Vasomodulator Mechanisms in Rat Carotid Artery and in Vessels from an Experimental Model of Heart Failure

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By

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Declaration

The work for this thesis was carried out by myself alone with the exception of histological work prepared by Dr. Ian Montgomery. The model of heart failure was prepared by M. Hicks and co-workers in the Royal Infirmary, Glasgow. Part of the work for this thesis has been published as listed below.

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Abbreviations

5-HT	5-Hydroxytryptamine
5MU	5-Methylurapidil
ACh	Acetylcholine
ADP	Adenosine 5'-diphosphate
AII	Angiotensin II
ATP	Adenosine 5'-triphosphate
Ca^{2+}	Calcium
CCRC	Cumulative concentration response curve
CEC	Chloroethylclonidine
CHF	Congestive heart failure
CRC	Concentration response curve
Cyclic AMP/cAMP	Adenosine-3', : 5' cyclic monophosphate
Cyclic GMP/cGMP	Guanosine-3' : 5' cyclic monophosphate
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetra acetc acid
Hb	Haemoglobin
Ins (1,4,5) p3	Inositol (1,4,5) triphosphate
K+	Potassium
KCl	Potassium chloride
L-NAME	N^{ω}-nitro-L-arginine methylester
LVD	Left ventricular dysfunction
NA	Noradrenaline
NO	Nitric oxide
NOS	Nitric oxide synthase
PE	Phenylephrine
PG	Prostaglandin
PGI_2	Prostacyclin
s.e.mean	Standard error of the mean
SNP	Sodium nitroprusside
U-46619	9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$
$[Ca^{2+}]_i$	Intracellular calcium

Summary

The purpose of the work presented here was to investigate: i) in the rat isolated common carotid artery the population of postjunctional α-adrenoceptors, subtypes of α_1-adrenoceptors and their interaction with nitric oxide. ii) in the rabbit isolated saphenous vein the subtypes of α_1-adrenoceptors. iii) in the rabbit coronary ligation model of heart failure endothelium-dependent and -independent relaxations, contraction to NA and effect of cocaine treatment on large blood vessels. The major findings are briefly summarised below:

Rat carotid artery

1 The dominance of α_1-adrenoceptors is shown by the high sensitivity of NA or PE to prazosin and the ineffectiveness of rauwolscine except in non-selective concentrations. UK-14304 produced contraction and it is theoretically possible that UK-14304 exerts its actions through combined α_1 and α_2 activation, but the effectiveness of prazosin and the ineffectiveness of rauwolscine except in non-selective concentrations, shows that even this effect is mediated through α_1-adrenoceptors. Thus we suggest that the population of postjunctional α-adrenoceptors mediating contraction of smooth muscle in the rat carotid artery is predominantly α_1.

2 Rat carotid artery showed moderate sensitivity to α_{1A}-selective antagonists and low sensitivity to CEC. The pA_2 values correlated best with the published affinities of these compounds for the expressed α_{1d}-adrenoceptor clone and poorly with those at either the expressed α_{1b}- or α_{1a}-adrenoceptor clones. This suggests negative evidence that contractions of the rat common carotid artery are mediated by non-α_{1A}, non-α_{1B}-adrenoceptors. The data is consistent with a functional α_{1D}-adrenoceptor such as was reported in rat aorta.

3 In the rat isolated carotid artery, UK-14304 induced relaxation of NA-induced tone (but not thromboxane-induced tone) which was independent of α_2-adrenoceptors, functional endothelium, production of NO and cyclo-oxygenase products. There was no specific evidence from our study that α_2-adrenoceptors can mediate the release of EDRF in rat carotid artery. We suggest that UK-14304 acted as a partial agonist at α_1-adrenoceptors in this artery.

4 Inhibition of NO synthesis by L-NAME results in significant vasoconstriction. Also, L-NAME prevented the relaxation of rat carotid artery by acetylcholine. Thus we suggest that both basal and stimulated release of nitric oxide can regulate vascular tone in this artery.

5 Mechanical disruption of the vascular endothelium (to an extent which prevented vasodilation by acetylcholine) reduced, but did not abolish the ability of L-NAME to produce contraction. This suggests an extra-endothelial site for nitric oxide synthesis in rat common carotid artery.

6 The effect of UK-14304 was significantly enhanced in the presence of L-NAME. Inhibition of nitric oxide synthase with L-NAME potentiates responses to PE and UK-14304 but not to NA. Mechanical disruption of the vascular endothelium mimicked the effect of L-NAME on contractile responses to UK-14304 and PE consistent with L-NAME inhibiting endothelium-derived nitric oxide synthase. There was one unexplained difference between L-NAME and denuding endothelium. Although L-NAME did not increase sensitivity to NA, mechanical disruption increases potency of NA. Overall the results suggest that constitutive NO activity has substantial inhibitory influence on vasoconstrictor responses to PE and UK-14304 but not to NA.

7 Since L-NAME greatly potentiates responses to UK-14304, a series of experiments were conducted to see whether other stimuli that produces submaximal contraction would have a similar synergistic effect. Submaximal

contraction with KCl increased responses significantly, but inducing tone with PE, U-46619 and 5HT had no effect on responses to UK-14304. The presence of AII increased responses though less than KCl. Overall the greatest influence found was blockade of nitric oxide synthase.

Rabbit saphenous vein

8 We investigated the α_1-adrenoceptor subtypes of rabbit saphenous vein which has a mixed functional population of α_1 and α_2-adrenoceptors. The strategy was based on using the α_1-adrenoceptor "selective" agonist, PE. Prazosin competitively inhibited contractile responses to phenylephrine with pA_2 value of 8, WB-4101 had a pA_2 of 8.6 but a low Schild plot slope, while low potency was found with 5MU (pA_2 7.2) and HV-723 (pA_2 7.97). This data is not consistent with a definitive for α_{1A} or α_{1N} and taken alone the evidence from prazosin is in favour of α_{1L} as defined by Muramatsu *et al.*, (1990). However the "selective" α_2-adrenoceptor antagonist delequamine inhibited phenylephrine-induced contractions. Overall the data is consistent with phenylephrine-induced contractions being mediated by α_1- and α_2-adrenoceptors. The best estimate of subtype of α_1-adrenoceptor mediating contraction is α_{1L} due to the relatively low absolute pA_2 values for prazosin.

"Heart failure" model

9 We investigated endothelium-dependent or -independent relaxations and sensitivity and neuronal uptake of NA at the level of larger vessels (thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein) in a model devised to mimic heart failure. The model presented here is the rabbit

coronary ligation model in which myocardial infarction was produced in male New Zealand white rabbits (2.6kg-3.0kg) by ligation of the marginal branch of the left descending coronary artery. It was prepared by Royal Infirmary, Glasgow. The development of chronic heart failure was allowed to proceed over either eight or sixteen weeks. The results of the haemodynamic measurements suggest that short-term (8 week) and long-term (16 week) coronary ligation in the rabbits each corresponds to a model of experimental asymptomatic left ventricular dysfunction or minimally symptomatic LVD rather than of clinical heart failure. LVD was confirmed by echocardiographic measurement of ejection fraction.

10 Acetylcholine was chosen as endothelium-dependent vasodilator and sodium nitroprusside as an endothelium-independent vasodilator. ATP and adenosine were also chosen as vasodilators to study the function of endothelium in addition to acetylcholine. We used cocaine (1μM) to inhibit neuronal reuptake of noradrenaline. The properties of vessels from ligated rabbits were compared with sham operated controls. The investigation was blind with respect to sham or ligated.

11 The results led to 3 major conclusions with respect to the model. First, the relaxation responses to acetylcholine, sodium nitroprusside, ATP and adenosine were not impaired. Second, vasoconstrictions to noradrenaline were unaltered. Third, contractions to KCl (125mM) were preserved in large vessels (arteries and veins) in coronary ligated rabbits after 8 or 16 weeks compared with a normal control population.

12 The results of our experiments in this model of heart failure suggest that vasoconstriction to noradrenaline and normal stimulation of endothelial NO are preserved in larger peripheral conduit vessels. The changes demonstrated here provide a useful model for studying the progression of Left ventricular dysfunction to heart failure.

13 In relaxation responses to acetylcholine ear vein, saphenous vein and aorta were most sensitive in terms of threshold and concentration producing 50% maximum relaxation of tone. Saphenous vein and aorta produced the greatest maximum relaxations (78-90%). Saphenous artery and ear artery had the poorest responses to acetylcholine.

14 In contraction responses to NA aorta, ear artery and ear vein were the most sensitive preparations (pD_2 values: 9.96, 7.04 and 7.8 respectively). Renal artery and aorta had relatively very large maximum responses to NA among the arteries (6.7 and 4.3g respectively) and saphenous vein had greatest maximum response among the veins (2.9g).

15 In effect of cocaine (1μM) only in ear artery and saphenous artery or vein were the 3rd CRC to NA i.e. in the presence of cocaine, shifted to the left indicating increased sensitivity to NA. These results suggest that among arteries and veins that were studied, ear artery and saphenous artery or vein have innervation which can influence sensitivity to NA. Also we investigated the network of sympathetic nerves stained by glyoxylic acid. In agreement with the functional results, it was shown that ear artery and saphenous artery or vein have dense innervation but in other preparations sparse innervation or in vena cava no nerves were observed.

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CHAPTER 1
General Introduction

1.1 Historical perspective

Oliver & Schafer demonstrated in (1895) that injection of extracts of adrenal gland caused a rise in arterial pressure *in vivo*. The initial view about adrenoceptors held by Elliot (1905) was that adrenaline was the most likely mediator of sympathetic neurotransmission. He suggested that adrenaline is liberated from sympathetic nerve ending and acts on the effector cells. Dale (1906) provided some of the earliest experimental evidence that adrenoceptors could be differentiated into two classes. He observed that excitatory responses of various organs to adrenaline and nerve stimulation were paralysed by the ergot alkaloids, whereas inhibitory responses were not. Barger and Dale (1910) demonstrated that noradrenaline mimicked the effects of sympathetic nerve stimulation more closely than adrenaline.

Cannon and Rosenblueth (1933) explained the fact that adrenaline could excite some smooth muscles but inhibit others by calling sympathetic transmitter sympathin, to emphasise that its actions were not identical to adrenaline. To explain why released sympathin or exogenous adrenaline could excite some smooth muscles but inhibit others they postulated that it combined with one of two hypothetical substances. These substances made the sympathin excitatory or inhibitory so that two sympathins could be produced: sympathin E (excitatory) or sympathin I (inhibitory).

In (1948) Alquist noted the effects of five catecholamines on eight different physiological functions and clearly showed that the order of potency of the catecholamines for five of the physiological functions was markedly different from the order of the potency for the other three functions. He concluded that the differences in potency orders could only be explained by assuming differences in the receptors. Therefore he classified adrenoceptors initially into α and β subtypes. Effector cells with α-adrenoceptors had a high sensitivity to adrenaline

and noradrenaline but were practically insensitive to isoprenaline. Those tissues with β-adrenoceptors were sensitive to isoprenaline but less sensitive to adrenaline or noradrenaline.

1.2 α-adrenoceptors

The α-adrenoceptors are intrinsic membrane glycoproteins that mediate a variety of important sympathetic nervous system responses. They mediate a variety of functions and have been of major interest for many years as targets for drug action, and implicated in many human diseases.

The original proposal for the subclassification of α-adrenoceptors was based on pharmacological differences observed using α-adrenoceptor blocking agents on peripheral presynaptic and postsynaptic α-adrenoceptors in the perfused cat spleen (Cubeddu *et al.*, 1974). The discovery of presynaptic α-adrenoceptors and their role in the modulation of noradrenergic neurotransmission provided the stimulus for the subclassification of α-adrenoceptors. This subclassification developed as a result of the pharmacological differences between presynaptic α_2-adrenoceptors that mediate inhibition of the release of noradrenaline from sympathetic nerve terminal and postsynaptic α_1-adrenoceptors. It was suggested that the postsynaptic receptor should be referred to as α_1 while the presynaptic receptor should be called α_2 (Langer, 1974). Therefore α-adrenoceptors were divided into two subtypes, termed α_1 and α_2 dependent on anatomical location and function.

Differences in relative potencies of agonists which stimulate and antagonists which block these receptors have led to the conclusion that postsynaptic receptors are qualitatively different from presynaptic receptors. Classically, identification of α-adrenoreceptors should be based on the relative affinity and potency of

different agonists and antagonists in effector tissues, and this was shown to be the case with these α_1 -and α_2-adrenoceptors which were originally found to be postsynaptically and presynaptically located respectively (Langer, 1974; Starke *et al.,* 1974).

1.3 Evidence for the existence of postsynaptic α_1- and α_2-adrenoceptors on vascular smooth cells

Subsequent work in this field, using a number of selective agonist and antagonist drugs, has confirmed the existence of α_1- and α_2-adrenoceptors postsynaptically and allowed characterisation of their location and function (Docherty & McGrath, 1980).

Although the demonstration of postjunctional α_2-adrenoceptor in isolated vascular smooth muscle preparations is difficult, particularly in arteries, there is clear evidence for the presence of postjunctional α_2-adrenoceptors as well as α_1-adrenoceptors on the smooth muscle of many vascular preparations (McGrath, 1982). Although the α_1-adrenoceptor subtype linked to vasoconstriction is the predominant postsynaptic receptor in vascular smooth muscle, the postsynaptic α_2-adrenoceptor subtype also mediates constriction of vascular smooth muscle (McGrath *et al.,* 1989).

1.4 α_2-adrenoceptors on vascular smooth muscle cells

The presence of postsynaptic α_2-adrenoceptors has been demonstrated in many studies *in vivo*, but only a limited number of isolated vascular tissues are known to exhibit a typical postsynaptic α_2-adrenoceptor-mediated response. The presence of postjunctional α_2-adrenoceptors on the smooth muscle of the rat tail artery has

been demonstrated in binding studies with selective radioligands (Weiss *et al.*, 1983) and activation of these α_2-adrenoceptors produces contractile responses (Rajanayagam & Medgett, 1987). Postsynaptic α-adrenoceptors possess the α_2-subtype in the rat isolated saphenous vein (Cheung, 1985), the rat isolated femoral vein (Downing *et al.*, 1986), the rabbit isolated saphenous vein (Schumann & Lues, 1983) and the rabbit isolated ear vein (Daly *et al.*, 1988), as defined by responsiveness to selective agonists and the competitive inhibition by selective antagonists with a high specificity. The existence of α_2-adrenoceptor-mediated vasoconstriction was also documented in peripheral human vessels (Taddei *et al.*, 1988).

1.5 α_2-adrenoceptors: *in vivo* and *in vitro*

Many haemodynamic studies of whole organ preparations have documented vasoconstriction to both α_1- or α_2-adrenoceptor agonists, which indicated the postsynaptic location of both receptor subtypes on vascular smooth muscle (Docherty & McGrath, 1980; O'Brien *et al.*, 1985). In spite of the availability of α-adrenoceptors agonists with proven selectivity for either subtype *in vivo*, there are few examples *in vitro* of blood vessels that possess a functional population of postjunctional α_2-adrenoceptors. Several explanations have been put forward to explain the elusive nature of this subtype *in vitro*. It has been suggested that postjunctional α_2-adrenoceptors are located on small arterioles which, because of their size, are not normally accessible for examination *in vitro* (McGrath, 1982). An additional explanation for the difficulty in demonstrating postjunctional α_2-adrenoceptors *in vitro* may be the lack of humoral agents normally present in the whole animal (Schumann & Lues, 1983).

1.6 Selectivity of some agonists and antagonists

In general, α-adrenoceptors of the α_1 type are most effectively activated by phenylephrine and antagonised by prazosin, selective for the α_1-adrenoceptors (Cambridge *et al.*, 1977). Responses to α-adrenoceptor agonists in the vast majority of isolated vascular preparations, particularly arterial vessels, have been shown to be sensitive to prazosin (McGrath, 1982). YM-12617 is a potent and highly selective α_1-adrenoceptor antagonist. The α_1-adrenoceptor antagonist activity of YM-12617 was found to be more potent than that of prazosin in isolated rabbit aorta, urinary bladder base, urethra and prostate (Honda *et al.*, 1985). In the isolated rabbit aorta, YM-12617 competitively antagonised noradrenaline- and phenylephrine-induced contraction with pA_2 values of 10.2 and 9.95, respectively. Based on the pA_2 and pK_i values of *in vitro* assay, YM-12617 has a greater than 5000 times higher affinity for α_1- than for α_2-adrenoceptors. This inhibitory action of YM-12617 against the α_1-adrenoceptor-mediated pressor response was over 2000 times greater than that against the α_2-adrenoceptor-mediated pressor effect of UK-14304 in pithed rats (Honda *et al.*, 1987). The rauwolfia alkaloids, rauwolscine and yohimbine were originally shown to be highly selective α_2-antagonists (Weitzell *et al.*, 1979).

The α-adrenoceptors classified as α_2 are preferentially stimulated by clonidine and inhibited by rauwolscine. Radioligand-binding studies have led to a comparable subclassification (Wood *et al.*, 1979). Although clonidine has some degree of selectivity for α_2-adrenoceptors (Starke *et al.*, 1974), it is not a full agonist and has partial agonist effects on α_1-adrenoceptor subtype (Grant & Scrutton, 1980). UK-14304 is a full agonist at α_2-adrenoceptors in various pharmacological preparations (Grant & Scrutton, 1980; Cambridge, 1981). Also BHT-920 has been used as a selective α_2-adrenoceptor agonist (Kobinger & Pichler, 1983).

Noradrenaline stimulation of both postjunctional α_1- and α_2-adrenoceptors can be demonstrated in pithed rabbits, using the sequential administration of the antagonists prazosin and rauwolscine, the combination of which produces a greater effect than either antagonist alone (McGrath *et al.*, 1982).

1.7 Location of postsynaptic α_2-adrenoceptors

The α_1-selective adrenoceptor antagonist prazosin was more effective in blocking the responses to noradrenaline released by nerve stimulation than in antagonising the same end-organ responses induced by exogenous noradrenaline (Langer & Shepperson, 1982). Similar results were obtained with the use of other α-selective antagonists (Yamaguchi & Kopin, 1980).

The fact that α_1-adrenoceptor antagonists were found to be more effective at blocking responses to sympathetic nerve stimulation than they were at blocking equivalent sized responses to exogenous noradrenaline, led to the suggestion that postjunctional α_1-adrenoceptors were neurogenic receptors, while α_2-adrenoceptors were located extrajunctionally, responding to circulating catecholamines (McGrath, 1982).

Exogenous noradrenaline can activate both α_1- and α_2-adrenoceptors mediating vasoconstriction, whereas the transmitter released by nerve stimulation elicits vasoconstriction through the activation of α_1-adrenoceptors (Langer & Shepperson, 1982). It is therefore possible that the α_1-adrenoceptor predominates in the adventitial-medial border, where most of the noradrenergic nerve terminals are present, whereas the postsynaptic α_2-adrenoceptors are located mainly near the intima and therefore may be the target of circulating catecholamines. The postsynaptic α_2-adrenoceptor subtypes also mediates contraction of vascular smooth muscle and appears to be located close to the intima of blood vessels,

where they may be the target of circulating catecholamines rather than neuronally released noradrenaline (Langer & Hicks, 1984).

1.8 Role of calcium in responses via α-adrenoceptors

Pressor responses to α_2-adrenoceptor agonist *in vivo* can be blocked by calcium channel blocking drugs such as verapamil, nifedipine and diltiazem (Cavero *et al.*, 1983; Dunn *et al.*, 1991b). Furthermore, contractions of isolated vascular smooth muscle elicited by α_2-adrenoceptor activation are reduced by lowering the calcium concentration and by calcium channel blocking drugs in the rat tail artery (Su, *et al.*, 1986). The enhancing effect of α_2-adrenoceptor agonists on responses of the rat tail artery to α_2-adrenoceptor agonists involves an increase in Ca^{2+}-influx into smooth muscle cells through Ca^{2+} channels that are opened when α_2-adrenoceptors are activated (Xiao & Rand, 1989).

In most instances, stimulation of postjunctional α_1-adrenoceptors elevates $[Ca^{2+}]_i$ by entry of extracellular Ca^{2+} into the cell and also the release of Ca^{2+} from intracellular stores. Contractions evoked by α_2-adrenoceptor stimulation are known to rely more heavily on the influx of extracellular calcium than those caused by α_1-adrenoceptors (Reid & McGrath, 1985).

1.9 Effect of different physiological factors

The presence of Bay K-8644 or inducing tone with prostaglandin $F_{2\alpha}$, enhanced responses to the selective α_2-adrenocetor agonist BHT-920 which were prazosin-resistant and rauwolscine-sensitive, in canine isolated saphenous artery and portal vein respectively (Sulpizio & Hieble, 1987). Furthermore, the physiological stimulant angiotensin II (AII), enhances postjunctional

α_2-adrenoceptor function in some venous preparation (Schumann & Lues, 1983; Daly *et al.*, 1988b).

In the pithed rat, the responses to the α-adrenoceptor agonists were differentially affected by respiratory alkalosis and acidosis. Responses to α_1-adrenoceptor agonist were greater in alkalosis; those to α_2-adrenoceptor agonist were better in acidosis (O'Brien *et al.,* 1985).

Significant potentiation of noradrenaline-induced contraction was observed in canine internal carotid arteries treated with prazosin (10nM), whereas yohimbine (10nM) attenuated the time-dependent potentiation. The contractile responses of isolated canine basilar and internal carotid arteries to noradrenaline are potentiated during the course of the experiment, which is likely to be related, in part, to an enhancement in α_2-adrenoceptor mediated contraction (Kawai *et al.*, 1991).

There is much evidence, especially from studies in pithed rats, that α_2-adrenoceptor-mediated responses are dependent on the physical environment. It has been suggested that the α_2-adrenoceptor exists in different conformational states depending on tissue or species, according to the presence of co-factors in the immediate vicinity of the receptor (Alabaster *et al.*, 1986). The possibility existed therefore that α_2-adrenoceptors could differ sufficiently to allow the identification of antagonists which could be targeted to different tissues or different α_2-sites. On balance the data are compatible with the view that α_2-adrenoceptor-mediated responses in vascular smooth muscle are more dependent on the physiological environment than are responses mediated by α_1-adrenoceptors (Alabaster & Davey, 1984).

1.10 Functional subclassification of vascular α_1-adrenoceptors

Since the establishment of two general subtypes of α-adrenoceptors (α_1 and α_2), a further subclassification for α_1-adrenoceptors has been suggested (McGrath, 1982). McGrath provided evidence that α_1-adrenoceptors are a heterogeneous group of receptors, and that postjunctional responses mediated by α_1-adrenoceptors, could not be explained adequately on the basis of a single receptor subtype. He proposed a subdivision of α_1-adrenoceptors into α_{1A} and α_{1B}. It was suggested that both phenethylamines and non-phenethylamines were efficacious agonists at the α_{1A} subtype but α_{1B}-adrenoceptors were activated only by phenethylamines. In 1985, Drew first suggested that the variety of pA_2 values for prazosin and yohimbine in many tissues may reflect a possible heterogeneity of α_1-adrenoceptors. Flavahan & Vanhoutte (1986) suggested that there were two distinct subtypes of α_1-adrenoceptors which could be distinguished by their affinities for both prazosin and yohimbine in the blood vessels (α_{1H} and α_{1L} according to their High or Low affinity for prazosin). α_{1H} has a high affinity for both drugs (pA_2 values greater than 9 for prazosin and greater than 6.4 for yohimbine), and the other (α_{1L}) has a low affinity for drugs (pA_2 values less than 9 and 6.2 respectively). Muramatsu and co-workers (1990) extended this functional subclassification of α_1-adrenoceptors of various vascular smooth muscle preparations with the use of five α-adrenoceptor competitive antagonists (phentolamine, yohimbine, WB-4101, prazosin and HV-723) and irreversible antagonist, chloroethylclonidine as show in the table. Their classification scheme for α_1-adrenoceptor subtypes is based primarily on differential affinities for prazosin.

Table 1.1

Characteristics of α_1-adrenoceptor subtypes in blood vessels proposed by Muramatsu *et al.*, (1990)

subtype	antagonist potency (pA_2 value)	susceptibility to chloroethylclonidine
α_{1H}	prazosin > HV-723,WB-4101>yohimbine (>9.5) (8.0-9.3) (>6.5)	sensitive (α_{1B})
α_{1L}	prazosin , HV-723, WB-4101>yohimbine (8.0-9.0) (6.4>)	insensitive
α_{1N}	HV-723>WB-4101>prazosin >yohimbine (>9.0) (>8.4) (8.3>) (>6.5)	insensitive

In some tissues such as rabbit thoracic aorta, both affinities for prazosin are detected, suggesting the existence of prazosin-high (α_{1H}) and -low (α_{1L})-adrenoceptors. In such cases, the slope of Schild plot for prazosin significantly deviates from unity (Oshita *et al.*, 1993).

In tissues with low affinity for prazosin, the pK_B values for prazosin are approximately 8.5, which is 10 times less in affinity than for α_{1H}-adrenoceptors. HV-723 (Kohno *et al.*, 1994) can subdivide the α_{1L}-adrenoceptor into two subtypes: α_{1L} (HV723-low, <1nM) and α_{1N} (neither α_{1H} or α_{1L}) (HV723-high). It is a useful antagonist for the subclassification of the prazosin-low affinity sites (Muramatsu *et al.*, 1995).

From the relative potencies of the α-antagonists, the α_{1H} subtype is the most sensitive to prazosin and the α_{1N} subtype is more sensitive to HV-723 and

WB-4101 than prazosin. The α_{1L} subtype shows equal affinity for prazosin, HV-723 and WB-4101. From the absolute pA_2 values for the α-antagonists, prazosin discriminates α_{1H} and α_{1L}, and HV-723 further distinguish between α_{1L} and α_{1N}. WB-4101 and 5-methylurapidil are selective for α_{1A}-adrenoceptor subtypes and spiperone has been reported to have a 10-fold higher affinity for α_{1B}- than for α_{1A}-adrenoceptors (Michel *et al.*, 1989).

α_{1H}-adrenoceptors were, at one point in time, subdivided into four subtypes: α_{1A}, α_{1B}, α_{1C} and α_{1D}. Since then the cloned α_{1c} has been renamed α_{1a} and it is believed to be equivalent of the pharmacologically defined, tissue type α_{1A} (Bylund *et al.*, 1994). The cloned, formerly named α_{1a} and α_{1d} ($\alpha_{1a/d}$) are now considered equivalent, called α_{1d} and considered the counterparts of the functionally defined α_{1D}. Thus, the new nomenclature of α_1-adrenoceptors with high affinity for prazosin recently acknowledged that the cloned α_{1c}-adrenoceptor corresponds to the classical α_{1A}-adrenoceptor, the cloned α_{1b}-adrenoceptor equivalent of α_{1B}-adrenoceptor and the cloned α_{1d}-adrenoceptor corresponds to the classical α_{1D}-adrenoceptor (Hieble *et al.*, 1995). Therefore in the new nomenclature α_{1H}-adrenoceptor can be subdivided into three subtypes: α_{1A}, α_{1B}, and α_{1D}.

In the dog carotid artery a high pK_B value (9.7) for prazosin was estimated. However, the pK_B values for WB-4101 and 5-methylurapidil were relatively low (8.2 and 7.2, respectively). Pretreatment with CEC, which produces relatively selective inactivation of the cloned α_{1B} and α_{1C} subtypes, remarkably attenuated the contractile response to noradrenaline. These results suggest that the α_1-adrenoceptor of dog carotid artery belongs in the α_{1H} group of the functional subclassification and further corresponds to the cloned α_{1B} subtype. The α_1-adrenoceptor with prazosin-high affinity (α_{1H}) of rabbit thoracic aorta (α_{1B}) is a similar subtype to that of dog carotid artery (Oshita *et al.*, 1993).

1.11 Subtypes of α_2-adrenoceptors

α_2-Adrenoceptor is defined as one that is sensitive to both the physiological catecholamine agonists, noradrenaline and adrenaline, as well as selective agonists, such as BHT-920 and UK-14304, and is antagonised by agents such as rauwolscine and yohimbine. α_2-Adrenoceptor subtypes can be divided into at least four subtypes: α_{2A}, α_{2B}, α_{2C} and α_{2D} based on ligand binding and molecular cloning studies. All known α_2-adrenoceptor can be activated by noradrenaline and adrenaline, and there is no evidence that these physiological catecholamines show significant selectivity between any of the known α_2-adrenoceptor subtypes. All subtypes can be blocked by yohimbine and rauwolscine, although the affinity can vary substantially between subtypes. Some α_2-adrenooceptor subtypes have a high affinity for prazosin, previously thought to interact only with α_1-adrenoceptors, and others have a relatively low affinity for rauwolscine, compared to other α_2-adrenoceptor antagonists. All α_2-adrenoceptors appear to be linked to inhibition of adenylate cyclase as one, but not only, mechanism of signal transduction. Prazosin and another α_1-adrenoceptor antagonist, ARC-239, have high affinity for α_{2B}-adrenoceptor, and low affinity for α_{2A}-adrenoceptor. The partial α-adrenoceptor agonist, oxymetazoline, is more selective to the α_{2A}-adrenoceptor than other subtypes. The α_{2C}-adrenoceptor is similar to the α_{2B}-adrenoceptor with respect to a relatively high affinity for prazosin, ARC-239, and spiroxatrine, but it has a higher affinity for rauwolscine. WB-4101 is more selective for α_{2C}- versus α_{2B}-adrenoceptors. α_{2D}-Adrenoceptor has a lower affinity for rauwolscine than the other subtypes and , like the α_{2A}-adrenoceptor, a low affinity for prazosin, spiroxatrine and ARC-239 (Ruffolo, *et al.,* 1993; Bylund *et al.,* 1994).

1.12 Signal transduction mechanisms

α_1-Adrenoceptors belong to that family of receptors which initiate their signals in target cells by increasing free cytosolic Ca^{2+} levels. Han and colleagues (1987b) proposed that α_{1A}- and α_{1B} adrenoceptors activated distinct signal transduction mechanisms to increase cytosolic Ca^{2+} level. They suggested that the α_{1B}-subtype activated formation of inositol (1,4,5) triphosphate and released stored intracellular Ca^{2+}, whereas the α_{1A}-subtype increased influx of extracellular Ca^{2+} through an inositol phosphate-independent mechanism.

Experiments with exogenous noradrenaline in rat vas deferens led to the view that the α_{1A} subtype activates calcium influx through dihydropyridine sensitive channels whereas the α_{1B} subtype activates phosphatidylinositol breakdown followed by calcium mobilisation from intracellular stores (Han *et al.*, 1987b). These receptors activate a G protein, which in turn activates a phosphoinositide specific phospholipase C. This enzyme cleaves inositol (1,4,5) triphosphate from a membrane phospholipid, and Ins (1,4,5)p3 mobilises stored pools of intracellular Ca^{2+}. Diacylglycerol is also released from this reaction, which acts synergistically with Ca^{2+} to activate protein kinase C. However, it is known that α_{1A}-adrenoceptors can also couple to InsP formation and mobilisation of intracellular Ca^{2+}, while the α_{1B}-adrenoceptor can couple to voltage-gated Ca^{2+} influx (Minneman, 1988). The relative antagonist potency observed with the S(+)- versus R(-)- isomer of niguldipine (approximately 30 fold) is fully consistent with reported characteristics of α_{1A}-adrenoceptors (Boer *et al.*, 1989).

1.13 Endothelium-Derived Relaxing Factor (EDRF)

Prostacyclin (PGI_2), a potent vasorelaxant, was the first endothelium-derived vasoactive substance discovered in the late 1970s (Moncada & Vane, 1979).

Later it was demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium (Furchgott & Zawadzki, 1980). They observed that acetylcholine relaxed rings or strips of rabbit aorta *in vitro*, but only if endothelium cells were present in the preparation. Later Furchgott (1983) provided evidence that this effect was mediated by a labile non prostanoid humoral factor, known as EDRF (endothelium-derived relaxing factor).

1.14 Agonists that stimulate production of EDRF

Endothelium-dependent relaxation occurs in response to a variety of substances including platelet derived products such as adenine nucleotides (ATP, ADP) (Furchgott & Zawadzki, 1980; De Mey & Vanhoutte, 1981). Other numerous compounds, such as substance P, histamine (Moncada *et al.*, 1991) can release EDRF. This observation has since been confirmed and extended, and it was recognised that release of EDRF was responsible for the dilator response to acetylcholine and bradykinin in a variety of animal and human arteries and veins (Thom *et al.*, 1987). Nitrovasodilators induce vascular relaxation by endothelium-independent mechanisms (Furchgott, 1984).

Release of EDRF was observed under basal conditions as well as after stimulation with acetylcholine (Martin *et al.*, 1985). Contrary to the PGI_2 synthesis, the production of ERDF is highly dependent on extracellular Ca^{2+}. An elevation in the intracellular free Ca^{2+} concentration ($[Ca^{2+})]_i$ has been shown to be an absolute prerequisite for the acute increase in the synthesis of EDRF in the endothelial cells induced by either receptor-dependent agonists such as acetylcholine, ATP, and bradykinin or receptor-independent compounds such as calcium ionophores or thimerosal (Luckhoff *et al.*, 1988).

1.15 Identification of EDRF as NO

Based on the similarities in the pharmacological behaviour of EDRF and NO generated from acidified NO_2, it was suggested in 1987 that EDRF may be NO. The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF released from vascular endothelial cells was detected by the chemical means used to identify NO. Much evidence strongly suggested that EDRF is NO (Palmer *et al.*, 1987).

The amino acid L-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells. Endothelial cells, cultured in the absence of L-arginine for 24 hour prior to the experiments, showed a decrease in the release of NO induced by bradykinin and A23187 which could be restored by L-but not D-arginine (Palmer *et al.*, 1988). NO is formed from L-arginine by NADH-dependent nitric oxide synthase, which is also a calcium-sensitive enzyme, and is located in the endothelial cytosol. Endogenous NO produced by endothelial cells diffuses passively into neighbouring vascular smooth muscle cells, where it binds to the heme component of guanylyl cyclase, thereby activating the enzyme, resulting in increased cyclic GMP production and relaxation by dephosphorylation of myosin light chain. Smooth muscle tone depends on the cytoplasmic calcium concentration and NO has been reported to inhibit calcium release from intracellular stores and calcium influx through receptor operated channels (Moncada *et al.*, 1991).

1.16 Physiology of NO

Three isozymes of NOS (NO synthase) have been characterised and all isoforms of nitric oxide synthase utilise L-arginine as the substrate. Despite substantial homology among the gene sequences of the three isozymes, their physiologic

actions differ markedly and the predominant isozyme in endothelial cells is a constitutive form. A second isozyme of NOS, which is predominantly found in activated macrophages, is also expressed in endothelial cells and vascular smooth muscle cells exposed to cytokines. This inducible form of NOS does not require calcium or calmodulin for its activity and, once expressed, produces NO in an unregulated fashion. The third isozyme of NOS is found predominantly in the central nervous system and has not been identified in endothelial cells (Dinerman *et al.,* 1993; Anggard, 1994).

The vascular endothelial cells are the only cells to possess both the constitutive and inducible NO synthases. The production of NO can be stimulated by several agonists acting on different cell-surface receptors and using distinct intracellular signal transduction pathways. NO is not stored but diffuses freely from its site of formation whereas classical mediators are frequently stored in granules and released specifically. It is rapidly metabolised to nitrite and nitrate in the presence of oxygen with a half-life of approximately 3-5 seconds; the half-life is shortened by the presence of free radicals such as superoxide anion and is, thus, prolonged by free radical scavengers such as superoxide dismutase. It is soluble both in water (up to 2mmol L at 20°C and one atmosphere) and lipid (Busse *et al.,* 1993).

1.17 Inhibition of NO

The effects of EDRF were shown to be inhibited by Hb, methylene blue, and other agents such as dithiothreitol and hydroquinone (Griffith *et al.*, 1984). Several analogues of L-arginine are inhibitors of vascular nitric oxide (NO) synthase. They inhibit NO synthase in an enantiomerically specific manner and act as competitive inhibitors of all three isozymes of NOS and, thus, have been used extensively in investigations of NO metabolism. NG-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO) and N^{ω}-nitro-L-arginine

methylester (L-NAME) are inhibitors of NO synthase in the vascular endothelium *in vitro* and *in vivo*. They are specific inhibitors of both the constitutive and inducible NO synthases (Rees *et al.*, 1990).

1.18 Vasodilation via α_2-adrenoceptors on endothelium

Stimulation of the release of endothelium-derived relaxing factor nitric oxide (EDRF), a powerful vasodilator, mediating relaxation through activation of soluble guanylate cyclase in neighbouring smooth muscle cells (Moncada *et al.*, 1991), has been shown to oppose the effects of α-agonists and of other vasoconstrictors. The receptors on the endothelium mediating the relaxation response to noradrenaline were tentatively classified as α_2-adrenoceptors (Cocks & Angus, 1983; Angus *et al.*, 1986).

In isolated arteries and veins endothelium dependent vasodilatory responses to clonidine and to other α_2-agonists can efficiently counteract their direct constrictor effects on vascular smooth muscle cells via this endothelial pathway. Removal of the endothelium in isolated arteries (Cocks & Angus, 1983; Tschudi, *et al.*, 1991) or exposure to methylene blue, an inhibitor of EDRF-mediated activation of guanylate cyclase (Gold *et al.*, 1990; Martin, 1985) may increase the vasopressor effects of clonidine by several orders of magnitude, emphasising the significance of α_2-mediated endothelial-dependent vasodilation in certain vascular beds.

It has been reported that α_2-adrenoceptor agonists produced vasodilation in isolated dog coronary arteries (Angus & Cocks, 1983; Angus *et al.*, 1986), in dog femoral and pulmonary arteries and veins (Miller & Vanhoutte, 1985) and in rat tail arteries (Matsuda *et al.*, 1985). It was postulated that this was due to activation by the agonists of receptors on the endothelium to release EDRF.

To illustrate the α_2-adrenoceptor-mediated activity directly, it is necessary to have intact endothelium, and α_1 -and β-adrenoceptor antagonists present. The signal is quite powerful in some circumstances as it has been demonstrated that this EDRF release can alter the maximum response and shift the concentration response curves for the contractile effects of noradrenaline in dog and in pig coronary arteries (Cocks & Angus, 1983).

In pig the contractions via α-adrenoceptors are small, and endothelium dependent relaxations via α_2-adrenoceptors are large, in the coronary and carotid arteries which perfuse the vital organs. It is suggested that in the state of activation of the sympathetic nervous system, arterial tone in response to α-adrenoceptor stimulation may be regulated not only by α-adrenoceptors on vascular smooth muscle but also by those on endothelium, through release of endothelium derived relaxing factor (EDRF) via α_2-adrenoceptors (Ohgushi *et al.*, 1993).

CHAPTER 2

Investigation of α_1-Adrenoceptor Subtypes and Effect of Nitric Oxide in Rat Common Carotid Artery

Section 1: Introduction

Much of the experimental work on the large arteries in rat has been concentrated on the aorta, which is the largest artery in the rat. However aorta contains a substantial component of collagen and elastin with few smooth muscle cells relative to its size. We wished to characterise a large artery which might contain relatively more smooth muscle cells and less connective tissue and therefore be more suitable for physiological analysis. We anticipated similar properties to the aorta for which there is more background information. Although some previous studies of the rat carotid artery made with perfusion-system, in which a carotid artery was perfused (Paterno *et al.,* 1994), there are other studies in rat carotid artery using ring preparations. Hongo and colleagues (1988) used rat carotid artery as a ring to investigate effects of aging and hypertension on endothelium-dependent vascular relaxation. They found that endothelium-dependent relaxation of the carotid artery is impaired in old rats and in hypertension.

Previous studies in rat carotid arteries concentrate more on effects of different disorders. Since sympathetic stimulation and production of nitric oxide have great influence on the cerebral circulation, our experiments were designed to demonstrate the population and subtypes of α-adrenoceptors and effects of nitric oxide in this artery.

There are many reports showing that contractions of vascular smooth muscle cells by α-adrenoceptor agonists are depressed in the presence of endothelium or basal release of NO (Carrier & White, 1985; Young & Vatner, 1986; Kaneko & Sunano, 1993; Maclean *et al.*, 1993). We investigated effects of removal of the endothelium and inhibition of basal nitric oxide on α-adrenoceptor agonists in this artery.

Considerable quantitative variation between different laboratories exists with regard to the demonstration of the vasoinhibitory influence of spontaneous basal release and agonist-stimulated release of EDRF. For example, in rat aorta contractile responses to some α-adrenoceptor agonists, particularly partial agonists such as clonidine, are sometimes found to be markedly potentiated by disruption of the endothelium, while other agonists, particularly those with greater intrinsic activity such as phenylephrine, are relatively unaffected (Egleme *et al.,* 1984). However, in an essentially similar study, Martin and co-workers (1986) found qualitatively similar but quantitatively different results where endothelial removal had less effect versus clonidine but more versus phenylephrine. Mechanical removal of the endothelium in the rat isolated thoracic aorta is associated with an increase in the maximum contraction to phenylephrine and an increase in both the maximum response and potency of various partial agonists at α-adrenoceptors (e.g. clonidine, BHT-920) (Lues & Schumann, 1984; Godfraind *et al.*, 1985).

Young and Vatner (1986) demonstrated that removal of the endothelium not only reversed the vasodilator response to adrenaline to vasoconstriction, in the conscious animal, but that it also enhanced α_1-adrenoceptor constriction in response to phenylephrine and noradrenaline but not to α_2-adrenoceptor stimulation with B-HT920. Removal of the endothelium in canine pulmonary and systemic blood vessels caused a shift to the left of the concentration-response curves to adrenaline, noradrenaline and the α_2-adrenoceptor agonist UK-14304, but not of that to the selective α_1-adrenoceptor agonist phenylephrine (Miller & Vanhoutte, 1985). Endothelial disruption was associated with an enhancement of contractile responses to NA, without a similar effect on responses to BHT-920 (McGrath *et al.,* 1990).

Initial experiments showed an unexpected series of effects from the supposedly selective α_2-adrenoceptor agonist UK-14304 which we pursued and which

eventually turned out to be due to the very effective α_1-adrenoceptor system in this artery rather than an involvement with α_2-adrenoceptors.

