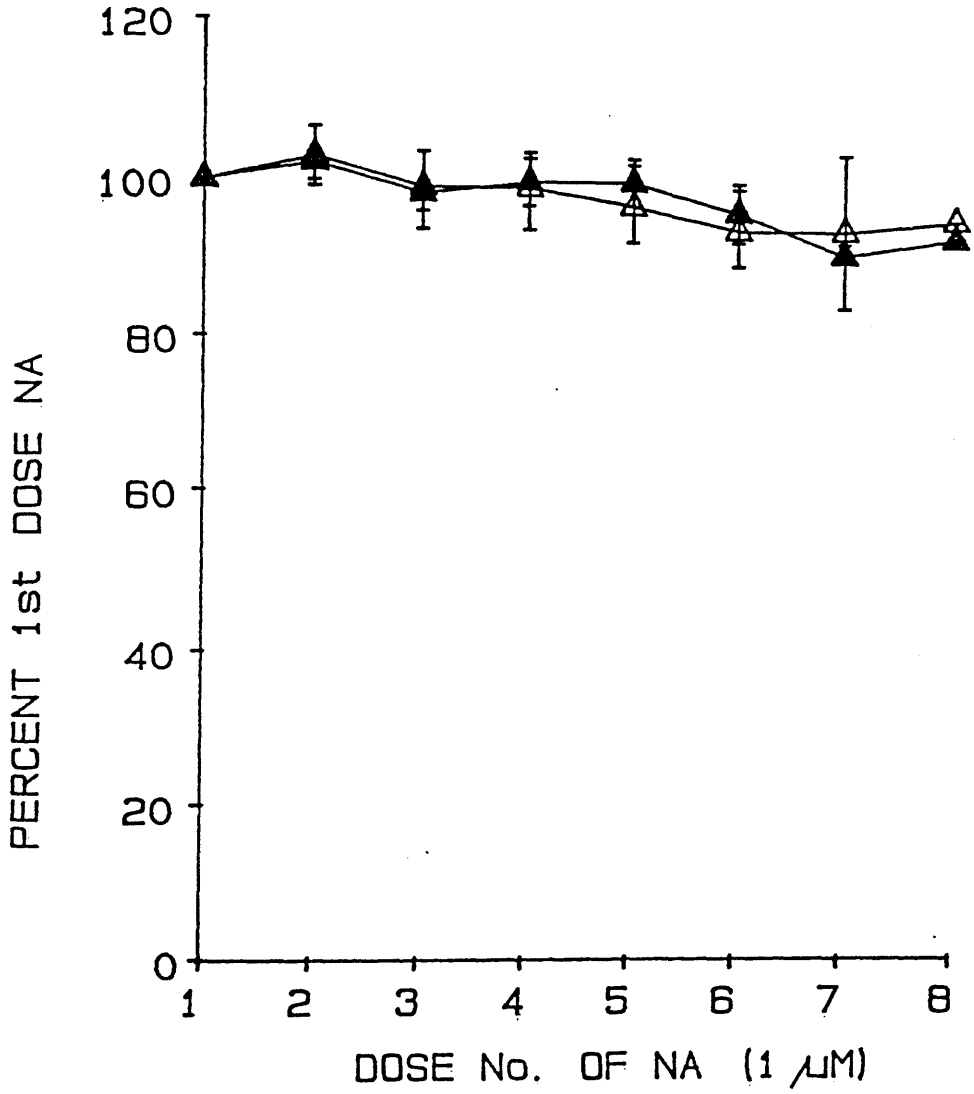


FIG 22 Effect of repeated administration of noradrenaline (1 uM) on the strength of the contraction produced by the isolated rat aorta. Responses are expressed as a percentage of the response to the first exposure of the tissue to noradrenaline. Open symbols represent the slow component of the response; closed symbols represent the fast component of the response. Vertical bars represent SEM (n=4). 100%: Fast = 0.94 ± 0.22g; Slow = 1.2 ± 0.08g

The second experiment examined the effect of the Tris solution, on the NA response. Tris solution is gassed with 100% O_2 and it is not always easy to ensure that it is being gassed at the same rate in each experiment. The solutions were gassed with two levels of O_2, "high" (pO_2=492 +/- 26 mmHg, n=3) and "normal" (pO_2=395 +/- 33 mmHg, n=3) and a third solution was not gassed at all (pO_2=178 +/- 3 mmHg, n=3). Two concentrations of noradrenaline were used to contract the aorta, $3x10^{-8}$M and 10^{-6}M. There was no significant difference between the responses to 10^{-6}M NA in the high and "normal" gassed solutions but the responses in the non-gassed solution were smaller, i.e. on average 21% +/- 3 smaller for the ITC and 32% +/- 5 smaller for the SSC, (ITC: $0.001<P<0.01$; SSC: $0.01<P<0.05$, n=4), table 2. Conversely, there were significant differences between the responses produced by $3x10^{-8}$M NA in the high and "normal" gassed solutions i.e. the highly gassed solutions reduced both phases of the response (ITC and SSC: $0.001<P<0.01$, n=4). The low O_2 solution significantly reduced only the SSC ($0.01<P<0.05$) for $3x10^{-8}$M NA. So, as long as 10^{-6}M NA was used and the solutions were bubbled to a reasonable degree, the NA responses would not be affected.

3.2) Intracellular and Extracellular Ca^{2+} Antagonists

3.2.a) Nifedipine

It is believed that the ITC is not as dependent on extracellular Ca^{2+}-entry as is the SSC, therefore it follows that nifedipine would exert its greatest effect on the

TABLE 2 Summarises the effects of varying the strength of bubbling, of the Tris buffer solution, on the contraction of the isolated rat aorta to noradrenaline (3×10^{-8} M–10^{-6} M). Responses are expressed as a percentage of the "normal" oxygen level response, mean values +/- SEM, (n=3).

	Response as % of "normal" O_2 response (395 ± 33mmHg)			
Dose of NA	3×10^{-8}M		10^{-6}M	
Component of response	Fast	Slow	Fast	Slow
High O_2 (492 ± 26mmHg)	71 ± 4	66 ± 4	89 ± 3	79 ± 4
Low O_2 (178 ± 3mmHg)	87 ± 7	68 ± 7	79 ± 3	68 ± 5

secondary component of NA's response. To examine this, nifedipine, in increasing concentrations (10^{-9}M-10^{-5}M), was added to the perfusate as NA was pulsed. Various tris solutions, with different calcium concentrations were used to examine the separate components of the NA response.

i) 2mM Ca^{2+} Solution:

Both components of contraction are produced in this "normal-Ca^{2+}" solution. Nifedipine however, did not inhibit the response to NA but significantly increased both the ITC and the SSC (e.g. with 10^{-6}M nifedipine, for both ITC and SCC: $0.001<P<0.01$, n=6) fig 23. The tissues were also tested with repeated concentrations of alcohol, the nifedipine vehicle, to ensure it was not this which was causing the increase; it had no such effect. A lower concentration of noradrenaline ($3x10^{-8}$M) was also tried to see if nifedipine could block either phase of the response it produced. 10^{-6}M nifedipine had no effect but $3x10^{-6}$M nifedipine produced an inhibition of both the fast and slow components of both concentrations of noradrenaline's aortic response, (fig 24). All graphs are drawn as the log concentration of nifedipine vs the response to NA as a % of the contraction before the addition of nifedipine.

ii) $[Ca^{2+}]_{free}$ Solution:

No Ca^{2+} salts were added to the solution and 5mM EGTA was used to chelate the remaining Ca^{2+} contaminants. The calcium concentration was calculated to be 10^{-10}M. These conditions produce a more dominant ITC so making the effect of nifedipine on it , easier to study. The slow component

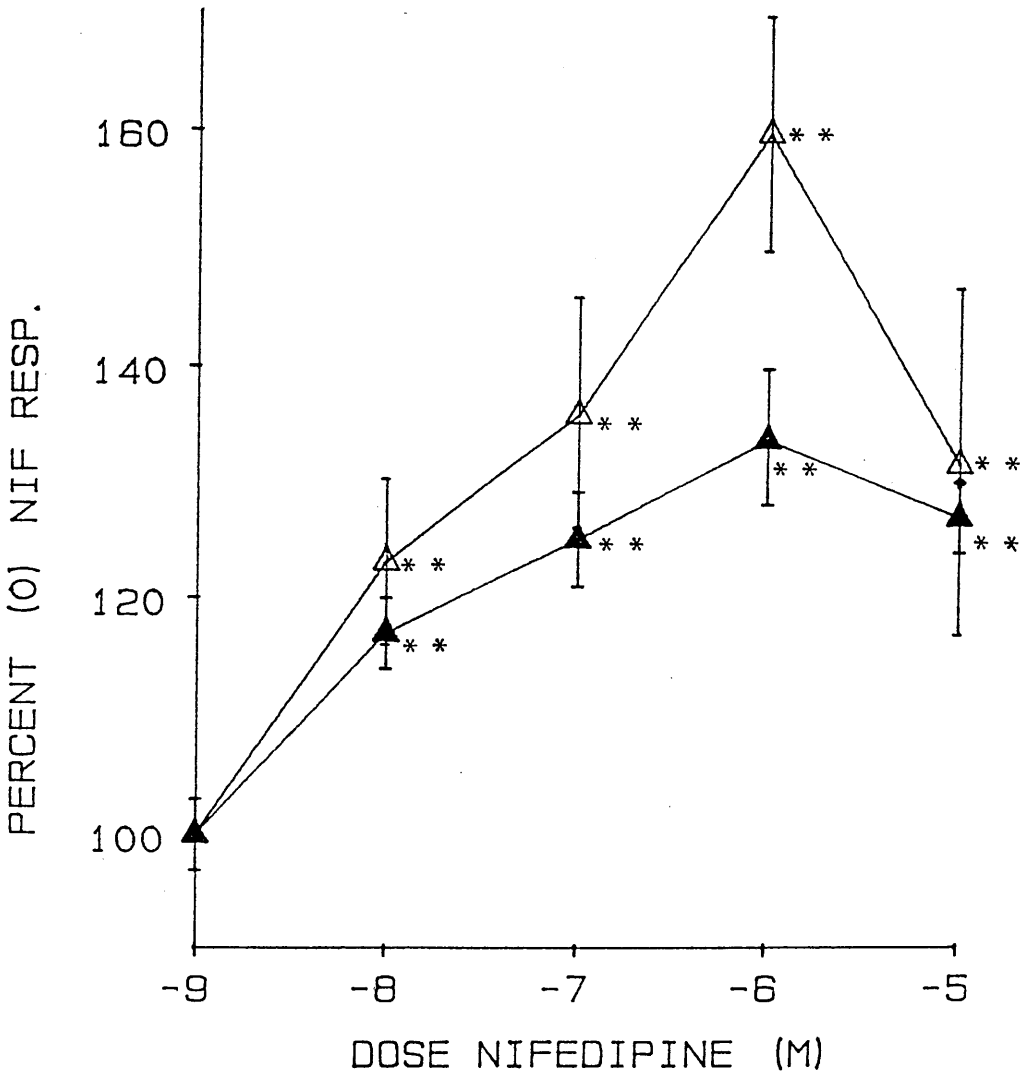
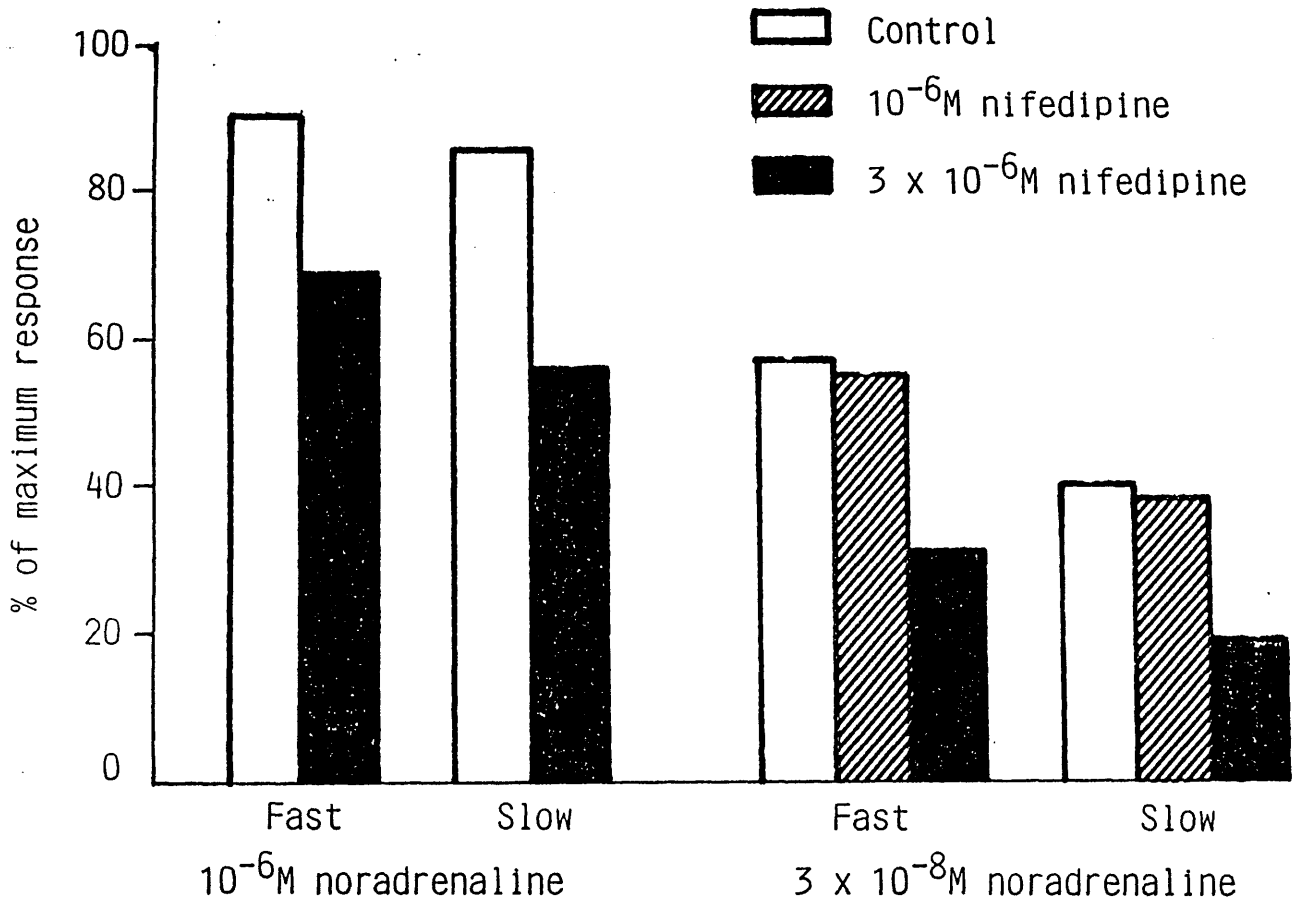


FIG 23 Effect of nifedipine (10^{-9}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no nifedipine present. Tissues are bathed in a 2mM Ca^{2+} Tris buffer solution, with no EGTA present. Statistical significance: ** 99% level. Vertical bars represent SEM (n=6). 100%: Fast = 0.68 ± 0.02g; Slow = 1.06 ± 0.11g.

FIG 24 Histogram displaying the percentage inhibition of the rat aorta contactile response to noradrenaline (10^{-6}M and $3x10^{-8}$M) by two different concentrations of nifedipine (10^{-6}M and $3x10^{-6}$M). Responses are expressed as a percentage of the maximum contractile response elicted by noradrenaline (10^{-5}M). Data is shown for both the fast and slow components of the response.


Control
10⁻⁶M nifedipine
3 x 10⁻⁶M nifedipine
% of maximum response
100
80
60
40
20
0
Fast
Slow
Fast
Slow
10⁻⁶M noradrenaline
3 x 10⁻⁸M noradrenaline

is not totally abolished when the tissues are bathed in "calcium-free" solutions. On average 35% +/- 4 (n=4) of the response remains. Fig 25 shows that there was no inhibition of either phase until 10^{-6}M nifedipine and above was used. Unexpectedly the ITC was reduced more than the SSC but even then the maximum inhibition was only 37% with the nifedipine concentration of 10^{-5}M (n=5).

iii) Reduced Ca^{2+} Solution:

Calcium chloride was added to the tris solution (with 1mM EGTA) to produce a concentration of free calcium of $5x10^{-8}$M. When the tissues were exposed to this saline, the SSC was more pronounced than in the $[Ca^{2+}]_{free}$ solution fig 26. This time nifedipine blocked the SSC (52% +/- 4 of maximum, n=6) significantly more than the ITC (92% +/- 5 of maximum, $P<0.001$ for the concentration 10^{-7}M, n=6) and had a significant effect with concentrations of 10^{-8}M and greater. The maximum inhibition of the SSC was 60%, with 10^{-5}M nifedipine, (n=6). Figure 27(a) shows representative traces of the effect of nifedipine on the contractile response to noradrenaline in normal (i) and reduced calcium solutions (ii).

iv) EGTA Adjusted Solutions:

The above solutions all contained varying amounts of EGTA, so in order to standardise the procedure, experiments i) and iii) were repeated with the same amount of calcium buffer as in ii), i.e. 5mM EGTA, and the calcium added was increased to maintain the $[Ca^{2+}]_{free}$ levels in i) and iii). The "normal Ca^{2+}" experiments, showed greatly different results,

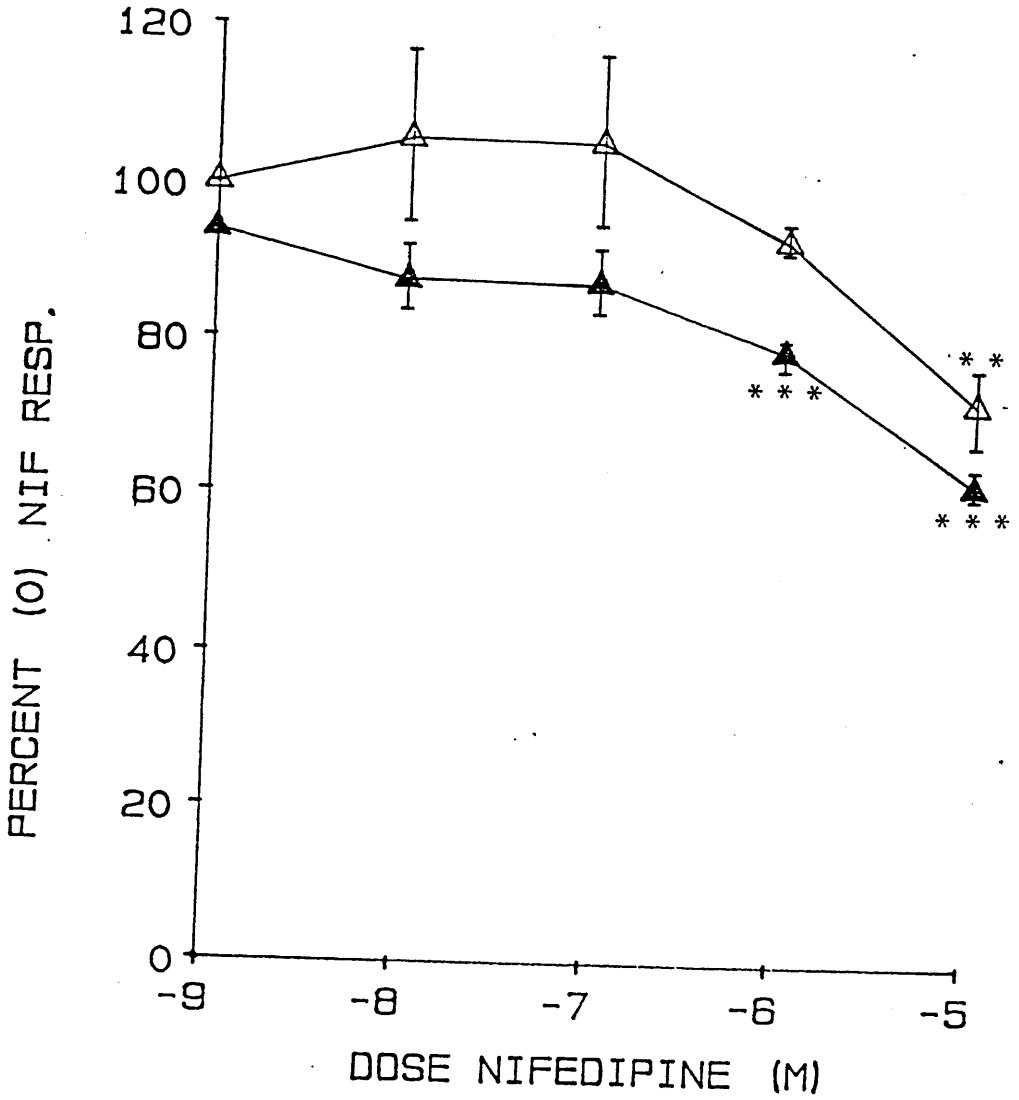


FIG 25 Effect of nifedipine (10^{-9}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no nifedipine present. Tissues are bathed in a calcium free Tris buffer solution, with 5mM EGTA present. Statistical significance: ** 99%, *** 99.9% level. Vertical bars represent SEM (n=5). 100%: Fast = 0.69 ± 0.02g; Slow = 0.68 ± 0.04g.

