A MECHANISTIC STUDY OF THE POSTJUNCTIONAL α-ADRENOCEPTORS OF THE FEMORAL VEIN OF THE RAT.

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A mechanistic study of the postjunctional α-adrenoceptors of the femoral vein of the rat.

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SUMMARY.

Contractile responses of the femoral vein of the rat to α-adrenoceptor agonists were investigated. The non-selective α-adrenoceptor agonist noradrenaline, the α_1-selective agonists cirazoline and phenylephrine and the α_2-selective agonists BHT 920 and UK 14,304 all elicited contractile responses. Maximum contractions to cirazoline, phenylephrine, BHT 920 and UK 14,304 were of similar size and were smaller than contractions to noradrenaline. Contractions to noradrenaline were inhibited by either the α_1-antagonist corynanthine or the α_2-antagonist idazoxan; those to cirazoline were antagonised by corynanthine but not idazoxan and those to BHT 920 by idazoxan but not corynanthine. These results indicate that the femoral vein of the rat possesses both postjunctional α_1- and α_2-adrenoceptors.

In calcium-free EGTA PSS, noradrenaline or cirazoline elicited transient contractions suggesting release of intracellular calcium; a calcium release component could not be demonstrated to BHT 920. Contractions to noradrenaline in calcium-free EGTA PSS were significantly greater in the presence of idazoxan than those in the presence of corynanthine. These results suggest that stimulation of α_1-adrenoceptors causes release of calcium from intracellular stores while stimulation of postjunctional α_2-adrenoceptors can not.

Study of receptor mediated changes in second messenger production revealed that stimulation of α_1-adrenoceptors by cirazoline or by noradrenaline in the presence of idazoxan produced a significant increase in polyphosphoinositide hydrolysis while stimulation of α_2-adrenoceptors by BHT 920 or noradrenaline in the presence of corynanthine did not. Conversely, stimulation of α_1-adrenoceptors had no effect upon tissue cAMP levels while stimulation of α_2-adrenoceptors caused a significant decrease in the intracellular cAMP content.

These results suggest that postjunctional α_1- and α_2-adrenoceptors of the femoral vein of the rat differ in their calcium mobilisation processes and post receptor coupling mechanisms.

Keywords:- vascular muscle, α-adrenoceptor subtypes, second messengers.

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I dedicate this thesis to my parents, who have always shown me the most extreme faith and trust, as the result of their endless encouragement, and to Carole, my wife-to-be and dearest companion.

Finally, I would like to begin this account of my work with a quotation from Goethe:-

> I have studied now Philosophy,
> And Jurisprudence, Medicine,
> And even, alas, Theology.
> From end to end, with labour keen;
> And here poor fool, with all my lore,
> I stand no wiser than before.
>
> Faust, I.

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5. GENERAL INTRODUCTION.

5.1. Historical background to adrenoceptor classification.

Current understanding of adrenoceptor pharmacology suggests two major classes of adrenoceptor, the α- and β-adrenoceptor, with two subtypes of each class. Ahlquist (1948) took the first step towards the differentiation of adrenoceptors when he determined the potency ratios for a range of sympathomimetic compounds acting on a variety of physiological tissues. It became apparent to Ahlquist that the tissues could be differentiated into two groups on the basis of their potency ratios; all the tissues in either group showed a similar order of potency ratios, but the orders of the two groups were different. This observation led Ahlquist to conclude that the sympathomimetics were exerting their effects by acting on two receptor types and he termed these the α- and β-adrenoceptors.

Lands and colleagues (1964, 1967) extended the work of Ahlquist using a larger range of sympathomimetics and tissues and he confirmed the conclusions of Ahlquist. However, Lands showed that those effects which Ahlquist had described to be mediated by β-adrenoceptors could themselves be divided into two distinct groups and he therefore suggested that two subtypes of the β-adrenoceptor existed. Lands termed these β_1- and β_2-adrenoceptors.

The first indication that two subtypes of α-adrenoceptor may also exist came in 1957 when Brown and Gillespie showed that the irreversible α-adrenoceptor antagonist phenoxybenzamine caused an increase in noradrenaline overflow following stimulation of the nerve supply to the perfused spleen of the cat. Brown and Gillespie interpreted this effect as a decrease in the effectiveness of the noradrenaline deactivating enzymes, although in hindsight it can be seen to be the consequence of the blockade of presynaptic, autoregulatory α_2-adrenoceptors. It was in 1970 that Langer suggested that the phenoxybenzamine-induced increase in noradrenaline

overflow from the isolated nictitating membrane of the cat and the rat vas deferens might be the consequence of blockade of autoinhibitory presynaptic α-adrenoceptors and this view was supported by Enero *et al* (1972). It was initially thought that pre- and postsynaptic α-adrenoceptors were homogenous but in 1974, Dubocovich and Langer demonstrated a different potency of phenoxybenzamine at these receptors, leading them to conclude that the receptors were different and thus, in 1974, Langer suggested that the prejunctional α-adrenoceptor be termed the α_2-adrenoceptor while the term α_1-adrenoceptor be reserved for the postjunctional α-adrenoceptor.

At this time it was considered that the postjunctional α-adrenoceptors mediating vasoconstriction in vascular muscle were a homogenous population of the α_1-subtype. However, in 1977, Bentley, Drew and Whiting presented preliminary reports suggesting that this may not be so and this was confirmed in 1979 when Drew and Whiting showed that the pressor response of the pithed rat to noradrenaline was not wholly antagonised by the potent α_1-antagonist prazosin. It was later reported by Flavahan and McGrath (1980) that this prazosin-resistant pressor response was blocked by the α_2-antagonist yohimbine, and this was substantial evidence that the two α-adrenoceptor subtypes could not be differentiated purely on an anatomical basis. Since then, an abundance of *in vivo* research has supported the original observation of Bentley, Drew and Whiting (1977) and shown that postjunctional α_1- and α_2-adrenoceptors coexist in the vasculature of a range of mammalian species. These include pithed rat (Timmermans, Kwa and van Zwieten, 1979; Docherty, Macdonald and McGrath, 1979), anaesthetised cat (Drew and Whiting, 1979), conscious rabbit (Hamilton and Reid, 1980), autoperfused hindlimb of the dog (Langer, Massingham and Shepperson, 1980), autoperfused hindlimb of the rabbit (Madjar, Docherty and Starke, 1980) and also in Man (e.g. van Brummelen *et al*, 1985).

Despite the volume of experimental data demonstrating the presence of postjunctional α_2-adrenoceptors *in vivo*, little *in vitro* evidence has become available to support this suggestion. Isolated blood vessels in which a

significant population of postjunctional α_2-adrenoceptors has been demonstrated are limited to human vessels including the palmar digital artery (Moulds *et al*, 1977) and saphenous vein (Docherty and Hyland, 1985) and to canine vessels including the saphenous vein (De Mey and Vanhoutte, 1981), mesenteric vein (Suzuki, 1981) and basilar artery (Sakakibara, 1982).

A great deal of research has been undertaken to determine the postjunctional α-adrenoceptor population of a range of isolated blood vessels from the rat. However, despite the fact that much of the *in vivo* evidence for the presence of postjunctional α_2-adrenoceptors has arisen from this species, no vessel has, to date, been shown conclusively to possess a significant population of these receptors. Medgett and colleagues (Medgett, Hicks and Langer, 1984; Hicks, Tierney and Langer, 1985; Medgett and Langer, 1984) have shown that postjunctional α_2-adrenoceptors are present in the perfused tail artery of the spontaneously hypertensive rat but such a population in normotensive rats appears to be limited. The rat saphenous vein is another possible exception (Cheung, 1985) but the small size of this vessel and the limited tension that it develops (only 5-10mg) make it unsuitable for detailed study in many laboratories. The demonstration of a blood vessel from the rat which possesses a population of postjunctional α_2-adrenoceptors would be a very significant pharmacological advance, since it would allow a more direct comparison of *in vivo* and *in vitro* research.

5.2. Physiological function of postjunctional α_1-and α_2-adrenoceptors.

In an attempt to determine the physiological functions of postjunctional α_1- and α_2-adrenoceptors, a number of workers have compared the effects of selective α_1- and α_2-antagonists on responses to exogenous agonists or neuronally released noradrenaline following electrical stimulation of vascular nerves both *in vivo* and *in vitro*. Yamaguchi and Kopin (1980) showed that the pressor response of the pithed rat to exogenous or circulating noradrenaline was antagonised by either α_1- or α_2-antagonists while the pressor response to sympathetic nerve stimulation was more readily blocked

by α_1-antagonists. This suggested to Yamaguchi and Kopin that a possible differential localisation of the two α-adrenoceptor subtypes within the smooth muscle of the blood vessel walls might occur and they have proposed that responses to released neurotransmitter are mediated via α_1-adrenoceptors close to the sympathetic nerve endings while those to exogenous or endogenous circulating agonists are mediated via either these α_1-adrenoceptors or α_2-adrenoceptors close to the luminal surface. Yamaguchi and Kopin (1980) have thus termed the α_1-adrenoceptors intrasynaptic and the α_2-adrenoceptors extrasynaptic. This proposal is supported by experiments *in vitro* made by Medgett and Langer (1984) who showed that the response of the rat isolated tail artery to noradrenaline is antagonised by either prazosin or idazoxan, the pA_2 values indicating action at α_1- and α_2-adrenoceptors respectively, while the contractile response to electrical stimulation was blocked by prazosin but not by idazoxan. A differential anatomical distribution of α_1- and α_2-adrenoceptors is also suggested by McGrath (1982) and van Meel *et al* (1983).

5.3. Role of the endothelium in vascular control.

In 1980, Furchgott and Zawadzki reported that the presence of vascular endothelium was necessary in order that acetylcholine may cause relaxation of rabbit isolated aortic rings preconstricted with noradrenaline. Removal of the endothelial cells by mechanical rubbing caused the complete abolition of the acetylcholine-induced relaxation and, in some cases produced potentiation of the noradrenaline contraction. Furchgott and Zawadzki (1980) suggested that acetylcholine was causing the release, from the endothelial cells, of a substance, or substances, which acted directly on the vascular muscle to cause relaxation. This substance was later termed endothelium derived relaxing factor or EDRF. The release of EDRF has since been studied extensively and has been reported to occur in a number of different blood vessels including the canine femoral artery (De Mey and Vanhoutte, 1982), the rat aorta (Lues and Schumann, 1984) and the rabbit coronary artery

(Griffith *et al*, 1984a). Also, agents other than acetylcholine have been shown to induce EDRF release from endothelial cells and these include bradykinin (Cherry *et al*, 1982), ATP, thrombin and arachidonic acid (De Mey and Vanhoutte, 1982).

Following the discovery of EDRF by Furchgott and Zawadzki, much research was directed to determine the exact nature of this vasoactive agent. Griffith *et al* (1984b) used a cascade system of rabbit aortic and perfused coronary artery strips to investigate the nature of EDRF; they showed that when the endothelium of the upper aortic strip was present, the effluent from this caused relaxation of the lower, preconstricted coronary artery. When the upper donor vessel was unstimulated the effluent caused a 17 ± 3% relaxation of the lower vessel, indicating that a basal release of EDRF occured. Addition of acetylcholine to the perfusate above the donor vessel increased the relaxation of the lower vessel to 62 ± 5% indicating an acetylcholine-induced increase in EDRF release.

Griffith *et al* (1984b) also determined the half-life of EDRF by altering the distance (and therefore the passage time of the eluate) between the donor and recipient vessels and this was found to be 6.3 ± 0.6 seconds. Since this report, other researchers have determined the half-life of EDRF in different vessels and variable values have been reported. These values range from 5 to 50 seconds (see Moncada *et al*, 1987) while Defeudis (1985) reports values of 24 and 49 seconds for EDRF from rabbit aorta and canine femoral artery respectively. It could be that these different half-life values are indicitive of species differences, but Forsterman *et al* (1984) suggest that the stability of EDRF is dependent upon the pO_2 and that differences in experimental protocol between different laboratories may therefore explain the discrepancies.

Furchgott and Zawadzki (1980) had made suggestions as to the chemical nature of EDRF, suggesting that it might be a humoral agent, a free radical or a lipoxygenase derivative. Griffith *et al* (1984b) concluded that EDRF was not a free radical nor a lipoxygenase derivative by the use of various chemical

agents and inhibitors and they indicated that it was a humoral agent. More recently, Furchgott (1987) suggested that EDRF may in fact be nitric oxide (NO) since the pharmacological properties of acidified NO_2^-, an NO generator, was similar to that of EDRF. This has been confirmed by Palmer, Ferridge and Moncada (1987) who compared various properties of NO with those of EDRF and found the two to be indistinguishable in terms of biological activity, stability and susceptibility to the same inhibitor (haemoglobin) and potentiator (superoxide dismutase).

Shortly after the initial report of Furchgott and Zawadzki in 1980, concerning the release of EDRF from vascular endothelium, Singer and Peach (1982) produced evidence that EDRF release in the rabbit aorta was calcium dependent. These workers showed that calcium removal or the calcium entry blockers verapamil and nifedipine decreased EDRF release while the calcium entry promoter calimycin (A23187) increased the release in a concentration dependent fashion. The calcium dependency of EDRF release has since been confirmed using a more direct approach by Long and Stone (1985a, 1985b) who have used a dual cascade system to allow them to decrease the calcium concentration to the donor vessel while that to the recipient remained constant. This latter work confirmed that both basal (unstimulated) and acetylcholine-stimulated release of EDRF is calcium dependent.

In many blood vessels, EDRF release is known to be accompanied by increased levels of cGMP and it has been suggested that it is this increase in the cyclic nucleotide which mediates the endothelium dependent relaxation. Rapoport and Murad (1983) showed that in the presence of intact endothelium, the relaxation of the rat aorta elicited by acetylcholine, calimycin or histamine was associated with a concentration dependent increase in cGMP. Following mechanical removal of the endothelium by gentle rubbing of the intimal surface, neither the relaxation nor the cGMP accumulation occured. Coincidentally, the nitro-vosodilators such as nitroprusside and nitroglycerin are also thought to exert their effects through the formation of cGMP (e.g. Katsuki, Arnold, Mittal and Murad, 1977). This is

particularly interesting in view of the reports, discussed above, concerning the possibility that EDRF is in fact NO since Katsuki *et al* (1977) and Arnold *et al* (1977) have suggested that the nitro-vasodilators activate guanylate cyclase via the production of NO. A role for increased cGMP levels as a mediator of endothelial induced relaxation is also supported by other workers including Griffith *et al* (1985). Also, Collins, Griffith, Henderson and Lewis (1986) have shown that the increase in cGMP brought about by EDRF inhibits both influx and intracellular release of calcium in the rabbit aorta. In relation to this, Lang and Lewis, (1988) have reported that EDRF (and also sodium nitroprusside) inhibits the hydrolysis of polyphosphoinositides and thus inositol trisphosphate (IP_3) formation in the rabbit aorta, and Saida and van Breemen (1987) have reported that IP_3-induced calcium release is dependent upon GTP. It therefore appears that EDRF (or NO) activates guanylate cyclase resulting in an increase in cGMP which causes a decrease in both intracellular calcium release and influx and thus muscle relaxation. Associated with the increase in cGMP would be a decrease in the concentration of GTP thus preventing IP_3-induced calcium release, contributing towards the relaxation.

Griffith *et al* (1984b) demonstrated that there is a constant basal release of EDRF, thus it might be expected that prevention of this release or removal of the endothelium may cause a potentiation of agonist induced contractions as a result of the removal of vasodilatation caused by EDRF. However, a number of reports have suggested that not all agonists are similarly affected by endothelial removal (e.g. Cocks and Angus, 1983 and Egleme, Godfraind and Miller, 1984). Egleme *et al* (1984) reported that in the rat isolated aorta with intact endothelium, the contractile response to the α_2-agonist clonidine was only approximately 10% of the size of the noradrenaline contraction. Following removal of the endothelium, the maximum response to noradrenaline tended to be higher, though not significantly so, while the contractile response to clonidine was greatly potentiated so that it became similar to that of noradrenaline. There was also an associated decrease in the EC_{50} concentration for each agonist indicating that the tissues became more

sensitive to both agonists. Egleme *et al* (1984) suggested that clonidine was stimulating receptors on the endothelium resulting in an increased release of EDRF. Thus, in endothelium-intact preparations, the contractile response to clonidine was decreased because of the inhibitory effect of this EDRF. Noradrenaline on the other hand did not stimulate these receptors to any extent and so little potentiation of the noradrenaline contraction occured following endothelial removal. Egleme *et al* (1984) thus concluded that the endothelium contained a population of α_2-adrenoceptors, stimulation of which increased EDRF release. Miller *et al* (1984) produced similar results for the α_2-agonist B-HT920 in the rat isolated aorta and also concluded that an α_2-like adrenoceptor was present on the endothelium, stimulation of which increased EDRF release, a view shared by Bullock, Taylor and Weston (1985).

However, it is interesting that Egleme *et al* (1984) and Miller *et al* (1984) interpreted the inhibitory effect of the endothelium on B-HT920 and clonidine responses to be the consequence of their α_2-agonistic properties and yet noradrenaline, which is also an agonist at α_2-adrenoceptors, was not affected by endothelial removal, suggesting that it did not increase EDRF release. This may indicate that it is not the α_2-stimulatory properties of clonidine and B-HT920 which render them sensitive to endothelial inhibition and Lues and Schumann (1984) showed that the addition of the α_2-antagonist rauwolscine to endothelium intact rat aortic rings did not mimic the effect of endothelium removal thus suggesting that the endothelial receptor causing EDRF release was not of the α_2-subtype. Lues and Schumann (1984) also investigated the effect of endothelial removal on the responsiveness of the rat aorta to a range of α_1- and α_2-agonists and they showed that the response of all partial agonists, both α_1- and α_2-selective, were affected by endothelial removal. Thus, following endothelium removal, the intrinsic activities of these agonists increased to that of noradrenaline. This led Lues and Schumann (1984) to conclude that it is those agonists with low intrinsic activities which are susceptible to endothelial inhibition while agonists with high intrinsic activities are little affected.

5.4. Calcium and vascular muscle.

In 1883, Ringer demonstrated that calcium is an essential component of the fluid bathing an isolated tissue. It is now well documented that an increase in the cytosolic free calcium concentration is necessary for contraction of vascular muscle. The extracellular concentration of calcium is in the region of 10^{-3}M and the intracellular concentration in the resting cell in the region of 10^{-7}M; this must rise to about 10^{-5}M in order for contraction of the vascular muscle to occur (Janis and Triggle, 1983).

Godfraind and Kaba (1969) investigated the effects of calcium removal from the bathing medium on responses of the rat aorta to KCl or adrenaline and observed that while responses to KCl were totally dependent on the presence of extracellular calcium, incubation in calcium free Krebs solution reduced but did not abolish the response to adrenaline. From these results, Godfraind and Kaba (1969) concluded that there are two possible sources of activator calcium in the rat aorta. The first is a limited supply of intracellular membrane bound calcium and the second is calcium located in the extracellular space. This concept is now generally accepted for many types of vascular muscle. The site of the intracellular store of calcium has been extensively studied and is generally considered to be the sarcoplasmic reticulum in vascular muscle (see Somlyo, Broderick and Somlyo, 1986). This store can be released by agonists such as noradrenaline and adrenaline and contributes to the contractile response. However, KCl, and other agents, cannot release the intracellular store and these agents are therefore dependent on extracellular calcium for contraction. The criteria that determine an agonists ability to release intracellular calcium will be discussed later.

The extracellular pool of calcium enters the cell down a concentration gradient through specific calcium channels following changes in the calcium permeability of the cell membrane. At present it is believed that two types of calcium channel exist, one being a potential dependent channel (PDC) which allows the influx of calcium following changes in the membrane potential

(e.g. following KCl depolarisation) and the second is a receptor operated channel (ROC) which allows calcium to enter the cell following receptor-agonist combination and is independent of the membrane potential.

In 1972, Godfraind and Kaba extended their observations of 1969 and reported that the biphasic form of the contractile response of the rat aorta to adrenaline was the consequence of the calcium contributing to the contraction arising from two different sources. Thus, the rapid rise in tension seen immediately following agonist addition was the result of a rapid rise in the intracellular calcium concentration due to calcium release, while the slow, maintained form of the contraction was dependent on calcium influx. Removal of extracellular calcium or addition of drugs that prevent calcium translocation had no effect on the initial phase of the contraction but markedly reduced the latter phase. In contrast, the response to KCl rose very rapidly but had no well-defined initial fast component, since this contraction relied on extracellular sources of calcium alone.

Recently, the source(s) of activator calcium for contractions of vascular muscle following stimulation of postjunctional α_1- or α_2-adrenoceptors has been under debate. *In vivo* reports have suggested that pressor responses of pithed rats to α_2-agonists are invariably sensitive to calcium entry blocking drugs while responses to α_1-agonists are generally resistant. Van Meel *et al* (1981) showed that the calcium entry blockers verapamil, nifedipine and D600 all antagonised the pressor response of the pithed rat to B-HT920 while responses to methoxamine were unaffected. These observations led van Meel *et al* (1981) to propose that the *in vivo* vasoconstriction mediated by α_2-adrenoceptors was totally dependent on calcium translocation while a portion of the α_1-mediated response was not dependent upon calcium entry. Cavero *et al* (1983) produced supportive evidence using cirazoline and M-7 as α_1- and α_2-agonists respectively and diltiazem and verapamil as calcium entry blockers. In addition, Cavero *et al* (1983) found that pressor responses to the α_1-agonist developed more rapidly than that to the α_2-agonist, and suggested that this too indicated different sources of activator calcium, α_1- but not α_2-

stimulation involving a release component.

In vitro evidence in support of the proposal of van Meel *et al* (1981) has been presented by Jim, DeMarinis and Mathews (1985), Jim *et al* (1983) and Jim and Mathews (1985). In the former report, Jim *et al* (1985) reported that contractions of the isolated canine saphenous vein to α_1-agonists involved both influx and release components. Whereas Jim *et al* (1983) had shown that contractions of the canine saphenous vein to B-HT920 were abolished following removal of extracellular calcium. Jim and Mathews (1985) extended this to show that the α_2-component of contractions to a number of α_2-agonists in the same vessel were abolished by incubation in PSS containing 5mM lanthanum.

Thus, both *in vivo* and *in vitro* evidence supports the hypothesis of van Meel *et al* (1981) that the ability of α-adrenoceptor agonists to release intracellular calcium is dependent upon the subgroup of α-adrenoceptor stimulated by the agonist. Stimulation of α_1-adrenoceptors is associated with both release and influx while agonists acting at α_2-adrenoceptors are totally dependent on calcium influx.

In contrast to this, Ruffolo *et al* (1984) reported a high inverse correlation between the intrinsic activity of a range of α_1-agonists and the degree to which their pressor responses are inhibited by the calcium entry blocker diltiazem. They too showed that in the pithed rat, responses to the α_1-agonist cirazoline were unaffected by the calcium entry blocker diltiazem, while responses to B-HT933 were inhibited. However, following the removal of a proportion of the spare α_1-adrenoceptors using the irreversible α_1-antagonist phenoxybenzamine, responses to cirazoline became susceptible to antagonism by diltiazem. Increasing the concentration of phenoxybenzamine, thereby decreasing receptor reserve, rendered the cirazoline pressor response progressively more sensitive to the calcium entry blocker. These results were paralleled when using a number of α_1-agonists with different intrinsic activities.

Ruffolo *et al* (1984) therefore proposed that it is the intrinsic activity of

the agonists, rather than the α-adrenoceptor subtype, that determines sensitivity to calcium entry blockers and therefore dependence on extracellular calcium. Thus, the responses induced by agonists with low intrinsic activities are susceptible while those to agonists with high intrinsic activities are not. Since the α_2-agonists used by van Meel *et al* (1981), Cavero *et al* (1983), Jim *et al* (1983) and Jim and Mathews (1985) are all partial agonists, their susceptibility to calcium entry blockers may be explained by this proposal.

The exact significance of both receptor subtype and intrinsic activity on the susceptibility of agonists to calcium removal or the prevention of calcium translocation still, as yet, remain unclear.

5.5. Polyphosphoinositide hydrolysis as a second-messenger generating system.

5.5.1. The phosphatidylinositol (PI) cycle.

In 1953, Hokin and Hokin first provided evidence to indicate a link between the effects of hormones and the metabolism of phosphoinositides. More recently, a wide variety of receptor mediated events have been shown to involve the stimulation of membrane bound phospholipase C and the subsequent hydrolysis of phosphoinositides (for reviews see Michell, 1975 and Berridge, 1981). The initial observation of Hokin and Hokin (1953) suggested that the prime target of phospholipase C was phosphatidylinositol (PI) but it is now generally considered that it is the polyphosphoinositide phosphatidylinositol-4,5-bisphosphate (PIP_2) which is hydrolysed following receptor stimulation resulting in the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge *et al*, 1983, Berridge, 1983). The cycle of events known as the PI cycle is shown diagrammatically in figure 5.1. Under resting conditions in the cell, a basal rate of PIP_2 hydrolysis occurs and this is accelerated following agonist-receptor combination resulting in an increase in the formation of the second messengers.

As can be seen from figure 5.1, PI is a precursor of PIP_2, the latter being synthesised by the successive phosphorylation of PI at the 4 and 5 positions of the inositol ring. Following the stimulation of phospholipase C, as a result of agonist-receptor combination, PIP_2 is hydrolysed to produce the two intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). The successive enzymatic dephosphorylation of IP_3 deactivates this messenger and produces IP_2, IP and eventually free inositol (see Figure 5.1). The deactivation of DAG results from its phosphorylation to phosphatidic acid (PA) and then to CDP-DAG. The two breakdown products (free inositol and CDP-DAG) are then combined to reform PI, thus completing the cyclic process.

An interesting discovery concerns the effect of Li^+ on the PI cycle. In 1971, Allison and Stewart reported that Li^+ decreased the concentration of inositol in the cerebral cortex of rats. It was then shown that this decrease was associated with an increase in the content of inositol-1-phosphate resulting from the inhibition, by Li^+, of *myo*-inositol-1-phosphatase (Hallcher and Sherman, 1980). In 1982, Berridge, Downes and Hanley reported similar results using rat brain and parotid gland slices and insect salivary glands. These results thus indicated that Li^+ was a useful pharmacological tool in the study of agonist-receptor effects linked to PIP_2 hydrolysis since it allowed amplification of the pharmacological response.

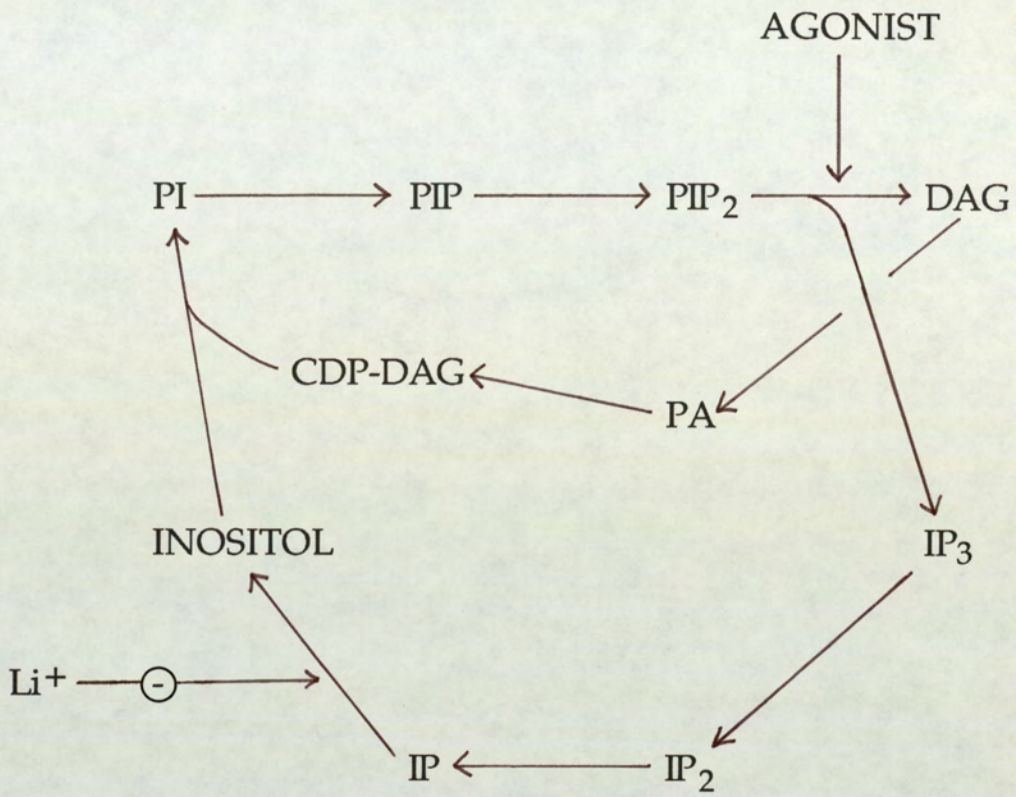


Figure 5.1. The phosphatidylinositol (PI) cycle.

Phosphatidylinositol (PI) is phosphorylated to produce phosphatidylinositol phosphate (PIP) and then phosphatidylinositol bisphosphate (PIP_2). The agonist acts at the receptor to stimulate the hydrolysis of PIP_2 resulting in the production of the two intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is then cycled back, via inositol bisphosphate (IP_2) and inositol phosphate (IP), to free inositol for resynthesis to PI. DAG is phosphorylated to form PA (phosphatidic acid) which interacts with CTP to form cytidine diphosphate diacylglycerol (CDP-DAG) which recombines with inositol to replenish the pool of PI. Li^+ inhibits the enzyme inositol-1-phosphatase preventing the conversion of IP to free inositol.

(Adapted from Berridge, 1984).

5.5.2. Second messengers associated with polyphosphoinositide hydrolysis.

Michell (1975) noticed that the hormones and agonists with which are associated an increase in intracellular calcium following cell activation are also those which stimulate hydrolysis of polyphosphoinositides. Michell thus suggested that there was a link between inositol phospholipid hydrolysis and calcium signalling, although the nature of the link was unknown. In 1983, Berridge indicated that hydrolysis of PIP_2 produced IP_3 and he suggested that the rapid accumulation of the trisphosphate following agonist stimulation may indicate a second messenger role for this in the control of the release of calcium from intracellular stores. This was confirmed by Streb, Irvine, Berridge and Schulz (1983) who reported that addition of micromolar concentrations of IP_3 to pancreatic acinar cells released calcium from a nonmitochondrial intracellular calcium store. This site is now considered to be the endoplasmic reticulum (ER) (Berridge, 1986). Further, the experiments of Irvine *et al* (1986) suggested that the calcium store released by IP_3 was the same as the agonist-releasable pool which was released by acetylcholine.

Streb *et al* (1983) also showed that the release of calcium from the intracellular store was specific for IP_3 since other inositol phosphates (IP_2, IP, cyclic IMP) and also free inositol failed to cause calcium release. The mechanism by which IP_3 causes release of intracellular calcium has been investigated. The need for only micromolar concentrations of IP_3 to cause maximum release of calcium suggests that IP_3 may be acting via a specific intracellular receptor and the specificity of IP_3 in causing this release (Streb *et al*, 1983) supports this theory. Burgess *et al* (1984) and Irvine *et al* (1986) have investigated the effects of analogues of IP_3 on calcium release and their results confirm the stereospecificity of IP_3-mediated calcium release supporting the theory of the existance of an intracellular IP_3-receptor. This is further supported by Spat *et al* (1980) who have investigated the characteristics of the IP_3 binding site in guinea pig hepatocytes and rabbit neutrophils and conclude that the properties of this site suggest it to be a

physiological IP_3 receptor. More recently, Nahorski, *et al* (1988) have described the effects of a synthetic analogue of IP_3, ($IP(S)_3$), which is a full agonist of intracellular calcium release and a potent displacer of ^3H-IP_3 binding to cerebellar membranes but is resistant to attack by inositol-5-phosphatase thus rendering it resistant to metabolism.

It is not yet clear whether the hydrolysis of membrane phospholipids also results in an increase in calcium influx across the plasma membrane and reports are conflicting. Michell *et al* (1977) have suggested that a product of inositol phospholipid hydrolysis may cause the opening of a membrane calcium channel and they have suggested that phosphatidic acid, a metabolite of DAG, may act as a calcium ionophore, a view shared by Putney *et al* (1980) and Salmon and Honeyman (1980). Serhan *et al* (1981) have suggested such a role for phosphatidic acid in liposomes while Holmes and Yoss (1983) provide evidence that this is not so. Also, Burgess *et al* (1984) have suggested that IP_3 may also cause calcium translocation across the cell membrane in a similar manner to its effect at the ER membrane, although Streb *et al* (1984) and Ueda *et al* (1986) suggest that this is not so. In relation to this, Slack *et al* (1986) have shown that injection of IP_3 into sea urchin eggs results in effects which are associated with calcium influx. This has led Putney (1986) to propose a mechanism by which IP_3 can cause calcium influx by an action on the ER, not the plasma membrane itself. He suggests (Putney, 1986) that the ER and plasma membrane share a close functional association and that calcium influx occurs via the ER. Thus, as the ER empties of calcium following IP_3 stimulation, extracellular calcium enters the ER across the plasma membrane and, providing IP_3 is still present, this will then pass into the cytosol. Further, it has been suggested (Taylor, 1986) that the passage of calcium from the extracellular medium into the ER may itself be regulated by a metabolite of IP_3, namely IP_4 (1,3,4,5 IP_4).

The second product of inositol phospholipid hydrolysis which has a second messenger function is DAG. Takai *et al* (1984) have shown that DAG activates protein kinase C, an enzyme present in an inactive state in the cell

cytoplasm. Activated protein kinase C can have a number of functions and one of these is the phosphorylation of myosin light chain (Naka *et al*, 1983) which is a necessary step towards contraction of vascular muscle. Protein kinase C also controls the cellular production of inositol phosphate and thus the production of DAG may act as a feedback inhibition system, working to decrease further inositol phospholipid hydrolysis (see Dillon, Murray, Uhing and Synderman, 1987).

5.5.3. Polyphosphoinositide hydrolysis and α-adrenoceptors.

A number of reports have indicated that stimulation of vascular α_1-adrenoceptors is associated with an increased hydrolysis of membrane polyphosphoinositides. Legan, Chernow, Parrillo and Roth (1985) have shown that stimulation of the α_1-adrenoceptors of the rat aorta with noradrenaline results in increased hydrolysis, a view shared by Chiu, Bozarth and Timmermans (1987). Other vessels in which α_1-stimulation is reported to be associated with increased hydrolysis of polyphosphoinositides are the rabbit mesenteric artery (Hashimoto *et al*, 1986), the rabbit aorta (Campbell, Deth, Payne and Honeyman, 1985) and the rat caudal artery (Fox *et al*, 1985).

5.6. The role of cAMP in vascular muscle control.

Cyclic adenosine monophosphate (cAMP) is a cyclic nucleotide formed from ATP following stimulation of the enzyme adenylate cyclase which is found in all cells except non-nucleated red blood corpuscles. The functions of cAMP as a second messenger are widespread and include increasing lipolysis in adipocytes, increasing glycogenolysis, decreasing the tone of the intestine and increasing the force of contraction of the heart.

In 1967, Robison *et al* suggested a link between adrenoceptor stimulation and changes in cellular cAMP content and Vulliemoz *et al* (1970) indicated that increased tissue cAMP was associated with relaxation of vascular muscle. It was then suggested by Triner *et al* (1971) that β-adrenoceptor stimulation of vascular muscle caused relaxation by accelerating the action of adenylate

cyclase resulting in increased intracellular cAMP.

The biochemical sequence of events which leads to vascular muscle contraction, and the influence of cAMP on these events is well established and is shown diagrammatically in Figure 5.2. In order for contraction to occur, the light chain of the myosin molecule (MLC) must be phosphorylated by the enzyme myosin light chain kinase (MLCK). This enzyme is only active when bound to the calcium-calmodulin complex. A rise in the intracellular concentration of calcium from the resting level (10^{-7}M) to 10^{-5}M results in the binding of calcium to calmodulin and thus initiates muscle contraction by binding to, and activating, MLCK (see Figure 5.2). cAMP regulates muscle contraction by its effect on MLCK; an increase in cAMP converts a cAMP-dependent protein kinase from an inactive to an active form and activated cAMP-dependent protein kinase phosphorylates MLCK. In its phosphorylated form, MLCK does not readily bind the calcium-calmodulin complex and therefore muscle contraction does not occur. Thus, the increase in cAMP caused by β-adrenoceptor stimulation of adenylate cyclase prevents MLC phosphorylation and therefore results in muscle relaxation.

The contractile effect of α-adrenoceptor stimulation was suggested by Robison and Sutherland (1970) to be the consequence of a decrease in tissue cAMP concentration by inhibition of adenylate cyclase. This would result in a decreased activity of cAMP-dependent protein kinase and promote unphosphorylated MLCK. This would readily combine with the calcium-calmodulin complex resulting in myosin phosphorylation and thus contraction. Volicer and Hynie (1971) reported that, in the rat aorta and tail artery, catecholamine stimulation resulting in relaxation (ie. the β-effect) was associated with increased tissue cAMP while contraction (the α-effect) was associated with decreased cAMP. However, Seidel *et al* (1975) reported that contractions of canine coronary arteries mediated via α-adrenoceptors were not associated with changes in tissue cAMP levels. Thus, the effect of α-adrenoceptor stimulation on tissue cAMP levels remains in dispute.