Section 2: Methods and Materials

2.2.1 Methods

Common carotid arteries (700 μm in lumen diameter) were obtained from male Wistar rats, weighing 320-400g, which were killed (i.p. injection) by overdose with pentobarbitone sodium. Although carotid artery is smaller than aorta a pair of common carotid arteries were easily dissected out and were placed in cold, oxygenated modified Krebs-Henselite solution (Krebs). The arteries were cleared of any extraneous connective tissue using fine scissors, when it was necessary under a dissecting microscope.

Each preparation was cut transversely in to 3-4mm rings and suspended between thick wire supports. During the preparation of the arterial ring segments, any contact with the luminal surfaces was avoided to preserve endothelial integrity. In some preparations the endothelial layer was removed mechanically by gently rolling the tissue around a thin wire. Removal of the endothelium was confirmed pharmacologically by a lack of relaxant response to the potent endothelium-dependent vasodilator acetylcholine. This ensured that endothelium removal had been successful. Fluorescent dyes were also used to examine histologically the structure of rubbed endothelial cells in some random preparations (Daly *et al.*, 1992). Each ring was suspended horizontally by means of two stainless-steel L-shaped hooks carefully passed through the lumen. The upper support was connected by cotton to an isometric transducer while the lower support was connected to a glass tissue holder. The arterial rings were mounted in 10ml isolated organ bath, bathed in Krebs maintained at 37°C and gassed with 95% O_2 plus 5% CO_2.

The rings were then placed under resting tension at 2.5-3g for each group of arterial rings of carotid artery, which was determined from the tension development curves to be optimal (Figure 2.1).

Isometric contractions were measured by a Grass FT03 transducer connected to a Linseis (TYP 7208) pen recorder. In all experiments, tissues were left to equilibrate for a 60 min period, during which time the tension was re-adjusted to a set value which was maintained constant throughout the rest of the experimental day. Each preparation was then exposed to NA (1μM) and allowed to contract for 5-10 min. This first contraction to an agonist minimises changes in the sensitivity of preparations to further addition of agonists. Following complete washout, an additional one hour equilibration period was allowed before commencement of any other experimental procedure

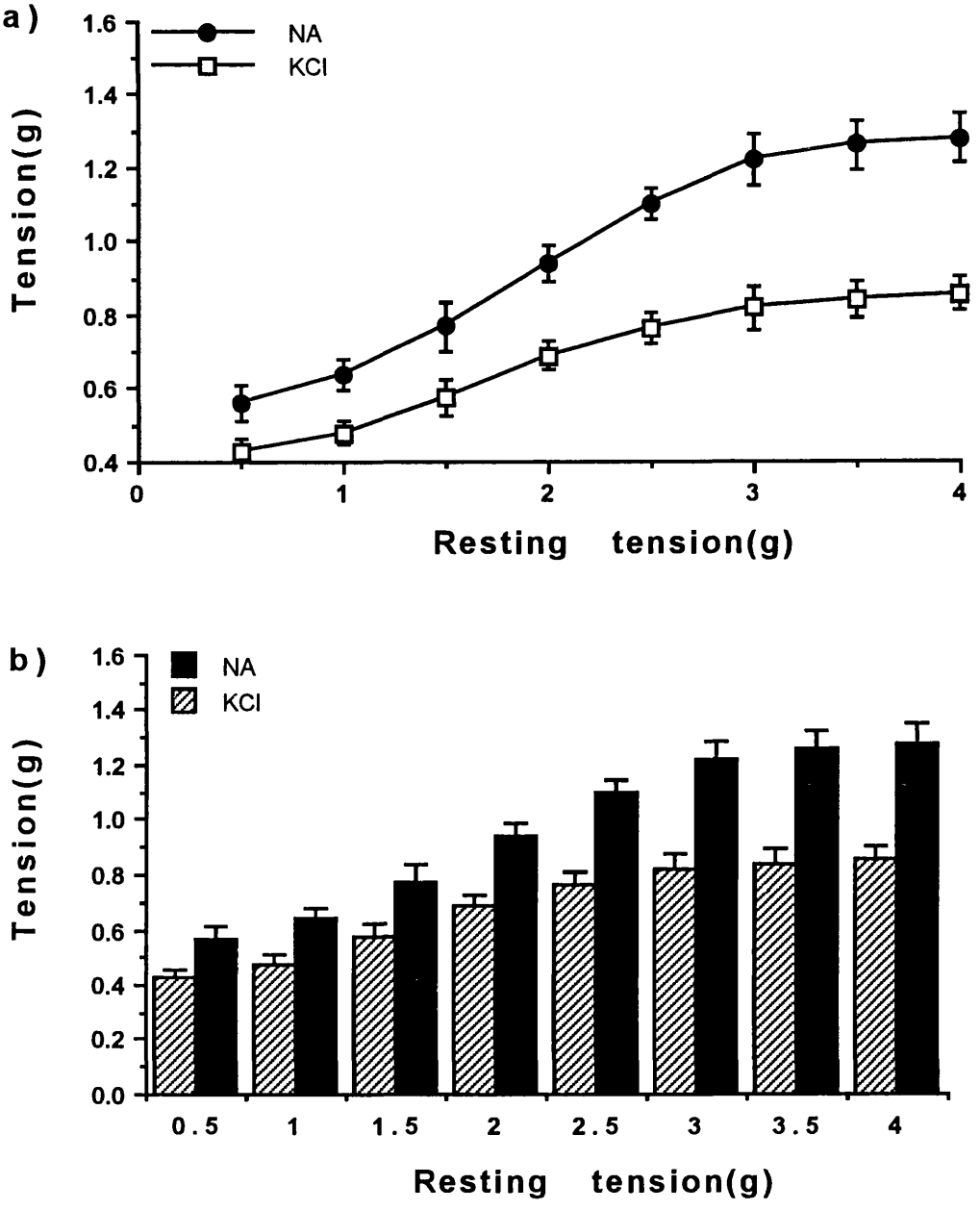


Figure 2.1

Tension-development curves of the effects of resting tension on active contractile force of the rat isolated common carotid artery in response to KCl (50mM) or NA (1μM). Optimal contractile force is demonstrated with resting tension of (2.5-3g) in all groups of arterial rings.

Each point or column represents mean ± s.e.mean (n=10).

2.2.2 Protocol

Concentration-response curves were constructed in a cumulative manner by increasing the concentration of the agonists in half-log increments. When responses to agonists were not maintained, addition of the next concentration was made as close to the peak as possible. An initial control CCRC, to any given agonist, was obtained in each preparation. Following attainment of the maximal control contraction, preparations were washed until complete relaxation was effected. The preparations were then left for a further period of 45-60 min before re-exposure to the agonist.

When the competitive antagonists like prazosin and rauwolscine were used, the preparations were incubated at least for 45 minutes with the drugs prior to the onset of a second CCRC.

When examining the effects of angiotensin II and L-NAME (an inhibitor of NO), these drugs were added approximately 10-15 min prior to the onset of CCRC to an agonist.

2.2.3 Statistical analysis

Results are expressed as mean ± standard error of mean (s.e.mean). Comparisons between two groups were performed using the paired or unpaired Student's t-test with values as follow: * $p<0.05$, ** $0.001 <p<0.0l$, *** $p < 0.001$. Comparisons among several groups were performed using one-way analysis of variance. A value of $p <0.05$ was taken as statistically significant.

2.2.4 Calculation of results

Responses to agonists are expressed as a percentage (mean ± s.e.mean) of maximum response of the first control CCRC to any given agonist or of the maximum response to NA (1μM) in the first of experiment. The potency of agonist was determined as the pD_2, which is the negative logarithm of the concentration causing half the maximal response. In examining the effect of antagonist, agonist concentration ratio values were determined from the concentrations producing 50% of the maximum response (EC_{50}) in the absence and presence of each concentration of antagonist. The EC_{50} value of the agonist was expressed as the pD_2 value which was calculated as the negative logarithm of the EC_{50} value (pD_2= -log EC_{50}). Schild analysis (Arunlakshana & Schild, 1959) was performed for competitive antagonists where appropriate by plotting log (DR-1) on the y-axis (where DR represents the concentration ratio (EC_{50} (in presence of antagonist) divided by EC_{50} (in absence of antagonist) against the log M [antagonist] on the x-axis and fitting using linear regression. The pA_2 value (-log M [antagonist] required to produce a concentration ratio of 2) was derived from the x-intercept which is equal to the antagonist dissociation constant (K_B) under equilibrium conditions. If antagonism is competitive a plot of the logarithm of dose ratio-l (DR-1) against the negative logarithm of the molar concentration of the antagonist yields a straight line whose slope is not significantly different from one. Antagonist was considered to be competitive if the 95% confidence limits for the slope of the Schild plot, drawn by linear regression, overlapped unity. Concentrations of antagonists which did not consistently produce greater than 2-fold rightward displacements of the agonist CCRC were excluded from quantitative Schild analysis.

2.2.5 Solutions and Drugs

The composition of the modified Krebs-Henselite solution was as follows: (in mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.6, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 11. Na_2EDTA (23μM) was also included in the Krebs in all experiments to prevent degradative oxidation of NA and propranolol (1μM) and cocaine hydrochloride (10μM) were also included to inhibit β-adrenoceptors and neuronal uptake of NA respectively. The following compounds were used:

prazosin HCl (Pfizer); rauwolscine (Roth); UK-14304 (Pfizer); (-)-phenylephrine HCl (Sigma); (-)-noradrenaline bitrate (Sigma); propranolol HCI (Sigma); cocaine HCl (Mac Carthys); Angiotensin II amide (Ciba); L-NAME (Sigma); L-Arginine (Sigma); U-46619 (Upjohn); $PGF_{2\alpha}$ (Upjohn); indomethacin (Sigma); adenosine (Sigma); adenosine triphosphate (Sigma); HV-723 (Gift from Dr. Muramatsu, Japan); 5-methylurapidil (Research Biochemicals International); isoprenaline (Sigma); acetylcholine (Sigma); WB-4101 (Research Biochemicals International); YM-12617 (Yamanouchi); sodium flurbiprofen (Boots); sodium nitroprusside (Sigma); 5HT (Sigma); GR-32191B (Glaxo); chloroethylclonidine (Research Biochemicals International); BMY-7378 dihydrochloride (Research Biochemicals International); Niguldipine (Research Biochemicals International); Delequamine (RS-15385-197, Syntex, Gift from Dr. Whiting)

All drugs except U-46619, indomethacin and niguldipine were dissolved in distilled water. U-46619 was initially dissolved in high-performance liquid chromatography-grade absolute ethanol, with subsequent dilutions made in distilled water. Niguldipine was initially dissolved in dimethyl sulphoxide (DMSO), with subsequent dilutions made in distilled water. The stock solution of indomethacin was dissolved in 10% of $NaHCO_3$. Solutions of indomethacin, sodium nitroprusside and niguldipine were protected from light and experiments

carried out in near darkness. All concentrations of the drugs used are expressed as final concentration in the organ bath.

Section 3: Results

2.3.1 Potency of agonists

The mixed α-adrenoceptor agonist NA (non-selective), PE (selective α_1) and UK-14304 (selective α_2) produced concentration-dependent contractions in the rat isolated common carotid artery. The rank order of potencies for these agonists were as follows: NA> PE > UK-14304 (Figure 2.2).

Consecutive CCRC's to NA, PE and UK-14304 were reproducible, there being a small and non significant change in the maximum response with time (Figure 2.3).

2.3.2 Effects of α_1-adrenoceptor agonists and antagonists

NA produced isometric contraction with a pD_2 of 7.97 and a maximum contraction of 1.13 ± 0.03g. PE produced isometric contraction with a pD_2 of 7.07 and a maximum contraction of 0.96 ± 0.02g. The maximum response to NA and PE were not significantly different, but to UK-14304 was smaller than for the other two agonists. Relative to NA its intrinsic activity was 0.37. UK-14304 did not clearly produce maximum.

Responses to these agonists were analysed using antagonists proposed to be selective as follows: prazosin, selective for α_1 c.f. α_2; 5MU, selective α_{1A} c.f. α_{1B}; WB-4101, selective α_{1A} c.f. α_{1B} and CEC irreversible antagonist at α_{1B} c.f. α_{1A} and α_{1D}.

Prazosin produced concentration dependent parallel rightward displacement of NA and PE CCRC (Figure 2.4a and 2.4b respectively). The pA_2 value for prazosin versus NA was 10.05 and the slope of Schild plot was not significantly different from one (1.045), indicating competitive antagonism (Table 2.1).

The pA_2 value for prazosin versus PE was 9.81 and slope of the Schild plot was very near to unity (0.98), indicating competitive antagonism (see Schild plot figures).

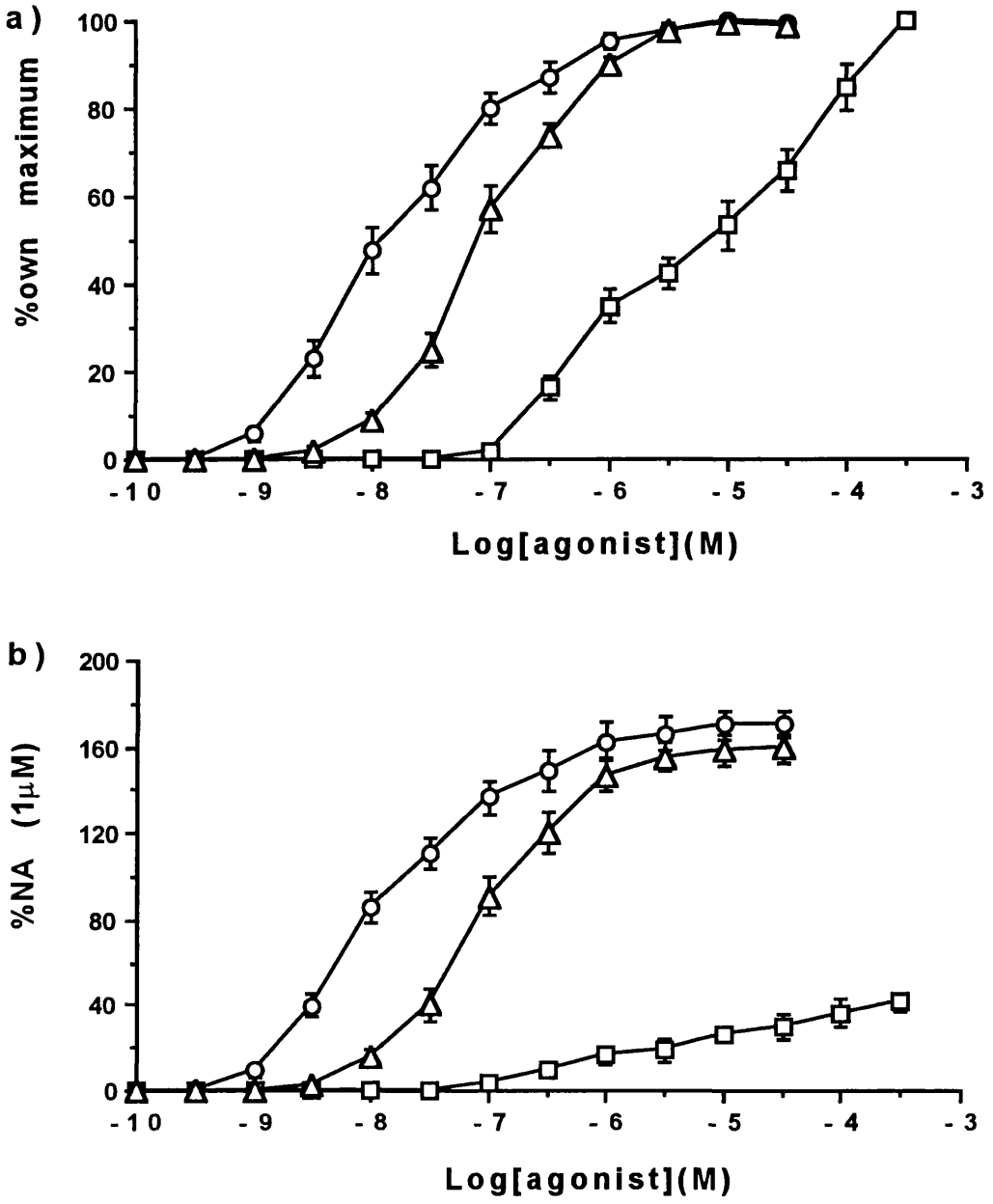


Figure 2.2

Cumulative concentration-response curves to α-adrenoceptor agonists: NA (○), PE (△) and UK-14304 (□) in the rat isolated common carotid artery . Results are expressed as: **a)** % of the maximum response of each individual CCRC to agonist or **b)** % of the maximum response to initial application of NA (1μM).

Each point represents the mean ± s.e.mean (n=8).

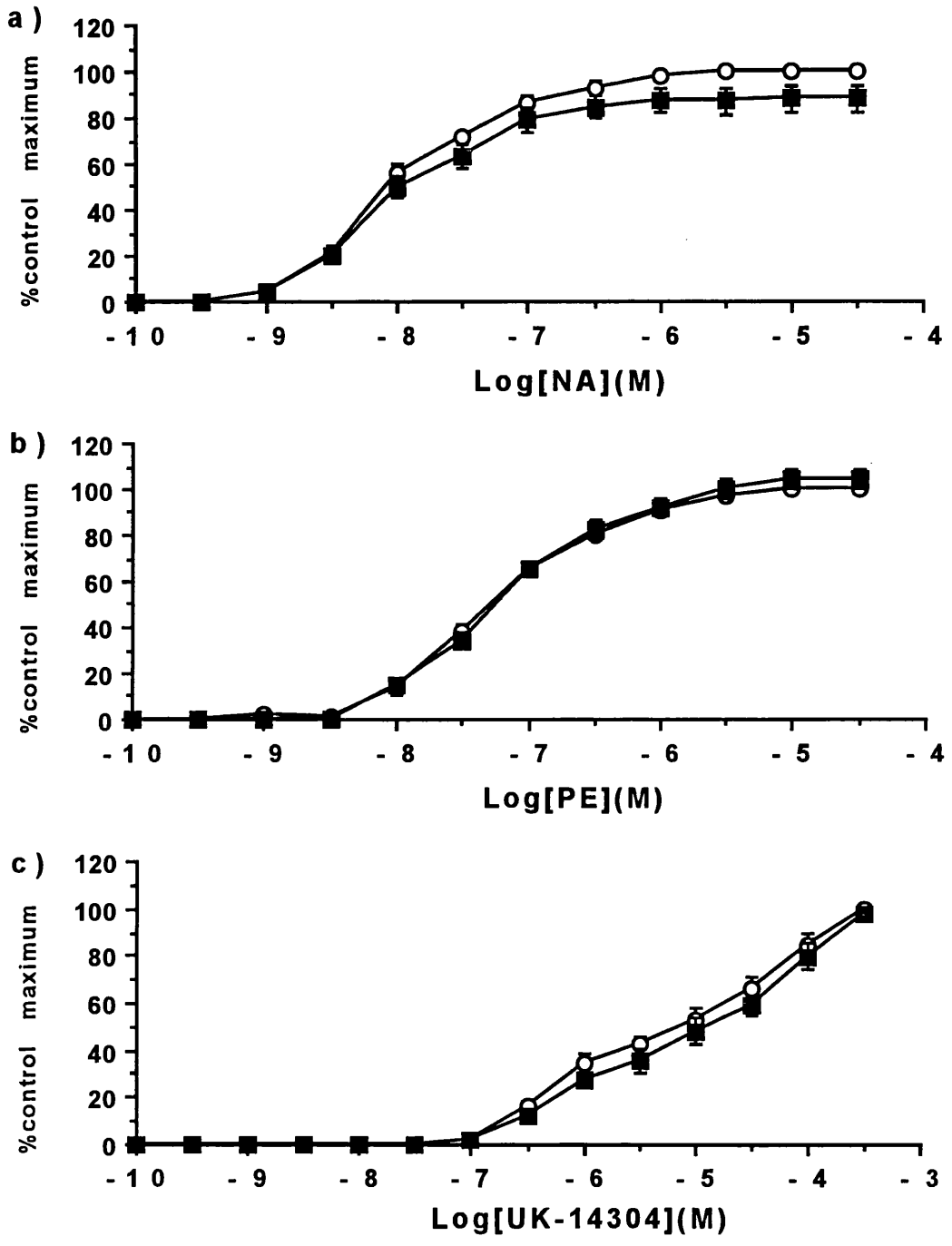
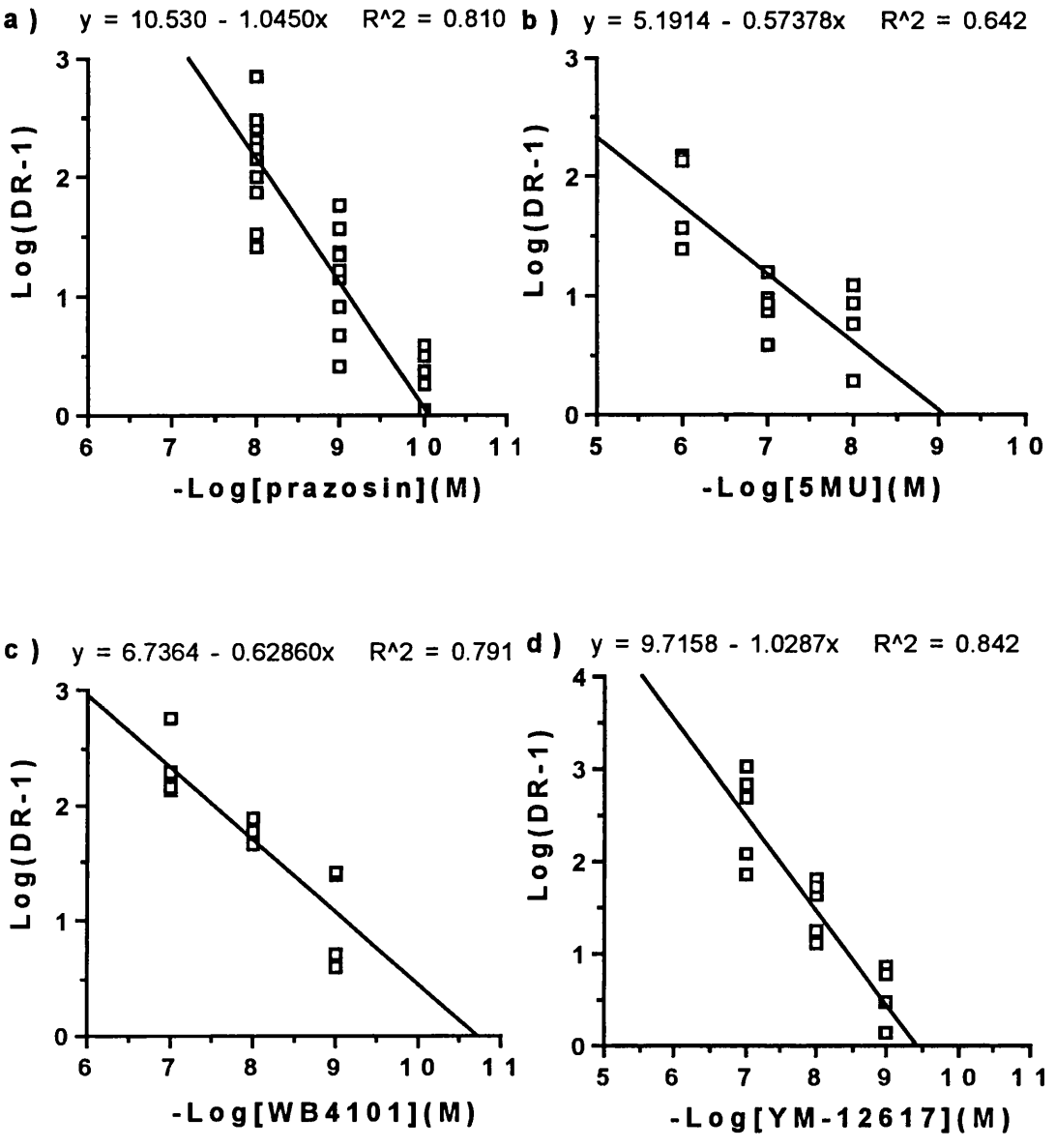


Figure 2.3

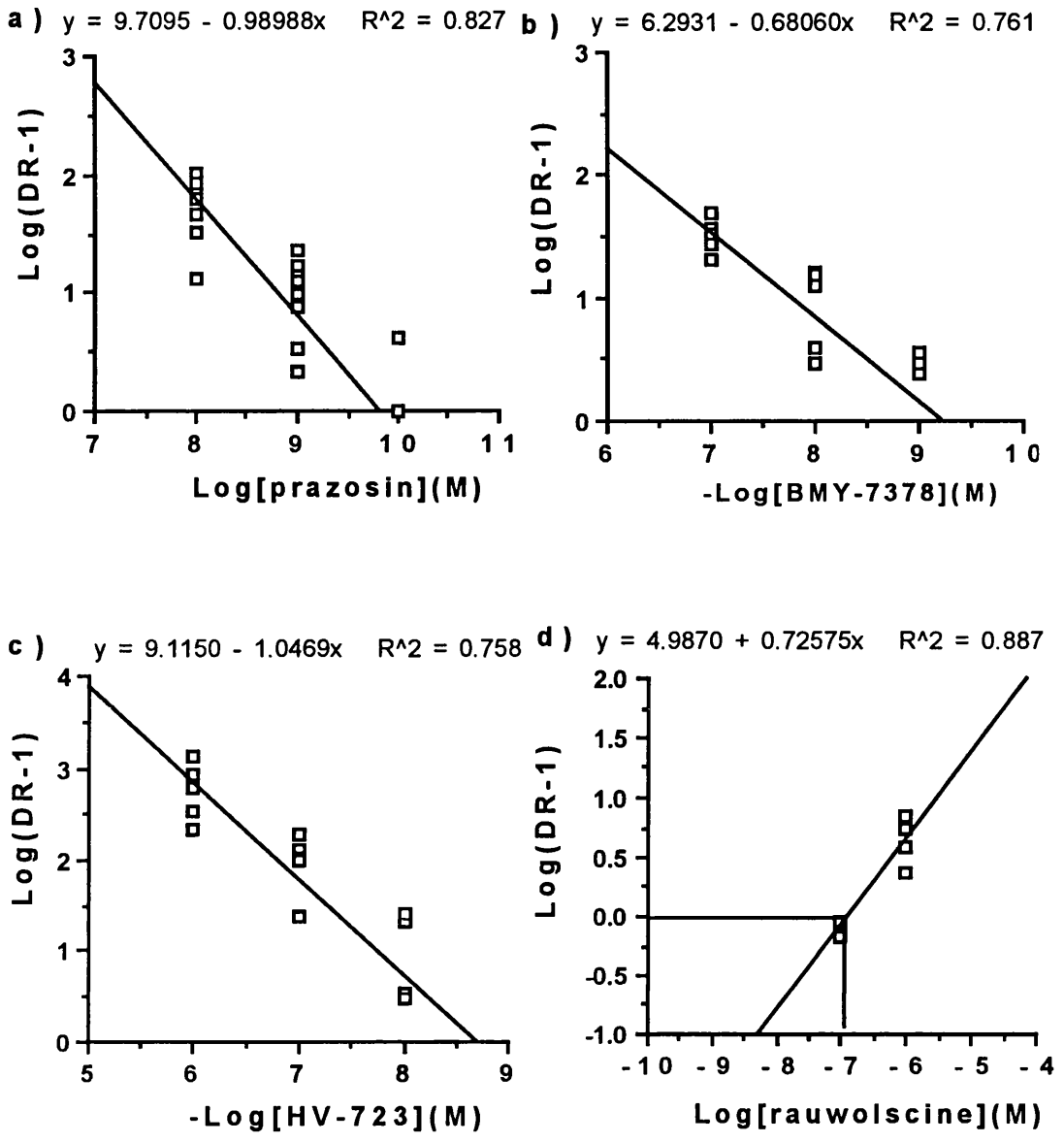
Effect of time on cumulative concentration-response curves to α-adrenoceptor agonists **a**) NA **b**) PE **c**) UK-14304, in the rat isolated common carotid artery. The first CCRC to agonist is represented by (○) while second CCRC to agonist represented by (■). Results are expressed as: % of the maximum response of the first CCRC to each agonist.

Each point represents mean ± s.e.mean (n=8).



pA_2 values obtained for the α_1-adrenoceptor antagonists using Schild analysis by plotting log (DR-1) on the y-axis against the log M [antagonist] on the x-axis and fitting using linear regression. The pA_2 value was derived from the x-intercept which is equal to the antagonist dissociation constant (K_B) under equilibrium conditions. **a**) prazosin **b**) 5-MU **c**) WB-4101 **d**) YM-12617 against contractions to noradrenaline in the rat isolated common carotid artery.

Each point represents an individual experiment.



pA_2 values obtained for the α_1-adrenoceptor antagonists using Schild analysis by plotting log (DR-1) on the y-axis against the log M [antagonist] on the x-axis and fitting using linear regression. The pA_2 value was derived from the x-intercept which is equal to the antagonist dissociation constant (K_B) under equilibrium conditions **a**) prazosin against contractions to phenylephrine **b**) BMY-7378 **c**) HV-723 **d**) rauwolscine against contractions to noradrenaline in the rat isolated common carotid artery.

Each point represents an individual experiment.

Table 2.1

antagonist	pA_2	slope
rauwolscine	6.9 (6.63-7.2)	0.72 (0.61-0.84)
prazosin	10.05 (9.8-10.32)	1.04 (0.94-1.1)
5MU	9.1 (8.61-9.61)	0.57 (0.46-0.69)
YM-12617	9.44 (9.24-9.64)	1.02 (0.905-1.15)
WB-4101	10.7 (10.27-11.15)	0.62 (0.53-0.71)
HV-723	8.7 (8.43-9)	1.04 (0.88-1.21)
BMY-7378	9.24 (8.86-9.4)	0.68 (0.57-0.78)

List of pA_2 values with the slopes of the Schild plots (With 95% confidence limits) for α-adrenoceptor antagonists against responses to noradrenaline in the rat isolated common carotid artery in the presence of propranolol (1μM) and cocaine (10μM).

pA_2 values were determined from a regression analysis of the logarithm of dose ratio-l against the negative logarithm of the molar concentration of the antagonist.

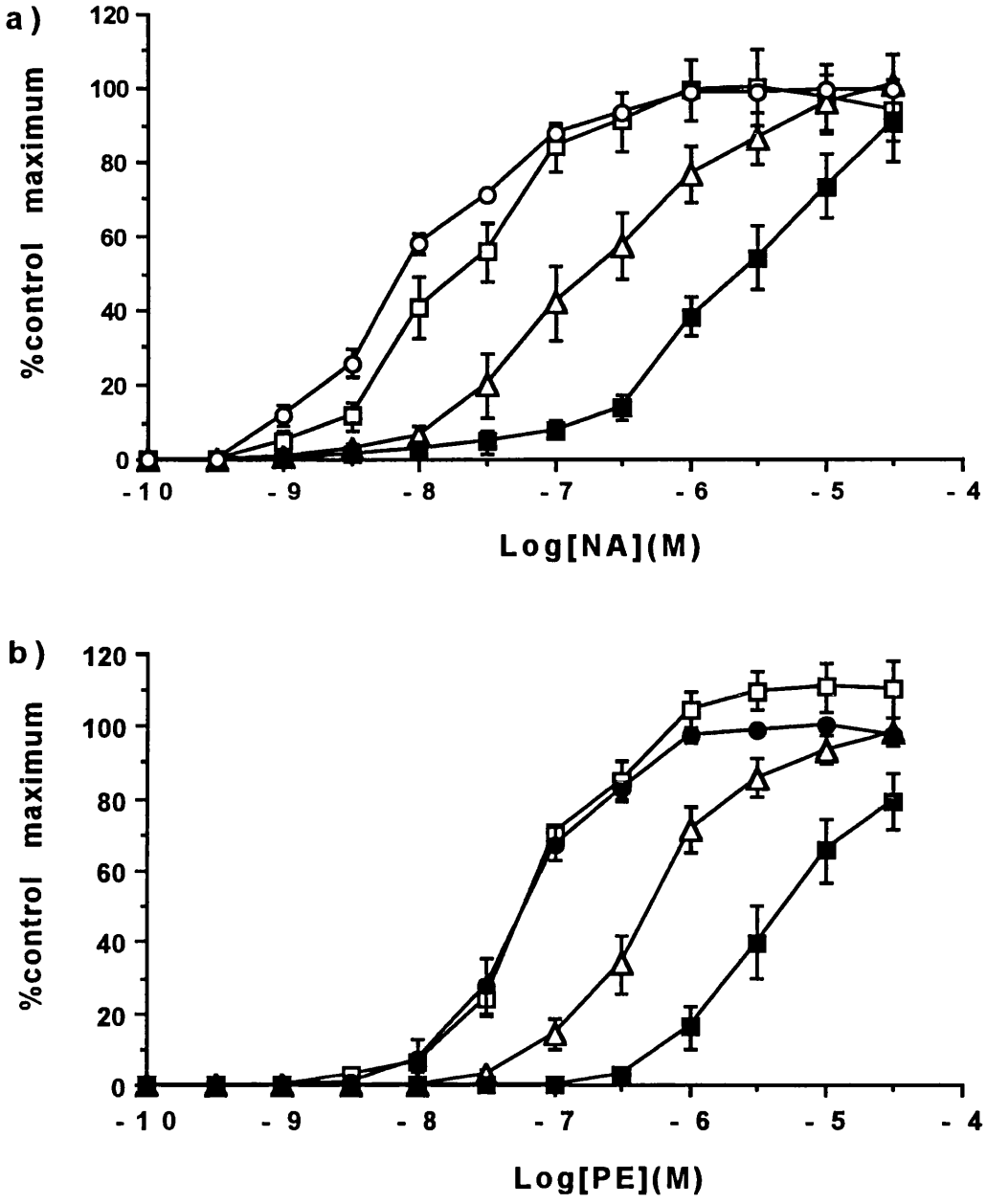


Figure 2.4

Effects of selective α_1-adrenoceptor antagonist prazosin 0.1nM (□), 1nM (△) or 10nM (■) on control responses to **a)** NA (○) **b)** PE (●) in the rat isolated common carotid artery. Responses are expressed as % of the maximum response of the control CCRC to NA or PE in the absence of antagonist.

Each point represents mean ± s.e.mean (n=6-8).

WB-4101 produced concentration-dependent shifts in the potency of NA without reducing the maximum response (Figure 2.5a). The pA_2 value for WB-4101 was 10.7 and slope of the Schild plot was 0.62, very different from unity, suggesting non-competitive antagonism (Table 2.1).

5-MU produced concentration-dependent shifts in the potency of NA without reducing the maximum response (Figure 2.5b). The pA_2 value for 5-methylurapidil was 9.1 and slope of the Schild plot was 0.57, very different from unity, indicating non-competitive antagonism (Table 2.1).

The irreversible antagonist chloroethylclonidine (100μM) that preferentially alkylates α_{1B}-adrenoceptors, failed to reduce significantly the maximum response to NA but produced parallel shifts in the potency of NA. The tissues were treated with CEC (100μM) for 30 minutes followed by washout of the irreversible antagonist for an additional 30-45 minutes. The shift in potency of NA produced by CEC was 125.9 times (Figure 2.6a).

HV-723 produced parallel shifts of the concentration-response curve to NA without reduction in maximum response (Figure 2.6b). Schild regression analysis yielded line with slope of 1.04, not significantly different from one and pA_2 value of 8.7 (Table 2.1).

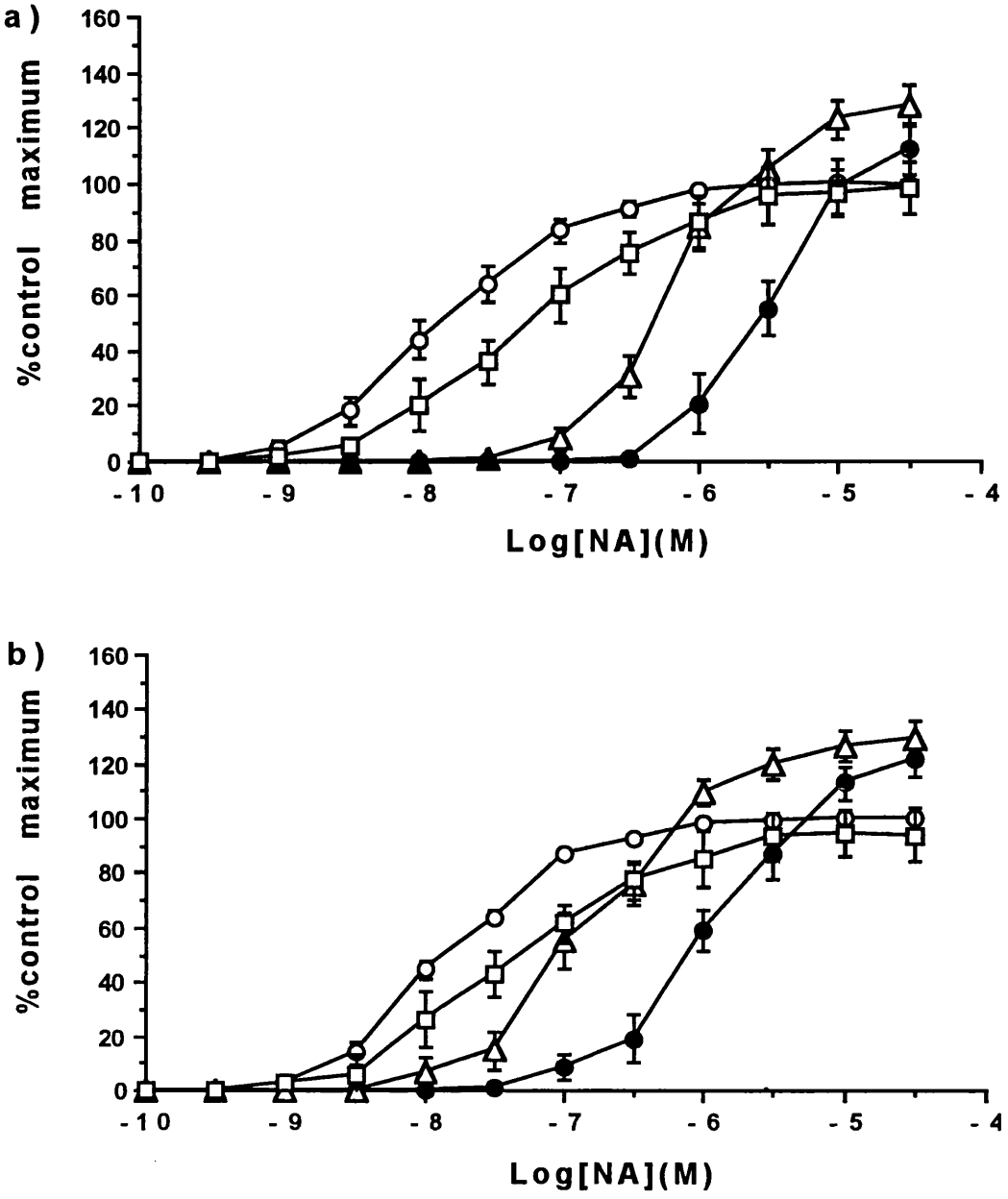


Figure 2.5

Effects of α_1-adrenoceptor antagonists **a**) WB-4101 1nM (□), 10nM (△) or 0.1μM (●) **b**) 5-methylurapidil 10nM (□), 0.1μM (△) or 1μM (●) on control responses to NA (○) in the rat isolated common carotid artery. Results are expressed as % of the maximum response of the control CCRC in the absence of antagonist.

Each point represents mean ± s.e.mean (n=6).

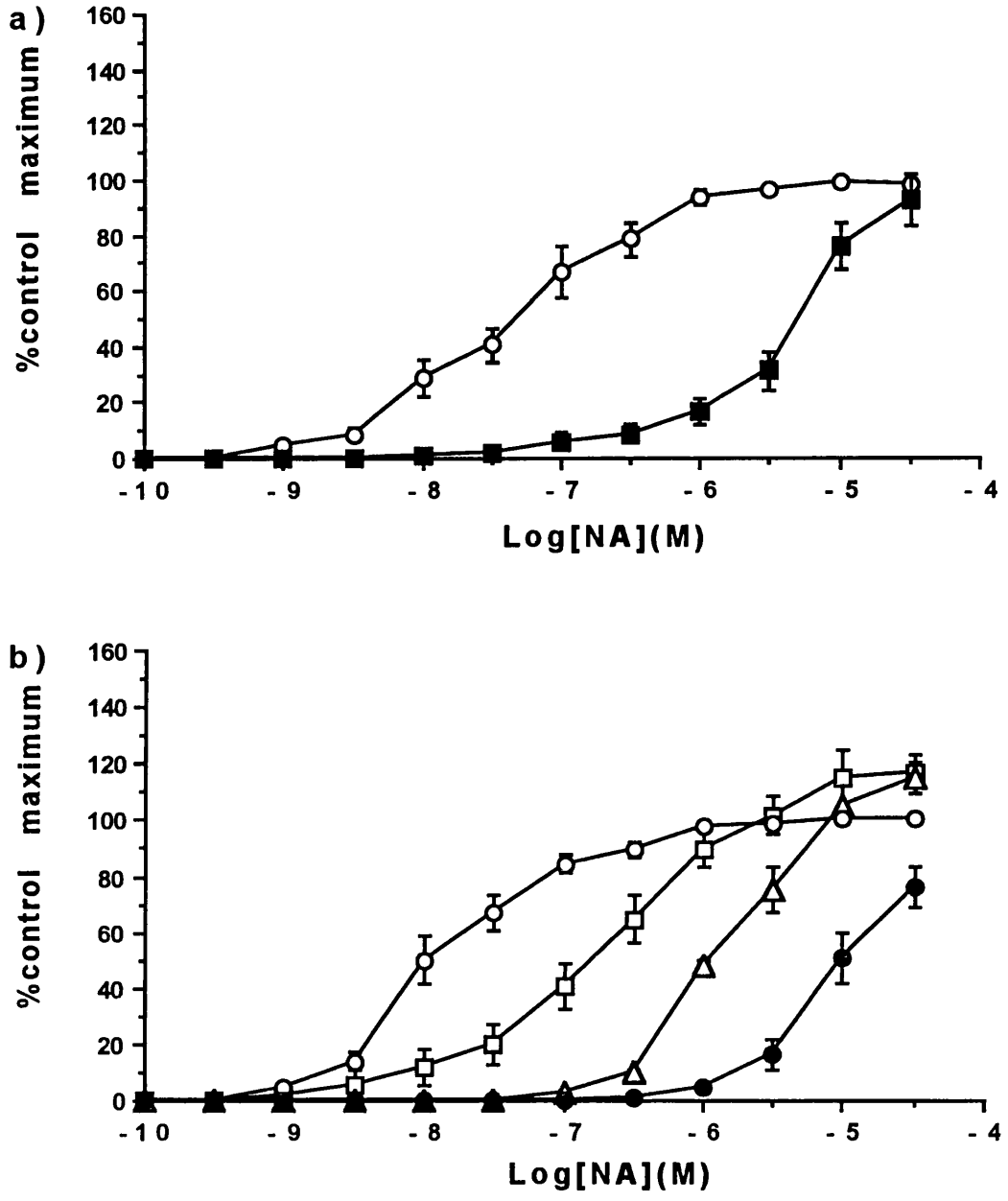


Figure 2.6

Effects of **a**) treatment with the alkylating drug chloroethylclonidine (■); tissues were treated with 100μM for 30 minutes followed by washout for an additional 30-45 minutes, **b**) α_1-adrenoceptor antagonist HV-723 10nM (□), 0.1μM (△) or 1μM (●), on control responses to NA (○) in the rat isolated common carotid artery. Results are expressed as % of the maximum response of the control CCRC in the absence of antagonist or chloroethylclonidine.