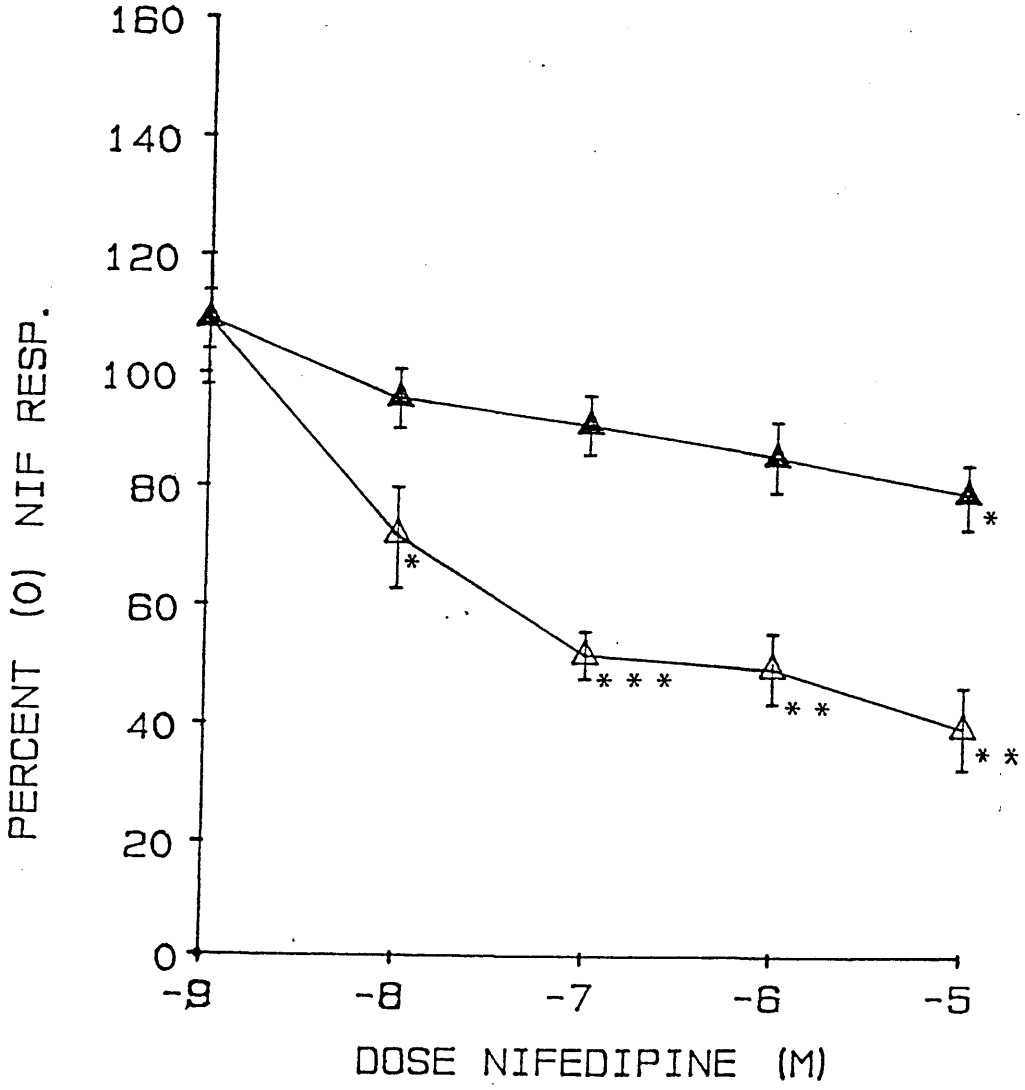


FIG 26 Effect of nifedipine (10^{-9}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no nifedipine present. Tissues are bathed in a reduced Ca^{2+} ($5x10^{-8}$M) Tris buffer solution, with 1mM EGTA present. statistical significance: * 95% level, ** 99% level, *** 99.9% level. Vertical bars represent SEM (n=6). 100%: Fast = 1.03 ± 0.06g; Slow = 1.20 ± 0.05g.

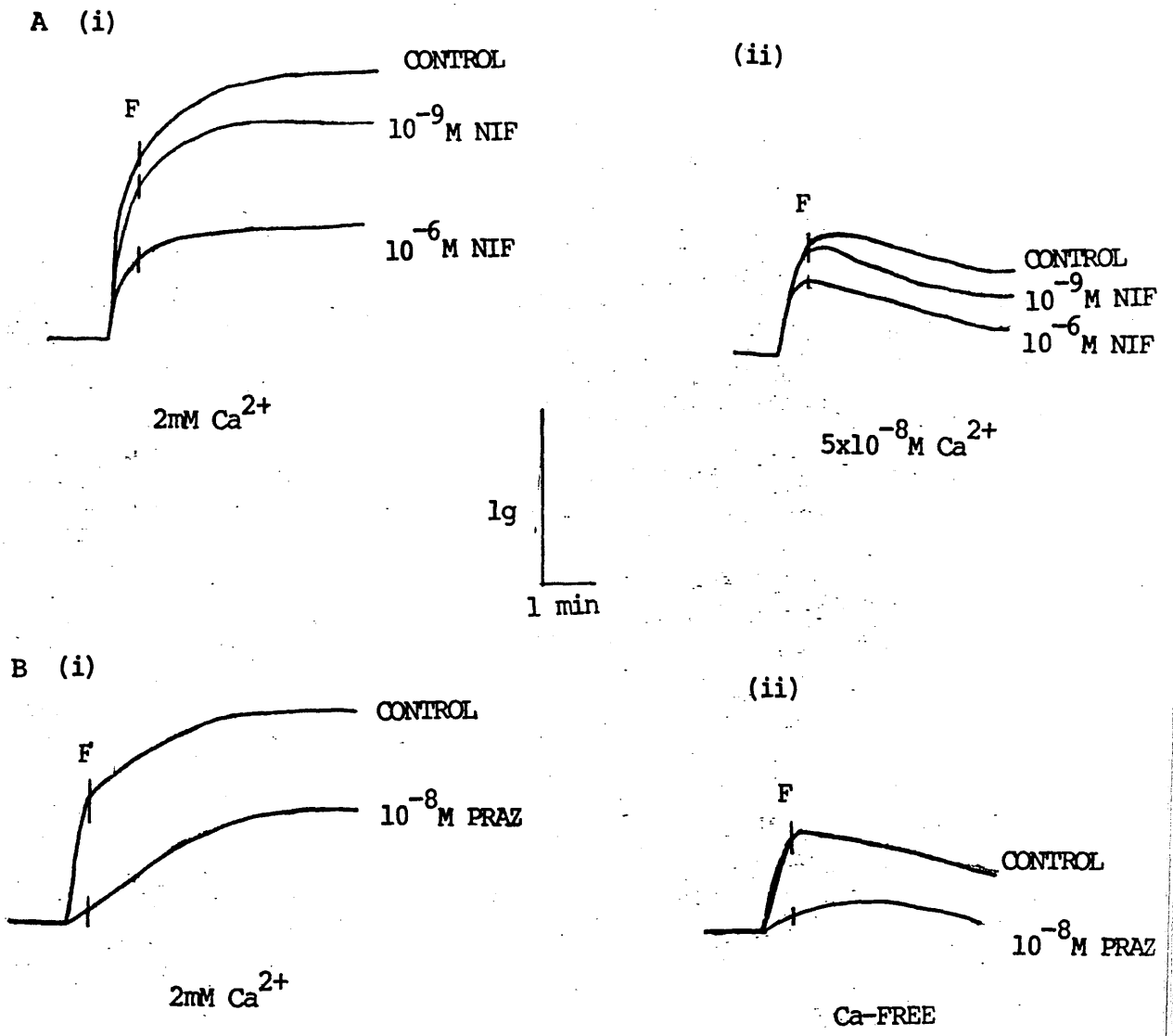


FIG 27 Representative traces of the effect of A) nifedipine in i) 2mM Ca^{2+} and ii) reduced calcium Tris buffer solution and B) prazosin in i) 2mM Ca^{2+} and ii) calcium-free Tris buffer solution on contractile responses to 10^{-6}M noradrenaline on the rat isolated thoracic aorta. F represents the fast (ITC) component.

fig 28.

Instead of potentiating the responses, nifedipine now produced a slight inhibition of both phases, significantly with 10^{-6}M (ITC: $0.05<P<0.1$; SSC: $0.01<P<0.05$, n=4). However, 10^{-5}M nifedipine produced no block. Adjusting the EGTA level in the "reduced Ca^{2+}" solution did not produce significantly different results from before (see (iii)). The SSC was blocked more than the ITC as expected and concentrations above 10^{-8}M nifedipine significantly inhibited this second phase of the response ($0.01<P<0.05$, n=4), fig 29.

3.2.b) Cadmium

Cadmium chloride was used next to see how it affected the response to noradrenaline, as an example of an inorganic calcium entry blocker. The concentrations used ranged between 10^{-5}M and 10^{-3}M.

i) 2mM Ca^{2+} Solution:

Cadmium produced an inhibition of both the ITC and SSC to the same extent. Concentrations greater than 10^{-4}M blocked the response to NA, eventually reducing it to baseline (n=9), fig 30.

ii) $[Ca^{2+}]_{free}$ Solution:

Under these conditions (producing a more dominant ITC), cadmium once again reduced both components of the NA response equally well, almost down to baseline (n=2), fig 31. It was discovered though, that this inhibition of the

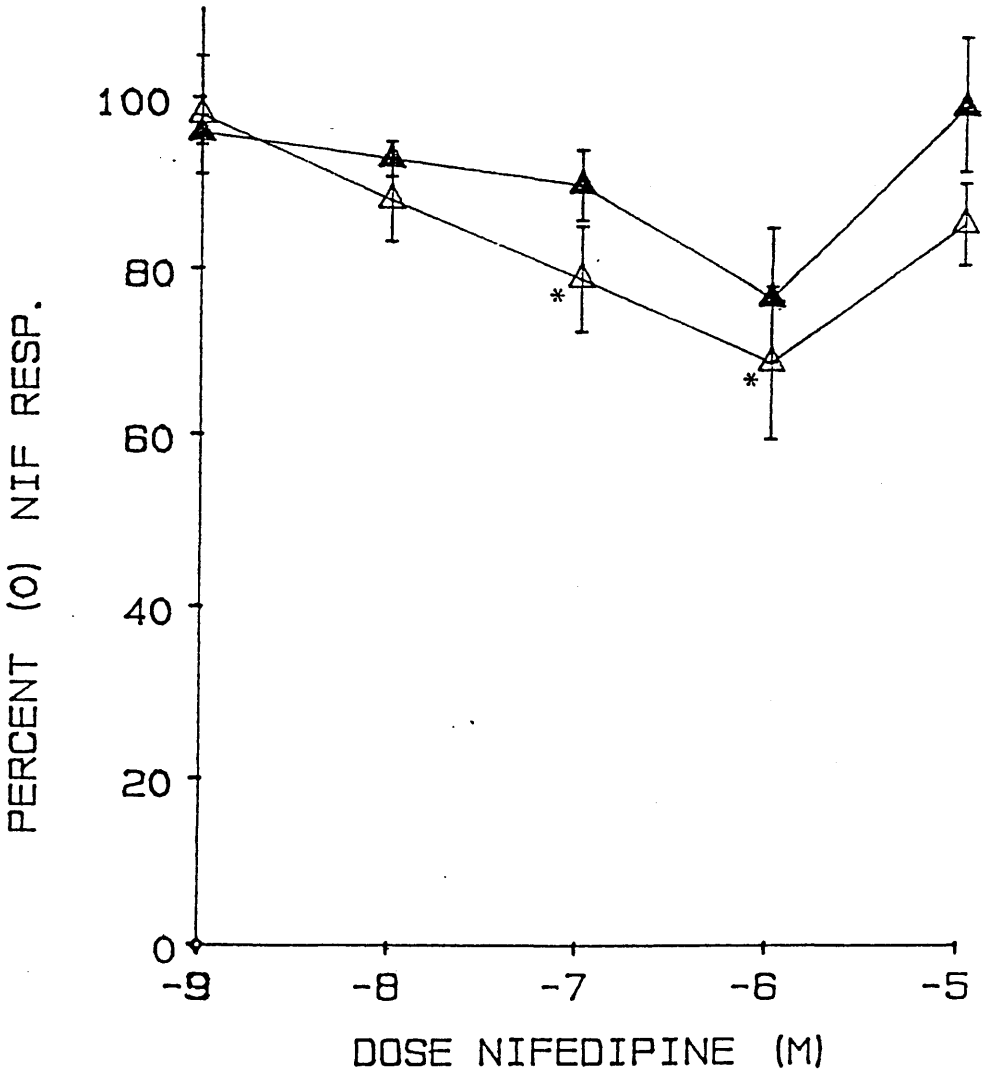


FIG 28 Effect of nifedipine (10^{-9}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no nifedipine present. Tissues are bathed in a 2mM Ca^{2+} Tris buffer solution, with 5mM EGTA present . Statistical significance: * 95% level. Vertical bars represent SEM (n=4). 100%: Fast = 0.9 ± 0.06g; Slow = 1.18 ± 0.08g.

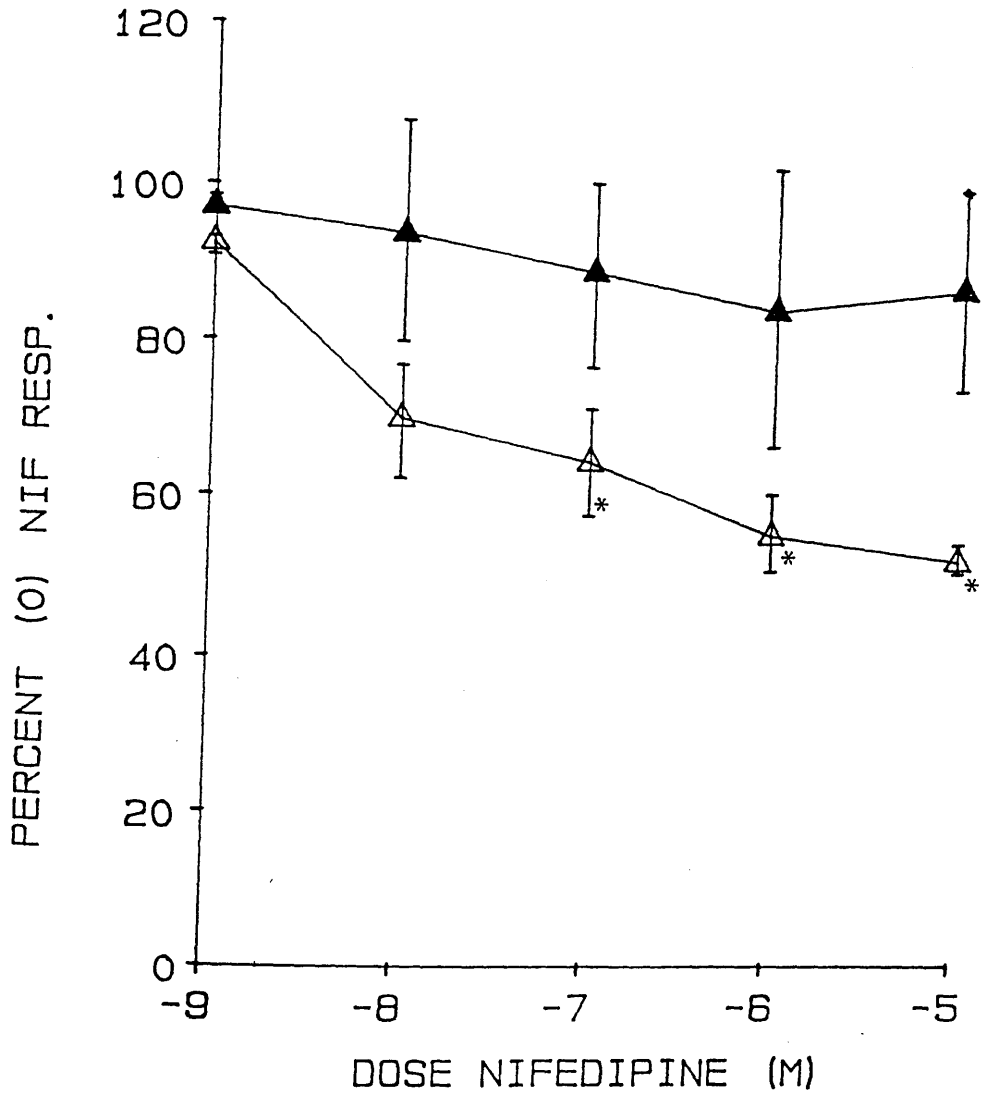


FIG 29 Effect of nifedipine (10^{-9}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no nifedipine present. Tissues are bathed in a reduced Ca^{2+} ($5x10^{-8}$M) Tris buffer solution, with 5mM EGTA present. Statistical significance: * 95% level. Vertical bars represent SEM (n=4). 100%: Fast = 0.77± 0.05g; Slow = 1.18 ± 0.14g.

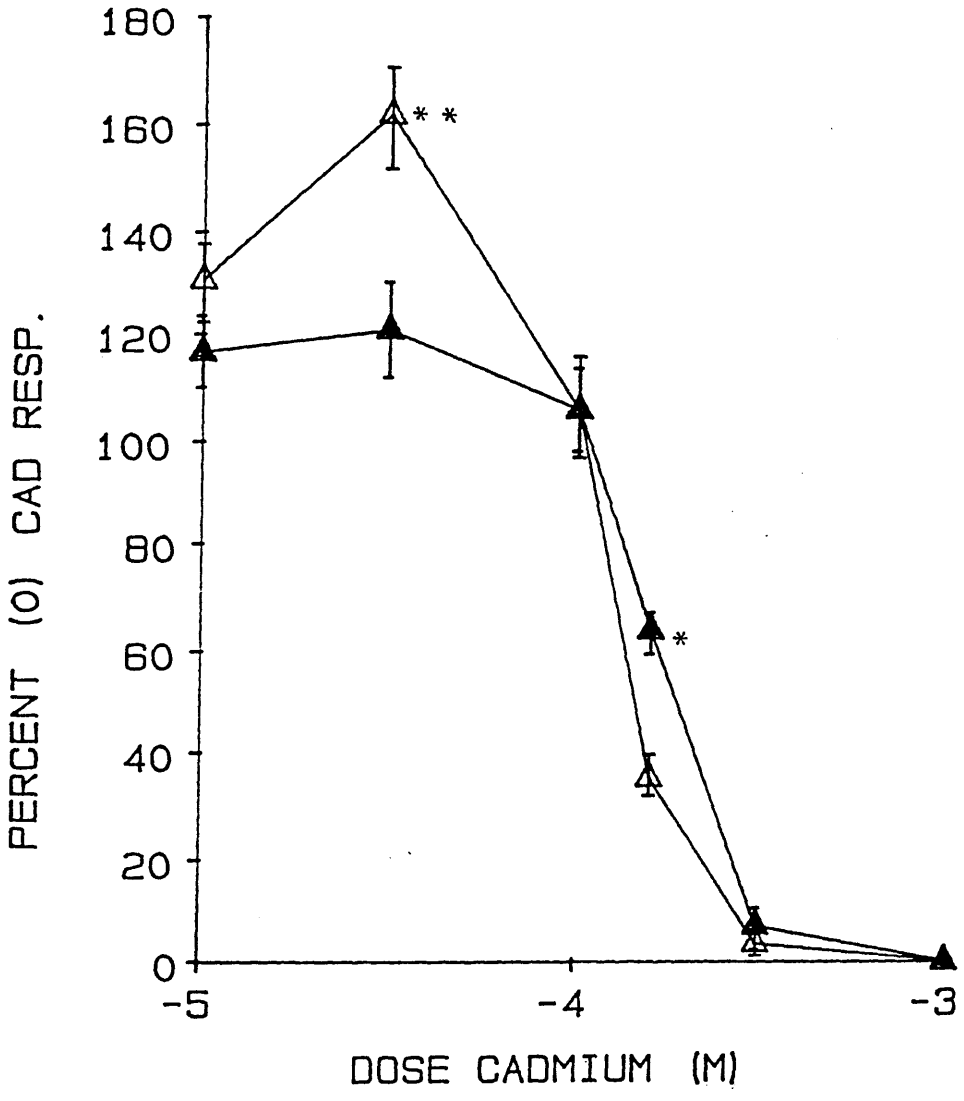


FIG 30 Effect of cadmium (10^{-5}M-10^{-3}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no cadmium present. Tissues are bathed in a 2mM Ca^{2+} Tris buffer solution. Statistical significance: * 95% level, ** 99% level. Vertical bars represent SEM (n=9). 100%: Fast = 0.8 ± 0.08g; Slow = 1.08 ± 0.13g.