In a number of non-vascular tissues, stimulation of α-adrenoceptors of the

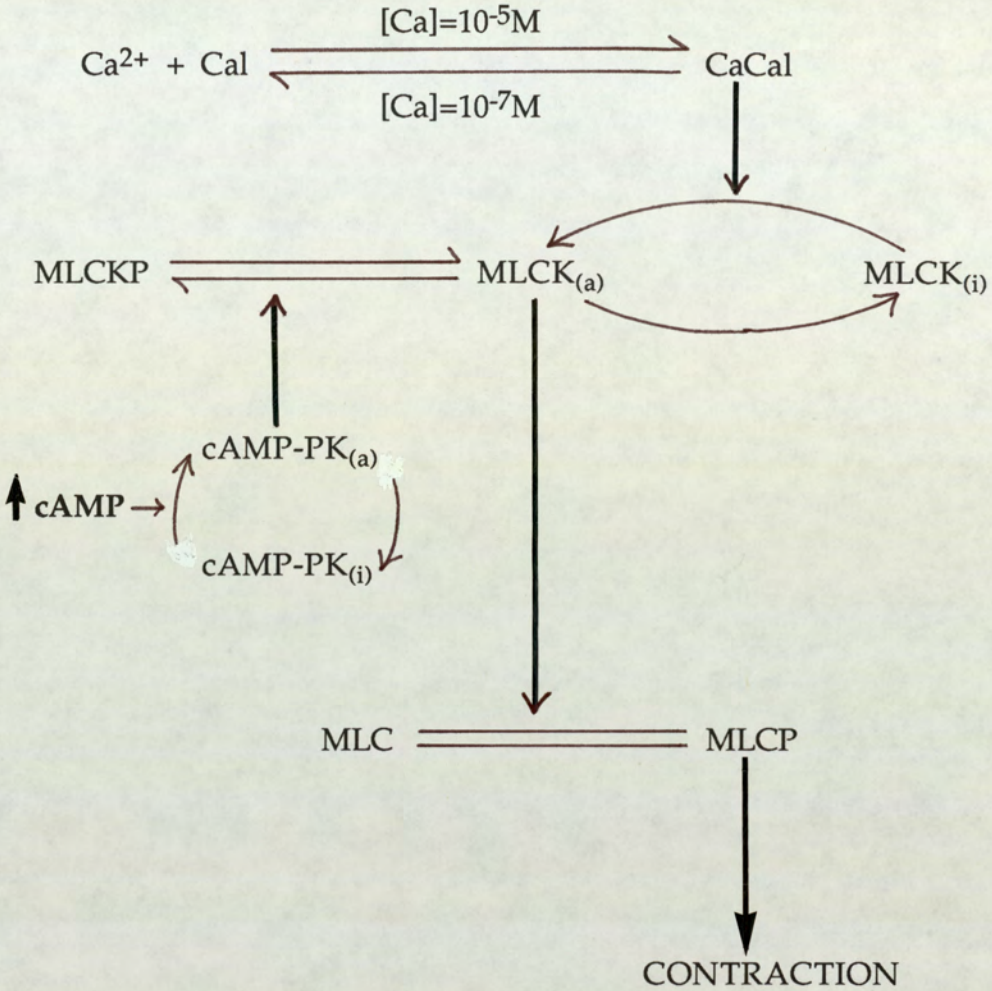


Figure 5.2. The influence of cAMP on vascular muscle contraction.

As the intracellular calcium concentration is increased, calcium binds to calmodulin (Cal) so forming the active calcium-calmodulin complex (CaCal). CaCal binds to myosin light chain kinase (MLCK) converting it from its inactive ($MLCK_{(i)}$) to its active $MLCK_{(a)}$) form. In its active form, $MLCK_{(a)}$ phosphorylates myosin light chain (MLC) leading to MLCP, resulting in muscle contraction.

In the presence of a high concentration of cAMP, the enzyme cAMP-dependentprotein kinase is converted from its inactive ($cAMP\text{-}PK_{(i)}$) to its active ($cAMP\text{-}PK_{(a)}$) form. $cAMP\text{-}PK_{(a)}$ phosphorylates $MLCK_{(a)}$ rendering the latter inactive and thus leads to muscle relaxation.

α_2-subtype has been reported to result in decreased cAMP content (e.g. human platelets, Salzman and Neri (1969); human adipocytes, Burns *et al* (1971); cat thyroid slices, Yamashita *et al* (1977)). The existance of a mixed population of α_1- and α_2-adrenoceptors may explain the conflict in the literature concerning the effect of α-adrenoceptor stimulation of vascular muscle on cAMP content. If stimulation of vascular postjunctional α_2-adrenoceptors causes decreased cAMP levels, then those studies which report such a decrease may arise from investigation of vessels possessing postjunctional α-adrenoceptors of the α_2-subtype. In contrast, those reports indicating no effect of α-stimulation on cAMP content may be from studies in vessels possessing a homogenous population of postjunctional α_1-adrenoceptors. However, Volicer and Hynie (1971) report a decrease in the cAMP content following stimulation of α-adrenoceptors with noradrenaline in the presence of propranolol in the rat aorta and by noradrenaline alone in the rat tail artery. This is interesting as the rat aorta has been shown to possess a homogenous population of α_1-adrenoceptors (e.g. Digges and Summers, 1983) and a population of postjunctional α_2-adrenoceptors in the rat tail artery of the normotensive rat has not been confirmed. The canine coronary artery studied by Siedel *et al* (1975) in which they report no changes in cAMP concentration may possess only α_1-adrenoceptors, thus explaining why no change in cAMP was seen.

Thus, in view of our present understanding of the pharmacology of the postjunctional α-adrenoceptors of vascular muscle, it would seem relevent to investigate the effects of the stimulation of α_2-adrenoceptors on cAMP, content using a suitable vessel (ie. one possessing postjunctional α_2-adrenoceptors) and selective agonists and antagonists rather than the non-selective catecholamines used in the early studies.

6. INTRODUCTION TO THE THESIS.

It is now well established that the postjunctional α-adrenoceptors of vascular muscle are not a homogenous population of the α_1-subtype but rather a mixed population of α_1- and α_2-adrenoceptors. Postjunctional α_2-adrenoceptors are well documented *in vivo* (e.g. Bentley, Drew and Whiting, 1979; Timmermans, Kwa and van Zwietten, 1979; Hamilton and Reid, 1980) but to date few reports have demonstrated conclusively the presence of this subtype *in vitro*. This is particularly true for the rat, despite the fact that much research has been undertaken to study these receptors of this species *in vivo*. Attempts have been made to characterise the α-adrenoceptor population of a number of isolated blood vessels from the rat (including, for example, the aorta, tail artery and portal vein) but studies suggest that these vessels probably possess a homogenous population of α_1-adrenoceptors.

The initial aim of the present study was to determine the postjunctional α-adrenoceptor population of the isolated femoral vein of the rat in the hope that this vessel would possess a heterogenous population of α-adrenoceptors. This vessel was chosen for study because it is anatomically close to the saphenous vein, a vessel which Cheung (1985) has shown to possess postjunctional α_2-adrenoceptors. However, the latter vessel is very small and the tension developed by it only in the region of 5 to 10 mg making it unsuitable for detailed study in many laboratories. It was therefore reasoned that the femoral vein may share some of the characteristics of the saphenous vein (i.e. a mixed postjunctional α-adrenoceptor population) but, that being larger than the saphenous vein, it would be more suitable for study.

Initial experiments determined the ability of a number of agonists, either non-selective or α_1- or α_2-selective, to cause a contractile response in the femoral vein. The postjunctional α-adrenoceptors were then further characterised using both selective α-agonists and antagonists with the aim of determining more conclusively the α-adrenoceptor population of the vessels.

Following the study of the effects of application of exogenous agonists to the tissues, the effect of transmural stimulation of the sympathetic nerves of the vessel was investigated. In particular, this part of the study aimed to determine whether the neurotransmitter released during transmural stimulation acted upon both α_1- and α_2-adrenoceptors or upon a single subtype and thus to determine the role of these receptors in the response of the tissues to nervous stimulation.

Over recent years, it has come to light that the endothelium of vascular tissue plays an important role in the regulation of vascular tone (see Furchgott, 1981, 1984). It has further been demonstrated that this influence of the endothelium is mediated by the release of vasoactive substances (see Furchgott and Zawadzki, 1980). Egleme and colleagues (1984) have suggested that the influence of EDRF, one such vasoactive agent, is greater on responses mediated by α_2-adrenoceptors than by α_1-adrenoceptors and have suggested that this is because α_2-adrenoceptors are found on the endothelium, stimulation of which causes EDRF release. Therefore, in the next part of this thesis the influence of the endothelium on responses of the rat femoral vein to α_1- and α_2-adrenoceptor stimulation was investigated.

Having established the presence of a mixed postjunctional population of α-adrenoceptors in the vessel, experiments were undertaken to determine the sources of activator calcium for either receptor subtype. The sources of activator calcium following α_1- and α_2-adrenoceptor stimulation is currently an area of some dispute in the literature. On the one hand, van Meel and colleagues (1981) suggest that responses to α_1-agonists are associated with an increase in the intracellular calcium that arises from both the extracellular space and from intracellular stores, while responses to α_2-agonists rely solely upon the translocation of extracellular calcium, these agonists being unable to release intracellular calcium. In contrast, Ruffolo and colleagues (1984) suggest that it is not the subtype of α-adrenoceptor *per se* which determines the dependence on extracellular calcium, but rather the intrinsic activity of the activating agonist.

Study of the form of the contractile response for each agonist provided initial indirect evidence that the sources of activator calcium might be different for the two adrenoceptor subtypes as the form was different for α_1- and α_2-adrenoceptor agonists. It was decided to investigate this further by determining the ability of the agonists to produce a contractile response in the presence of calcium-free PSS containing the calcium ion chelator, EGTA. In addition, the effect of the calcium entry blocking agent, VER on responses of the femoral vein to the agonists was also investigated. These experiments indicated that while stimulation of α_1-adrenoceptors was associated with the release of intracellular calcium, stimulation of α_2-adrenoceptors resulted in an increase in the intracellular calcium concentration arising from the translocation of extracellular calcium alone, no component of this contraction being dependent on intracellular sources of calcium.

In view of the observation that α_1-adrenoceptor stimulation could be coupled to the release of intracellular calcium while α_2-stimulation was not, it was considered that this might be the the consequence of differences in the excitation-contraction coupling processes for the two α-adrenoceptor subtypes. It has recently been suggested that inositol trisphosphate (IP_3) an immediate product of the action of phospholipase C on membrane phosphoinositides causes the release of calcium from its intracellular store in the E.R. (see Streb *et al*, 1983). It was therefore considered worthwhile to investigate the effect of α-adrenoceptor stimulation of the femoral vein of the rat on the breakdown of polyphosphoinositides and the production of inositol phosphates, with the aim of determining whether the ability of α_1-agonists to release intracellular calcium is the consequence of a stimulatory action of phospholipase C. Having confirmed that α_1-adrenoceptor agonists do increase polyphosphoinositide hydrolysis in the femoral vein of the rat, an attempt was then made to determine which inositol phosphates were produced following α-adrenoceptor-mediated stimulation of phospholipase C in order to ascertain the phosphoinositide hydrolysed by phospholipase C.

In the final section of the study, the effect of α_1- and α_2-adrenoceptor

stimulation on the cAMP content of the femoral vein was investigated. It is known that stimulation of the β-adrenoceptors of vascular muscle is associated with an elevated concentration of tissue cAMP as a consequence of the β-mediated stimulation of adenylate cyclase. It has also been shown that stimulation of the α_2-adrenoceptors of a number of non-vascular tissues is associated with a decrease in the tissue cAMP content (e.g. human platelets (Salzman and Neri, 1969) and human adipocytes (Burns *et al*, 1971)). In the light of these reports, it was decided to investigate the effect of both α_1- and α_2-adrenoceptor stimulation on the cAMP levels of the femoral vein of the rat in order to determine whether inhibition of adenylate cyclase and changes in tissue cAMP content were asociated with stimulation of either subtype.

It is hoped that this thesis will provide further information regarding, in particular the excitation-contraction coupling mechanisms of α_1- and α_2-adrenoceptors and these are discussed in relation to the calcium handling properties of these receptors.

7. MATERIALS AND METHODS.

ORGAN BATH STUDIES.

7.1. Tissue preparation.

Male Wistar rats (200-300g) were stunned and killed by cervical dislocation. The skin was removed from the upper leg region to expose the femoral vein and artery, the vein was carefully separated from the artery and, under a dissecting microscope (Zeiss Jena) was cleaned of loosely adherent tissue. A small incision was made at the distal end of the vein and a triangle made of steel wire (0.15mm O.D., Coopers Needleworks) inserted into the lumen of the vessel. The wire was inserted into the vessel *in situ* to minimise damage to the vein and endothelium. A 5mm section of the vein was dissected free and placed in physiological salt solution (PSS). Any remaining adherent tissue was then removed and a second steel wire triangle inserted into the lumen. One triangle was attached to a transducer and the other to the base of a 20ml organ bath containing PSS gassed with 5% CO_2 in O_2 and maintained at 37°C. A resting tension of 0.5g was applied to each tissue. All tissues were allowed to equilibrate for a period of 60 minutes prior to addition of drugs and during this time the bathing medium was changed 6 times, the tension being reset as required.

Contractile responses were recorded with isometric force transducers (type UF1, Pioden) coupled to a preamplifier (Ormed) and displayed on Ormed MX4 chart recorders.

7.1.1. Composition of the physiological salt solution (PSS).

The PSS was of the following composition (mM): NaCl, 118.3; KCl, 4.7; $MgSO_4.7H_2O$, 1.2; KH_2PO_4, 1.19; $NaHCO_3$, 25; $CaCl_2$, 2.5; (D)-glucose, 11.7. PSS also contained propranolol (1μM) to inhibit β-adrenoceptors, cocaine (10μM) to block neuronal uptake mechanisms, ascorbic acid (50μM) to prevent

oxidation of catecholamines and ethylene diamine-tetra-acetic acid (EDTA, 10μM) to chelate heavy metal impurities.

7.2. Experimental methods.

7.2.1. Effects of agonists on the femoral vein of the rat.

In initial experiments the response of the femoral vein was determined to a number of α-adrenoceptor agonists. In these experiments, after the 60 minute equilibration period, each tissue was exposed to four agonists added sequentially in the form of a Latin Square. In some experiments two agonists were added together to investigate whether summation of responses occured.

In other experiments, following equilibration, reproducible responses were elicited to a single agonist (10^{-6}M) and then after 30 minutes a non-cumulative concentration-response curve was constructed. To account for changes in tissue sensitivity due to time, a series of responses were determined to a 1μM concentration of each agonist.

In all cases the agonist was left in contact with the tissues for a period of 5 minutes and successive doses of agonist were added at 20 minute intervals, the tissues being washed every 5 minutes. A contact time of 5 minutes was used throughout since all agonists reached peak tension within this time.

7.2.2. Effect of selective α_1- and α_2-adrenoceptor antagonists on agonist concentration-response curves.

After equilibration, reproducible responses were elicited to a single agonist (10^{-6}M) and 15 minutes was then allowed before antagonist was added to the bathing PSS. The antagonist was left in contact with the tissues for 30 minutes before the determination of a non-cumulative concentration-response curve to the agonist. During the 30 minute antagonist incubation period the bathing medium was changed 4 times. The time cycle of agonist addition and the contact time for agonists were as described above and all washes were made with the antagonist present in the PSS. In all cases, only a single concentration of antagonist was studied per tissue.

In other experiments a 30 minute rest period was allowed after the first concentration-response curve and then a second concentration-response curve was determined to the agonist.

7.2.3. The effect of electrical stimulation on the femoral vein of the rat.

In these experiments cocaine was omitted from the PSS to allow uptake mechanisms to function and refilling of nerve terminals to occur.

A single contraction was elicited to 60mM KCl in order to determine the maximum response of the tissue against which responses to electrical stimulation could be compared. The tissues were then washed and allowed to rest for a period of 30 minutes. The tissues were then electrically stimulated with platinum field electrodes. In initial experiments a range of stimulation frequencies, voltages and pulse widths were used to determine the optimum stimulation parameters for stimulation of the nerve supply without direct muscle stimulation.

In some experiments, after the determination of the response to KCl, a cumulative frequency-response curve (pulse width 0.1ms, stimulating voltage 95V) was determined. The tissue was stimulated at a low frequency and when the contraction reached plateau, the frequency was increased. This was repeated until further increases in stimulation frequency caused no further increases in tension. Tissues were then allowed to rest for a period of 45 minutes before a second frequency-response curve was determined.

In some experiments antagonist was added to the tissues 30 minutes before the determination of the second frequency-response curve and in other experiments tetrodotoxin (10^{-6}M) was added 15 minutes before the determination of the second frequency-response curve.

7.2.4. Effect of endothelium on responses to agonists.

In some experiments removal of the endothelium by mechanical rubbing using a small wooden stick or a piece of cotton was attempted. However, this procedure was found to drastically reduce the size of the contractile response

to any agonist and it was concluded that this procedure was causing damage to the smooth muscle of the vessels. It was also attempted to remove the endothelium by flushing the vessels with distilled water, but this produced similar results. It was then found that leaving the tissues mounted in the organ bath for a long period of time (over 20 hours) resulted in the absence of a relaxatory effect following the addition of ACh to precontracted tissues and visible signs of endothelial damage in preparations stained by the method of Griffith *et al*, (1984) (see below). It is suggested that this is because lack of oxygen and nutrients to the intimal surface causes the death of the endothelial cells.

To test for release of EDRF following the equilibration period, reproducible responses were elicited to a single agonist (10^{-6}M). A further response was then elicited and when the peak height of contraction was attained, acetylcholine (ACh, 10^{-6}M) was added. A relaxant response to this concentration of ACh was taken as an indication of the existance of a functional endothelium (Furchgott and Zawadzki, 1980). A rest period of 25 minutes was then allowed before agonist was reintroduced. The effect of ACh on this contraction was again ascertained. This procedure was performed repeatedly to investigate the effects of time on the contractile response to agonists and the effect of ACh on these responses, the tissues being left for periods of over 24 hours in some cases.

Tissues were also examined histologically for the presence of endothelium as described by Griffith, Henderson, Edwards and Lewis, (1984a). Tissues were bathed in 1% glutaraldehyde for 5 minutes and then in 1% silver nitrate for 2 minutes followed by a further 2 minutes in 3% cobalt bromide plus 3% ammonium bromide. Tissues were then rinsed in PSS before mounting and were examined under a light microscope.

7.2.5. Effects of calcium removal or calcium antagonists on responses to agonists.

Reproducible responses were elicited to a single agonist and then a 40

minute period was allowed during which time the preparations were washed 5 times. The bathing medium was then changed for calcium-free PSS containing 0.2mM EGTA and 2 minutes later agonist was added for 5 minutes after which normal PSS was reintroduced for 25 minutes to allow refilling of the intracellular calcium stores and then a further control response was elicited. Following a 15 minute rest period verapamil (VER, 10^{-5}M) was added to the bath. VER was left in contact with the tissues for 30 minutes before readdition of agonist. During the preincubation time in VER the bathing medium was changed 4 times, all washes being made in the presence of VER.

In some experiments the effect of calcium-free PSS without EGTA was studied before the effect of VER, using a procedure identical to that above.

In another set of experiments, the effect of VER on the agonist concentration-response curves was studied using the same protocol as in section 7.2.2.

STUDIES OF PHOSPHOINOSITIDE HYDROLYSIS.

7.3. Tissue preparation.

Tissues were dissected as described in section A.1 except that for this part of the study 10mm sections of veins were used. Following dissection and cleaning, the vessels were cut open to form flat sheets, lightly blotted and weighed.

7.4. Experimental methods.

Phosphoinositide hydrolysis was studied using an adaptation of the method of Best and Bolton (1986).

7.4.1. Incorporation of myo-^{3}H-inositol into the tissues.

The veins were washed in PSS and 24 vessels were incubated in 2 ml PSS containing myo-^{3}H-inositol (specific activity, 30μCi/ml) at 37°C gassed with 5% CO_2 in O_2 for 3 hours, in a shaking water bath. Tissues were then rinsed 4 times with PSS containing 10mM lithium chloride (lithium-PSS) and 2 vessels were placed into each of 12 test tubes containing 900μl lithium-PSS.

7.4.2. Stimulation of tissues.

After loading the tissues with myo-^{3}H-inositol for 3 hours, 100μl agonist or vehicle was added to the tubes which were then incubated for a further 60 minutes under the above conditions. At the end of this incubation period, stimulation was terminated by the addition of 3ml $CHCl_3$:CH_3OH:HCl (200:100:1).

In some experiments, designed to investigate the effect of antagonists on agonist-induced hydrolysis of phosphoinositides, antagonist was added 15 minutes before the addition of agonist.

7.4.3. Extraction of inositol phosphates.

Following the addition of $CHCl_3$:CH_3OH;HCl, the tubes were vortexed and then allowed to stand at room temperature for a period of 20 minutes and after 10 minutes the tubes were vortexed again for 20 seconds. The

mixture was then centrifuged at 500 x g for 5 minutes. A 1ml aliqout of the upper aqueous phase containing the inositol phosphates was removed, added to 3ml distilled H_2O and applied to a Dowex chromatography column (formate type, 1 x 2, 200 mesh) for inositol phosphate elution.

7.4.4. Elution of inositol phosphates.

Columns were washed with 10 ml distilled H_2O before inositol phosphates were eluted.

a). For determination of total inositol phosphate content, 15 ml of a solution of 0.1M formic acid / 1M ammonium formate was applied to the column and the eluate collected.

b). For determination of individual inositol phosphate content, 10 ml aliqouts of solutions of 0.1M formic acid containing 0.2, 0.4, 0.8 or 1.2M ammonium formate were added sequentially to elute the inositol mono-, bis-, tris-, and tetrakisphosphates respectively (Berridge *et al*, 1982). The eluates were collected in individual vials.

7.4.5. Determination of inositol phosphate content.

A 2 ml sample of the eluate was added to 10 ml scintillant (Optiphase) and the contents were mixed. Tritiated inositol phosphate content was measured by liquid scintillation counting for 10 minutes in a Beckman LS230 liquid scintillation counter. A background correction was made in duplicate in each experiment by counting 2 vials containing scintillant alone.

STUDIES OF cAMP CONTENT.

7.5. Tissue preparation.

10mm sections of veins were dissected and prepared as described previously (section 7.3). The vessels were blotted, weighed and each tissue was placed on a fine triangular stainless steel holder attached to a length of cotton to facilitate transfer of the tissues between solutions.

7.6. Experimental methods.

Studies of the effects of receptor stimulation on tissue cAMP content were made using an adaptation of the method of Itoh *et al* (1982).

The tissues were placed into test tubes containing 2ml PSS at 37°C gassed with 5% CO_2 in O_2 for 60 minutes in a shaking water bath. After this period agonist or vehicle was added to the tubes and after 3 minutes the tissues were removed and immersed in liquid N_2 to stop all reactions. Tissues were stored frozen until assayed for cAMP content. Tissues were homogenised with 1ml of 6% trichloroacetic acid and then centrifuged at 2500g for 15 minutes. The supernatant was extracted with 5 volumes of water-saturated ether and then evaporated to dryness under a stream of air. Tissue cAMP content was determined by radioimmunoassay using a commercially available cAMP radioimmunoassay kit (RIANEN).

In some experiments aimed to investigate the effect of antagonists on agonist-induced changes in cAMP levels, antagonist was added 15 minute before the addition of agonist. In other experiments the effect of shorter agonist contact times was studied.

7.7. Calculation of results and statistical analysis.

All results have been expressed and shown graphically as the mean ± s.e.m. of (n) experiments. Statistical analysis of data has been made using Students t-test for unpaired data, the Mann-Whitney test, or analysis of variance, as appropriate. The differences between two means were considered significant if $p<0.05$.

Agonist dose-ratio values were determined from the EC_{50} values of concentration-response curves in the presence and absense of antagonist. Least squares analysis was used to fit the best line through the linear portion of the curve and the EC_{50} value of each curve taken from this line. Curves were considered parallel if the 95% confidence limits for the slopes of these lines overlapped.

Initial experiments revealed that following the determination of the first agonist concentration-response curve, a subsequent curve was significantly different, indicating that a change in tissue sensitivity had occurred. For this reason, only one curve was determined in each tissue and data was therefore unpaired. In order to allow comparison of agonist response curves in the absence of antagonist against those in the presence of antagonist, the initial sensitising responses were compared and, when these were found not to be significantly different, the two sets of tissues were determined to be from the same population and therefore comparisons between these valid.

The type of antagonism was determined from the slope of Arunlakshana and Schild plots (Arunlakshana and Schild, 1959). When a plot of the logarithm of [dose ratio - 1] against the negative logarithm of the molar concentration of antagonist yielded a straight line with a slope of unity, blockade was considered competitive. The slope of the line was determined using least squares analysis. Under conditions of competitive antagonism, the pA_2 of the antagonist was determined as the intercept along the abscissa.

7.8. Materials, drugs and solutions.

Drugs and chemicals used in the study, and their suppliers are listed below:

Drugs and chemicals.	**Supplier.**
Acetylcholine Chloride	Sigma
Ammonium formate	Fisons
L-Ascorbic acid	BDH
B-HT920 hydrochloride	Boehringer Ingelheim*
Calcium chloride (aqueous)	BDH
cAMP radioimmunoassay kit	NEN
Cirazoline	Synthelabo*
Cocaine hydrochloride	Thornton and Ross
Corynanthine hydrochloride	Sigma
Ethylenediamine tetra-acetic acid (EDTA)	BDH
Ethyleneglycol tetra-acetic acid (EGTA)	Sigma
Formic acid (90%)	Fisons
(D)-glucose	BDH
Idazoxan hydrochloride	Reckitt and Colman*
myo-^{3}H- Inositol	Amersham
Lithium chloride	BDH
Magnesium chloride	BDH
Magnesium sulphate heptahydrate	BDH
(-) Noradrenaline bitatrate	Sigma
Optiphase scintillant	LKB
(L)-Phenylephrine hydrochloride	Sigma
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH
Prazosin hydrochloride	Pfizer*
(DL)-Propranolol hydrochloride	Sigma

Sodium chloride	BDH
Sodium hydrogen carbonate	BDH
ST587	Boehringer Ingelheim*
Tetrodotoxin	Sigma
Theophylline	Sigma
Trichloroacetic acid	Fisons
UK14,304	Pfizer*
(±)-Verapamil hydrochloride	Sigma / Abbot*

* indicates drugs generously donated as gifts.

All drugs were obtained as pure powders. Prazosin was prepared as a 10mM stock solution in 5% glucose / 5% glycerol and dilutions were made as required. All other drugs were prepared as 10mM stock solutions in distilled water and were frozen until required. Fresh stock solutions were prepared at the beginning of each week. Dilutions of all drugs were made in PSS (organ bath and cAMP studies) or lithium-PSS (inositol phosphate studies). All drugs were kept on ice throughout the course of the experiments and were discarded at the end of the day. Potassium chloride was prepared as a 3M stock solution.

All solutions of catecholamines contained 100μM ascorbic acid to reduce drug oxidation.

8. RESULTS SECTION I

CHARACTERISATION OF THE α-ADRENOCEPTORS OF THE FEMORAL VEIN OF THE RAT.

8.1. INTRODUCTION.

Postjunctional α_2-adrenoceptors are well documented *in vivo* (e.g. Drew and Whiting, 1979; Yamaguchi and Kopin, 1980). However, to date few *in vitro* preparations of vascular muscle have been shown to possess these receptors, although De Mey and Vanhoutte (1980) have reported that the canine saphenous vein possesses a heterogenous population of postjunctional α-adrenoceptors.

The evidence for the existence of postjunctional α_2-adrenoceptors in vascular muscle of the rat has arisen from *in vivo* studies in pithed or anaesthetised animals (e.g. Drew and Whiting, 1979; Timmermans, Kwa and Van Zwietten, 1979) and there is still no conclusive evidence that these receptors can be seen in *in vitro* preparations from the rat. The rat saphenous vein is a possible exception (Cheung, 1985) but this vessel is very small and the tension developed by it is only 5-10 mg, rendering it unsuitable for the detailed study of its characteristics in many laboratories. A rat vessel shown to possess a postjunctional population of α_2-adrenoceptors *in vitro* would be a very useful pharmacological asset since it would allow mechanistic studies, not possible *in vivo*, to be performed and also allow a more direct comparison to be made between *in vivo* and *in vitro* findings.

The initial aim of this study was to classify pharmacologically the postjunctional α-adrenoceptors of the femoral vein of the rat, making use of the selectivity and specificity of a number of α-adrenoceptor agonists and antagonists, in the hope that a population of postjunctional α_2-adrenoceptors could be demonstrated. This vessel was chosen for study because of its anatomical proximity to the saphenous vein, suggesting that it may share some of the characteristics of this vessel.

The effect of α_1- and α_2-antagonists on responses of the femoral vein to electrical stimulation of the nerves of the vessel was then investigated to determine whether the actions of endogenous, nerve-released catecholamines are mediated by both α_1- and α_2-adrenoceptors or by a single subtype.

Finally the role of the endothelium in the regulation of responses to α_1- and α_2-adrenoceptor stimulation was examined as it has been reported that the inhibitory effect of the endothelium is more pronounced against responses to α_2- than to α_1-adrenoceptor agonists (e.g. see Cocks and Angus, 1983 and Egleme *et al*, 1984) and Egleme and colleagues (1984) also suggest that α_2-adrenoceptors are present on the endothelium of the rat aorta, stimulation of which results in the release of EDRF (see General Introduction).

8.2. RESULTS.

8.2.1. Effects of α-adrenoceptor agonists on the femoral vein of the rat.

Initial experiments aimed to determine whether agonists of varying selectivities for α_1- and α_2-adrenoceptors caused contractile responses in the femoral vein of the rat. It was found that the non-selective α-adrenoceptor agonist noradrenaline (NA), the preferential α_1-adrenoceptor agonist phenylephrine (PE), the α_1-selective agonist cirazoline (CIR) and the α_2-selective agonists B-HT920 (BHT) and UK 14,304 (UK) all produced concentration-dependent increases in tension in the vessel (see Figure 8.1). Figure 8.1 shows that the time course of the contractile response to each agonist was dependent on the stimulant used, the differences between contractile forms showing a tendancy to be most marked at low concentrations. Responses to NA developed rapidly and were sustained; peak tension was attained approximately 1 minute following agonist addition. A rapid rise in tension was also seen following CIR addition but these contractions showed a transient form, the tension often falling to baseline levels within the 5 minute period of agonist contact. Contractions to PHE showed a form intermediate between those of NA and CIR; a rapid rise in tension was seen but the response was less well maintained than that to NA though less transient than the response to CIR.

In contrast to the rapid rise in tension seen for NA, CIR and PHE, the response of the femoral vein to BHT or UK developed slowly, taking up to 2.5 minutes to reach peak tension. Following the attainment of peak tension, the response was then relatively well maintained.

Figure 8.2 shows concentration response curves for those agonists used in the study. NA was a full agonist in this preparation and produced a maximum response of 1.03 ± 0.08 g at a concentration of 5×10^{-6}M. In contrast, the maximum responses observed for PHE, CIR, BHT, and UK were less than that to NA, suggesting that these may all be partial agonists in this preparation. The $E_{max.}$ values of the agonists, relative to that of NA, are

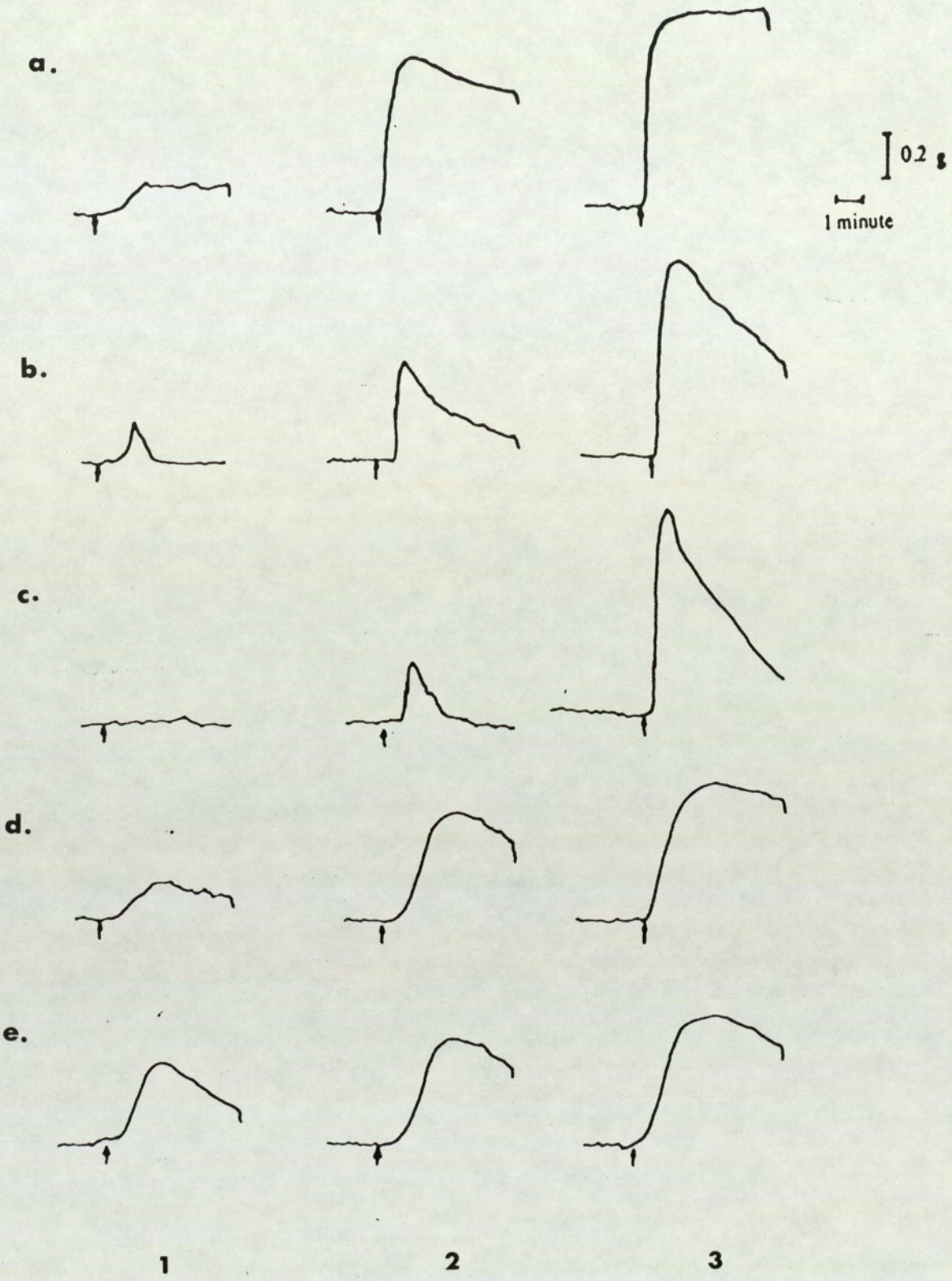


Figure 8.1. Representative traces showing contractile responses of the femoral vein to a) noradrenaline, b) phenylephrine, c) cirazoline, d) B-HT920 and e) UK 14,304. Agonist was added at the following concentrations; 1) $5x10^{-8}$M, 2) $1x10^{-7}$M and 3) $1x10^{-6}$M. The arrow indicates where agonist was added.