Each point represents mean ± s.e.mean (n=6).

The S(+)-isomer of the dihydropyridine Ca^{2+} channel antagonist, niguldipine caused a shift of the concentration-response curve of NA to the right and reduced the maximum response. The shift in potency of NA produced by niguldipine was 4.07 times (Figure 2.7a).

The selective α_{1D}-adrenoceptor antagonist BMY-7378 produced a parallel shift of the concentration-response curve to NA with a large change in maximum response (Figure 2.7b). Schild regression analysis yielded a line with a slope of 0.68, and pA_2 value of 9.24 (Table 2.1).

Increasing concentrations of α_1-adrenoceptor antagonist YM-12617 shifted NA concentration-response curve to the right in a parallel manner with a large change in maximal response (Figure 2.8a). The pA_2 value for YM-12617 was 9.44 and slope of the Schild plot was 1.02, not different from unity, indicating competitive antagonism (Table 2.1).

Increasing concentration of the selective α_2-adrenoceptor antagonist rauwolscine produced small rightward displacement of NA CCRC on high concentration (1μM) (Figure 2.8b). The pA_2 value was 6.9 and slope of the Schild plot was 0.72, significantly different from unity, indicating non-competitive antagonism (Table 2.1).

The rank order of potencies for these α-adrenoceptor antagonists was as follows: WB-4101> prazosin> YM-12617> BMY-7378> 5MU> HV-723> rauwolscine (Table 2.1).

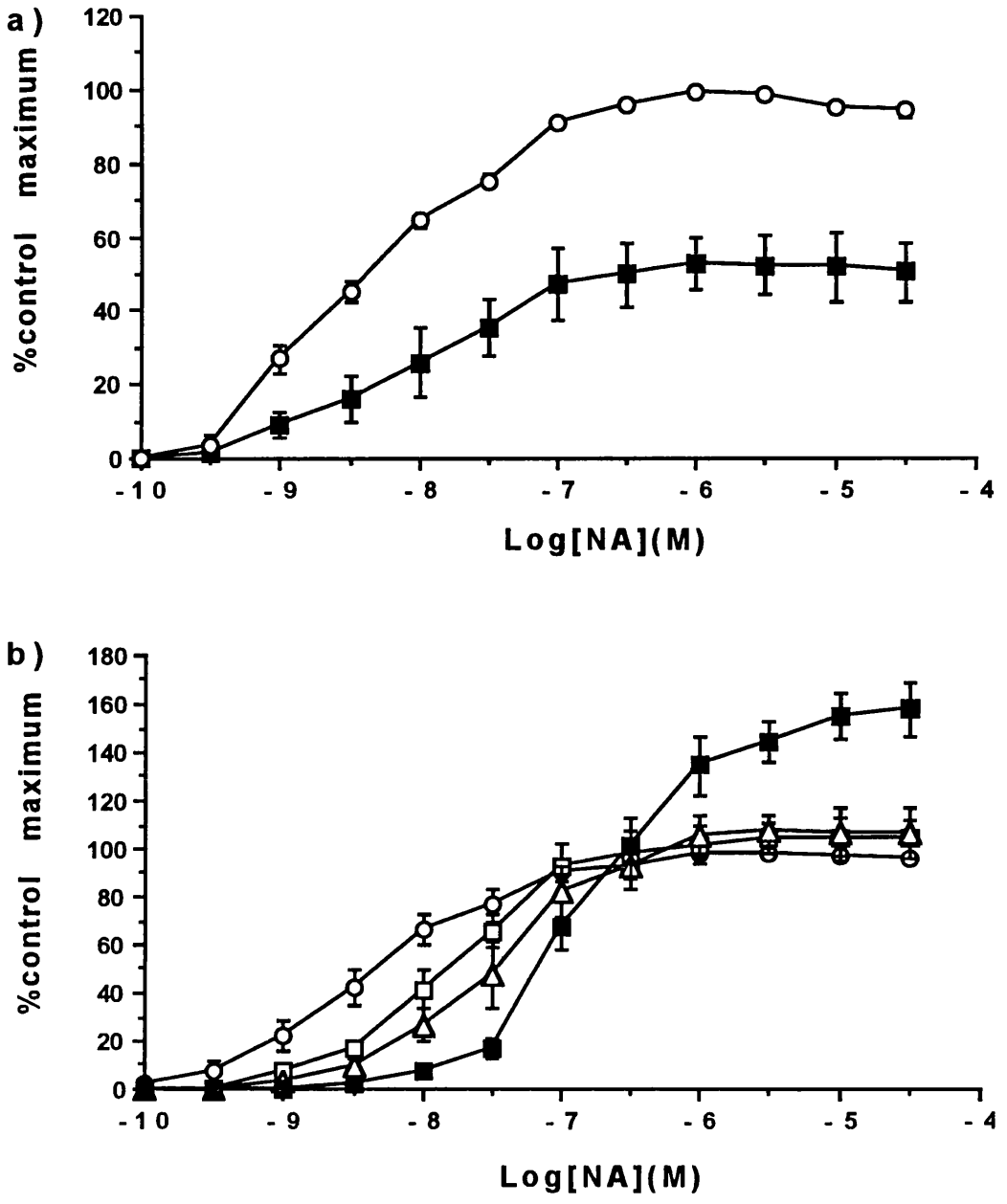


Figure 2.7

Effects of **a**) treatment with the niguldipine (1μM) (■) **b**) selective α_{1D}-adrenoceptor antagonists BMY-7378 1nM (□), 10nM (△) or 0.1μM (■) on control responses to NA (○) in the rat isolated common carotid artery. Results are expressed as % of the maximum response of the control CCRC in the absence of antagonist or niguldipine.

Each point represents mean ± s.e.mean (n=6).

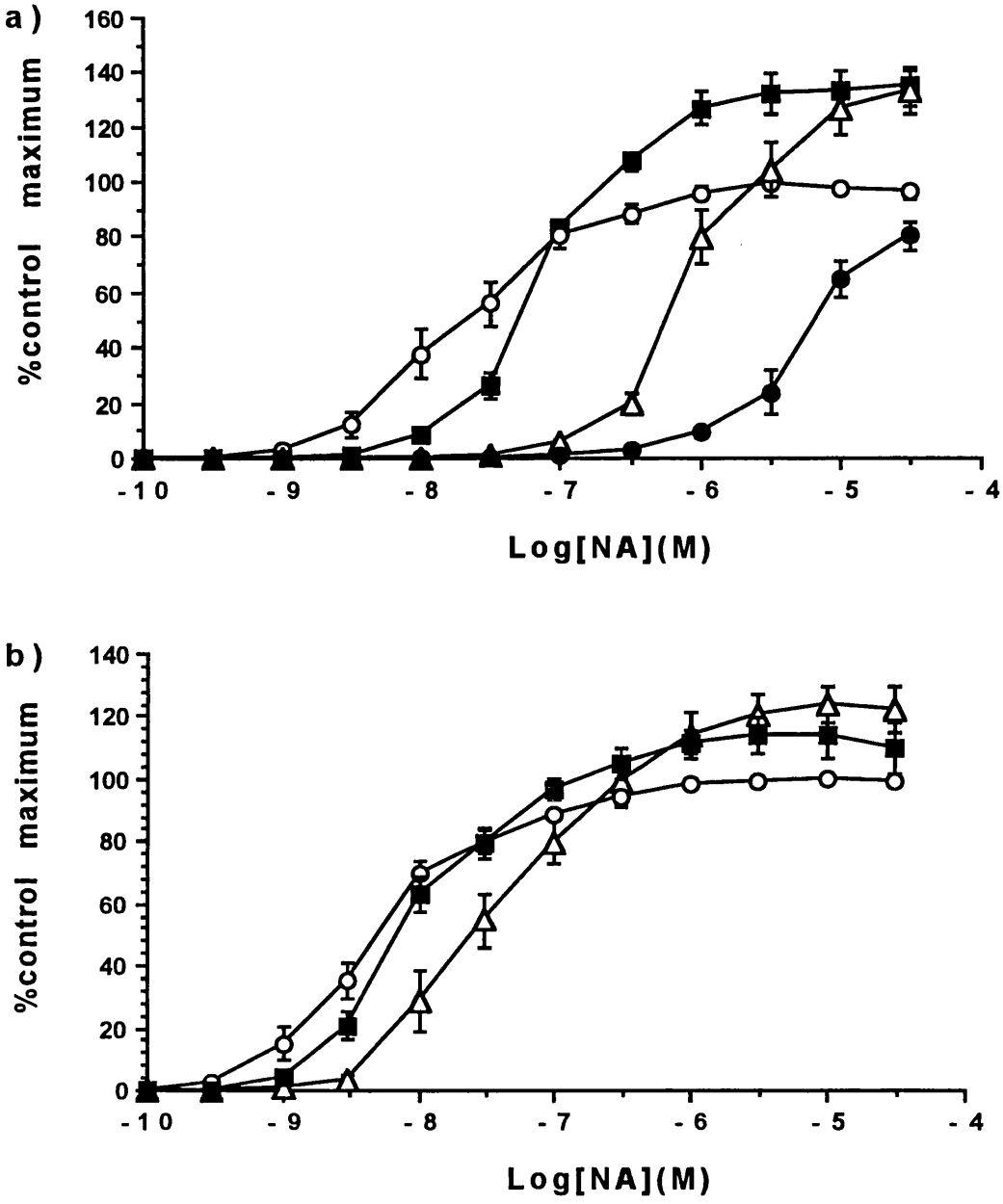


Figure 2.8

Effects of α-adrenoceptor antagonists **a)** selective α_1–adrenoceptor agonist YM-12617 1nM (■), 10nM (△) or 0.1μM (●) **b)** selective α_2–adrenoceptor antagonist rauwolscine 0.1μM (■) or 1μM (△) on control responses to NA (○) in the rat isolated common carotid artery. Results are expressed as % of the maximum response in the absence of antagonist.

Each point represents mean ± s.e.mean (n=6).

2.3.3 UK-14304-induced contraction and relaxation

UK-14304 produced isometric contraction with a pD_2 of 5.1 and maximum contraction of 0.44 ± 0.01g. Rauwolscine produced a small rightward displacement of the UK-14304 CCRC (Figure 2.9a).

Contraction to UK-14304 was inhibited by prazosin (1nM, 10nM). Lack of a true maximum contraction prohibits a pA_2 value for prazosin versus UK-14304, but the high sensitivity to prazosin strongly implicates α_1 agonism (Figure 2.9b).

Preparations were precontracted with PE (1μM) or NA (1μM) to induce contraction. When the contraction reached a plateau, cumulative concentration-effect curves to UK-14304 were obtained by increasing its concentration in half-log increments (1nM-300μM). UK-14304 dose-dependently induced a relaxation in preparations preconstricted by NA or PE. Versus PE (1μM); pIC_{50} for UK-14304 was 4.91 ± 0.14 (Figure 2.10a); versus NA (1μM) was 4.75 ± 0.16 (Figure 2.13).

In preconstricted preparations induced by NA, rauwolscine produced relaxation in high concentration, pIC_{50} was 5.76 ± 0.13 (Figure 2.10b).

In preconstricted preparations induced by PE (1μM), prazosin produced relaxation with a pIC_{50} of 8.14 ± 0.17 (Figure 2.12).

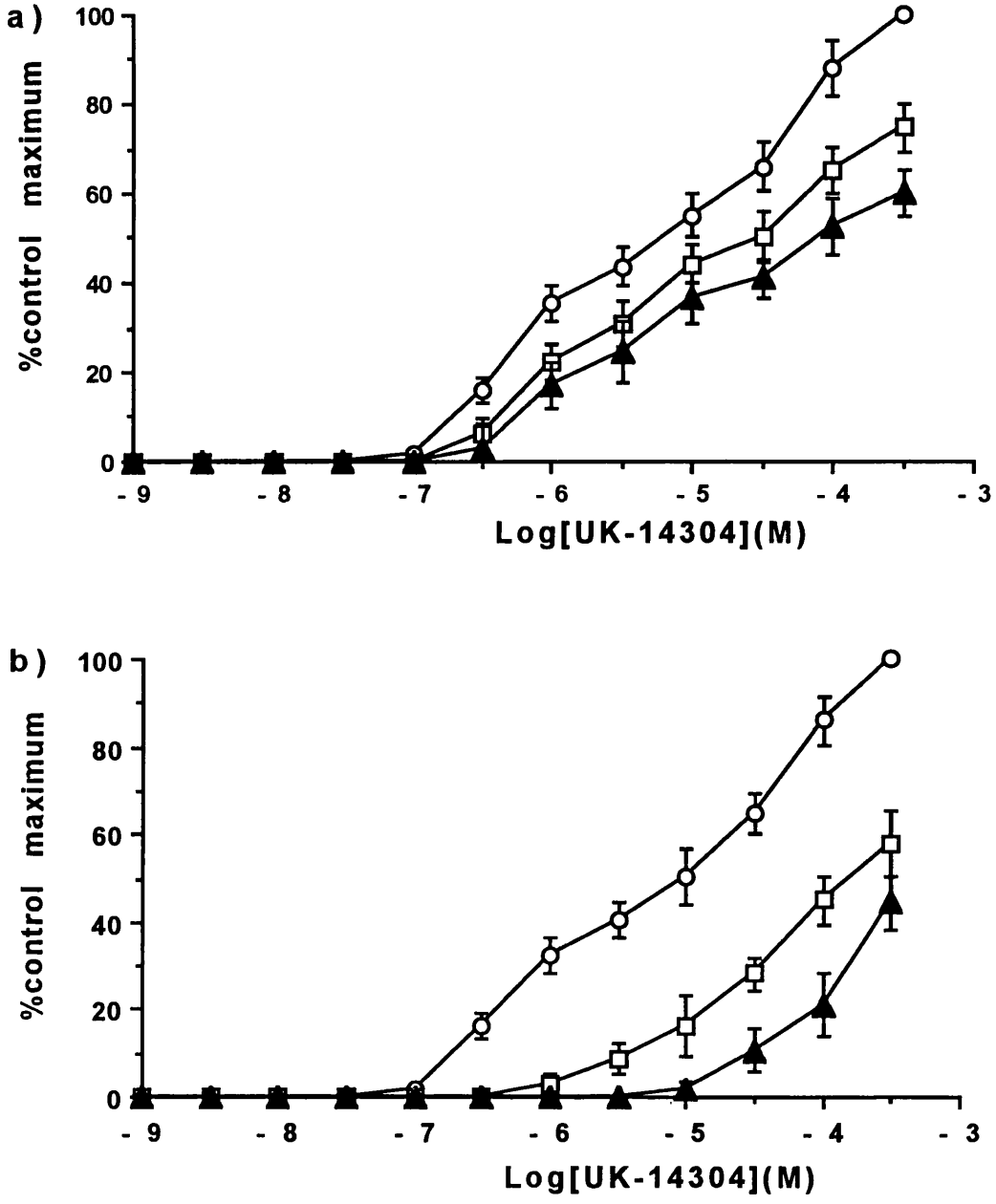


Figure 2.9

Effects of **a**) selective α_2-adrenoceptor antagonist rauwolscine 0.1μM (□) or 1μM (▲) and **b**) selective α_1-adrenoceptor antagonist prazosin 1nM (□) or 10nM (▲) on control responses to UK-14304 (○) in the rat isolated common carotid artery. Responses are expressed as % of the maximum response of the control CCRC in the absence of antagonist.

Each point represents mean ± s.e.mean (n=6).

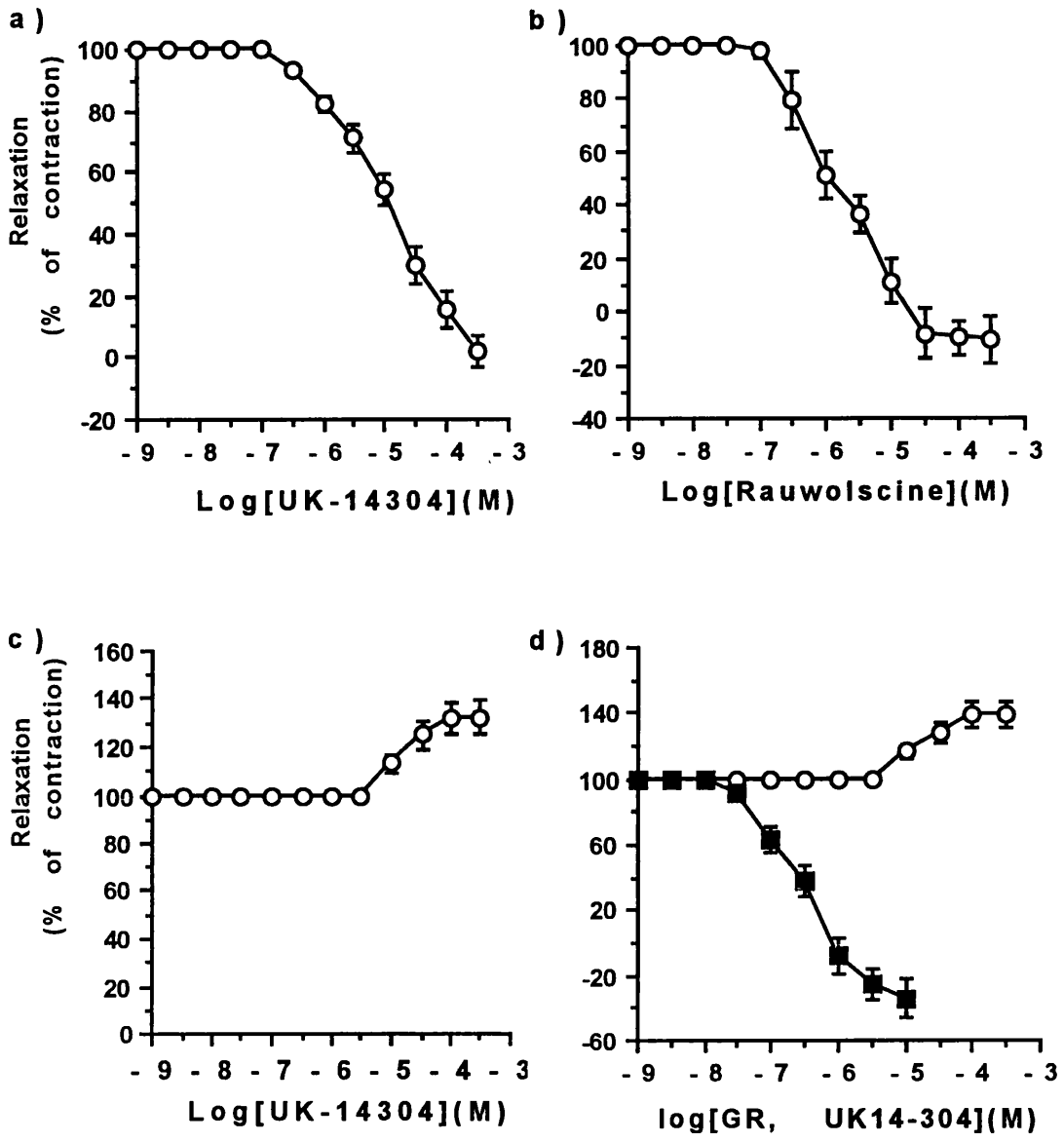


Figure 2.10

Relaxation effects to **a)** UK-14304; preconstricted by PE (1μM); **b)** rauwolsine, preconstricted by NA (1μM); **c)** UK-14304, preconstricted by $PGF_{2\alpha}$ (1μM); **d)** UK-14304 (○), or specific thromboxane A_2 receptor antagonist, GR-32191B (■) preconstricted by U-46619 (0.1μM). Results are expressed as % of maximum response to constrictor.

Each point represents the mean ± s.e.mean (n=8).

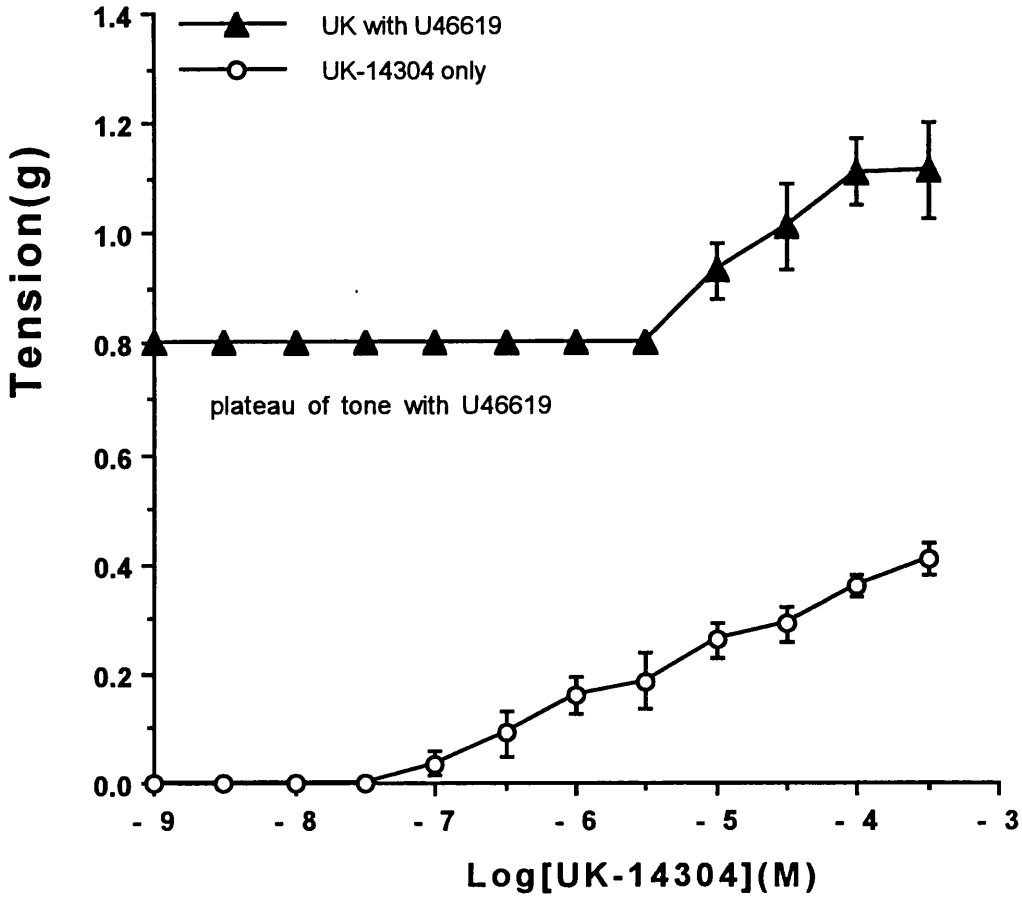


Figure 2.11

Cumulative concentration-response curves to the UK-14304 in the absence and presence of tone with U46619 (0.1μM). Results are expressed as absolute tension (g).

Each point represents the mean ± s.e.mean (n=6).

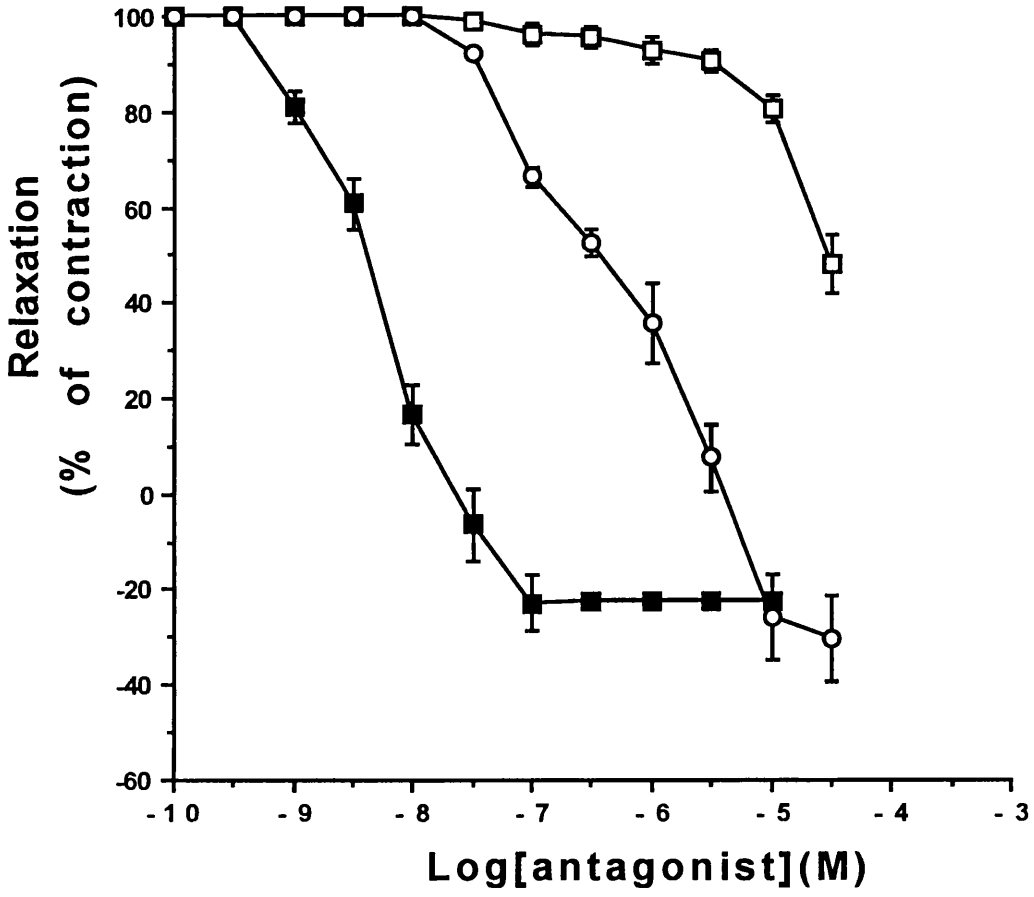


Figure 2.12

Relaxation effects of CCRC to (■) prazosin, (○) rauwolscine or (□) delequamine in preparations preconstricted by PE (1μM). Results are expressed as % of maximum response to PE (1μM) used for inducing tone.

Each point represents the mean ± s.e.mean (n=5-6).

In preconstricted preparations induced by PE (1μM), selective α_2-adrenoceptor antagonist delequamine produced poor relaxation in high concentrations compared with rauwolscine (Figure 2.12).

Contrast to α-adrenoceptor agonists, the specific thromboxane A_2 receptor antagonist, GR-32191B, in preconstricted preparations induced by U-46619 (0.1μM) produced relaxation that pIC_{50} was 6.5 (Figure 2.10d).

When tone was induced with $PGF_{2\alpha}$ (1μM) or U-46619 (0.1μM) UK-14304 did not produce relaxation but in high concentrations produced contraction (Figure 2.10c, d).

Neither functional disruption of the endothelium by rubbing nor inclusion of L-NAME (100μM), rauwolscine (1μM) or flurbiprofen (1μM) had any effect on UK-14304-induced relaxation (Figure 2.13).

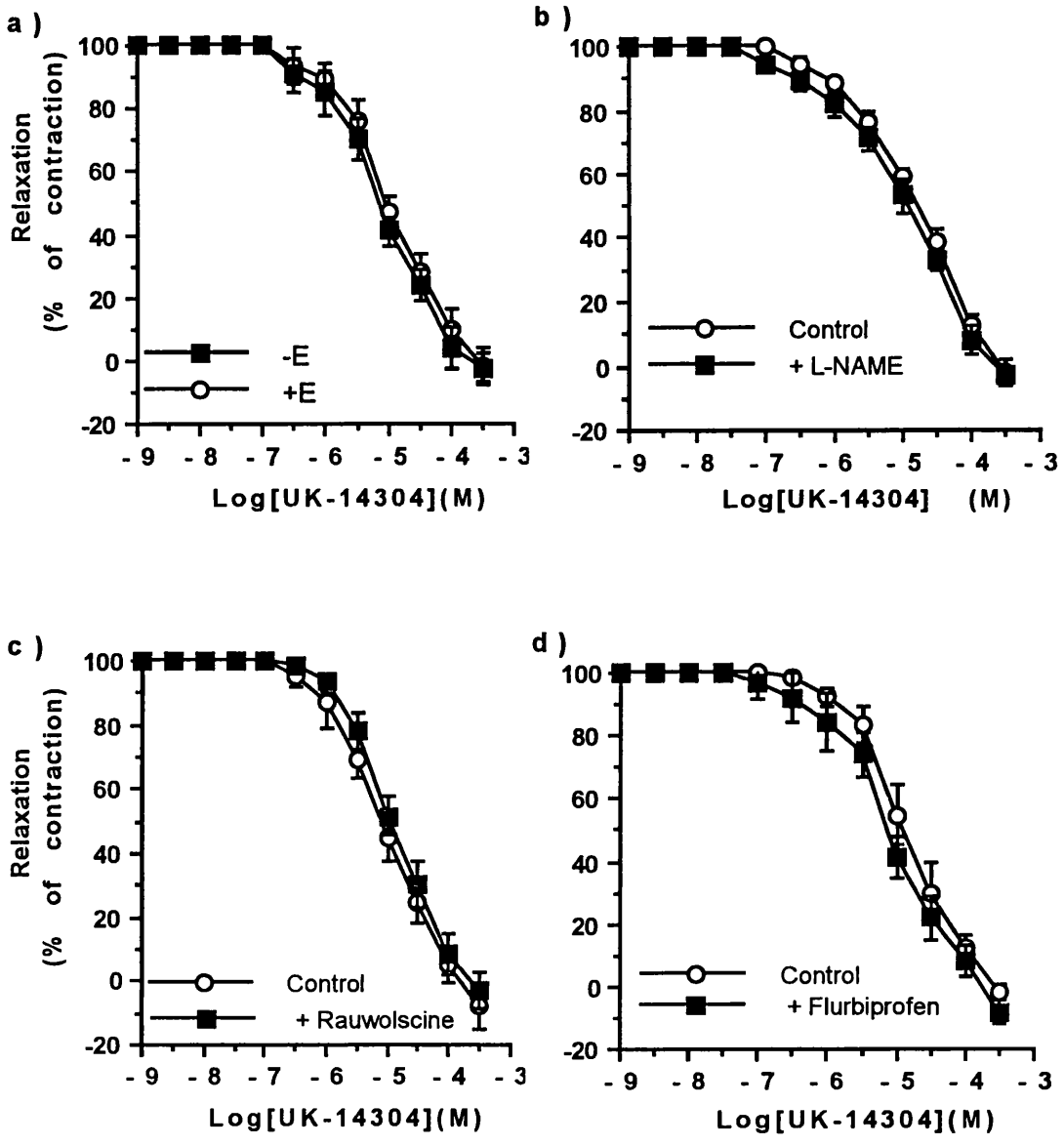


Figure 2.13

a) Effects of endothelium removal that preparations have intact endothelium (+E) or endothelium removed (-E) **b)** an inhibitor of NO synthase (L-NAME) 100μM **c)** selective α_2-antagonist rauwolscine (1μM), **d)** an inhibitor of cyclo-oxygenase flurbiprofen (1μM) on relaxation of CCRC to α_2-agonist UK-14304 in preconstricted preparations induced by NA (1μM). Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents the mean ± s.e.mean (n=6).

2.3.4 UK-14304 as an antagonist

The concentration-response curve to PE was shifted in parallel without decreasing the maximum response by pretreatment of UK-14304 (3, 10, 30μM) for 5 or 45 minutes. The Schild plot of the data yielded a slope of 0.75, which was significantly different from unity, suggesting non-competitive antagonism or at least, not providing direct evidence of competitive antagonism. pA_2 values against PE were 5.98 (Figure 2.14a) and 5.89 (figure 2.14b) respectively.

Prazosin caused rightward shifts of PE CCRC. Slope was 0.98 not different from unity, indicating competitive antagonism. The pA_2 value was 9.81 (see Schild plot figures).

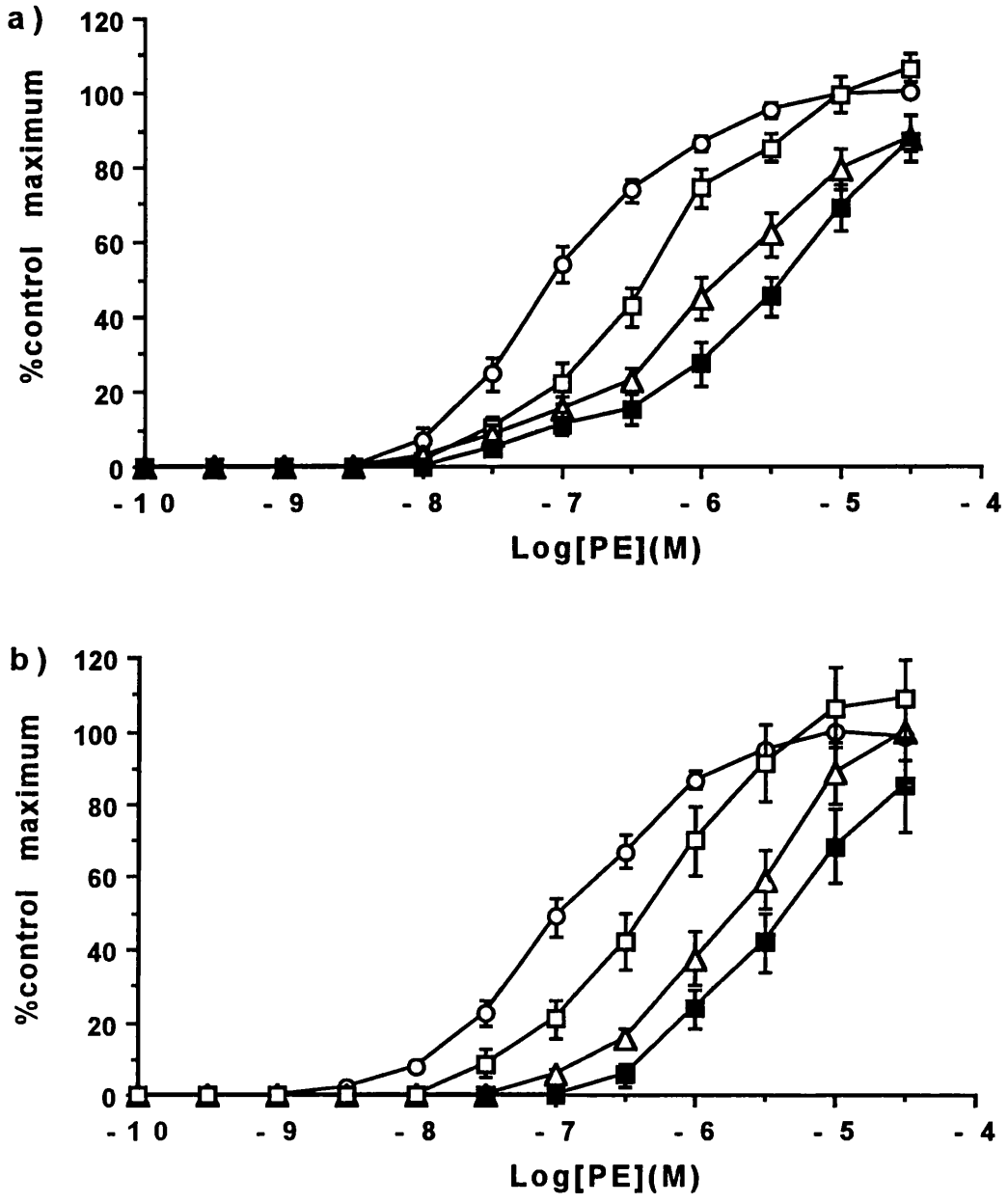


Figure 2.14

Effect of incubation time on the ability of UK-14304 to antagonise PE 3μM (□), 10μM (△) or 30μM (■). UK-14304 was added either **a)** 5 minutes or **b)** 45 minutes before starting CCRC to PE (○). Responses are expressed as % of the maximum response of the control CCRC to PE in the absence of UK-14304.

Each point represents the mean ± s.e.mean (n=6).

2.3.5 Effects of (L-NAME) on responses to α-adrenoceptor agonists

L-NAME itself in unrubbed preparations caused a large sustained increase in vascular tone that was 58 ± 9.5% of the contraction to NA (1μM).

Inclusion of L-NAME (100μM) resulted in small reduction of the "maximum" response to NA (Figure 2.16a). pD_2 was not changed significantly 8.07 compared with 7.89 (Table 2.2).

Inclusion of L-NAME (100μM) resulted in increase of the maximum response and sensitivity to PE (Figure 2.16b). pD_2 increased significantly to 8.02 compared with 7.03 ($p<0.05$) (Table 2.2).

Figure (2.16c) shows the effect of L-NAME on response to UK-14304. It increased the potency of UK-14304 as well as increasing the maximum tension that it was able to achieve in these vessels by 336% of control absolute maximum tensions. pD_2 increased to 6.63 compared with 5.06 ($p<0.01$) (Table 2.2).

Surprisingly, mechanical disruption of the vascular endothelium (to an extent which prevented vasodilation by an agonist acting via the endothelium-derived nitric oxide system) reduced, but did not abolish the ability of L-NAME to produce contraction. Addition of L-NAME to rubbed preparations produced a significantly smaller contraction than in unrubbed (20 ± 6% of NA 1μM).

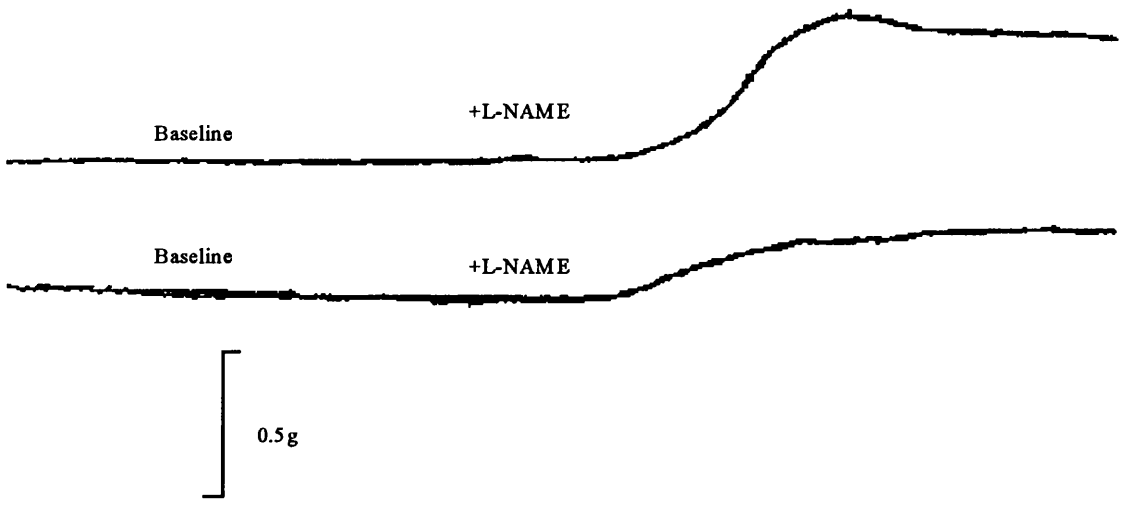


Figure 2.15

Representative trace recording of inclusion of L-NAME (100μM) on the isolated rat common carotid artery in unrubbed preparations. First the preparations were placed under resting tension at 2.5-3g for each group of arterial rings of carotid artery. Then tissues were left to equilibrate for a 60 min period, during which time the tension was re-adjusted to a set value which was maintained constant throughout the rest of the experimental day. L-NAME was added to inhibit NO synthase and in these preparations produced contraction.