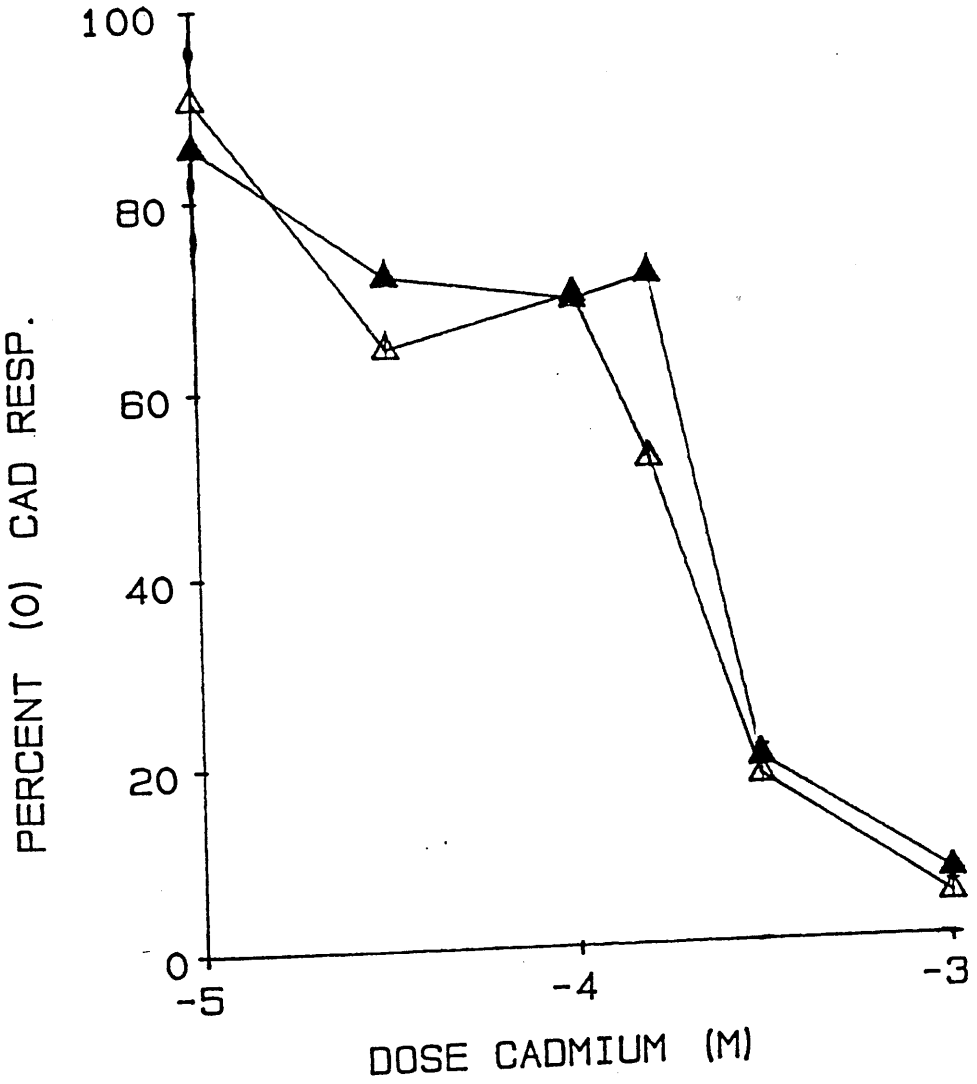


FIG 31 Effect of cadmium (10^{-5}M-10^{-3}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no cadmium present. Tissues are bathed in a Ca^{2+} free Tris buffer solution, with 5mM EGTA present. 100%: Fast = 1.28; Slow = 0.74, (n=2).

response may in fact be due to a poisoning of the tissues rather than a blockade of the Ca^{2+}-entry. When the EGTA chelates the cadmium in the solution (as it does in preference to calcium), there is a release of H^+ ions which lowers the pH of the solution to between 7.3 and 7.2 depending on the concentration of cadmium. It may be this lowering of the pH which is affecting the response.

Therefore the experiment was repeated, but EGTA was eliminated, fig 32. This meant that Ca^{2+} contaminants were present in the solution, a typical concentration being 20uM. Now, a preferential inhibition of the SSC could be seen, especially with $5x10^{-5}M$ cadmium, where the ITC isn't affected and the SSC is blocked by 47% ($0.001<P<0.01$, n=6).

3.2.c) Dantrolene sodium

Dantrolene sodium is believed to be an intracellular calcium blocker and so it was used in an attempt to inhibit the mobilisation of the internal Ca^{2+} ions, thus, presumably, preferentially blocking the ITC. The results were not as expected. The concentrations used were between $10^{-9}M$ - $10^{-5}M$.

i) 2mM Ca^{2+} Solution:

When "normal" calcium was present in the solution, dantrolene sodium inhibited both phases of the response equally well with concentrations higher than $5x10^{-8}M$ (n=6), fig 33. The maximum inhibition was achieved with $10^{-5}M$ dantrolene sodium ,49% and 60% for the ITC and SSC

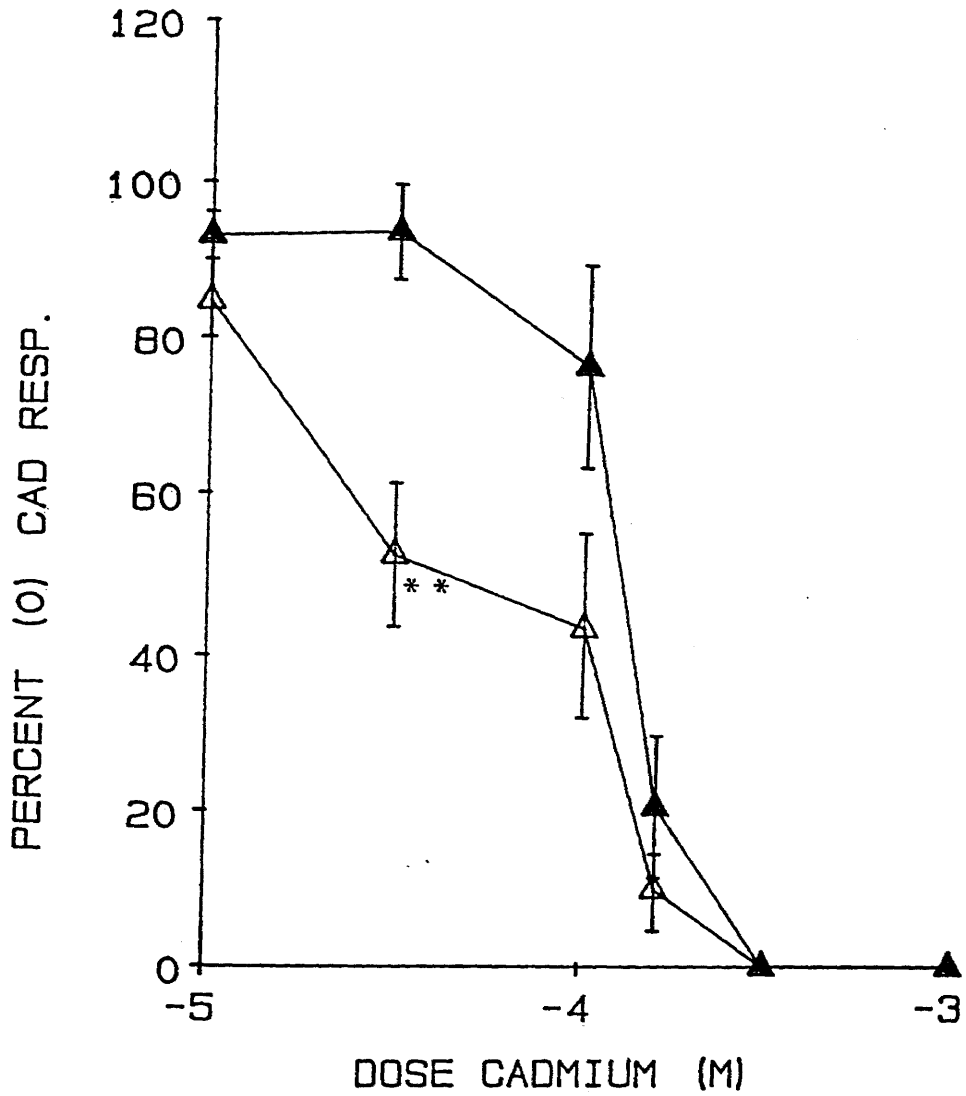


FIG 32 Effect of cadmium (10^{-5}M-10^{-3}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no cadmium present. Tissues are bathed in a Ca^{2+} free (20 uM) Tris buffer solution, with **NO** EGTA present. Statistical significance: ** 99% level. Vertical bars represent SEM (n=6). 100%: Fast = 0.91 ± 0.13g; Slow = 0.78 ± 0.1g.

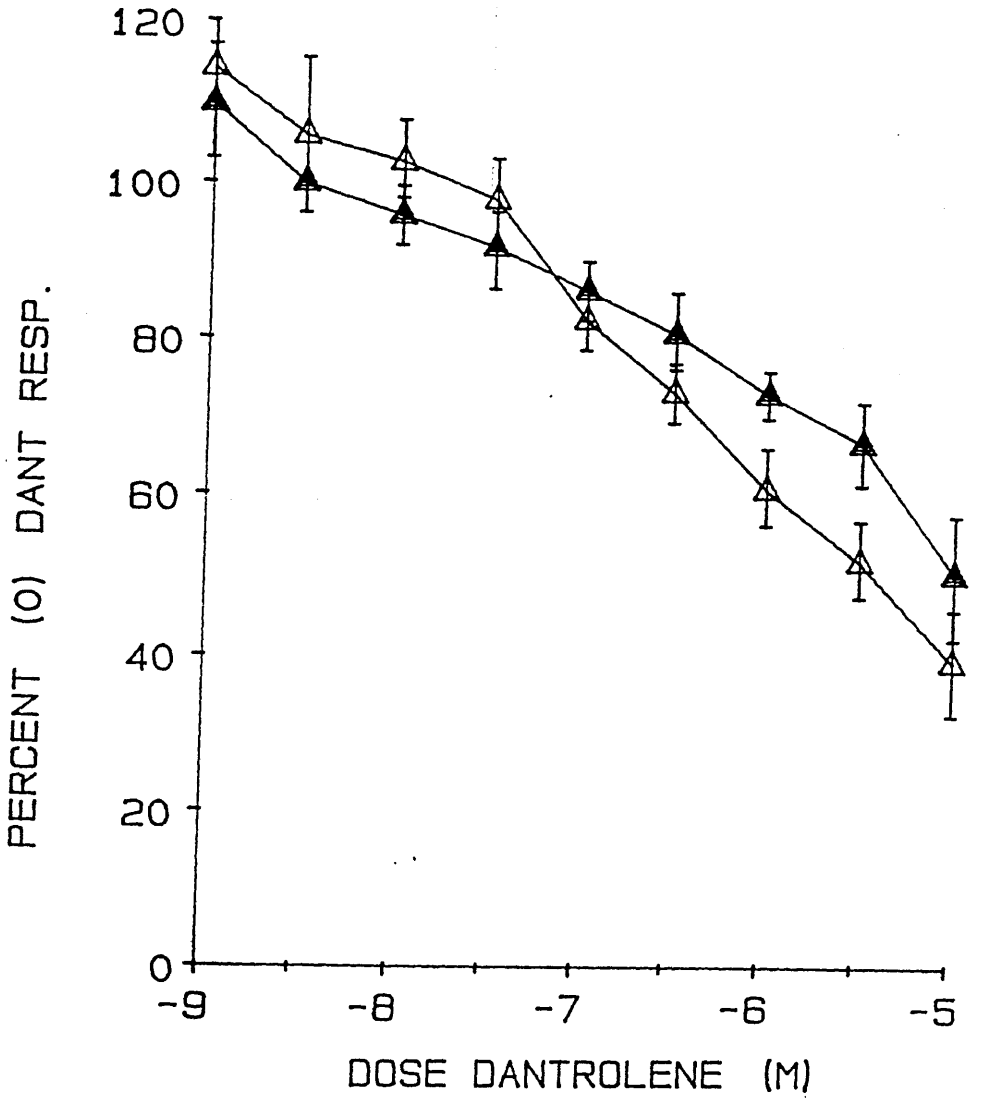


FIG 33 Effect of dantrolene (10^{-9}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no dantrolene present. Tissues are bathed in a 2mM Ca^{2+} Tris buffer solution. Vertical bars represent SEM (n=6). 100%: Fast = 0.93 ± 0.16g; Slow = 1.1 ± 0.21g.

respectively.

ii) $[Ca^{2+}]_{free}$ Solution:

There was no significant blockade of the ITC when calcium was omitted from the solution, fig 34. The SSC was reduced at every concentration of dantrolene tried, increasing from 29% +/- 2 with 10^{-9}M to 53% +/- 9 with 10^{-5}M (n=6).

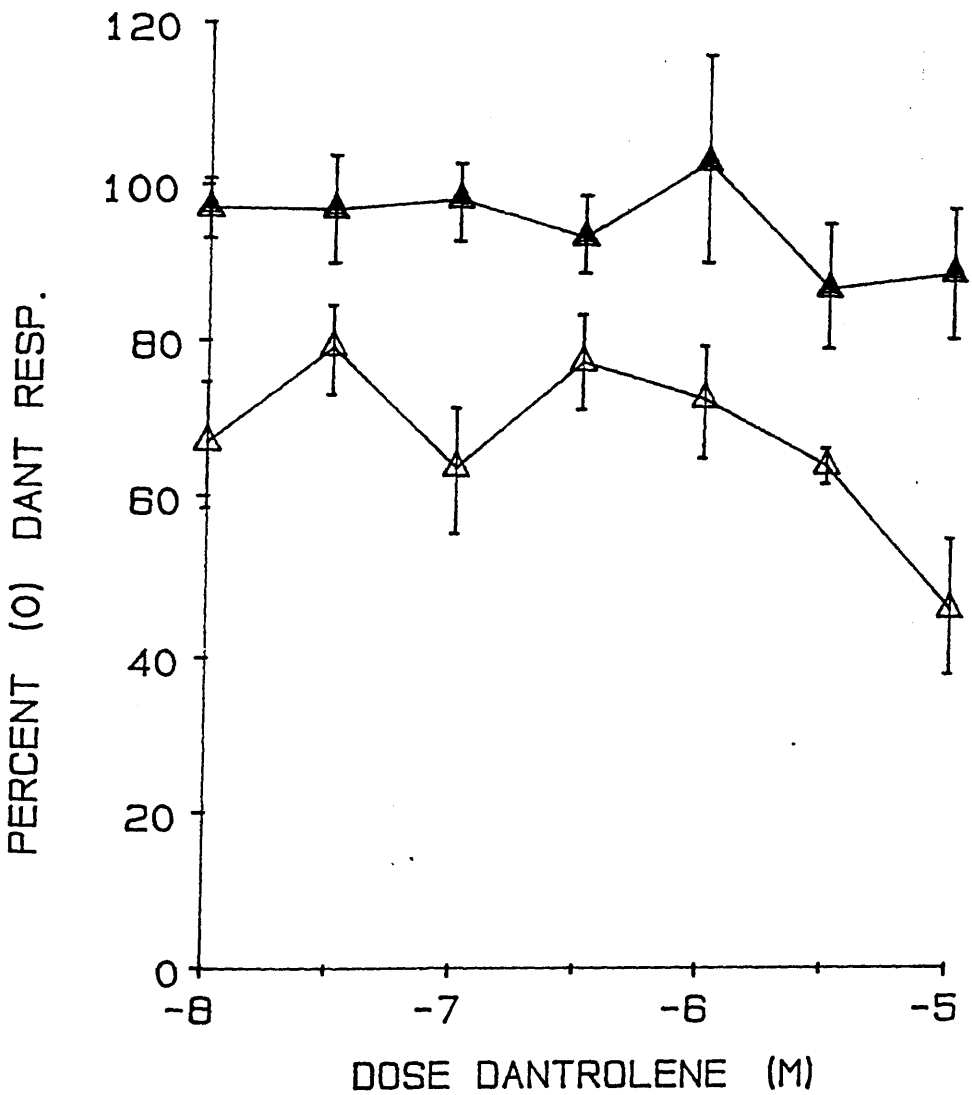


FIG 34 Effect of dantrolene (10^{-8}M-10^{-5}M) on the fast component (closed symbols) and slow component (open symbols) of the isolated rat aorta response to noradrenaline (10^{-6}M). Responses are expressed as a percentage of the response elicited with no dantrolene present. Tissues are bathed in a Ca^{2+} free Tris buffer solution, with 5mM EGTA present. Vertical bars represent SEM (n=6). 100%: Fast = 1.02 ± 0.11g; Slow = 0.67 ± 0.05g.

3.3) Calcium Re-entry

The main purpose of this section was to examine the effect of the removal and re-introduction of calcium into the Tris solution and its effects on the different components of the NA response. Several different methods were tried.

3.3.a) First of all, calcium chloride was added to the solution in increasing concentrations and pulsed through the tissues for 4 mins before the NA was introduced (1/2 hr between pulses). The ITC was not much affected by the re-introduction of calcium but the SSC was, fig 35. It was reduced to 42% +/- 7 of its original value by omitting calcium from the solution.

The SCC was increased, but not gradually as expected. When the free-calcium in the solution was calculated this result was not surprising. There had been a 10,000 fold increase in the concentration of calcium from 3.8×10^{-8}mM to 3.4×10^{-4}mM, (n=8). 1mM EGTA was present in the solution and one explanation for the above result maybe that, when the total calcium concentration exceeded this value the $[Ca^{2+}]_{free}$ concentration would rapidly increase, disproportionately. This rapid increase in the $[Ca^{2+}]_{free}$ concentration would therefore allow this "all-or-nothing" SSC response when the $[Ca^{2+}]_{total}$ reached a critical value of the same order of concentration as the buffer.

3.3.b) The threshold for the SSC response appeared to lie somewhere between 10^{-4}M and 10^{-3}M calcium, so perhaps the

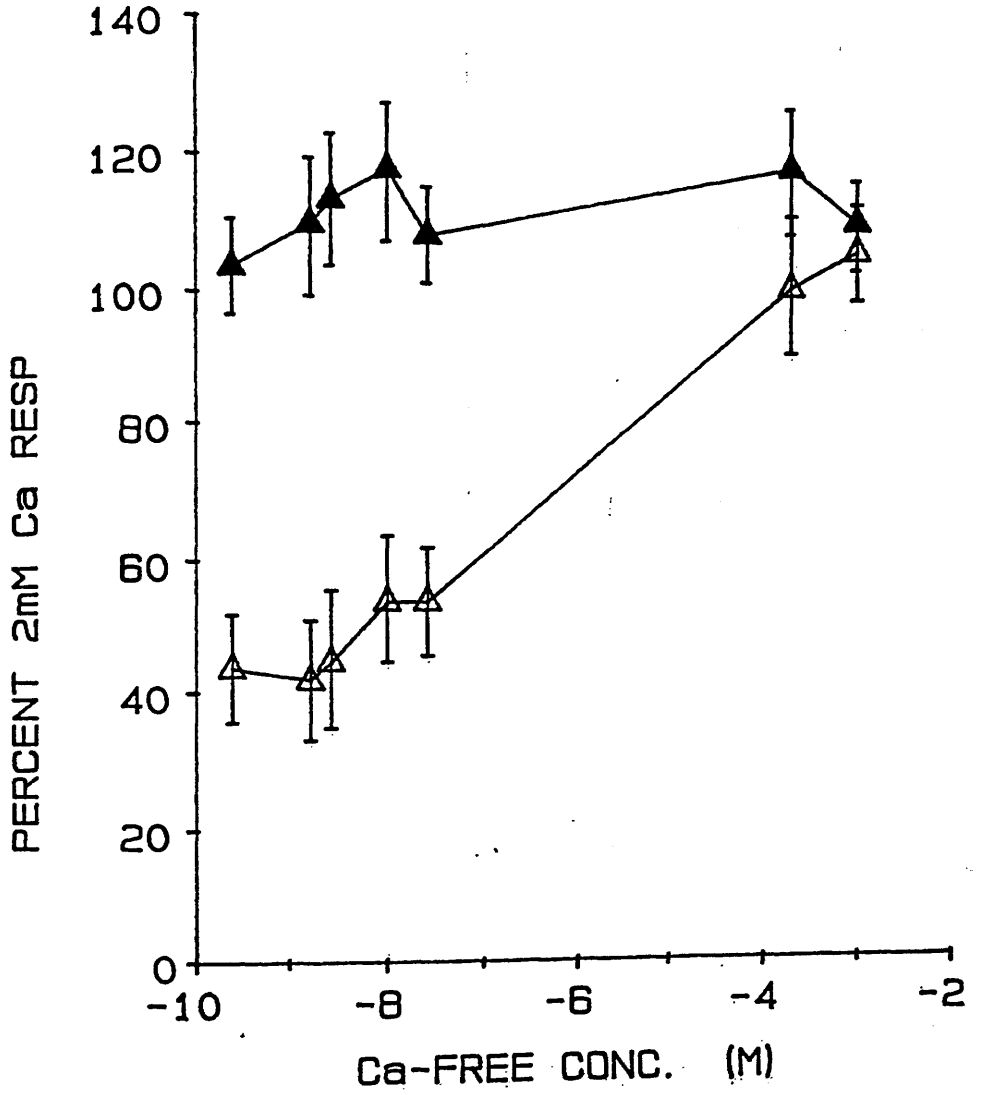


FIG 35 Calcium concentration/response curve constructed in Tris buffer with 1 mM EGTA present. Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in pulsed experiments: fast response (closed symbols); slow response (open symbols). Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. Vertical bars represent SEM (n=8). 100%: Fast = 1.02 ± 0.11g; Slow = 1.01 ± 0.12g.

calcium could be re-introduced without buffers present. This method proved to be much more successfull, fig 36. 5 mM EGTA was used to produce a $[Ca^{2+}]_{free}$ solution but the other solutions contained no calcium buffers. The first contained only Ca^{2+}-contaminants and the others contained increasing concentrations of calcium chloride, (10^{-4}mM - 10^{-2}mM). Now the SSC could be seen to increase gradually as the concentration of calcium increased. The Ca^{2+} pD_2 value was 4.73 +/-0.09, n=4.