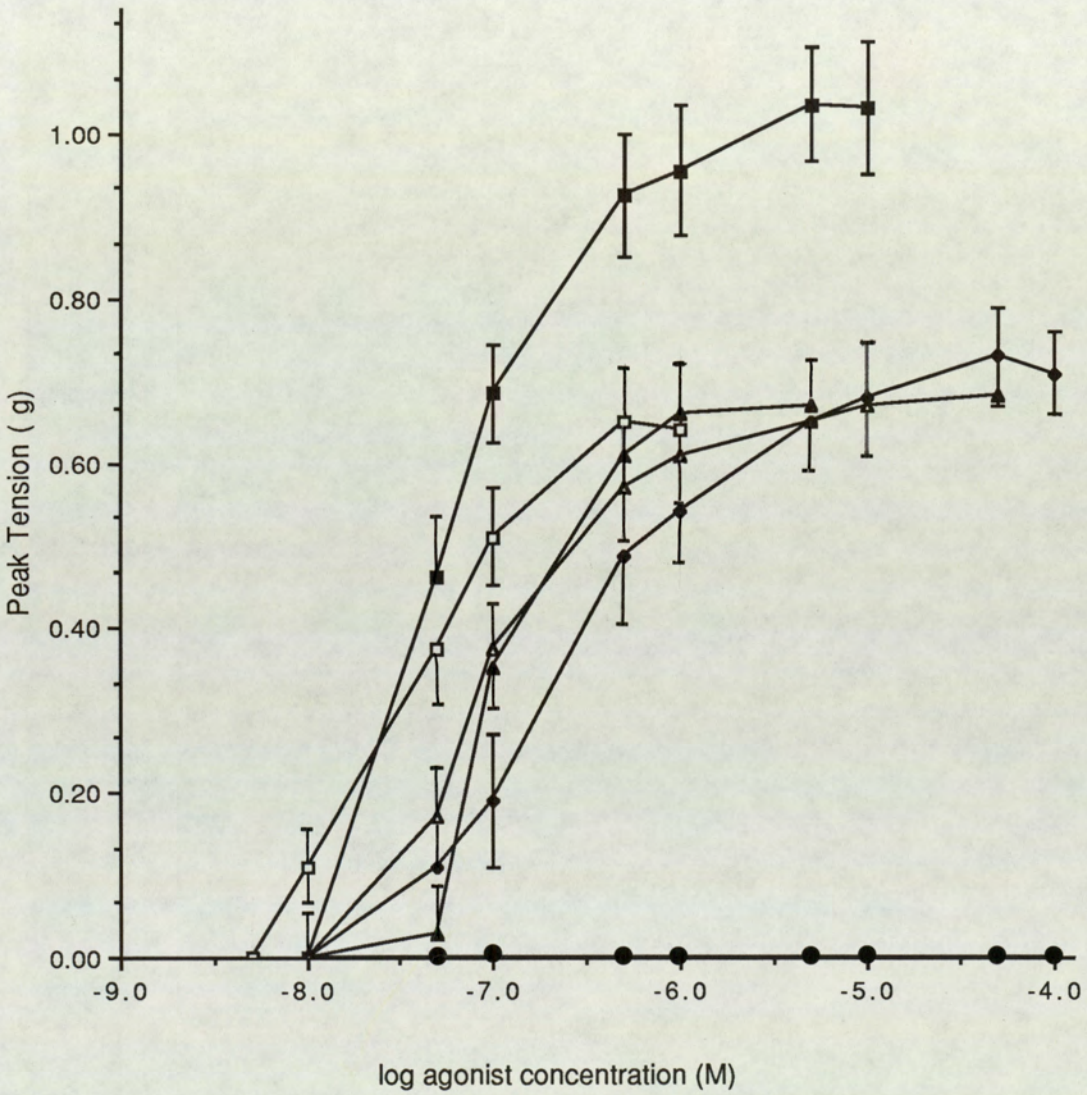


Figure 8.2. The response of the femoral vein to noradrenaline (■), phenylephrine (△), cirazoline (♦), B-HT920 (▲), UK14,304 (□) and ST587 (●).

Each point is the mean of 6-8 experiments and bars represent s.e.m..

shown in Table 8.1.

In the rat aorta, ST587 has been shown to be a partial agonist at α_1-adrenoceptors (e.g. Timmermans *et al*, 1983b). However, in the present study ST587 produced no changes in tension at concentrations up to 10^{-4}M.

These preliminary results suggest that the femoral vein of the rat may contain a postjunctional population of both α_1- and α_2-adrenoceptors.

The potencies of the agonists, expressed as EC_{50} values, were determined from Figure 8.2 and are shown in Table 8.1. The results indicate that the order of potencies of the agonists in the vessel is UK>NA>CIR=BHT>PHE, PHE being significantly less potent than the other agonists.

The addition of an α_1-selective agonist with an α_2-selective agonist did not cause summation of contractions (Figure 8.3), contractions to the combined agonists being significantly smaller than those to NA. The large contraction to NA thus may reflect the high efficacy of this agonist in addition to its ability to stimulate both α_1- and α_2-adrenoceptors, although an alternative suggestion could be that there may be competition between the two agonists when these are in contact with the tissue together.

8.2.2. The effects of antagonists on agonist induced contractions of the femoral vein of the rat.

These initial experiments indicated that the femoral vein of the rat may possess a significant population of postjunctional α_2-adrenoceptors. However, stronger evidence in support of this can be obtained from the use of agonists in combination with selective antagonists.Therefore, in the following sections, experiments aimed to investigate the effect of selective antagonists on responses of the tissues to agonists selective for either the α_1- or the α_2-adrenoceptor and also to non-selective α-adrenoceptor agonists.

	NA	PHE	CIR	BHT	UK	ST
EC50 ($x10^{-8}M$)	6.08 ±0.65	39 ±12	9.9 ±2.1	10.2 ±4.4	3.6 ±0.8	0
$E_{max.}$ (g)	1.03 ±0.08	0.74 ±0.07	0.67 ±0.03	0.68 ±0.04	0.65 ±0.05	0
$E_{max.}$ relative to NA.	1.0	0.72	0.65	0.66	0.63	0

Table 8.1. The EC_{50} values, E_{max} values and E_{max} values relative to NA of the agonists used in the study.

Values of EC_{50} and E_{max} are mean ± s.e.m..

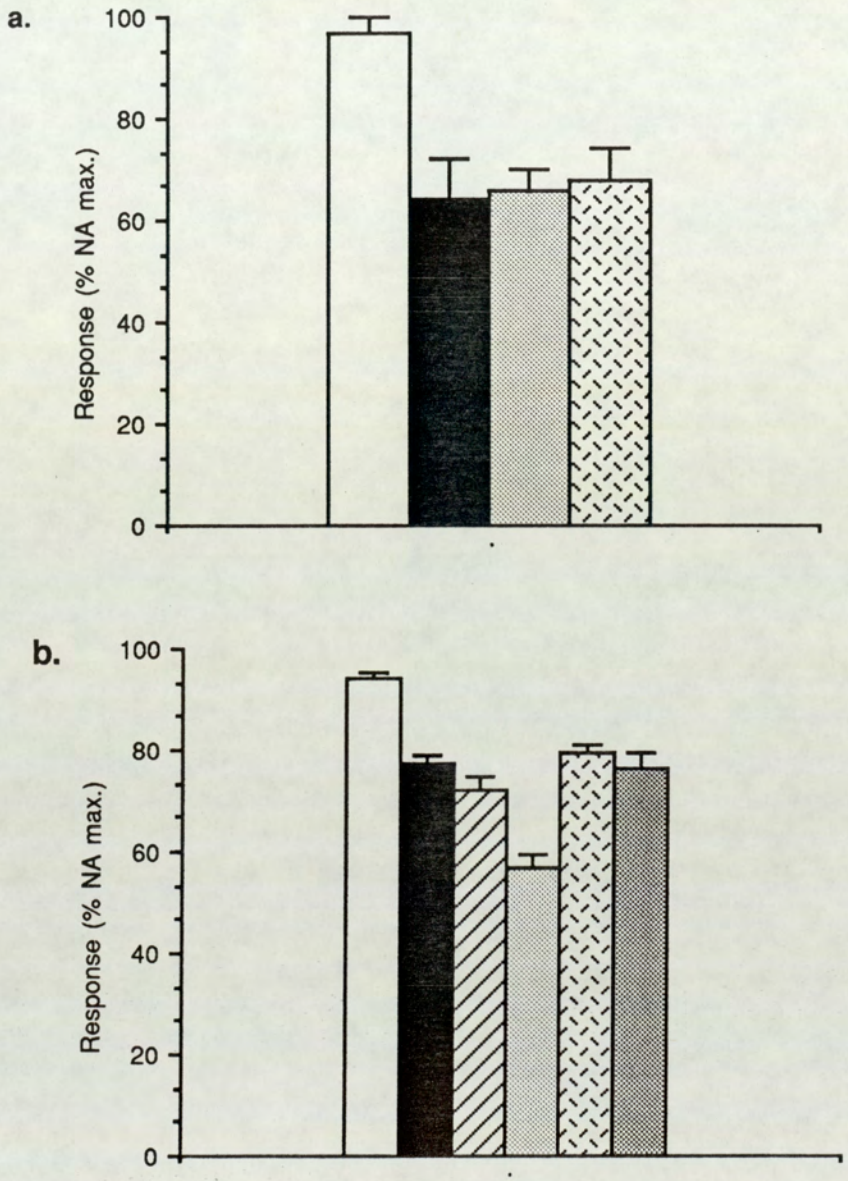


Figure 8.3. The magnitude of the response of the femoral vein of the rat to noradrenaline (□), phenylephrine (■), cirazoline (▨), B-HT920 (▩), phenylephrine + B-HT920 (▩) and cirazoline + BHT 920 (▩).

Figure a) shows responses for agonists added at 10^{-6}M while Figure b) those for the concentration of agonists producing the maximum effect as determined from Figure 8.2.

Columns are the mean of 6-8 experiments and the bars represent s.e.m..

8.2.2.1. Effect of idazoxan.

In these experiments the effect of the selective α_2-antagonist idazoxan upon contractions of the rat femoral vein to the non-selective agonists NA and PHE, the α_1-selective agonist CIR or the α_2- selective agonist BHT was determined.

Idazoxan caused a parallel shift of the concentration-response curves to BHT and NA to the right in a concentration dependent fashion with no decrease in the maximum response (Figures 8.4 and 8.5). The dose-ratio for each antagonist concentration was determined from Figures 8.4 and 8.5 and these were used to construct a Schild plot (Figure 8.6), the lines of the plot being fitted by least squares analysis using a microcomputer. This plot is linear for each agonist and the slopes approximated to unity which indicated that the antagonism shown by idazoxan was competitive in nature. pA_2 values for idazoxan acting against BHT and NA were determined from the Schild plots as 7.6 and 7.12 respectively. These values are similar to others cited in the literature for idazoxan acting at α_2-adrenoceptors (e.g. see Waterfall, Rhodes and Lattimer, 1985) and the results therefore indicate a population of postjunctional α_2-adrenoceptors in the vessel. The pA_2 values and slopes of the Schild plots are shown in Table 8.2.

Idazoxan also caused a parallel concentration-related shift of the concentration-response curve to PHE to the right (Figure 8.7). The dose-ratio for each antagonist concentration was determined and was used to construct a Schild plot (see Figure 8.6). The plot is linear with a slope different to unity (Table 8.2) indicating non-competitive antagonism. In contrast, ID ($5x10^{-6}M$) caused no significant shift in the concentration-response curve to CIR (see Figure 8.8) which indicated that CIR had no action at the α_2-adrenoceptors of this vessel. This concentration of antagonist was used because it represents a concentration greater than the pA_2 concentration as determined for BHT and NA in the earlier part of this section (see Figure 8.6).

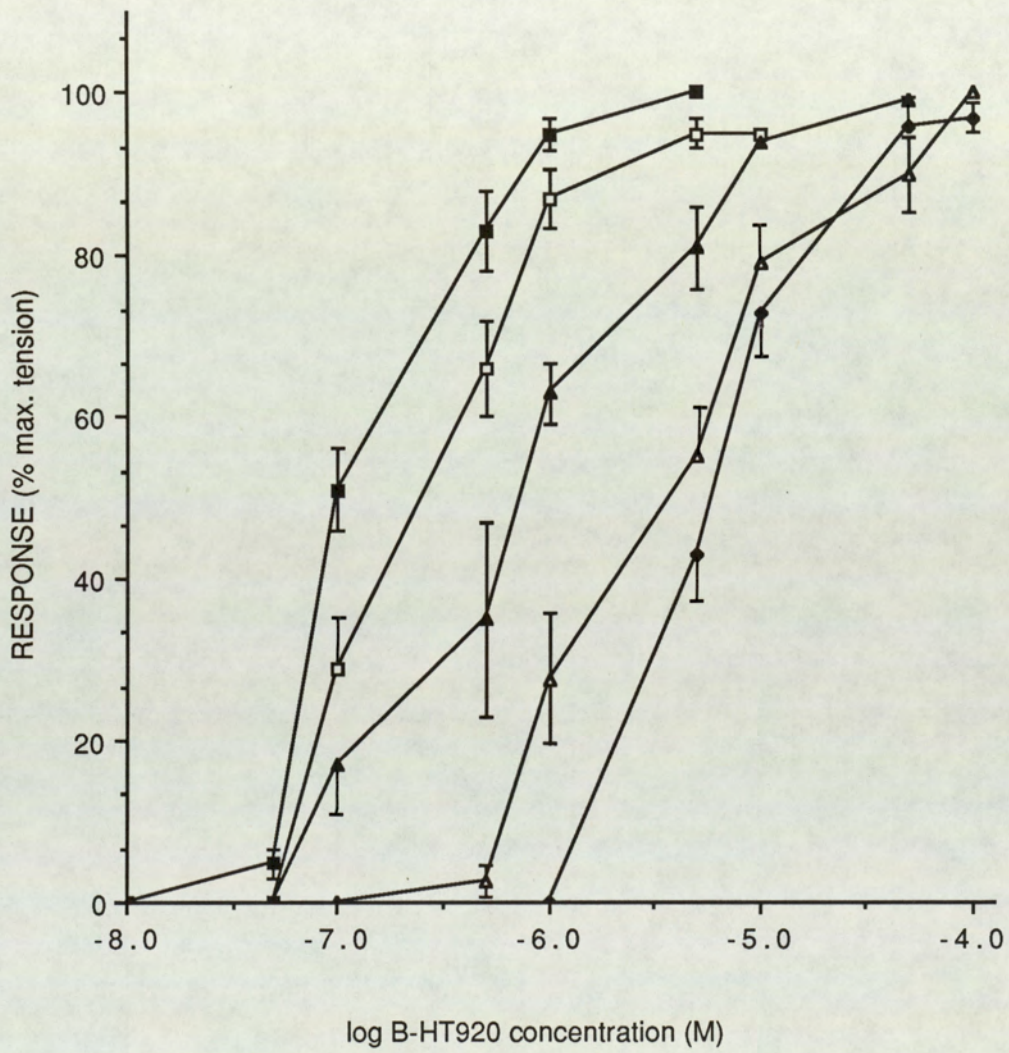


Figure 8.4. Log concentration response curves showing the effect of idazoxan on responses of the femoral vein of the rat to BHT.

Curves are BHT alone (■) and in the presence of ID at concentrations of $1x10^{-7}$M (□), $3x10^{-7}$M (▲), $5x10^{-7}$M (△) and $5x10^{-6}$M (◆).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

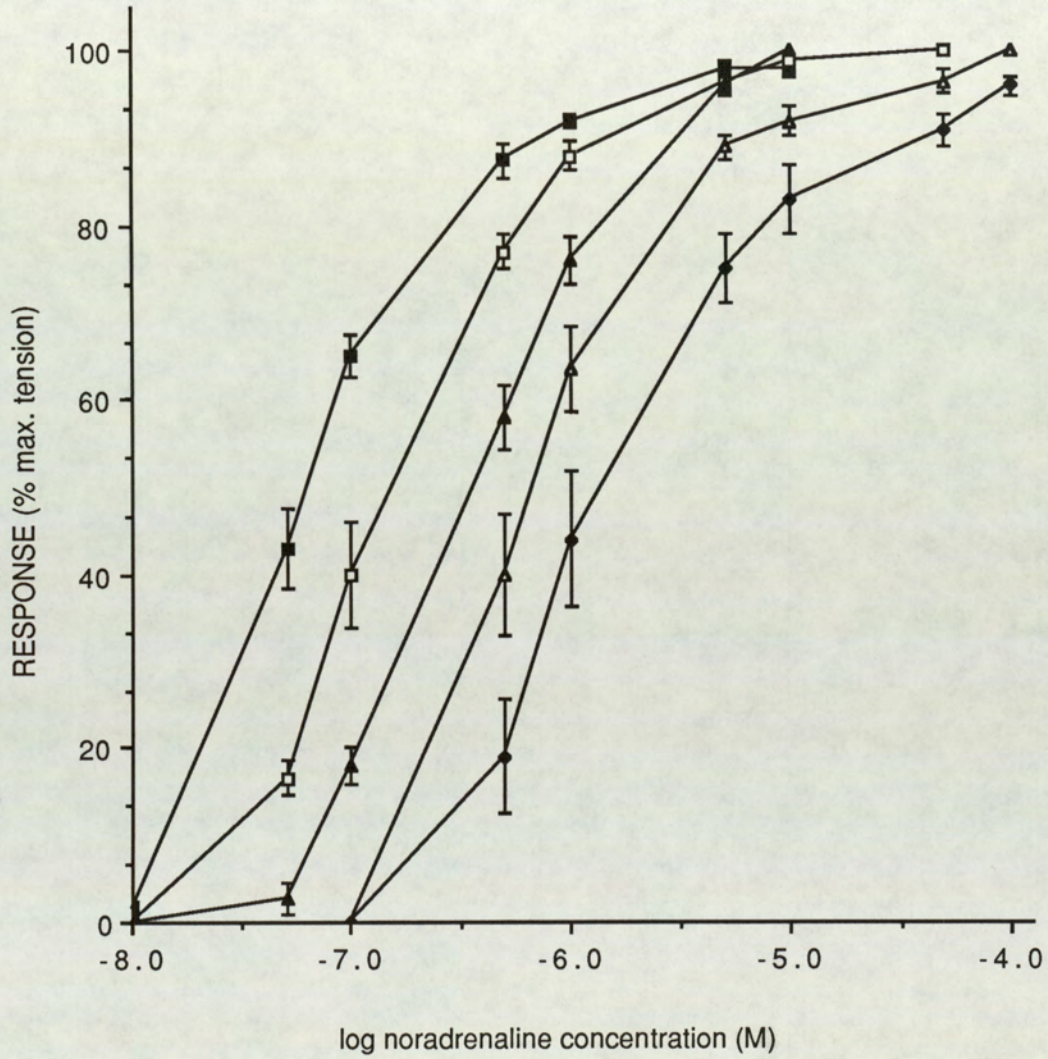


Figure 8.5. Log concentration response curves showing the effect of idazoxan on responses of the femoral vein of the rat to noradrenaline.

Curves are NA alone (■) and in the presence of ID at bath concentrations of $2x10^{-7}$M (□), $5x10^{-7}$M (▲), $2x10^{-6}$M (△) and $5x10^{-6}$M (◆).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

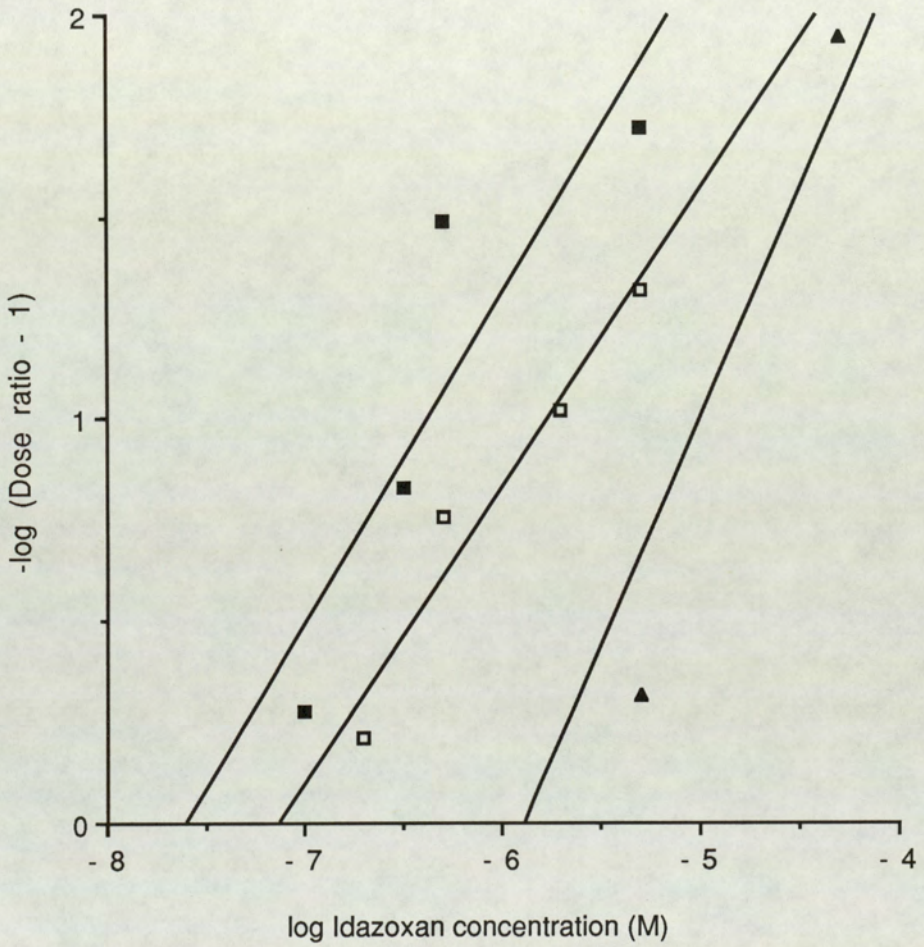


Figure 8.6. Schild plots showing the effect of idazoxan on responses of the femoral vein of the rat to BHT920, noradrenaline and phenylephrine.

Plots are BHT (■), NA (□) and PHE (▲) and are determined from Figures 8.4, 8.5 and 8.7 respectively.

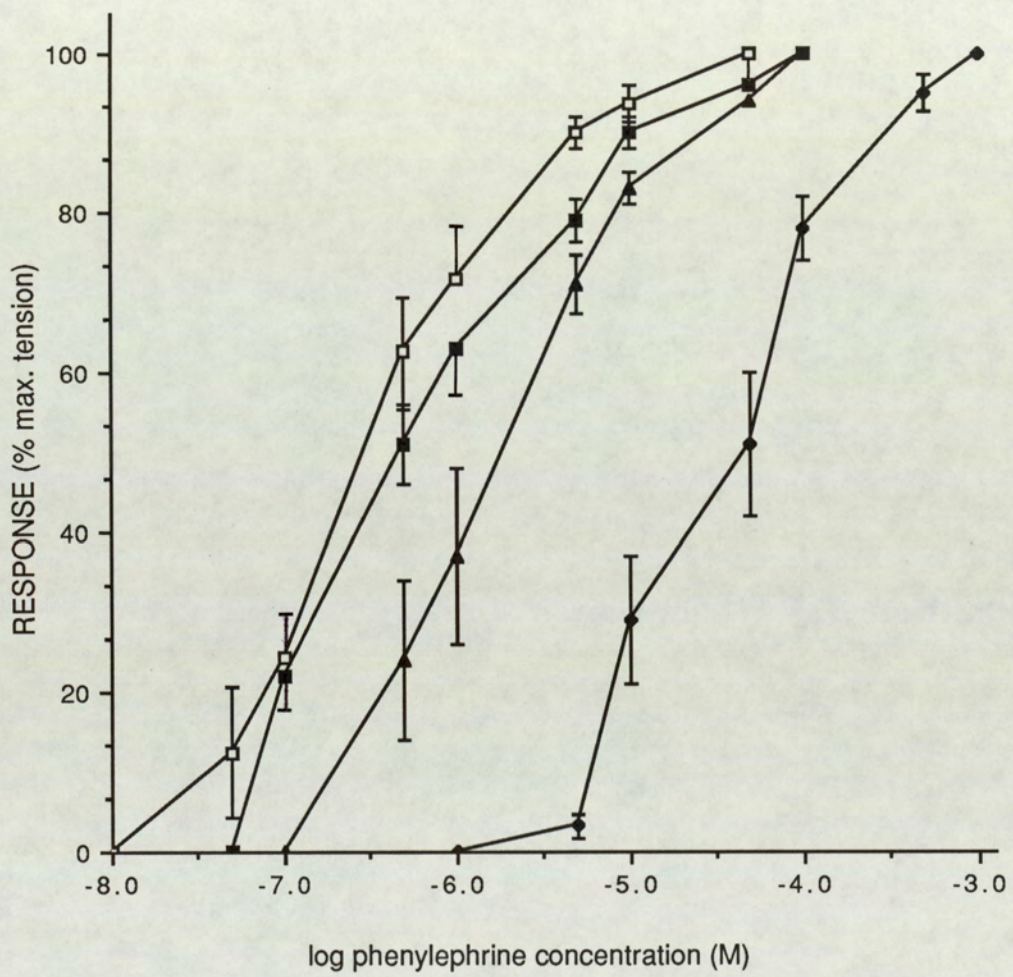


Figure 8.7. Log concentration response curves showing the effect of idazoxan on responses of the femoral vein of the rat to phenylephrine.

Curves are PHE alone (□) and in the presence of ID at bath concentrations of $5x10^{-7}$M (■), $5x10^{-6}$M (▲) and $5x10^{-5}$M (◆).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

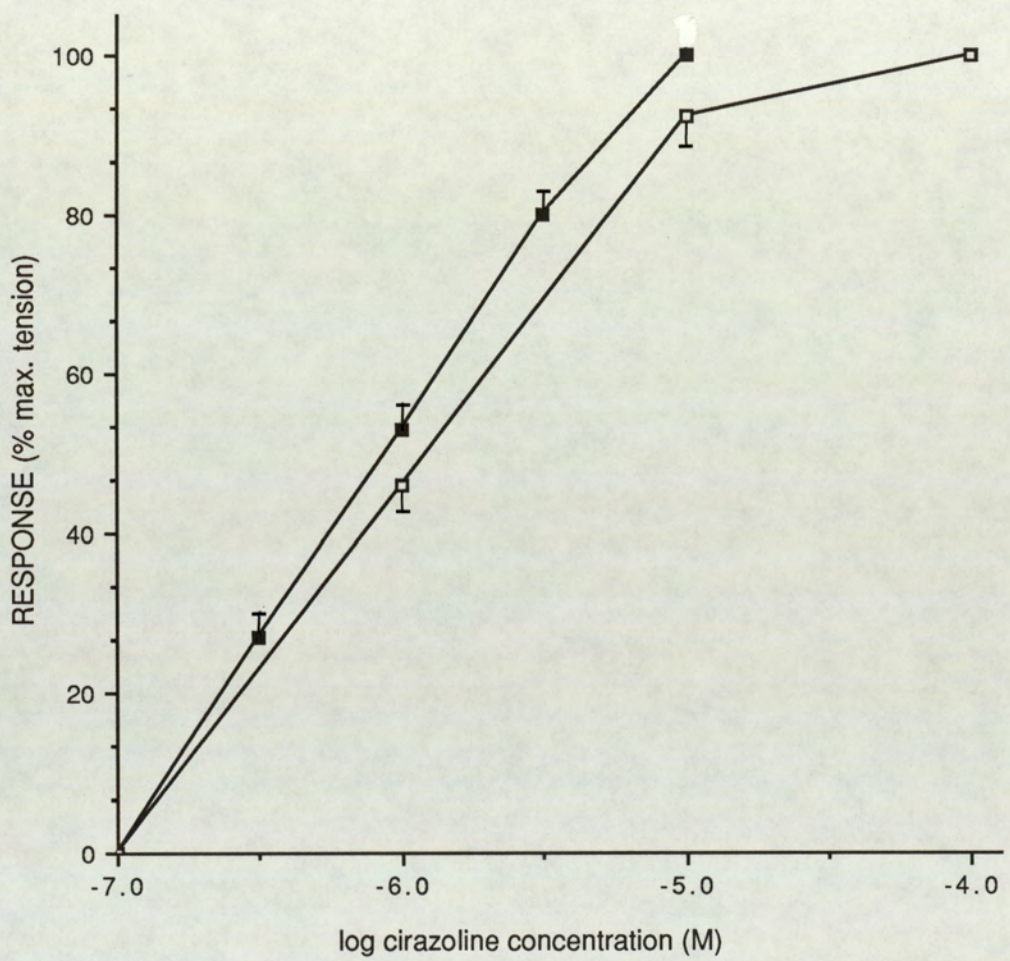


Figure 8.8. Log concentration response curves showing the effect of idazoxan on responses of the femoral vein of the rat to cirazoline.

Curves are CIR alone (■) and in the presence of ID $5x10^{-6}$M (□).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

ANTAGONIST	AGONIST	SLOPE	pA_2	r
IDAZOXAN	BHT 920	1.01	7.6	0.99
	NA	0.97	7.12	0.96
	PHE	1.13		0.94
CORYNANTHINE	NA	1.05	6.8	0.98
	CIR	1.02	6.67	0.96

Table 8.2. Values of pA_2 and the respective slope of the Schild plot and correlation coefficient for the agonists and antagonists used in the study.

(ii) Effect of α_1-antagonists.

The objective of this part of the study was to confirm that the α_2-agonists were eliciting contractile responses through stimulation of α_2- and not α_1-adrenoceptors.

The effect of the α_1-selective antagonist PZ on responses of the tissues to NA are shown in Figure 8.9. At a concentration of $1x10^{-8}$M, PZ caused a parallel shift of the concentration-response curve for NA to the right. However, higher concentrations of antagonist ($2x10^{-8}$M or $5x10^{-8}$M) had no effect on the NA concentration-response curve. PZ also had a similar effect on the concentration-response curve to PHE (Figure 8.10). Low concentrations of PZ (1 to $10x10^{-9}$M) caused a shift of the concentration-response curve to the right but increasing the concentration of antagonist to $5x10^{-8}$M resulted in a leftward displacement of the concentration-response curve so that this was now similar to control.

Because of these unexpected findings with PZ the effects of an alternative selective α_1-antagonist, corynanthine (COR) were examined on responses of the rat femoral vein to CIR, an α_1-agonist which had previously been shown to have no actions at the α_2-adrenoceptors of the tissue (Figure 8.8) and also to NA. The effect of COR on the concentration-response curves for CIR is shown in Figure 8.11. It can be seen that COR caused a parallel shift of the concentration-response curve to this agonist to the right in a concentration-dependent fashion. The dose-ratio for each antagonist concentration was determined from Figure 8.11 and these were used to construct the Schild plot shown in Figure 8.12. The plot is linear and the slope approximates to unity indicating that the antagonism shown by COR is competitive in nature (see Table 8.2). The pA_2 value for COR acting against CIR determined from the Schild plot was 6.67 (see Table 8.2).

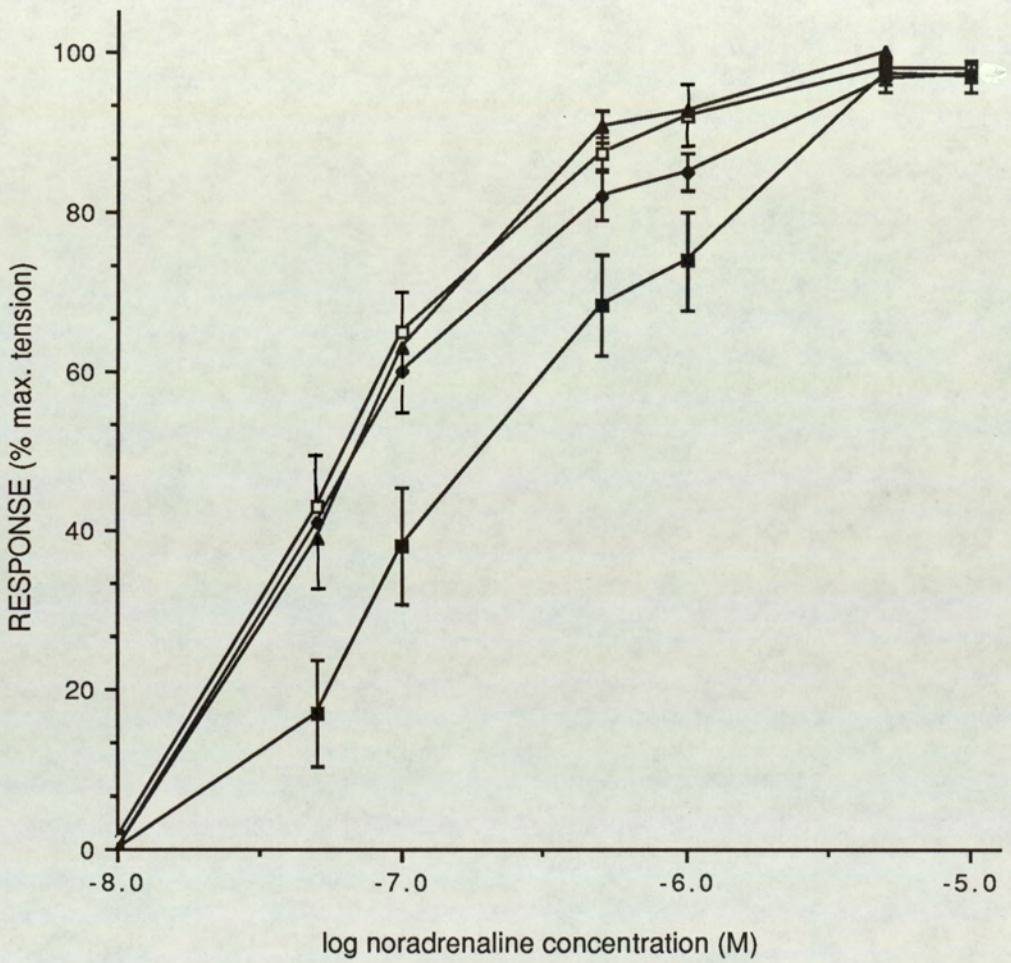


Figure 8.9. Log concentration response curves showing the effect of prazosin on responses of the femoral vein of the rat to noradrenaline.

Curves are NA alone (□) and in the presence of PZ at bath concentrations of $1x10^{-8}$M (■), $2x10^{-8}$M (▲) and $5x10^{-8}$M (◆).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

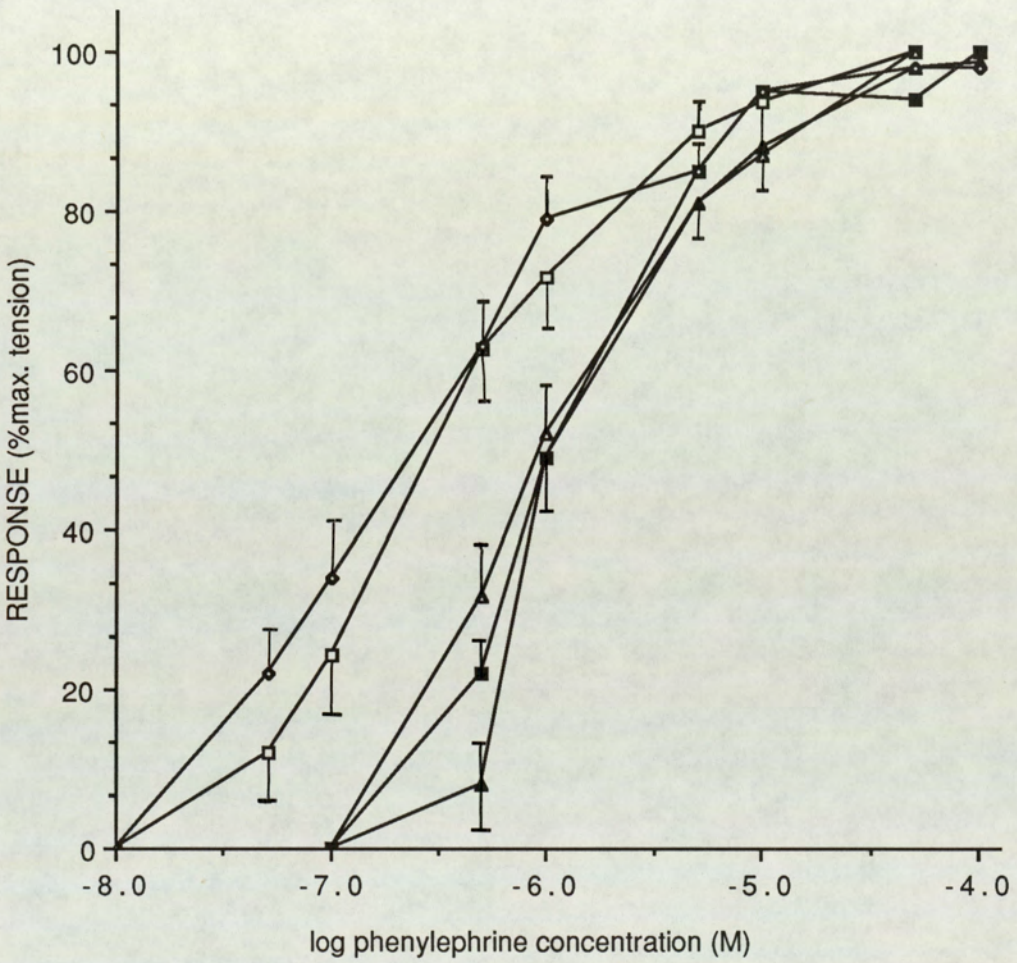


Figure 8.10. Log concentration response curves showing the effect of prazosin on responses of the femoral vein of the rat to phenylephrine.