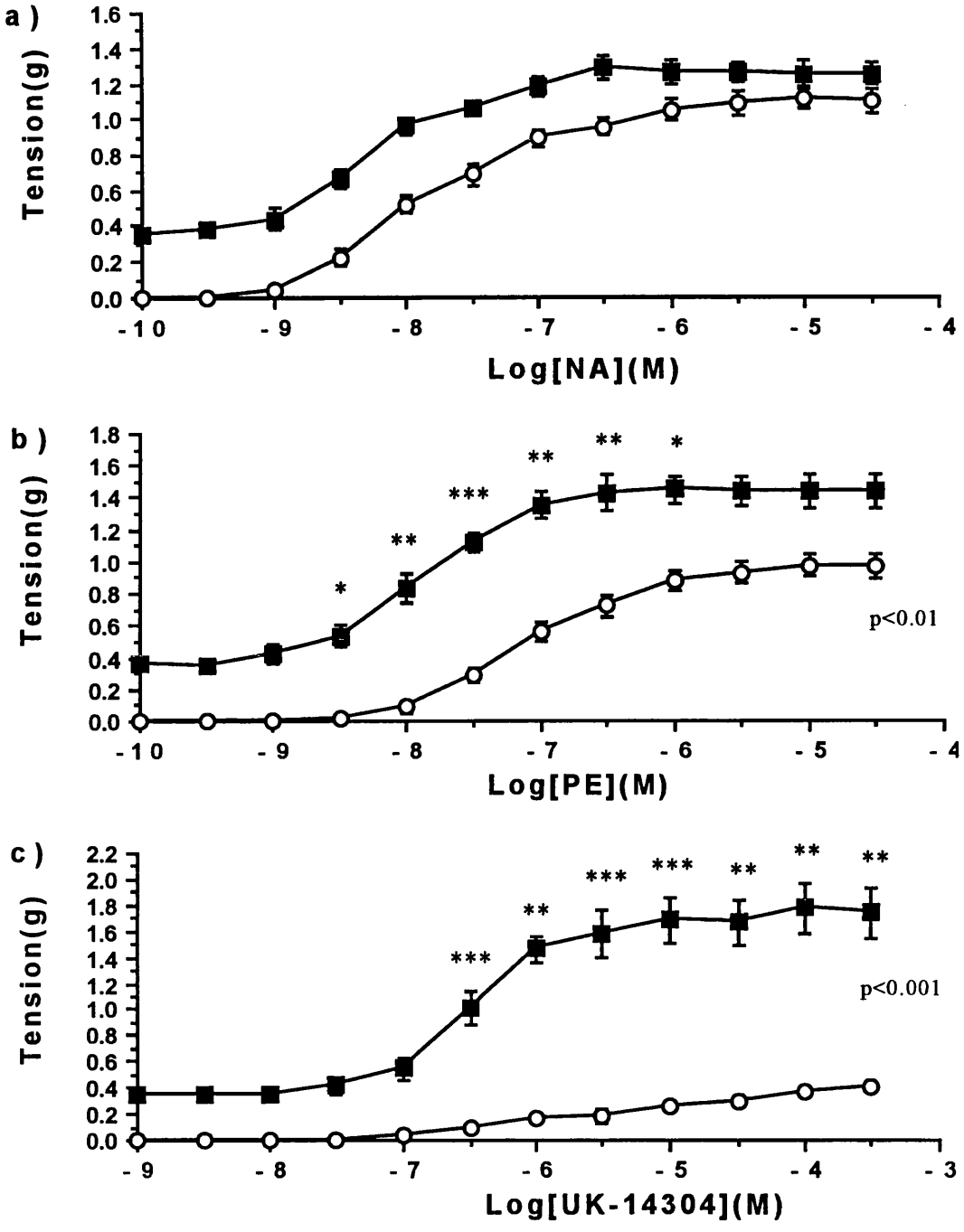


Figure 2.16

Effects of L-NAME (100μM) (■) in the preparations with intact endothelium on control (○) CCRC to α-adrenoceptor agonists, **a**) NA **b**) PE **c**) UK-14304 in the rat isolated common carotid artery. Results are expressed as tension (g).

Each point represents mean ± s.e.mean (n=10). Statistically significant differences are represented by * $p<0.05$, ** $0.001<p<0.01$, *** $p< 0.001$, paired Student's t-test. Value of p shows comparison of two curves by one way ANOVA.

2.3.6 Effects of endothelium removal on α-adrenoceptor agonists

In some preparations the endothelial layer was removed mechanically by gently rolling the tissue around a thin wire. Removing the endothelium increased responses to NA at low concentrations, but the maximum response was not changed (Figure 2.17a). pD_2 increased to 8.68 compared with 7.85 ($p<0.05$) (Table 2.3) .

Removing the endothelium increased responses to PE at low concentrations (Figure 2.17b). pD_2 increased to 7.83 compared with 7.1 ($p<0.05$) (Table 2.3).

In preparations with rubbed endothelium maximum response to UK-14304 increased (Figure 2.17c). pD_2 increased to 5.76 compared with 5.06 ($p<0.05$) (Table 2.3).

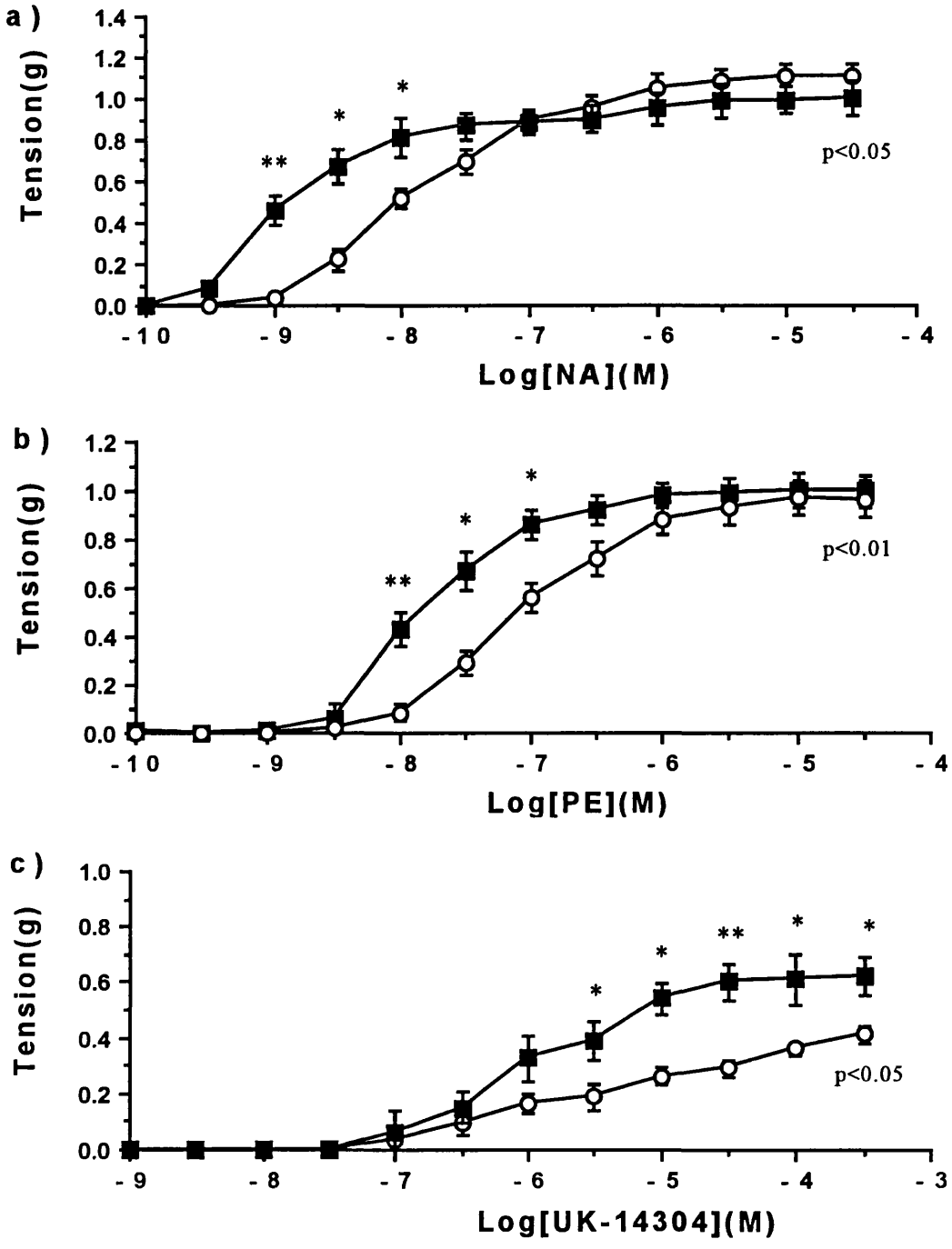


Figure 2.17

Effects of removing the endothelium (■) on cumulative concentration-response curves to the α-adrenoceptor agonists in preparations with endothelium (○) **a)** NA **b)** PE **c)** UK-14304 in the rat isolated common carotid artery. Results are expressed as tension (g).

Each point represents mean ± s.e.mean (n=10). Statistically significant differences are represented by * $p<0.05$, ** $0.001<p<0.01$, unpaired Student's t-test. Value of p shows comparison of two curves by one way ANOVA.

Table 2.2

The effect of N^{ω}-nitro-L-arginine methylester (L-NAME) on responses to α–adrenoceptor agonists in the rat isolated common carotid artery

Agonist	Effect of L-NAME	pD_2	n	Maximum response (g)
Noradrenaline	Control	7.89 ± 0.11	12	1.13 ± 0.035
	+ L-NAME	8.07 ± 0.1	12	0.88 ± 0.024
Phenylephrine	Control	7.03 ± 0.09	10	0.98 ± 0.021
	+ L-NAME	8.02 ± 0.1 *	10	1.15 ± 0.014
UK-14304	Control	5.06± 0.08	12	0.44 ± 0.018
	+ L-NAME	6.63 ± 0.12 **	12	1.45 ± 0.08 ***

pD_2 is expressed as the -log of the EC_{50} (concentration producing 50% of the maximum response). n shows the number of animals. Data are expressed as mean ± s.e.mean. Statistical comparisons with controls were carried out using paired Student's t test., * $p<0.05$, ** $0.001<p<0.01$, *** $p<0.001$.

Table 2.3

The effect of mechanical disruption of the vascular endothelium on responses to α-adrenoceptor agonists in the rat isolated common carotid artery

Agonist	Endothelium	pD_2	n	Maximum response (g)
Noradrenaline	Present	7.85 ± 0.14	8	1.1 ± 0.01
	Absent	8.68 ± 0.1 *	8	1 ± 0.03
Phenylephrine	Present	7.1 ± 0.06	8	0.96 ± 0.013
	Absent	7.83 ±0.04 *	8	1 ± 0.021
UK-14304	Present	5.18 ± 0.07	8	0.41 ± 0.03
	Absent	5.76 ± 0.09 *	8	0.62 ± 0.052 **

pD_2 is expressed as the -log of the EC_{50} (concentration producing 50% of the maximum response). n shows the number of animals. Data are expressed as mean ± s.e.mean. Statistical comparisons with controls were carried out using unpaired Student's t test., *$<p<0.05$, ** $0.001<p<0.01$.

2.3.7 Effect of inducing tone with KCl, PE, U-46619 or 5HT on responses to UK-14304

Since L-NAME greatly potentiates responses to UK-14304, a series of experiments were conducted to see whether other stimuli that produces submaximal contraction would have a similar synergistic effect.

2.3.8 KCl

KCl (10-15mM) produced a contraction, equivalent to 32 ± 6.4% of the NA contraction (1μM) in each preparation. There was a significant leftward shift and increase in maximum response in the CCRC to UK-14304 in the presence of tone with KCl (10-15mM) ($p<0.01$) (Figure 2.18a).

2.3.9 Phenylephrine

A low concentration of phenylephrine (10nM) produced a sustained contraction equivalent to 16.8 ± 3.3 % of the NA contraction (1μM). After inducing tone, exposure of the preparation to UK-14304, resulted in concentration dependent contractions to this agonist. The sensitivity and maximum response of the preparation to UK-14304 were not changed (Figure 2.18b).

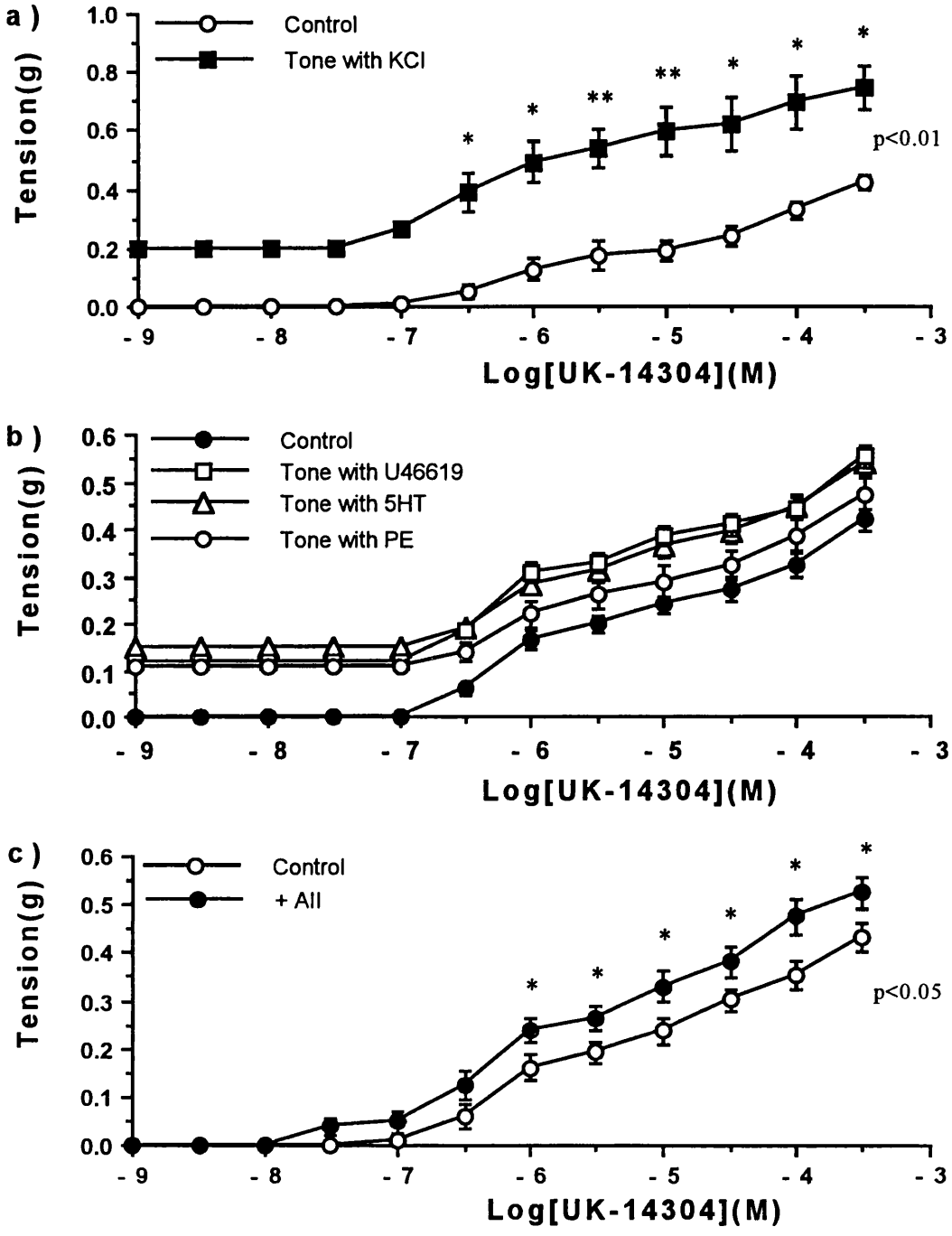


Figure 2.18

Effects of inducing tone **a**) with KCL (10-15mM) **b**) with 5HT (0.5-1μM), PE (10nM) or U-46619 (1nM) **c**) effect of presence of angiotensin AII (10nM) on responses to UK-14304 in rat isolated common carotid artery. Results are expressed as tension (g).

Each point represents the mean ± s.e.mean (n=6-8). Statistically significant differences are represented by *$p<0.05$, **$0.001<p<0.01$, using paired Student's t-test. Value of p shows comparison of two curves by one way ANOVA.

2.3.10 U-46619

The thromboxane A_2 mimetic agent, U-46619 (1nM) produced a sustained contraction, equivalent to 17.19 ± 6.14% of the NA contraction (1μM) in the rat isolated carotid artery. In the presence of U-46619, the sensitivity and maximum response of the preparation to UK-14304 were not changed (Figure 2.18b).

2.3.11 5-Hydroxytryptamine (5HT)

5HT (0.5-1μM) produced a contraction equivalent to 22.4 ± 5.4% of NA contraction (1μM). Inducing tone with this agent was not associated with a significant enhancement or uncovering of responses to UK-14304 (Figure 2.18b).

2.3.12 Effects of AII on responses to UK-14304

AII (0.01μM) produced a transient contraction, which returned to baseline after 5-10 min, in the rat isolated common carotid artery. This response was equivalent to 97 ± 3.23% of NA contraction (1μM) in each preparation. There was a small but significant leftward shift in the CCRC to α_2-adrenoceptor agonist UK-14304 in the presence of AII ($p<0.05$) (Figure 2.18c).

2.3.13 UK-14304 mediated contraction in present of AII or L-NAME is α_1

Since either L-NAME or AII could potentiate the contractile effect of UK-14304, the α-adrenoceptor antagonists prazosin and rauwolscine were re-examined to see whether UK-14304's main action remained via α_1.

Figure 2.19 shows the effects of rauwolscine (1μM), prazosin (1, 10nM) or delequamine (1μM) on the concentration-response curves to UK-14304 in preparations pretreated with L-NAME. Rauwolscine produced a small shift in the UK-14304 concentration-response curve. This was a surmountable antagonism with no shift in maximum. It was however characterised by a shallower CCRC which contrasted with the parallel shifts produced by prazosin (Figure 19). Delequamine did not significantly altered the pD_2 for UK-14304. However the maximum response was increased. The results show that responses to UK-14304 in the presence of L-NAME were rauwolscine- and delequamine-resistant but prazosin-sensitive in this artery.

In the presence of prazosin (10nM) and AII (10nM), responses to UK-14304 were not significantly different from preparations that had prazosin alone although there was a tendency to increase. By adding rauwolscine (1μM) in tissues that had prazosin (10nM) and AII (10nM), the concentration-response curve to UK-14304 had a small shift to the left (Figure 2.20). This is similar to the situation without AII or in the presence of L-NAME (see Figure 2.19).

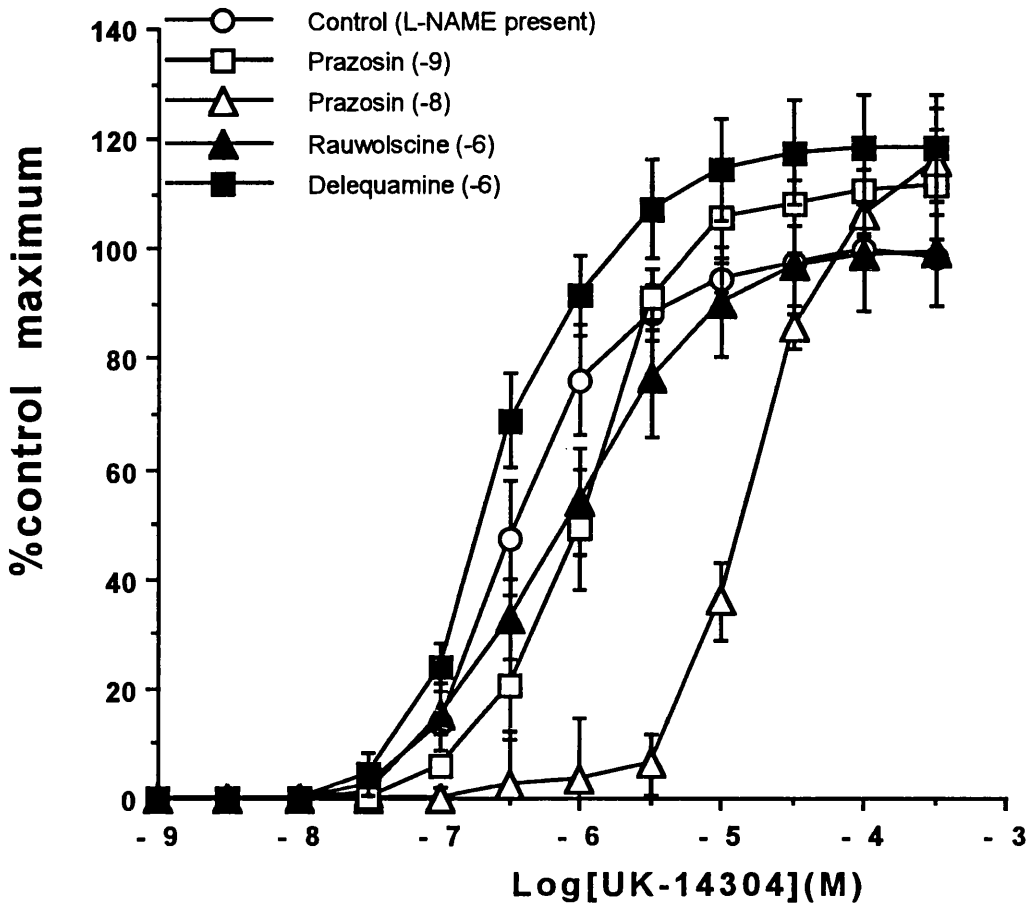


Figure 2.19

Analysis of α-adrenoceptor contractile response to UK-14304 after potentiation with L-NAME (100μM). Responses are expressed as % of the maximum response of the control CCRC to UK-14304 in the presence of L-NAME. Concentration is shown as Log(M) in the graphs.

Each point represents mean ± s.e.mean (n=6).

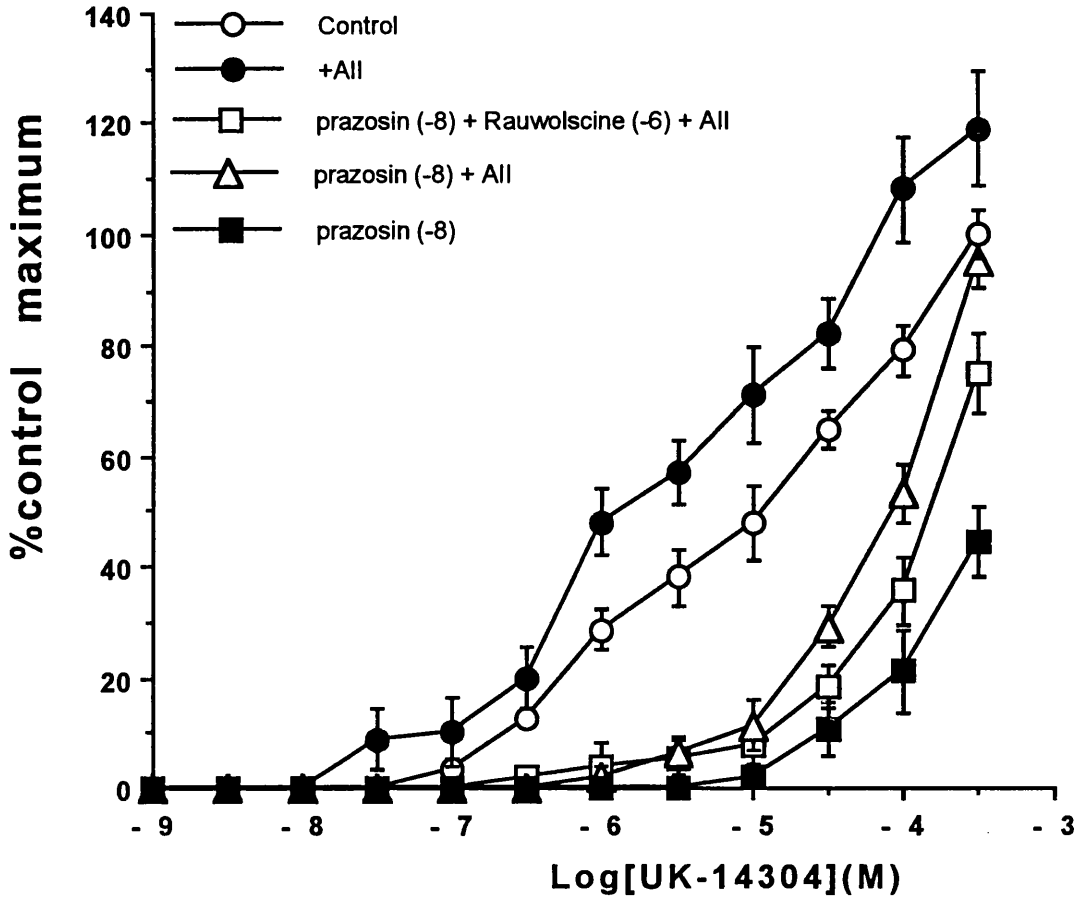


Figure 2.20

Analysis of α-adrenoceptor contractile response to UK-14304 after potentiation with AII (10nM) and in presence of prazosin or rauwolscine on responses to UK-14304. Responses are expressed as % of the maximum response of the control CCRC to UK-14304 in the absence of antagonist or AII. Concentration is shown as Log(M) in the graphs.

Each point represents mean ± s.e.mean (n=6).

2.3.14 Relaxations to ACh, ATP, adenosine or isoprenaline

ACh relaxed preparations with intact endothelium. The maximum relaxation was 59% of NA (1μM) induced tone and pIC_{50} was 7.04. Treatment with L-NAME (100μM) 10-15 minutes prior to ACh abolished any relaxation to ACh. In preparations without endothelium there was no relaxation in response to ACh (Figure 2.21a).

Isoprenaline relaxed preparations both with and without endothelium. Maximum relaxations were 55.72% and 52.41% of U-46619 (0.1μM) induced tone in unrubbed and rubbed preparations respectively. pIC_{50} values were 6.95 and 6.88 in tissues with endothelium and without endothelium respectively. Since the relaxation was not affected significantly by removal of the endothelium, this suggests that relaxation was due to stimulation β-adrenoceptors located on the smooth muscle cells (Figure 2.21b).

Adenosine and ATP both produced relaxations versus NA (1μM), but in high concentrations. For adenosine the pIC_{50} value was 4.15 with endothelium, 3.98 without (not significantly different) (Figure 2.21c).

For ATP pIC_{50} value was 3.97 with endothelium, without 3.82 (not significantly different) (Figure 2.21d).

It is likely that relaxations to ATP and adenosine in rat carotid artery were largely due to stimulation of purinoceptors located on the smooth muscle cells not on the endothelium.

5HT produced isometric contraction with a pD_2 of 5.82 and a maximum contraction of 0.57 ± 0.07g (Figure 2.22).

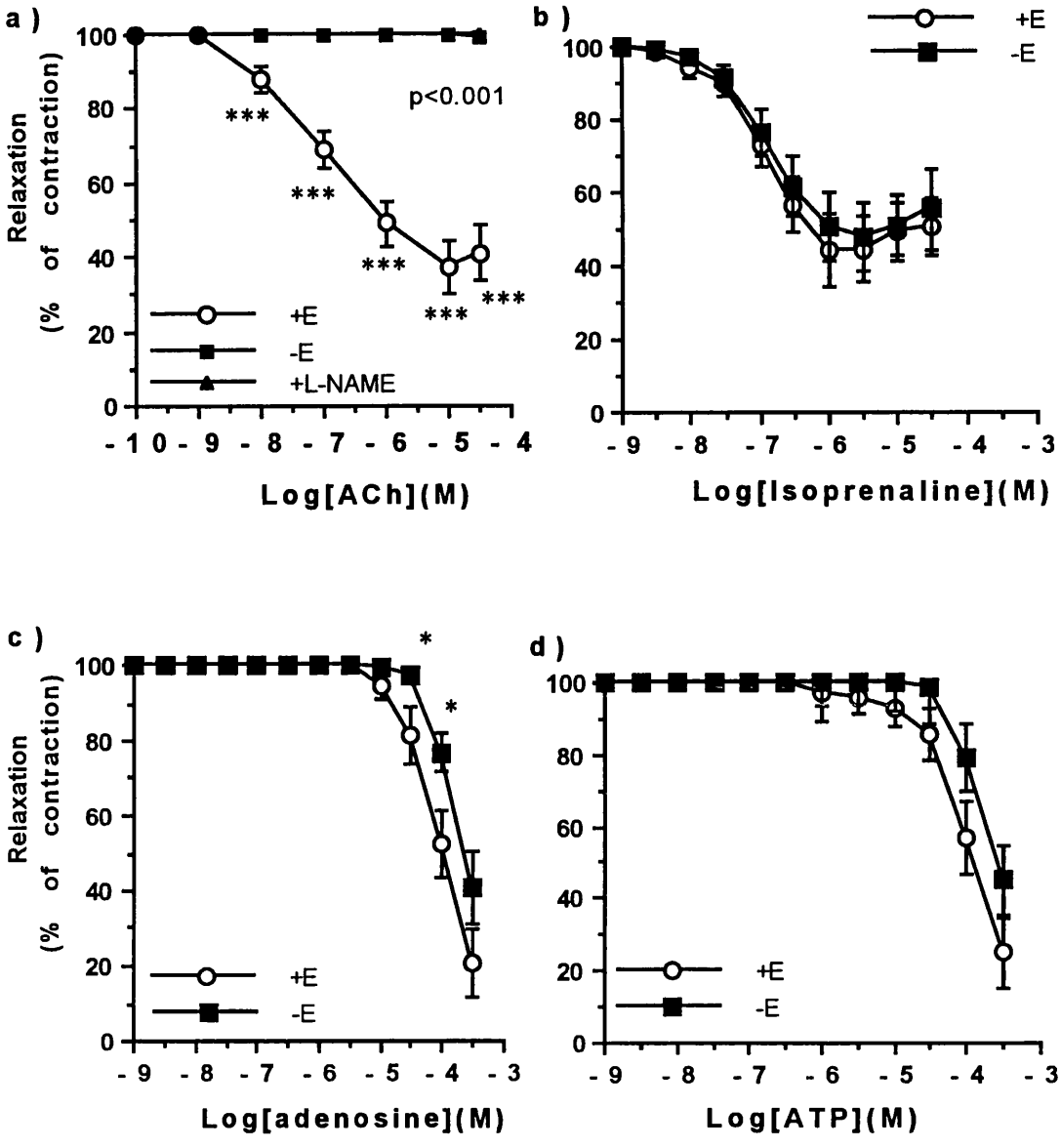


Figure 2.21

Relaxation effects of endothelium-dependent or independent agonists. Preparations have intact endothelium (+E) or endothelium removed (-E) by gently rolling the tissue around a thin wire **a)** ACh, also with endothelium but in presence of L-NAME (100μM); preconstricted by NA (1μM) **b)** β-adrenoceptor agonist isoprenaline preconstricted by U-46619 (0.1μM) **c)** and **d)** adenosine or ATP respectively preconstricted by NA (1μM). Results are expressed as % of maximum response to constrictor.

Each point represents the mean ± s.e.mean (n=8). Statistically significant differences are represented by * $p<0.05$, *** $p< 0.001$, paired (effect of L-NAME) or unpaired (effect of adenosine) Student's t-test. Value of p shows comparison of two curves by one way ANOVA.

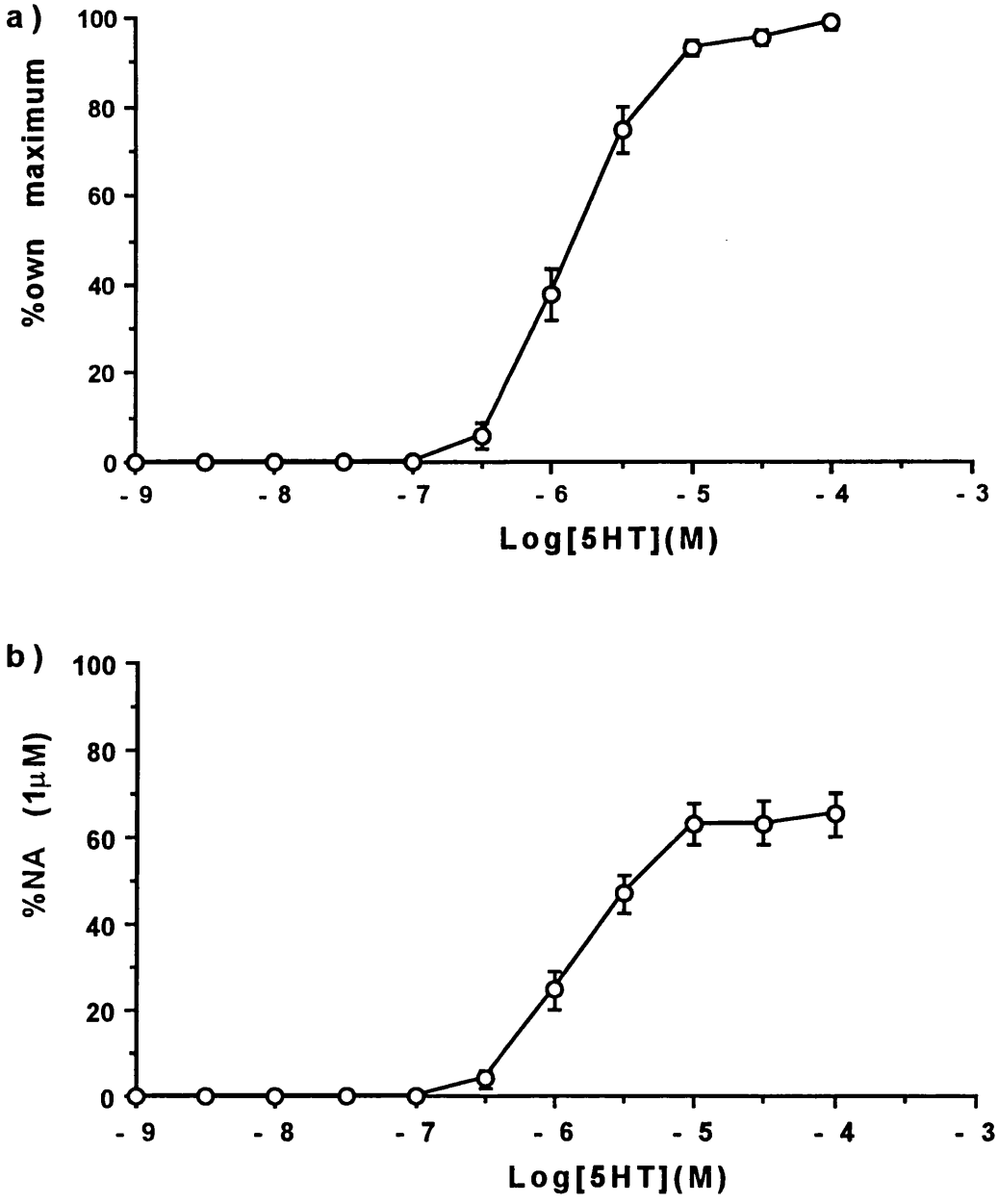


Figure 2.22

Cumulative concentration-response curves to 5HT (○) in the rat isolated common carotid artery . Results are expressed as: **a**) % of the maximum response of CCRC to agonist or **b**) % of the maximum response to NA (1μM) in the first of experiment.

Each point represents the mean ± s.e.mean (n=5).

Section 4: Discussion

Our study demonstrates that in the rat common carotid artery: i) the population of postjunctional α-adrenoceptors mediating contraction of smooth muscle is α_1. ii) the subtype of α_1-adrenoceptor mediating contraction is non-α_{1A}-, non α_{1B}-adrenoceptor. iii) UK-14304 is an α_1-adrenoceptor partial agonist.

2.4.1 Contractions to α-adrenoceptor agonists are mediated by α_1-adrenoceptors

It has been difficult to demonstrate contraction to postjunctional α_2-adrenoceptors in isolated large arteries. The present study clearly shows dominance of α_1-adrenoceptors. Although there are a few old reports of the presence of α_1 -and α_2-adrenoceptors in arterial preparations and α_2-adrenoceptors in the veins (Ruffolo, 1986), our finding of α_1-adrenoceptors in this large artery is consistent with most of the intervening literature.

It has long been known that phenylephrine is a relatively selective α_1-adrenoceptor agonist c.f. α_2 (Starke *et al.*, 1975) and that prazosin is a highly selective α_1-adrenoceptor antagonist with a more than 250 times higher affinity for the α_1-adrenoceptor than for the α_2-adrenoceptor (McGrath & Daly, 1995). In this study contractions to NA and PE were relatively sensitive to low concentrations of prazosin (1nM). Responses to NA were insensitive to the selective α_2 antagonist rauwolscine. In high concentration rauwolscine (1μM) caused only a small shift to the right of the NA CRC.

UK-14304 is a selective α_2-adrenoceptor agonist in a variety of preparations (Cambridge, 1981). UK-14304 produced a concentration-dependent contraction with a maximum 41.3 ± 4.47% of NA (1μM). In high concentration rauwolscine (1μM) caused only a small shift on responses mediated by UK-14304.

2.4.2 Subtype of α_1-adrenoceptor mediating contraction is non-α_{1A}-, non α_{1B}-adrenoceptor

Affinity constants (pK_B) for prazosin in various tissues can be roughly subdivided into two groups. One with high pK_B values close to 10 (α_{1H}), another with lower values less than 9, α_{1L}(Muramatsu *et al*,.1990). Since the pA_2 value for prazosin in this preparation is 10.05, this indicates α_{1H} subtype.

The α_{1A}-adrenoceptor subtype shows high affinity for WB-4101 and 5-methylurapidil and is relatively insensitive to the alkylating agent, chloroethylclonidine, while the α_{1B}-adrenoceptor subtype has low affinity for the antagonists mentioned above and is potently inactivated by CEC. Both α_{1A} - and α_{1B}-adrenoceptor subtypes have a high affinity for non-subtype selective antagonist prazosin (Han *et al.*, 1987a). 5-methylurapidil is highly selective (50- to 100-fold) but WB-4101 show less selectivity (10- to 20-fold) for α_{1A}-adrenoceptor subtype over α_{1B} (Hanft & Gross, 1989).

In our study in addition to prazosin, all α_1-antagonists that were used (5-MU, WB-4101, HV-723, YM-12617 and BMY-7378) showed high potency. The slope of Schild plots for prazosin was near to unity.

The S(+)-isomer of the dihydropyridine-type Ca^{2+} channel antagonist, niguldipine is the most selective ligand available for the α_{1A}-adrenoceptor subtype. It is at least 50- to 100-fold selective for the α_{1A}-adrenoceptor over α_{1B}-adrenoceptor subtype (Boer *et al.*, 1989). Niguldipine produced a shift of the concentration-response curve of NA to the right and reduced the maximum response (50%). The shift in potency of NA produced by a high concentration of niguldipine (1μM) was 4.07 times.

The α_{1A}-adrenoceptor subtype is considered insensitive to the alkylating agent, chloroethylclonidine which is a reactive derivative of clonidine, but in this study

CEC produced a parallel shift in the potency of NA. Together these observations suggest that contractions of the rat common carotid artery are not mediated by α_{1A}. In rat vas deferens, the competitive antagonists prazosin, WB-4101 and 5MU inhibited contractions to NA with pA_2 values of 9.26, 9.54, and 8.43 respectively. CEC (100μM) failed to affect contractions to NA. It was concluded that contractions of rat vas deferens are mediated by α_{1A}-adrenoceptors as shown by the high potency of α_{1A}-adrenoceptor selective antagonists and the lack of effect of CEC (Aboud *et al.*, 1993).

CEC has been classified as a selective, irreversible α_{1B}-adrenoceptor antagonist (Minneman, 1988) and has been used to subdivide α_1-adrenoceptor subtypes. CEC did not reduce the maximum response to NA, but produced a parallel shift in the potency of NA. While the α_{1B}-adrenoceptor subtype has low affinity for the WB-4101 and 5MU, it is potently inactivated by CEC. In rat spleen, the competitive antagonists prazosin, WB-4101 and 5MU inhibited contractions to PE with pA2 values of 9.56, 8.85, and 6.62 respectively. CEC (100μM) significantly reduced the maximum contraction to PE. Contractions of rat spleen are mediated by α_{1B}-adrenoceptors since α_{1A}-selective antagonists showed low potency and CEC significantly reduced the maximum response to PE (Aboud *et al.*, 1993).

The cloned α_{1D}-adrenoceptor, characterised by its low affinity for 5-methylurapidil, oxymetazoline and (+)-niguldipine and its relative resistance to alkylation by CEC, is without a clear-cut functional correlate (Ford *et al.*, 1994). BMY-7378, is a selective α_{1D}-adrenoceptor antagonist (Saussy *et al.*, 1994).

In the classical α_1-adrenoceptor preparation, rat aorta, the potencies of WB-4101 and 5-MU against contractions to noradrenaline in rat aorta were 9.21 and 8.12 respectively. CEC (100μM) shifted potency of CCRC to NA 26.1 times. CEC did not reduce the maximum response and the shift in potency of NA was parallel. They concluded that the subtype of α_1-adrenoceptor have may represent

functional α_{1D} (Aboud *et al.*, 1993). Affinity estimates for α_1-adrenoceptor ligands prazosin, WB-4101, 5-MU and S(+)-niguldipine were 9.9, 9.6, 8 and 7.1 respectively at rat cloned α_{1d}-adrenoceptor (Clarke *et al.*, 1995).

In this study the selective α_{1D}-adrenoceptor antagonist BMY-7378 produced parallel shift of concentration-response curve to NA with a pA_2 value of 9.24, very near to the value obtained in rat aorta. In functional assays using rat aorta, the phenylephrine-induced increase in tension was inhibited in a competitive manner by selective α_{1D}-adrenoceptor antagonist BMY-7378 with a pA_2 value of 9.1 (Saussy *et al.*, 1994).

Comparison of pA_2 values for the antagonists with their published pK_i on cloned subtypes (Burt *et al.*, 1995).

	pK_i on cloned α_1-adrenoceptors expressed in cells			
antagonist	α_{1b}	α_{1a}	α_{1d}	**rat carotid artery**
prazosin	9.6 ± 0.2	9.2 ± 0.2	9.4 ± 0.2	10.05
WB-4101	8.2 ± 0.1	9.5 ± 0.3	9.2 ± 0.1	10.7
5-MU	6.8 ± 0.3	8.8 ± 0.1	7.3 ± 0.3	9.1
BMY-7378	7.2	6.6	9.4	9.24

Taken together, these data suggest that contractions of the rat common carotid artery are mediated by non-α_{1A}, non-α_{1B}-adrenoceptors, due to the high potency of the α_{1A}-selective antagonists and sensitivity to CEC. The pA_2 values correlated best with the published affinities of these compounds on the expressed

α_{1d}-adrenoceptor clone and poorly with those at either the expressed α_{1b}- or α_{1a}-adrenoceptor clones.

Functional potencies on rat aorta and common carotid artery (pA_2/pK_B) and binding affinities (pK_i) to cloned α_{1d} adrenoceptors also suggest α_{1D} for rat carotid artery.

antagonist	**α_{1d} binding**	**rat aorta**	**rat carotid artery**
prazosin	9.5 ± 0.1	9.8 ± 0.2	10.05
WB-4101	9.2 ± 0.06	8.9 ± .08	10.7
5-MU	7.8 ± 0.03	7.8 ± 0.19	9.1
BMY-7378	8.2 ± 0.1	8.3 ± 0.1	9.24

The effect of BMY-7378 suggests a functional α_{1D}-adrenoceptor such as was reported in rat aorta (Perez *et al.,* 1991; Saussy *et al.,* 1994), perhaps with the presence of at higher concentrations of noradrenaline of a receptor with relatively low affinity for the antagonists.