3.3.c) Another method of examining the SSC was to omit returning the tissues to normal calcium for 1/2hr between reduced calcium solutions and just to go straight from one level of calcium to the next. In this way only the SSC is present. The reintroduction of the SSC was not quite as gradual as would have been expected, however the pD_2 value obtained was 3.85 +/- 0.07, n=2, fig 37.

3.3.d) In order to get a more accurate estimate of the pD_2 value for calcium, an improved buffering system was used. This method of buffering the calcium had already been used extensively on the anococcygeal muscle (McGrath et al, 1984) and produced even increments of calcium which could be accurately calculated. Buffer 6 - Buffer 1 produced concentrations of calcium between 10^{-6}M - $3x10^{-4}$M and when pulsed through the tissues produced a SSC which increased gradually fig 38a. The pD_2 using this method was measured as 4.41 +/- 0.11, n=4. Once again the ITC was not much affected by the removal and re-addition of calcium ions from the bathing medium, (fig 39).

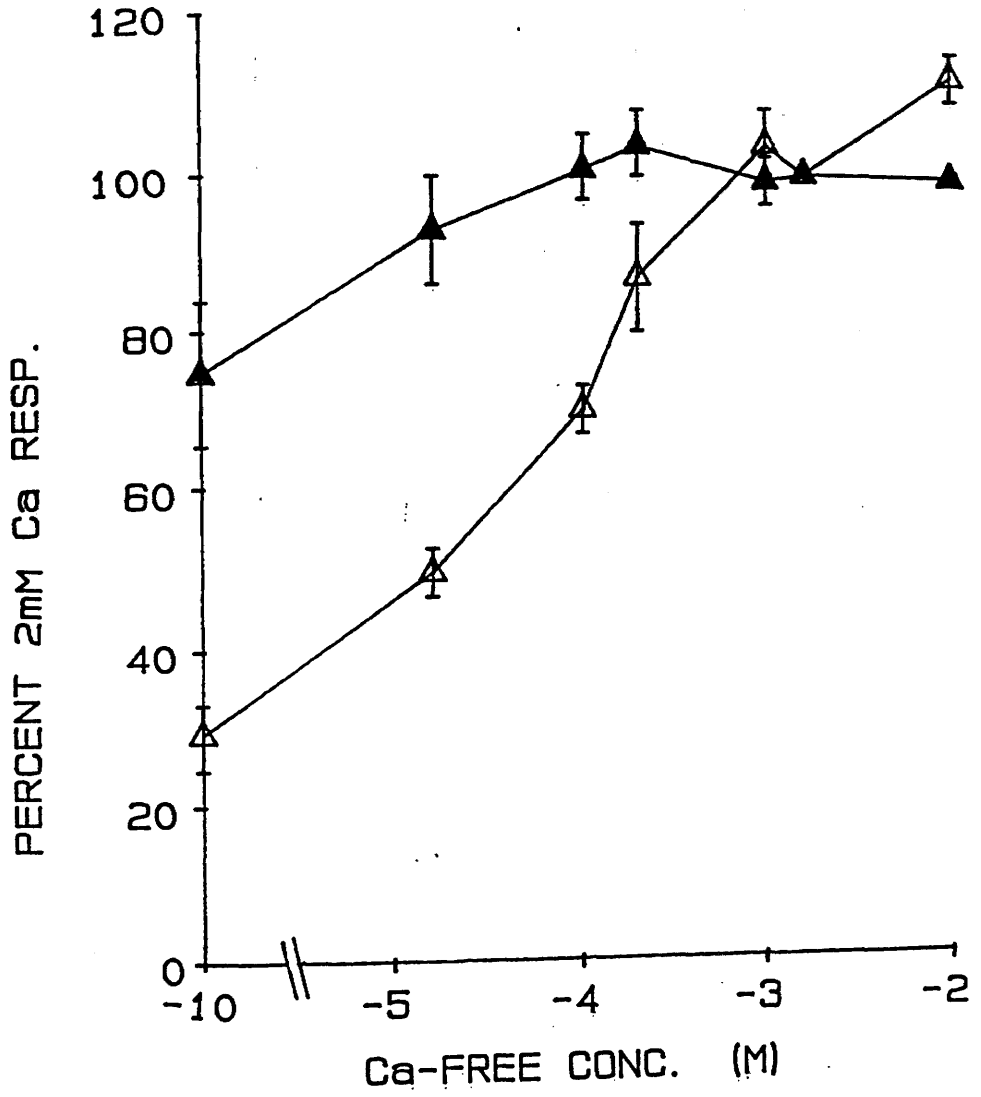


FIG 36 Calcium concentration/response curve constructed in Tris buffer with no EGTA present (5mM EGTA was present for the first dose of noradrenaline only, to give a calcium free response). Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in pulsed experiments: fast response (closed symbols); slow response (open symbols). Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. Vertical bars represent SEM (n=4). 100%: Fast = 0.9 ± 0.17g; Slow = 1.18 ± 0.12g.

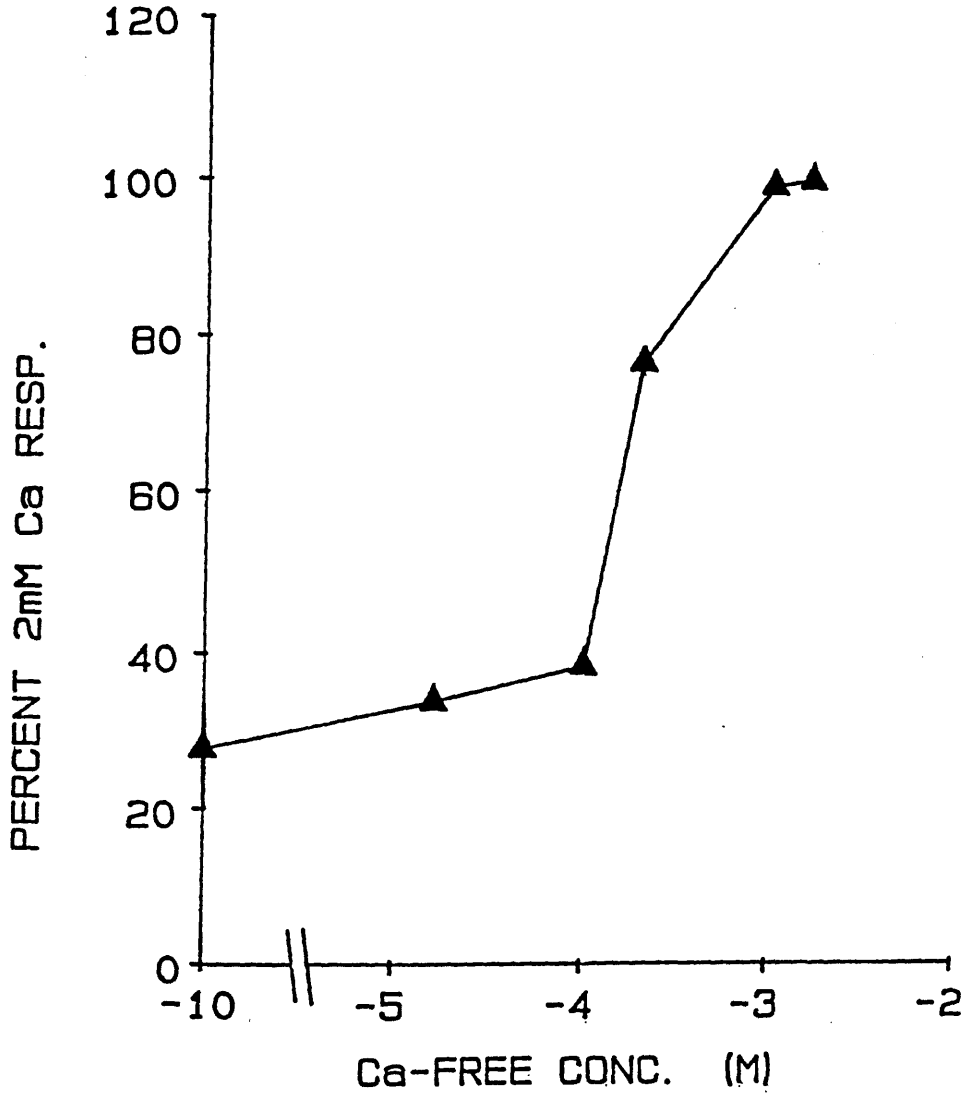


FIG 37 Calcium concentration/response curve constructed in Tris buffer with no EGTA present (5mM EGTA present to give 10^{-10}M calcium free concentration). Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in "continuous" experiments. Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. 100% = 2.25g, (n=2).

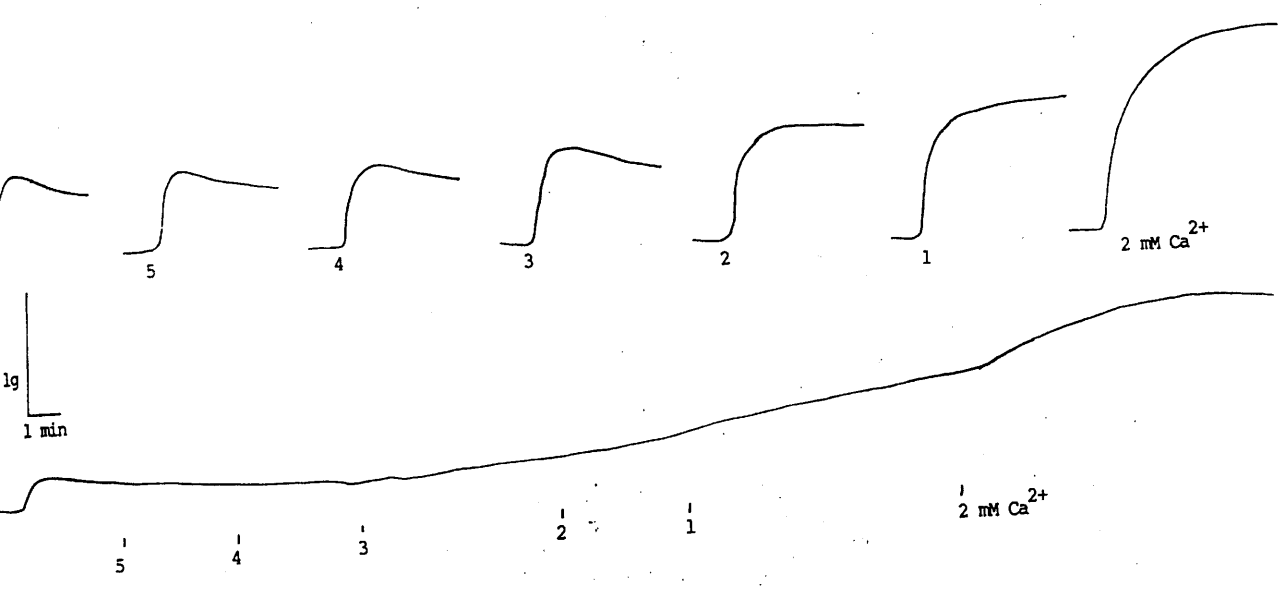


FIG 38 Representative traces of the contraction to 10^{-6}M noradrenaline on the rat aorta showing the responses elicited when the buffers (no.'s 6 - 1) and 2mM Ca^{2+} Tris solution are a) pulsed or b) sequentially perfused through the tissues.

3.3.e) The above experiment was repeated but this time the buffers were not pulsed. Buffer 6 to Buffer 1 were sequentially perfused through the tissues, the next buffer only being used when the previous one had produced its maximum NA response. This became known as the "continuous" method of perfusing the buffers (see fig 38b). As explained before this displays only the SSC, which increased as the calcium concentration increased, fig 40. The pD_2 value measured was 3.93 +/- 0.10, n=4.

3.3.f) As the previous work done with these buffers had been carried out in bicarbonate and not tris physiological saline solutions, experiments d) and e) were repeated using bicarbonate solution to check that the results would be the same, figs 41 & 42.

In both the pulsed and the continuous buffer experiments the SSC could be seen to increase as the calcium concentration increased (pD_2= 4.34 +/- 0.14 and 3.74 +/- 0.14 respectively, n= 4 for each expt.) Unexpectedly though, in the pulsed experiment, the ITC was reduced by the low calcium buffer and increased with increasing calcium (pD_2= 4.74 +/- 0.14, n=4).

It appeared that it made no difference to the pD_2 values for the SSC whether these experiments were carried out in tris or bicarbonate solutions. However it did affect the ITC which became reduced in low-calcium, bicarbonate-buffered solutions. However, the two methods of introducing the low calcium buffers, i.e. pulsed or continuous did make

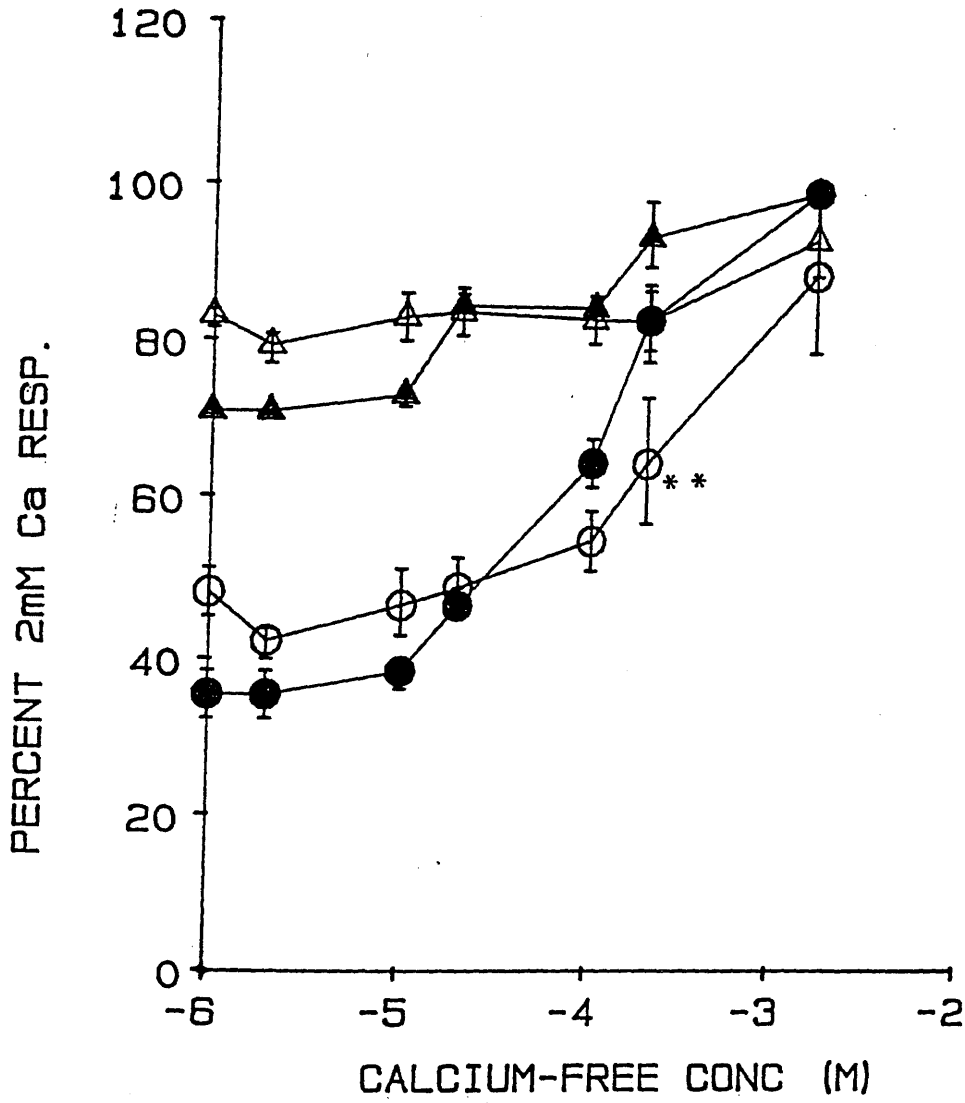


FIG 39 Calcium concentration/response curves constructed in Tris buffer using the EGTA/NTA calcium buffering system, with and without nifedipine (10^{-6}M) present. Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in pulsed experiments: fast response (triangles); slow response (circles). Control responses are represented by the closed symbols and the open symbols represent responses elicited in the presence of nifedipine. Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. Statistical significance of the inhibition of the SSC by nifedipine: ** 99% level. Vertical bars represent SEM (n=4). Control 100%: Fast = 0.92 ± 0.16g; Slow = 1.4 ± 0.35g. Nifedipine control 100%: Fast = 1.44 ± 0.22g; Slow = 2.0 ± 0.37g.

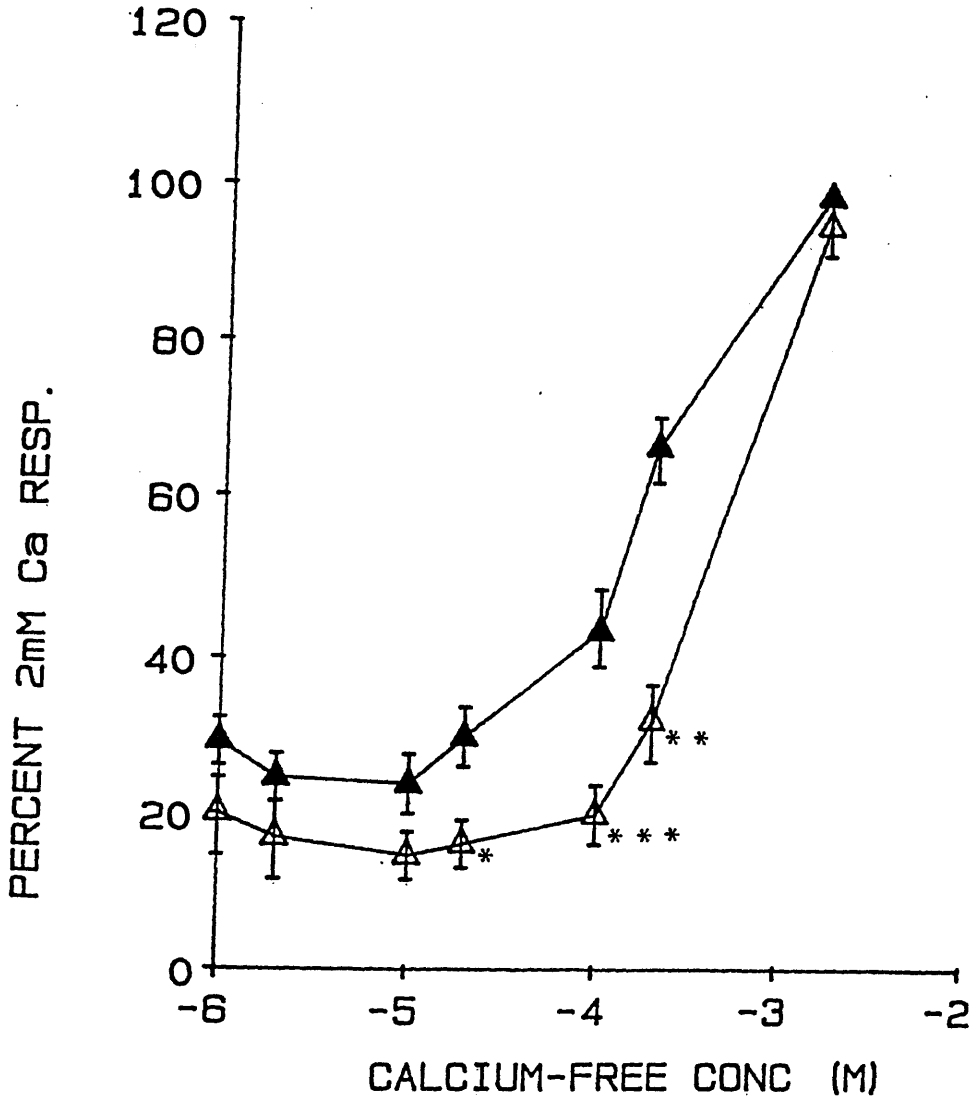


FIG 40 Calcium concentration/response curve constructed in Tris buffer using the EGTA/NTA calcium buffering system, with and without nifedipine (10^{-6}M) present. Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in "continuous" experiments: Closed symbols represent the control responses; open symbols represent responses in the presence of nifedipine. Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. Statistical significance: * 95% level; ** 99% level; *** 99.9% level. Vertical bars represent SEM (n=4). Control 100%: 1.12 ± 0.2g; Nifedipine control 100%: 1.99 ± 0.37g.