Curves are PHE alone (□) and in the presence of PZ at concentrations of $1x10^{-9}$M (■), $5x10^{-9}$M (▲), $1x10^{-8}$M (△) and $5x10^{-8}$M (◆).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

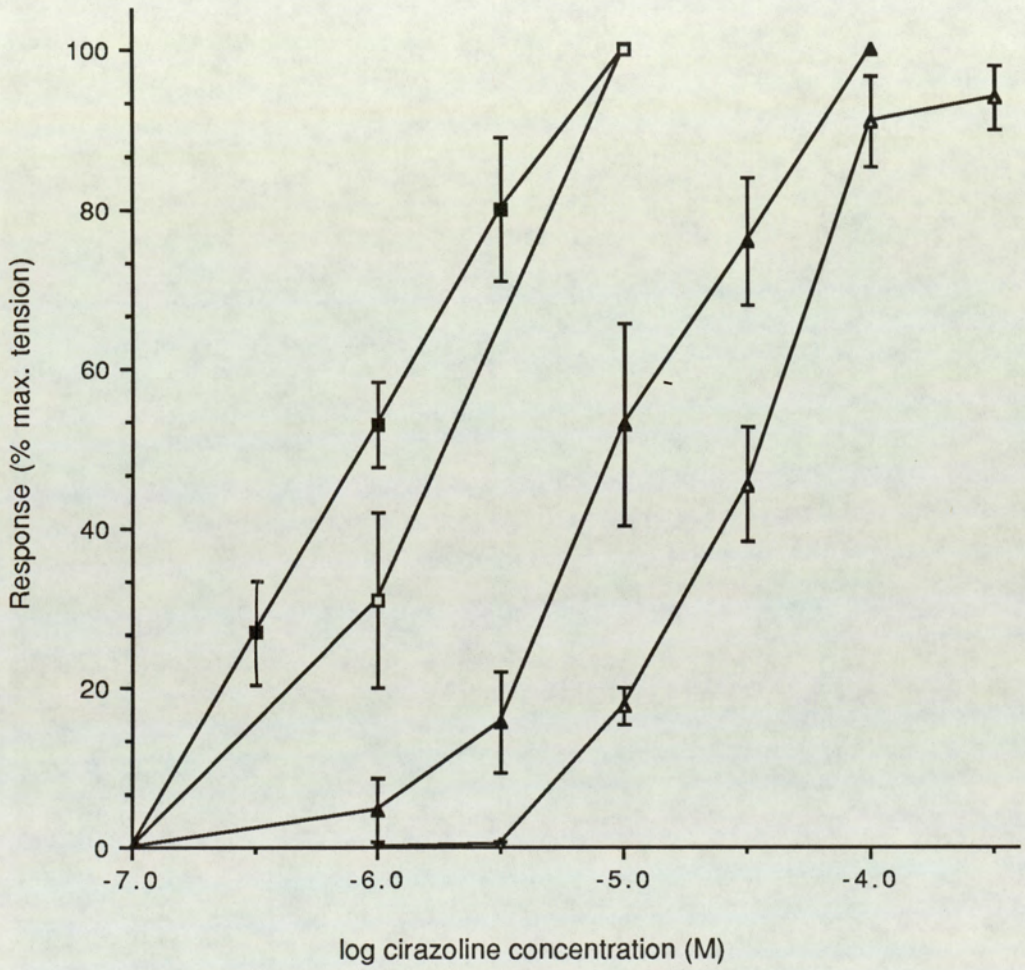


Figure 8.11. Log concentration response curves showing the effect of corynanthine on responses of the femoral vein of the rat to cirazoline.

Curves are CIR alone (■) and in the presence of COR at bath concentrations of $5x10^{-7}$M (□), $1x10^{-6}$M (▲) and $5x10^{-6}$M (△).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

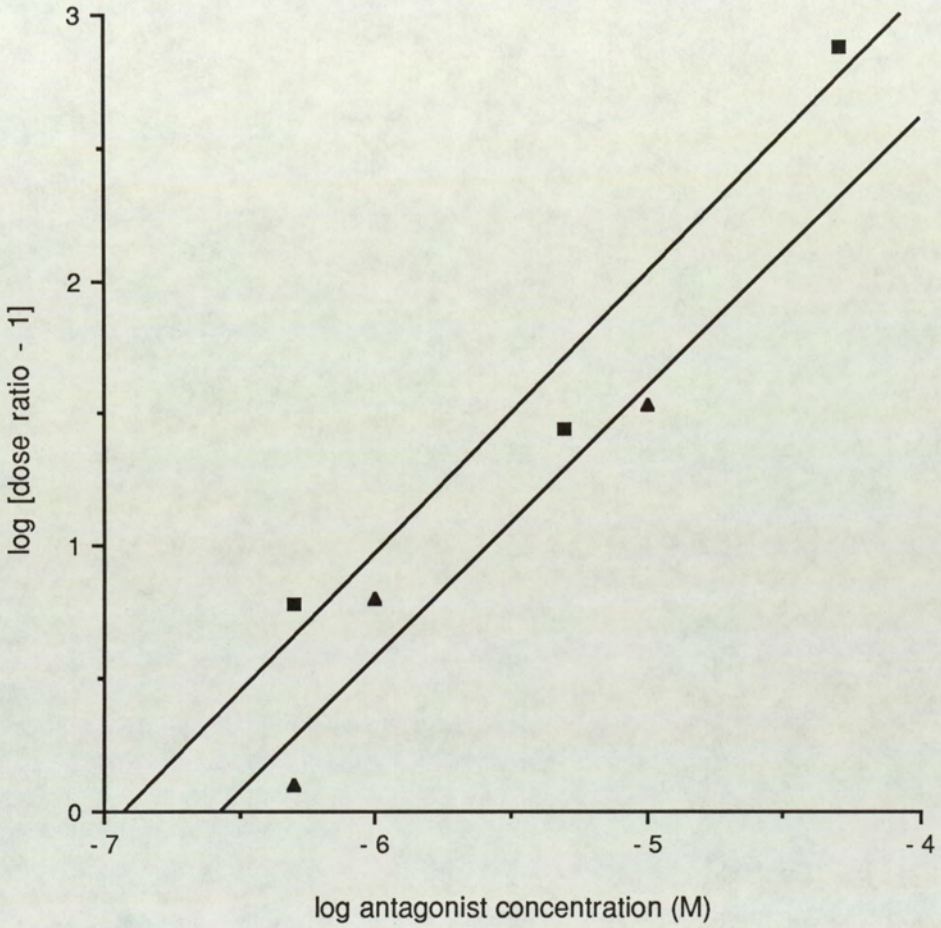


Figure 8.12. Schild plots showing the effect of corynanthine on responses of the femoral vein of the rat to noradrenaline and cirazoline.

Plots are CIR (▲) and NA (■) and are determined from Figures 8.11 and 8.13 respectively.

The effect of COR on the response of the tissue to NA is more difficult to interpret (see Figure 8.13). It can be seen from Figure 8.13 that COR produced a rightward shift of the NA concentration-response curve in a concentration-dependent fashion, but this shift did not appear to be of a simple parallel nature. There appears to be a kink in the concentration response curves obtained in the presence of higher concentrations of antagonist and this is at a different position for each antagonist concentration. Thus, as the concentration of COR is increased from $5x10^{-6}M$ to $5x10^{-5}M$, the position of the kink on the curve descends from 55 to 25% of the maximum response. This may be evidence of two different inhibition slopes for COR against the α_1- and α_2-mediated components of the NA response with the α_1-component being responsible for the upper portion of each slope; i.e. that portion greatly antagonised by COR. Thus, the lower portion of each curve represents the α_2-mediated component which is less affected by COR and is therefore shifted to a lesser degree (see Figure 8.13).

The dose-ratio for each antagonist concentration was taken at the 50% level and these were used to construct a Schild plot (Figure 8.12). This plot is linear with a slope near to unity indicating competitive antagonism at this level of the curve. A pA_2 value for COR acting against NA was determined from the Schild plot and this was 6.8. It is possible that if more concentration-response curves were available to contribute points to the Schild plot, then there would be a deflection point on the plot, enabling two straight lines to be drawn. One of these would have a slope of unity and would represent the α_1-antagonism of COR (corresponding to the line shown in Figure 8.12), while the second would have a slope less than unity and would represent the antagonism of the α_2-component of the NA response. Further, this second line would cross the x-axis at a significantly higher point than the first line.

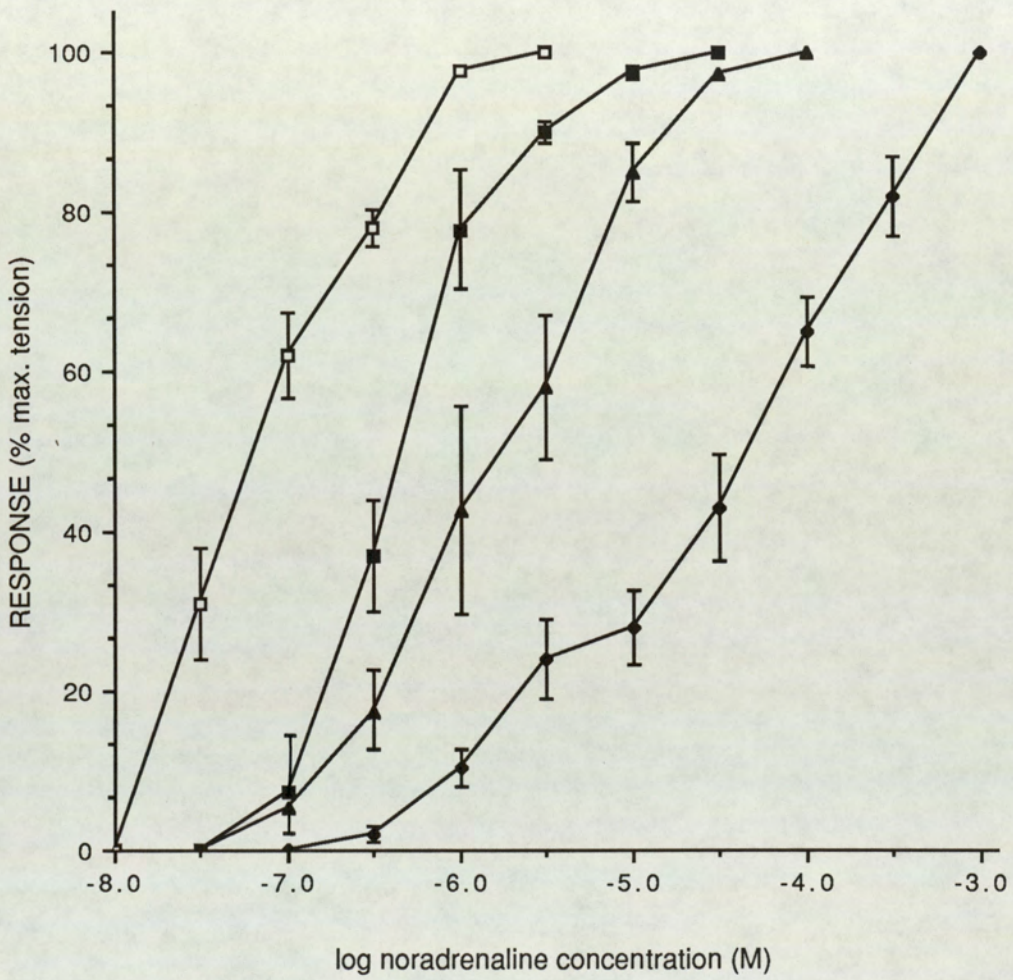


Figure 8.13. Log concentration response curves showing the effect of corynanthine on responses of the femoral vein of the rat to noradrenaline.

Curves are NA alone (□) and in the presence of COR at bath concentrations of $5x10^{-7}$M (■), $5x10^{-6}$M (▲) and $5x10^{-5}$M (◆).

Curves are the mean of at least 6 experiments and bars represent s.e.m.

Figure 8.14 shows the effect of two α_1-antagonists upon the concentration-response curve to the selective α_2-agonist BHT. Neither prazosin ($1x10^{-8}M$) nor corynanthine ($1x10^{-6}M$) had any significant effect on the concentration-response curve to BHT. These concentrations of antagonist were chosen because earlier experiments (see above) had shown them to antagonise the response of the tissue to the selective α_1-agonist CIR or the non-selective agonists NA and PHE (see Figures 8.9, 8.10 and 8.11). EC_{50} values obtained from these curves were ($x\ 10^{-8}M$) 9.9 ± 0.21 (n=7), 5.47 ± 1.2 (n=8) and 7.8 ± 1.6 (n=8) for BHT alone and in the presence of PZ or COR respectively.

8.2.3. Effect of antagonists on contractions of the tissues induced by electrical stimulation.

The results obtained with exogenous agonists and antagonists indicated that contractile responses of the femoral vein of the rat could be induced by stimulation of α_1- or α_2-adrenoceptors. In order to determine whether responses to catecholamines released from sympathetic nerves were mediated by both α-adrenoceptors or by a single subtype, the effect of selective α_1- or α_2-adrenoceptor antagonists on contractions induced by field stimulation was investigated.

Pilot experiments were made to determine the optimum stimulation parameters for the tissue (i.e. those parameters which gave maximal contractions of the tissue via nerve stimulation without causing direct stimulation of the vascular muscle). The parameters chosen were a stimulating voltage of 95V and a pulse width of 0.1ms. Figure 8.15.a shows representative traces for two consecutive frequency-response curves from a single tissue and in Figure 8.15.b the combined results from 8 tissues are represented graphically, the results being plotted as a percentage of the response to axogenous potassium chloride (60mM).

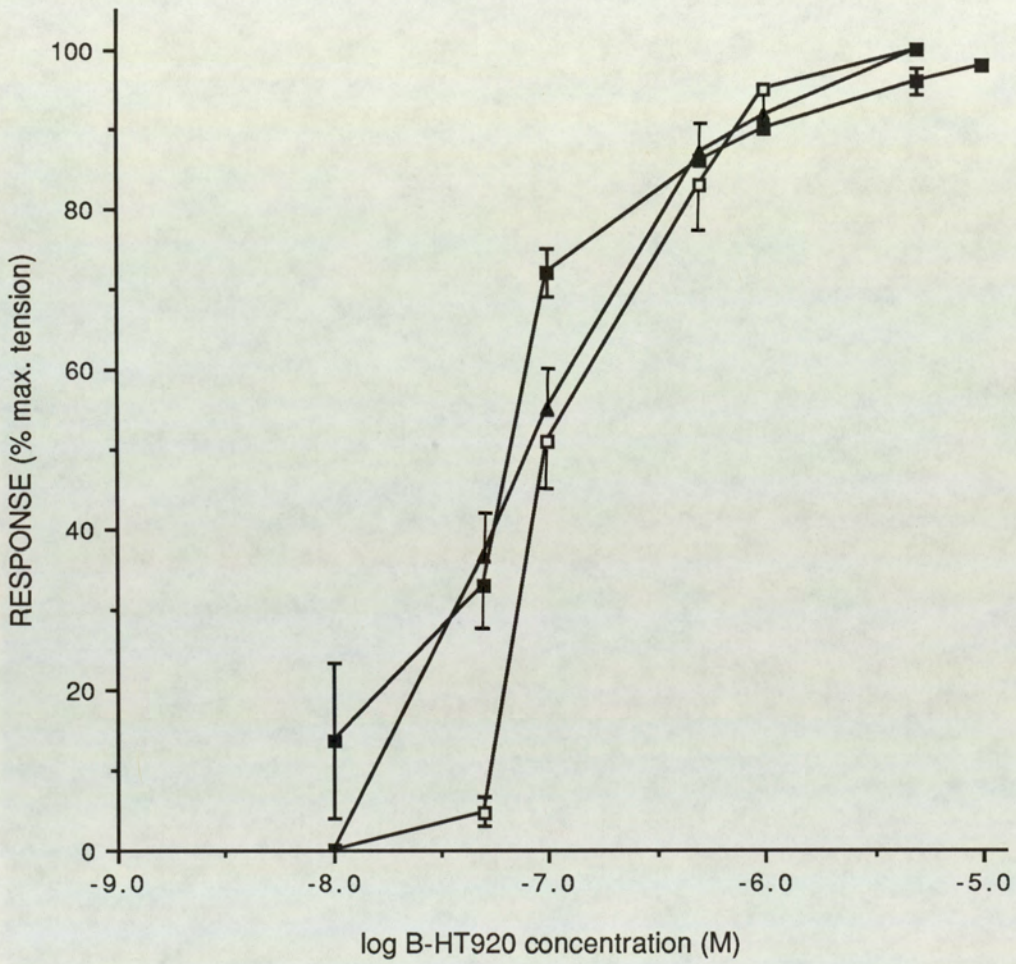


Figure 8.14. Log concentration response curves showing the effect of two α_1-antagonists on responses of the femoral vein of the rat to BHT920.

Curves are BHT alone (□) and in the presence of PZ ($1x10^{-8}$M, ■) and COR ($1x10^{-6}$M, ▲).

Curves are the mean of at least 6 experiments and bars represent s.e.m..

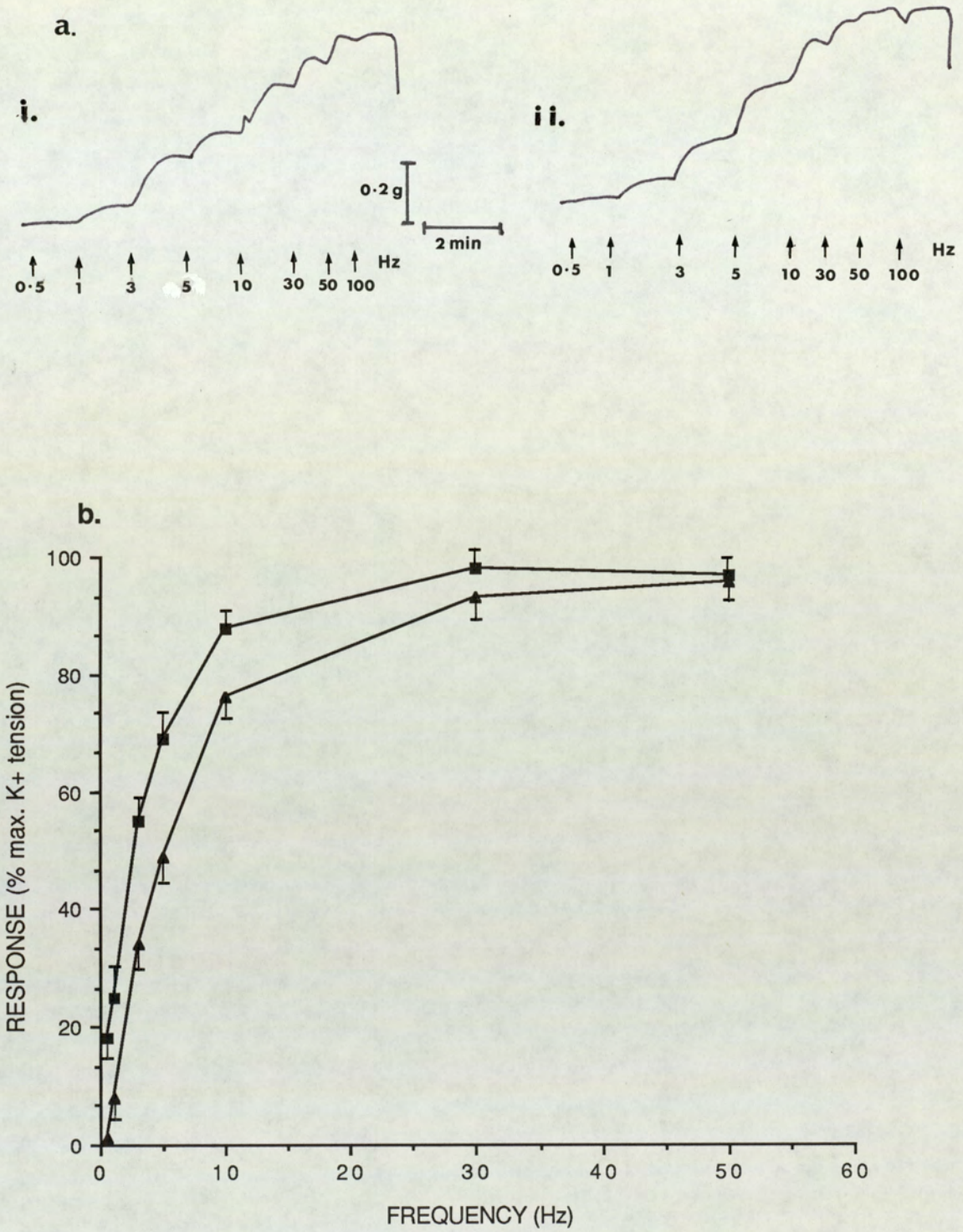


Figure 8.15. The effect of field stimulation on the contractile response of the femoral vein of the rat.

a) shows representative traces for two consecutive frequency-response curves from a single tissue and b) is a graphical representation showing the combined results from 8 tissues for curves 1 (■) and 2 (▲).

In b) each point is the mean of 8 experiments and bars represent s.e.m..

It can be seen that there is a threshold frequency of 0.5 Hz and that the maximum contraction occurs at a frequency of 10-30 Hz. It can also be seen that the responses were reproducible i.e. that the second frequency-response curve, determined 40 minutes after the first curve was not significantly different. Addition of tetrodotoxin ($1x10^{-6}M$) 15 minutes before the determination of the second frequency-response curve caused complete abolition of contractions in response to transmural stimulation indicating that the contractions were caused by endogenous catecholamines released from the sympathetic nerves and were not the consequence of direct stimulation of the smooth muscle fibres.

The effect of the selective α_1-antagonist corynanthine on the responses of the tissues to electrical stimulation is shown in Figure 8.16. COR ($5x10^{-7}M$ and $1x10^{-6}M$) caused a slight shift of the frequency-response curve to the right although this appeared to be of a similar magnitude for both concentrations of antagonist. However, this shift is similar to that seen for the second control curve (Figure 8.15) suggesting that COR caused no significant shift of the stimulation curve. A depression of the maximum response of the tissue was however seen at the higher concentration of antagonist. Figure 8.17 shows the effect of the selective α_2-antagonist idazoxan on responses to electrical stimulation. ID (1 and $5x10^{-7}M$) caused a dose-dependent shift of the frequency-response curve to the right and a depression of the maximum response occured at both antagonist concentrations.

These results therefore indicate that the response of the tissues to field stimulation is inhibited by either α_1- or α_2-antagonists, this conclusion being based on the depression of the maximum seen with the α_1-antagonist COR. This suggests that endogenous catecholamines released from sympathetic nerves act at both receptor subtypes and it would seem that this effect is greater at the α_2- rather than the α_1- adrenoceptor.

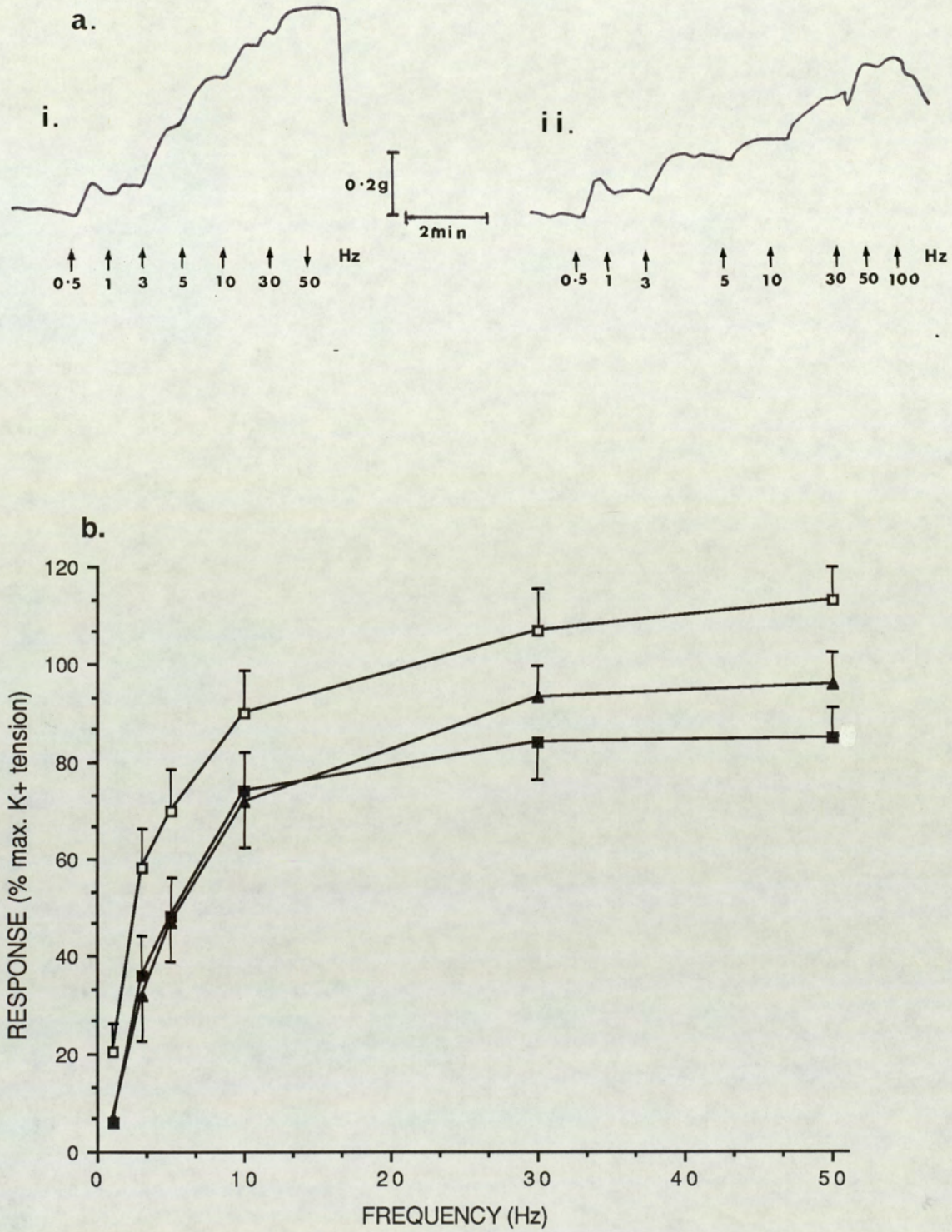


Figure 8.16. The effect of the α_1-antagonist COR on the contractile response of the femoral vein of the rat to field stimulation.

a) shows representative traces for two consecutive frequency-response curves; (i) control and (ii) in the presence of $1x10^{-6}$M COR, and in b) curves are control (□) and in the presence of COR at a bath concentration of $5x10^{-7}$M (▲) and $1x10^{-6}$M (■).

Each point is the mean of 8 experiments and bars represent s.e.m..

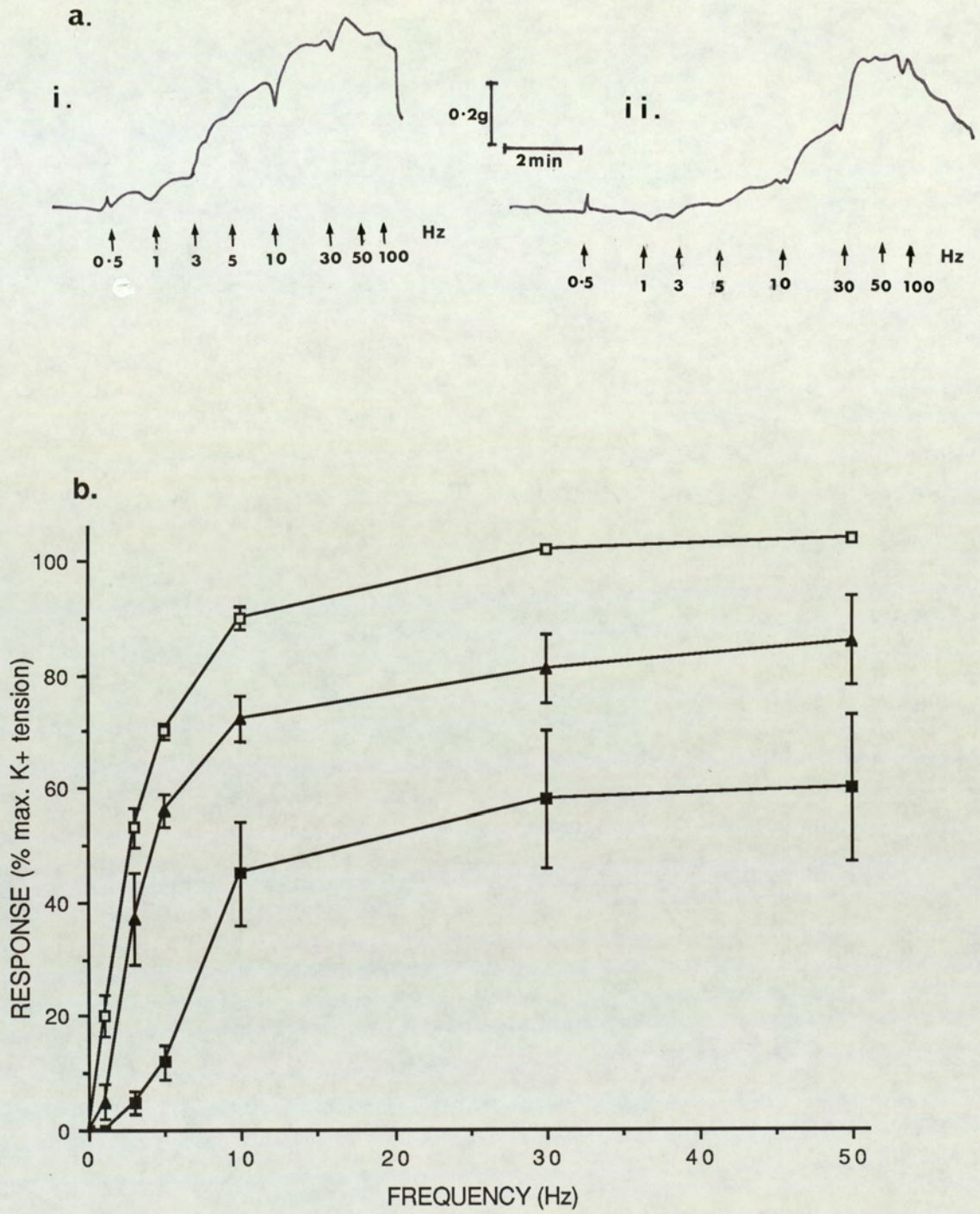


Figure 8.17. The effect of the α_2-antagonist ID on the contractile response of the femoral vein of the rat to field stimulation.

a) shows representative traces for two consecutive frequency-response curves; (i) control and (ii) in the presence of $5x10^{-7}$M ID, and in b) Curves are control (□) and in the presence of ID at a bath concentration of $1x10^{-7}$M (▲) and $5x10^{-7}$M (■).

Values are the mean of 7 experiments and bars represent s.e.m..

8.2.4. Influence of the endothelium on responses of the vessel to exogenous agonists.

The importance of the vascular endothelium as a regulating influence on vascular reactivity has become increasingly obvious over the past few years. Under certain conditions, the endothelium has been shown to exert either a contractile (via the release of endothelin, see Yanagisawa *et al*, 1988) or a relaxant (via EDRF, see Furchgott and Zawadzki, 1980) effect on vascular muscle. It has been suggested that EDRF may exert a greater effect on responses of tissues to partial rather than to full agonists (e.g. see Egleme *et al*, 1984) and Egleme *et al* (1984) have also suggested that a population of α_2-adrenoceptors is present on the endothelium of vascular muscle, stimulation of which results in the release of EDRF (see General Introduction).

It was decided to investigate the effect of ACh (as an indicator of endothelial viability) on responses to NA, BHT and CIR in the rat femoral vein in order to determine whether the endothelium has a more pronounced regulatory role on α_2-mediated responses than on those mediated by α_1-agonists. At a concentration of $1x10^{-6}$M, ACh produced a 50 ± 4.4% relaxation of the contractile response of the femoral vein to NA (10^{-6}M) and a relaxation of the contractile response to BHT (10^{-6}M) and CIR (10^{-6}M) of 79 ± 3.6% and 83 ± 6% respectively. Attempts were made to remove the endothelial layer by mechanical rubbing of the intimal surface of the vessels (e.g. with small wooden sticks or cotton), and also by infusion of the vessels with distilled water. However, these procedures were found to drastically reduce the magnitude of the contractile response to NA and it seemed likely that this was due to damage to the smooth muscle of the vessels. It was therefore decided to leave the vessels set up in the organ baths for a long period of time (20 hours) in the hope that the lack of oxygen to the intimal surface would result in the death of the endothelial layer. Challenge of tissues maintained in this way with ACh did not result in a relaxatory response. Histological examination of vessels both before and after prolonged incubation revealed that viable endothelium was not present on the latter tissues (see Plates 1 and

2).

Over a period of 5 hours, tissues were repeatedly contracted with a single agonist and, at peak contraction, challenged with ACh (10^{-6}M). Figures 8.18, 8.19 and 8.20 show how the size of the contraction and the degree of relaxation caused by ACh varied with time for the three agonists studied. The magnitude of the contractile response to NA increased steadily over the first 90 to 130 minutes of the experiment and then remained constant for the remainder of the experiment (see Figure 8.18.a). Figure 8.18.a also shows how the relaxant effect of ACh remained constant for the first 150 minutes of the experiment and then steadily fell over the next 180 minutes. After a period of 20 hours, ACh caused no relaxation of NA-contracted vessels (see Figure 8.18.b). The diminishing size of the relaxation to ACh was taken to be indicative of decreased endothelial viability and a histological study of tissues confirmed that 20 hours after mounting, there was no viable endothelium (see Plates 1 and 2). Figure 8.18.b shows representative traces demonstrating the effect of ACh on contractions to NA at 60 minutes, 300 minutes and 20 hours. It is worthy of note at this point that the period of the experiment over which the magnitude of the response to 10^{-6}M NA increased (i.e. the first 150 minutes) coincided with the sensitising period of previous experiments.

Figures 8.19.a and b show similar responses for CIR. The contractile response of the tissue increased steadily over the initial stages of the experiment (0 to 100 minutes) and then remained relatively constant. Also, the relaxatory response to CIR was fairly constant over the early stages of the experiment and then fell sharply over the latter stages. After 20 hours, the challenge of CIR-contracted tissues with ACh caused no relaxation (see Figure 8.19.b). Representative traces showing the effects of CIR and of ACh at 50 minutes, 300 minutes and 20 hours are presented in Figure 8.19.b.

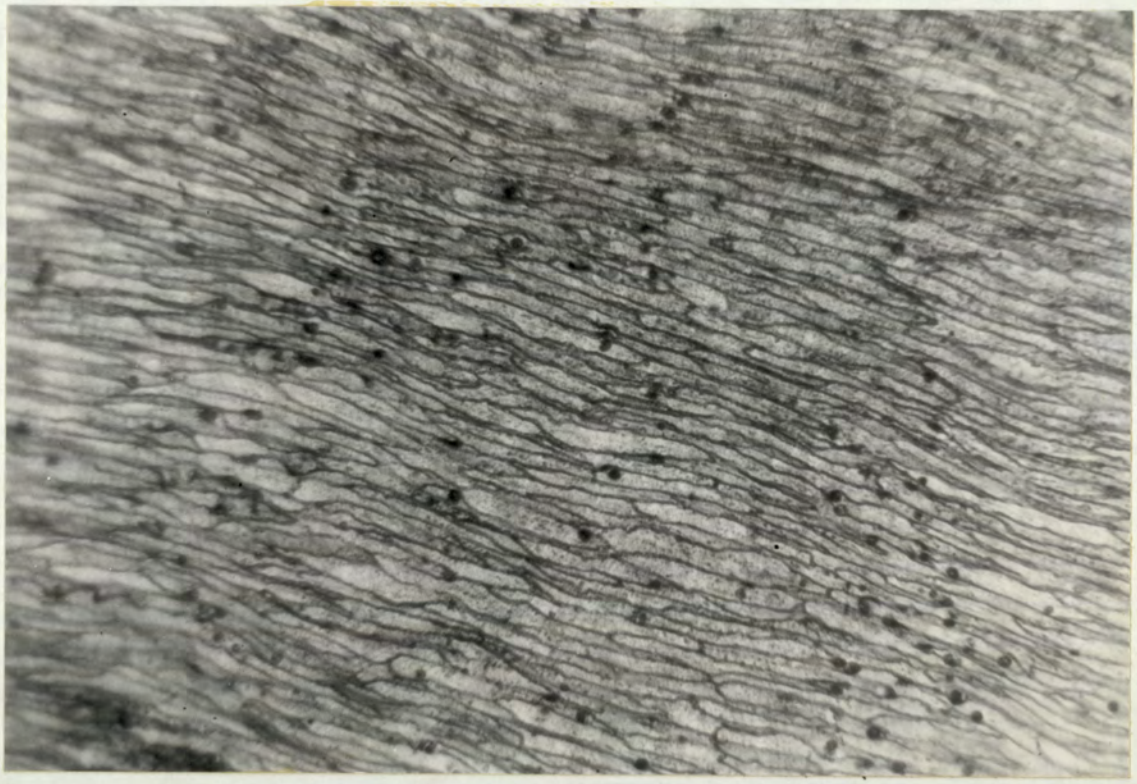


Plate 1

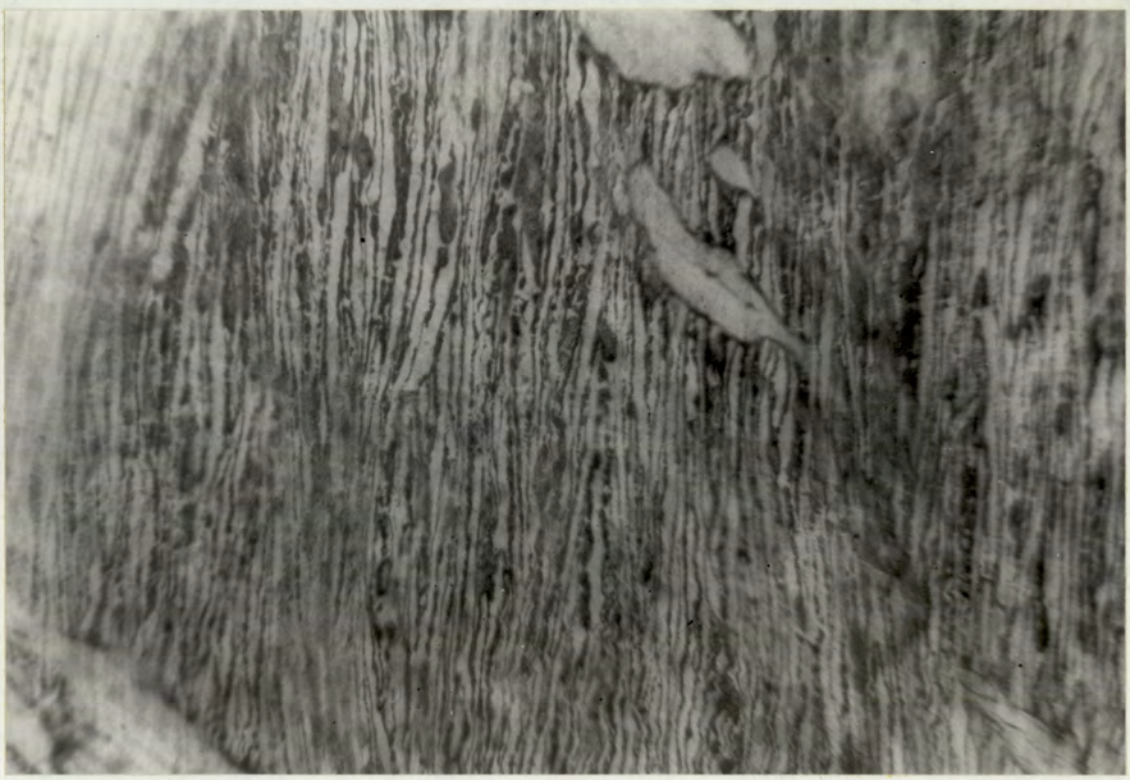


Plate 2.