2.4.3 Analysis of the vasorelaxation produced by UK-14304

It is well-known that stimulation of postsynaptic α_2-adrenoceptors can initiate not only a vasoconstriction (Van Meel *et al.*, 1981; McGrath, 1982) but also a relaxation (Cocks & Angus, 1983; Miller & Vanhoutte, 1985; Angus *et al.*, 1986). In the present study, our initial observations showed that UK-14304 induced relaxation in isolated rat common carotid artery preconstricted by NA or PE which would have been consistent with α_2-adrenoceptors-mediated stimulation of endothelial cells leading to a relaxation of the artery. However UK-14304

-induced relaxation was not influenced by the absence of the endothelium or inclusion of L-NAME (100μM).

It quickly became apparent that a UK-14304-induced relaxation of NA-induced tone was independent of endothelium and due to its occupation of some α_1-adrenoceptors. However a number of possible α_1-mediated actions also has to be excluded since we are seeking as complete as possible an analysis of α-adrenoceptor-mediated actions in this vessel.

Furthermore UK-14304-induced relaxation was not inhibited by the selective α_2-adrenoceptor antagonist rauwolscine. Rauwolscine has been shown to be a selective α_2-antagonist (Weitzell *et al.*, 1979) and has been used widely as an α_2-antagonist in assessing α_1- and α_2-adrenoceptor-mediated contractile functions and adrenoceptor properties in vascular smooth muscle (McGrath, 1982). The present study indicates that UK-14304 induced relaxation is not mediated by activation of α_2-adrenoceptors in the rat common carotid artery.

UK-14304-induced relaxation was not modified by flurbiprofen (1μM) which inhibits cyclo-oxygenase. Therefore relaxation does not involve PGI_2.

Since propranolol (1μM) was always present in the Krebs, UK-14304-induced relaxation was not mediated by β-adrenoceptors.

In preparations preconstricted by $PGF_{2\alpha}$ or U-46619, UK-14304 did not induce any relaxation but rather in high concentration produced contraction. When tone was raised with U-46619 (0.1μM), the specific thromboxane A_2 receptor antagonist, GR-32191B produced relaxation.

In the present study UK-14304 dose-dependently induced relaxation in preparations preconstricted by NA or PE but not in those preconstricted by $PGF_{2\alpha}$ or U46619. This suggests that UK-14304 acts by antagonism of α_1-adrenoceptors. Known antagonists produce similar effects. Both prazosin and

rauwolscine dose-dependently induced relaxation in preparations preconstricted by PE or NA. Rauwolscine like prazosin produced relaxation, but in high concentrations, consistent with blocking of α_1-adrenoceptors. The selective α_2-adrenoceptor antagonist delequamine (Brown *et al.,* 1993) produced a small relaxation compared to rauwolscine, consistent with its greater selectivity for α_2-adrenoceptors.

Concentration-dependent relaxation versus phenylephrine (1μM) yielded a pIC_{50} for of UK-41304 4.91 ± 0.14, for prazosin 8.14 ± 0.21 and for rauwolscine versus NA was 5.76 ± 0.13.

When tested as an antagonist UK-14304 produced parallel shifts of concentration-response curves to PE. Slope of the Schild plot was different from unity so purely competitive antagonism can not be established. However, since UK-14304 does produce contraction, evidence for competitive antagonism may be unrealistic.

These results with prazosin, rauwolscine and GR-32191B clearly show when tone is induced by an agonist, antagonist activity of a compound can produce relaxation by antagonism of receptors occupied with agonist. Therefore, it is likely that UK-14304-induced relaxation in preparations preconstricted by NA or PE is due to its antagonistic action against activated α_1-adrenoceptors. Previously, α_2-adrenoceptor agonists have been reported to behave as antagonists at α_1-adrenoceptors in the isolated and perfused hindquarter of the rat (Kobinger & Pichler, 1983) and in rabbit aorta (Lues & Schumann, 1984).

There was no evidence from our study that α_2-adrenoceptors can mediate the release of EDRF in rat carotid artery. This is consistent with attempts to induce endothelium-dependent relaxation in rat aorta after blockade of α_1-adrenoceptors by prazosin, in which clonidine failed to cause relaxation (Godfraind *et al.*, 1985; Martin *et al.*, 1986). Furthermore, no α_2 binding sites could be detected on the rat

aortic smooth muscle or its endothelium by use of [^{3}H]-rauwolscine binding combined with autoradiography (Jacobs & Dashwood, 1986).

2.4.4 Influence of endothelium removal and L-NAME

The contribution of NO to the regulation of vascular tone in rat common carotid artery has been demonstrated by showing that inhibition of its synthesis by L-NAME results in significant vasoconstriction. Also, L-NAME prevented the relaxation of rat carotid artery by endothelium-dependent vasodilator acetylcholine. As reported by Rees and co-workers (1989) inhibition of NO can induce an endothelium-dependent and enantiomerically specific contraction of the vascular rings, confirming that there is a continuous use of L-arginine for the basal release of NO. The magnitude of this basal release can be determined indirectly by measuring the degree of contraction. In our study L-arginine (300μM), which is a substrate for EDRF (Palmer *et al.,* 1988), totally reversed the effects of L-NAME. L-arginine had no effect on its own (unpublished data).

Mechanical disruption of the vascular endothelium (to an extent which prevented vasodilation by an agonist acting via the endothelium-derived nitric oxide system) reduced, but did not abolish the ability of L-NAME to produce contraction. This suggests an extra-endothelial site for nitric oxide synthesis in rat common carotid artery. One site could be the vascular smooth muscle cell, as already suggested for rat ileac artery and rabbit pulmonary artery (Gonzalez *et al.,* 1992; Maclean *et al.,* 1993b). In rabbit pulmonary artery removal of the vascular endothelium increased the maximum contractile responses to electrical field-stimulation (EFS) but did not inhibit the ability of L-NAME to potentiate contractile responses to EFS. Other sites of synthesis could be cells in the adventitia.

In the present study, the effect of UK-14304 was significantly enhanced in the presence of L-NAME. Mechanical disruption of the vascular endothelium mimicked the effect of L-NAME on contractile responses to UK-14304 and PE consistent with L-NAME inhibiting endothelium-derived nitric oxide synthase. In agreement with this study, Joly and co-workers (1992) found that removal of the endothelium either by balloon catheter or mechanically by a small metal wire in rat carotid artery results in an increase of the contractions evoked by phenylephrine.

The results show that inhibition of nitric oxide synthase with L-NAME potentiates responses to PE and UK-14304 in rat common carotid artery. In the case of NA the result was less clear out. Although L-NAME did not increase sensitivity to NA, mechanical disruption increases potency of NA without effect on maximum response. The vascular endothelium produces and releases many other factors such as the relaxant prostacyclin (Moncada *et al.*, 1978) and contractile factor endothelin (Yanagisawa *et al.*, 1988). As the response to NA was augmented by endothelium disruption, but not by L-NAME this could imply an endothelial cell derived vasoactive factors other than NO also exert effects on the adrenoceptor activation.

The endothelium-mediated depression of the responses is likely to be due to the basal release of endothelium-derived nitric oxide by the endothelial cells. It was concluded that constitutive NO activity has substantial inhibitory influence on vasoconstrictor responses to PE and UK-14304 but not to NA. This effect of L-NAME on NA is in contrast to other reports in some other vessels (Thompson & Weiner, 1993; Maclean *et al.*, 1993a). The link between L-NAME and noradrenaline-induced contraction is unexplained and requires further investigation. The reason for this discrepancy is unclear.

Quantitative differences in the effectiveness of endothelium removal and NO-inhibition in various isolated blood vessels on α-adrenoceptors can arise from a variety of factors, e.g. a concomitant stimulatory action of these factors on the smooth muscle, the release of different endothelium-dependent vasoconstrictor substances and the release of different endothelium-dependent vasodilator substances.

2.4.5 Effects of contractile agents on responses to UK-14304

Attempts to potentiate responses to UK-14304 with synergistic agents showed: i) KCl increased responses significantly; ii) AII increased responses though less than KCl; iii) inducing tone with PE, U-46619 and 5HT had no effect on responses to UK-14304.

Since the original demonstration of postjunctional α_2-adrenoceptors in the pithed rat (Drew & Whiting, 1979; Docherty *et al.*, 1979), identification of this subtype in isolated vascular preparations, (i.e.) responding to NA and resistant to prazosin or a suitable α_1-adrenoceptor antagonist, has proved very difficult. It has been reported that activation with a physiological stimulant, namely AII, reveals a quiescent population of α_2-adrenoceptors in the saphenous artery although the response was mediated entirely by α_1-adrenoceptors in the absence of AII. This is associated with a marked increase in the sensitivity of the preparation (up to 300 fold) to the α_2-adrenoceptor agonist UK-14304 (Dunn *et al.*, 1989). Similarly, in the perfused isolated tail of the rat α_2-adrenoceptor-mediated responses are observed only after raising tone with, for example vasopressin or endothelin (Templeton *et al.*, 1989; Maclean & McGrath, 1990). Similarly, phenylephrine-induced tone has been shown to uncover responses to B-HT920 in the canine portal vein (Furuta, 1988). Furthermore, α_2-adrenoceptor-mediated

responses to BHT-920 in the plantaris artery of the dog are only apparent in the presence of Bay K-8644 (Sulpizio & Hieble, 1987).

In the present study activation of isolated common carotid artery with AII (10nM), caused a small but significant increase in the sensitivity of the preparation to UK-14304. However, analysis using antagonists showed that responses to UK-14304 after potentiation by L-NAME or angiotensin were prazosin-sensitive, rauwolscine-resistant, and therefore by definition α_1-adrenoceptor-mediated. This is in contrast to earlier studies in which inducing tone with phenylephrine or endothelin introduced rauwolscine-sensitive responses to UK-14304 in the isolated vascular bed of rat tail (Templeton *et al.*, 1989; Maclean & McGrath, 1990). In the saphenous artery under normal circumstances stimulation of postjunctional α_1-adrenoceptors provide the positive input required for the expression of postjunctional α_2-adrenoceptors thereby rendering all responses to α_1-adrenoceptor agonists possessing any α_1 efficacy sensitive to prazosin. The facilitatory action of AII on postjunctional α_2-adrenoceptor-mediated responses is apparent only if α_1-adrenoceptors are blocked by prazosin or phenoxybenzamine (Dunn *et al.*, 1991a). In this investigation potentiation effect of AII on UK-14304-mediated responses in the presence of prazosin was rauwolscine-resistant, consist out with α_1-adrenoceptor activation. In conclusion with regard to potentiation by prior exposure to AII or L-NAME, it is theoretically possible that UK-14304 exerts its actions through combined α_1 and α_2 activation, but the effectiveness of prazosin and the ineffectiveness of rauwolscine except in non-selective concentrations makes the simplest explanation that it is working entirely via α_1.

Inducing tone with PE, U-46619 or 5HT had no effect on responses to UK-14304. However KCl increased responses. The simplest explanation for the potentiation of the response to UK-14304 is the of slight depolarisation induced by KCl affects the receptor-response coupling of UK-14304, as suggested by Lues and

Schumann (1984) in potentiation of BHT-920 in rabbit aorta. The potentiating effect of KCl is consistent with this possibility. In agreement with this possibility Hondeghem and colleagues (1986) showed that partial depolarisation by KCl potentiated noradrenaline-induced contraction in the rabbit aorta and porcine coronary artery. The effect of angiotensin can be explained by assuming that angiotensin acts on a common pathway linked to different receptors which is part of the excitation-contraction coupling mechanism. These results have parallels with previous results showing that a stimulating factor (the presence of a stimulating agent e.g. AII or inhibition of NO or partial depolarisation by KCl) is necessary for expression of the contractile responses to α_2-adrenoceptor agonist.

CHAPTER 3

Subtypes of α1-Adrenoceptors in Rabbit Saphenous Vein

3.1 Introduction

The rabbit lateral saphenous vein has a mixed functional population of α_1 and α_2-adrenoceptors (Purdy *et al.*,1980; Schumann & Lues, 1983). It represent a tissue which has a mixed population of postjunctional α-adrenoceptors where α_2-adrenoceptors dominate to a greater extent (Daly *et al.*, 1988a).

In carotid artery where only α_1-adrenoceptors are present, we classified α_1-adrenoceptor subtypes using selective α_1-adrenoceptor agonists and antagonists. We chose saphenous vein to characterise the α_1-adrenoceptor subtypes which co-exist with α_2-adrenoceptors in this tissue. It is possible that different selective α_1-adrenoceptor agonists and antagonists interact with α_2-adrenoceptors. The aim of this study is to examine and interpret pharmacological subclassification of α_1-adrenoceptors proposed by Muramatsu and co-workers (1990) in this atypical preparation that has both postjunctional α_1 and α_2-adrenoceptors.

Methods

Vessel isolation and preparation are as described in section 4.2.2.

Results

3.2 Potency of PE

PE produced concentration-dependent contractions in the isolated lateral saphenous vein. PE produced isometric contraction with a pD_2 value of 5.84 and a maximum contraction of 3.36 ± 0.15g.

Consecutive CCRC's to PE were not reproducible, there being a significant increase in the maximum response with time (Figure 3.1)

3.3 Effects of α-adrenoceptor antagonists

Prazosin produced concentration dependent rightward displacement of PE CCRC (Figure 3.2a). The pA_2 value for prazosin was 8 and the slope of Schild plot was close to unity (0.98), indicating competitive antagonism (Table 3.1).

HV-723 produced parallel shifts of concentration-response curve to PE (Figure 3.2b). Schild regression analysis yielded line with pA_2 value of 7.97 and slope of 0.61, different from unity, indicating non-competitive antagonism (Table 3.1).

5MU produced concentration-dependent shifts in the potency of PE without reducing the maximum response (Figure 3.3a). The pA_2 value for 5MU was 7.2 and slope of the Schild plot was 0.81, different from unity, indicating non-competitive antagonism (Table 3.1) .

Figure 3.3b demonstrates the effect of various concentration of the selective α_1-adrenoceptor antagonist WB-4101 on responses to PE. The pA_2 value for WB-4101 was 8.6 and slope of the Schild plot was different from unity (0.74), indicating non-competitive antagonism (Table 3.1).

The irreversible antagonist chloroethylclonidine (100μM) that preferentially alkylates α_{1B}-adrenoceptors, failed to reduce significantly the maximum response to PE but produced small shifts in the potency of PE. The tissues were treated with CEC (100μM) for 30 minutes followed by washout of the irreversible antagonist for an additional 30-45 minutes. The shift in potency of PE produced by CEC (100μM) was 12.8 times (Figure 3.4a).

α_2-adrenoceptor antagonist delequamine produced shift of concentration-response curve to PE (Figure 3.4b). The pA_2 value was 8.3 and slope of the Schild plot was different from unity (0.7), indicating non-competitive antagonism (Table 3.1).

The rank order of potencies for these α-adrenoceptor antagonists were as follows: WB-4101>delequamine>prazosin >HV-723>5MU (Table 3.1).

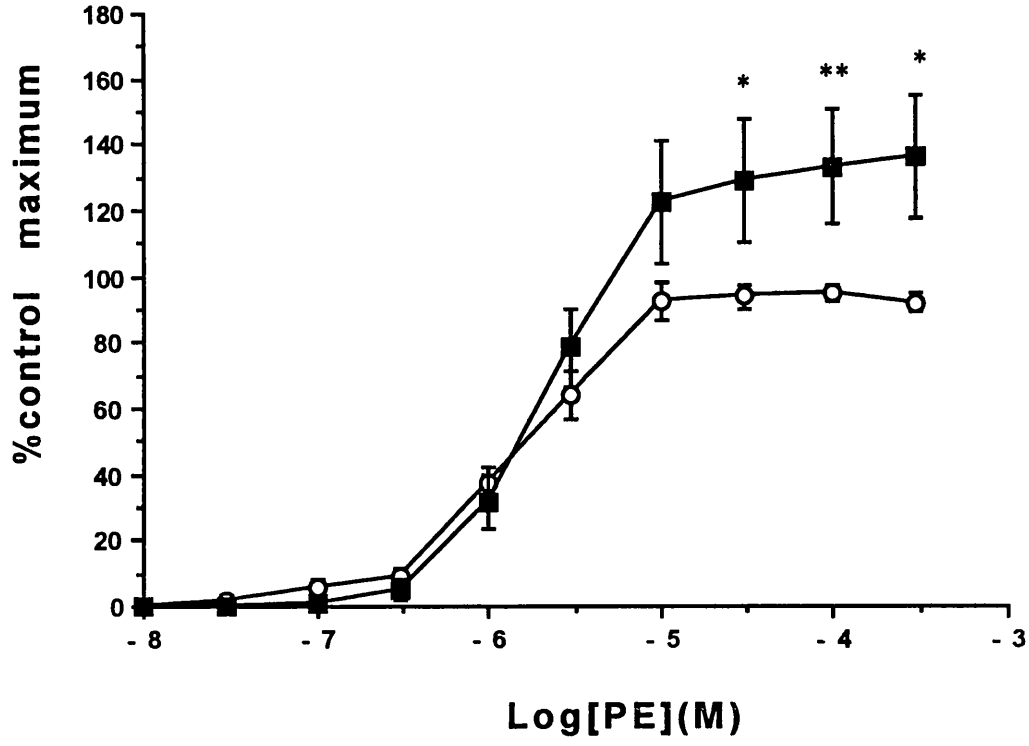


Figure 3.1

Effect of time on α-adrenoceptor agonist PE cumulative dose response curve. The first CCRC to agonist is represented by (○) while second CCRC to agonist represented by (■). Results are expressed as % of the maximum response of the first control CCRC to PE. Statistically significant differences are represented by * $p<0.05$, ** $0.001<p<0.01$, paired Student's t-test.

Each point represents mean ± s.e.mean (n=6).

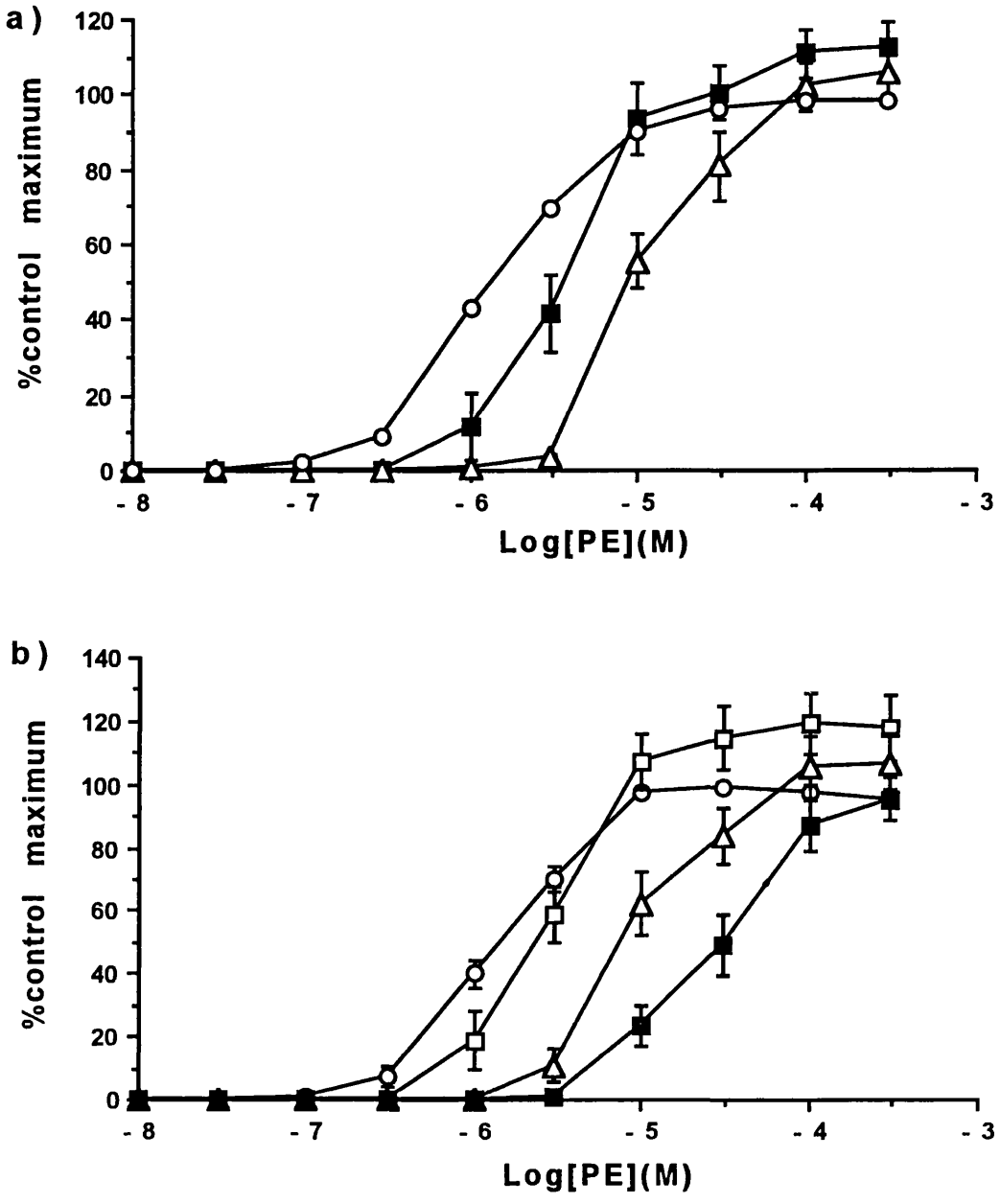


Figure 3.2

Effects of α_1-adrenoceptor antagonists a) prazosin 0.01μM (■) or 0.1μM (△) b) HV-723 0.01μM (□), 0.1μM (△) or 1μM (■) on responses to PE (○) in the rabbit isolated lateral saphenous vein. Results are expressed as % of the maximum response of the control CCRC in the absence of antagonist.

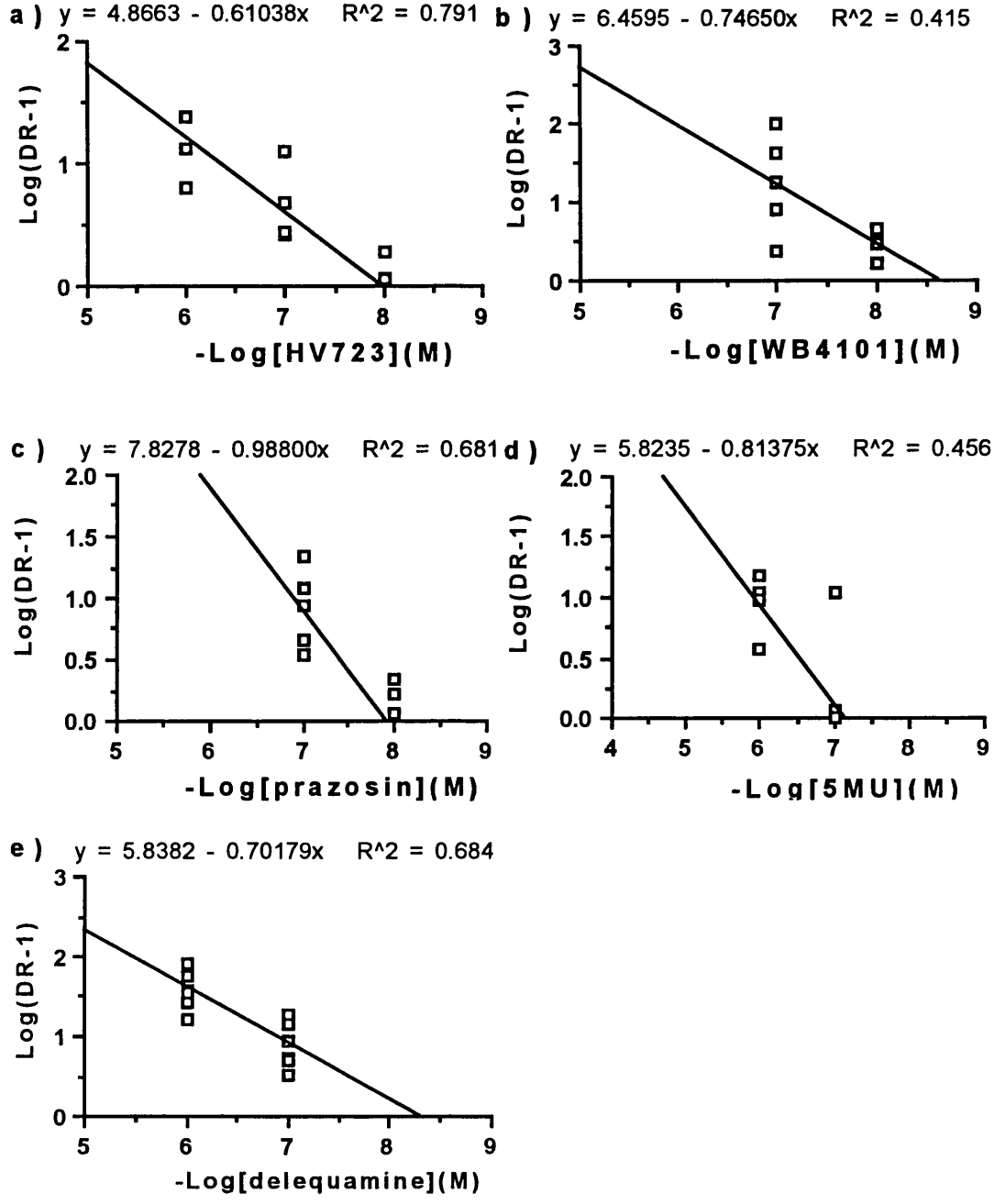
Each point represents mean ± s.e.meam (n=6).

Table 3.2.1

antagonist	pA_2	slope
prazosin	8 (7.6-8.95)	0.98 (0.93-1.1)
5MU	7.2 (6.77-8.85)	0.81 (0.74-0.95)
WB-4101	8.6 (7.93-9.37)	0.74 (0.63-0.91)
HV-723	7.97 (7.75-8.19)	0.61 (0.502-0.706)
delequamine	8.31 (7.94-8.69)	0.71 (0.56-0.83)

List of pA_2 values with the slopes of the Schild plots (With 95% confidence limits) for α-adrenoceptor antagonists against responses to phenylephrine in the rabbit isolated lateral saphenous vein.

pA_2 values were determined from a regression analysis of the logarithm of dose ratio-1 against the negative logarithm of the molar concentration of the antagonist.



pA_2 values obtained for the α-adrenoceptor antagonists using Schild analysis by plotting log (DR-1) on the y-axis against the log M [antagonist] on the x-axis and fitting using linear regression. α_1-adrenoceptor antagonists **a**) HV723 **b**) WB4101 **c**) prazosin and **d**) 5-MU and α_2-adrenoceptor antagonist **e**) delequamine aganist contractions to phenylephrine in the rabbit isolated lateral saphenous vein.

Each point represents an individual experiment.

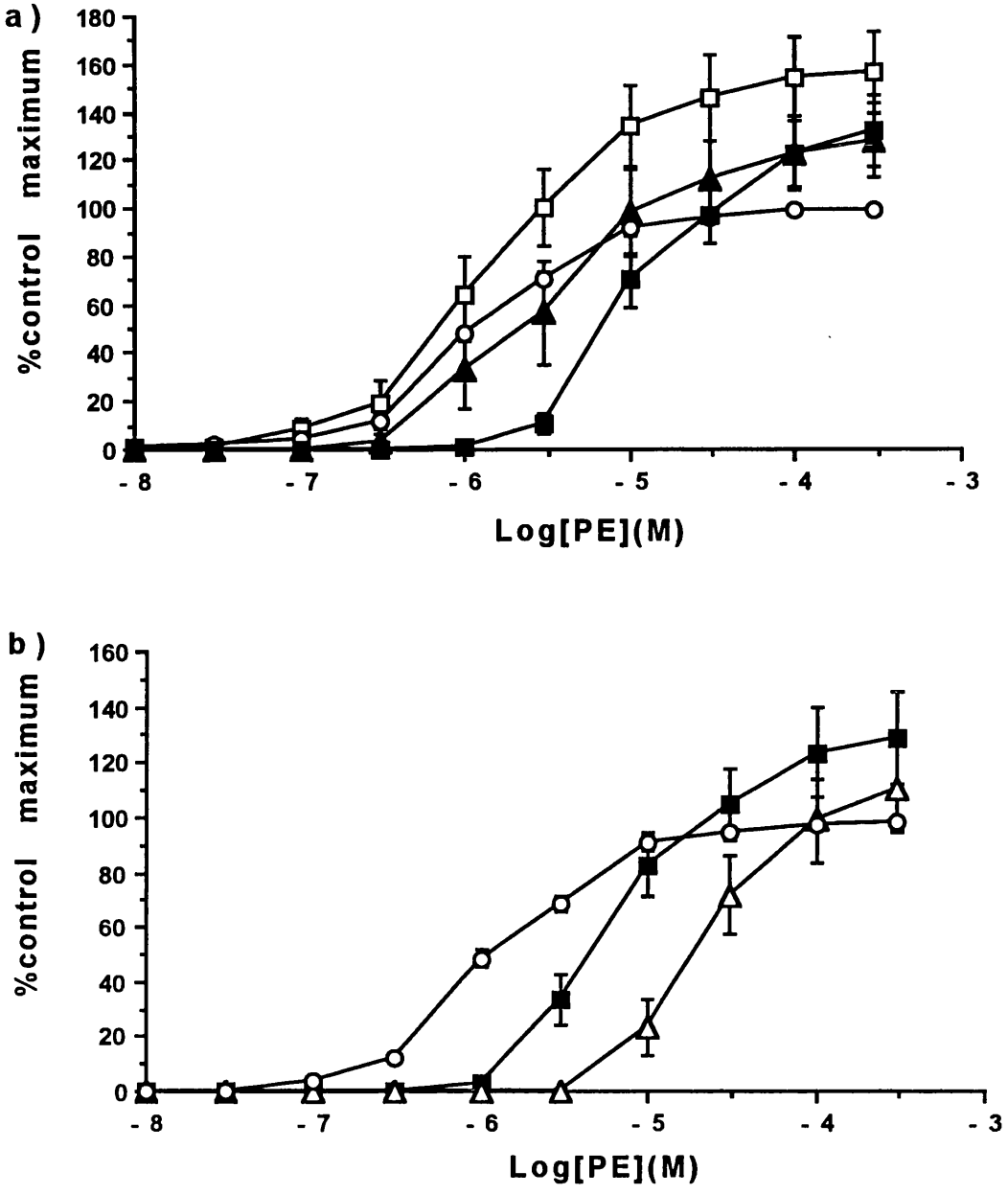


Figure 3.3

Effects of α_1-adrenoceptor antagonists **a)** 5-methylurapidil 0.01μM (□), 0.1μM (▲) or 1μM (■) **b)** WB-4101 0.01μM (■) or 0.1μM (△) on control responses to PE (○) in the rabbit isolated lateral saphenous vein. Results are expressed as % of the maximum response of the control CCRC in the absence of antagonist.

Each point represents mean ± s.e.meam (n=6).

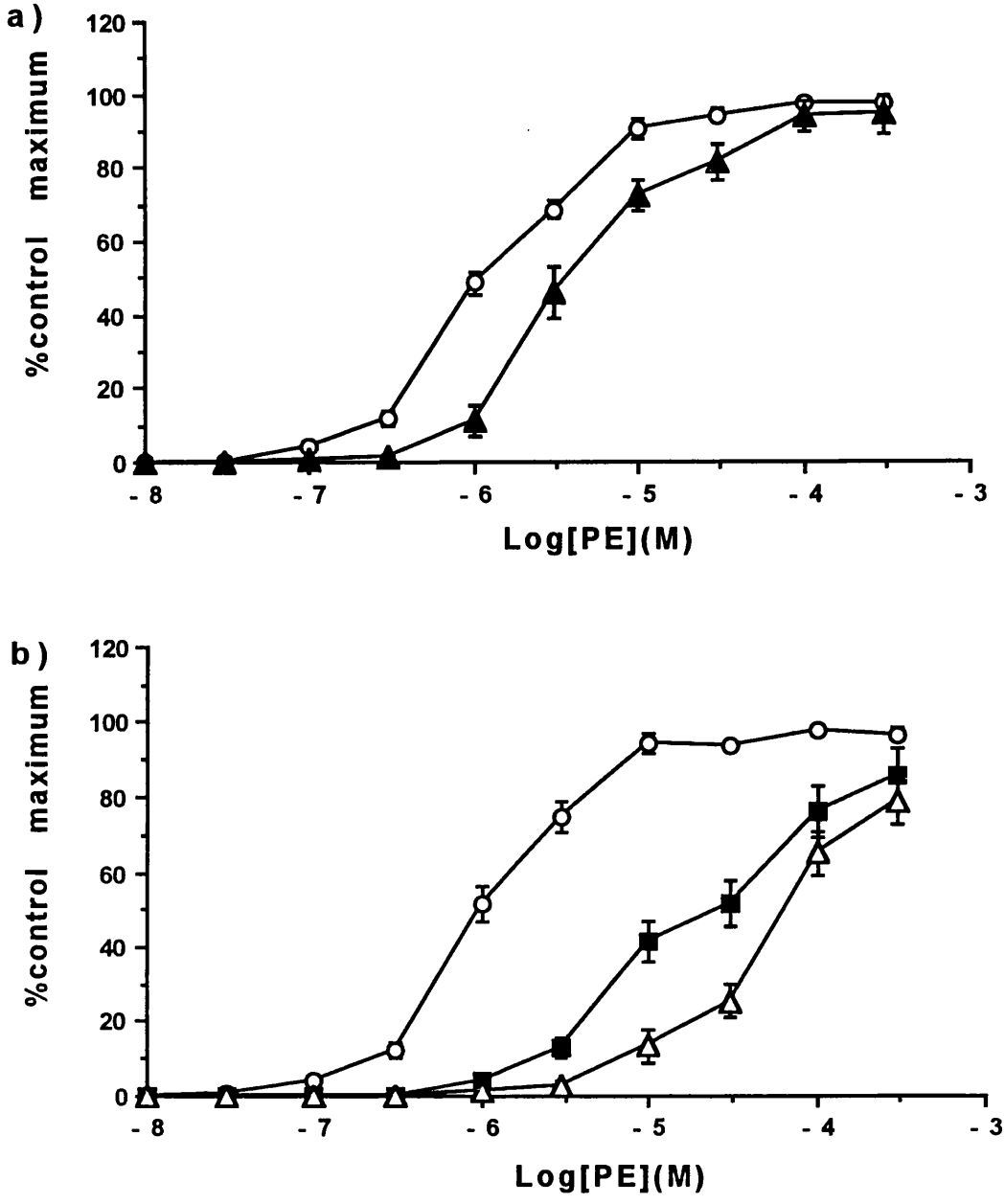


Figure 3.4

Effects of **a)** treatment with chloroethylclonidine (▲); tissues were treated with 100μM for 30 minutes followed by washout for an additional 30-45 minutes **b)** selective α_2-adrenoceptor antagonist delequamine 0.1μM (■) or 1μM (△) on control response to PE (○) in the rabbit isolated lateral saphenous vein. Results are expressed as % of the maximum response of the first control CCRC to PE in the absence of CEC or delequamine.

Each point represents mean ± s.e.mean (n=6).

Discussion

Although phenylephrine is considered to be a selective α_1-adrenoceptor agonist (Starke *et al.,* 1975) and has been used to activate the postjunctional α_1-adrenoceptors, the potency of phenylephrine in the rabbit saphenous vein is low (with pD_2 value of 5.84) and it is possible that phenylephrine mediates contraction by activation of α_2-adrenoceptors. A difficulty is that postjunctional α_1 and α_2-adrenoceptors do not co-exist in a simple manner and they interact at the level of a common post-receptor site in the events leading to contraction. Schumann and Lues (1983) reported that phenylephrine responses were insensitive to prazosin but sensitive to rauwolscine in the rabbit saphenous vein. In another report rauwolscine competitively antagonised the contractile responses to phenylephrine in the rabbit saphenous vein with pA_2 of 7.16 (Alabaster *et al.,* 1985).

In the current study prazosin competitively inhibited contractile responses to phenylephrine with a pA_2 value of 8. The selective α_2-adrenoceptor antagonist delequamine (Brown *et al.,* 1993) inhibited phenylephrine-induced contractions. The pA_2 and slope were 8.3 and 0.7 respectively. If it is accepted that delequamine is highly selective for α_2-adrenoceptors then this would be consistent with phenylephrine contraction being mediated by α_1- and α_2-adrenoceptors and corollary is that its responses can be attenuated by antagonists of either receptor type.

In dog saphenous vein concentration-dependent contractile response curves obtained to selective α_2-adrenoceptor agonist BHT-920 were progressively displaced to the right of controls by delequamine. Schild analysis of these data gave a pA_2 of 10 with slope of 0.85. Pretreatment of the tissues with phenoxybenzamine at a concentration (10nM) which irreversibly inactivates the α_1-adrenoceptors did not modify the antagonist effects of delequamine. In

rabbit aorta delequamine was a weak antagonist of phenylephrine-induced contractions. pA_2 was 6.05 with slope of 0.9 (Brown *et al.,* 1993). Also in the present study in preconstricted preparations of rat carotid artery induced by NA delequamine produced weak relaxation of NA-induced contractions compared with rauwolscine. Thus delequamine does appear to be highly selective for α_2.

3.3.2 Subtype of α_1-adrenoceptor mediating contraction is consistent with α_{1L}

In this preparation there is evidence for phenylephrine-induced contraction being mediated by α_1- and α_2-adrenoceptors and synergistic effect of α_1- and α_2-adrenoceptors. The relatively low absolute pA_2 values for prazosin, in rabbit saphenous vein is consistent with blockade of the α_{1L} type as defined by Muramatsu and co-workers (1990). An alternative explanation is that all antagonists appear to have low potency due to the synergism between α_1- and α_2-adrenoceptors but the α_{1L} hypothesis will be discussed further.

The α_{1L}-adrenoceptors are also less sensitive to WB-4101 and 5-MU (pK_B: approximately 8) and are relatively resistant to CEC. HV-723 (Muramatsu *et al.,* 1990) can subdivide the α_{1L}-adrenoceptor into two subtypes: α_{1L} (HV723-low, <1nM) and α_{1N} (neither α_{1H} or α_{1L}) (HV723-high). In this study pA_2 of HV-723 was 7.97 that is less than 9. Therefore the subtype of α_1-adrenoceptor mediating contraction in this preparation can be interpreted as the α_{1L} type. Lack of inhibition of phenylephrine-induced contraction by CEC supports that the response is mediated by a subtype other than α_{1B} or α_{1D} subtypes. The same pattern was seen when α_1-adrenoceptor mediated contraction of the guinea-pig ileum by phenylephrine was examined. Phenylephrine-mediated contraction was not affected by treatment with CEC. In addition both WB-4101 and 5-MU antagonised the α_1-adrenoceptor mediated contraction with low affinity (Abel *et al.,* 1995).

In the present investigation, prazosin, WB-4101, 5-MU and HV-723 antagonised the phenylephrine-mediated contraction with lower affinity than we expected for α_1-adrenoceptors. In the classical α_1-adrenoceptor preparation, rat aorta, WB-4101 and 5-MU competitively antagonised contractions to NA. The pA_2 values were 9.21 and 8.12 respectively (Aboud *et al.*,1993). HV-723 competitively inhibited the contractile responses induced by phenylephrine in rabbit thoracic aorta and rat aorta. The pA_2 values were 8.71 and 9.21 respectively (Muramatsu *et al.*, 1990). Although they are low for α_2, the affinities of these antagonists are much higher than we would expect for α_2-adrenoceptors. This indicates that in this preparation the relative resistance of responses to PE shown by the selective α_1-adrenoceptor antagonists may be because PE interacts not only with the α_1-adrenoceptors. However the data can support the presence of the prazosin-low affinity sites (α_{1L}-adrenoceptors) which have also been detected in many other tissues: human, dog and rabbit prostates, human coronary vein, rat vas deferens, rat anococcygeus muscle, rat portal vein, dog femoral artery and vein (Muramatsu *et al.*, 1995). The α_{1L}-adrenoceptor is found in vascular smooth muscle (the thoracic aorta) of the guinea-pig and is insensitive to inactivation by CEC. In addition to having a low affinity for prazosin (>1nM), they also have a relatively low affinity (1-10nM) for WB-4101 (Abel *et al.*, 1995).

The present data may fit with the α_{1L} subtype within the α_{1H}, α_{1L} and α_{1N} subclassification proposed by Muramatsu and co-workers (1990), although this subtype has not yet been identified by molecular cloning techniques. In conclusion, our study demonstrates that in the rabbit isolated lateral saphenous vein: **i)** subtype of α_1-adrenoceptor mediating contraction is α_{1L}. **ii)** contraction induced by the selective α_1-adrenoceptor agonist phenylephrine is mediated via α_1- and α_2-adrenoceptors.

CHAPTER 4

Investigation of the Rabbit Coronary Ligation Model of Heart Failure

Section 1: Introduction

4.1.1 Introduction to heart failure

Chronic heart failure is a clinical syndrome characterised by the inability of the heart to provide adequate nutrient supply to metabolically active tissues. Or in pathophysiological terms, heart failure can be defined as an inability of the heart to deliver blood (oxygen) at a rate commensurate with the requirements of the metabolising tissues despite normal or increased ventricular filling pressures (Dargie & Mcmurray, 1993).

Sudden cardiac death claims an estimated 350 000 lives per year in the United States and between 50 000 and 100 000 lives a year in the United Kingdom. There are numerous underlying diagnoses in patients suffering sudden cardiac death. In 75% of cases, the underlying pathology causing heart failure in patients with sudden cardiac death is coronary heart disease (Pye & Cobbe, 1992). In the Framingham study, initiated in 1949, hypertension was the major etiological factor in 75% of cases of chronic heart failure (CHF), with coronary heart disease (CHD) accounting for only 10% of cases (Mckee *et al.,* 1971). Recent data from USA, in particular from the very large SOLVD (Study Of Left Ventricular Dysfunction) registry and treatment groups, show that coronary artery disease is now the major cause of CHF. Coronary disease accounts for three-quarters of CHF in white male patients. Hypertension is a much more common cause of heart failure in blacks (36% of cases) and hypertension and dilated cardiomyopathy are more common in females (Teerlink *et al.*, 1991).