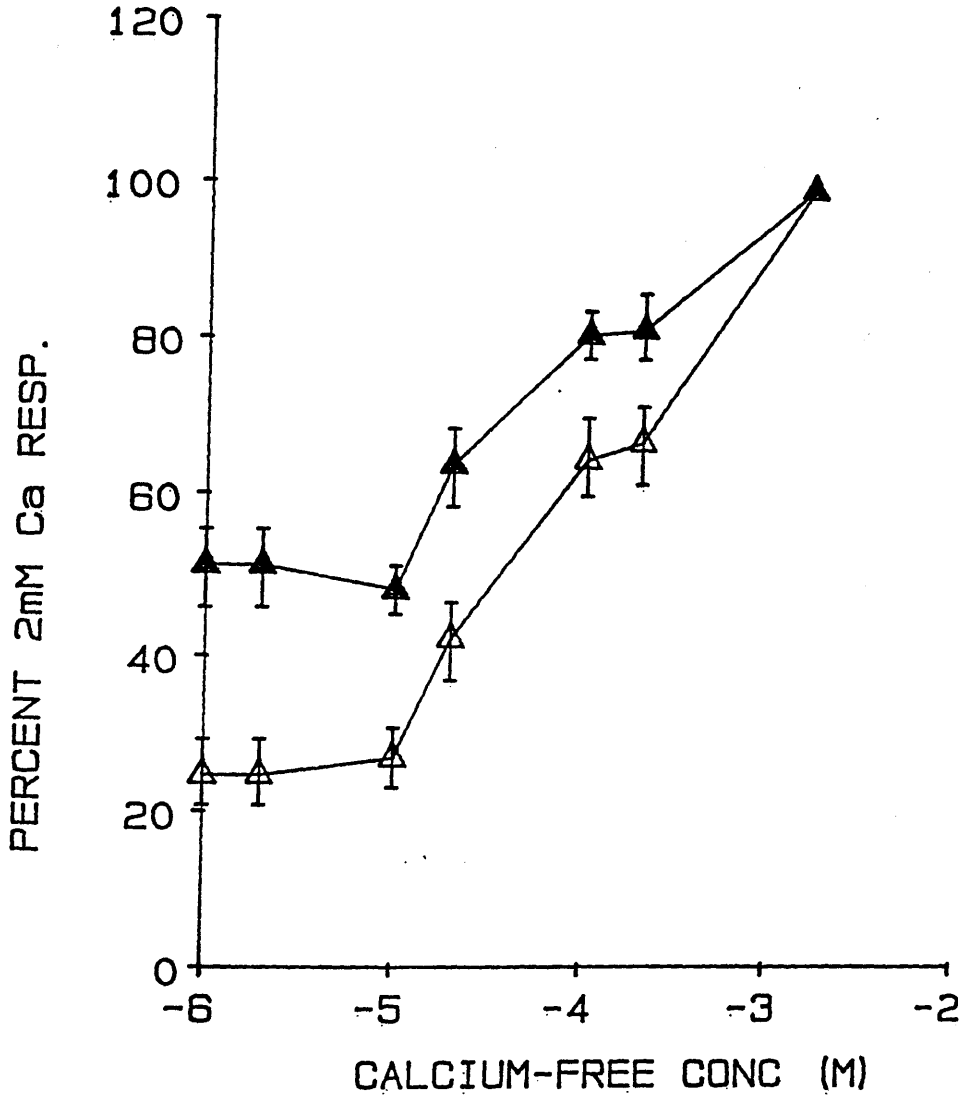


FIG 41 Calcium concentration/response curve constructed in bicarbonate buffer with the EGTA/NTA calcium buffering system. Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in pulsed experiments: fast response (closed symbols); slow response (open symbols). Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. Vertical bars represent SEM (n=4). 100%: Fast = 0.95 ± 0.12g; Slow = 0.81 ± 0.09g.

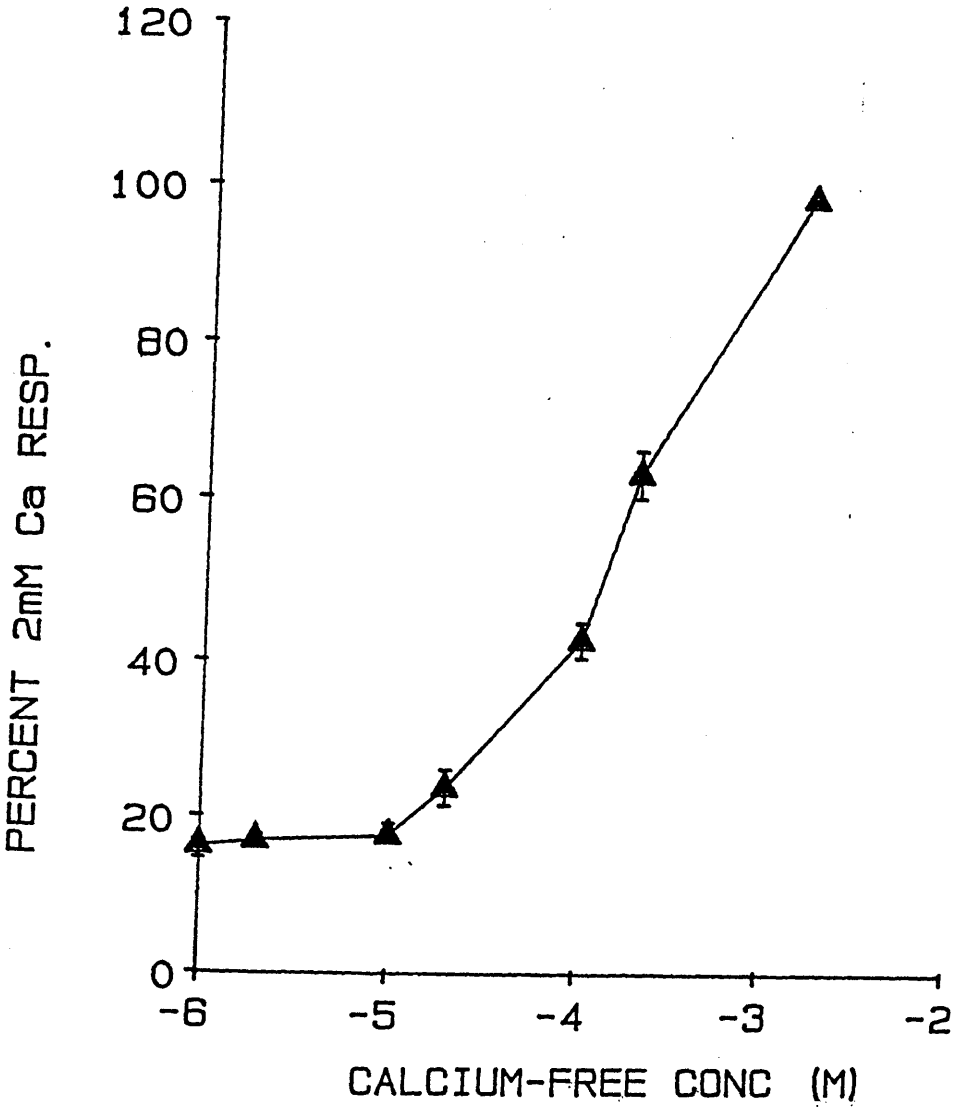


FIG 42 Calcium concentration/response curve constructed in bicarbonate buffer with the EGTA/NTA calcium buffering system. Shows effect of reintroducing calcium into the buffering saline, on the rat aortic response to noradrenaline (10^{-6}M) in "continuous" experiments. Responses are expressed as a percentage of the aortic response to noradrenaline (10^{-6}M) in 2mM calcium. Vertical bars represent SEM (n=4). 100% = 1.60 ± 0.28g.

a difference to the SSC pD_2 value obtained. The pD_2 value for the SCC was higher in the "pulsed" experiments than in the "continuous" experiments. pD_2 value for "pulsed"= 4.59 +/- 0.13 and for "continuous"=3.84 +/- 0.06 respectively, ($0.001<P<0.01$, n=3).

3.3.g) Finally, the effect of nifedipine on calcium re-entry was investigated, see figs 39 & 40. 10^{-6}M nifedipine was present throughout the Ca^{2+}-re-entry experiments using both pulsed and continuous Buffers 6-1. In the pulsed experiments, nifedipine had no effect on the ITC and did not inhibit the SSC until 10^{-4}M Ca^{2+} and above and then only for a maximum of 18% (with $3x10^{-4}$M Ca^{2+}, $0.001<P<0.01$, n=4). pD_2= 4.44 +/- 0.31, (pD_2:control= 4.41 +/- 0.11) therefore no significant inhibition was achieved. However, when the buffers were perfused contiuously, the SCC was inhibited by nifedipine, for a maximum of 34% when the calcium concentration was $3x10^{-4}$M ($0.001<P<0.01$, n=4). The pD_2 value was reduced to 3.30 +/- 0.07, from 3.93 +/- 0.10.

3.4) Prazosin

As a final set of experiments on the NA response in vascular smooth muscle, prazosin, an $alpha_1$ antagonist, was used to see if it had any differentiating effect on the ITC or SSC, figs 43 & 44. Doses between 10^{-11}M and 10^{-7}M were used to block the response to 10^{-6}M NA. In both the normal calcium and the calcium-free solutions, the ITC was reduced significantly more than the SSC (e.g. for the dose 10^{-9}M prazosin, 2mM Ca^{2+}: $0.01<P<0.05$ and for Ca^{2+}free:

$0.001<P<0.01$, n=4) until 10^{-7}M prazosin, when both responses were reduced to baseline. Fig 27(b) shows typical traces of the effect of prazosin on noradrenaline's response on the rat aorta in normal (i) and calcium-free (ii) solutions. It can be seen that prazosin preferentially inhibits the fast phase of the response.

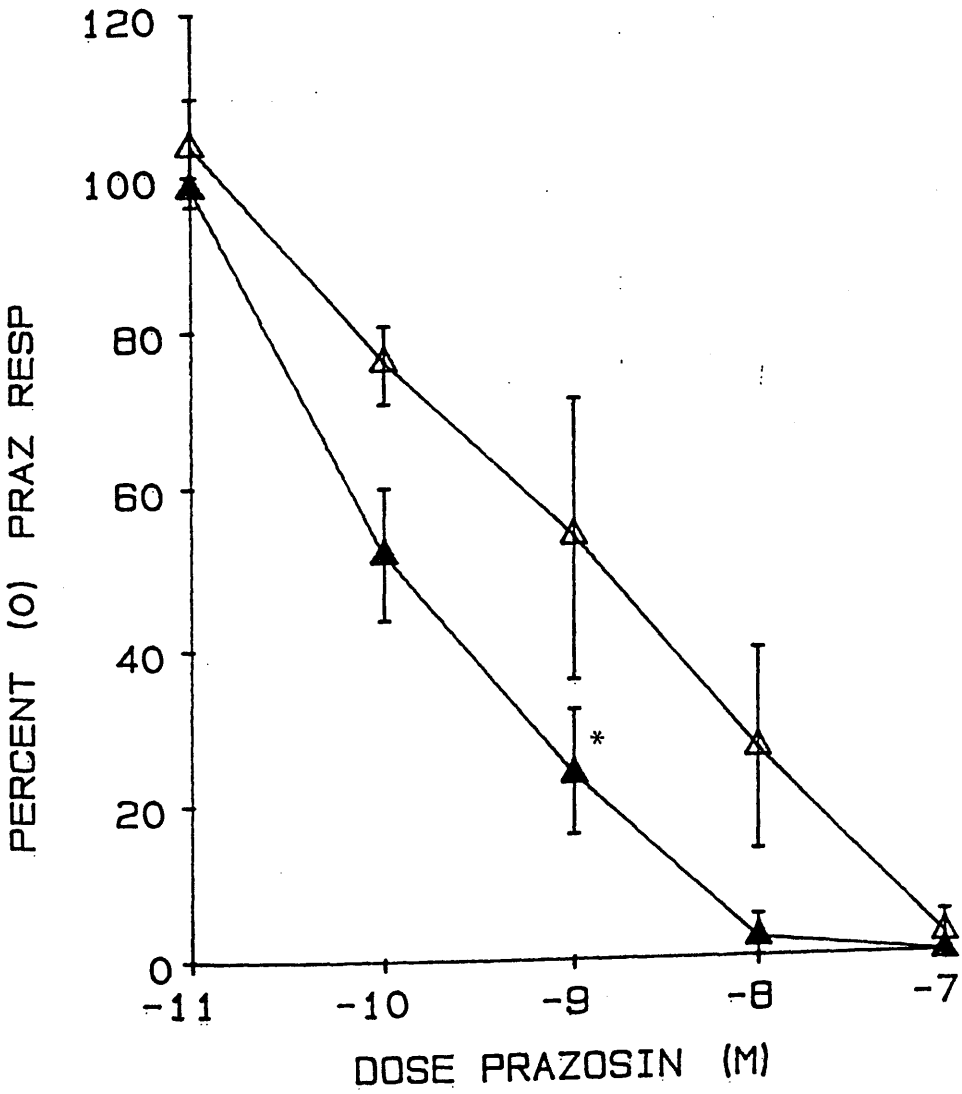


FIG 43 Effect of prazosin (10^{-11}–10^{-7}M) on the rat aortic contractile fast response (closed symbols) and slow response (open symbols) to noradrenaline (10^{-6}M). Tissues are bathed in a 2mM calcium Tris buffer solution. Responses are expressed as a percentage of the aortic response in the absence of prazosin. Statistical significance: * 95% level. Vertical bars represent SEM, (n=4). 100%: Fast = 1.23 ± 0.09g; Slow = 1.67 ± 0.17g.

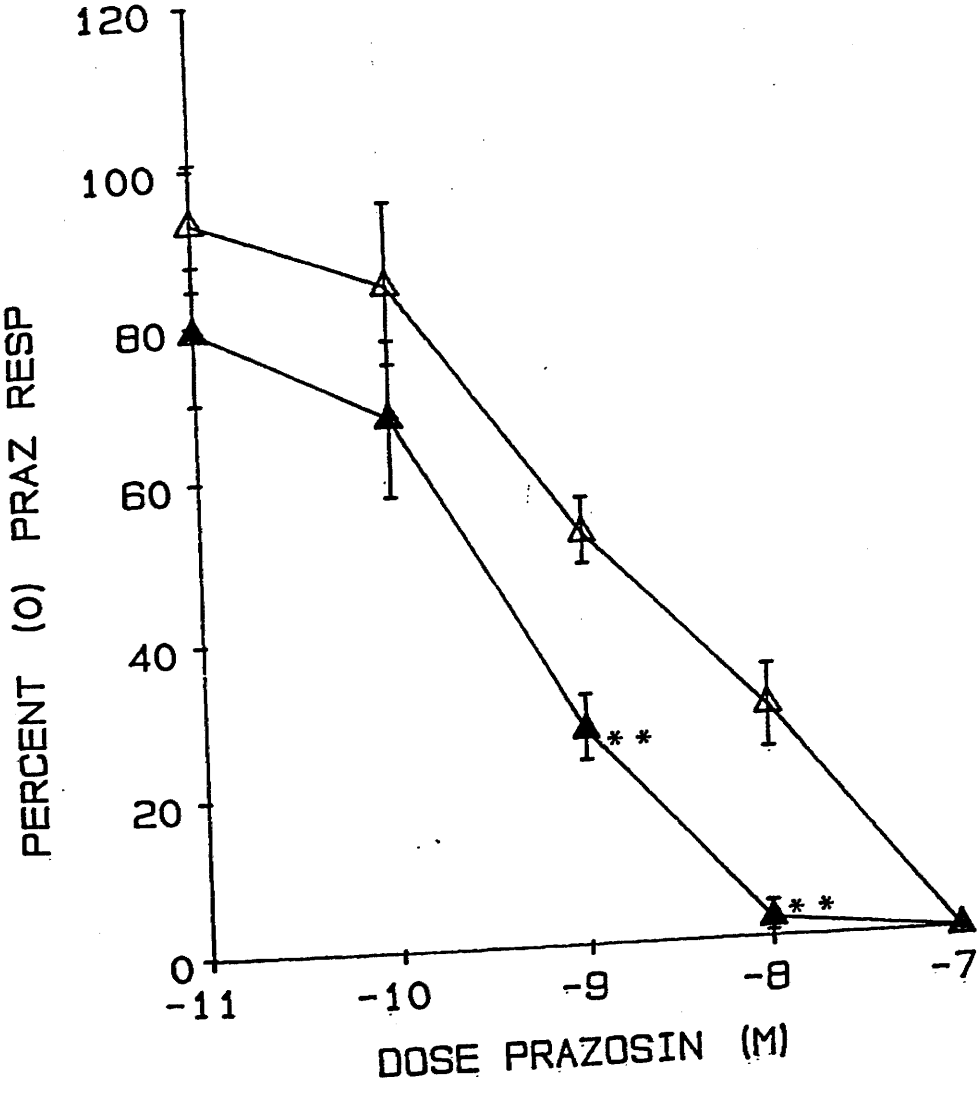


FIG 44 Effect of prazosin (10^{-11}-10^{-7}M) on the rat aortic contractile fast response (closed symbols) and slow response (open symbols) to noradrenaline (10^{-6}). Tissues are bathed in a calcium free solution (10^{-10}M), with 5mM EGTA. Responses are expressed as a percentage of the response in the absence of prazosin. Statistical significance: ** 99% level. Vertical bars represent SEM, (n=4). 100%: Fast = 1.06 ± 0.1g; Slow = 0.87 ± 0.07g.