Plate 1. Endothelial surface of a segment of femoral vein stained after 1 hour equilibration in an organ bath.

Plate 2. Endothelial surface of a segment of femoral vein stained after 20 hours equilibration in an organ bath.

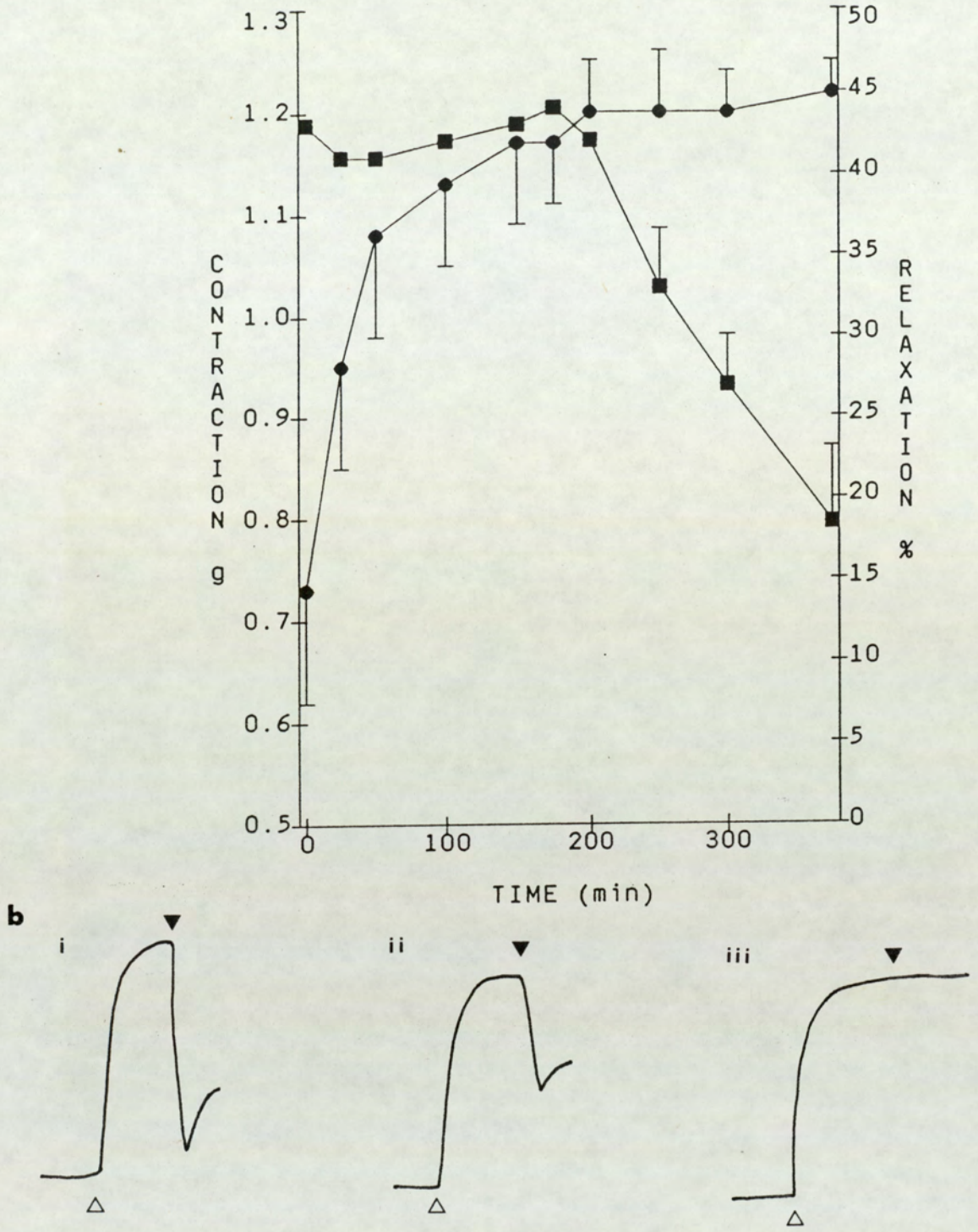
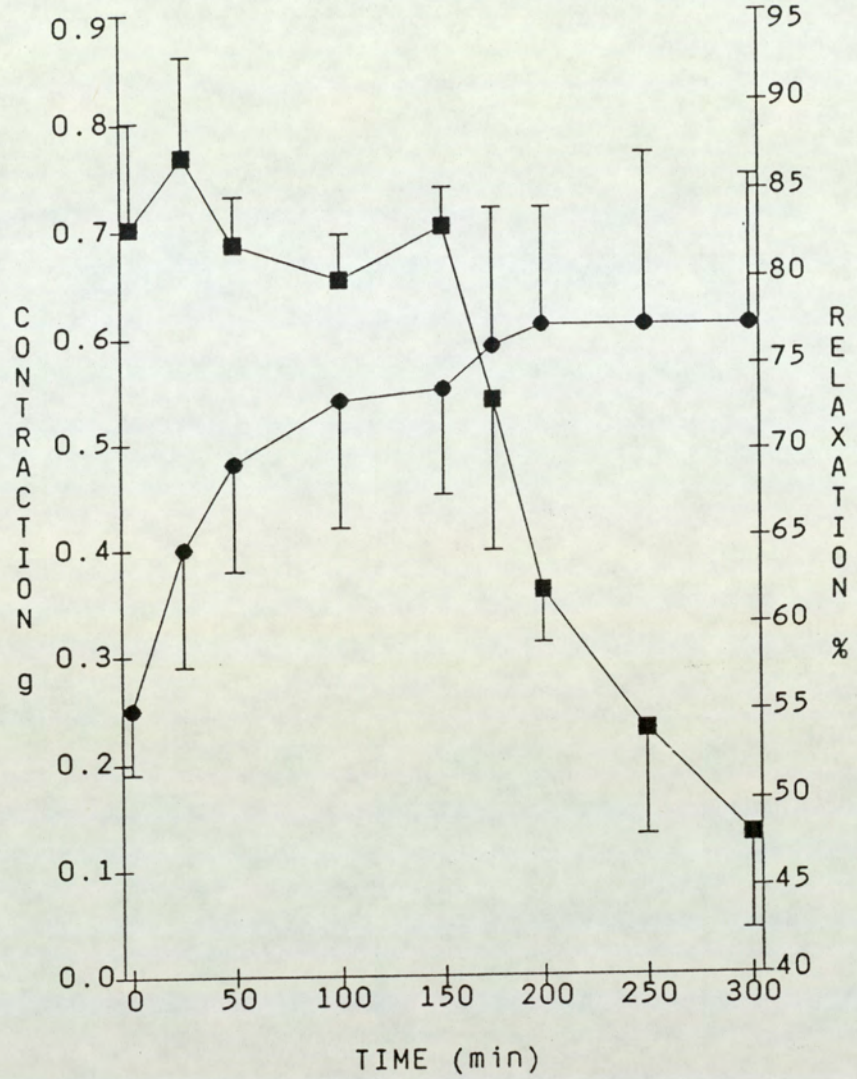


Figure 8.18. The effect of time upon the contractile response of the femoral vein of the rat to NA and upon the relaxation of this response induced by Ach (10^{-6}M).

a) shows graphically mean responses ± sem for 8 tissues. ● indicates contractions to NA (10^{-6}M) and ■ the relaxation of the NA response induced by ACh.

b) shows representative traces at i) 60 minutes, ii) 300 minutes and iii) 20 hours after mounting of the tissues. △ indicates addition of NA (10^{-6}M) and ▼ indicates addition of ACh.



b

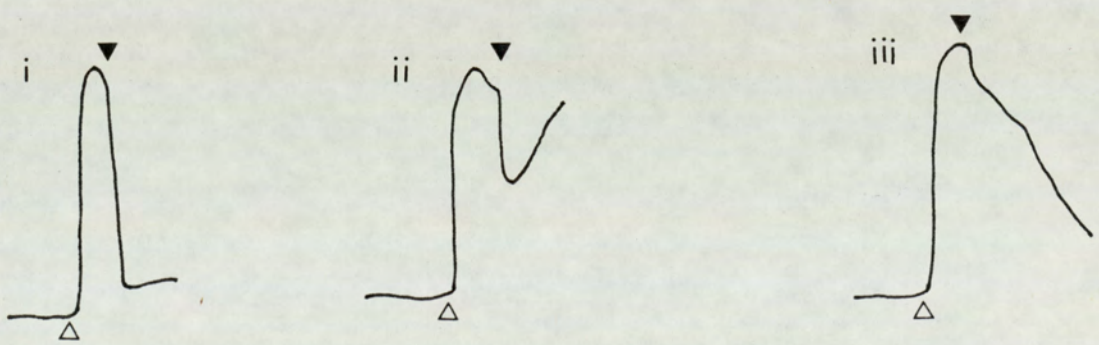


Figure 8.19. The effect of time upon the contractile response of the femoral vein of the rat to CIR and upon the relaxation of this response induced by Ach (10^{-6}M).

a) shows graphically mean responses ± sem for 8 tissues. ● indicates contractions to CIR (10^{-6}M) and ■ the relaxation of the CIR response induced by ACh.

b) shows representative traces at i) 60 minutes, ii) 300 minutes and iii) 20 hours after mounting of the tissues. △ indicates addition of CIR (10^{-6}M) and ▼ indicates addition of ACh.

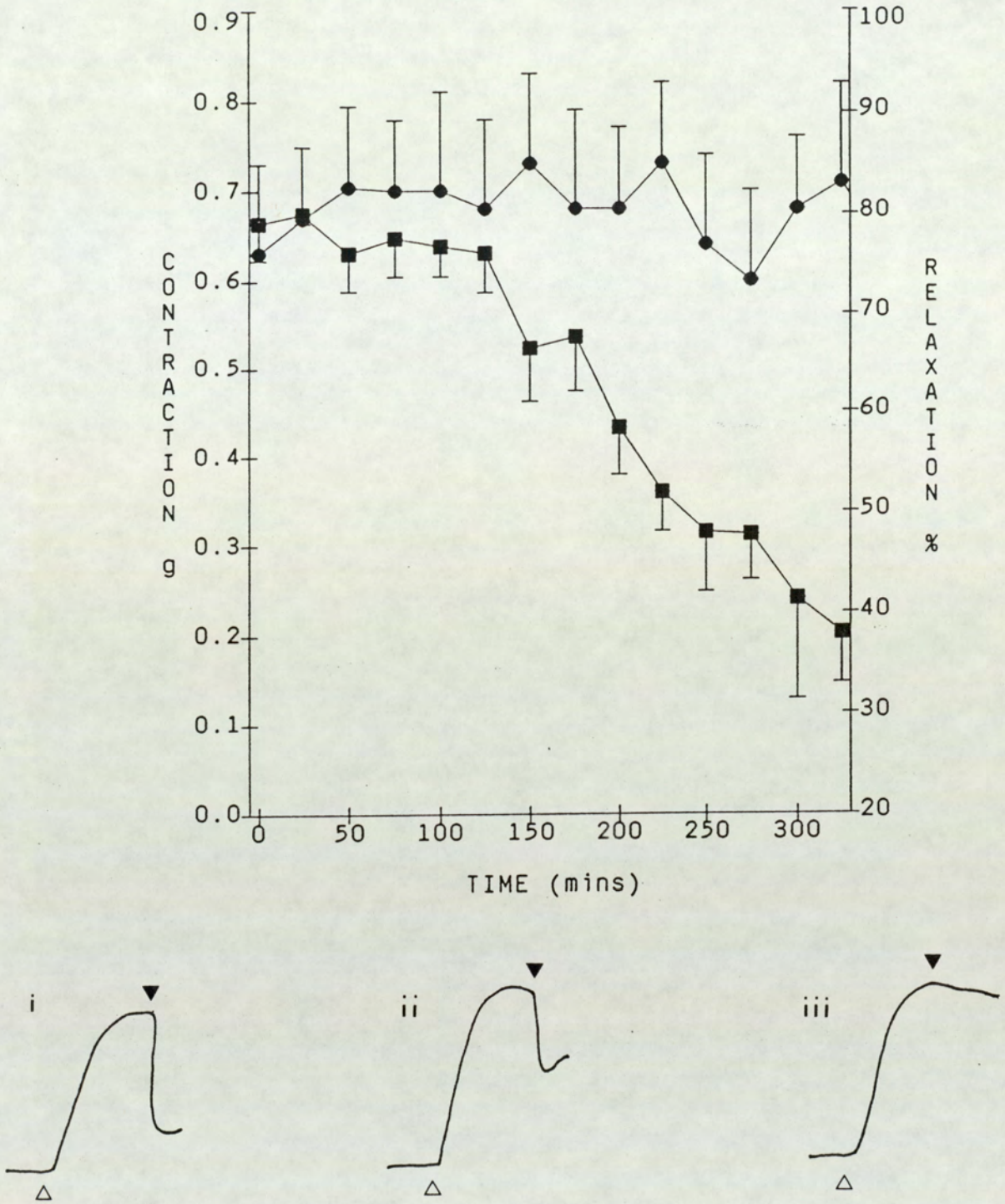


Figure 8.20. The effect of time upon the contractile response of the femoral vein of the rat to BHT and upon the relaxation of this response induced by Ach (10^{-6}M).

a) shows graphically mean responses ± sem for 8 tissues. ● indicates contractions to BHT (10^{-6}M) and ■ the relaxation of the BHT response induced by ACh.

b) shows representative traces at i) 60 minutes, ii) 300 minutes and iii) 20 hours after mounting of the tissues. △ indicates addition of BHT (10^{-6}M) and ▼ indicates addition of ACh.

The effects of repeated challenge of the tissues with BHT and of the effect of ACh on these contractions are shown as representative traces in Figure 8.20.b and graphically in Figure 8.20.a. It can be seen that the contractile response of the tissues to BHT remained steady throughout the duration of the experiment and the degree of relaxation of the BHT-induced contraction caused by ACh remained constant for the first 100 minutes of the experiment and then steadily diminished thereafter. In common with the effects of both NA and CIR, after 20 hours, BHT produced a contractile response but ACh was without effect on this indicating loss of viable endothelium (Figure 8.20.b).

It is interesting to note that for all three agonists, as the effect of ACh (and therefore endothelial viability) decreased, there was no change in the size of the contractile response to the agonist. Further, after 20 hours when there is no viable endothelium on the tissues, the size of the contractile response to any agonist studied was similar to the early responses. This would suggest a limited influence of the endothelium on responses to all three agonists.

8.3. DISCUSSION.

One of the major problems encountered in the study of vascular postjunctional α_2-adrenoceptors has been the demonstration of a suitable isolated vessel which possesses a significant population of these receptors (see General Introduction, Chapter 5). To date most *in vitro* research into vascular α_2-adrenoceptors has been performed on the isolated canine saphenous vein, a vessel which does possess both α-adrenoceptor subtypes (De Mey and Vanhoutte, 1980). In the pithed rat, a postjunctional population of α_2-adrenoceptors has been demonstrated by a number of workers (e.g. Drew and Whiting, 1979 and Timmermans, Kwa and Van Zwietten, 1979) and this preparation has been used extensively in the *in vivo* study of the pharmacology of α_2-adrenoceptors (see General Introduction, Chapter 5). However, attempts to demonstrate postjunctional α_2-adrenoceptors in isolated blood vessels from the rat has met with little success and to date no suitable isolated vessel from this animal has been shown to possess a significant population of these receptors.

The initial aim of this study was to investigate the postjunctional α-adrenoceptors of the femoral vein of the rat. This vessel was chosen for study because it is anatomically close to the saphenous vein, a vessel in which Cheung (1985) demonstrated a population of postjunctional α_2-adrenoceptors. Because the femoral vein is larger than the saphenous vein it was hoped that this vessel would prove more suitable for study than the saphenous vein.

In the femoral vein of the rat, all agonists studied, with the exception of ST, produced concentration dependent changes in tension. NA was a full agonist in the preparation and produced a maximum tension of 1.03 ± 0.08 g, while PHE, CIR, BHT and UK all produced maximal contractions of similar magnitudes which were significantly less than that for NA, the Emax. values of these agonists, as compared to NA, falling between 0.63 and 0.72.

The Emax. values of CIR or BHT suggest that these agonists may be partial agonists in this tissue. However, this can not be confirmed since these values

are obtained by comparing agonists which act on a single receptor subtype with NA which has activity at both α-adrenoceptor subtypes.

The time course of the contractile response for each agonist was characteristic for the type of agonist used, α_1-agonists causing a rapidly developed, transient contraction while responses to α_2-agonists developed slowly and were well maintained. Responses to non-selective α-adrenoceptor agonists showed characteristics of both subtypes, these being rapidly developed and well maintained. Differences between responses were most marked at lower agonist concentrations. Similar findings have been reported by Cavero *et al* (1983) who showed that the pressor response of the pithed rat to CIR developed very rapidly while that to the α_2-agonist M7 took almost twice as long to reach maximum. Furthermore, following the attainment of the peak pressor response, the response to CIR fell rapidly while that to M7 was better maintained. Timmermans and van Zwietten also reported in 1980 that the pressor response of the pithed rat to α_2-agonists developed more slowly than that to α_1-agonists.

One explanation to account for the differences in the shapes of the contractile responses to α_1- and α_2-agonists may be differences in the utilisation of intracellular and extracellular sources of calcium for contractions mediated by the two α-adrenoceptor subtypes (van Meel *et al*, 1981). Thus, the rapid rise in tension seen following stimulation of α_1-adrenoceptors may arise from a rapid rise in the concentration of intracellular free calcium as a result of release of calcium from intracellular stores. In contrast, the maintained phase of the α_1-response and the whole of the α_2-mediated response may reflect influx of calcium from the extracellular space. These concepts are investigated further in the following chapter of this thesis (Chapter 9).

The findings with selective α-adrenoceptor agonists suggested that the femoral vein of the rat possesses a significant population of postjunctional α_1- and α_2-adrenoceptors and this was confirmed by the use of selective antagonists. The selective α_2-antagonist ID antagonised the response of the

tissues to both BHT and NA in a competitive fashion. The pA_2 valus, determined from the Schild plot, of 7.6 and 7.12 are consistent with values cited in the literature for ID acting at α_2-adrenoceptors (e.g. Waterfall, Rhodes and Lattimer (1985) reported a pA_2 of 7.24 for ID acting against BHT 933 in the canine saphenous vein). These results therefore indicate that both BHT and NA are acting upon a population of postjunctional α_2-adrenoceptors in this vessel.

ID also caused antagonism of the response to PHE although this was non-competitive in nature, indicating that PHE is not acting as a selective α_1-agonist in the vessel. Thus, in the femoral vein of the rat PHE acts as a non-selective agonist or perhaps as a preferential α_1-agonist with additional action at the α_2-adrenoceptors of the vessel. In contrast, responses of the tissues to CIR were unaffected by ID indicating that CIR had no action at the α_2-adrenoceptors of this vessel. For this reason, CIR was chosen as the selective α_1-agonist for use in further experiments.

The Schild plot determined for COR acting against both NA and CIR indicated competitive antagonism and the pA_2 values of 6.8 and 6.67 determined from the Schild plot are consistent with pA_2 values for agonists acting at α_1-adrenoceptors in other tissues (e.g. 7.04 against PHE in the rat tail artery, Su *et al*, 1984 and 6.6 against NA in the rabbit pulmonary artery, Weitzell *et al*, 1979). These results therefore support the hypothesis that both NA and CIR act at α_1-adrenoceptors in the vessel.

The effect of the α_1-antagonist PZ on responses to NA and PHE were unexpected, and the concentration-independent shift of the concentration response curves is difficult to explain. It may be the consequence of non-specific effects of PZ, unrelated to its action as an α_1-adrenoceptor antagonist. One known property of PZ other than its α_1-antagonistic properties is phosphodiesterase inhibition reported by Hess (1974). Indeed, PZ was originally considered clinically useful because of this activity. Phosphodiesterase inhibition would result in an increased intracellular concentration of both cAMP and cGMP although this would be expected to

cause relaxation of the vessels (see General Introduction, section 5.6 and Figure 5.2). Thus, this property of PZ would not be expected to serve as an explanation of the unexpected effects of the antagonist observed in the present study.

In order to confirm that BHT was not acting as a partial agonist at α_1-adrenoceptors, the effect of α_1-antagonists on the response to BHT was investigated. Responses to BHT were unaffected by either PZ or COR. Little conclusion can be drawn from the effect of PZ in view of its atypical actions against NA and PHE in this preparation (see above). The effect of COR against NA and the α_1-agonist CIR suggest that this antagonist is exerting selective antagonism at the α_1-adrenoceptors of this tissue. Therefore results with COR are probably the most reliable indicator of action at α_1-adrenoceptors. These results indicate that BHT is indeed acting not at α_1- but at α_2-adrenoceptors.

It is interesting that the Schild plots for both ID and COR acting against NA are linear with a slope of unity, in view of the fact that NA is a non-selective agonist acting in a preparation which appears to have two receptor subtypes. This has already been discussed in relation to COR (see section 8.2.2) when it was proposed that a deflection in the Schild plot may have become apparent were there more points on the plot. This is particularly obvious in consideration of the effect of COR upon the shape of the concentration-response curve to NA (see Figure 8.13), in which a kink in the curves can be seen in the presence of antagonist.

However, the shift of the NA concentration-response curve by ID does appear to be a parallel shift (see Figure 8.5). This would suggest that NA has a similar affinity for both the α_1- and α_2-adrenoceptor and this seems unlikely since a parallel shift was not seen with NA in the presence of COR. An alternative suggestion would be that the α_2-adrenoceptor is the predominant site of action of NA in this vessel. However, this also seems unlikely as COR produced a marked shift of the NA concentration-response curve indicating a significant action at α_1-adrenoceptors (see Figures 8.13 and 8.12).

Despite the problems encountered in the characterisation of the receptors

using mixed agonists, as mentioned earlier, the most convincing evidence concerning the nature of the receptors must come from the analysis of the effects of selective antagonists upon the actions of selective agonists. The results of this section have therefore indicated that the femoral vein of the rat possesses a population of both postjunctional α_1- and α_2-adrenoceptors, and that the contractile response of the vessel to exogenous α-adrenoceptor agonists may be mediated via either subtype.

It has been suggested that while the pressor response of the pithed rat to exogenous α-adrenoceptor agonists is mediated by either α_1- or α_2-adrenoceptors, the response to endogenous catecholamines released following the electrical stimulation of sympathetic nerves is mediated by only α_1-adrenoceptors (e.g. Yamaguchi and Kopin, 1980; see General Introduction, Chapter 5 section 2). Medgett and Langer (1984) have arrived at the same conclusions from investigations of the α-adrenoceptors of the isolated tail artery of the rat. These findings suggest a differential anatomical distribution of the two receptor subtypes. This was investigated further in the femoral vein of the rat by studying the effect of α-adrenoceptor antagonists on contractions of this vessel mediated by electrical stimulation. It was found that the electrical response was antagonised by either the α_1-antagonist COR or by the α_2-antagonist ID indicating that neuronally released catecholamine acts at both receptor subtypes. Indeed the results of the present study suggest a greater block by the α_2-antagonist than by the α_1-antagonist. These results are in conflict with those of Yamaguchi and Kopin (1980) and of Medgett and Langer (1984) and suggest that both α_1- and α_2-adrenoceptors occur intrasynaptically in the vessel. Alternatively, the neuronally released catecholamine may not be acting solely upon synaptic receptors and a significant portion of the released catecholamine may leak out of the synapse to act at extrasynaptic receptors. This could be investigated further by studying the overflow of labelled noradrenaline from the sympathetic nerves following nerve stimulation.

The experiments in the final section of this chapter aimed to investigate

the influence of the endothelium on responses of the femoral vein of the rat to various α-adrenoceptor agonists. A relaxation of precontracted tissues following the addition of ACh (10^{-6}M) was taken as an indication of endothelial viability (see Furchgott and Zawadzki, 1980).

Maximal contractions of the tissues to NA relaxed by 50 ± 4.4% following ACh addition. This degree of relaxation is less than values cited in the literature for the ACh-mediated relaxation of NA contractions of arterial vessels (e.g. Miller *et al* (1985), rat aorta, 67.9 ± 5.8%; Lues and Schumann (1984), rat aorta, 87.3 ± 3%; Furchgott and Zawadzki (1980), rabbit aorta, 90-100%) and greater than that reported for venous tissues (e.g. De Mey and Vanhoutte (1982) reported values of 20-25% in canine femoral and saphenous veins, while the canine pulmonary vein gave no relaxations to ACh). Furchgott (1983) suggested that in general, veins are less affected by ACh than arteries and that this may be because the endothelium-mediated relaxation of veins is masked by an ACh-mediated contraction. In the present study, endothelium-free vessels gave no such contraction to ACh which may explain why these vessels do give a relatively large relaxation to ACh. This relaxation was less than that of arteries which is consistent with the theory of Furchgott that the endothelium may play less of a regulatory role in veins than in arteries.

The influence of the endothelium on responses of this vessel to α-adrenoceptor agonists was investigated further by observing changes in the ACh response with time (see General Introduction and section 8.2.4). The presence of viable endothelium appears to have little effect on the size of the contractile response to NA, since, after the first 90 minutes of the experiment, the amplitude of the contraction to NA remained constant even as the viability of the endothelium decreased (see Figure 8.18). In addition, after 20 hours, the size of the contraction was equal to that after 90 minutes even though there was no viable endothelium remaining at this time. This is in agreement with observations of other workers (e.g. Egleme *et al*, 1984) who have shown that mechanical removal of the endothelium has little effect on

responses to NA.

Responses of the tissue to CIR showed a similar pattern to those of NA (Figure 8.19) in that as the function of the endothelium decreased, there was no change in the amplitude of the contractile response to CIR. Further, when there was no endothelium present, the contractile response of the tissue was of the same magnitude as that in the presence of fully intact endothelium. This suggests a limited influence of the endothelium on responses of the tissues to CIR. Similar results were obtained in this study with the α_2-agonist BHT (see Figure 8.20). This is an interesting observation since it has been shown by a number of workers that removal of the endothelium causes a marked increase in the magnitude of the contractile response to partial α_2-agonists such that the response becomes similar in size to that of full agonists (e.g. see Egleme *et al*, 1984). Egleme *et al* (1984) suggest that this occurs because a population of α_2-adrenoceptors is found on the endothelium, stimulation of which causes EDRF release which opposes contraction. Thus, removal of the endothelium prevents this α_2-mediated release causing potentiation of the contractile response. Since removal of the endothelium did not potentiate the α_2-response in the present vessel, then this would suggest that either there are no α_2-receptors on the endothelium of this vessel, or that stimulation of these does not cause EDRF release. An alternative explanation would be that BHT is acting as a full agonist at the α_2-adrenoceptors of the femoral vein and thus the agonist is not susceptible to endothelial inhibition.

These results therefore suggest that the influence of the endothelium on responses mediated by both α_1- and α_2-adrenoceptors is limited and this is in contrast to the findings of Egleme *et al* (1984) and of Miller *et al* (1984). The disparity of the present observations with those of Egleme *et al* (1984), Miller *et al* (1984) and of Lues and Schumann (1984) is difficult to explain. However, the observations of all these authors were derived from experiments in the rat aorta, a vessel which possesses no α_2-adrenoceptor population (or at least a very small and non-significant population) and therefore the results for

responses of this tissue to α_2-agonists may be of little value and simply reflect partial α_1-agonism. It may alternatively be the case that the influence of the endothelium is dependent on the type of vessel such that its influence on venous tissue is different to that on arterial tissue. This may be extended even further to suggest that the endothelial regulatory function is tissue dependent and that different vessels are influenced to different extents by the endothelium. Alternatively, it may be that slight variations in the conditions under which the experiment is performed can have a profound influence on the effect of the endothelium.

8.4 Summary.

The results of this chapter have shown the following:

1. NA, PHE, CIR, BHT and UK all produced contractile responses in the femoral vein of the rat. NA was a full agonist while the remaining agonists produced maximum responses which were less than that for NA and the Emax. values ranged from 0.63 to 0.72.

2. The form of the contractile response for each agonist was dependent upon the subgroup of adrenoceptor activated by that agonist.

3. The use of selective antagonists revealed that the vessel possessed a population of both postjunctional α_1- and α_2-adrenoceptors. Responses to BHT were mediated by the α_2-subtype, responses to CIR by the α_1-subtype and responses to NA and PHE by both α-adrenoceptor subtypes.

4. NA released from the sympathetic nerves by electrical stimulation was shown to act at both α_1- and α_2-adrenoceptors.

5. The endothelium was shown to exert little influence upon responses mediated by NA, CIR or the α_2-agonist BHT.

9. RESULTS SECTION II

CALCIUM UTILISATION PROCESSES OF α_1- AND α_2-ADRENOCEPTORS.

9.1. Introduction.

The results described in the previous chapter provide support for the view that the femoral vein of the rat is a suitable vessel for the study of postjunctional α_1- and α_2-adrenoceptors. As discussed, the shape of the contractile response depended upon the adrenoceptor subgroup mediating the response. A similar effect has been reported *in vivo* where it has been shown that the pressor response of the pithed rat to B-HT920 is slow in onset taking 1-2 minutes to reach maximum while that to phenylephrine takes only 15 seconds (Timmermans and van Zwietten, 1980). Similar results have been reported by Cavero, Shepperson, Lefevre-Borg and Langer (1983) for the α_1-agonist cirazoline and the α_2-agonist M7 in the pithed rat.

One possible explanation for the differences in the forms of contraction may be the source of activator calcium utilised by the two receptor subgroups. As discussed in the general introduction (Chapter 5) the contractile response of a number of isolated blood vessels (e.g. the rat aorta, Godfraind and Kaba, 1972) to exogenous catecholamines can be differentiated into two components: an initial rapid rise in tension which is transient in nature and reflects release of calcium from intracellular stores, and a second slowly attained tonic component reflecting translocation of extracellular calcium.

Accordingly, Timmermans and van Zwietten (1987) have proposed that the *in vivo* vasoconstriction mediated via α_2-adrenoceptors is totally dependent on influx of calcium from the extracellular space while the α_1-mediated response is not wholly dependent on calcium influx and a proportion of the pressor response is mediated via calcium arising from intracellular stores. Further, *in vivo* reports have shown that pressor responses of pithed rats to α_2-adrenoceptor agonists are invariably sensitive to calcium entry blocking drugs while those to α_1-agonists are generally

insensitive (e.g. van Meel, de Jonge, Kalkman, Wilffert, Timmermans and van Zwietten, 1981; Cavero, Shepperson, Lefevre-Borg and Langer, 1983; for review see Timmermans, Chiu and Thoolen, 1987).

In sharp contrast to this, Ruffolo, Morgan and Messick (1984) have reported a high inverse correlation between the intrinsic activities of α_1-adrenoceptor agonists and the degree to which their pressor responses are inhibited by the calcium entry blocker DIL. Ruffolo, Morgan and Messick (1984) therefore suggest that it is the intrinsic activity of the agonist rather than the α-adrenoceptor subtype that determines the sensitivity to CEBs and therefore the dependence on extracellular calcium.

The aim of the present study was to investigate the sources of activator calcium for responses mediated by α_1- and α_2-adrenoceptors *in vitro* and to see whether the different forms of the contractile responses of the femoral vein of the rat to α_1- and α_2-adrenoceptor agonists (Figure 8.1, section 8.2.1) are a consequence of differences in the sources of activator calcium for the two receptor subtypes.

9.2. RESULTS.

9.2.1. The effect of calcium-free PSS on contractile responses to α-adrenoceptor agonists.

This part of the study aimed to determine the ability of the agonists to release intracellular calcium as judged by their ability to induce a contraction in the absence of extracellular calcium. In these experiments, control contractions to the agonists were compared with the responses obtained following incubation in calcium-free PSS containing the calcium ion chelator EGTA (the EGTA-resistant response or ERR). This ensures that all free calcium is removed from the bathing medium and any contraction remaining under these conditions is taken to be indicative of calcium released from intracellular stores (Cauvin and Malik, 1984). In the femoral vein of the rat, this procedure completely abolished the response to 60mM KCl indicating that extracellular calcium was absent.

Figure 9.1.a shows that 10^{-6}M NA produced an ERR which was 31 ± 5% of the control contraction in normal PSS. An increase in the concentration of NA to 10^{-5} or 10^{-4}M caused no significant increase in the size of the ERR (Figure 9.1.a). In order to ascertain whether the ERR for NA was mediated by α_1- or α_2-adrenoceptors, the effect of the α_1-antagonist COR and the α_2-antagonist ID was investigated. In Figure 9.1.b it can be seen that the ERR for NA in the presence of 10^{-6}M COR (the α_2-mediated response) was very small (6.9 ± 1.6% of the control value) and was significantly less than the ERR in the presence of $5x10^{-7}$M ID (22 ± 3.6% of control)($p<0.05$). These two concentrations of antagonist give similar (approximately 30%) reductions in the magnitude of the control NA response in normal Krebs buffer. These observations suggested that the ERR produced by NA may be α_1-mediated. In order to substantiate this conclusion, the response of the selective α_1-agonist CIR and the α_2-selective agonist BHT were studied. Figure 9.1.c shows that the effect of CIR was similar to that of NA; 10^{-6}M CIR caused an ERR which was 34 ± 5% of control values and was not increased when the concentration of

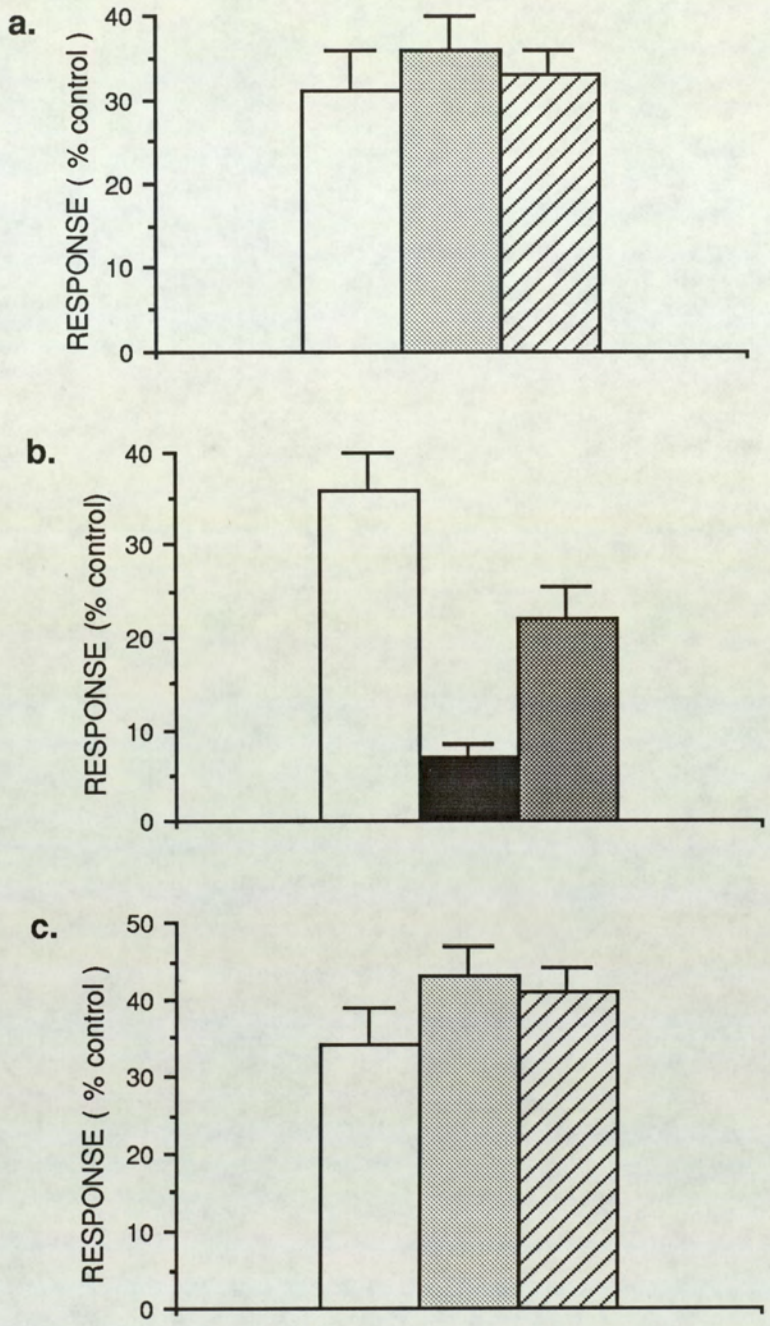


Figure 9.1. The effect of incubation of the tissues in calcium-free PSS containing 0.2mM EGTA on responses to (a) increasing concentrations of NA, (b) NA in the presence of $1x10^{-6}$M COR (filled) or $5x10^{-7}$M ID (heavy stipled) and (c) increasing concentrations of CIR.