4.1.2 Basic changes and adaptations in heart failure

The enhanced peripheral resistance seen in advanced heart failure results from increased sympathetic tone, stimulation of vascular α_1-adrenoceptors by increased circulating catecholamines, and increased levels of their circulating humoral vasoconstrictors (Zelis *et al.*, 1981). The increased sympathetic tone in heart failure is associated with a loss of myocardial surface β-adrenoceptors with a selective loss of the myocardial contractile response to β-adrenoceptor stimulation (Bristow *et al.*, 1986).

Plasma noradrenaline levels are elevated in CHF patients and correlate with increased mortality. Endogenous catecholamines such as noradrenaline can act as potent vasoconstrictors, which mediate vasoconstriction via vascular α-adrenoceptors, ensuring blood flow to vital vascular beds (Cohn *et al.*, 1984). Elevated levels of circulating noradrenaline may result from exaggerated adrenomedullary release of the catecholamine, increased efferent sympathetic outflow from the central nervous system, facilitated release of noradrenaline from adrenergic nerve terminals, impaired peripheral reuptake of this catecholamine, or a combination of these mechanisms. The impairment of pump performance in chronic heart failure leads to the activation of various neurohumoral compensatory mechanisms, aimed at maintaining peripheral vascular tone and tissue perfusion (Davis *et al.*, 1988; Jenning & Esler, 1990).

A reduction in cardiac output is often accompanied by an increase in ventricular filling pressures and by alterations in systemic and pulmonary arterial pressures. Abnormalities in vasomotor tone are a well-known component of chronic heart failure (Francis, 1987). It has been described as a condition of generalised neurohumoral excitation, characterised by activation of the sympathetic nervous and renin-angiotensin systems, increases in plasma vasopressin concentration, and parasympathetic withdrawal. Although the endogenous release of neurohormones

can exert deleterious haemodynamic and metabolic effects, these substances also play an important beneficial role in the support of systemic blood pressure, cardiac contractility and glomerular filtration rate. Furthermore, prolonged neurohormonal activation is accompanied by changes in end-organ responsiveness that can significantly modify the effects of neurohormonal agonists. Finally abnormally high concentrations of circulating hormones, neurotransmitters and other toxins may also injure remaining muscle, giving rise to the concept of a self-perpetuating vicious cycle of deteriorating cardiac function (Swedberg *et al.*, 1990).

In heart failure, circulating adrenaline is increased much less than is noradrenaline, although if one combines all studies that have measured adrenaline in heart failure there is a two to three-fold increase. The magnitude of neurohormonal activation appears to adversely affect the prognosis of patients with heart failure, at least with respect to circulating levels of plasma noradrenaline and renin activity. Although the precise mechanism responsible for these relations are unclear, it is known that neurohormonal activation is a progressive phenomenon in patients with heart failure. Contemporary concepts suggest that heart failure is associated with a maladaptive overcompensation of neurohormonal response to impairment of cardiac function and the initiation of a vicious cycle of progressive neurohormonal activation, further decline in cardiac function, etc. (Bristow, 1993).

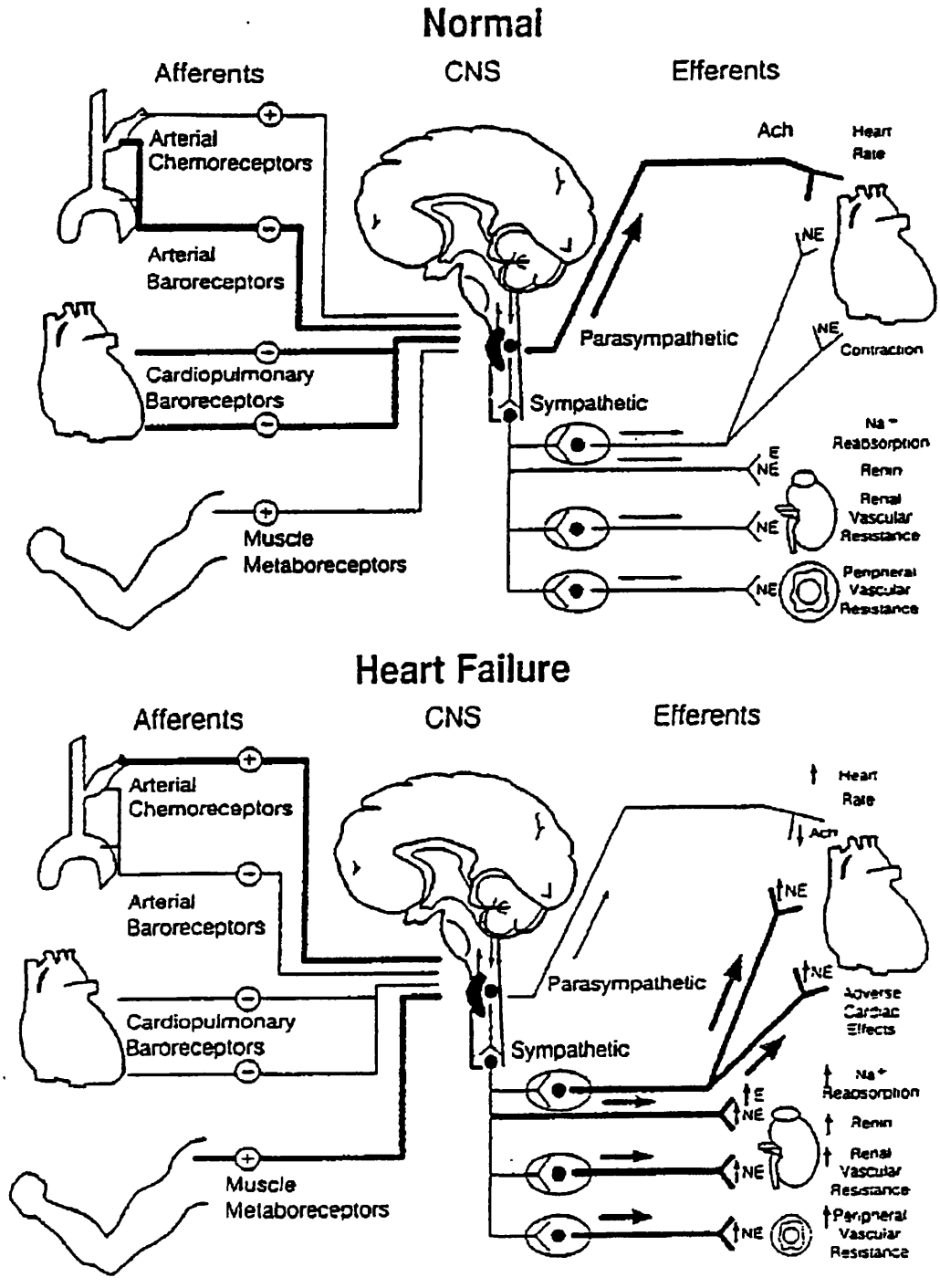


Figure 4.1

Mechanisms for generalised sympathetic activation and parasympathetic withdrawal in heart failure. ACh= acetylcholine; CNS= central nervous system; E= epinephrine; Na^+= sodium; NE= norepinephrine, (From Floras, J.S., 1993)

4.1.3 Changes in vascular system

Zelis and co-workers (1968) found that the ability of the resistance vessels of patients with congestive heart failure compared to normal patients to respond to a wide variety of endogenous vasodilator stimuli (reactive hyperaemia, hand exercise and local heating) and exogenous vasodilator stimuli (intra-arterial sodium nitrite) was reduced. In CHF the peripheral vascular response is characterised by increased peripheral vasomotor tone at rest that has been attributed to multiple factors including increased sodium and water content in the arterial wall, neurohormonal activation, reconditioning, and intrinsic abnormalities in vascular smooth muscle. Systemic vascular resistance thought to increase as a compensation mechanism to maintain arterial pressure and organ perfusion (Zelis & Flaim, 1982). It is also conceivable that a rise in total systemic vascular resistance is a late event in the pathogenesis of heart failure. Since that the smooth muscle cells of the vessel wall can generate considerable intrinsic tone, independent of neural or hormonal input, it is possible that the onset of the rise in vascular resistance is delayed, in part, by opposing changes in intrinsic vascular tone (Pawlowski & Morgan, 1992).

4.1.4 Animal models of heart failure

Several animal models of human congestive heart failure have been developed in attempts to reproduce these features to study the pathogenic mechanisms involved in this disease. The coronary artery occlusion model of heart failure in the rat has been extensively studied. The model has been validated by the measurement of haemodynamic variables (Drexler *et al.*, 1985).

The rat with aorta-caval fistula appears to be a relevant experimental model in comparison with the high cardiac output heart failure models, and has similar

humoral characteristics but different local haemodynamic characteristics (Flaim *et al.*, 1979). Rats with aortocaval (A-V) fistula, an experimental model of CHF, display alterations in renal handling of salt and water that closely mimic those of patients with cardiac failure (Winaver *et al.*, 1988).

Rapid ventricular pacing in the dog has been shown to fulfil the clinical, radiographic and haemodynamic definitions of congestive heart failure (Armstrong *et al.*, 1986). In this model of heart failure, plasma noradrenaline gradually increases with advancing symptoms, becoming significantly elevated after 1 week of pacing and progressively enhanced as heart failure develops (Moe *et al.*, 1989).

Pigs share similar cardiac anatomy and coronary vasculture to that of humans, which makes them suitable for the study of human disease processes. Chronic pacing results in systemic and diastolic dysfunction with reduced collagen support for adjoining myocytes (Spinale *et al.*, 1992).

Adriamycin is a widely used anthracycline antibiotic and possesses cardiotoxic properties. This toxic side-effect has been utilised in a number of studies to induce heart failure in rabbit. The method involves administration of a toxic dose of adriamycin over a prolonged time period sufficient to produce heart failure. Rabbits treated chronically with adriamycin developed symptoms of heart failure such as lower CO, increased heart weight/body weight ratio (Minatoguchi & Majewski, 1994).

Cardiomyopathic male hamsters of the BIO TO-2 strain, a unique experimental model of CHF characterised by progressive myocytolytic necrosis of cardiac muscle are available in study of CHF (Sole, 1986).

The rabbit coronary ligation model is a relatively straightforward model of left ventricular dysfunction. In coronary ligation model it has become recognised that

collateral flow is the most important determinant of the rate and extent of cell death within an ischaemic zone. Collateral flow in the rabbit has been shown to be essentially very poor, similar to the human and pig (Maxwell *et al.,* 1987). Since in 75% of cases, the underlying pathology causing heart failure in patients with sudden cardiac death is coronary heart disease (Maurice & Cobbe, 1992), this model produces similar circumstance to coronary heart disease.

Section 2: Methods and Materials

4.2.1 Preparation of the model

The model was prepared by M. Hicks and co-workers in the Royal Infirmary, Glasgow. Myocardial infarction was produced in male New Zealand white rabbits (2.6kg-3.0kg) by ligation of the marginal branch of the left descending coronary artery. Male New Zealand white rabbits are pre-medicated with intra-muscular Hypnorm (0.3mg /kg, fluanisone (10mg/ml); fentanyl citrate (0.315mg/ml). The anterior and left lateral chest wall was shaved and cleaned with Hibidil in isopropyl alcohol. The animals were further sedated with intravenous midazolam (0.25-0.5mg/kg) via the marginal ear vein to allow edotracheal incubation. Anaesthesia was maintained using an inhaled mixture of 1% Halothane, 1% nitrous oxide and 1% oxygen. The animal was placed on aprewarmed table and body temperature maintained by means of a thermostatically controlled heating mat. The left circumflex artery (which supplies a significant part of the left ventricle) was identified. This artery was ligated with an Ethicon suture at the mid-point between the atrioventricular groove and the cardiac apex. The infarcted region rapidly becomes blue and is easily seen. The infarcted region could be assessed and if it was not large enough a second suture was placed more proximal to the first until a satisfactory infarct was obtained. Sometimes the infarct size was so large that cardiac output fell sufficiently to produce a marked reduction in peripheral pulse volume. Intractable ventricular fibrillation would follow soon after and only by untying the coronary ligature could there be any hope of the animal recovering. Such animals rarely survived and are not included in analysis. Animals were treated with intravenous quinidine (15mg/kg, marginal ear vein) 5 minutes prior to ligation of the coronary artery to reduce the incidence of arrhythmia.

The chest wall was closed using three 2/0 interrupted catgut sutures placed through the chest wall around the ribs adjacent to the wound and drawn firmly together thus splinting the ribs. Antibiotics were administered at the end of the procedure by intramuscular injection (ampicilin 25mg/kg and cephalexidine 15mg/kg). Post-operative analgesia was administered (0.2mg/kg intramuscular buprenorphine) at 30 minutes and 6-8 hours after the procedure, and as considered to be required. Intravenous fluids were administered at the end of the procedure (15-20ml normal saline).

4.2.2 Duration of coronary ligation in rabbit

The development of chronic heart failure was allowed to proceed over either eight or sixteen weeks. Sham operated animals underwent a similar procedure but no ligation was performed. Procedures were performed in pairs with one sham-operated and one ligation animal placed onto the protocol at weekly intervals so that they reached the end of protocol at the same time. In eight weeks coronary ligated rabbits two groups were studied with the same protocol . In the first group (group 1), for preliminary work, four isolated arteries and veins (aorta, vena cava, renal artery and renal vein) of sham operated rabbits with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits with mean ejection fraction of (49.83 ± 2.7), as determined by echocardiography, were investigated. We called this group of ligated rabbits as group 1 of LVD (left ventricular desfunction).

In the second group (group 2) four pairs of arteries and veins (thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein) of the sham operated with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits with mean ejection fraction of (46.5 ± 4.4) were studied. We called this group of

ligated rabbits as group 2 of LVD. In sixteen weeks coronary ligated rabbits one group was studied with the same protocols as in eight weeks coronary ligated rabbits. In this group four isolated arteries and veins (aorta, vena cava, renal artery and renal vein) of the sham operated with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits with mean ejection fraction of (39.17 ± 2.91) were investigated.

Arteries and veins were carefully removed with as little connective tissue as possible and placed in cold physiological salt solution (PSS). The arterial and venous rings were mounted in 10ml isolated organ baths, bathed in Krebs maintained at 37°C and gassed with 95% O_2 plus 5% CO_2.

The rings were then placed under different resting tensions: aorta (2-2.5g), renal artery (1.5g), lateral saphenous artery (1g), ear artery (1g), lateral saphenous vein (1.5g), vena cava (0.5g), renal vein (0.5g) and ear vein (0.5g) which were determined by contraction to NA (1μM) from some preliminary experiments. They were allowed to equilibrate for 1 hour before the experiments. Blood vessels were either used immediately or stored at 4°C in Krebs-Henselite solution overnight, since Ito and Chiba (1985) have shown that canine intermediate auricular artery do not lose activity due to this storage technique. We used preparations immediately for relaxation study, but stored overnight for contraction study. Our preliminary experiments showed that preparations used in this study did not lose their activity on responses to NA or KCl due to storage in 4°C overnight.

Protocols: Individual vessels were subjected to one of two protocols as follows.

4.2.3 Protocol I: relaxation study

It was the aim of the present study to assess whether endothelium-dependent or -independent relaxations play an important role at the level of larger vessels in a

model devised to mimic heart failure. However, few data are available on the function of conductance vessels throughout the large artery and vein trees in heart failure. It is known that large arteries impose low resistance to blood flow. Futhermore, large arteries contribute to cardiac load by the conversion of pulsatile flow to continuous tissue perfusion. We wished to study the function of larger vessels that serve as conduits to deliver oxygenated blood to the body organs in arteries and return blood to the heart in veins. Acetylcholine was chosen as endothelium-dependent vasodilator and sodium nitroprusside as an endothelium-independent vasodilator. To further investigate ATP and adenosine were also chosen as vasodilators to study the function of endothelium in addition to acetylcholine.

After initial application of tension tissues were left to equilibrate for a 60 min period, during which time the tension was re-adjusted to a set value which was maintained constant throughout the rest of the experimental day. Each preparation was then contracted with KCl (Krebs solution, Na free and high KCl, 125 mM) and allowed to contract for 5-10 min. Following complete washout with normal Krebs, an additional 30 minutes equilibration period was allowed. Then all tissues were precontracted with NA (1μM). This induced submaximal contraction in all vessels with the exception of the ear vein (see CRC's to NA, Figure 4.23). When the NA-induced contraction reached a plateau, cumulative concentration-response curves to ACh, SNP, ATP or adenosine were obtained by increasing the concentration of the vasodilators in half-log increments. Between each concentration-response curve to vasodilator, vessels were washed repeatedly and allowed to re-equilibrate for at least one half hour. All concentrations are expressed as the final concentration in the organ bath fluid.

4.2.4 Protocol II: contraction study

The aim of this study was to test sensitivity and neuronal uptake of NA in this model of heart failure. The intracellular uptake of noradrenaline is mediated by at least two processes, named uptake$_1$ and uptake$_2$. Uptake$_1$ has been identified as neuronal uptake of catecholamines into the sympathetic nerve terminals and is believed to be the main process terminating the actions of noradrenaline released as a neurotransmitter. We used cocaine (1μM) to inhibit neuronal reuptake of noradrenaline.

Initially all tissues were exposed to cumulative concentration of NA (1nM-300μM). Following complete washout, the preparations were left 45 minutes to re-equilibrate. Then all preparations were contracted with KCl (Krebs solution, Na free and high KCl, 125mM) and allowed to contract for 5-10 min. Following complete washout with normal Krebs, an additional 30 minutes equilibration period was allowed. Again, tissues were contracted with KCl (Krebs solution, Na free and high KCl, 125mM) and allowed to contract for 5-10 minutes. Following complete washout with normal Krebs, an additional 30 minutes equilibration period was allowed. Then preparations were exposed cumulatively to NA. Following complete washout, the preparations left 45 minutes to re-equilibrate. After preincubation with cocaine (10μM) for 10-15 minutes to inhibit neuronal uptake of NA, a final NA CRC was conducted cumulatively.

4.2.5 Histological studies

First experimental arteries and veins were dissected out and cleared of any extraneous connective tissue using fine scissors, as much as possible. Glyoxylic acid technique (Bjorklund *et al.,* 1972 and Dr. Ian Montgomery as personal communication) was used to demonstrate axons and varicoses of sympathetic

nerves on these isolated blood vessels. Specimens photographed using a Zeiss Axiophot microscope fitted with epifluorescence and a barrier filter block at 390-420μm. Photomicrographs were taken using Fujichrome Provia 1600 colour reversal film.

4.2.6 Calculations and Statistics

The relaxation response to vasodilator (ACh, SNP, ATP or adenosine) was expressed as percentage of the contraction generated by NA (1μM) against which it was tested. Response to each contractile agonist is expressed as absolute tension (g). Response to first NA CRC also was expressed as % of first KCl (125mM). This would give three useful comparisons: i) it allows some comparison between the vessels; ii) it lets us see how good NA is at achieving the maximum that KCl can pull; iii) the full contractile potential of each vessel. All data are given as mean ± s.e.mean. Significance was always accepted at the 0.05 level of probability.

Section 3: Results of experimental heart failure (group 1)

4.3.1 Relaxation to ACh

Acetylcholine induced relaxations in both arteries and veins. Renal artery was the least sensitive preparation to acetylcholine (Figure 4.3). At a few individual concentrations, in the two veins, there was a significant smaller relaxation in the shams. This affected the threshold in the vena cava but not the renal vein. There was no pattern of difference between coronary ligated and sham operated rabbits after 8 weeks in group 1 (Table 4.1).

4.3.2 Relaxation to SNP

Sodium nitroprusside produced vasorelaxations in all tissues (Figure 4.4). There was no difference between coronary ligated and sham operated rabbits after 8 weeks in group 1 (Table 4.1).

4.3.3 Relaxation to ATP

This agent could induce some relaxations in both arteries and veins (Figure 4.5). The veins relaxed more than arteries whose responses were trivial, but in every case the response to ATP compared to ACh or SNP was poor in terms of both threshold concentration and maximum relaxation attained with in the concentration range tested. In relaxation to ATP, there was no difference between coronary ligated and sham operated rabbits after 8 weeks in group 1 .

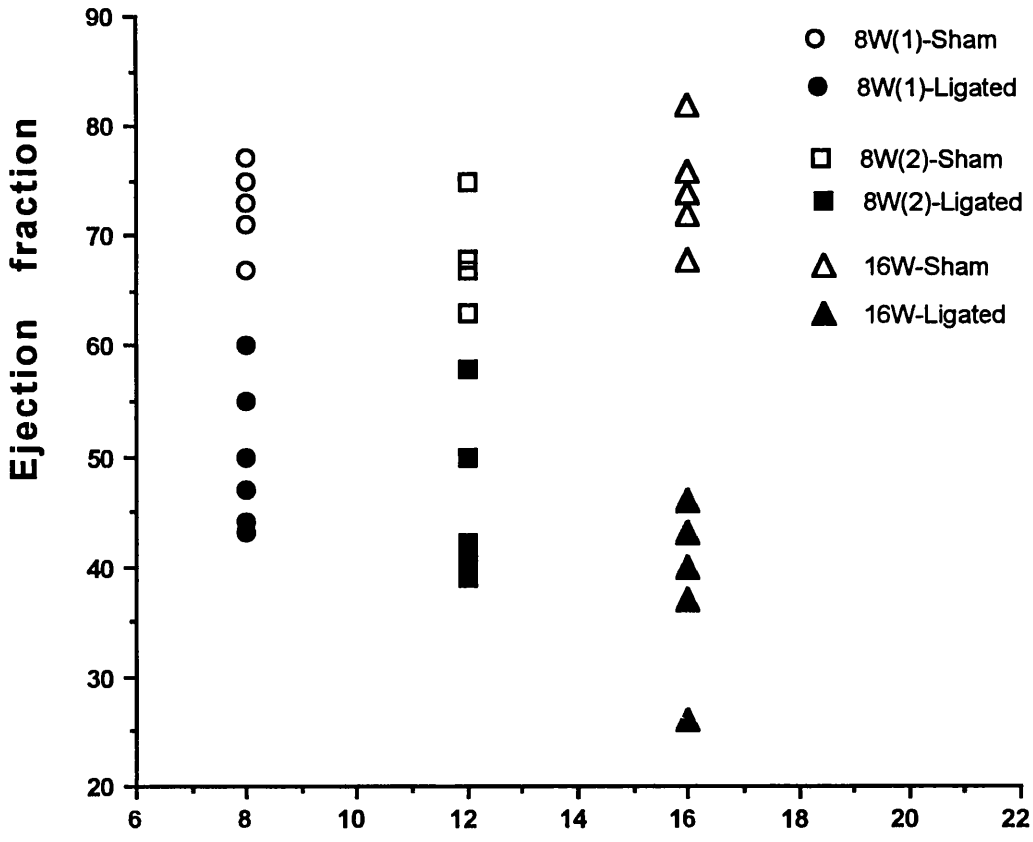


Figure 4.2

Left ventricular ejection fraction values from sham and coronary ligated rabbits 8 or 16 weeks after operation.

8W(1): rabbits after 8 weeks sham operation or coronary ligation in group 1

8W(2): rabbits after 8 weeks sham operation or coronary ligation in group 2

16W: rabbits after 16 weeks sham operation or coronary ligation

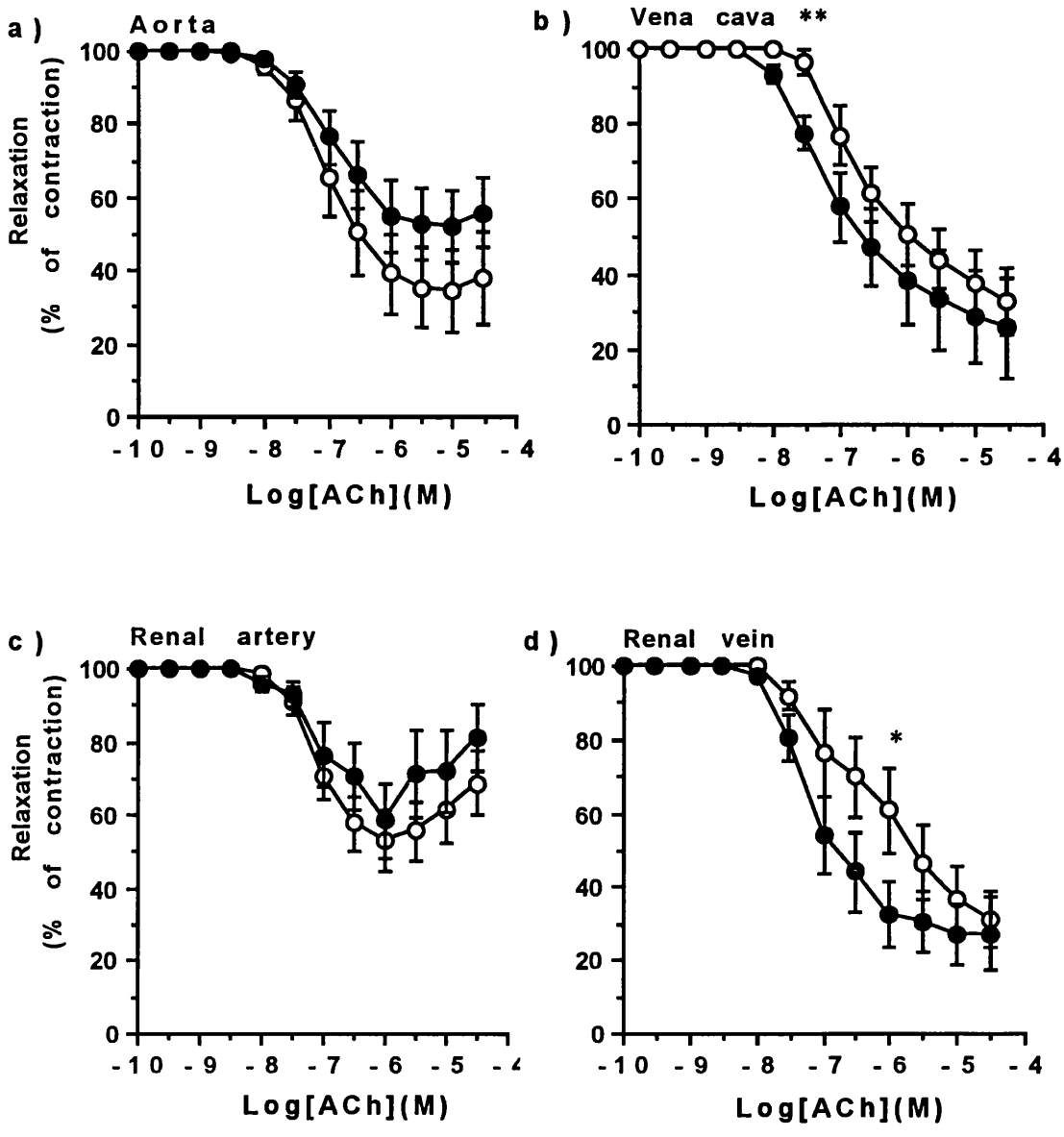


Figure 4.3

Relaxation to ACh in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, ** $0.001<p<0.01$, Student's t-test.

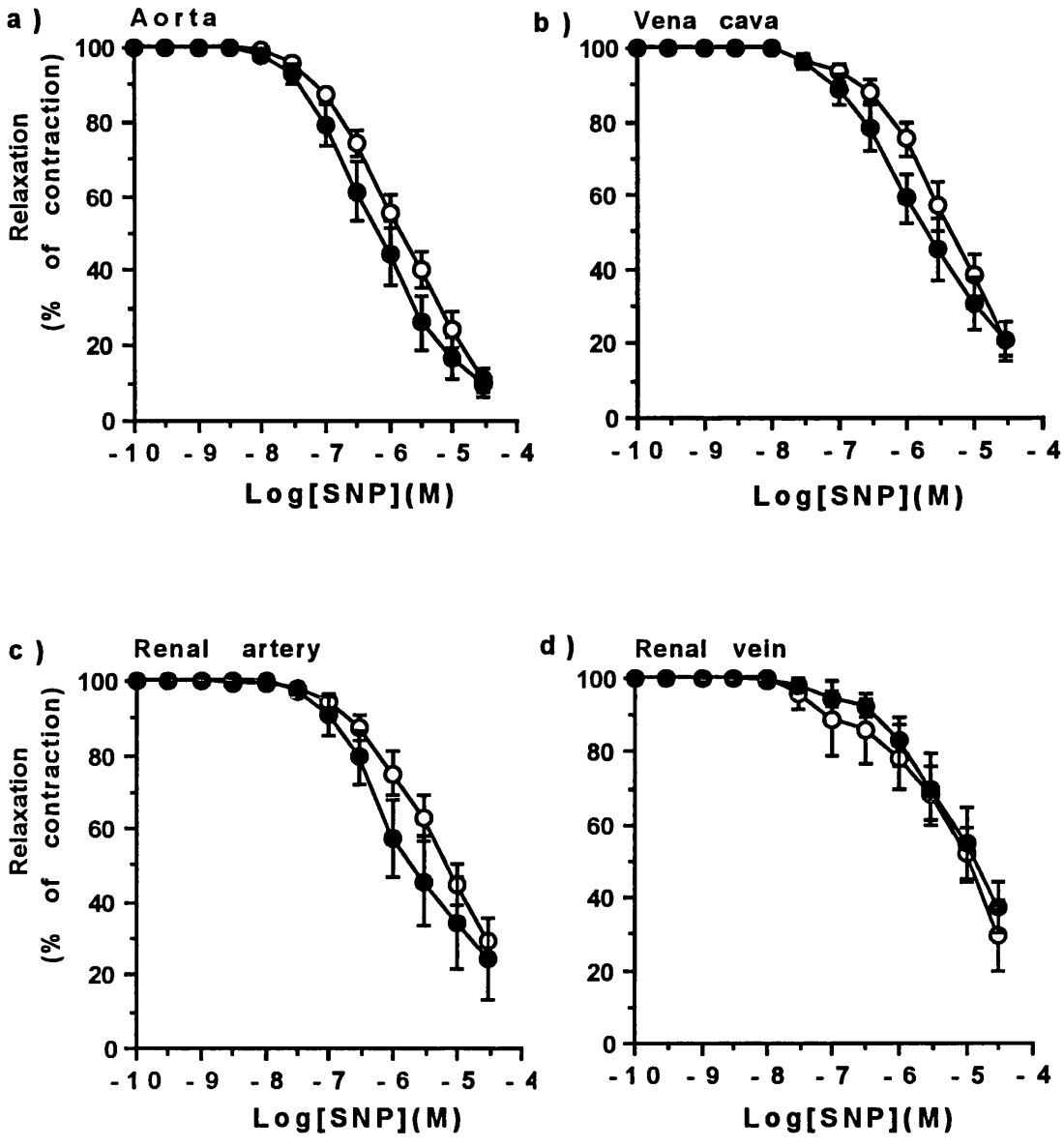


Figure 4.4

Relaxation to SNP in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

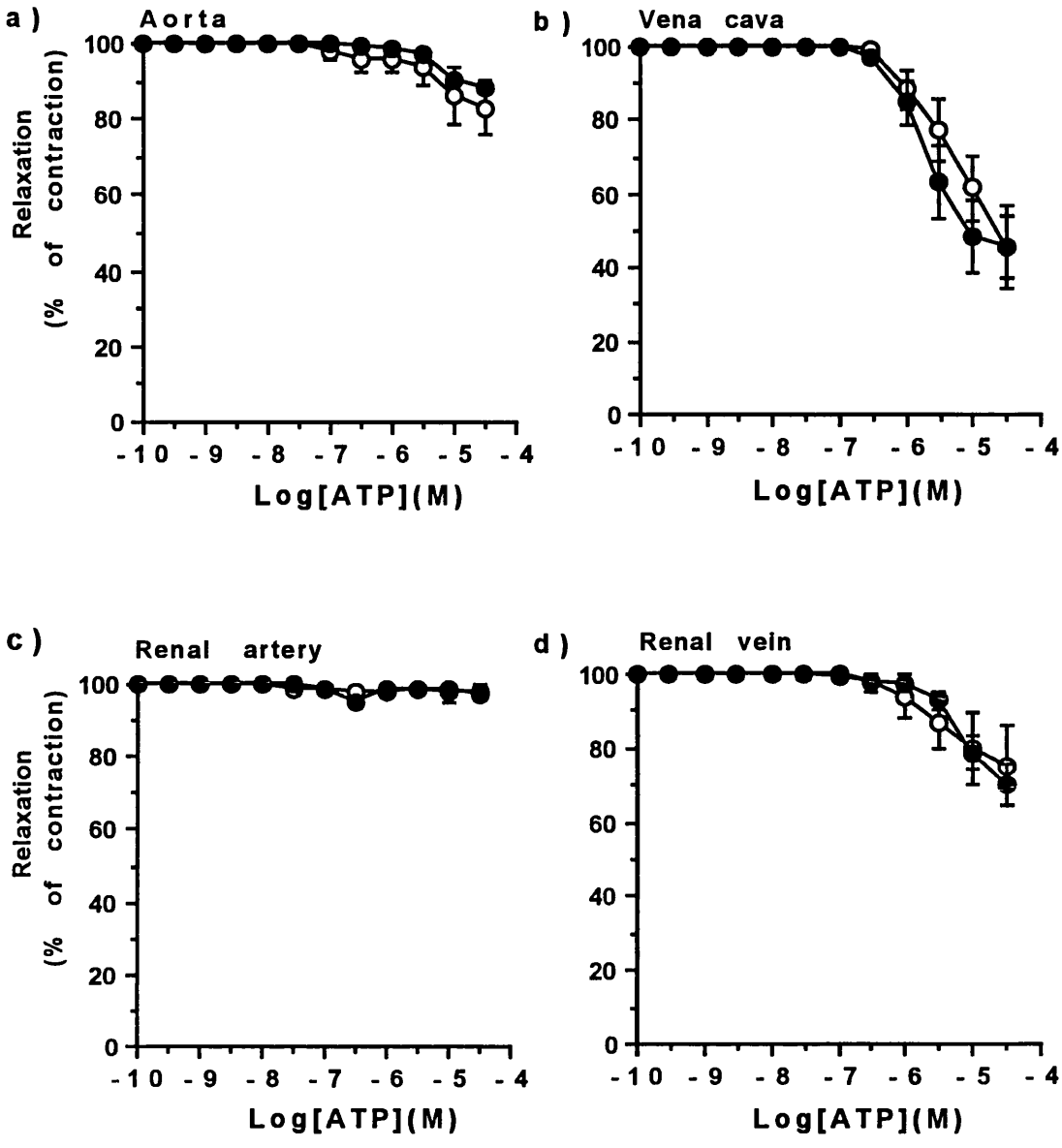


Figure 4.5

Relaxation to ATP in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

Table 4.1

Vessel	pIC_{50} (Ach) sham	pIC_{50} (Ach) Ligated	pIC_{50} (SNP) sham	pIC_{50} (SNP) Ligated
Aorta	6.88 ± 0.15	6.51 ± 0.36	5.97 ± 0.1	5.98 ± 0.17
Vena cava	6.72 ± 0.12	7.02 ± 0.13	5.58 ± 0.15	6.25 ± 0.06
Renal artery	7.05 ± 0.07	6.76 ± 0.2	5.57 ± 0.12	5.8 ± 0.3
Renal vein	6.31 ± 0.36	7.26 ± 0.16	5.74 ± 0.37	5.28 ± 0.34

Comparison of pIC_{50} expressed as the -log of the IC_{50} (concentration producing 50% of the maximum relaxation attained within the range tested) in sham and coronary ligated rabbits in response to ACh and SNP after 8 weeks operation in group 1.

Data are expressed as mean ± s.e.mean (n=6).

Table 4.2

Vessel	pIC_{25} (Ach) sham	pIC_{25} (Ach) Ligated	pIC_{25} (SNP) sham	pIC_{25} (SNP) Ligated
Aorta	7.03 ± 0.3	6.8 ± 0.23	6.52 ± 0.11	6.78 ± 0.2
Vena cava	6.77 ± 0.2	7.09 ± 0.35	6.08 ± 0.12	6.41 ± 0.22
Renal artery	6.93 ± 0.18	6.89 ± 0.24	5.9 ± 0.18	6.4 ± 0.46
Renal vein	6.51 ± 0.42	7.24 ± 0.15	5.9 ± 0.43	5.55 ± 0.21

Comparison of pIC_{25} expressed as the -log of the IC_{25} (concentration producing 25% of the maximum relaxation to NA (1μM) used for inducing tone) in sham and coronary ligated rabbits in response to ACh and SNP after 8 weeks operation in group 1.

Data are expressed as mean ± s.e.mean (n=6).

4.3.4 Relaxation to adenosine

Adenosine induced small relaxations in both arteries and veins (Figure 4.6). The veins relaxed more than arteries, but generally response to adenosine compared to ACh or SNP was poor. In relaxation to adenosine, there was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 1.

4.3.5 Relaxation to β-adrenoceptor agonist isoprenaline

Isoprenaline induced relaxations in both arteries and veins (Figure 4.7). In every case response obtained to isoprenaline within the range tested compared to ACh or SNP was smaller. Renal artery was the most sensitive one and produced the maximum relaxation (20-26%). In relaxation to β-adrenoceptor agonist isoproterenol, there is no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 1.

4.3.6 Contraction to NA

Initially all tissues were exposed to cumulative concentrations of NA (1 nM-300μM). Renal artery and aorta had much greater maximum responses to NA compared with the veins (Figure 4.8). In vasoconstriction to NA, there was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 1.