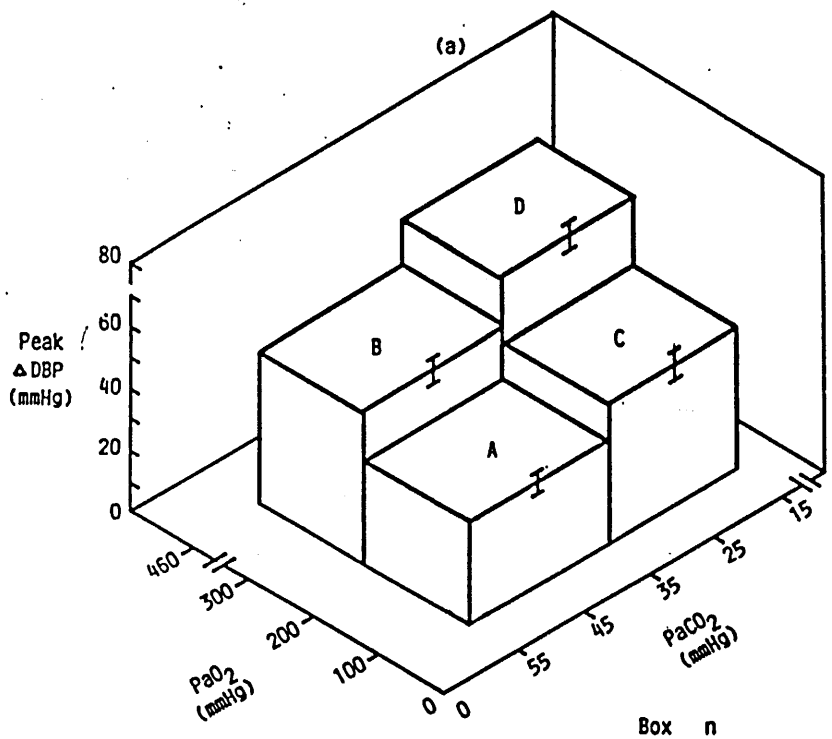
DISCUSSION

THE INFLUENCE OF BLOOD GASES ON $ALPHA_1$ AND $ALPHA_2$ ADRENOCEPTOR-MEDIATED PRESSOR RESPONSES IN THE PITHED RAT

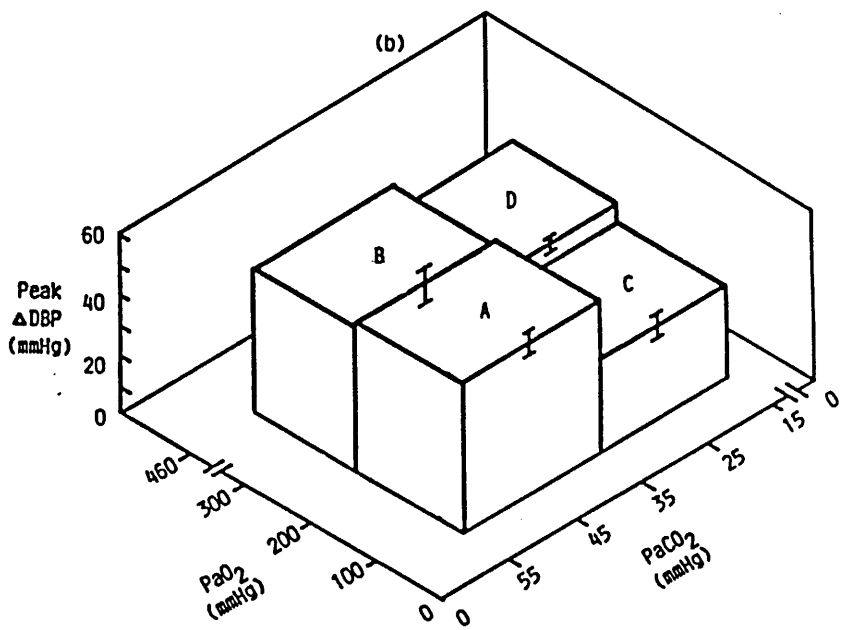
The results indicate distinct effects of blood gases on responses mediated by different alpha-adrenoceptor sub-types. Section one show that, (i) increased $PaCO_2$ decreases responses to phenylephrine whereas those to xylazine are increased; (ii) increased PaO_2 increases responses to phenylephrine whereas those to xylazine are unaffected. Thus the responses to phenylephrine in air-ventilated rats show greater susceptibility to increased CO_2 indicating synergism between hypercapnia and hypoxia in depressing the response. This becomes apparent when all the data is grouped in ranges of $PaCO_2$ and PaO_2 and plotted as a 3-dimensional histogram (fig 45). Within the physiological range of blood gases, responses to phenylephrine are depressed by decreasing O_2 or increasing CO_2. In the case of xylazine there is no reduction as PaO_2 decreases but increasing the $PaCO_2$ produces increases in response in each range of PaO_2.

This corroborates and extends the earlier interpretation from adrenaline's pressor response that respiratory acidosis favours $alpha_2$ and diminishes $alpha_1$-mediated pressor responses in the pithed rat (McGrath et al, 1982). The earlier experiments did not detect the additional effect of PaO_2 since they employed ventilation with 100% O_2 in an attempt to maintain PaO_2 at supra-physiological levels even in the face of considerable reductions in ventilation stroke volume. With air ventilation, the additional depressant

FIG 45 Three dimensional histograms of the mean peak diastolic pressor responses obtained within "ranges" of PaO_2 and $PaCO_2$ to a) phenylephrine (3.0 ug/kg) and b) xylazine (0.1 mg/kg). Vertical bars represent SEM.



Box	n
A	11
B	16
C	30
D	12



Box	n
A	11
B	4
C	13
D	3

effect of low PaO_2 on the response to phenylephrine emerged. With xylazine, any depression by hypoxia was offset since the responses were potentiated by hypercapnia. Consequently, in conditions of hypoxia with hypercapnia, the phenylephrine response was greatly depressed whereas the response to xylazine was still near to its optimum.

When the $PaCO_2$ was elevated by addition of CO_2 to the inspired gas and the PaO_2 was maintained at physiological levels, the effect on the response to xylazine remained the same as with altered ventilation, increasing with $PaCO_2$. This suggests that for xylazine, the effect of CO_2 alone is unequivocal. However, in the case of phenylephrine, when O_2 was kept constant at normal "physiological" levels, there was no longer a clear depression as $PaCO_2$ increased. When $PaCO_2$ was held constant and PaO_2 was varied, in either acidotic or alkalotic conditions, the response to phenylephrine increased as the O_2 levels increased. This suggests that, around physiological levels, PaO_2 was more critical for phenylephrine's response but that at supraphysiological PaO_2, there was a greater influence from $PaCO_2$.

It was clear in preliminary experiments that the rats ventilated with air gave poor reponses to xylazine compared to those found when ventilating with O_2. The reasons for this became apparent when the effects of blood gases were analysed. To keep the preparation "viable", i.e. not hypoxic, on air, large stroke volumes were necessary. This caused alkalosis and, hence, decreased xylazine's response.

If the stroke volume was reduced to achieve "normal" pH and $PaCO_2$, the rats became hypoxic and the preparation was non-viable. The basis of this blood-gas imbalance seems to be a straightforward diffusion problem in the lungs, presumably as a result of the massive sympathetic discharge on pithing, which produces, simultaneously, large increases in cardiac output and peripheral resistance and leads to pulmonary congestion. This diffusion problem can be circumvented by ventilating with pure O_2 or a mixture where air is supplemented with O_2. "Physiological" blood gases can be mimicked by ventilating the rats with 40% O_2, 60%N_2 at 2.5ml/stroke and 60 strokes/min. These would seem to be the optimal conditions for studying physiological adrenergic mechanisms in the pithed rat.

The earlier results, which showed opposite effects of acidosis on $alpha_1$- and $alpha_2$-mediated responses to adrenaline, suggested a simple functional distinction between the two subtypes of alpha-adrenoceptor which might enable them to play distinct physiological roles; $alpha_2$ but not $alpha_1$ could survive a combination of hypoxia and hypercapnia. Hence, $alpha_1$ could be overridden in ischaemic conditions, while $alpha_2$ could survive and become the dominant mediator of vasoconstriction, perhaps, on the arterial side, as a final means of sacrificing certain beds to preserve circulation to vital areas and, on the venous side, to maintain venoconstriction and hence sustain circulating volume. The present study extends this by showing that with short term responses oxygen tension is the

main influence on $alpha_1$ and carbon dioxide/pH on $alpha_2$. These observations provide clues to the excitation-contraction coupling processes involved in the two responses. However, it should be borne in mind that it is only transient, $alpha_1$-responses that are differently affected by blood gases. This applies also to the influence of calcium entry blockers and angiotensin II (Flavahan & McGrath, 1982).

Apart from the attenuation caused by hypoxia, it may also be of physiological relevance that one of the sub-types of receptor is facilitated by "high" PaO_2, since vasoconstriction is appropriate in such circumstances. It is also appropriate that this can be overridden by increased $PaCO_2$. Thus teliological arguments can be found to justify the properties uncovered here.

The main purpose of this investigation was to further examine the findings of McGrath et al (1982) who had found that varying the blood gases changed the susceptibility of adrenaline's pressor response to alpha-blockers. The choice of phenylephrine and xylazine as mimics of adrenaline's $alpha_1$ and $alpha_2$ stimulation is based not only on their receptor selectivities but also on the time-courses of the responses which they initiate. Adrenaline has a biphasic response whose first, rapid, transient phase is mainly $alpha_1$ and whose second, slower-onset, more prolonged phase is mainly $alpha_2$ (Flavahan & McGrath, 1981c). Phenylephrine mimics the first phase and has a poor second phase. Xylazine has a dominant second-phase, which is similar to or

slightly more prolonged than an equivalent response to adrenaline (Flavahan, 1983). We have, therefore, tried to use these agents to mimic adrenaline's alpha$_1$ and alpha$_2$ responses without recourse to antagonists, which is the alternative approach (Flavahan & McGrath, 1981c; McGrath et al, 1982).

The suitability of these agonists is discussed below. Phenylephrine has been widely used to characterise alpha$_1$-adrenoceptors since it was shown to be relatively more potent at these receptors than at alpha$_2$ receptors (Starke et al, 1975; Drew, 1977; Wikberg, 1978 and Bylund & U'Prichard, 1983). (Xylazine on the other hand, consistently demonstrated greater potency for pre- rather than post-junctional alpha adrenoceptors, i.e. alpha$_2$ receptors (Drew, 1977; Wikberg, 1978).) The suitability of using phenylephrine may be put into doubt by the findings of McDonald and McGrath (1980) who were worried that phenylephrine might not be a very suitable "selective" alpha$_1$-adrenoceptor agonist when they showed that it failed to produce as great a maximum as other agonists in the rat vas deferens. When they investigated this further they found that phenylephrine exhibited pre-junctional alpha$_2$ agonism and pre- and post-junctional beta-agonism each of which lay within the range of its alpha$_1$ concentration/response curve. These other effects would of course distort the alpha$_1$ relationship.

Examination of another "selective" alpha$_1$ agonist,

amidephrine, confirmed these suspicions. This agonist had been found to contract rabbit aorta and to produce no inhibition of the indirectly evoked twitch of the rat vas deferens. It displayed no $beta_1$ or $beta_2$ activity and no $alpha_2$-adrenoceptor effects, unlike phenylephrine (Dungan et al., 1965; Buchthal & Jenkinson, 1970; Butler & Jenkinson, 1978 and Flavahan & McGrath, 1981).

It is also known that phenylephrine is very susceptible to low doses of prazosin in the pithed rat, unlike noradrenaline (Drew & Whiting, 1979). This may be due to the fact that phenylephrine has greater affinity for $beta_2$ adrenoceptors. The increased level of $beta_2$ mediated vasodilation will tend to exaggerate the degree of antagonism by reducing the response which remains after partial blockade (McGrath, 1982).

The post-junctional $alpha_2$ activity of xylazine has been demonstated in *in vivo* preparations (Docherty et al., 1979; Docherty & McGrath, 1980) and is resistant to prazosin. No such $alpha_2$ activity is demonstrable in either *in situ* or *in vitro* rat anococcygeus experiments although a prazosin resistant component can be found. McGrath concluded in his 1982 review that these observations may be critical for the study of post-junctional $alpha_2$-adrenoceptors. The $alpha_2$receptor may be more easily demonstrated in blood perfused tissues than in isolated tissues. The conditions under which such receptors can be demonstrated in vitro may provide a key to the cellular mechanisms which they mediate

and hence their physiological role.

Section one shows that $alpha_1$ and $alpha_2$adrenoceptor responses are influenced by blood gases. This may explain quantitative differences between the observations of different groups of workers who are using similar preparations. A slight variation in experimental technique may be the cause of these discrepancies, and should be considered when comparing results. Each receptor sub-type may have its own physiological role in determining vascular resistance and regulating blood flow and this will be dependent on the physiological or pathological state of the system. Despite the demonstration of alkalosis-induced depression of the $alpha_2$-receptor mediated pressor responses, air-respired rats are used frequently to study post-junctional $alpha_2$-receptors (Drew & Whiting, 1979; Timmermans et al., 1979; Gerold & Hausler, 1983).

This study concerns itself with the effects of respiratory induced changes in acid-base balance, however Flavahan achieved qualitatively similar effects from the influence of metabolic or lactic acidosis on alpha-adrenoceptor mediated vascular responses in the dog hind-limb (Flavahan, 1983). It therefore appears likely that localised ischaemic conditions may differentiate between alpha-adrenoceptor sub-type mediated vasoconstriction.

It is already known that the myogenic tone and the actual constrictor or dilator responses of a given blood vessel to a given vasoactive agent depend on the local physical

(distension level and temperature) and the chemical (eg. PO_2 or pH) conditions to which it is exposed, either acutely or chronically (Vanhoutte, 1981; Shepherd, 1967). The amount of blood flowing through a tissue as well as the distribution of this blood within the tissue is dictated by its metabolic demands (Kjellmar, 1965), however, this varies between vessels as resistance vessels are dominated by vasodilator metabolites and capacitance vessels are dominated by nervous supply, (Mellander & Lewis, 1962). Hyperosmolarity and acidosis decrease adrenergic neurotransmission in isolated veins i.e. metabolic changes in the vicinity of the neuroeffector junction are capable of decreasing the output of neurotransmitter to the blood vessels (Verhaeghe, 1978). This could mean that if the sympathetic post-junctional adrenoceptors located in vascular resistance vessels are mainly $alpha_1$, then acidosis or hypoxia could cause a functional sympathectomy.

The similarity of phenylephrine's and xylazine"s response to adrenaline is not shared by all "alpha-adrenoceptor" agonists. For example, when a bolus of an agonist can produce a prolonged $alpha_1$-mediated pressor response, the relationship established here for blood gases no longer holds. This can be demonstrated with amidephrine: this produces an extremely persistent $alpha_1$-mediated pressor response, which is potentiated by respiratory acidosis (Flavahan, 1983). In contrast to the short-lasting $alpha_1$-response to adrenaline or phenylephrine but similar to the second phase of the response to xylazine, this prolonged

alpha$_1$-response is susceptible to calcium entry blockers (Flavahan & McGrath, 1982). This provides further support for the proposal that it is the excitation-contraction coupling rather than the receptor *per se* that is the critical element influenced by blood gases. It also emphasises the need to consider the time course of responses in pithed rats, which never represent an equilibrium but rather show a series of linked events reflecting the rise and fall of the blood concentration of the drug (Docherty & McGrath, 1980c).

It remains possible that increased pH might affect the ionisation and, hence, the distribution of the agonists. This could be the basis of the increase in the prolonged part of the response to adrenaline, amidephrine and xylazine in acidotic conditions. As yet, there is no evidence on which to asses this.

CALCIUM-ENTRY BLOCKADE ON PRESSOR RESPONSES TO ALPHA-AGONISTS IN THE PITHED RAT.

The results discussed in the previous section suggest that the excitation-contraction coupling for the early and late phases of the alpha-adrenergic response, may be differently influenced by outside factors. This is reinforced by the effects of nifedipine on the various alpha agonists used in section 2.

Amidephrine was the first $alpha_1$ agonist used to show that not just $alpha_2$ agonists were affected by calcium-entry blockade (Flavahan & McGrath, 1982). Under the respiratory conditions used to potentiate the secondary component of the response, nifedipine was once again shown to preferentially block the slow phase of the response. However, when amidephrine was infused, there was very little inhibition of the response. Indeed, nifedipine had no effect until 10 minutes into the infusion. A possible explanation of this is that acidosis inhibits calcium influx (Deth & van Breemen, 1974) and as such would be expected to cause attenuation of the nifedipine sensitive response. However this does not agree with the results from most of the other alpha agonists tested.

Table 3 summarises the effect of nifedipine on the alpha agonists. Except for methoxamine (where the bolus is little inhibited) all the $alpha_1$ agonists can be blocked, to some extent by nifedipine. The maximum peak responses of the bolus responses are little affected but it is the secondary

TABLE 3 Summarises the maximum responses and the percentage inhibition, by nifedipine (3ug/kg), of the bolus and infusion responses of the various alpha agonists tested in section 2. Data is expressed as the mean values +/- SEM for the maximums and as the percentage inhibition of the area under the graph (A.U.G.) for the nifedipine inhibition.

Bolus					Infusion		
Drug	Selectivity	Dose µg/kg	Maximum response mmHg	% inhibition by nifedipine A.U.G.	Dose µg/kg/min	Maximum response mmHg	% inhibition by nifedipine A.U.G.
Amidephrine	α_1	10	58 ± 3	26	1	56 ± 8	19
Methoxamine	α_1	40	60 ± 5	31	25	63 ± 5	30
Oxymetazoline	α_1/α_2	0.05	64 ± 3	45	0.5	64 ± 3	34
Noradrenaline	α_1/α_2	1	76 ± 8	46	1	58 ± 5	31
Phenylephrine	α_1	3	75 ± 14	54	3	54 ± 5	59
SGD101/75	α_1	500	63 ± 6	55	250	58 ± 7	22
M7	α_2	10	57 ± 2	57	6	69 ± 5	52
BHT 933	α_2	250	55 ± 4	66	100	47 ± 2	50
Cirazoline	α_1	1	70 ± 5	68	1	57 ± 6	58
Xylazine	α_2	100	51 ± 4	77	250	71 ± 3	56

phase of the responses which are inhibited. The $alpha_1$ agonist bolus responses are reduced between 26%-68% (measured as area under the graph, a.u.g.) whereas the infusion responses are reduced between 19%-59% (a.u.g.).

Both the initial, maximum pressor response and the slower, secondary component of the $alpha_2$ agonists tested are inhibited by nifedipine. The infusion response to these agonists is also inhibited. The bolus responses are reduced between 57%-77% and the infusion responses are reduced between 52%-58%. The ability of nifedipine to block an alpha pressor response does not therefore appear to depend on whether the drug is classified as acting at $alpha_1$ or $alpha_2$ receptors but on the duration of the response produced. Nifedipine tends to block slow prolonged responses better than the rapid initial responses produced by some drugs. It would appear that the prolonged phase is associated with the nifedipine-sensitive calcium channel observed in vitro in isolated blood vessels and which allows the entry of extracellular calcium (Godfraind et al., 1982).

The initial fast component is more difficult to classify. As it is transient it can in some ways be compared to the initial, transient component (ITC) of isolated smooth muscle. This ITC is resistant to Ca^{2+}-entry blockers and to reduction of $[Ca^{2+}]_o$ and is thought to be due to the release of bound intracellular calcium (see next section). It is not possible to test this, however, with the whole animal preparation. Even a moderate reduction in calcium concentration would stop the heart and sufficient reduction

of $[Ca^{2+}]_o$ even in a perfused organ would be extremely difficult to achieve. The role of intracellular calcium release cannot be completely ruled out but, since maintained responses to continuous infusion of alpha agonists can be produced in the presence of nifedipine it is possible that a nifedipine-resistant Ca^{2+}-entry is involved (see McGrath, 1985). Since it is possible to avoid the initial fast component by injecting the agonist more slowly i.e infusing it (see fig 8), it can be assumed that this response is due to the initial high concentration of the agonist, which contacts the blood vessels as the bolus makes its "first pass" through the ciculation (Docherty & McGrath, 1980).

The difference in the time course of the $alpha_1$ and the $alpha_2$ bolus responses appears to be mainly that $alpha_2$ agonists have a slower onset of action and a longer lasting second phase than $alpha_1$-agonists (excepting amidephrine). However, there is a much greater difference between the time courses of their infusion responses. The $alpha_1$ agonists appear unable to produce a maintained plateau during the infusion time. Phenylephrine produces a maximum response which then declines, whereas oxymetazoline, cirazoline and methoxamine never reach a maximum during the 20 minutes infusion. The $alpha_2$ agonists on the other hand, show a very consistent pattern in their infusion responses. All rapidly achieve a plateau which is maintained throughout the administration of the drug. There is obviously a difference in the way $alpha_1$ and $alpha_2$ agonists produce their infusion (and bolus) responses but from where does this difference

stem?

The results so far do not appear to support the hypothesis that $alpha_1$- and $alpha_2$-adrenoceptor mediated responses involve different activation systems. The effects of factors which influence excitation-contraction coupling correlate with the ability of the agonist to produce the two phases of the response rather than with the alpha-adrenoceptor subtype involved. Thus the responses of SGD 101/75, a drug difficult to classify under the $alpha_1/alpha_2$ split, can be better understood and categorised. Timmermans et al (1983), however, believe that drugs such as SGD 101/75 stimulate a subtype of the $alpha_1$-adrenoceptor, one which initiates vasoconstriction dependant upon extracellular calcium.

It is interesting to note that the other $alpha_1$ agonist which is currently accepted as being susceptible to calcium-entry blockade, i.e. amidephrine, shows a similarity in pattern to the SGD 101/75 bolus response. Both these drugs produce a rapid maximum pressor response and a secondary component which has a pronounced "hump". The overall response produced by both drugs is longer lasting than the other $alpha_1$ agonists. It is the "hump" of the response which is most inhibited by nifedipine; however their infusion responses are very little affected by calcium-entry blockade.