In b) the open column represents the control response of the tissue to NA (10^{-6}M) in calcium-free PSS. In (a) and (c), the agonist concentration in each instance is 10^{-6}M (open), 10^{-5}M (stipled) or 10^{-4} M (hatched).

Columns are the mean of at least 6 experiments and bars are s.e.m..

CIR was raised to 10^{-5} or 10^{-4}M whereas concentrations of BHT up to 10^{-4}M failed to elicit an ERR. These results therefore indicate that stimulation of α_1-adrenoceptors can elicit release of intracellular calcium while stimulation of α_2-adrenoceptors appears unable to do so.

Representative traces showing the effect of calcium-free EGTA PSS on contractions to NA, CIR and BHT are shown in Figure 9.2. It can be seen that the ERR to NA or CIR was transient with peak tension being achieved rapidly (within 1 minute) followed by a fall to baseline tension within the 5 minute period of agonist contact.

9.2.2. The effect of the calcium entry blocker verapamil on responses to NA, CIR and BHT.

The dependence of NA, CIR and BHT-induced contractions on extracellular calcium was further investigated using the calcium entry blocker, VER. In the presence of VER (10^{-5}M) maximal contractile responses to NA, CIR and BHT were reduced to 78 ± 2%, 80 ± 4% and 34 ± 10% of control values respectively (see Figure 9.3). VER thus caused a significantly greater reduction of the contractile response to BHT than of that to either NA or CIR ($p<0.05$). However, the response to BHT was not completely abolished by VER. Coincidently, VER also failed to completely abolish the response to KCl (60mM) leaving a residual response of 26 ± 5%. This is in contrast to the effect of incubation in calcium-free PSS which caused total abolition of the response to 60mM KCl and to BHT. These observations therefore suggest that 10^{-5}M VER does not totally block the influx of calcium into this vessel.

It is interesting that the response to BHT in the presence of 10^{-5}M VER was not significantly different from that of KCl under these conditions (34 ± 10% and 26 ± 5% respectively, $p>0.05$). This suggests that BHT, like KCl, is dependent upon extracellular sources of calcium and is consistent with the results of the previous section which showed that BHT failed to elicit an ERR.

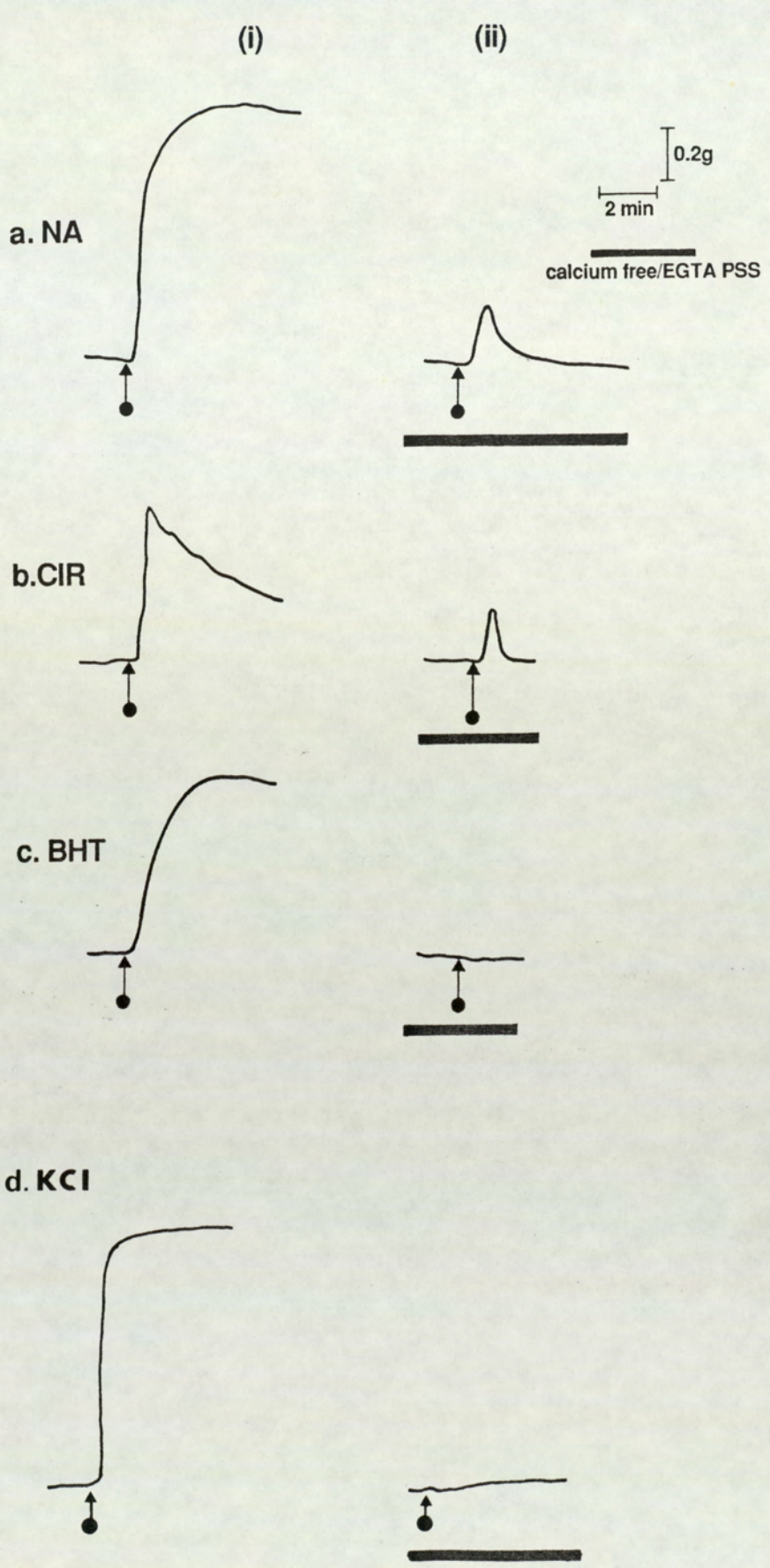


Figure 9.2. Representative traces showing the effect of 10^{-5}M (a) NA, (b) CIR and (c) BHT and of 60mM KCl on the contractile response of the rat femoral vein in (i) normal PSS and (ii) calcium-free PSS containing 0.2 mM EGTA.

shows addition of agonist and the bar shows the presence of calcium-free PSS containing EGTA.

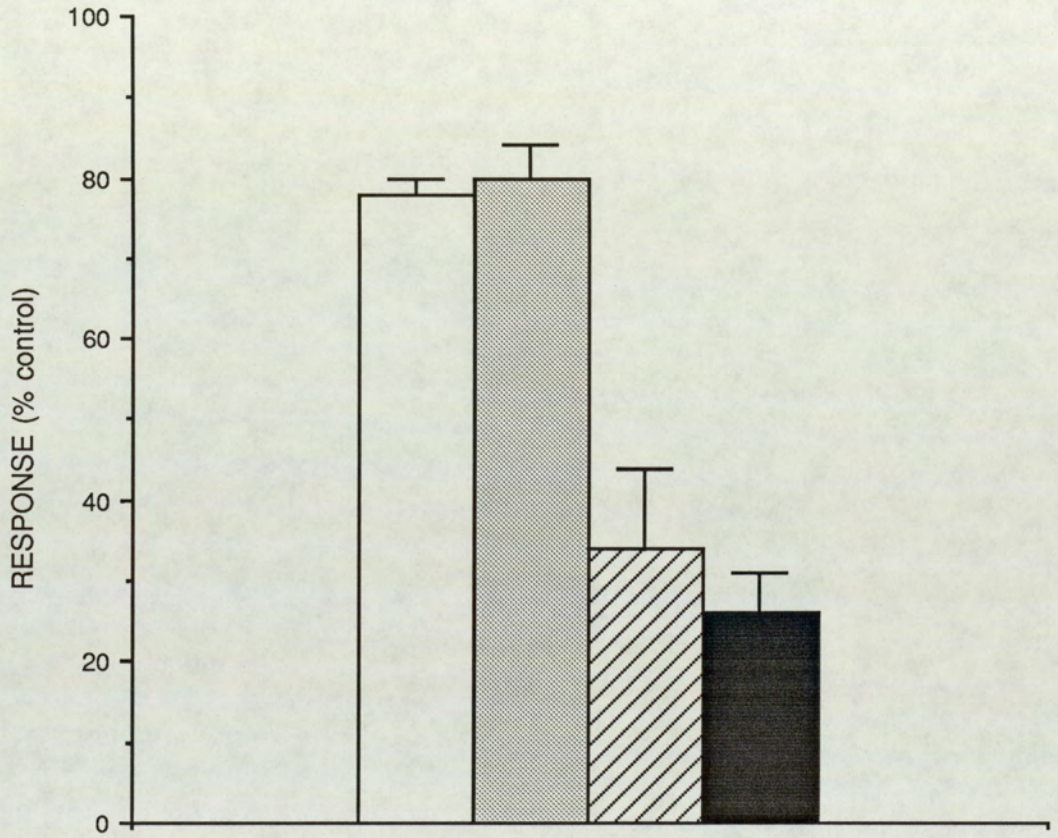


Figure 9.3. Effect of 10^{-5}M VER on the contractile response of the femoral vein to NA (open column), CIR (stipled column), BHT (hatched column) and KCl (filled column). All agonist concentrations are 10^{-5}M and KCl is 60 mM. Results are shown as percent of control value (i.e. in absence of VER). Columns are the mean of at least 6 experiments and the bars are s.e.m..

9.3 DISCUSSION.

The objective of the present study was to investigate the sources of activator calcium for contractions to α-adrenoceptor agonists and the initial aim was to determine the ability of selective α_1- and α_2-adrenoceptor agonists to elicit an ERR. Contractions produced in calcium-free PSS containing EGTA (ERR) have been widely used as an indication of the release of intracellular calcium (eg. Cauvin and Malik, 1984) and in the present study, the response to 60mM KCl was completely abolished by incubation in calcium-free PSS containing 0.2mM EGTA.

Following 2 minutes incubation in calcium-free EGTA-PSS the full non-selective α-adrenoceptor agonist NA elicited a response of 31 ± 5% of the control value in the femoral vein of the rat. Similarly, Cauvin and Malik (1984) have studied the effect of calcium-free EGTA PSS on contractions to NA in rat mesenteric vessels and have showed that an ERR of 64 ± 7% of control values was elicited. In the same study Cauvin and Malik also showed that NA produced an ERR in the rat aorta. Similar results have also been reported by Godfraind, Miller and Lima (1982) who reported ERRs in the rat aorta to both NA and PHE which were 60 - 70% of control values. Godfraind, Miller and Lima (1982) also examined the ability of agonists to release intracellular calcium by investigating the effect of agonist stimulation on ^{45}calcium efflux. They showed that both NA and PHE stimulated ^{45}calcium efflux into calcium-free PSS indicating release of intracellular calcium by these agonists. The values above may indicate a similar amount of releasable calcium stored in the aorta and in the mesenteric resistance vessels, and based on the present findings, the size of this store would appear to be greater than that in the femoral vein. It is important to note that the α-adrenoceptors of the rat mesenteric resistance vessels and of the rat aorta are a homogenous population of the α_1-subtype (Cauvin and Malik, 1984). In the present study, the ERR elicited by NA was inhibited by the α_1-antagonist COR to a greater degree than by the α_2-antagonist ID, suggesting that this ERR is mediated via

the α_1- rather than the α_2-subgroup. This suggestion was confirmed by investigating the effects of calcium removal on responses to the selective α_1-agonist CIR (Lefevre, Deportere and Cavero, 1976) and the selective α_2-agonist BHT. CIR elicited an ERR equal to that of NA while BHT failed to elicit an ERR.

The results are consistent with the findings of Cauvin and Malik (1984) and of Godfraind, Miller and Lima (1982) who suggest that stimulation of α_1-adrenoceptors is coupled to release of intracellular calcium. Similar findings have also been made by Jim, de Marinis and Matthews (1985) and Jim and Matthews (1985). In the former study, Jim, de Marinis and Matthews used calcium-free PSS containing lanthanum and showed that in the canine saphenous vein, a vessel possessing both α_1- and α_2-adrenoceptors, activation of postjunctional α_1-adrenoceptors causes both influx of calcium and release of intracellular calcium. In contrast to this, in the latter study the effects of calcium removal on responses to α_2-agonists was investigated, and these experiments revealed that α_2-stimulation depends primarily upon extracellular sources of calcium.

Skarby, Hogestatt and Andersson (1985) investigated the ability of NA to elicit contractions under calcium-free conditions in the presence of EGTA in a number of vessels possessing a predominant population of α_1-adrenoceptors, a mixed α-adrenoceptor population or predominant α_2-adrenoceptor population. They showed that in those vessels possessing α_1-adrenoceptors, a component of the contractile response remained after removal of calcium. This was not so for a vessel possessing a predominant α_2-population i.e. the cat middle cerebral artery. Thus, Skarby, Hogestatt and Andersson (1985) conclude that the α_2-mediated contractions of the cat middle cerebral artery are totally dependent on extracellular calcium while responses elicited by α_1-stimulation in the other vessels studied involved a release-mediated component.

It is interesting to note that in some vascular preparations stimulation of α_1-adrenoceptors elicits a contraction which does not involve the release of

intracellular calcium (e.g. the rabbit basilar artery, Bevan *et al* (1986) and the rat femoral artery, Waters (1988)). This may indicate that there is no intracellular store of calcium in these vessels or may imply an inability of the agonists to release such a store. Reasons for this are discussed later.

An alternative approach taken to investigate the dependence of agonists on extracellular sources of calcium is the use of calcium entry blockers which prevent or reduce calcium influx through transmembrane calcium channels. In the present study, the effect of VER on responses to NA, CIR and BHT was studied and the results lend support to the conclusions drawn from the observations in calcium-free EGTA PSS discussed above. Thus, the response to BHT was inhibited by VER to the same degree as that to KCl indicating total dependence on extracellular calcium for BHT responses, while responses to NA and CIR were only partially inhibited by VER, indicating that responses to these agonists are less dependent on extracellular calcium. It is surprising that the response to KCl was only reduced by 75% by VER since most reports indicate 100% block with 10^{-5}M VER (see van Breemen *et al*, 1982). Most studies using VER in the literature have used arterial preparations and relatively few report an effect of VER upon venous tissue; therefore the discrepancy in this study may reflect a difference between arterial and venous tissue.

These results are consistent with the findings of van Meel, de Jonge, Kalkman, Wilffert, Timmermans and van Zwietten (1981) who reported that the pressor responses of the pithed rat to α_1-agonists were resistant to the calcium entry blockers verapamil, D600 and nifedipine while these agents inhibited the pressor response to the α_2-agonist B-HT920. From their observations van Meel *et al* (1981) concluded that influx of calcium is necessary for the vasoconstriction *in vivo* initiated by stimulation of α_2-adrenoceptors while vasopressor responses to α_1-adrenoceptor stimulation are not dependent on calcium influx and a proportion of the component is mediated by calcium release.

There are many other reports that calcium entry blockers inhibit α_2- but

not α_1-responses *in vivo* (e.g. Cavero, Shepperson, Lefevre-Borg and Langer, 1983; see van Zwietten *et al*, 1985) but *in vitro* evidence concerning the effects of calcium entry blockers is generally lacking. Langer and Shepperson (1981) have investigated the effects of the calcium entry blockers VER and diltiazem on contractile responses of the isolated canine saphenous vein to NA, PHE and the α_2-agonist M7 and they reported that both calcium entry blockers increased the EC_{50} for NA and PHE but did not affect the maximum response. In contrast, the calcium entry blockers both increased the EC_{50} and reduced the maximum response to the α_2-agonist M7. Cavero *et al* (1983) have also investigated the effects of calcium entry blockers on the α_1- and α_2-mediated responses of the canine saphenous vein and report inconclusive results. Contractions to the α_2-agonist M7 were inhibited by the calcium entry blockers VER and diltiazem while the α_1-agonist CIR elicited contractile responses which were insensitive to the calcium entry blocker diltiazem but sensitive to VER.

The results presented here seem, therefore, to provide support for the hypothesis of van Meel *et al* (1981) that α_1-adrenoceptors are linked to both release and influx of calcium while responses to α_2-agonists are associated with influx only.

Hamilton, Reid and Sumner, (1983) reported that there were differences in the receptor reserve of α_1- and α_2-adrenoceptors in the conscious rabbit and Ruffolo and Yaden (1984) reported similar findings in the pithed rat. Both groups of workers found that there was a large receptor reserve for postjunctional α_1-adrenoceptors while that for α_2-adrenoceptors was relatively small. Ruffolo, Morgan and Messick, (1984) also noted a high correlation between the intrinsic activity of α_1-agonists and the degree to which their pressor response was inhibited by the calcium entry blocker DIL. They showed that in the untreated pithed rat, the pressor response to the full α_1-agonist CIR was unaffected by calcium entry blockade while the α_2-mediated response was inhibited. Following the removal of spare α_1-receptors by phenoxybenzamine treatment, the response to CIR became

susceptible to calcium entry blockers. Similarily, responses to partial α_1-agonists were affected by the calcium entry blocker. This led these workers to propose that the calcium entry blockers inhibit α_2-mediated responses to a greater degree than α_1-mediated responses because of this difference in the receptor reserve rather than differences in the calcium utilization of the agonists. The reasoning behind this suggestion was based on the fact that noncompetitive antagonists such as the calcium entry blockers cause greater inhibition of systems lacking receptor reserve than those with receptor reserve (see Ariens and van Rossum, 1957). Ruffolo, Morgan and Messick (1984) thus suggested that the resistance of the α_1-response to calcium entry blockade resulted from the spare receptor population acting as a buffer to this blockade, rather than a low dependence of the α_1-response on extracellular calcium and proposed that it is the intrinsic activity of the agonists rather than the receptor subtype which determines the susceptibility of the agonists to removal of extracellular calcium. This view is supported by Jim, de Marinis and Matthews (1985) who used α_1-agonists of different intrinsic activities to investigate the effect of calcium removal on responses in the canine saphenous vein and proposed that the intrinsic activity of the α_1-agonists was an important determinant of the ability of agonists to release calcium. Interestingly, the α_2-agonists used by Jim and Matthews (1985) are all partial agonists compared to the full α_1-agonist PHE, and all were inhibited by calcium removal. Timmermans *et al* (1983a,b) also showed that in pithed rats, responses to the partial α_1-agonist Sgd 101/75 or the partial α_1-agonist ST587 were inhibited by nifedipine while responses to full α_1-agonists were not.

From the accumulated evidence discussed above, there appears to be an association between the intrinsic activity of the agonists and the effectiveness of the calcium entry blockers as antagonists (or ability of agonists to release intracellular calcium) although the link is unproven. Ruffolo, Morgan and Messick (1984) suggest that calcium entry blockers are more effective antagonists against agonists of low intrinsic activity but that this effectiveness

is not indicative of an inability of α_2-adrenoceptors to release intracellular calcium, and Jim, de Marinis and Matthews (1984) suggest that the intrinsic activity of α_1-agonists determines the ability of the agonists to release intracellular calcium.

In the present study, attempts were made to investigate the influence of intrinsic activity upon the ability to release intracellular calcium by investigating the effects of ST587 on the femoral vein. However, this proved unsuccessful since ST587, which is reported to be a partial α_1-agonist (Timmermans *et al*, 1983b), did not produce a contractile response in the vessel.

The conclusion of the present study that it is the receptor subtype stimulated which determines the ability to release intracellular calcium appears to be in conflict with the hypothesis proposed by Ruffolo, Morgan and Messick (1984) that it is the intrinsic activity of the agonist which is the important determinant of this ability. However, it is important to note that Ruffolo, Morgan and Messick (1984) showed that under control conditions, in which the receptor populations are unaltered from the physiological situation, the receptor subgroup *is* a determinant of the susceptibility to calcium entry blockers. Thus, under these conditions, the pressor response to the full α_1-agonist was unaffected by calcium entry blockade while the α_2-mediated response was inhibited. It was only after the removal of spare α_1-receptors that the response to CIR became susceptible to calcium entry blockers.

It may therefore be hypothesised that the initial determinant of the ability of an agonist to release intracellular calcium - and thus the dependence on influx of calcium - is the subgroup of α-adrenoceptor stimulated by the agonist and that α_2-adrenoceptor stimulation is incapable of eliciting this release. However, it would appear that with α_1-adrenoceptor stimulation, the intrinsic activity of the agonist does determine the ability of agonists to release calcium. α_1-agonists with high intrinsic activities are capable of releasing the calcium store while those with low intrinsic activities are not

(e.g. Ruffolo, Morgan and Messick, 1984 and Timmermans *et al*, 1983a and 1983b). It could be argued that the lack of the ability of α_2-agonists to cause intracellular calcium release may be because all α_2-agonists studied appear to be partial agonists. If full α_2-agonists were made available, then the study of these agonists might confirm the general inability of α_2-adrenoceptor stimulation to cause calcium release.

That α_1-adrenoceptor stimulation releases intracellular calcium while α_2-adrenoceptor stimulation is unable to do so may reflect a difference in the excitation-contraction coupling mechanisms of α_1- and α_2-adrenoceptors. Recent evidence indicates that many receptor mediated responses involve the activation of phospholipase C and the hydrolysis of membrane-bound polyphosphoinositides (Michell, 1975). One of the products of the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) is inositol-1,4,5-trisphosphate (IP_3) and this has been shown to cause release of calcium from intracellular stores (Streb, Irvine, Berridge and Schulz, 1983). It has also been shown by Legan, Chernow, Parrillo and Roth (1985) and by Chiu, Bozarth and Timmermans (1987) that stimulation of the α_1-adrenoceptor of the rat aorta results in increased hydrolysis of PIP_2. Further, Chiu, McCall, Thoolen and Timmermans (1986) have shown that stimulation of the α_1-adrenoceptors of the rat aorta by full agonists such as NA, PHE or CIR results in contraction and a coincident production of IP_3 resulting in release of intracellular calcium. In contrast to this, Chiu *et al* (1986) have shown that the contraction to the partial α_1-agonist Sgd 101/75 is not associated with the production of IP_3 and thus no release of intracellular calcium occurs. It may therefore be suggested that the ability of an α_1-agonist to release intracellular calcium is related to its intrinsic activity since this determines the ability of the agonist to release IP_3. These concepts are discussed further, and in relation to the current work, in the next chapter.

Since the release of intracellular calcium depends upon the ability of agonists to hydrolyse membrane phosphoinositides (Streb *et al*, 1983) and since it appears that α_2-agonists do not release calcium (this study, Jim and

Matthews, 1985) it would be expected that α_2-adrenoceptor stimulation does not cause the hydrolysis of phosphoinositides. However, to date, little information is available from studies in vascular muscle concerning the effects of α_2-stimulation on this hydrolysis. The next step in this study was therefore to investigate the effects of α_1- and α_2-adrenoceptor stimulation on phosphoinositide hydrolysis in the femoral vein of the rat.

9.4. Summary.

1. The contractile response of the vein to 60mM KCl was completely abolished by incubation in calcium-free PSS. NA and CIR elicited ERRs in the vessel but the α_2-agonist BHT failed to elicit an ERR at concentrations up to 10^{-4}M.

2. The ERR for NA was inhibited by the α_1-antagonist COR to a greater degree than by the α_2-antagonist ID suggesting that the ERR for NA is mediated via the α_1-adrenoceptor.

3. The calcium entry blocker VER inhibited the contractile response to BHT but had a similar and relatively small effect on the responses to NA and CIR.

4. The ERR for CIR was of a similar size as that of NA inspite of the differences in the Emax. values of the two agonists. The Emax. values of CIR and BHT are similar but calcium removal had different effects on the responses to the two agonists.

It is concluded that the receptor subtype at which an agonist acts is an important determinant of the ability of that agonist to release intracellular calcium. Stimulation of α_1-adrenoceptors can cause release of calcium while stimulation of α_2-adrenoceptors cannot. In addition, the intrinsic activity of α_1-adrenoceptor agonists determines the ability of these agonists to release intracellular calcium.

10. RESULTS SECTION III

α-ADRENOCEPTOR STIMULATION AND POLYPHOSPHOINOSITIDE HYDROLYSIS.

10.1 INTRODUCTION.

As previously discussed, the different dependencies of α_1- and α_2-adrenoceptor mediated responses on intra- and extracellular sources of calcium may reflect a difference in the excitation-contraction coupling mechanisms of the two receptor subgroups. Experiments described in this chapter were designed to investigate the effect of α_1- and α_2-adrenoceptor stimulation on the hydrolysis of membrane phosphoinositides in order to determine whether this hydrolysis mediates the pharmaco-mechanical coupling for either α-adrenoceptor subtype.

The importance of the cyclic process of events known as the phosphatidyl inositol cycle, and its biochemistry and pharmacological significance has already been discussed (see General Introduction). In the present study, hydrolysis of membrane polyphosphoinositides (PPIs) was determined by the measurement of the incorporation of radioactive label into inositol phosphates. Tissues were preloaded with radioactive *myo*-inositol which was labelled with tritium on the inositol moeity. Following the addition of agonist, the amount of label incorporated into the water soluble inositol phosphates was determined.

The first question addressed by this study was whether stimulation of α_1- and/or α_2-adrenoceptors was coupled to stimulation of phospholipase C and acceleration of the hydrolysis of polyphosphoinositides. It has been shown that the stimulation of postjunctional α_1-adrenoceptors induces polyphosphoinositide hydrolysis in vascular muscle (Legan, Chernow, Parrillo and Roth (1985), rat aorta; Chiu, Bozarth and Timmermans (1987), rat aorta; Hashimoto, Hirata, Itoh, Kanmura and Kuriyama (1986), rabbit mesenteric artery), although few reports to date concern the effects of

stimulation of α_2-adrenoceptors on polyphosphoinositide hydrolysis.

The second aim was to investigate the significance of intrinsic activity on the ability of agonists to hydrolyse polyphosphoinositides. In Chapter 9 it was shown that NA and CIR both produced an ERR in the femoral vein, and this ERR was of similar size for both agonists. It has been proposed that IP_3, one of the immediate products of PIP_2 hydrolysis causes release of calcium from intracellular stores (Streb, Irvine, Berridge and Schulz, 1983), and so the production of an ERR would be expected to be associated with polyphosphoinositide hydrolysis (see General Introduction). However, Chiu, McCall, Thoolen and Timmermans (1986) and Chiu, Bozarth and Timmermans (1987), have shown that α_1-agonists with high intrinsic activities produce large contractions and a coincident production of IP_3 while α_1-agonists with low intrinsic activities produce small contractions which are associated with neither a release of intracellular calcium nor IP_3 production. Although in the present study, ST587 produced no contractile response, it was considered worthwhile to investigate whether this agonist produced any changes in polyphosphoinositide turnover.

A third objective was to investigate the effect of KCl on stimulation of polyphosphoinositide hydrolysis. The contractile response of the femoral vein of the rat to KCl is similar in size to that of NA and in the previous section it was shown that the contraction to KCl is mediated entirely via calcium influx (see Figure 9.2). This influx is a direct result of membrane depolarisation, and a link with polyphosphoinositide hydrolysis may not be expected. Reports in the literature concerning the effect of KCl stimulation on polyphosphoinositide hydrolysis are scarce and contradictary. Thus Chiu, Bozarth and Timmermans (1987) have reported that KCl does not induce polyphosphoinositide hydrolysis in tha rat aorta whereas Nishizuka (1984) has reported a stimulatory effect of KCl on polyphosphoinositide hydrolysis.

The aim of final experiments was to identify which inositol phosphates are produced by α-adrenoceptor stimulation in this preparation.

10.2. RESULTS.

10.2.1 Effect of NA on inositol phosphate accumulation.

The aim of initial experiments was to investigate the effect of NA on the accumulation of inositol phosphates in the femoral vein of the rat. Results have been expressed as counts per minute (cpm) per tissue and the mean weight of the tissues was 4.82 ± 0.02 mg. In later experiments the tissues were weighed following incubation in ^{3}H-inositol before the tissues were placed into individual vials. Thus, in later experiments, results could be expressed as cpm per tissue or as cpm per mg tissue. Expressing the results in either way led to the same conclusions (e.g. see Figures 10.4 and 10.5).

The basal accumulation of inositol phosphates over the 1 hour incubation period was 1577 ± 78 cpm per tissue (n=7). The addition of 10^{-5}M NA during the incubation period caused a significant increase in inositol phosphate accumulation to 2874 ± 388 cpm per tissue (n=6). All further experiments included a NA test group and a significant increase in inositol phosphate accumulation over control levels in this group was taken as an indicator of experimental validity.

10.2.2. Effect of antagonists on the NA-stimulated accumulation of inositol phosphates.

Chiu, Bozarth and Timmermans (1987) and Hashimoto *et al* (1986) have shown that the NA-induced increase in PPI hydrolysis in both the rat aorta and the rabbit mesenteric artery is mediated via α_1-adrenoceptors. The next step in this part of the study aimed to determine whether the accumulation of inositol phosphates induced by NA in the femoral vein of the rat was mediated via both α_1- and α_2-adrenoceptors or by a single subgroup.

Figure 10.1.a shows the effect of the selective α_1- and α_2-antagonists PZ and ID on the contractile response of the femoral vein to NA (10^{-5}M), and Figure 10.1.b shows the effect of these antagonists on the NA-stimulated accumulation of inositol phosphates in this vessel.

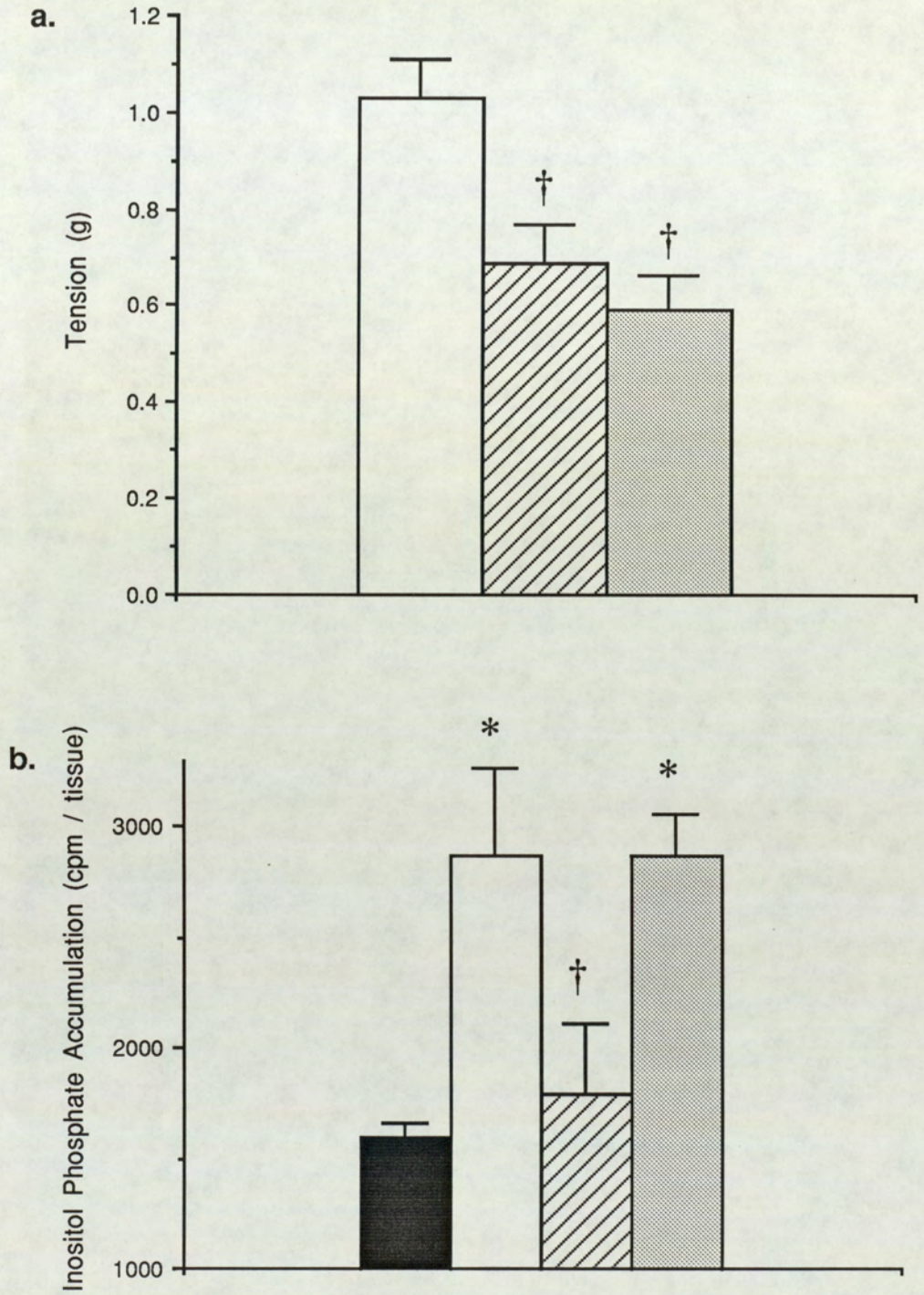


Figure 10.1. The effect of PZ ($1x10^{-8}$M, hatched columns) and ID ($5x10^{-6}$M, stipled columns) on (a) the peak tension, and (b) the accumulation of inositol phosphates, induced by 10^{-5}M NA (open columns) and in unstimulated tissues (filled columns).

Columns are the mean of at least 6 experiments and bars represent s.e.m..

* $p<0.05$ compared to control. † $p<0.05$ compared to NA.

It can be seen that both PZ and ID reduced the contractile response to NA, whereas only PZ reduced the NA-induced accumulation of inositol phosphates, ID having no effect on this accumulation. It is interesting that in the present series of experiments PZ caused a significant decrease in the magnitude of the contractile response to 10^{-5}M NA since in earlier experiments this antagonist had no effect on the contractile response to this concentration of NA (see Figure 8.9). It is probable that these later experiments are more reliable and the unexpected effect of PZ discussed in Chapter 8 may, at least in part, reflect the inexperience of performing initial experiments on a venous preparation which is more fragile than the more common arterial preparations.

These results therefore suggest that while the contractile response of the femoral vein to NA is mediated via both α_1- and α_2-adrenoceptors, as shown in Chapter 8, the NA-induced accumulation of inositol phosphates is mediated via α_1- but not α_2-adrenoceptors.

10.2.3. Effect of selective α_1- and α_2-agonists on inositol phosphate accumulation.

The results described in the previous section indicated that the NA-induced increase in the accumulation of inositol phosphates in the femoral vein is mediated via stimulation of α_1- but not α_2-adrenoceptors. The aim of this section was to extend this evidence by investigating the effect of the selective α_1-agonist CIR and the selective α_2-agonist BHT on inositol phosphate accumulation. As can be seen in Figure 10.2.a, at a concentration of 10^{-5}M, NA, CIR and BHT all caused a contraction of the femoral vein of the rat. The contraction to NA was significantly greater than that for the other two agonists while the contractions to CIR and BHT were of similar size and equal to 60-70% of the NA value.

Figure 10.2.b shows that both NA and CIR caused a significant increase in the accumulation of inositol phosphates compared to control levels.

However, the accumulation seen in the presence of CIR was significantly less than that seen for NA. In contrast, the α_2-agonist BHT caused no significant increase over control levels of inositol phosphate accumulation.

It can therefore be seen that although both CIR and BHT elicit contractile responses in the femoral vein of the rat, only the α_1-agonist CIR causes an increase in the hydrolysis of polyphosphoinositides.

These results therefore confirm the conclusions of the previous section that stimulation of postjunctional α_1- but not α_2-adrenoceptors causes accumulation of inositol phosphates and thus hydrolysis of polyphosphoinositides in the femoral vein of the rat.

ST587 is a partial agonist at α_1-adrenoceptors in many tissues (e.g. rat aorta, Timmermans *et al*, 1983b) although in the femoral vein of the rat it caused no contractile response at concentrations up to 10^{-4}M (see Figure 8.2). However, it was considered worthwhile to investigate the effects of ST on inositol phosphate accumulation in this vessel. Figure 10.2 shows that ST caused no accumulation of inositol phosphates in the femoral vein of the rat.