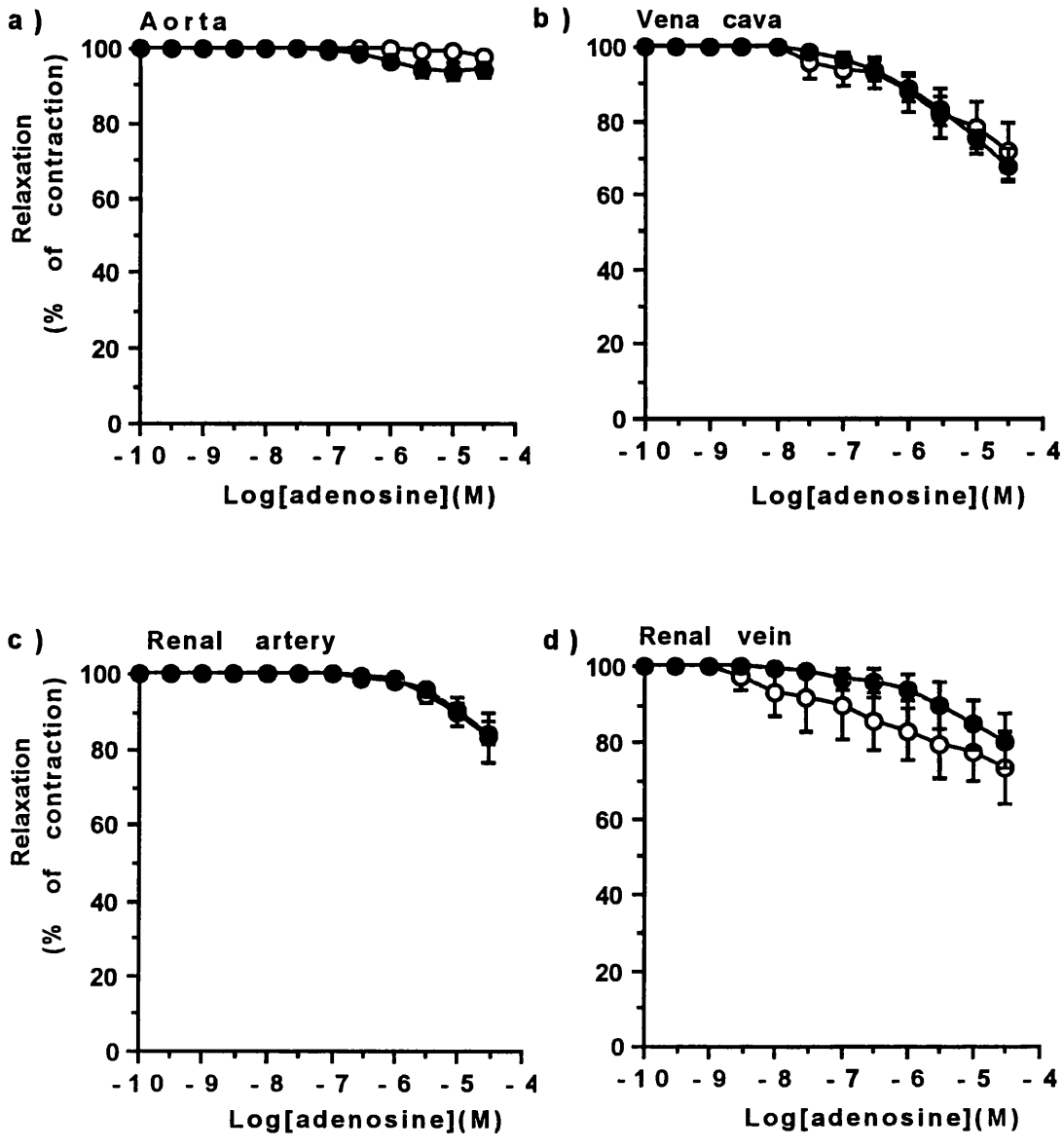


Figure 4.6

Relaxation to adenosine in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

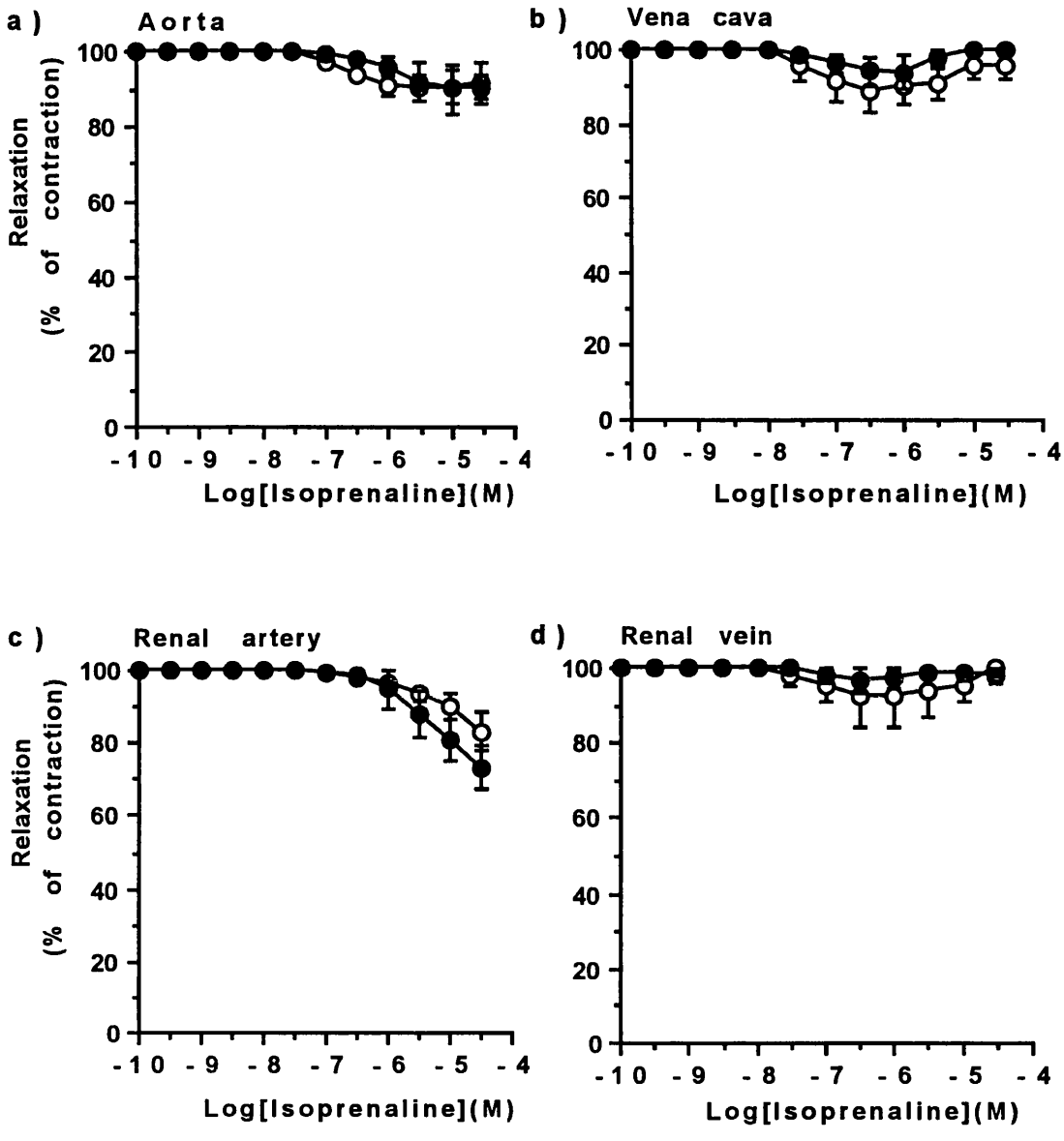


Figure 4.7

Relaxation to isoprenaline in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Results are expressed as % of maximum response to U-46619 (0.1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

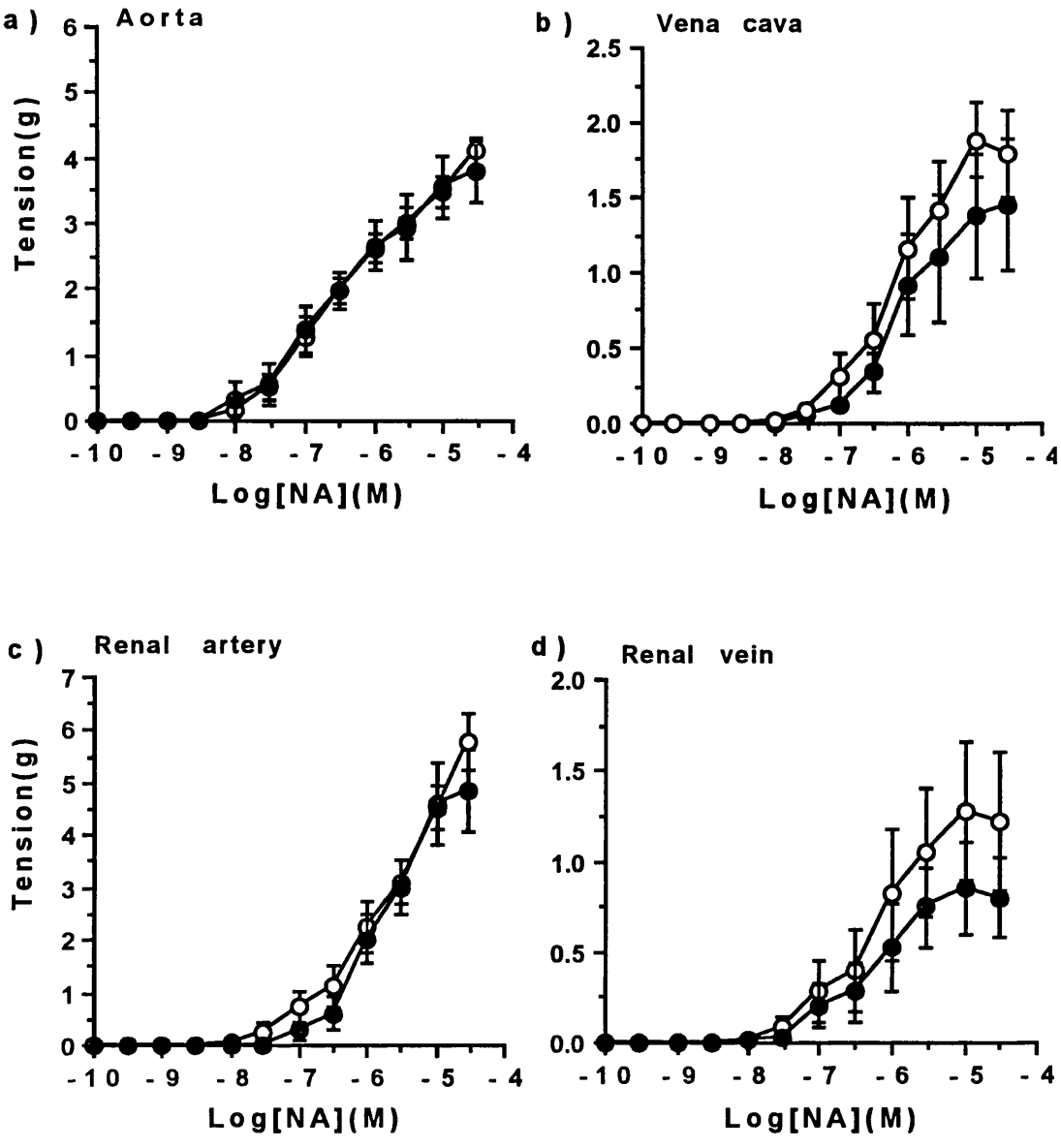


Figure 4.8

First CCRC to NA in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Responses are expressed as tension (g).

Each point represents mean ± s.e.mean (n=6).

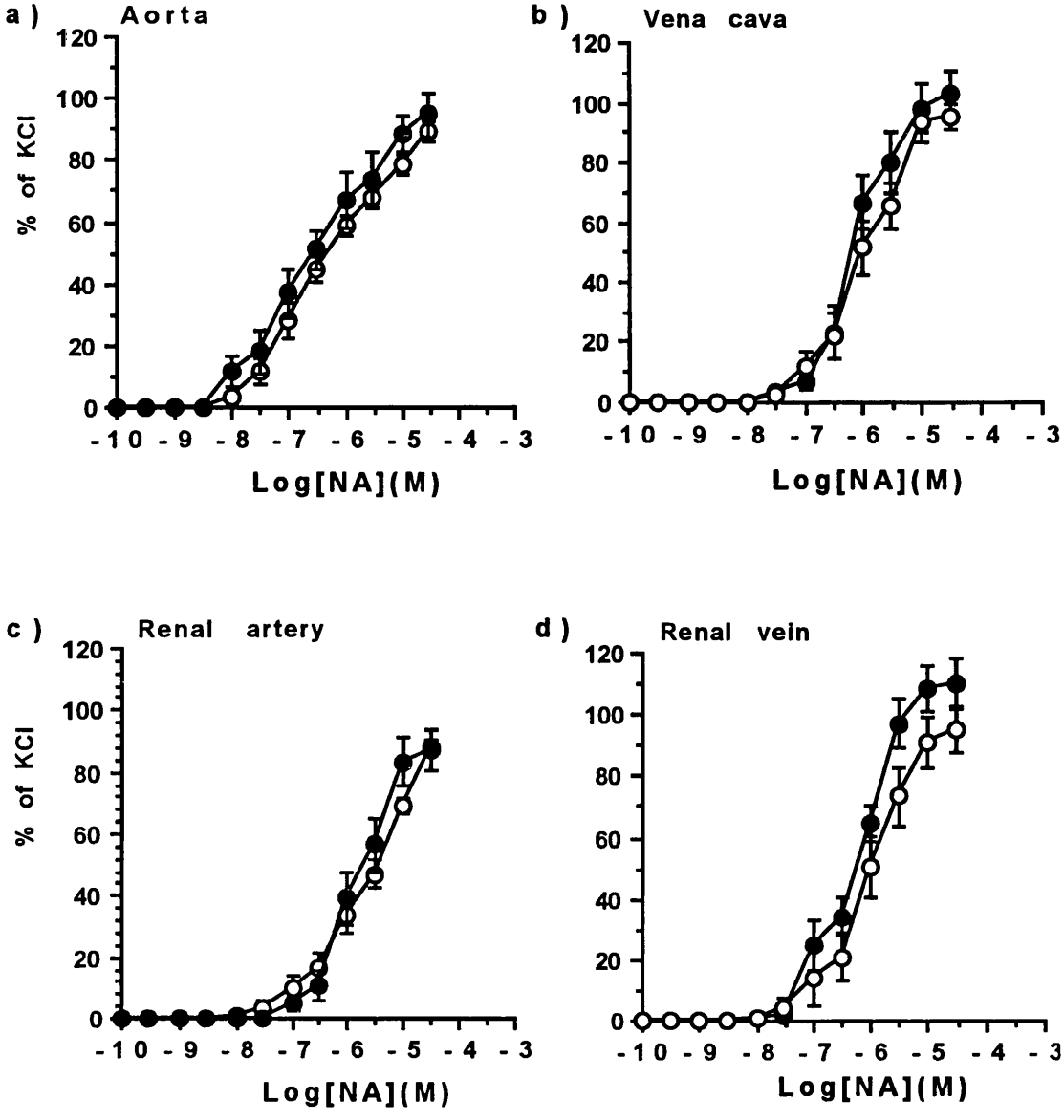


Figure 4.9

First CCRC to NA in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits (●) with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. Results are expressed % of the maximum response to KCl (125mM) after CCRC to NA in each preparation.

Each point represents mean ± s.e.mean (n=6).

4.3.7 Response to KCl

All preparation were contracted with KCl (Krebs solution, Na free and high KCl, 125mM) and allowed to contract for 5-10 min. Contraction to KCl in the first or second test showed no significant difference, and arteries had greater maximum contractions compared with veins. However, response to KCl was similar in coronary ligated and sham operated rabbits after 8 weeks operation in group 1 (Figure 4.10).

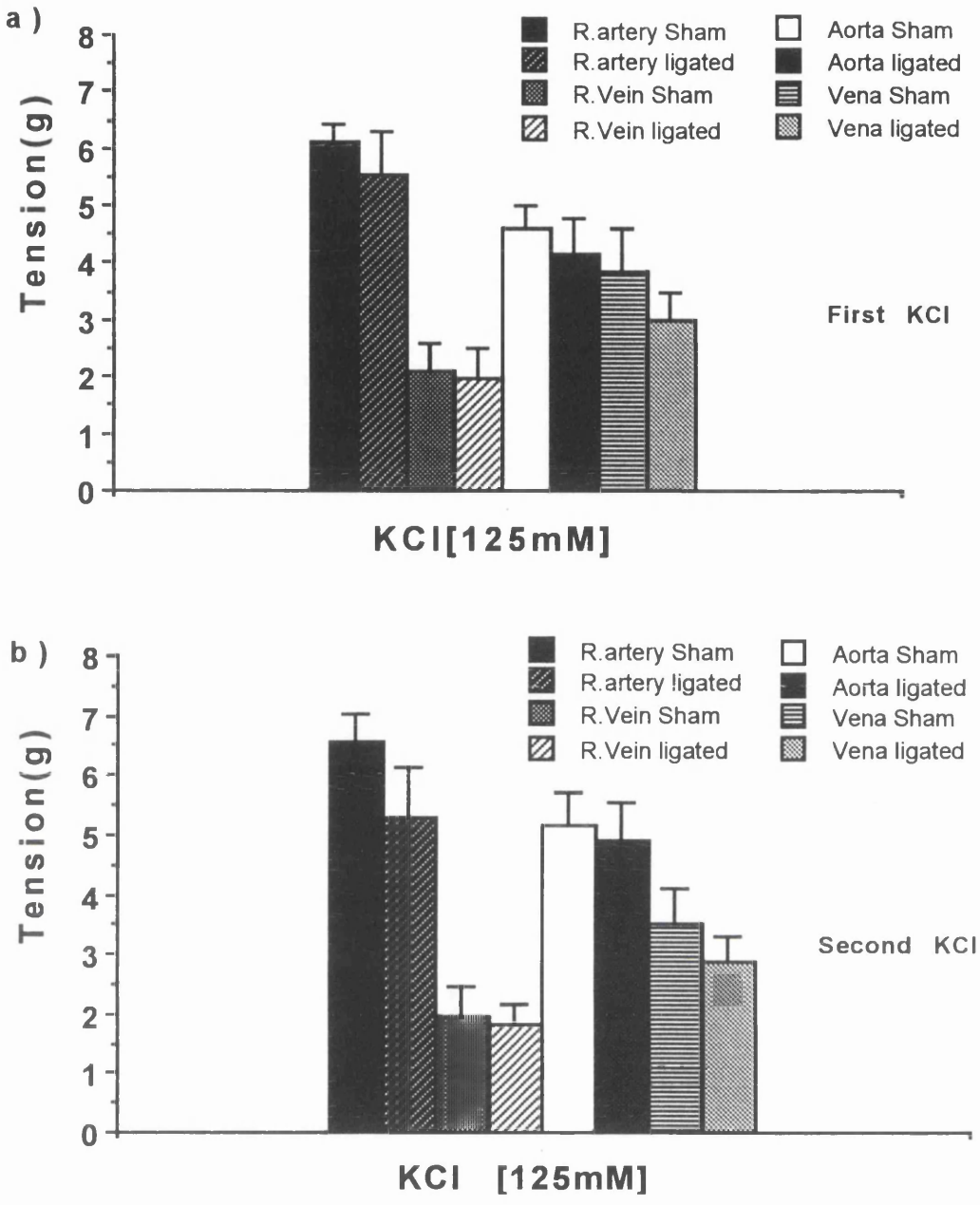


Figure 4.10

Response to KCl in the four isolated arteries and veins of the sham operated with mean ejection fraction of (69.67 ± 3.25) and coronary ligated rabbits with mean ejection fraction of (49.83 ± 2.7) after 8 weeks operation in group 1. **a**) response to KCl after CCRC to NA **b**) response to KCl after first KCl.

Each point represents mean ± s.e.mean (n=6).

Section 4: Results of experimental heart failure (group 2)

In experiments on tissues from this group of rabbits the data was essentially similar to group 1 where the same tissues and protocols were employed. The protocols were similar selected for an extension of all concentration range for NA since in group 1. The greatest concentration used did not produce a convincing maximum in the CRC to NA in some of the tissues tested in group 1 (compare Figure 4.8 with Figures 4.22 and 4.23). This follows a straightforward description of the data from group 2 followed by an our all consideration of groups of 1 and 2.

4.4.1 Relaxation to ACh

Acetylcholine induced relaxations in all arteries and veins. Ear vein, saphenous vein and aorta were most sensitive in terms of threshold and concentration producing 50% maximum relaxation of tone. Saphenous vein and aorta produced the greatest maximum relaxations (78-90%). In renal artery and ear vein high concentration of acetylcholine (>1μM) produced paradoxical contraction. We investigated mechanism of this contraction in renal artery. Indomethacin (1μM), which blocks the production of prostaglandins, did not affect the contraction evoked by acetylcholine in renal artery. In the presence of L-NAME (100μM) acetylcholine produced poor relaxation but L-NAME failed to affect contraction produced by acetylcholine in high concentrations. Atropine an antagonist of muscarinic receptors, blocked the contraction produced by acetylcholine. These results suggest that contraction to ACh in this artery is related to muscarinic receptors located in smooth muscle cells which are sensitive to high concentration of ACh.

Saphenous artery and ear artery had the poorest response to acetylcholine. It is possible that one reason for this is that narrow lumens in these preparations make it

difficult to avoid damage to the endothelium when stainless-steel L-shaped hooks are passed through the lumen. However relaxation to the endothelium-independent agent SNP was also poor in these preparations (see Figure 4.12 and 4.15).

In ear and renal veins the concentration response curves to ACh were clearly not monophasic, in the ear vein a rebound contraction occurred at high concentration of ACh and in the renal vein, in contrast there was a further relaxation phase at high concentration of ACh. In relaxation to acetylcholine, there was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 2 (Figures 4.12 and 4.13, Table 4.3).

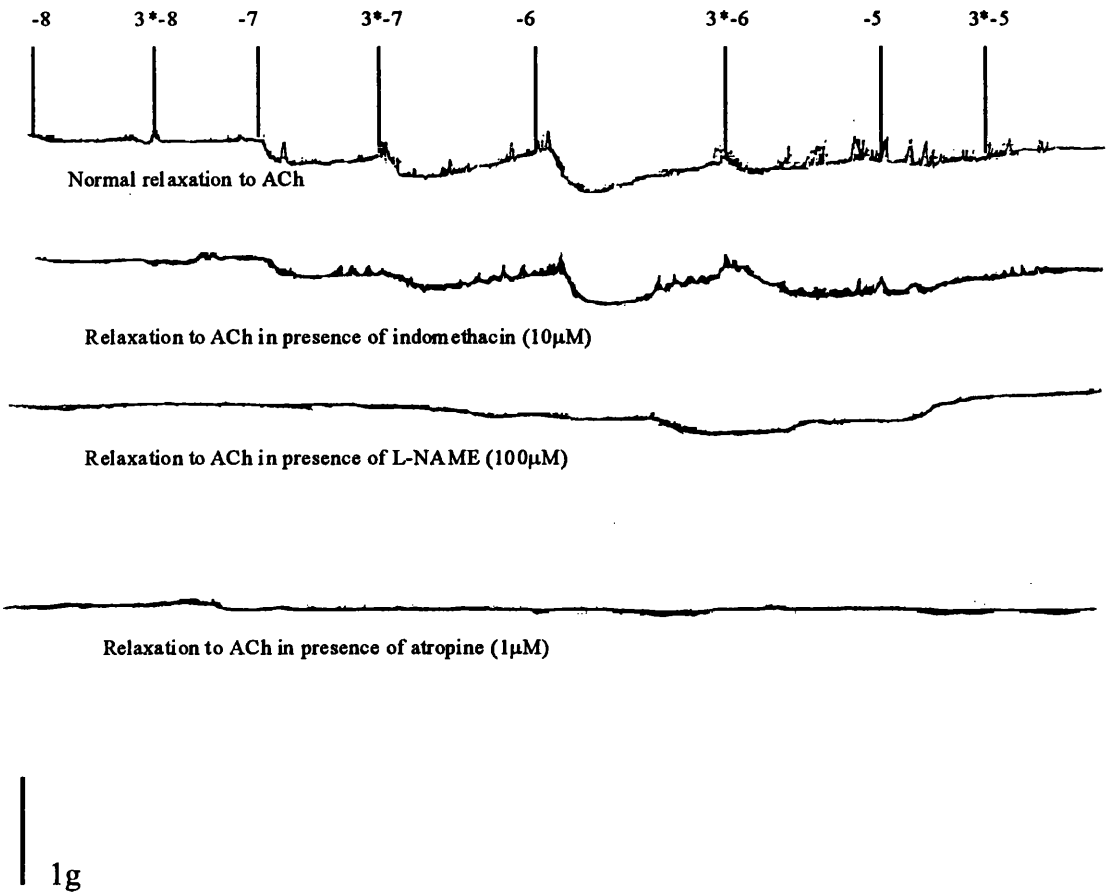


Figure 4.11

Representative trace recording of cumulative relaxation responses to ACh in the rabbit isolated renal artery and effects of an inhibitor of cyclooxygenase indomethacin (1μM), an inhibitor of NO synthase L-NAME (100μM) and an antagonist of muscarinic receptors atropine (1μM). Renal artery was incubated 45 minutes with indomethacin and atropine but L-NAME was added 10-15 minutes prior to the onset of CCRC to acetylcholine.

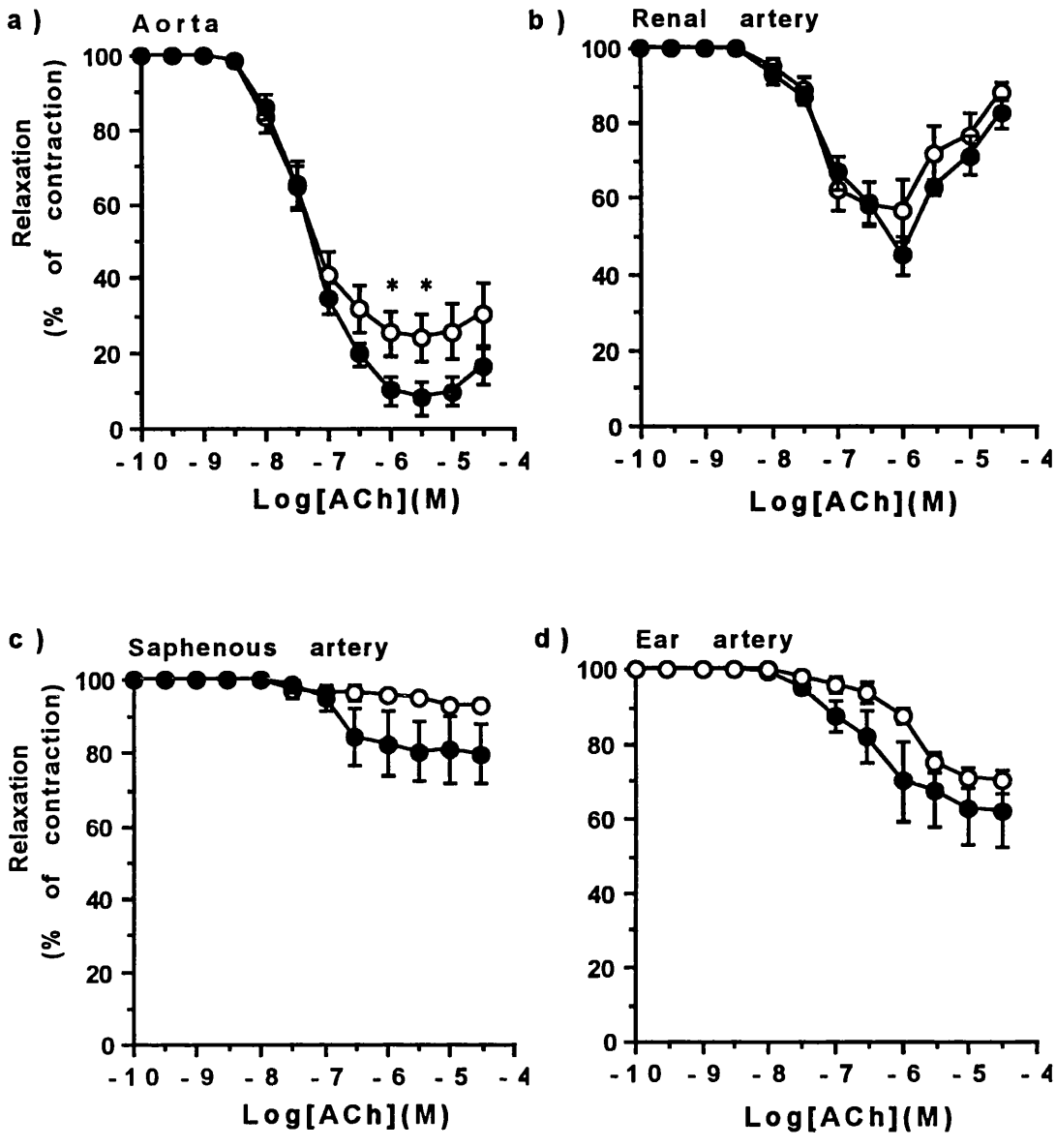


Figure 4.12

Relaxation to ACh in the four isolated arteries of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired Student's t-test.

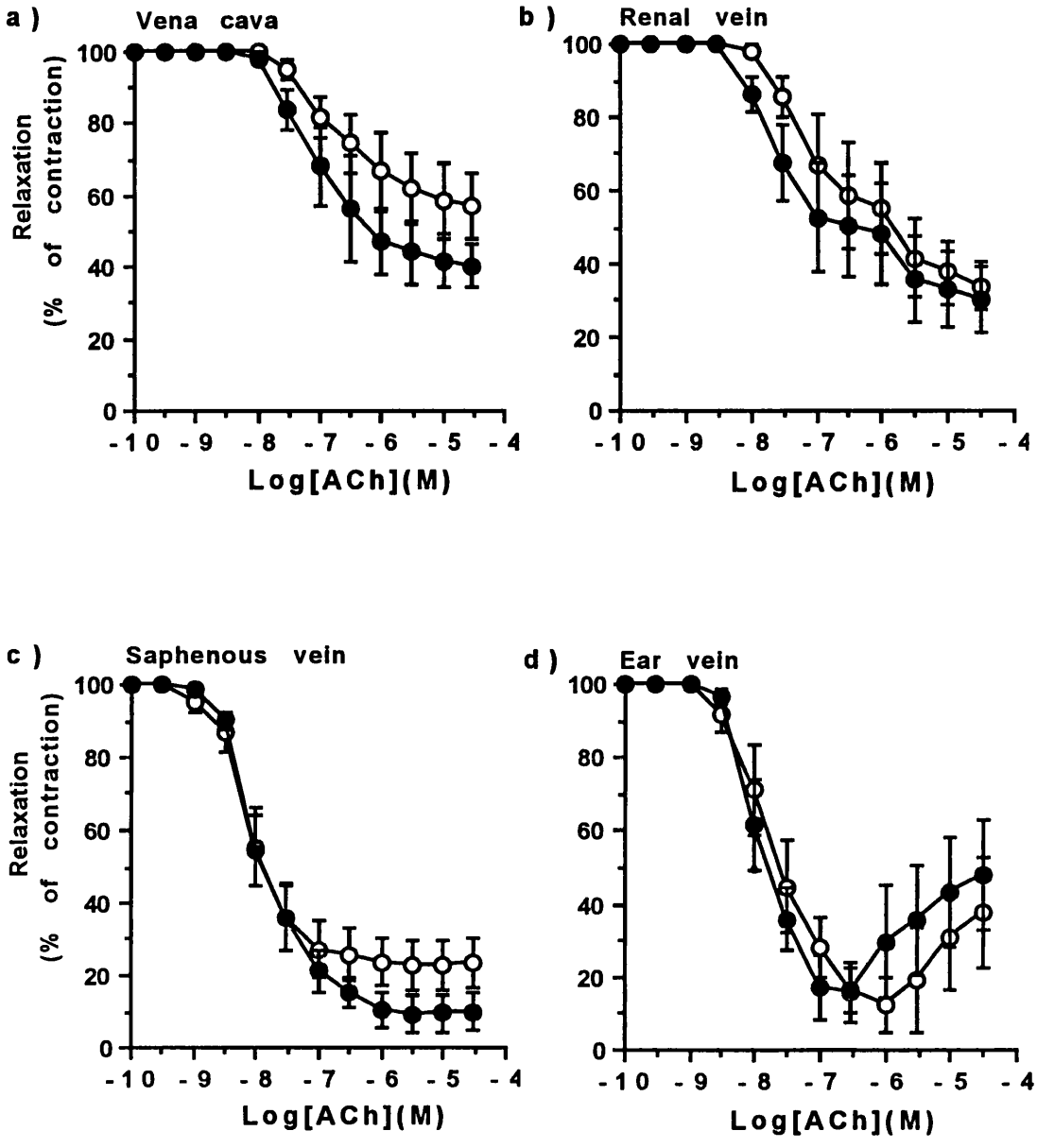
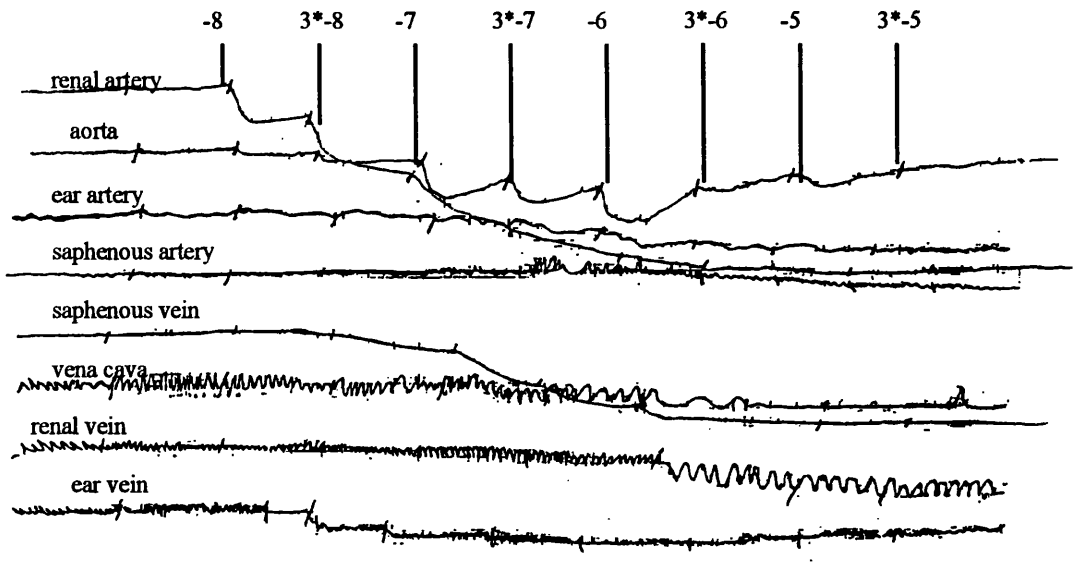


Figure 4.13

Relaxation to ACh in the four isolated veins of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).



1g (saphenous vein, renal artery and aorta)

0.5g (other preparations)

Figure 4.14

Representative trace recording of cumulative relaxation responses to ACh in the rabbit isolated four pairs of arteries and veins: thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein. All tissues were precontracted with NA (1μM). This induced submaximal contraction in all vessels with the exception of the ear vein. When the NA-induced contraction reached a plateau, cumulative concentration-response curves to ACh was obtained by increasing the concentration of the vasodilator in half-log increments.

Table 4.3

Vessel	pIC_{50} (Ach) sham	pIC_{50} (Ach) Ligated	Maximum relaxation % sham	Maximum relaxation % Ligated
Aorta	7.44 ± 0.06	7.35 ± 0.09	76.7 ± 6.11	91.97 ± 4.33 *
Vena cava	6.56 ± 0.3	6.93 ± 0.41	44.23 ± 9.59	59.39 ± 6.01
Renal artery	6.54 ± 0.67	7.09 ± 0.09	43.66 ± 6.05	55.02 ± 5.31
Renal vein	6.75 ± 0.37	7.13 ± 0.39	66.21 ± 6.72	69.55 ± 8.92
Saphenous artery	6.53 ± 0.74	6.13 ± 0.42	7.88 ± 1.35	20.79 ± 8.82
Saphenous vein	8.02 ± 0.11	7.89 ± 0.16	77.02 ± 6.8	90.43 ± 5.1
Ear artery	6.44 ± 0.18	6.33 ± 0.16	31.51 ± 2.85	38.08 ± 9.5
Ear vein	7.71 ± 0.23	7.89 ± 0.1	87.6 ± 7.54	84.15 ± 8.36

Comparison of pIC_{50} that is expressed as the -log of the IC_{50} (concentration producing 50% of the maximum relaxation attained within the range tested) in four pairs of arteries and veins of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2. Maximum relaxation is expressed as % of NA (1μM) used for inducing tone.

Data are expressed as mean ± s.e.mean (n=6). Statistical comparisons with controls were carried out using unpaired Student's t-test * $p<0.05$.

4.4.2 Relaxation to SNP

Sodium nitroprusside produced vasorelaxations in all tissues. As for acetylcholine, ear vein, aorta and saphenous vein were the most sensitive and also produced the greatest maximum relaxations within the concentration range tested. Again saphenous artery and ear artery had the poorest response to sodium nitroprusside. Together with the poor response to ACh this suggests that these tissues are relatively insensitive to the effects of NO whether it is done by SNP or released by endothelium. In general sensitivity to SNP was one log order less than to ACh in the shams. In ear vein and renal vein differences were 0.4 and 1.5 log units respectively. There was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 2 in response to sodium nitroprusside (Figures 4.15 and 4.16, Table 4.4).

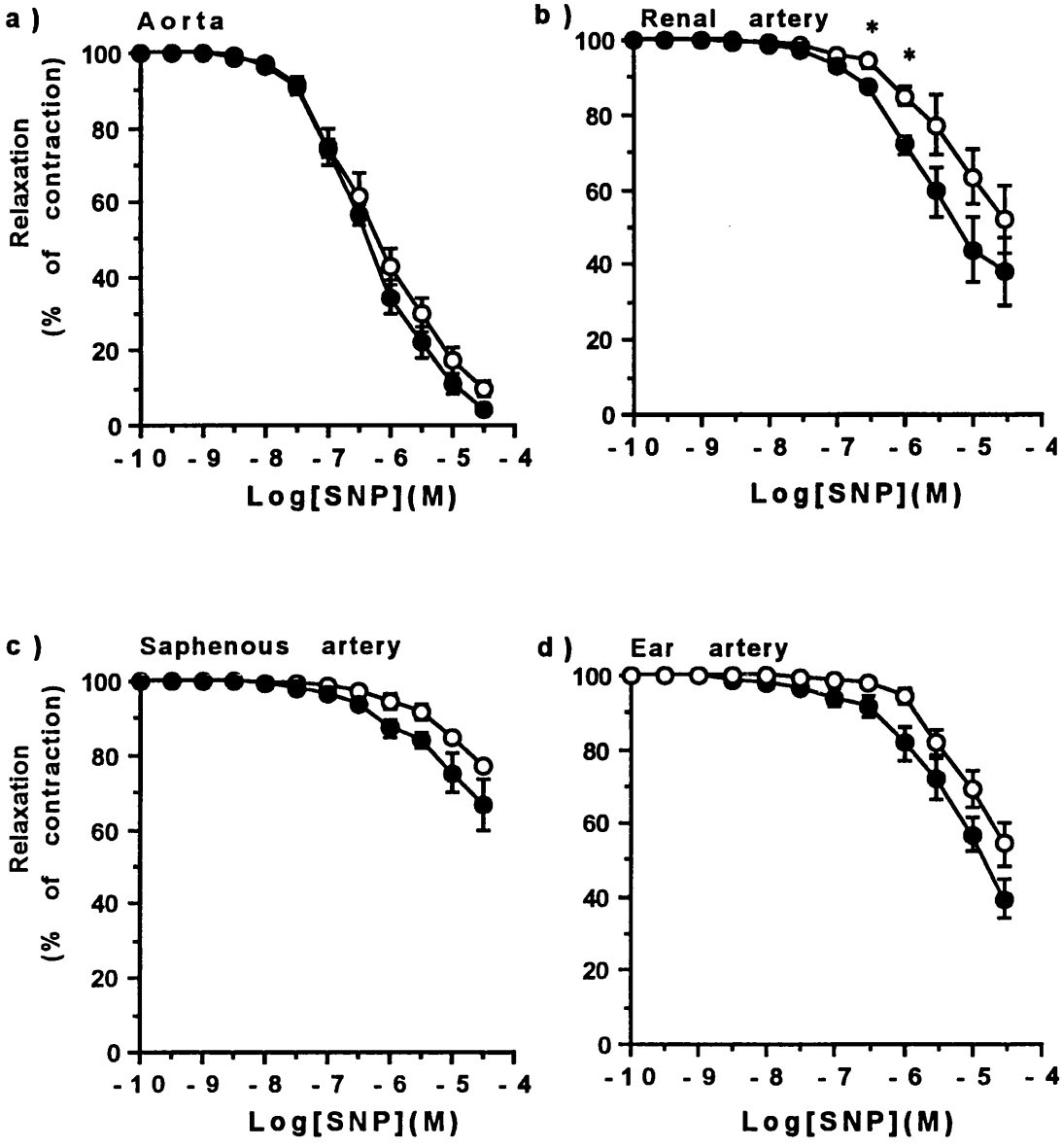


Figure 4.15

Relaxation to SNP in the four isolated arteries of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired Student's t-test.

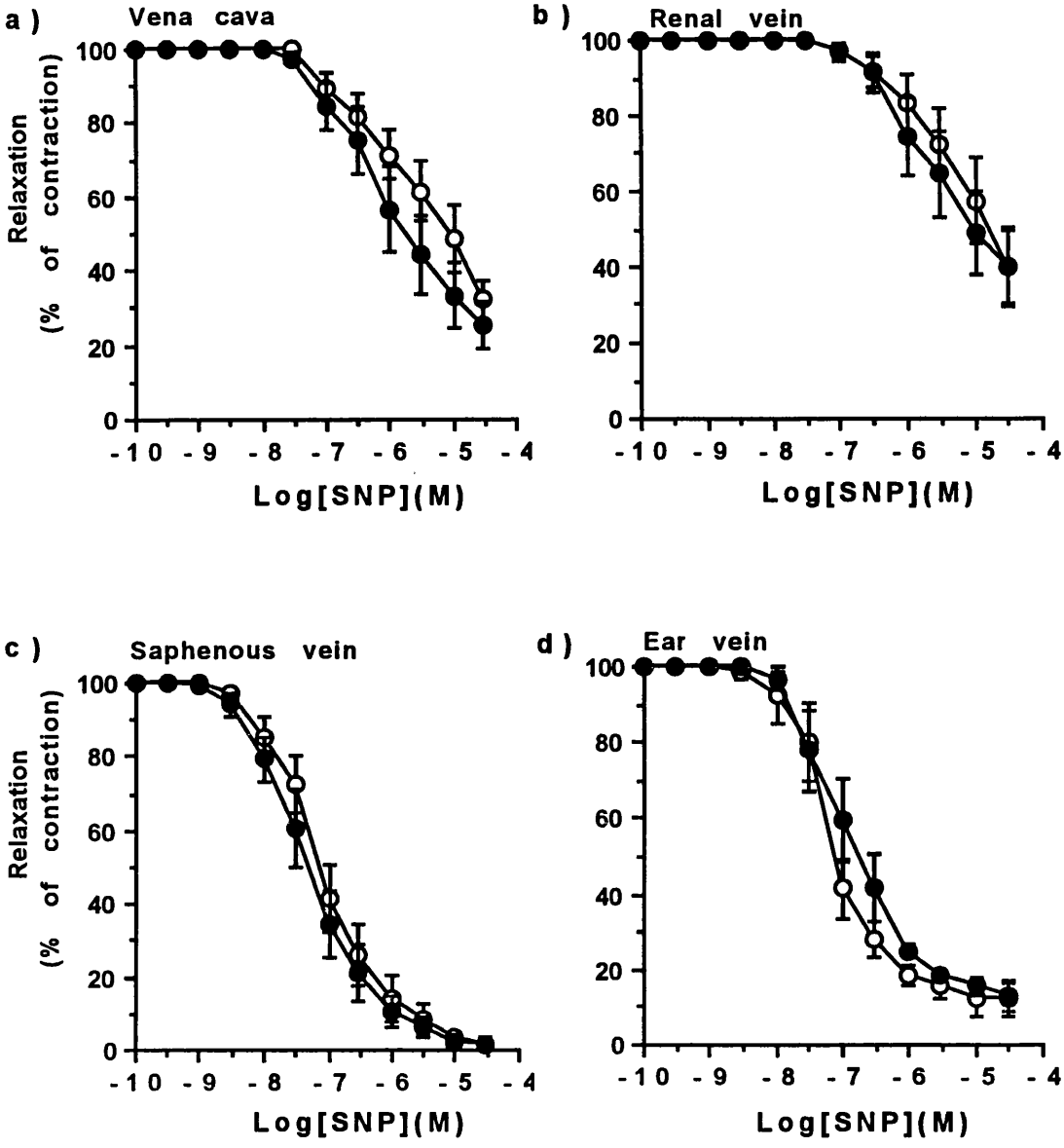
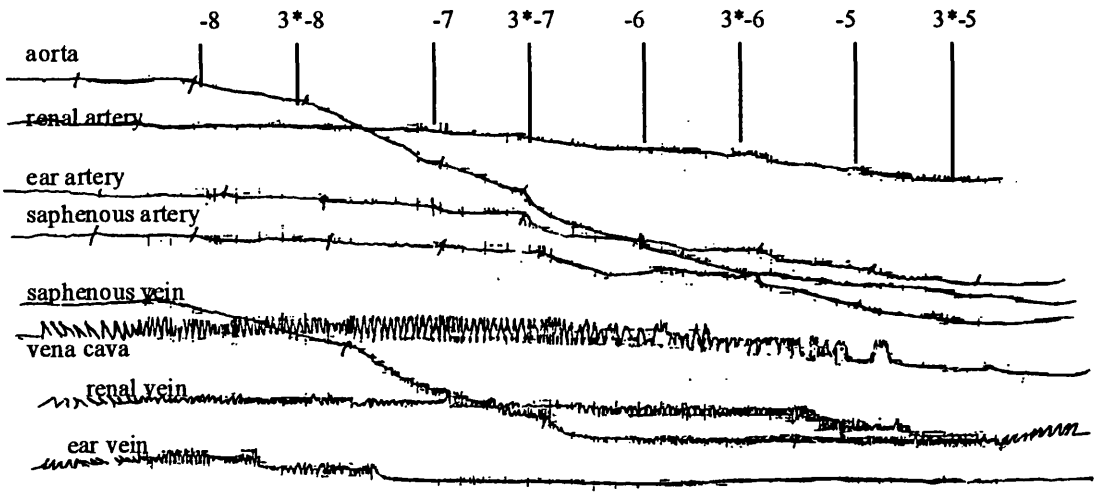


Figure 4.16

Relaxation to SNP in the four isolated veins of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).