If it is not receptor subtype which confers the ability to produce these different types of responses - what does? The

fact that many of the synthetic alpha$_2$-adrenoceptor agonists were shown to be partial agonists, led Cauvin et al.,(1983) to suggest that sensitivity to calcium entry blockade was related to the degree of activation induced by the agonist. McGrath (1985) found that xylazine and SGD 101/75 were relatively more susceptible to nifedipine inhibition than were phenylephrine, amidephrine or methoxamine in the rat anococcygeus muscle. He proposed agonist "efficacy" as an explanation of this i.e. agonists with low efficacy produce the prolonged response which is nifedipine sensitive. If this were correct, then there should exist certain tissues where the ability to respond to alpha$_1$ or alpha$_2$ agonists will depend on their receptor reserve and efficiency of coupling of the agonists with the different receptor subtypes. This possibility is encouraged as the absolute potency of alpha$_1$ and alpha$_2$ agonists does vary enormously between preparations.

The view of McGrath (1985) is supported by the findings of Ruffolo et al. (1984). He suggested a possible relationship between receptor reserve and the differential antagonism of alpha$_1$- and alpha$_2$-adrenoceptor mediated pressor responses by the calcium channel antagonist diltiazem, in the pithed rat. He found that pressor responses of agonists with high intrinsic activities (large receptor reserve) were resistant to antagonism by diltiazem, whereas the alpha$_1$ adrenoceptor-mediated pressor responses of partial agonists with low intrinsic activities (no receptor reserve) were highly sensitive to antagonism by diltiazem. This suggests that

the apparent resistance of the $alpha_1$-adrenoceptor mediated pressor response to antagonism by the calcium channel blocking agents may result, at least in part, from a large $alpha_1$-adrenoceptor reserve that serves to "buffer" $alpha_1$-adrenoceptor mediated pressor responses from inhibition by noncompetitive antagonists, such as the calcium channel antagonists.

Unfortunately the above observations were based on the assumption that the $alpha_1$ pressor response is resistant to calcium entry block. Ruffolo used cirazoline as an example of an $alpha_1$ agonist unaffected by calcium entry block. However, table 3 shows that cirazoline is in fact one of the best blocked $alpha_1$ agonists (68% a.u.g.). Ruffolo's observations were probably based on measuring the peak response, which is less affected than the overall response. In support of the findings summarised in table 3 are papers by Alabaster and Solca (1985) and Janssens and Verhaeghe (1984), see below.

Interpretation of data from studies in vivo can be complicated by the fact that the blood pressure response to injected agonists is the net result of direct and indirect effects on both the heart and vasculature. To avoid this, propranolol (1mg/kg) was administered before any drugs which might affect the heart rate. Alabaster and Solca (1985) found that pressor responses to phenylephrine, alpha-methyl-noradrenaline and angiotensin II were all antagonised by nifedipine in the anaesthetised cat after administration of propranol and atropine. (The propranolol and atropine were

given to prevent agonist-induced changes in cardiac output by reflex activation or direct effect.)

According to Cauvin et al, (1983) the $alpha_1$ agonists in table 3 which are full agonists would not be susceptible to calcium-entry blockade but this is obviously not the case. The findings of Janssens and Verhaeghe, (1984), increase the evidence that differentiation of responses, in vascular smooth muscle, by postreceptor mechanisms relating to calcium utilization, does not correlate with an $alpha_1/alpha_2$ classification. They found that in the dog saphenous vein, both $alpha_1$- and $alpha_2$-adrenoceptor activation induced an influx of calcium ions and a release of intracellular calcium.

The observation that nifedipine reduces the response to infused agonists has also been made in man. Reid et al. (1983) showed that systemic pressure increases to infusions of noradrenaline and of angiotensin II could be antagonised by nifedipine. It becomes obvious therefore that some form of calcium-entry is involved in the activation of both receptor subtypes. It could be proposed that there is in fact only one receptor type involved in alpha-adrenergic pressor responses. This single receptor type may then change its main excitation-contraction coupling process when presented with different agonists and/or agonist concentrations for reasons as yet not understood. However, the extensive work done with antagonists such as prazosin and rauwolscine still suggests that there are at least two distinct types of postsynaptic alpha-adrenoceptor (for a review see McGrath, 1982).

The changes with nifedipine are not specific to alpha-adrenoceptors, and in the pithed rat, nifedipine must be exerting other effects in addition to the reduction of the alpha-adrenergic response. Calcium-entry blockers will interfere with any process which involves the transport of calcium into the cell via the slow channels. Calcium-antagonists, such as nifedipine, are also very effective at blocking excitation-secretion coupling and although of little importance in the pithed animal, they can for example, block the release of oxytocin and vasopressin from the depolarised neurohypophysis or the release of insulin from excited B-cells in the Islets of Langerhans (Devis et al., 1975; Matthews & Sakamoto, 1975). It must also be remembered that the classic study involving calcium-entry blockers was carried out on heart muscle where their antagonism of beta-adrenoceptor activity was studied. Nifedipine does not block beta receptors on the heart (Fleckenstein, 1977; Kroneberg, 1973; Schumann et al., 1975), but does antagonise the action of beta agonists by interfering directly with the transmembrane supply of calcium. Thus calcium antagonists are used clinically as antianginal, antiarrhythmic, antihypertensive or cardioprotective drugs.

How calcium antagonists ultimately produce these benefits is probably a mixture of the three following therapeutically important factors; a) they reduce myocardial energy expenditure and therefore oxygen demand, b) they indirectly decrease cardiac oxygen requirement by facilitating heart work at a reduced level of arterial blood pressure and c)

they improve myocardial oxygen supply due to vasodilator or spasmolytic effects particularly on extramural vessels. Nifedipine is therefore a most specific and powerful calcium-antagonist (Fleckenstein et al., 1972) with extremely important clinical applications, and is not just a useful pharmacological tool, as employed in this study.

An interesting observation in this series of experiments was the apparent decline in the pressor response to infusions of phenylephrine and noradrenaline: both these drugs showed a marked reduction in response after 10 minutes of infusion. Usually successive applications of the same dose of a powerful agonist produce constant responses (within the limits of biological variation) provided that modest concentrations are used, the exposure time is not too prolonged and a sufficiently long rest period is allowed to enable the tissue to recover from the response. However, with some drugs the successive responses become smaller i.e tachyphylaxis occurs. It has been known for many years that repeated i.v. doses of certain sympathomimetic amines elicit smaller and smaller cardiovascular responses (Chen & Meek, 1926), but noradrenaline has been found to produce little or no tachyphylaxis (Day & Rand, 1963).

This waning of the infusion response does not appear to be due to any general exhaustion of the contractile response as increased infusion rate and injection of other alpha agonists are still able to produce a pressor response. So why is the response declining when the drug is still being

administered?

The decline in pressor response to infusions of phenylephrine and noradrenaline was similar to an earlier observation with adrenaline (Gillespie & Muir, 1966). Each response showed a marked decline after 10 minutes of infusion. The decline in response, for noradrenaline at least, is not due to the removal of the drug from the blood as the levels of noradrenaline present in the plasma, before, during and after infusion of the drug were measured using an HPLC system. The results were interesting for several reasons: 1) the peak pressor effect for that dose of noradrenaline was achieved after 5 minutes when the concentration of noradrenaline in the arterial plasma had not achieved its maximum (N.B. the pressor response to the infusion itself was sub-maximal). 2) when the pressor effect was waning the concentration of noradrenaline was still rising in the arterial blood, and 3) the concentration of noradrenaline in the venous plasma showed very little change from control values throughout the infusion. 1) and 2) show that there is some form of tachyphylaxis occuring and 3) shows that noradrenaline does not pass through the systemic vascular beds to circulate.

A large proportion of the noradrenaline infused into the jugular vein seems to have been lost in its passage through the heart and pulmonary bed to the carotid sampling site. From the Fick Principle, with this rate of infusion and the arterial levels found, cardiac output would need to be approximately 100 to 150 ml/min if no noradrenaline was

lost. Since the best estimate of this in pithed rats of this size are approximately 15 to 25 ml/min (Kaufman & Vollmer, 1985; Gerold & Haeusler, 1983), this suggests that at least 75% of intravenously infused noradrenaline is lost before it reaches the arterial side of the systemic circulation.

This rapid loss of noradrenaline, first 75% in the lungs and then the remainder in the systemic beds, is in marked contrast to clonidine, which is present in plasma up to 40 minutes after bolus injection of a sub-maximal pressor dose (Docherty & McGrath, 1980a). High levels of clonidine were found in the venous blood, indicating that the drug was re-circulating. The pressor response to clonidine lasts no longer than those to many of the agonists tested here so it is likely that many of them recirculate. Conversely, the short lived alpha-agonists may all experience a strong "first pass" effect and have very low venous concentrations. This implies that only the longer lasting alpha-agonists could exert an alpha-mediated venomotor action, which by raising venous return could increase cardiac output. Such an action of endogenous angiotensin II has already been demonstrated in pithed rats (Kaufman & Vollmer, 1985).

This may mean that long lived, recirculating agonists will raise the blood pressure by a combination of resistance (pre-capillary tone) and cardiac output (post-capillary tone). Since blood pressure is proportional to both then the actions at the two sites will be geometric rather than

additive and a small change in each will produce a disproportionately large change in blood pressure. This, rather than any real difference in the excitation process may be the reason for prolonged responses being more susceptible than responses due to transient acute increases in resistance. It would also be likely that only re-circulating drugs would build up high concentrations in the tissues. This may be another contributing factor to the difference in the duration of response produced. Several further factors may lead to individual profiles of susceptibility to blockade, e.g.a) any differences in distribution of alpha-adrenoceptor sub-types between pre- and post-capillary vessels, b) differences in the agonist potency series at these different sites even at the same receptor (such as can arise when receptor response varies) or c)different sensitivity of the excitation-contraction coupling processes in the different vessels to drugs producing "physiological" antagonism, e.g. nifedipine.

It is of some interest that nifedipine does not produce a maintained lowering of the blood pressure on its own, although it did attenuate responses to the agonists. Since vascular tone in the pithed rat (mainly venous) seems to be maintained by a high circulating level of angiotensin II (Kaufman & Vollmer, 1985), and since the pressor response to infusion of angiotensin II is blocked by the dose of nifedipine used in this study (Grant & McGrath, personal communication), it might be expected that nifedipine would reduce pressure and that consequently any vasopressor response would be reduced. Since it did not, analysis of

the results is simplified at least in this respect but it may be concluded that the effects of the endogenous angiotensin II are relatively resistant to nifedipine.

It would be useful to examine the levels of phenylephrine in the plasma during the infusion to see if they follow the same pattern as noradrenaline. Williams et al. (1984) have now discovered that phenylephrine is an endogenous substance and can be found in various animal and human urine samples and tissues. It is interesting that it is only the three natural phenylethanolamines (adrenaline, noradrenaline and phenylephrine), which when infused, displayed the decline in pressor response. Perhaps it is only endogenous sustances which achieve a true equilibrium concentration in the blood. Once this equilibrium has been attained, further drug reaching the receptors at a constant rate, only serves to modify the response. This modification takes the form of a decline in response presumably as some sort of protection against overworking the tissue. Presynaptic receptors modify the release of neuronally released noradrenaline but are of no use in regulating the amount of circulating agonist activating the postsynaptic receptor. The above hypothesis may indicate that there is some form of postsynaptic control over the response produced by circulating agonists. Whatever the receptor system that is involved, it is interesting that the non-physiological agonists do not replicate the phenomenon.

Perhaps the reason that increasing the infusion rate is

still able to increase the pressor response, is that it is the change in rate of the drug reaching the receptor, rather than the absolute concentration of the drug itself, which affects the strength of response. A bolus injection of another drug type (one which is not able to activate this postsynaptic control) would still be able to produce a response, as was observed with methoxamine, cirazoline, M7 and angiotensin II (see section 2.2.b.).

There has been much work examining the phenomenon that after long-term infusion of noradrenaline in conscious animals the blood pressure falls below baseline when the infusion is terminated (Casals-Stenzel et al., 1982; Blacket et al., 1950). Unfortunately, in this study the blood pressure levels after the infusion of noradrenaline was stopped, were not recorded but it would be interesting to see if this still occurred. It is thought that the decline in baseline levels may be linked to the release of prostaglandins by the circulating noradrenaline (Hedqvist, 1977), or that the alpha-receptors become saturated or reduced in number (Burn & Rand, 1959; Romero et al., 1975). The decline in infusion response and the decline in baseline levels after infusion may be linked in some mechanistic way. If this were so, then although the alpha-adrenergic receptors would not be reduced in number during the short 20 minute infusion used here, they may be becoming saturated.

Ca^{2+}-DEPENDENCE OF NORADRENALINE-INDUCED CONTRACTION IN R AORTIC SMOOTH MUSCLE

In a wide variety of excitable cells the transmembrane exchange of monovalent cations Na and K can be considered to be the substantial basis of bioelectric membrane activity, whereas Ca^{2+} ions are required as mediators when intracellular reactions such as muscular contraction, glandular secretion or liberation of transmitter substances are initiated (Ebashi & Endo, 1968; Rubin, 1970). Calcium ions can exert this messanger function either by entering the cell across the cell membrane or by being released from intracellularly located endoplasmic stores.

One way by which calcium may enter the cell is via the potential operated channel (POC). It opens permitting the inflow of extracellular calcium when the transmembrane potential decreases and it closes when the potential increases. The membrane potential of vascular smooth muscle is depolarised by either action potentials or graded depolarisation. This channel is the one most readily blocked by calcium channel blockers (Andersson et al., 1984).

However, calcium may also enter the cell by a channel that is not influenced by membrane potentials. These channels are opened by specific agonists' occupancy of receptors, hence they are called receptor operated channels (ROC). Noradrenaline, which was used in this study, operates such a channel and it was Andersson and co-workers (1984) who demonstrated that when vascular smooth muscle is treated

with nifedipine the contractile response to noradrenaline is still large. Godfraind (1981) found that this residual response in rat aorta after exposure to the calcium-entry blocker flunarizine was 50% of the maximum contraction. This value was similar to the response evoked in Ca^{2+}-free solution. In this study, on average, 34% of the response remained when the response was evoked in a calcium-free solution. Godfraind concluded from calcium efflux studies that this intracellularly released calcium is responsible for the sustained contraction evoked by noradrenaline when the noradrenaline-dependent calcium influx is blocked. It was suggested that flunarizine does not alter the refilling of calcium stores sensitive to noradrenaline. The calcium stores could be replenished by a Ca^{2+}-Ca^{2+} exchange occurring through "leak channels" (Flaim, 1982).

A third source of calcium involved in mediating vascular muscular contraction are the internally bound stores. A large amount of calcium is bound to or stored in the sarcoplasmic reticulum, the plasma membrane and the mitochondria. Calcium from the first two sites plays a role in the contractile response to most agonists (Saida & van Breemen, 1984; Loutzenhiser & van Breemen, 1983). These stores were thought to be responsible for the initial rapid response whereas the maintained, slow component of the response results from activator calcium arising from the external source. The aorta derives a large portion of its activator calcium from internal sources whereas the activator calcium for small mesenteric resistance vessels arises largely from external sources (Cauvin et al., 1984).

Godfraind and Kaba (1969) observed that vascular smooth muscle bathed in a Ca^{2+}-free solution was still contracted by noradrenaline whereas K^{+} depolarisation was no longer able to activate the contraction. These results indicated that alpha-adrenergic stimulation acted on two calcium pools (one extracellular and the other intracellular). It also suggested that depolarisation of the membrane is not required for alpha adrenegic activation of vascular smooth muscle. This was confirmed when Casteels (1980) showed that noradrenaline evokes a contraction without changes in membrane potential.

The contractile response to noradrenaline in the rat aorta is biphasic and it was Godfraind and Kaba (1972), who first suggested that the initial fast component was mediated by the release of an intracellular source of activator calcium and the slow component by entry of extracellular calcium. The results in section 3 do indeed show that nifedipine, the calcium-entry blocker, differentiates between the fast and slow components. However the results are not as clear cut as they had originally suggested.

When the rat aorta is stimulated by noradrenaline, the slow component is not totally abolished when the tissues are bathed in "calcium-free" solutions i.e. 10^{-10}M calcium. On average 35% +/- 4 (n=4) of the response remains, fig 1. This portion of the slow response may not be the result of calcium-entry and would therefore be nifedipine insensitive.

However it could be presumed that the other 65% would be susceptible to calcium entry block. This was not the case. When the tissues were perfused with "normal" calcium solutions (2mM) not only did nifedipine not have any inhibitory effect but the responses to noradrenaline were seen to increase as the concentration of nifedipine increased. Nifedipine appeared to be having a potentiating effect on the NA response (the tissues were also tested with repeated concentrations of alcohol, the nifedipine vehicle, to ensure it was not this which was causing the increase; it had no such effect).

When the calcium was chelated from the solution using 5mM EGTA, nifedipine had no effect, possibly because this portion of the SSC remaining is not due to calcium entry. The only concentration of nifedipine tested which had an effect was 10^{-5}M, and it inhibited the ITC and the SSC equally. It could be concluded that this concentration of nifedipine must be having other effects, rather than calcium-entry block, perhaps alpha receptor blockade.

In a reduced calcium solution ($(Ca^{2+})_{free}=5.2x10^{-8}$M), the "expected" results were achieved. Nifedipine blocked the SSC significantly more than it did the ITC. Somehow, reducing the free calcium in the solution had rendered the aorta sensitive to calcium-entry blockade. Perhaps the level of extra-cellular calcium is a very important factor in deciding whether nifedipine has an affect or not. If there is an excess of calcium, nifedipine is ineffective until it reaches very high concentrations (unspecific

action). That is, enough calcium must still be entering the cells to elicit a response, presumably by channels other than the ones nifedipine blocks. These channels may be nifedipine insensitive or they may be channels which are susceptible to nifedipine but which are part of the proportion not blocked at a particular instant. On the other hand, if the extra-cellular calcium is reduced, the levels are no longer high enough to "swamp" the action of nifedipine and the SSC is reduced.

A possible explanation as to why no blockade of the noradrenaline response is seen in normal calcium solutions could be because of the concentration of noradrenaline used (10^{-6}M). McGrath (1985) found that high concentrations of agonist, those producing more than 80% of the maximum response in the rat anococcygeus muscle, had no nifedipine-sensitive component. In the aorta, 10^{-6}M noradrenaline produces a SSC of 86% +/- 6 of maximum. This, does not explain why the nifedipine-sensitive component reappears in low calcium solutions or why no blockade of the response was achieved when a lower concentration of noradrenaline was used, fig 24. However, increasing the concentration of nifedipine does produce an inhibition but is this totally as a result of calcium-entry blockade?

Another unexplained observation is the effect on calcium entry blockade, of increasing the concentration of EGTA in the normal calcium solution, fig 28. When the concentration of free-calcium is kept at 2mM, and the concentration of EGTA

is increased from zero to 5mM, nifedipine no longer causes an increase in the noradrenaline response. As the concentration of calcium is maintained and there is no other alteration to the physiological solution, it must be the addition of EGTA which is producing this effect. The high concentration of EGTA may be having a poisonous effect on the tissues. However no such poisonous effect is seen if the EGTA concentration is similarly increased in the low calcium solution, fig 29.

When cadmium was used as an example of an inorganic calcium-entry blocker, it reduced both the fast and slow components equally well in the normal and calcium-free solutions. When it was tested in a reduced calcium solution i.e. one containing only calcium contaminants (20uM), the SSC appeared more blocked than the ITC until both were abolished at a concentration of $5x10^{-4}$M cadmium. This could mean that calcium-entry is involved in both components but more so in the SSC. As mentioned in the results section, pH could be playing a part in the inhibition of the responses. As more cadmium is added, it chelates with the EGTA in the calcium-free solutions; the pH falls and poisons the tissue response.

Dantrolene sodium, is reputed to be an intra-cellular calcium blocker. It impairs the release of Ca^{2+} from the sarcoplasmic reticulum, the primary step being inhibition of the release of trigger calcium (Pinder et al., 1977). As such it should preferentially block the ITC. However, both the fast and slow components were reduced in a

concentration-dependent manner, in the normal calcium solution. In the calcium-free solution no such reduction was seen. This indicates that the dantrolene sodium is inhibiting calcium-entry in the normal calcium solutions, and as a result, is reducing both components of the response (as found with the cadmium). There does not appear to be any blockade of the intracellular calcium by dantrolene so perhaps the role of intracellular calcium could be better investigated using another blocker, e.g. ryanodine or PRMDI.