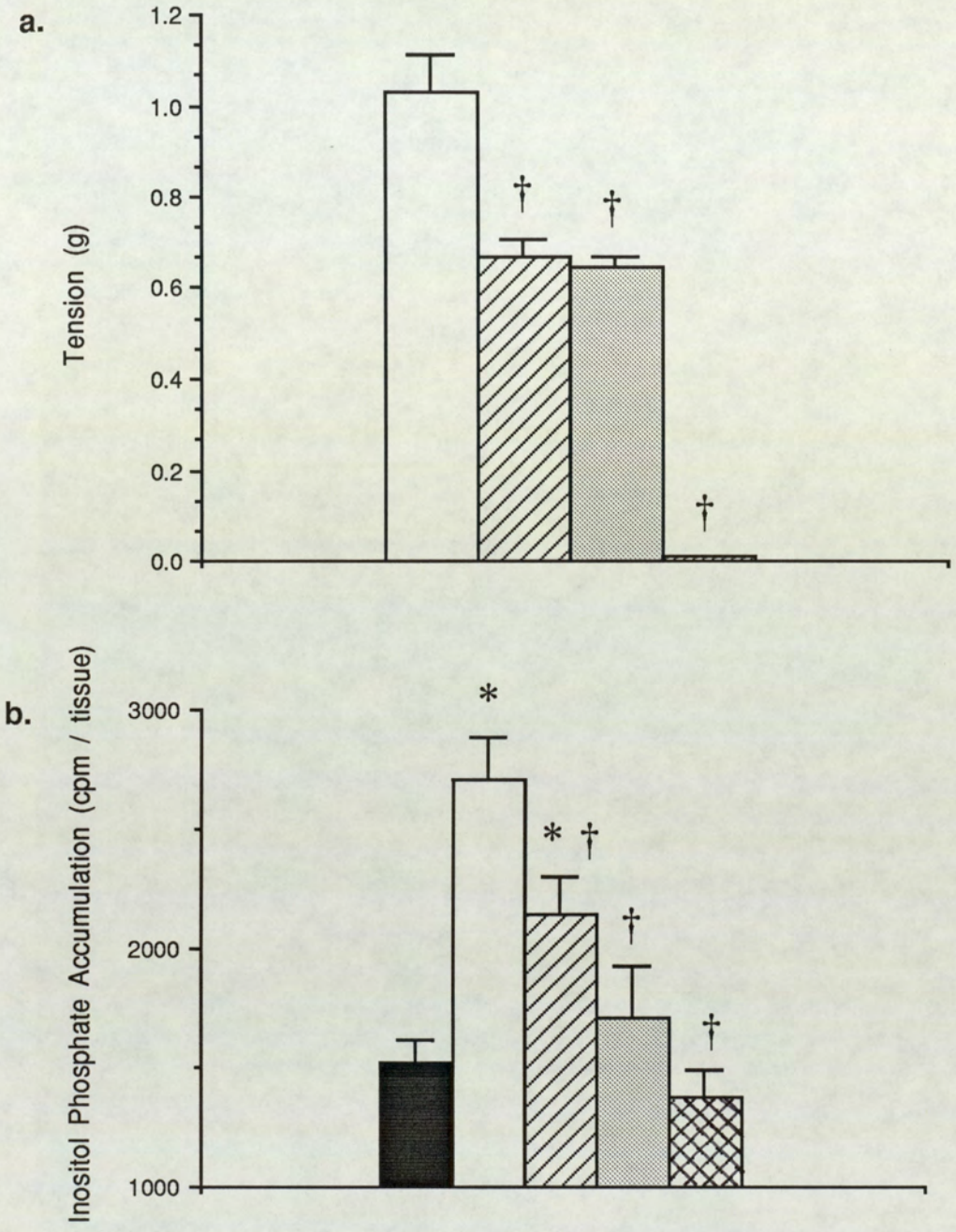


Figure 10.2. Section (a) shows the peak tension of the femoral vein of the rat to NA (open columns), CIR (hatched columns), BHT (stipled columns) and ST (cross-hatched columns) and section (b) shows the accumulation of inositol phosphates induced by the same agonists and the accumulation under control conditions (filled column).

All agonist concentrations are 10^{-5}M. The columns are the mean of at least 6 experiments and the bars represent s.e.m..

* $p<0.05$ compared to control. † $p<0.05$ compared to NA.

10.2.4. Effect of membrane depolarisation induced by KCl on inositol phosphate accumulation.

In the femoral vein of the rat, 60 mM KCl elicited a contractile response of 1.08 ± 0.05g. This is of similar size as that of the full α-adrenoceptor agonist NA (1.03 ± 0.08g) (Figure 10.3.a). It has previously been shown (Figure 9.2) that the contractile response for KCl is mediated entirely by calcium influx and this influx is generally accepted to be a direct consequence of the membrane depolarisation caused by KCl. This influx would not therefore be expected to be associated with increased PPI hydrolysis. However, reports in the literature concerning the effect of KCl depolarisation on PPI hydrolysis are equivocal (see Introduction, section 10.1). It was therefore considered worthwhile to investigate the effects of KCl on inositol phosphate accumulation in the femoral vein of the rat.

Figure 10.3.b shows that incubation in PSS containing 60mM KCl had no effect on inositol phosphate accumulation and inositol phosphate levels were similar to basal levels. Thus, KCl-induced depolarisation did not elicit PPI hydrolysis in the rat femoral vein and it can further be concluded that an influx of calcium across the cell membrane does not itself trigger PPI hydrolysis.

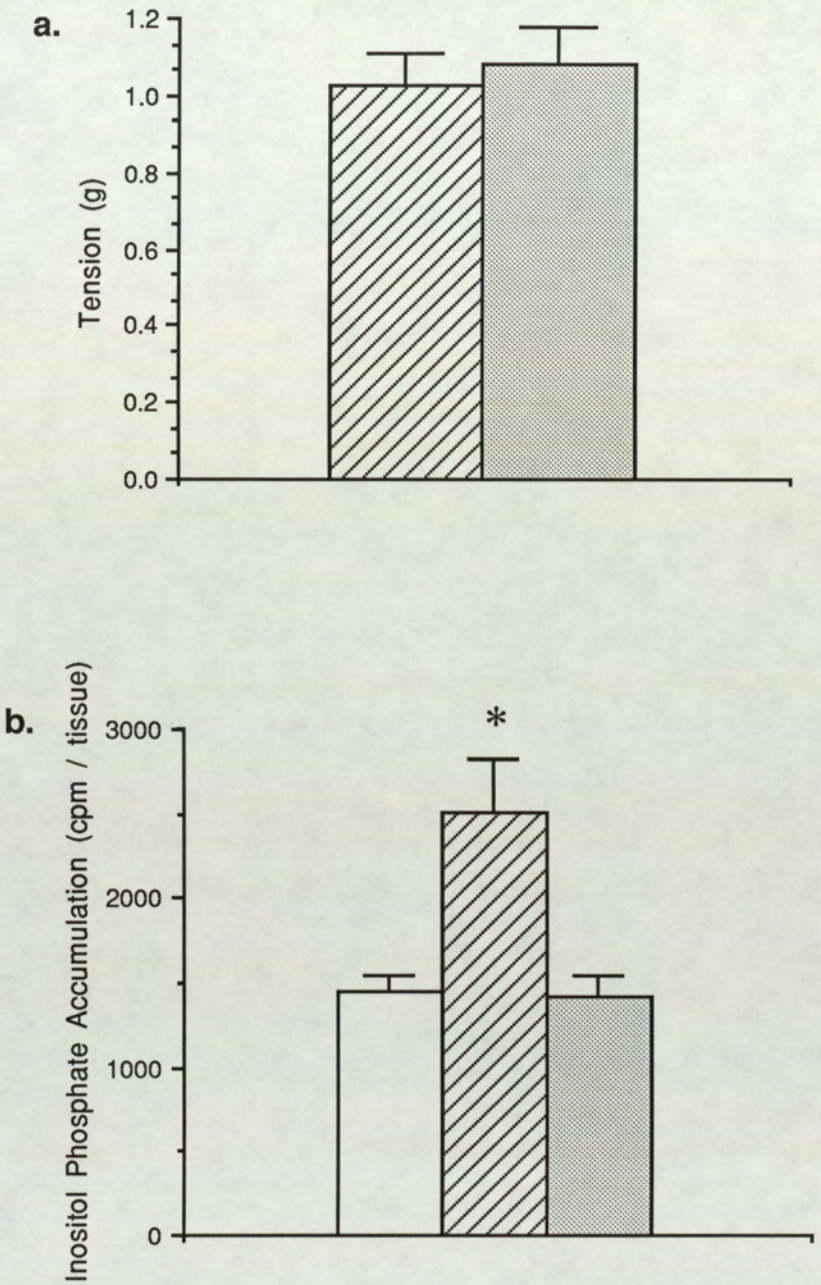


Figure 10.3. Section (a) shows the peak tension of the femoral vein of the rat to 10^{-5}M NA (hatched columns) and 60mM KCl (stipled columns) and section (b) shows the accumulation of inositol phosphates induced by the same agents and the control accumulation (open column).

The columns are the mean of at least 6 experiments and the bars represent s.e.m.. * $p<0.05$ compared to control.

10.2.5. Study of the effect of NA on the accumulation of inositol mono-, bis-, tris- and tetrakisphosphates.

In all previous experiments, the results presented refer to the total inositol phosphate accumulation. The next step in this part of the study was to attempt to determine which of the individual inositol phosphates (i.e. the mono-, bis-, tris- or tetrakisphosphates) accumulate after NA-stimulation. Figure 10.4 shows the accumulation of each of the four phosphates after 1 hour incubation in PSS and after 1 hour incubation in PSS containing 10^{-5}M NA. It can be seen from Figure 10.4 that there was a large increase in the levels of inositol monophosphate following NA stimulation and a small, but significant increase in the accumulation of inositol bisphosphate. The levels of inositol trisphosphate and inositol tetrakisphosphate were both unchanged following 1 hour stimulation by NA. It would thus appear that, over the time of the study, the increase in the inositol phosphate accumulation observed following NA stimulation was probably due to an increase in inositol monophosphate. However, the increase in inositol bisphosphate indicates that a membrane phospholipid more highly phosphorylated than phosphatidylinositol is hydrolysed, suggesting that PIP or PIP_2 is the target of enzyme hydrolysis.

Hashimoto *et al* (1986) have shown that the increase in IP_3 seen following stimulation of rabbit mesenteric artery by NA is transient in nature, falling to baseline levels within 120 seconds of tissue stimulation. In contrast, the increase in IP_2 and IP accumulation follows that of IP_3 and continues to rise after the IP_3 levels return to baseline reflecting the breakdown of IP_3 to IP_2 and IP. It would appear therefore that in the experiments presented above, the NA contact time of 1 hour is too long to allow the increase in IP_3 to be seen. In view of this it seemed worthwhile to repeat the experiment using a shorter contact time and the results for a contact time of 15 seconds are shown in Figure 10.5.

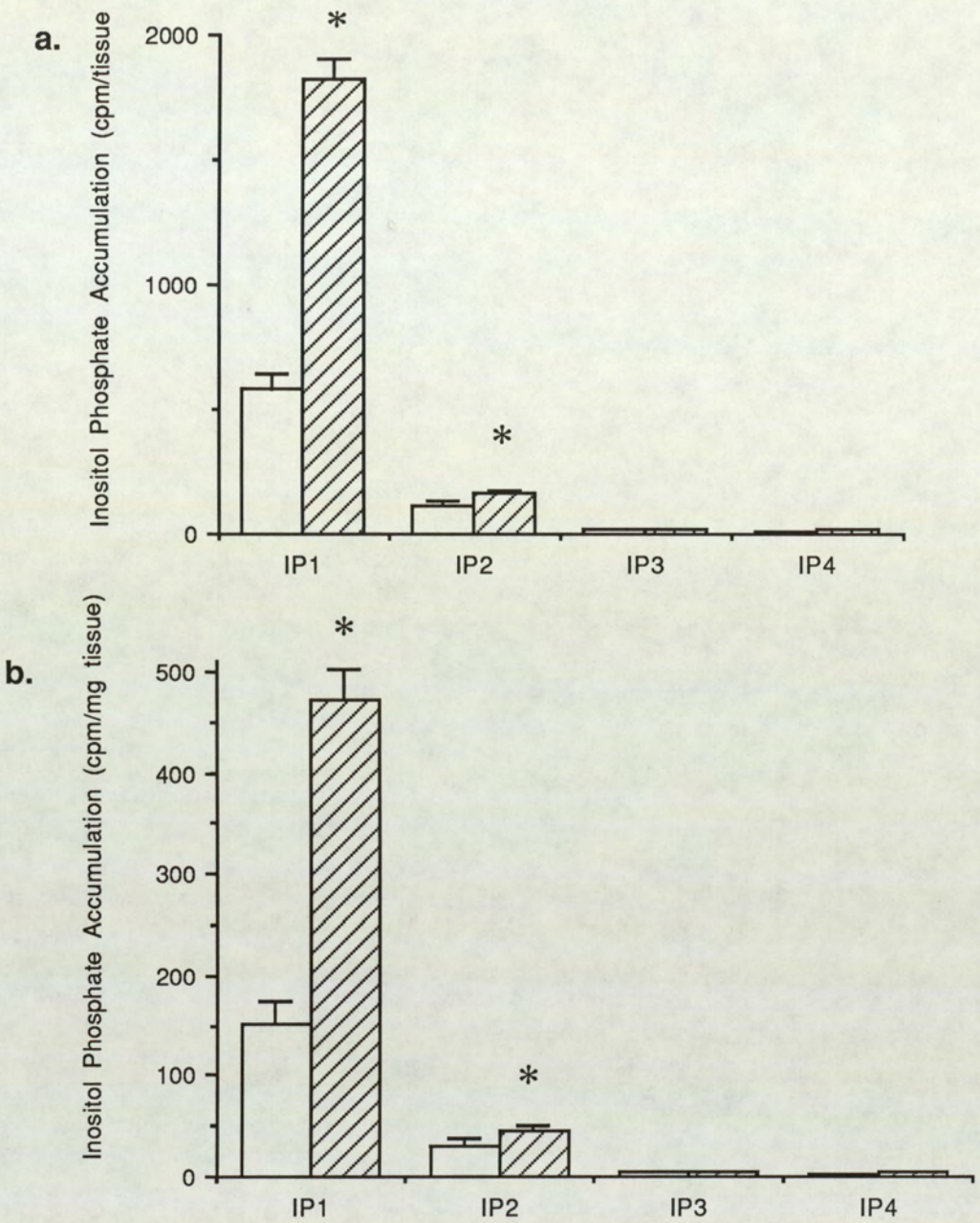


Figure 10.4. The effect of NA on the accumulation of the individual inositol phosphates. Open columns represent the accumulation under control, unstimulated conditions and hatched columns represent that in the presence of 10^{-5}M NA. Figure (a) shows the accumulation expressed as cpm per tissue and in (b) this is expressed as cpm per mg tissue. Inositol phosphates are inositol monophosphate (IP1), inositol bisphosphate (IP2), inositol trisphosphate (IP3) and inositol tetrakisphosphate (IP4).

Columns are the mean of at least 6 experiments and bars are s.e.m..

* $p<0.05$ compared to control.

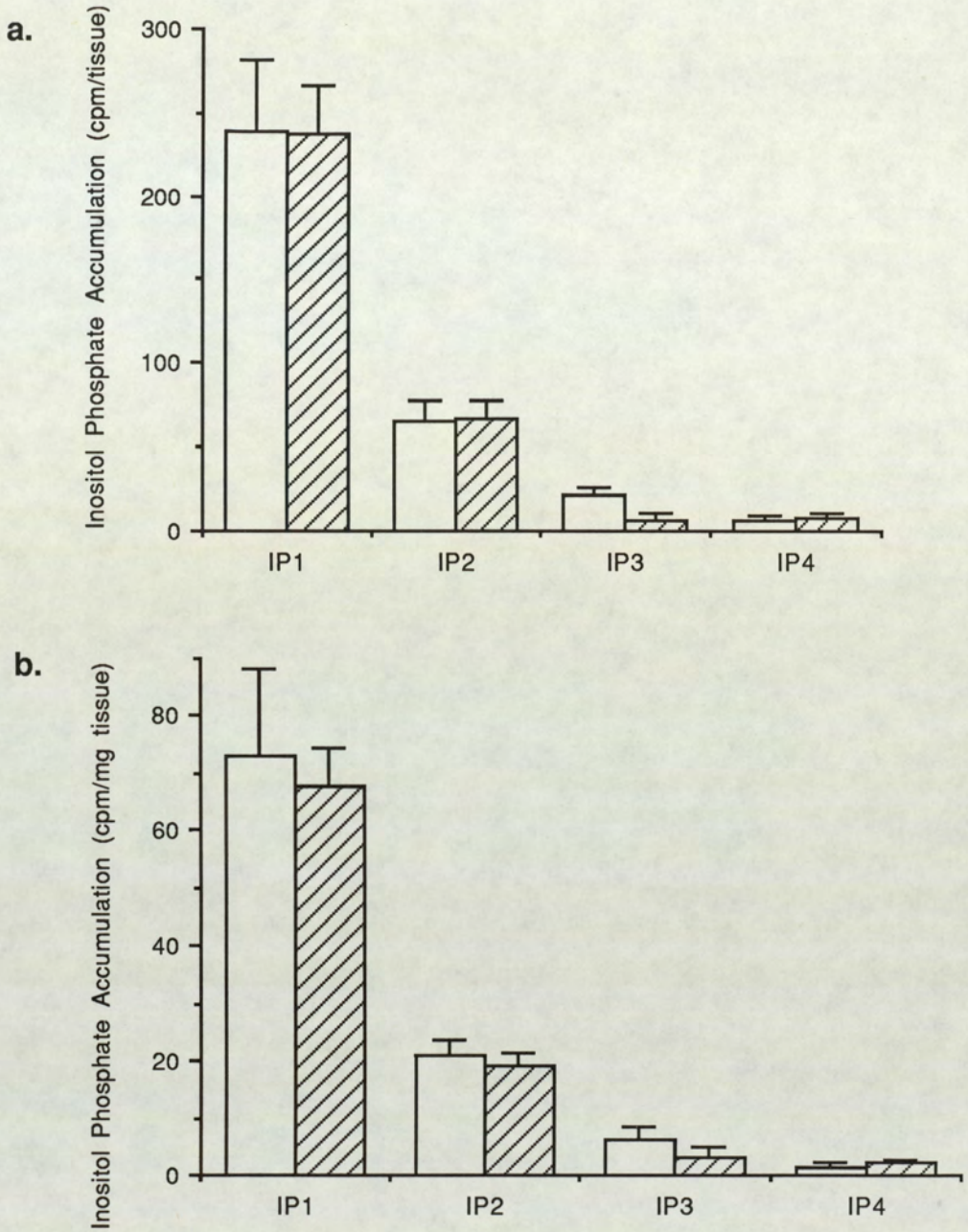


Figure 10.5. The effect of 15 second incubation in NA on the accumulation of the individual inositol phosphates. Open columns represent the accumulation under control, unstimulated conditions and hatched columns represent that in the presence of 10^{-5}M NA. Figure (a) shows the accumulation expressed as cpm per tissue and in (b) this is expressed as cpm per mg tissue. Inositol phosphates are inositol monophosphate (IP1), inositol bisphosphate (IP2), inositol trisphosphate (IP3) and inositol tetrakisphosphate (IP4).

Columns are the mean of at least 6 experiments and bars are s.e.m..

* $p<0.05$ compared to control.

As can be seen, there is no increase in any of the inositol phosphates at a contact time of 15 seconds. This may be because a contact time of 15 seconds may be too short for this vessel in that the small change in inositol phosphate accumulation over a 15 second period is too small to be detected by the assay method.

10.3 DISCUSSION.

Receptor-mediated hydrolysis of polyphosphoinositides has been reported in a variety of tissues and for a number of receptor subtypes (see General Introduction, Chapter 5). This study is concerned with the postjunctional α-adrenoceptors of vascular muscle and it is relevant that a number of workers have reported that the stimulation of postjunctional α_1-adrenoceptors of vascular muscle is associated with increased hydrolysis of polyphosphoinositides (e.g. Legan *et al* (1985), Chiu, Bozarth and Timmermans (1987) and Hashimoto *et al* (1986)). However, to date little is known about the effect of α_2-adrenoceptor stimulation on polyphosphoinositide hydrolysis, and all the studies cited above investigate the effect of agonists in vascular muscle which is devoid of postjunctional α_2-adrenoceptors (i.e. rat aorta and rabbit mesenteric artery). The lack of reports concerning the effect of α_2-stimulation on polyphosphoinositide hydrolysis may reflect a general belief that the excitation-contraction coupling processes of α_2-adrenoceptors are mediated via changes in cAMP levels and thus polyphosphoinositide hydrolysis must be unimportant. Alternatively, it may simply reflect the lack of a suitable vessel in which to study postjunctional α_2-adrenoceptors since there are relatively few vessels in which these receptors have been convincingly demonstrated. It was therefore considered worthwhile to investigate the effects of α_1- and α_2-adrenoceptor stimulation on polyphosphoinositide hydrolysis in the femoral vein of the rat in order to determine which receptor subgroups are linked to their hydrolysis.

At a concentration of 10^{-5}M, NA significantly increased inositol phosphate accumulation (approximately 2-fold). Other reports have also shown a NA-induced increase in inositol phosphate accumulation although a greater than 2-fold increase usually occurs; c.f. Chiu *et al* (1987) who showed a 13-fold increase over unstimulated control values in the rat aorta. The smaller increase seen in this study may reflect slight differences in experimental protocol or tissue differences or it may be that a smaller turnover of

polyphosphoinositides occurs in venous as compared with arterial tissue. However, the relevant point is that a significant increase in polyphosphoinositide hydrolysis occurs following NA-stimulation.

From Figure 10.1 it can be seen that the NA-induced increase in inositol phosphate accumulation is inhibited by the α_1-antagonist PZ, but not the α_2-antagonist ID which suggests that the NA-induced accumulation of inositol phosphates is mediated via the α_1-subtype. This conclusion is supported by the results shown in Figure 10.2 which show that the α_1-agonist CIR produced an increase in inositol phosphate accumulation over basal levels while an equieffective concentration (regarding the contractile response) of the α_2-agonist BHT had no effect on basal inositol phosphate accumulation. It can thus be concluded that while the contractile response of the femoral vein of the rat can be mediated via either α_1- or α_2-adrenoceptors, it is only the α_1-mediated response which is associated with an increased hydrolysis of polyphosphoinositides, and the production of inositol phosphates and DAG.

The effect of NA and CIR on the production of their second messengers, the inositol phosphates, sheds more light onto the nature of these agonists. It has already been discussed (Chapter 8) that the contractile response to NA was greater than that to CIR but that this does not reflect differences in the intrinsic activity of these agonists. Similarly, NA and CIR produced ERRs which were of similar magnitude.

However, when considering the effect of these agonists on polyphosphoinositide hydrolysis, NA did produce a significantly greater response than did CIR. NA produced an increased accumulation of inositol phosphates equal to 1183 ± 174 cpm per tissue over basal levels (see Figure 10.2.b) and CIR produced a significant increase which was equal to 622 ± 156 cpm per tissue. The increase produced by CIR was significantly less than that for NA, and these results therefore support a view that CIR may not be acting as a full agonist in the tissue. Unfortunately, no conclusion can be drawn from the effect of ST587 which produced neither a contractile response nor changes in polyphosphoinositide hydrolysis in the vessel. It thus would seem

that ST587 does not act as an agonist in the tissue.

The results are, in part, consistent with the conclusions of Chiu, McCall, Thoolen and Timmermans (1986) and Chiu, Bozarth and Timmermans (1987) who demonstrated that CIR acts as a partial agonist in the rat aorta and who showed that CIR induced polyphosphoinositide turnover, but that this was less than that for NA and was equal to only 25% of the accumulation for NA. Chiu *et al* (1987) have also shown in the rat aorta that PHE has a high intrinsic activity of 0.94 and that this agonist elicited polyphosphoinositide hydrolysis which was greater than that for CIR but less than that for NA (41% of NA value). It would appear from these observations that intrinsic activity is an important determinant of the ability of α_1-adrenoceptor agonists to hydrolyse polyphosphoinositides, with those agonists which have low intrinsic activities inducing smaller degrees of hydrolysis. It could also be envisaged that a "threshold" intrinsic activity value may exist such that α_1-agonists with intrinsic activities less than this value are unable to hydrolyse polyphosphoinositides. An agonist which falls into this category would be Sgd 101/75 which Chiu and colleagues (1986) have shown to have an intrinsic activity of 0.41 in the rat aorta and which does not cause polyphosphoinositide hydrolysis (Chiu *et al*, 1986).

The study then aimed to determine the effect of KCl-induced depolarisation on polyphosphoinositide hydrolysis in the femoral vein. As can be seen in Figure 10.3.b, 60mM KCl, which caused a maximum contractile response in the vessel (Figure 10.3.a) had no effect on the accumulation of inositol phosphates. Since it has already been shown that the contraction elicited by KCl is totally dependent on calcium influx, it is not surprising that KCl failed to stimulate polyphosphoinositide hydrolysis. However, a further conclusion that can be drawn from this study is that the translocation of calcium itself does not cause polyphosphoinositide hydrolysis.

The final aim of this section of the study was to determine which of the individual inositol phosphates contribute to the increase in total inositol phosphate measured in previous experiments. Figure 10.4 shows that NA

(10^{-5}M) produced the greatest increase in inositol monophosphate. This is as expected since lithium blocks the action of *myo*-inositol-1-phosphatase, the enzyme which converts inositol monophosphate to inositol (Hallcher and Sherman, 1980). Figure 10.4 also shows that an increase in inositol bisphosphate occured and this may be the result of the inositol monophosphate accumulation affecting the equilibrium between inositol monophosphate and inositol bisphosphate such that inositol-1-4-bisphosphate phosphatase, the enzyme which converts inositol bisphosphate to inositol monophosphate was inhibited. Since an increase in inositol bisphosphate occured following receptor stimulation, this would suggest that the phospholipid hydrolysed was not PI but rather PIP or PIP_2. However, no increase in IP_3 or IP_4 were seen and so it cannot be confirmed which polyphosphoinositide was actually hydrolysed.

Berridge (1983) has shown that following stimulation of the fly salivary gland by 5HT, a very rapid accumulation of IP_3 and IP_2 occurs, equivalent to a 5-fold increase, within 5 seconds. However, no change in the level of IP occured. Similarily, Hashimoto *et al* (1986) have shown that following stimulation of the rabbit mesenteric artery, with NA, a large transient increase in IP_3 occured, this increase reaching peak within 10 seconds and falling to baseline level by 120 seconds. In contrast the increase in total phosphates continued to rise after 10 seconds and was still rising after 120 seconds. These results indicate that the increase in IP_3 occurs rapidly and is transient in nature. Thus, in the present study, any increase in IP_3 would already have occured within the 1 hour of NA contact and so it would not be measurable. It was therefore considered worthwhile to investigate the effects of NA-stimulation on individual inositol phosphate accumulation at a shorter time of 15 seconds. Figure 10.5.a reveals that at this time period, no increase in any of the four inositol phosphates are seen. This may be because the time of 15 seconds is too short for this tissue or it may be that the changes in inositol phosphate accumulation that occur over 15 seconds are so small that they cannot be accurately measured in such a small amount of tissue as

used in these experiments.

In conclusion however, the results obtained following 1 hour stimulation with NA suggest that a **poly**phosphoinositide is hydrolysed and reports from the literature (e.g. Berridge, 1983; Hashimoto *et al*, 1986) would indicate that this is PIP_2.

10.4. Summary.

1. This section has shown that in the femoral vein of the rat, although stimulation of both postjunctional α_1- and α_2-adrenoceptors elicited contraction, only stimulation of α_1-adrenoceptors caused accelerated hydrolysis of polyphosphoinositides.

2. The degree of polyphosphoinositide hydrolysis induced by NA is greater than that produced for CIR, despite the fact that these two agonists have previously been shown to elicit ERRs of similar size.

3. KCl-depolarisation was not associated with polyphosphoinositide hydrolysis and the influx of calcium did not trigger increased polyphosphoinositide hydrolysis.

4. Polyphosphoinositides and not phosphatidylinositol are hydrolysed, and the hydrolysis of PIP_2 rather than PIP would be expected.

11. RESULTS SECTION IV

cAMP AND α-ADRENOCEPTOR STIMULATION.

11.1. INTRODUCTION.

In the previous section, it was shown that contractions of the femoral vein mediated by postjunctional α_1-adrenoceptors are associated with the hydrolysis of membrane polyphosphoinositides whereas the receptor-effector coupling mechanisms mediating the contractions for α_2-adrenoceptors do not induce this hydrolysis. Consequently, α_2-adrenoceptor activation must utilise an alternative transduction mechanism.

It is well established that an elevation of cAMP content of vascular muscle causes relaxation (Bolton, 1979) and that the β-adrenoceptor mediated relaxation of vascular muscle is the result of adenylate cyclase activation resulting in increased cAMP production. Robison, Butcher and Sutherland (1967) suggested that α-effects may be mediated by inhibition of adenylate cyclase and a subsequent decrease in cAMP content. Such a decrease has been shown to occur in a number of non-vascular tissues following α_2-stimulation (e.g. human platelets, Salzman and Neri (1969) and Hoffman *et al* (1982); human adipocytes, Burns *et al* (1971); hamster adipocytes, Hittelman *et al* (1973)).

It was therefore considered worthwhile to study the effect of stimulation of postjunctional α_1- and α_2-adrenoceptors on the cAMP content of the femoral vein of the rat in order to determine whether stimulation of either subtype is associated with changes in the content of this nucleotide. The particular aim of the experiment was to determine whether stimulation of the α_2-subtype resulted in a decrease in cAMP content.

A further aim of the study was to investigate the effect of calcium influx on agonist-induced decreases in cAMP content, since an agonist-induced influx in calcium *per se* might cause decreased intracellular cAMP content (e.g. see Rasmussen and Barrett, 1984). This was investigated by determining

the effect of K^+-depolarisation on tissue cAMP content and also by investigating whether the removal of extracellular calcium prevents any agonist induced decreases in tissue cAMP.

11.2. RESULTS.

11.2.1. Basal cAMP content and the effect of α- and β-adrenoceptor stimulation.

Following incubation of tissues in PSS for 60 minutes, the basal cAMP content was determined and was found to be 0.209 ± 0.05 ρmol cAMP per mg tissue (ρmol/mg) (n=6). It is well established that stimulation of β-adrenoceptors causes an increase in tissue cAMP content due to stimulation of the activity of adenylate cyclase (e.g. Meisheri and van Breemen, 1982, rabbit aorta). The effect of the β-adrenoceptor agonist isoprenaline was therefore determined in this vessel. At a concentration of 10^{-6}M, isoprenaline caused a significant increase in cAMP content to 1.56 ± 0.86 ρmol/mg (n=5) (Figure 11.1).

In contrast to this finding, NA (10^{-5}M) caused a significant reduction in the cAMP content of the tissues to 0.068 ± 0.016 ρmol/mg (n=6) (see Figure 11.1).

Thus, following stimulation of the β-adrenoceptors of the vessel, cAMP content is increased while stimulation of the α-adrenoceptors causes a reduction in the cAMP content.

11.2.2. Effect of selective α-adrenoceptor antagonists on the NA-induced decrease in tissue cAMP content.

In an attempt to determine which subgroup of α-adrenoceptor mediates the NA-induced decrease in tissue cAMP content described above, the effect of the α_1-antagonist PZ and the α_2-antagonist ID was determined on the response to NA. Figure 11.2.a shows that both antagonists significantly reduced the contractile response to NA (n=8) which confirms that this response is mediated via both receptor subtypes (see also Chapter 8).

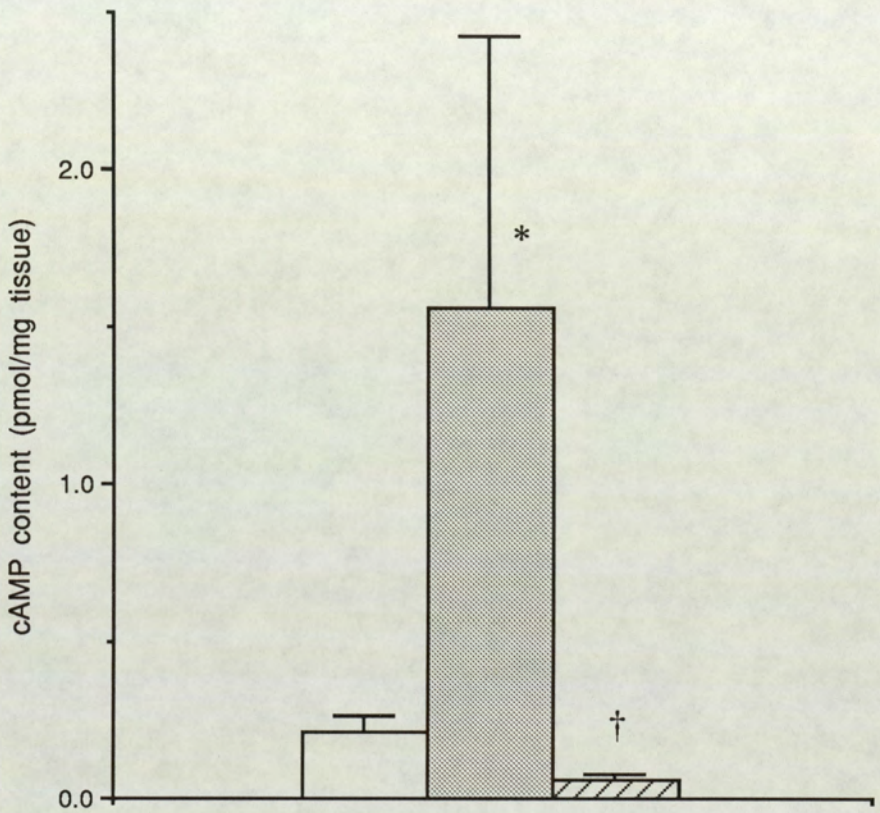


Figure 11.1. The effect of 10^{-6}M ISO (stipled columns) and 10^{-5}M NA (hatched columns) on the cAMP content of the tissues. Open columns represent cAMP content of control, unstimulated tissues.

Columns are the mean of at least 5 experiments and bars are s.e.m..

* significantly greater and † significantly less than control, $p<0.05$.

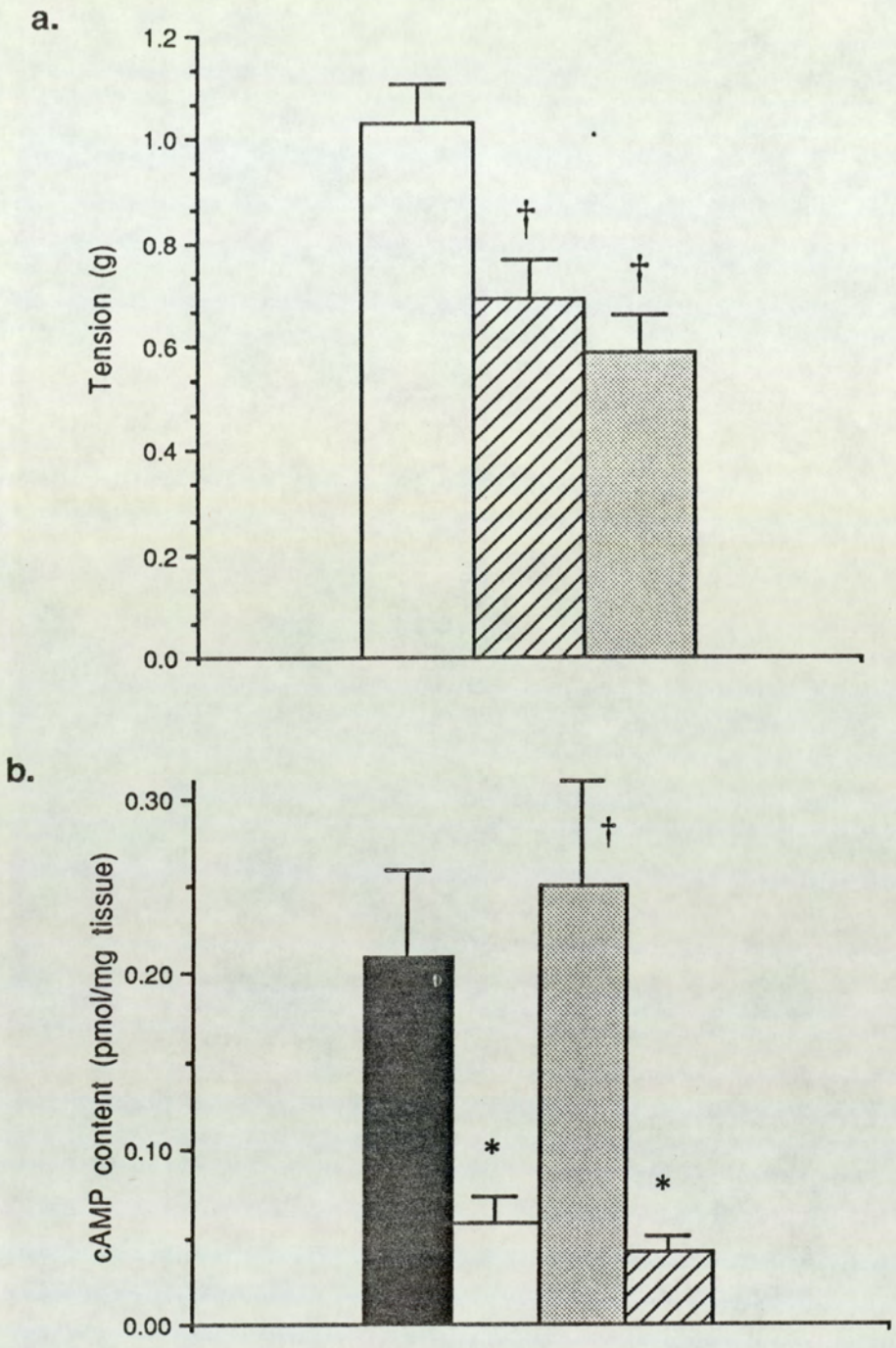


Figure 11.2. Section (a) shows the effect of PZ ($1x10^{-8}$M, hatched columns) and ID ($5x10^{-6}$M, stipled columns) on the contractile response of the tissues to NA (10^{-5}M, open columns) and section (b) shows the effect of these antagonists on the NA-induced decrease in cAMP content. In (b), the filled column represents the control level of cAMP in the tissues.

Columns are the mean of at least 6 experiments and bars are s.e.m..

* $p<0.05$ compared to control. † $p<0.05$ compared to NA.

It can be seen in Figure 11.2.b that when NA was added in the presence of ID, the cAMP content of the tissues was 0.25 ± 0.06 ρmol/mg which was not significantly different from control values. However, in the presence of PZ, NA caused a significant reduction in cAMP content, compared with control values, to 0.042 ± 0.009 ρmol/mg. The NA induced decrease in cAMP content observed in the presence of PZ was not significantly different from the decrease observed for NA alone.

These results show that the NA-induced decrease in tissue cAMP content is antagonised only by ID which suggests that the decrease in cAMP content evoked by NA is mediated by the α_2- but not the α_1-adrenoceptor.

11.2.3. Effect of selective α_1- and α_2-adrenoceptor agonists on tissue cAMP content.

To further investigate the above hypothesis, the effects of the α_1-selective agonist CIR and the α_2-selective agonist BHT were investigated on tissue cAMP content. Figure 11.3.b shows that at a concentration of 10^{-5}M, BHT caused a significant decrease in tissue cAMP content to 0.033 ± 0.006 ρmol/mg ($n=6$, $p<0.05$), which is not significantly different to that seen in the presence of NA (Figure 11.3.).

In contrast, the α_1-agonist CIR (10^{-5}M) caused no change in tissue cAMP content as compared to the content of control tissues (Figure 11.3.). The cAMP content of tissues exposed to CIR for 3 minutes was 0.196 ± 0.039 pmol/mg. It has previously been shown in this study (Figure 8.2) that the shape of the contractile response to CIR is transient in nature, reaching a peak tension approximately 1 minute after agonist addition and then falling to baseline, or near baseline, levels. It could therefore be argued that the second messenger levels may also follow a similar pattern and, if this were so, then any change in cAMP content would have passed at 3 minutes and would not be detected in the assay.

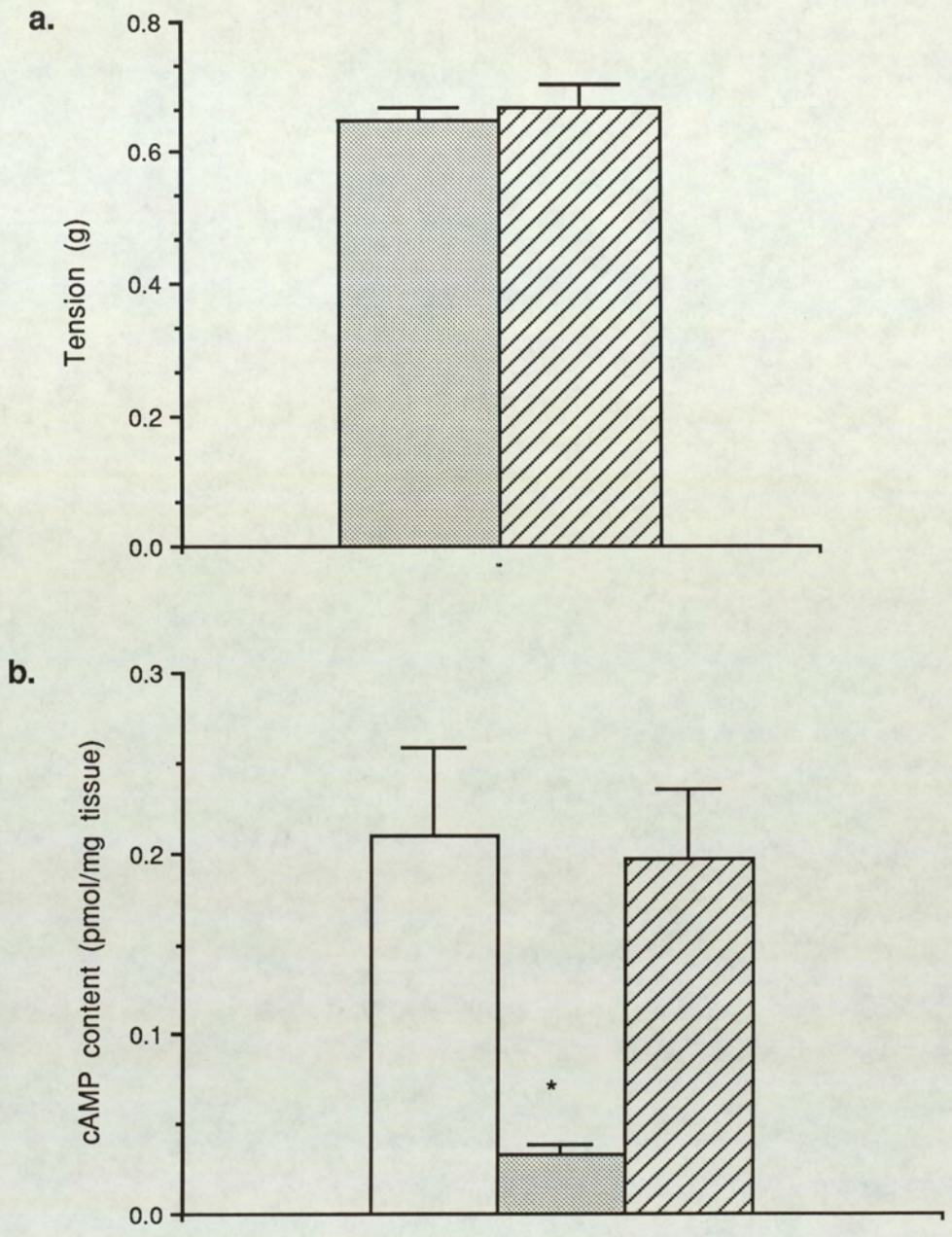


Figure 11.3. Section (a) shows the peak tension of the tissues to BHT (10^{-5}M, stipled columns) and CIR (10^{-5}M, hatched columns) and section (b) shows the effect of these agonists on the cAMP content of the tissues. In (b), the open column represents the control cAMP content of the tissues.

Columns are the mean of at least 6 experiments and bars are s.e.m..

* $p<0.05$ compared to control. † $p<0.05$ compared to NA.

For this reason, the effect of shorter agonist contact times for CIR was investigated. At a contact time of 1 and 2 minutes, tissue cAMP content was 0.267 ± 0.056 ρmol/mg (n=5) and 0.26 ± 0.03 pmol/mg (n=6) respectively. Neither value is significantly different to the value of cAMP content after 3 minutes of CIR contact, nor from basal cAMP content.

These results therefore provide further support for the suggestion that stimulation of α_2-adrenoceptors causes a decrease in the cAMP content of the tissues while stimulation of α_1-adrenoceptors has no effect.

11.2.4. Effect of incubation in calcium-free PSS and K^+-depolarisation on cAMP content.

To determine whether the BHT-induced decrease in cAMP content was a consequence of the increase in intracellular calcium, the effect of BHT was determined following incubation of the tissues in calcium-free PSS containing 0.2 mM EGTA. Figure 11.3.b showed that in normal PSS, following BHT-stimulation, the cAMP content of the tissues was reduced to 0.033 ± 0.006 ρmol/mg. Incubation of the tissues in the calcium-free/EGTA medium had no effect on the cAMP level of unstimulated tissues which was 0.209 ± 0.05 ρmol/mg in PSS containing calcium and 0.215 ± 0.02 ρmol/mg (n=5) after 2 minutes incubation in calcium-free PSS containing EGTA. Following incubation of the tissues in calcium-free PSS, BHT caused a reduction in tissue cAMP content to 0.027 ± 0.003 ρmol/mg (n=8). There was no significant difference between the cAMP content of BHT-stimulated tissues in normal or calcium-free PSS and therefore removal of calcium from the PSS did not prevent the α_2-induced decrease in tissue cAMP content. These results therefore suggest that the BHT-mediated decrease in cAMP content is not the consequence of a rise in the intracellular calcium concentration.

The effect of an increase in the intracellular calcium concentration on the tissue cAMP content was further studied by investigating the effect of 60 mM KCl. 60 mM KCl caused contraction of the tissues by increasing calcium influx

and if an increase in the intracellular calcium of the tissues resulted in a decrease in the tissue cAMP content, then such a decrease would also be expected following KCl stimulation. However, after 3 minutes incubation of the tissues in 60 mM KCl, the cAMP content of the tissues was 0.21 ± 0.024 ρmol/mg (n=6) and this is not different to the control level. KCl thus caused no change in the tissue cAMP content and this supports the previous results that an increase in intracellular calcium does not itself cause a decrease in tissue cAMP.

11.3. DISCUSSION.

In the previous chapter of this thesis evidence was presented for a role for polyphosphoinositide hydrolysis in the α_1-mediated response of the rat femoral vein, but it was shown that contractions mediated by α_2-adrenoceptors are not associated with polyphosphoinositide hydrolysis. An alternative receptor-effector transduction mechanism must therefore be considered for vascular postjunctional α_2-adrenoceptors and evidence from studies of non-vascular α_2-adrenoceptors suggest a possible link between α_2-stimulation and inhibition of adenylate cyclase activity.

In 1967, Robison, Butcher and Sutherland proposed that both α- and β-adrenoceptors were coupled to adenylate cyclase, the α-adrenoceptor being coupled in an inhibitory fashion while stimulation of the β-adrenoceptor was excitatory. It is worth noting that these observations pre-date the demonstration that postjunctional α-adrenoceptors are not homogenous and may be divided into two subclasses (α_1 and α_2) (Drew and Whiting, 1979). A β-mediated increase in tissue cAMP content is now well established in vascular and non-vascular tissues while in a number of non-vascular tissues, stimulation of α_2-adrenoceptors has been shown to cause a decrease in tissue cAMP content (e.g. human platelets, Salzman and Neri (1979) and hamster adipocytes, Hittelman, Wu and Butcher (1973)). However, since early studies investigating the effect of α-adrenoceptor stimulation in vascular muscle did not take into account the existance of a dual population of postjunctional α-adrenoceptors, interpretation of results is difficult. It is not possible to assess whether any of the response of early experiments is mediated by α_2-adrenoceptors and therefore early work investigating the effects of α-adrenoceptor stimulation on the cAMP content of vascular tissues has produced conflicting results. In the canine coronary artery (Seidel, Schnarr and Sparks, 1975) it has been reported that stimulation of α-adrenoceptors is not associated with decreased cAMP levels whereas in rat aorta and rat tail artery (Volicer and Hynie, 1971) and bovine mesenteric artery (Anderson,

1972) α-stimulation has been associated with a decrease in cAMP content.

These apparent discrepancies may be explained by the existance of postjunctional subpopulations of the α-adrenoceptor. For example, a failure to demonstrate a decrease in cAMP in some of these early reports could be the result of a lack of α_2-adrenoceptor stimulation either because no α_2-adrenoceptors were present on the tissues used, or the agonists employed had little α_2-stimulatory activity. Alternatively, the discrepancies may arise from methodological differences, arising possibly from the low sensitivity and selectivity of the radioimmunoassay used. For example, it is interesting that in the study of Volicer and Hynie (1971) it was shown that stimulation of the α-adrenoceptors of the rat aorta by noradrenaline caused a decrease in cAMP content and yet more recent reports have indicated that this vessel possesses no population of postjunctional α_2-adrenoceptors. Therefore, the change in cAMP content reported by these workers would not be expected to be the consequence of α-adrenoceptor stimulation and it is suggested that these may be either a misinterpretation of results or a lack of reliability of the methods used.

Since the late 1970s in only a few vessels have postjunctional α_2-adrenoceptors been demonstrated conclusively (see General Introduction) and this has been a major difficulty for the study of these receptors *in vitro*. As a result of this, there have been no studies reported of the effect of stimulation of vascular postjunctional α_2-adrenoceptors on tissue cAMP levels. However, the present study has shown the femoral vein of the rat to be a suitable vessel for the study of both postjunctional α-adrenoceptor subgroups, thus enabling the investigation of the effect of stimulation of both receptor subtypes to be made, and on the same vessel. Also, in recent years both agonists and antagonists with increased selectivities and specificities for either α_1- or α_2-adrenoceptors have become available making the study of the mechanisms of these receptors more precise and the emergence of new radioimmunoassays utilising double antibody reactions gives a more highly specific and selective determination of cAMP content.

The basal cAMP content of the femoral vein of the rat (i.e. the content of cAMP in unstimulated tissues) was found to be 0.209 ± 0.05 ρmol/mg. The protocol used was that of Itoh, Izumi and Kuriyama (1982) who reported a basal cAMP content of 2.2 ± 0.3 ρmol/mg tissue in the guinea-pig mesenteric artery. This higher value may reflect tissue differences or a difference between arterial and venous tissue. Challenge of the guinea-pig mesenteric artery with isoprenaline caused relaxation of K^+-contracted vessels and produced a significant 2-fold increase in tissue cAMP content (Itoh *et al*, 1982). In the present study, isoprenaline caused relaxation of the isolated femoral vein pre-contracted by NA and also produced a significant increase in the cAMP content of the vessels (from 0.209 to 1.56 ρmol/mg; a 7.5-fold increase). Furthermore, the non-selective α-agonist noradrenaline (10^{-5}M) caused a significant decrease in the cAMP content reducing the content to 0.068 ± 0.016 ρmol/mg. These results therefore confirm that the β-agonist isoprenaline causes an increase in the cAMP content of the femoral vein of the rat while the α-agonist NA produces a decrease in cAMP content.

The next step in this part of the study was to determine which subgroup(s) of α-adrenoceptor mediate the NA-induced decrease in cAMP content. Figure 11.2 shows the effect of the selective α_1-antagonist PZ and the selective α_2-antagonist ID on NA-induced contractile responses (11.2.a) and changes in cAMP content (11.2.b). Both PZ and ID caused a significant reduction in the contractile response to NA. However, only ID was found to antagonise the NA-mediated decrease in cAMP content (Figure 11.2.b). These results therefore indicate that the NA-induced decrease in tissue cAMP content is mediated via α_2-adrenoceptors.

This conclusion is substantiated by the results obtained with the selective α_2-agonist BHT and the selective α_1-agonist CIR. Both agonists produced a contractile response in the femoral vein but only BHT produced a decrease in the tissue cAMP content.

The final objective of this study was to investigate the effect of a depolarising solution of K^+ on tissue cAMP content and to determine

whether the BHT-induced decrease in cAMP content was a direct effect of BHT or the consequence of BHT-mediated influx of calcium. The exact mechanism by which α_2-adrenoceptor stimulation results in decreased intracellular cAMP is unknown, but is thought to be the result of inhibition of adenylate cyclase. However, an alternative is that the decreased cAMP is a consequence of an increase in intracellular calcium. Thus, if the α_2-adrenoceptor is linked directly to a calcium channel, the increase in the intracellular calcium concentration could be responsible for a decrease in cAMP (see General Introduction, Chapter 5 and Rasmussen and Barrett, 1984). Since α_1-adrenoceptor stimulation also causes a rise in the intracellular calcium concentration but does not decrease cAMP content, then it would seem likely that the BHT-induced decrease in cAMP is not the result of increased intracellular calcium, although it was considered worthwhile to investigate this further.

The effect of incubation of the tissues in calcium-free PSS on the cAMP content was determined and it was shown that this incubation had no effect on basal, unstimulated cAMP content (section 11.2.4.). To study the effect of α_2-adrenoceptor stimulation on cAMP content, tissues were preincubated in calcium-free PSS containing EGTA before the addition of BHT, in order to prevent a BHT-induced influx of calcium. Since no calcium is then present in the bathing medium, any changes in tissue cAMP content that occur cannot be the consequence of calcium influx and must therefore be the consequence of stimulation of the α_2-adrenoceptors. Following incubation in calcium-free PSS, BHT caused a significant decrease in tissue cAMP content from a control calcium-free content of 0.215 ± 0.02 pmol/mg to 0.027 ± 0.003 pmol/mg. This decrease is not significantly different to that seen for BHT in normal PSS and this indicates that the decrease in cAMP produced by BHT is not the consequence of a BHT-mediated increase in intracellular calcium.

To further substantiate this, tissues were exposed to a depolarising concentration of KCl (60mM) in PSS containing calcium. This concentration of KCl caused a maximal contractile response of the tissues and this response

was mediated entirely by calcium influx (see Chapter 9). The cAMP content of tissues following exposure to 60mM KCl (0.21 ± 0.024 ρmol/mg) was not significantly different to the basal unstimulated content (0.209 ± 0.05 ρmol/mg) indicating that the influx of calcium mediated by KCl did not cause a decrease in tissue cAMP content.

11.4. Summary.

1. The cAMP content of the femoral vein of the rat was determined under unstimulated (basal) conditions and in the presence of α- and β-adrenoceptor agonists. Selective α_1- and α_2-agonists and antagonists were used to determine whether stimulation of either α-adrenoceptor subtype caused changes in cAMP content of the vessels.

2. In this vessel, the basal, unstimulated cAMP content is 0.209 ± 0.05 ρmol/mg tissue wet weight. The β-agonist isoprenaline caused a significant increase in cAMP content while the α-agonist NA caused a significant decrease in cAMP content.

3. The NA-induced decrease in cAMP content was inhibited following 15 minutes incubation of the tissues in $5x10^{-6}$M ID while incubation in 10^{-8}M PZ had no effect on this decrease.

4. The selective α_2-agonist BHT produced a decrease in tissue cAMP content while the α_1-selective agonist CIR had no effect on cAMP content.

5. Incubation of the tissues in calcium-free PSS had no effect on basal cAMP content and did not prevent the BHT-mediated decrease in cAMP. Also, 60mM KCl failed to decrease cAMP content and thus the decrease in cAMP content mediated by BHT was the consequence of α_2-adrenoceptor stimulation rather than of calcium influx.

It is concluded that stimulation of the postjunctional α_2-adrenoceptors of the femoral vein of the rat causes a decrease in the cAMP content of the vessel while stimulation of the postjunctional α_1-adrenoceptors has no effect on the cAMP content.

12. GENERAL DISCUSSION.

12.1. Rationale behind the study.

In 1979, Drew and Whiting reported that the postjunctional α-adrenoceptor population of both the anaesthetised cat and the pithed rat comprised of a mixed population of α_1- and α_2-adrenoceptors. An abundance of *in vivo* evidence has since supported this observation (e.g. pithed rat; Timmermans *et al*, 1979 and conscious rabbit; Hamilton and Reid, 1980), although to date few isolated blood vessels have been conclusively shown to possess both postjunctional α-adrenoceptor subtypes. This is true for the rat despite the fact that a great deal of *in vivo* research has been performed on the postjunctional α_2-adrenoceptors of this animal. Cheung (1985) has suggested that the saphenous vein of the rat possesses postjunctional α_2-adrenoceptors, however, the small size of this vessel makes it unsuitable for detailed pharmacological study. The initial aim of the present study was to investigate the postjunctional α-adrenoceptor population of the femoral vein of the rat, a vessel chosen for study because of its anatomical proximity to the saphenous vein. Section 8 of this thesis has shown the femoral vein to possess a postjunctional population of both α_1- and α_2-adrenoceptors making it suitable for the further study of these receptors *in vitro*.

It has been shown that stimulation of the postjunctional α_1-adrenoceptors of a number of isolated blood vessels is associated with hydrolysis of polyphosphoinositides (e.g. Legan *et al*, 1985 and Chiu *et al*, 1987). Such a phenomenon has not been reported following stimulation of vascular α_2-adrenoceptors, although stimulation of the α_2-adrenoceptors of a number of non-vascular tissues (e.g. Saltzman and Neri, 1969; human platelets) is associated with an inhibition of adenylate cyclase and a subsequent decrease in tissue cAMP levels. The major theme of this thesis is a comparison of the excitation-contraction coupling processes of the postjunctional α_1- and α_2-adrenoceptors of the femoral vein of the rat and the thesis attempts to

determine the second messengers associated with each adrenoceptor and to relate these to the calcium mobilisation processes associated with contraction following stimulation of each α-adrenoceptor subtype.

12.2. The second messengers associated with each α-adrenoceptor subtype.

The results presented in Chapter 10 of this thesis indicate that stimulation of the postjunctional α_1-adrenoceptors of the femoral vein of the rat by either selective α_1-agonists or by the non-selective agonist noradrenaline in the presence of an α_2-antagonist result in an accelerated hydrolysis of membrane polyphosphoinositides as determined by the increased incorporation of radiolabel into inositol phosphates. These results therefore support the observations of Legan *et al* (1985) and Chiu *et al* (1987) who reported a similar effect following stimulation of the α_1-adrenoceptors of the rat aorta. In contrast to this observation, stimulation of postjunctional α_2-adrenoceptors by either selective α_2-agonists or by noradrenaline in the presence of an α_1-antagonist had no effect on this hydrolysis. It was further shown that the degree of polyphosphoinositide hydrolysis elicited by α_1-stimulation was dependent on the activating agonist since the hydrolysis for noradrenaline was greater than that for CIR.

It was shown in Chapter 11 that stimulation of the α_2-adrenoceptors of the femoral vein was associated with a decrease in the intracellular levels of cAMP of the tissues while stimulation of the α_1-adrenoceptors was without effect on this parameter.

12.3. The link between agonist stimulation and calcium release.

An area of much debate in the field concerns the sources of activator calcium utilised by stimulation of either α-adrenoceptor subtype and the ability of each subgroup to cause the release of intracellular calcium. This has been discussed previously in Chapter 9 of this thesis. In this chapter, two general hypotheses concerning the utilisation of intracellular and extracellular calcium were discussed; the first of these is the hypothesis of van

Meel and colleagues who suggest that it is the subtype of α-adrenoceptor stimulated *per se* which determines the source(s) of activator calcium utilised by an agonist. Thus, van Meel *et al* (1984) suggest that activation of α_1-adrenoceptors causes both the release of calcium from intracellular organelles and the influx of calcium across the cell membrane, while stimulation of α_2-adrenoceptors causes influx only and is incapable of eliciting calcium release.

This is not the sole hypothesis forwarded to explain the ability of some agonists to release intracellular calcium and Ruffolo and coworkers suggest that it is not the subgroup of receptor stimulated by the agonist which determines the ability of an agonist to release intracellular calcium, but rather a property of the agonist itself, which Ruffolo *et al* (1987) suggest to be the agonist intrinsic activity.

Indirect evidence to support the suggestion that α_1-adrenoceptor stimulation results in the release of calcium from intracellular stores while α_2-stimulation is unable to do so arises from the study of the form of the contractile response produced following α_1- and α_2-stimulation (see Figure 8.1). It can be seen from Figure 8.1 that the contractile response following α_1-adrenoceptor stimulation developed rapidly and was transient in nature. In contrast, contractions following α_2-adrenoceptor stimulation developed relatively slowly but were better maintained (see Chapter 8, section 8.2.1). As mentioned previously in the General Introduction, Janis and Triggle (1983) have reported that the intracellular calcium concentration of the smooth muscle cell must rise from a resting level of 10^{-7}M to a concentration of 10^{-5}M in order for contraction to occur. It is possible therefore that the rapid rise in tension seen following α_1-adrenoceptor stimulation (see Figure 8.1) reflects a rapid rise in the intracellular calcium concentration while the slowly developed contraction mediated by α_2-stimulation is associated with a slow increase in the concentration of intracellular calcium.

Such differences in the rate of rise of the intracellular calcium concentration for α_1- and α_2-adrenoceptor stimulation may be explained by different sources

of activator calcium for the two adrenoceptor subtypes. Thus, the rapid rise in tension seen following α_1-stimulation is the result of a rapid increase in the intracellular calcium concentration following the release of calcium from the intracellular store in the endoplasmic reticulum, which may become rapidly depleted, hence the transient form. In contrast, since α_2-stimulation does not result in the release of this calcium store but relies solely on the influx of calcium from the extracellular space, the increase in intracellular calcium concentration is not as rapid, as reflected by the slowly developed tension seen following stimulation of these receptors.

The possibility that either α-adrenoceptor subtype or agonist intrinsic activity determines the ability of an agonist to release intracellular calcium was investigated more directly in Chapter 9. It was shown that both the non-selective α-adrenoceptor agonist NA and the α_1-selective agonist CIR were able to cause release of intracellular calcium (as determined by the ability to produce a contraction in calcium-free PSS containing EGTA) while the selective α_2-agonist BHT was unable to do so. This therefore suggests that the α-adrenoceptor subtype stimulated is a determinant of the ability to utilise intracellular calcium. However, as discussed in Chapter 9 of this thesis, a number of workers in other laboratories have shown that α_1-agonists with low intrinsic activities are incapable of causing the release of intracellular calcium and are thus dependent on extracellular calcium alone for contraction. For example, Timmermans *et al* (1983a) showed that the pressor response of the pithed rat to the partial α_1-agonist Sgd 101/75 was inhibited by nifedipine while responses to full α_1-agonists were not.

It therefore appears that the initial determinant of the ability of an agonist to release intracellular calcium is the subgroup of α-adrenoceptor stimulated such that α_2-agonists are unable to cause this release while α_1-agonists may be able to do so. Timmermans *et al* (1986) have shown that a range of α_2-agonists of differing efficacies and potencies are all equally susceptible to blockade by nifedipine suggesting that efficacy or potency are unimportant in determining the susceptibility of these agonists to calcium entry blockers and

so it does appear likely that all α_2-agonists, irrespective of the intrinsic activity, are incapable of releasing intracellular calcium. However, regarding the α_1-adrenoceptor, evidence from the literature suggests that the intrinsic activity of the agonist does appear to determine whether the agonist can release intracellular calcium and α_1-agonists with high intrinsic activities can do so while those with low cannot. In the present study, NA and CIR produced a full ERR response but ST587, which is a partial α_1-agonist in the rat aorta (Timmermans *et al*, 1983b) produced no contractile response in this tissue. This may suggest that a minimal receptor reserve of α_1-adrenoceptors exists in the femoral vein of the rat, although no experiments were performed to investigate the receptor reserve of this vessel. Due to the limitations of time, no other 'partial' α_1-agonists were investigated in the present study and therefore this study provides no information as to the influence of the intrinsic activity of α_1-agonists upon the ability of the agonist to release calcium from intracellular stores.

It is interesting to question why a differentiation in the utilisation of activator calcium is seen for α_1- and α_2-adrenoceptors and it would appear that this is related to the different excitation-contraction coupling mechanisms linked to α_1- and α_2-adrenoceptors. Berridge (1983) proposed that an immediate product of polyphosphoinositide hydrolysis, namely IP_3, acts as an intracellular messenger to cause the release of calcium from its intracellular store in the endoplasmic reticulum and this view is now widely accepted. Streb *et al* (1983) suggested that a population of receptors specific for IP_3 exist on the membrane of the E.R., stimulation of which causes the release of the contents of the calcium store from the E.R.. It can therefore be seen that the ability of agonists to release intracellular calcium is related to their ability to hydrolyse polyphosphoinositides, and from this it can be seen that α_1-agonists would be expected to cause this release while α_2-agonists would not. Thus, the ability of α_1-agonists to hydrolyse polyphosphoinositides and produce the intracellular messenger IP_3 explains why stimulation of α_1-adrenoceptors is associated with the release of intracellular calcium while

stimulation of α_2-adrenoceptors is not.

The results presented in Chapter 10 of this thesis thus support the hypothesis of van Meel *et al* (1981) that it is the subgroup of adrenoceptor which determines the ability of an agonist to release intracellular calcium. However, the alternative hypothesis of Ruffolo *et al* (1984) that agonist intrinsic activity is an important determinant of the ability to release intracellular calcium need not be immediately refuted. As discussed previously, this study provides no evidence to support the hypothesis that release of intracellular calcium (as measured from the amplitude of the ERR) is a function of the intrinsic activity of the agonist. However, the results in Chapter 10 do suggest that α_1-agonists do not all elicit a similar turnover of polyphosphoinositides. Thus, as shown in Chapter 10, the IP accumulation after stimulation of the tissues by CIR was only about 50% of that following stimulation by NA.

It is noteworthy that despite the difference in IP accumulation seen for NA and CIR, the two agonists were equally effective at causing release of intracellular calcium as determined by the ERRs which were of similar magnitude (see Figure 9.2). This suggests that the relationship between polyphosphoinositide hydrolysis and release of intracellular calcium is more complex than a simple linear relationship and a number of possible suggestions as to why a linear relationship does not exist can be forwarded. It is possible that such is the efficacy of IP_3 for the E.R. membrane IP_3 receptor that a large population of spare receptors exists and that both NA and CIR elicit a turnover of polyphosphoinositides which is great enough to produce a full calcium-release response; i.e. both are above the threshold value for IP_3 release. Alternatively, a spare receptor population may be absent, but a large excess of IP_3 may be produced following both NA and CIR mediated stimulation of α_1-adrenoceptors.

Alternatively one can consider the calcium movements following α_1-stimulation, and suggest that it may be that the amount of intracellular calcium released by NA is indeed greater than that released by CIR, but that

both agonists exhibit the maximum "intracellular calcium response" and release an excess of calcium, this excess being greater for NA.

Clearly this area needs further study and the concepts mentioned above could be further investigated through the use of α_1-agonists with varying intrinsic activities. It would be expected that an α_1-agonist which produced an ERR less than that for CIR or NA would only elicit a very small turnover of polyphosphoinositides. Alternatively, the use of antagonists selective for the IP_3 receptor would be expected to prevent the release of intracellular calcium following α_1-stimulation and therefore abolish the ERR for these agonists. Further, such an antagonist should alter the shape of the contractile response for α_1-agonists while proving to be ineffective against α_2-agonists.

Recently, Nahorski and colleagues (1988) have reported the effects of a synthetic analogue of IP_3 which displaces IP_3 from its binding site on cerebellar membranes and is a full agonist of intracellular calcium release. This analogue, $IP(S)_3$ however is resistant to attack by inositol-5-phosphatase and is therefore resistant to metabolism. It might be that a synthetic IP_3 analogue which has antagonistic properties could be reported in the near future and this may serve to be a useful pharmacological asset.

In conclusion, it can be seen that the findings of this thesis lend support to a hypothesis which combines those of van Meel *et al* (1984) and Ruffolo *et al* (1987). As suggested by the former group of workers, an initial determinant of the ability of an α-agonist to release intracellular calcium is the subgroup of adrenoceptor stimulated by the agonist such that α_1-agonists may release intracellular calcium while α_2-agonists cannot do so. This differentiation is related to the ability of α_1-agonists to stimulate polyphosphoinositide hydrolysis resulting in the formation of IP_3 which is itself the intracellular messenger responsible for calcium release. Agonists acting at α_2-adrenoceptors do not cause polyphosphoinositide hydrolysis and therefore do not produce IP_3 nor release intracellular calcium. The suggestion of Ruffolo and coworkers that the agonist intrinsic activity is an important determinant of the ability to release intracellular calcium can thus be applied to extend the

present hypothesis; the intrinsic activity of α_1-agonists determines their ability to release calcium because this property determines the degree of polyphosphoinositide hydrolysis elicited by the agonist. However, α_2-agonists cannot cause calcium release irrespective of their intrinsic activity.

To date, little is known concerning the induction and regulation of calcium influx, although a further role for polyphosphoinositide hydrolysis is suggested as being the regulation of calcium influx following stimulation of some receptor subtypes. It has been suggested that DAG, a product of polyphosphoinositide hydrolysis may act as a calcium ionophore (Campbell *et al*, 1985) and phosphatidic acid, a metabolic product of DAG has also been suggested to have a similar role although this is desputed in both cases. A common pathway to cause both calcium release and influx is attractive in both metabolic and functional terms and an alternative role of the PI cycle in the regulation of calcium influx has been suggested by Putney (1986). It would seem unlikely that IP_3 itself acts at the cell membrane to cause calcium influx directly since it has been shown that the addition of IP_3 to purified plasmalemmal vessicles failed to release ^{45}calcium although such a release was observed following addition of IP_3 to E.R. vessicles (e.g. see Streb *et al*, 1984 and Ueda *et al*, 1986). Putney has thus proposed a hypothesis to explain how products of polyphosphoinositide hydrolysis may regulate calcium influx and has termed this the capacitative model of calcium entry. Putney suggests that following calcium release from the E.R. and in the presence of a continueing high concentration of IP_3, extracellular calcium passes across the cell membrane and into the E.R. thus refilling the latter. As a consequence of the high levels of IP_3, this calcium is then released from the E.R. into the cytosol leaving this empty and available for refilling. Thus, calcium enters the cell via the continued emptying and refilling of the E.R.. This hypothesis would require the close anatomical proximity of the E.R. and the plasma membrane and in vascular muscle the two are separated by only a narrow cleft.

This model suggested by Putney has since been extended by Taylor (1987) in

the light of an observation of Irvine and Moor (1986) who reported that the emptying of the E.R. is not itself a trigger for calcium influx. They suggest a role for IP_4, a metabolite of IP_3, as an intracellular second mesenger which acts to regulate the influx of calcium at the plasma membrane. Taylor (1987) hypothesises that IP_3 causes calcium release from the E.R. and that IP_4 then causes the passage of calcium from the narrow cleft into the E.R. from whence it would be available for release into the cytoplasm. It is thus the combined action of IP_3 and IP_4 which cause calcium entry via the E.R..

A major drawback of the model proposed by Putney and Taylor is that no provision is made for circumstances in which calcium entry is not associated with a previous release of calcium from the E.R., such as the contractions of the rat aorta which occur following a slow gradual increase in the concentration of α_1-agonist and those which occur to α_1-agonists of low intrinsic activity. Presumably the slow gradual activation of α_1-adrenoceptors or stimulation by agonists of low intrinsic activity do not result in a rapid increase in intracellular IP_3 and thus do not cause intracellular calcium release. Interestingly, some reports have suggested that IP_3 can indeed stimulate the passage of calcium across the plasma membrane of certain cells. Penner *et al* (1988) have used simultaneous patch-clamp and fura-2 techniques to show that calcium influx is induced by IP_3 in rat mast cells, and Kuno and Gardner (1987) have shown that IP_3 can stimulate calcium movement across the plasma membrane of T-lymphocytes. In addition, in contrast to the capacitance model of calcium entry suggested by Putney, a number of workers have shown that the passage of calcium from the extracellular space into the cytosol occurs *before* the release of calcium from the intracellular store (e.g. see Sage and Rink, 1987 and Sage, 1988). This passage therefore does not depend upon the release of calcium from this store. Further, a role for IP_4 in calcium entry into some sea urchin eggs is in fact disputed by Crossley *et al* (1988).

It can thus be seen that the regulation of calcium entry into the cytosol is a complex and multi-faceted system and that differences in this regulation exist

between cell types and possibly for the same cell under different physiological conditions. It would appear that in some cells calcium entry is regulated by IP_4 while in others this inositol phosphate plays no role. Further to this, the route of calcium entry would appear to be different between cells such that in some cells this route is indirect and via the E.R. (which may or may not involve IP_4) while in others, a direct route into the cytosol is followed.

12.4. The classification of α-adrenoceptors.

As a final comment, it is worthy to briefly discuss a number of points regarding receptor, and in particular α-adrenoceptor, classification.

This thesis has relied substantially on the results of the initial section of the study, namely the characterisation of the α-adrenoceptors of the femoral vein of the rat and the demonstration that a population of both α_1- and α_2-adrenoceptors co-exist postjunctionally on the smooth muscle cells of the vessel. In the interpretation of the results from which this conclusion was drawn, certain experiments were weighted against; i.e. those experiments involving the α_1-selective antagonist PZ were considered less reliable than those involving COR, and the reasons for this decision were discussed in Chapter 8. However, it is understandable that the decision not to place much emphasis upon the results of experiments involving PZ (which is often cited as the first choice for a selective α_1-antagonist) may be looked upon with some scepticism, and it could be argued that the early decision to designate the α-adrenoceptor population of this vessel as being mixed α_1 and α_2 was errenous. In retrospect however it can be seen that later experiments confirm a dual α-adrenoceptor population (i.e. the differential sensitivity to calcium removal and the demonstration of different second messenger systems for the two subtypes). Thus, the reliance of the interpreter upon results obtained using agonists and antagonists to characterise receptors may, in some instances, not be as suitable as is generally considered. It may therefore be suggested that alternative, or perhaps supplementary, approaches to receptor characterisation ought to be considered in some cases. Such a view has

recently been highlighted in the literature whereby Kenakin (1989) points out that the classical methods of receptor classification may not be ideal.

In particular, with modern advances in biochemical techniques, it may be that in certain circumstances the identification of receptor populations by the second messenger produced may be a more exacting option to follow. This would not hold for all cases of receptor characterisation (since for example the appropriate second messenger systems associated with the receptor under study would need to be known) but the present study has shown that such a step would be ideal for the characterisation of postjunctional α_1- and α_2-adrenoceptors since these two receptors have different, and very distinct, second messenger systems.

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