Calcium-entry blockers inhibit contractile responses to $alpha_1$ adrenoceptor agonists in venous smooth muscle as De Mey and Vanhoutte (1981) found in the dog saphenous vein. Nifedipine also blocks NA-induced contractions in the rat portal veins which appears to have only $alpha_1$ adrenoceptors (Vanhoutte & Rimele, 1982). From the wealth of evidence, it appears that, depending on the blood vessel (De Mey & Vanhoutte, 1981; Vanhoutte & Rimele, 1982) or the species (Van Meel et al., 1981a; Van Meel et al., 1981b; Jetley & Weston, 1980; Van Breemen et al, 1981) studied equally convincing data can be obtained to favour the interpretation that either $alpha_1$- or $alpha_2$- adrenoceptors modulate the entry of extracellular calcium in vascular smooth muscle.

As the investigation of the involvement of calcium in the rat aorta response to noradrenaline using calcium-entry blockers had produced puzzling and inconclusive results, the more direct approach of altering the levels of calcium in the solution was then tried. Using low levels of EGTA (1mM)

and re-adding calcium to the solutions did not produce sufficient buffering over the high levels of calcium. As the total (Ca^{2+}) was gradually increased from $0.64x10^{-3}M$ to $1.28x10^{-3}M$, there was a sudden increase in the amount of free calcium in the solution from $10^{-7}M$ to $10^{-4}M$, which increased the SSC from baseline (calcium-free) levels to a full response. It did show that the ITC relied less on external calcium for its activation than did the SSC.

The threshold level of calcium for the SSC appeared to lie between $10^{-4}M$ and $10^{-3}M$, and as calcium contaminants in a solution measure roughly $2x10^{-5}M$ (Miller & Smith, 1984) it was thought that the calcium should be added step-wise without a buffer present. This proved more successful as a more gradual increase in the SSC could be produced when the "pulsed" method was used. This "pulsed" method of introducing the tissues to the different levels of calcium is described in the Materials and Methods section. It involves perfusing the tissues for half an hour in 2mM Ca^{2+} buffer solution before exposing them to the reduced calcium solutions for 10 mins. After 10 minutes the noradrenaline is added and the tissues allowed to respond for approximately 5 minutes before being returned to the normal (2mM Ca^{2+}) solution. After half an hour the whole procedure is repeated with a different reduced calcium solution. This method produces both a fast and a slow response from the tissues.

The other method of exposing the tissues to different levels of calcium, is the "continous" method. This way the

tissues are not returned to a normal calcium solution between the low calcium buffers. In other words, they are continuously exposed to a reduced level of calcium as one buffer is replaced by another until the 2mM level is reached.

When the "continuous" method was used, the increase in the SSC was not so gradual. This is possibly reflecting a difference in the actual amount of calcium present immediately outside the cell. In the "continuous" method, the tissue is sitting in low levels of calcium for a longer time and probably reaches an equilibrium situation with them. The levels of calcium which were calculated probably reflect more accurately the equilibrium situation i.e. the extra-cellular concentrations in the "continuous" experiments. The calcium levels in the "pulsed" tissues, on the other hand, are unlikely to have reached an equilibrium within their short exposure time. This may mean that the actual levels of calcium surrounding the "pulsed" tissues are higher than those calculated and therefore could explain why the pD_2 values appear different for the pulsed and continuous experiments.

Despite this inaccuracy, the "pulsed" experiments still provide a useful tool for examining the fast and slow components of the aorta's response under different conditions. If the tissues are bathed in a calcium-free solution for longer than the 10 minutes used throughout section 3, for example 30 minutes, no fast response is seen

- implying once again that calcium-entry is involved in the ITC. A recent paper by Leijten et al. (1985), using the rabbit aorta and mesenteric artery, states that there are two processes responsible for the ITC. One process is the release of calcium from a small labile store on the inner plasmalemma. This calcium acts as a trigger and releases the activator calcium from the sarcoplasmic reticulum. The other process involved was found to happen simultaneously. Calcium from an extracellularly bound store is released and enters the cell through receptor linked channels.

When the revised buffering system was used, using the EGTA/NTA mix, a gradual increase in the SSC was achieved for both the pulsed and continuous experiments, while the ITC remained relatively unaffected. This system was obviously an improvement on any tried so far. Previously these buffers had been made up in bicarbonate solutions and used on the anococcygeus muscle (McGrath et al. 1984). It was this previous research and a paper by Altura et al. (1982) about the adverse effects on contractility of Tris buffer on arterial smooth muscle which prompted the repeating of these experiments in bicarbonate solution. The continuous experiments gave exactly the same results. Surprisingly the ITC in the bicarbonate pulsed tissues was significantly more reduced by the low calcium solutions than in the Tris pulsed tissues (e.g. for buffer 6: $0.001>P>0.01$, $n=4$).

Nifedipine once again gave mixed results. It inhibited the SSC in the continuous experiments but not in the pulsed. This may be because as proposed earlier, the level of

external calcium is critical to whether of not nifedipine exerts an effect. Perhaps in the pulsed experiments the levels of calcium remain too high immediately outside the cell for nifedipine to work. Nifedipine may show a better inhibition if the tissues are pulsed for longer with the reduced calcium solutions. This hypothesis is not in agreement however with the in vivo results. In the pithed rat, nifedipine was able to inhibit the pressor response to perfused noradrenaline where the calcium levels had not been (intentionally) tampered with. This may indicate that nifedipine does not exert its vasodilator effect by the mechanisms demonstrated on major blood vessels such as the aorta but rather exerts its effect by quite different processes found in the smaller blood vessels such as the arterioles.

Another explanation for the apparent difference in the inhibition of the pulsed and continuous experiments by nifedipine may be simply due to time course. In the pulsed experiments the noradrenaline is only allowed to react with the tissue for approximately 5 minutes. Nifedipine is more effective at inhibiting prolonged responses so perhaps no significant inhibition can be detected in this short time. In the continuous experiments noradrenaline is in contact with the tissue for much longer. The result is a greatly prolonged response and now nifedipine can be seen to have an effect.

The endothelium was not intentionally removed from these

tissues so, presumably it was present and intact during all the experiments (although it was not checked histologically). Furchgott and Zawadski dicovered in 1980 that if an isolated artery is pre-contracted by eg. noradrenaline, then the relaxation caused by acetylcholine is strictly dependent on the presence of endothelium cells. This was used as a quick test, pre-experimentally, to check that the endothelial cells were intact in similar work subsequently carried out by the author but unconnected with this thesis. This may hopefully be taken in retrospect to confirm the presence of endothelium during this series of experiments, since the methods used were similar.

The presence of endothelium was thought at first to facilitate the contractile response to noradrenaline in most blood vessels (De Mey & Vanhoutte, 1982) but it is now known that if the endothelial cells are removed carefully, there is no loss of sensitivity of the preparation to the contracting action of noradrenaline (Furchgott, 1983).

However external calcium levels do have an effect on the endothelium dependent relaxation of the aorta to various stimuli. Furchgott et al. (1981), hypothesised that perhaps an increase of calcium ions in the region of some key Ca^{2+}-activated enzyme (perhaps a phospholipase) might be an early step in the sequence of reactions mediating the release of EDRF by acetylcholine. This theory was based on work with the calcium ionophore A23187. This drug is an antibiotic which can form a lipophilic complex with divalent cations,

such as Ca^{2+}, and function as an ionophore. It produces rapid equilibration of the Ca^{2+} gradients by facilitating diffusion across the membrane (Scarpa et al., 1972).

Furchgott and co-workers found that A23187 was 10 - 30 times more potent than acetylcholine in producing relaxation of pre-contracted rabbit aorta and that its mode of action was also dependent on endothelial cells (Zawadski et al., 1980; Furchgott, 1981; Furchgott et al., 1983). Indeed they found so many similarities in what inhibited both A23187- and acetylcholine-induced relaxation that they believe both these drugs release the same EDRF.

The only drug found to differentiate between A23187 and acetylcholine in this system is quinacrine which can inhibit the action of acetylcholine but not A23187 (Furchgott & Zawadski, 1980; Singer & Peach, 1983). It was postulated that quinacrine may be interfering with alterations in ion fluxes or Ca^{2+} coupling set off by an agonist acting on the muscarinic receptor of endothelial cells and as a result has no effect on the action of A23187. Factors which do affect the A23187 response are calcium free solutions and the presence of nifedipine.

Singer and Peach (1983) showed that when rabbit aortic rings were contracted with phenylephrine, a calcium-free solution reduced the A23187 induced relaxation by 93%. Nifedipine reduced it by 47%. This is evidence to show that Ca^{2+} mediates or regulates the endothelium dependent

relaxation. This inhibition of A23187's action by nifedipine was unexpected but it may be that there is some direct interaction between the two compounds.

It is still too early to evaluate the physiological significance of EDRF but it must be considered as a contributing factor in experiments such as those in this study, where it has not been (intentionally) removed. Consideration should be given to the possibility that altering experimental conditions eg. calcium levels, will affect the endothelial cells and subsequently the EDRF. It may also be possible that certain drugs could produce vasodilation by acting via endothelial cells. For example, Spokas et al. (1983) reported that hydrallazine is dependent for part of its action on the presence of endothelium.

It could be postulated that a factor that causes smooth muscle relaxation could inhibit smooth muscle contraction and be released from the endothelium by alpha-adrenoceptor agonists. Godfraind, (1985) found an increased sensitivity to nifedipine in the isolated rat aorta if the endothelium was present. He proposed that removal of the endothelium causes a slight depolarisation of smooth muscle cells and this could be responsible for the increase in exchangeable intracellular calcium. This would allow more calcium to be liberated by $alpha_1$-agonists so that the contraction resistant to calcium-entry blockers is higher in the absence than in the presence of endothelium. In view of this, the presence of the endothelium should have made the aorta more sensitive to nifedipine than it appeared to be.

It is likely that the action of noradrenaline is not affected by the presence or lack of endothelium (Furchgott, 1983), although some controversy does exist on this point. Godfraind (1985), stated that the removal of the endothelium enhances the response to noradrenaline whereas De Mey & Vanhoutte (1982) believed that removal of the endothelium reduces the response to noradrenaline. This, along with other points such as the buffering of calcium in solutions and its effect on endothelial cells, needs closer investigation, e.g. is the endothelium an aid or a barrier to calcium fluxes? The endothelial cells are obviously an important factor in the responses of blood vessels to agonists and it must be noted that even in the most careful of dissections and setting-up of isolated tissue prepararions, a proportion of the cells will inevitably be lost. Perhaps there is some linkage between the difficulty of demonstrating the presence of $alpha_2$-receptors in isolated blood vessels and this drawback in experimental technique?

This study looked at calcium channels using calcium-entry blockers but another popular method is that of measuring ^{45}Ca movements. Direct measurements of ^{45}Ca are not feasible in smooth muscle because they are not indicative of changes that could occur within the cell. Several attempts have been made to identify the biologically active calcium fraction and the most successful are those based on the use of lanthanum (Van Breemen et al., 1972; Godfraind, 1976). Lanthanum replaces Ca^{2+} on superficial binding sites and does not penetrate the cell. It blocks transmembrane fluxes of calcium, and because of this, the calcium content of a tissue bathed in La^{3+} can provide an estimate of cellular calcium (Godfraind, 1984).

The ^{45}Ca fluxes in rat aorta can be estimated by measuring the ^{45}Ca turnover in the La^{3+}-resistant calcium fraction. This fraction corresponds to the amount of calcium that is not displaced when the tissue is bathed in a 50 mM La^{3+} solution (Godfraind, 1976). In this solution most of the calcium displacement occurs during the first 5 mins and Ca^{2+} efflux from intracellular stores is apparently delayed. NA increases the rate of uptake of ^{45}Ca into this calcium fraction. However, there is no net gain in tissue calcium as calcium efflux increases in a similar manner. The increase in the rate of ^{45}Ca uptake is dose dependent and is due to alpha-adrenoceptor activation (ROC). High K^{+} solutions also allow an increased influx of ^{45}Ca (POC) and the slow turnover of intracellular calcium at rest is likely to occur through Ca^{2+} "leak" channels (Godfraind, 1984).

Although studying ^{45}Ca fluxes can produce information about all three types of La^{3+}-sensitive pathways (ROC, POC and passive leak channels) these experiments are generally performed in rather non-physiological conditions. Changes in intracellular ^{45}Ca can only be assessed after replacing it with La^{3+} at $0^{o}C$ for example (Godfraind, 1976).

Slow calcium channel blockers only inhibit VOC-dependent Ca^{2+} influx (Weiss, 1982) and as such can only be used to study these particular channels. The more complex experimental approach of tracer studies can be used to confirm and extend the simpler muscle contractility studies. However, this approach has its problems and limitations too. For instance, K^{+}-induced ^{45}Ca accumulation in whole cells may be affected by other factors, such as intracellular Na^{+} concentration, also some VOCs are inactivated by a rise in cytoplasmic Ca^{2+} and agents which produce this effect may indirectly decrease the number of conducting VOCs (Spedding & Cavero, 1984).

The other method of studying the properties of the calcium channels is by the use of calcium-entry blockers, such as nifedipine, used in this study. The interaction of calcium-entry blockers with calcium channels may be examined in binding studies (Bolger et al., 1983; Gould et al., 1982) and in functional studies such as in this thesis. Calcium-entry blockers cause marked inhibition of the catecholamine-induced contraction in arterial or splanchnic venous smooth muscle but have little effect on cutaneous veins (Vanhoutte,

1981). Differences in the efficacy of these compounds in inhibiting a specific effect in a given blood vessel must reflect the kinetic properties of their interaction with and their access to the Ca^{2+} channels of the vascular smooth muscle membrane, i.e. the confusing results with nifedipine may be an access problem due to endothelial cells or surrounding fat or connective tissue (although the vessels were cleaned on removal from the animal). This could apply not only to the antagonist, nifedipine, but also to the different Ca^{2+} levels where exposure time in the pulsed experiments may not be long enough to allow sufficient access.

Vanhoutte and Rimele (1982) suggested that when examining such conflicting interpretations the logical conclusion is that the varying degrees of dependency of alpha-adrenoceptor activation on calcium-entry are determined by the functional differentiation of the vascular smooth muscle cells rather than by apparent pharmacological characteristics of their alpha-adrenoceptors. Blood vessels such as the arterioles exhibit myogenic activity that relies heavily on the availability of extracellular calcium. In these vessels, the other major determinant of vascular smooth muscle tone, NA, also stimulates the entry of Ca^{2+}. In cutaneous veins, the muscle is controlled mainly by sympathetic nerves and relies more heavily on cellular mobilisation of the activator ion (Vanhoutte, 1980).

It is possible that in a given tissue the same receptor may trigger the increase in cytoplasmic Ca^{2+} either by

liberating intracellularly bound Ca^{2+} and/or translocating extracellular Ca^{2+} through calcium channels sensitive to calcium-entry blockers (Bolton et al., 1981).

Godfraind's more recent work is echoed in the results of this study, that the calcium story is not as clear cut as he first proposed, (Godfraind et al., 1982). In a paper using both calcium-entry blockers and ^{45}Ca he has shown that while the $alpha_2$ agonists, oxymetazoline and clonidine, stimulate contractions that are totally dependent on extracellular calcium; noradrenaline and phenylephrine stimulate contracions that are partly dependent on extracellular calcium and partly dependent on intracellular calcium stores.

The final set of experiments looked at the ability of the $alpha_1$-blocker, prazosin, to inhibit noradrenaline's contractile response on the rat aorta. It could be supposed that as prazosin appears to preferentially inhibit the fast phase of the noradrenaline contraction, this phase is produced by a separate receptor. In other words, two receptors are involved in the noradrenaline response, one of which is preferentially inhibited by prazosin. That this is not the case, is the subject of some controversy.

It was Cohen, Wiley and Slater (1979), who demonstated prazosin could competatively block noradrenaline induced contraction in the rat aorta. Unfortunately they used cumulative concentration response curves which do not allow a separate assessment of the fast component. Downing et al. (1983) used non cumulative concentration response curves and they were able to show that prazosin selectively reduced the fast component. They concluded however that as other selective $alpha_1$ antagonists, corynanthine and trimazosin, (Constantine & Hess, 1981; McGrath, 1982a) did not selectively reduce the fast component, this action of prazosin was unlikely to be related to its selectivity for $alpha_1$-adrenoceptors. As phentolamine could completely reverse the effects of prazosin, it was seen as evidence that it was acting at the same $alpha_1$ receptor, and its ability to selectively reduce the fast component was a function of the way in which it combined with the receptor.

Randriantsoa and co-workers (1981), however, suggested that there were two distinct alpha-receptors differentially

antagonised by prazosin but Wilson, (1983) believes only one receptor is present on the aorta. He tested a wide range of alpha-adrenoceptor agonists and not one displayed any selectivity for the slow component of the contractions to noradrenaline. He also found that doxasosin, WB-4104, tiodazosin, UK-18596 and chlorpromazine could preferentially inhibit the fast component of contractions to noradrenaline, all of which possess selectivity for post-junctional $alpha_1$-adrenoceptors (Buyniski et al., 1980; Timmermans et al., 1981b; Drew, 1982; U'Prichard, 1977).

Wilson also observed that the concentration of noradrenaline required to elicit the fast component of contractions, in the absence of extra-cellular calcium was greater than that required for the stimulation of the slow component and that prazosin had a greater effect on these EGTA-resistant responses (as was also observed in this study). It appeared that the stimulus for the release of cellular bound calcium is greater. Thus the preferential antagonism of noradrenaline induced release of calcium by prazosin may be attributable to the ability of the antagonist to suppress the initial stimulus for contraction.

In the guinea-pig aorta, Eglen et al. (1985) discovered that prazosin (at concentrations reported to selectively abolish the fast component in the rat aorta) had no effect on the EGTA-resistant contractions. In this study it was found that prazosin did indeed inhibit the ITC more than the SSC, in normal and calcium-free solutions. Both components were abolished with 10^{-7}M prazosin.

Controversy exists as to the nature of the alpha-adrenoceptor sub-type present on the rat aorta. Some researchers claim both $alpha_1$- and $alpha_2$-adrenoceptors are to be found (Ruffolo et al., 1981; Godfraind et al., 1982; Scarborough & Carrier, 1984) while perhaps the more recent and widely accepted belief is that only $alpha_1$-adrenoceptors are present (Downing et al., 1983; Digges & Summers, 1983; Ruffolo 1985). So, if noradrenaline is acting on only one type of receptor, the $alpha_1$, it rules out the possibility that prazosin produces its effect by selectively blocking a receptor subtype responsible for the fast response. One hypothesis is that if the ITC relies at least partially on calcium-entry, then prazosin somehow preferentially inhibits this source - this would also offer an explanation as to why the SSC is also inhibited as it relies more heavily on calcium-entry.

However, possibly the most likely explanation is one of equilibrium between the high affinity antagonist and the receptor. The ITC component may need a short, sharp burst of receptor occupation, by an agonist, in order to be produced. Prazosin is a high affinity antagonist and therefore occupies the receptor for a relatively long period of time. This means that no such short, sharp burst of agonist activity is possible and the ITC appears preferentially blocked. The SSC is the result of an equilibrium situation between the agonist and the receptor, it takes longer to be produced, and therefore remains

relatively unaffected.

Although not suggested as a possible explanation by Downing et al. (1983), their results do corroborate this last hypothesis. They found that corynanthine, a low affinity alpha$_1$ antagonist, did not preferentially inhibit the ITC. This antagonist only occupies the receptor for short periods of time. This is not long enough to prevent the agonist from producing a short, sharp burst of activity and hence the ITC remains relatively unaffected. A danger in analysing this type of experiment is that the antagonist may not have reached an equilibrium. Two separate Schild plots could be constructed for each phase of the response as evidence of the presence of two distinct receptors. However, a separate Schild plot cannot be constructed for the first phase as this is not at an equilibrium, and if it were the slope would undoubtedly be greater than one (Furchgott, 1972).

In conclusion, this thesis was an attempt to examine various parameters which affect the activation of the postsynaptic alpha-adrenoceptors. It appears that the blood gases are important for the relative contribution of alpha$_1$- and alpha$_2$-adrenoceptors to vascular tone and that their involvement may be either under- or over-estimated depending on the arterial blood gases. The other parameter investigated was the involvement of calcium in the two receptor subtype responses. The results suggest that calcium entry occurs during the secondary component of the alpha pressor response and can be initiated by either

$alpha_1$- or $alpha_2$-adrenoceptor activation. That is, calcium-entry is related to the time course of the response and does not correlate with an $alpha_1/alpha_2$ classification.

Finally, when the involvement of calcium in the $alpha_1$-adrenoceptor response was investigated, it appeared that calcium entry may occur during both the fast and slow components of the response. This was further evidence that it is the time course of the response which is important, when studying the effects of factors influencing alpha-adrenergic pressor responses, rather than the pharmacological name-tag given to the receptor.

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