1g (saphenous vein, renal artery and aorta)

0.5g (other preparations)

Figure 4.17

Representative trace recording of cumulative relaxation responses to SNP in the rabbit isolated four pairs of arteries and veins: thoracic aorta and vena cava; left renal artery and left renal vein; lateral saphenous artery and lateral saphenous vein and finally central ear artery and marginal ear vein. All tissues were precontracted with NA (1μM). This induced submaximal contraction in all vessels with the exception of the ear vein. When the NA-induced contraction reached a plateau, cumulative concentration-response curves to SNP was obtained by increasing the concentration of the vasodilator in half-log increments.

Table 4.4

Vessel	pIC_{50} (SNP) sham	pIC_{50} (SNP) Ligated	Maximum relaxation% sham	Maximum relaxation% Ligated
Aorta	6.33 ± 0.13	6.38 ± 0.07	90.29 ± 2.14	95.81 ± 1.20
Vena cava	5.66 ± 0.26	6.07 ± 0.23	67.89 ± 5.4	74.39 ± 6.43
Renal artery	5.46 ± 0.1	5.82 ± 0.16	47.97 ± 6.39	63.67 ± 8.1
Renal vein	5.37 ± 0.2	5.61 ± 0.21	59.96 ± 9.29	59.99 ± 8.84
Saphenous artery	5.36 ± 0.17	5.71 ± 0.28	22.87 ± 1.16	33.47 ± 6.99
Saphenous vein	7.09 ± 0.18	7.3 ± 0.2	98.26 ± 0.77	98.39 ± 1.73
Ear artery	5.21 ± 0.09	5.72 ± 0.16	46.713 ± 5.93	60.81 ± 5.27
Ear vein	7.23 ± 0.2	6.92 ± 0.26	87.63 ± 4.54	87.25 ± 3.9

Comparison of pIC_{50} that is expressed as the -log of the IC_{50} (concentration producing 50% of the maximum relaxation attained within the range tested) in four pairs of arteries and veins of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2. Maximum relaxation expressed as % of NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

Table 4.5

Vessel	pIC_{25} (Ach) sham	pIC_{25} (Ach) Ligated	pIC_{25} (SNP) sham	pIC_{25} (SNP) Ligated
Aorta	7.76 ± 0.13	7.7 ± 0.11	6.88 ± 0.12	7.01 ± 0.08
Vena cava	6.57 ± 0.28	7.18 ± 0.35	6.17 ± 0.33	6.43 ± 0.3
Renal artery	7.2 ± 0.08	7.14 ± 0.13	5.47 ± 0.16	6.07 ± 0.12 *
Renal vein	7.24 ± 0.41	7.69 ± 0.31	5.57 ± 0.32	5.91 ± 0.29
Saphenous artery			4.5 ± 0.14	5 ± 0.36
Saphenous vein	8.25 ± 0.14	8.15 ± 0.16	7.6 ± 0.18	7.76 ± 0.17
Ear artery	5.4 ± 0.25	6.1 ± 0.26	5.23 ± 0.22	5.69 ± 0.18
Ear vein	8.03 ± 0.18	8.12 ± 0.11	7.46 ± 0.02	7.36 ± 0.21

Comparison of pIC_{25} expressed as the -log of the IC_{25} (concentration producing 25% of the maximum relaxation to NA (1μM) used for inducing tone) in four pairs of arteries and veins of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2. Relaxation expressed as % of NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6). Statistical comparisons with controls were carried out using unpaired Student's t-test * $p<0.05$.

4.4.3 Relaxation to ATP

This agent induced relaxations in both arteries and veins. The veins relaxed more than arteries, but in arteries responses to ATP compared to ACh and SNP were smaller. Relaxation CRC's to ATP were of similar form to those for ACh and SNP in veins, but on a molar basis sensitivity was less. Saphenous vein was the most sensitive and produced the maximum relaxation (45%). Among the veins, as for ACh and SNP the two veins tested in group 2, ear and saphenous veins show the greatest relaxations. In relaxation to ATP, there was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 2 (Figures 4.18 and 4.19).

4.4.4 Relaxation to adenosine

Adenosine induced relaxations in both arteries and veins. The veins relaxed more than arteries, but generally responses to adenosine compared to ACh or SNP were poor. In relaxation to adenosine, there was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 2 (Figures 4.20 and 4.21).

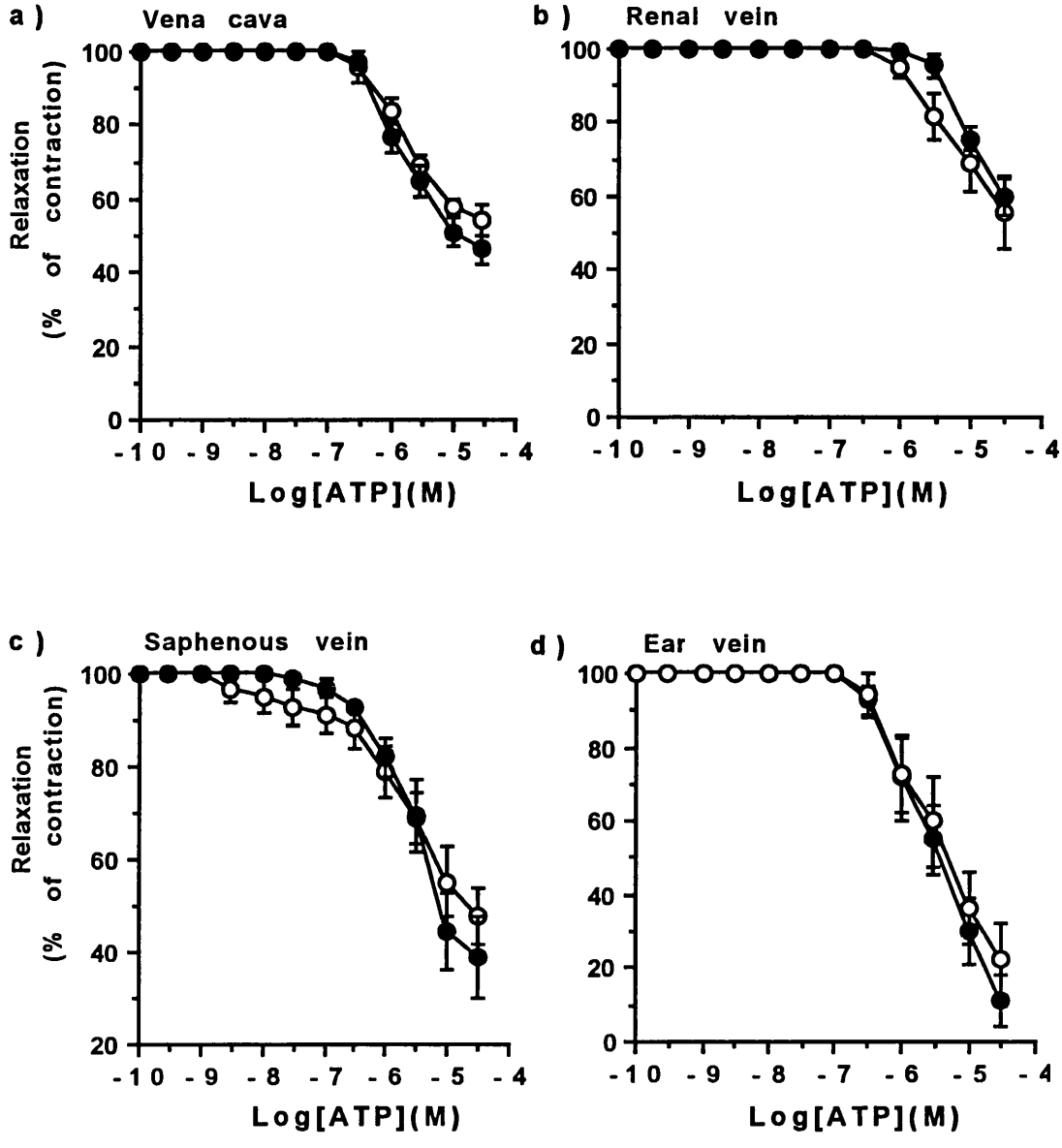


Figure 4.18

Relaxation to ATP in the four isolated veins of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

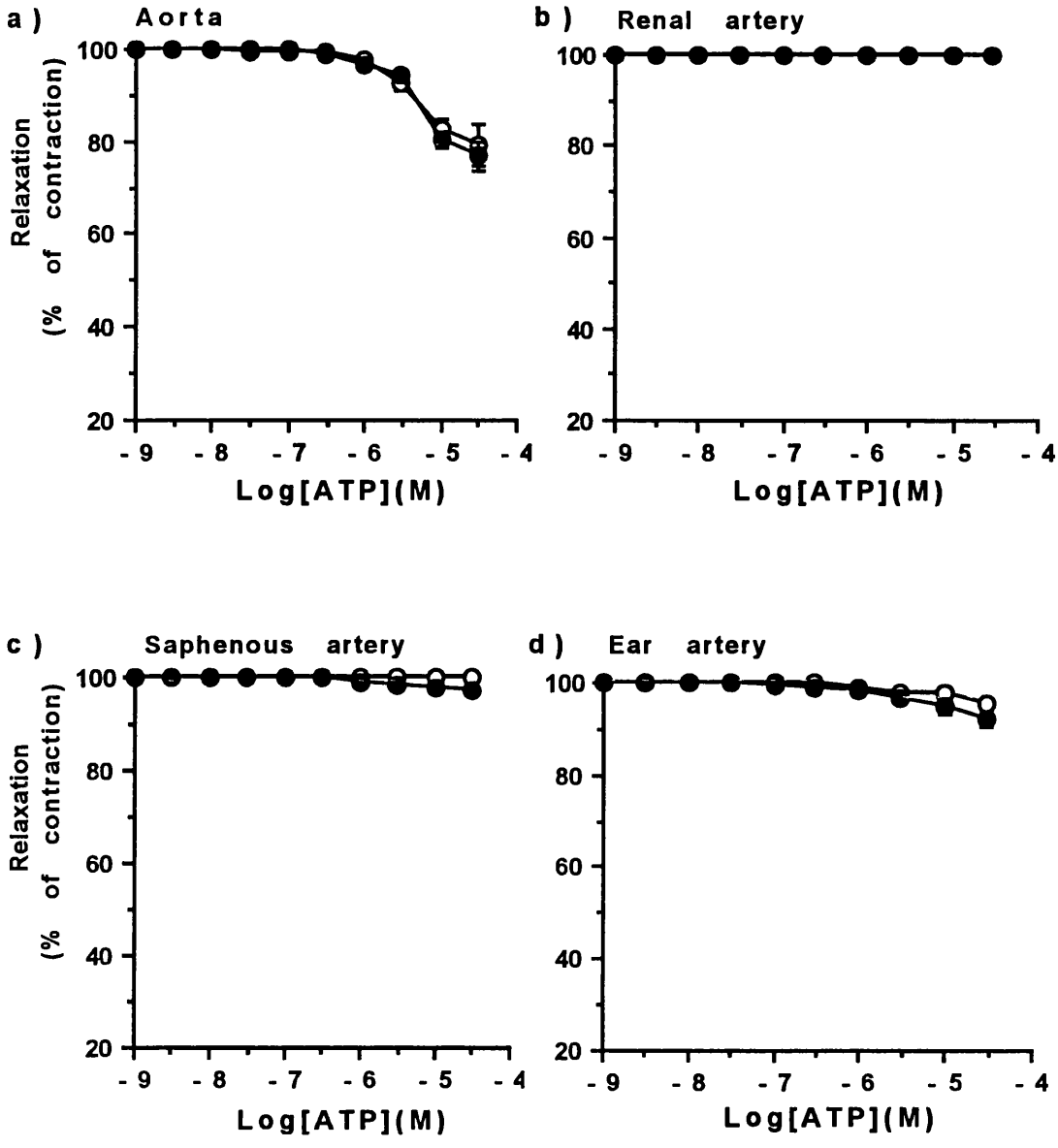


Figure 4.19

Relaxation to ATP in the four isolated arteries of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

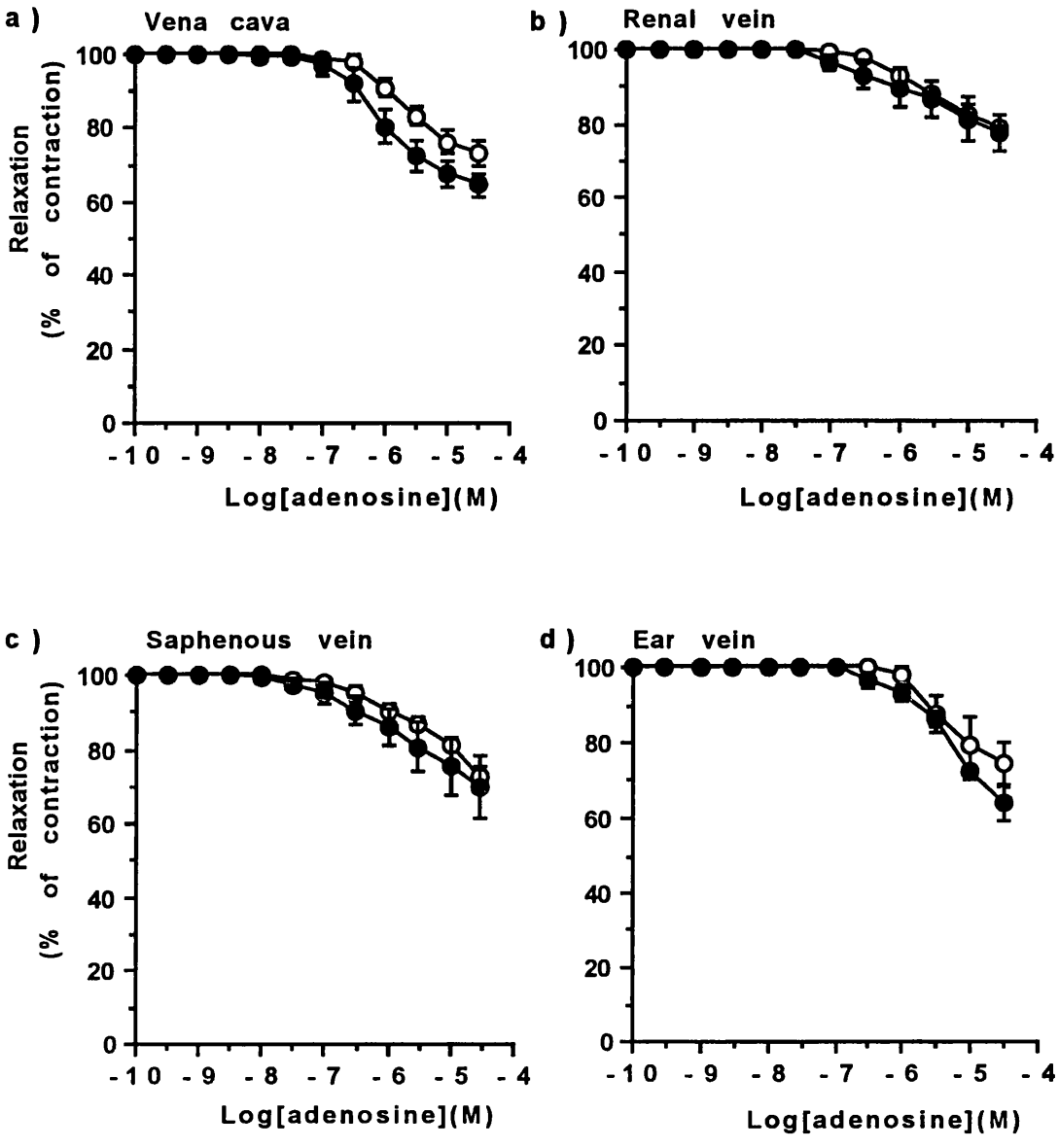


Figure 4.20

Relaxation to adenosine in the four isolated veins of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

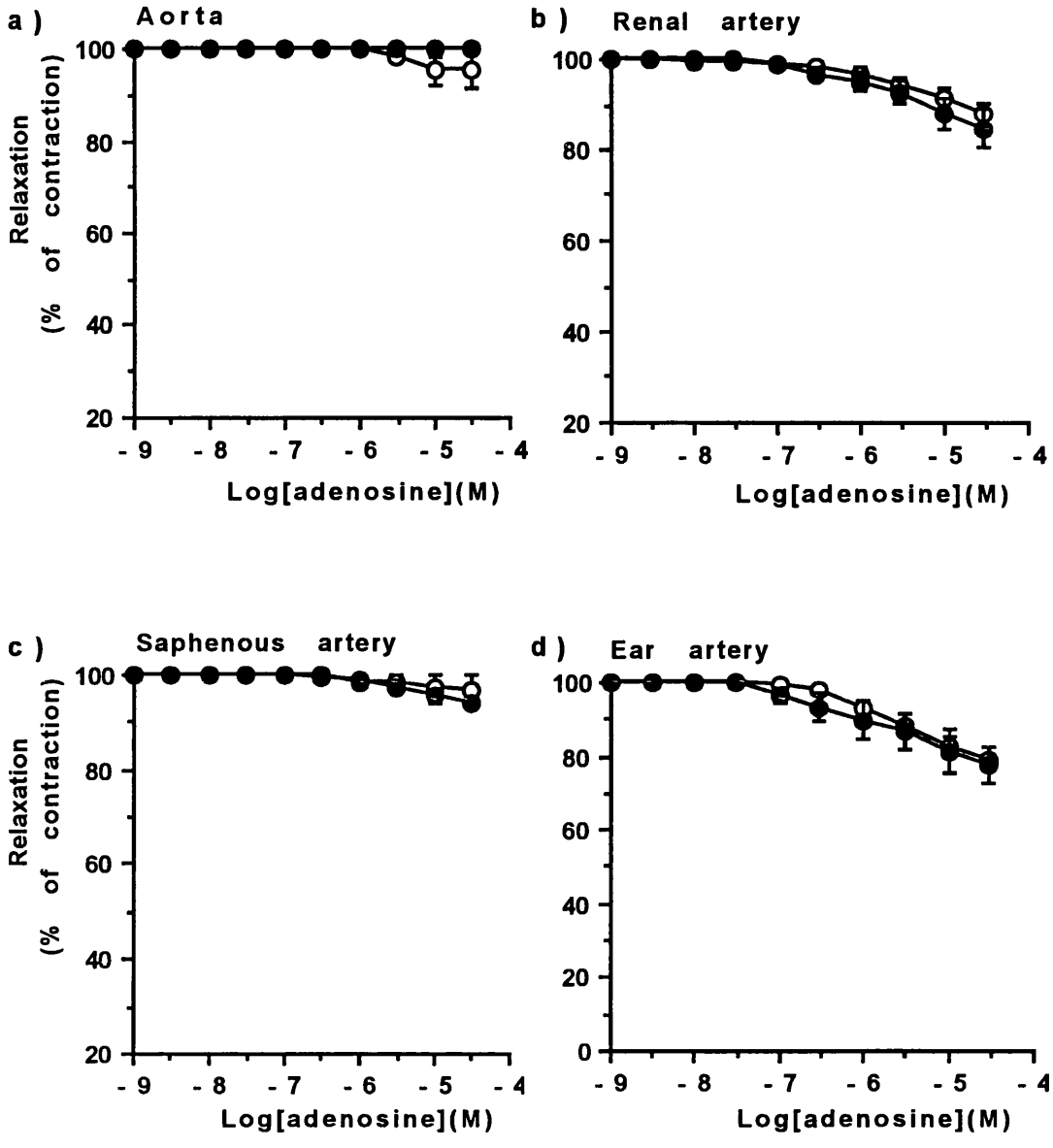


Figure 4.21

Relaxation to adenosine in the four isolated arteries of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (●) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

4.4.5 Contraction to NA

Initially all tissues were exposed to cumulative concentrations of NA (1 nM-300μM). Aorta, ear artery and ear vein were the most sensitive preparations (pD_2 values: 9.96, 7.04 and 7.8 respectively). Renal artery and aorta had relatively very large maximum responses to NA among the arteries (6.7 and 4.3g respectively) and saphenous vein had greatest maximum response among the veins. In vasoconstriction to NA, there was no difference between coronary ligated and sham operated rabbits after 8 weeks operation in group 2 (Figures 4.22 and 4.23, Table 4.6).

4.4.6 Response to KCl

All preparation were contracted with KCl (Krebs solution, Na free and high KCl, 125mM) and allowed to contract for 5-10 min. Contraction to KCl in the first or second test showed no significant difference and arteries except saphenous vein had greater contractions compared with corresponding veins. Renal artery, saphenous vein and aorta had the greatest maximum contraction (7, 5 and 4.2g respectively). However, the response to KCl was similar in coronary ligated and sham operated rabbits after 8 weeks operation in group 2 (Figure 4.26, Table 4.9).

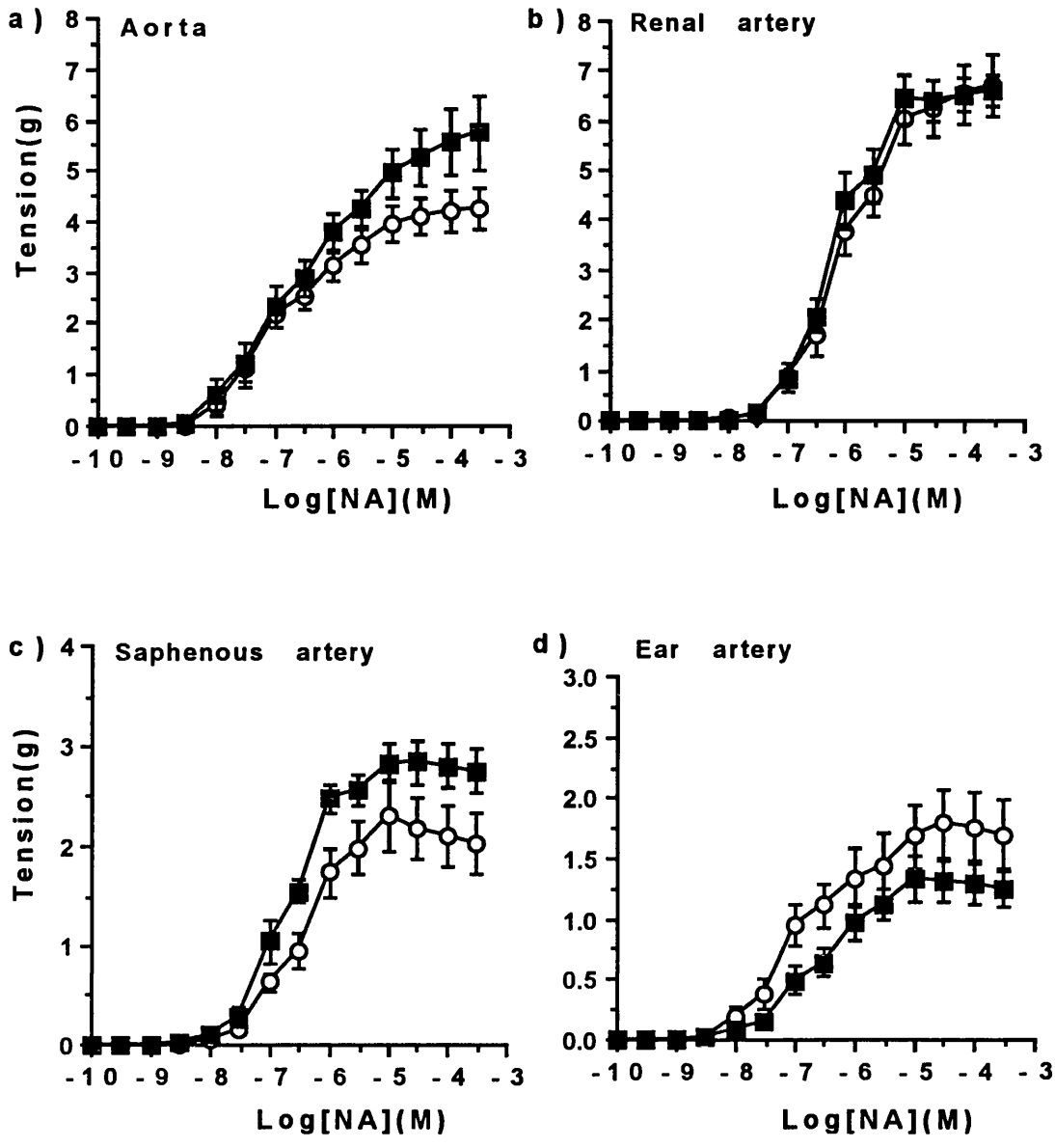


Figure 4.22

First CCRC to NA in the four isolated arteries of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (■) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

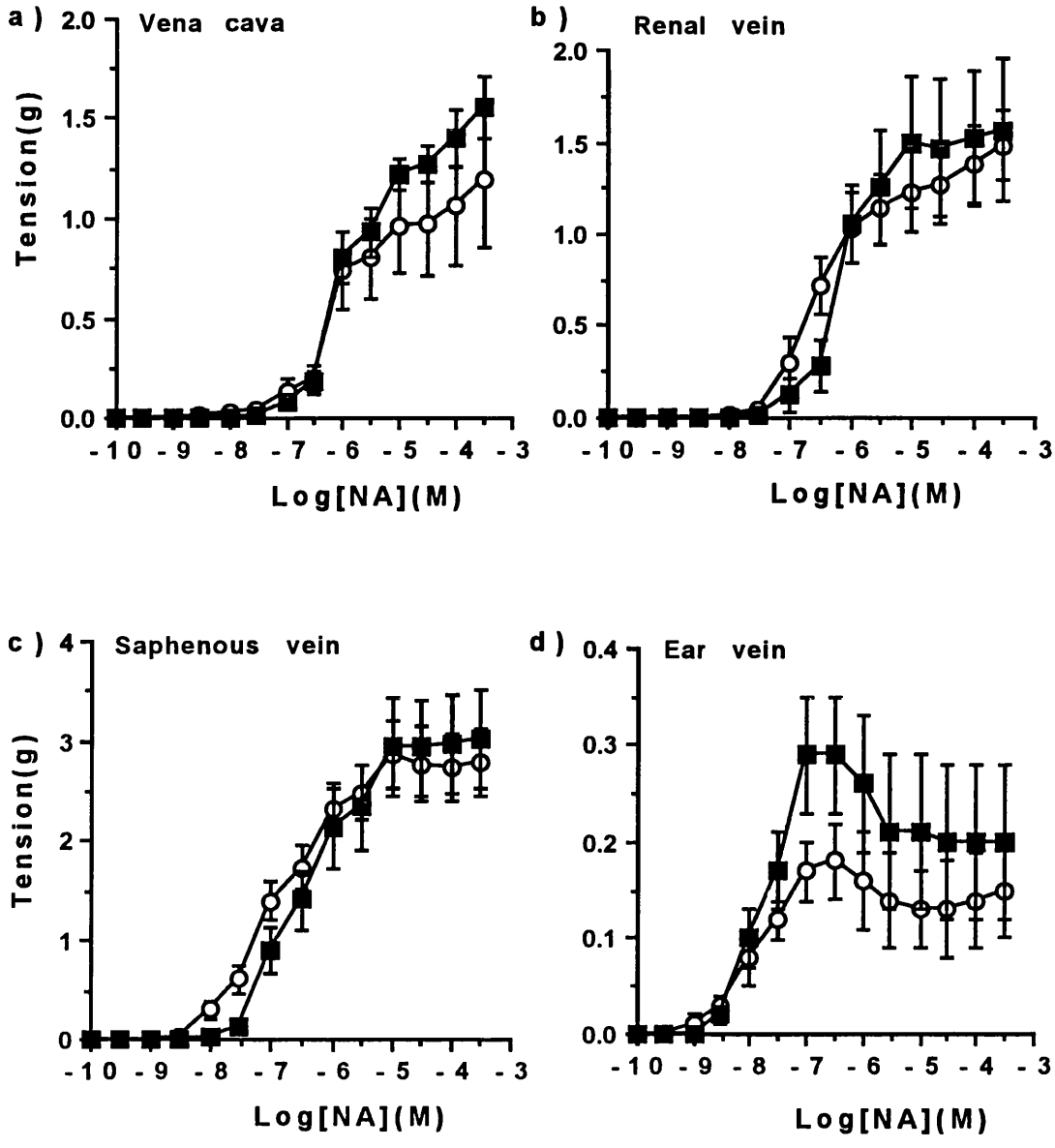


Figure 4.23

Comparison of first CCRC to NA in the four isolated veins of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (■) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

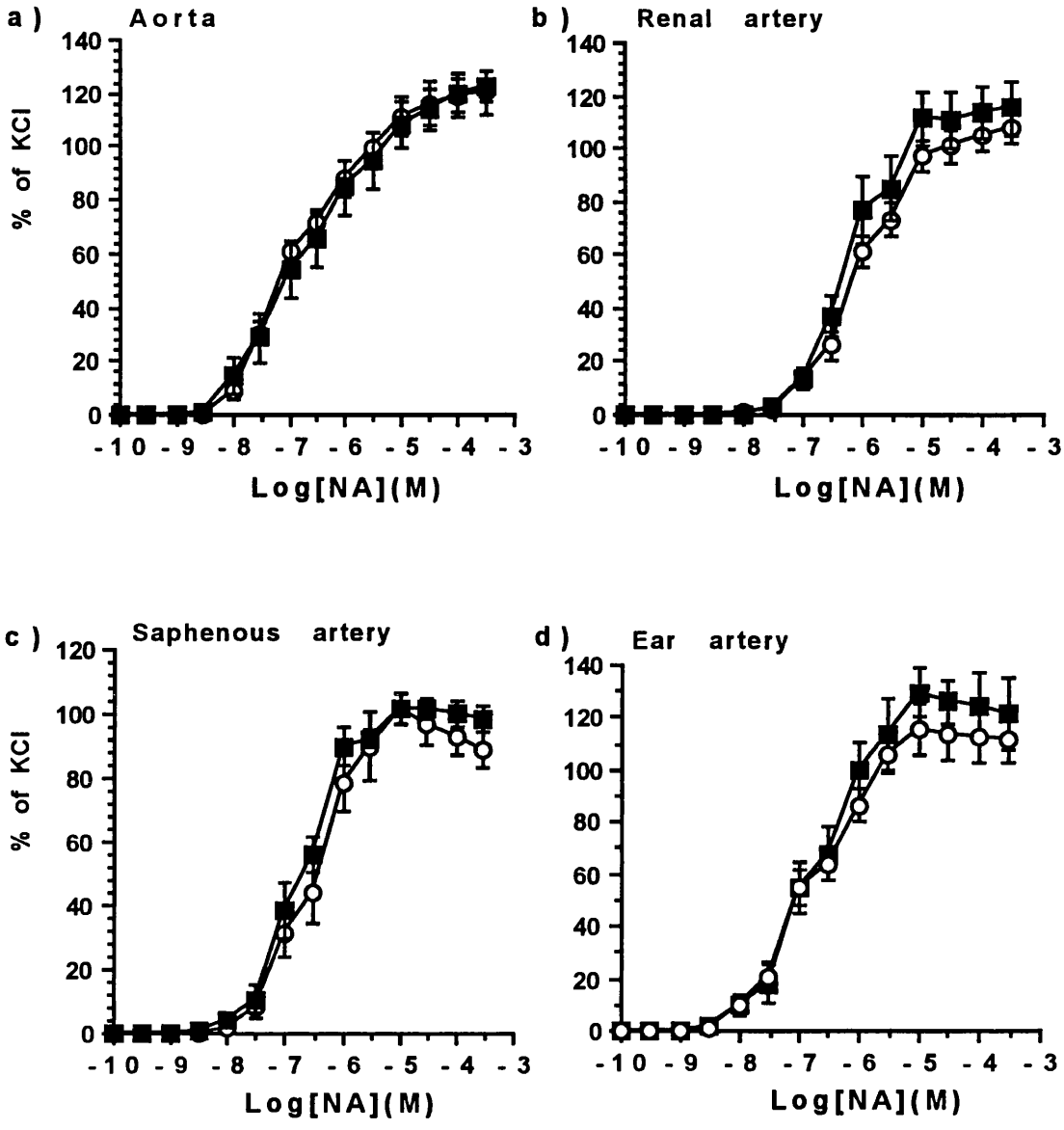


Figure 4.24

Comparison of first CCRC to NA in the four isolated arteries of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (■) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed % of the maximum response to KCl (125mM) after CCRC to NA in each preparation.

Each point represents mean ± s.e.mean (n=6).

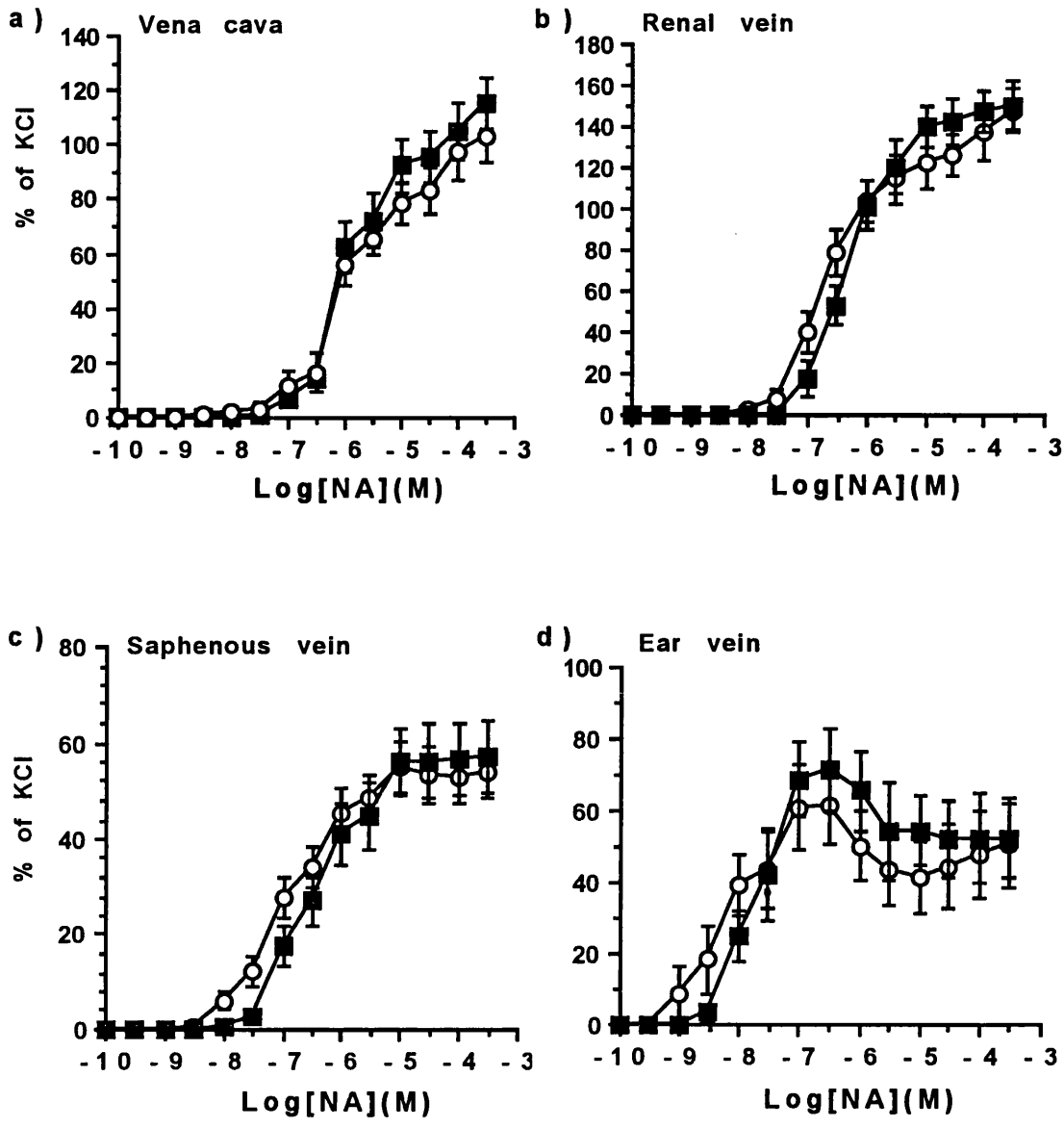


Figure 4.25

Comparison of first CCRC to NA in the four isolated veins of the sham operated (○) with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits (■) with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. Results are expressed % of the maximum response to KCl (125mM) after CCRC to NA in each preparation.

Each point represents mean ± s.e.mean (n=6).

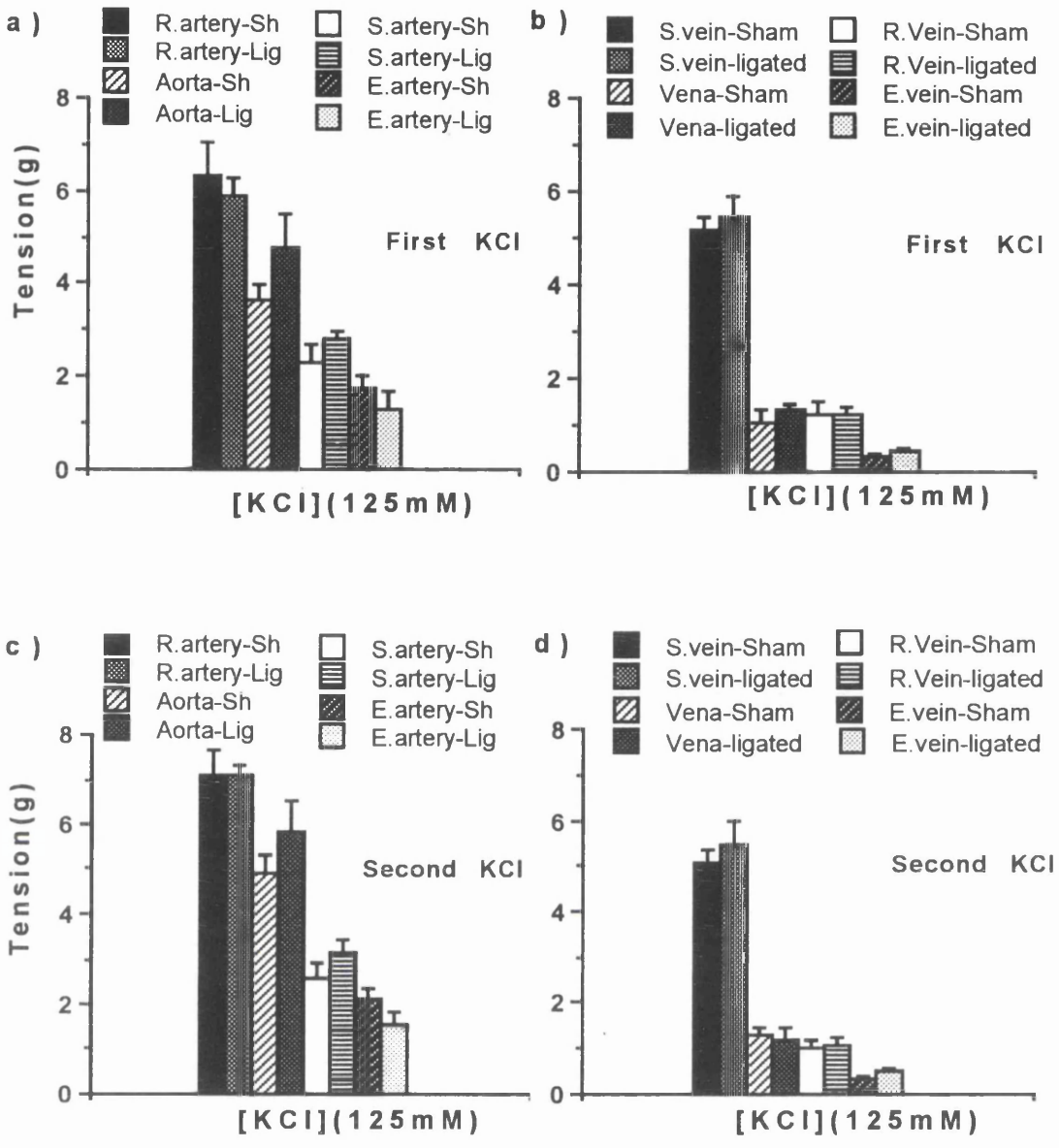


Figure 4.26

Response to KCl on the four isolated arteries and veins of the sham operated with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. **a**) response to KCl after CCRC to NA in the four arteries **b**) response to KCl after CCRC to NA in the four veins **c**) response to a second exposure to KCl after first KCl in the four arteries **d**) response to a second exposure to KCl after first KCl in the four veins.

Each point represents the mean ± s.e.mean (n=6).

Table 4.6

Vessel	pD_2 1st CCRC to NA sham	pD_2 1st CCRC to NA Ligated	pD_2 2nd CCRC to NA sham	pD_2 2nd CCRC to NA Ligated	pD_2 3rd CCRC to NA +cocaine sham	pD_2 3rd CCRC to NA +cocaine Ligated
Aorta	6.96 ± 0.08	6.72 ± 0.28	7.09 ± 0.07	6.98 ± 0.25	6.88 ± 0.09	6.72 ± 0.18
Vena cava	6.23 ± 0.18	5.95 ± 0.15	6.33 ± 0.23	5.8 ± 0.22	6.28 ± 0.11	5.89 ± 0.23
R. artery	6.07 ± 0.08	6.11 ± 0.16	6.34 ± 0.11	6.57 ± 0.22	6.28 ± 0.1	6.48 ± 0.2
R. vein	6.46 ± 0.21	6.29 ± 0.17	6.51 ± 0.15	6.09 ± 0.1	6.39 ± 0.12	6.34 ± 0.17
S. artery	6.43 ± 0.13	6.72 ± 0.18	6.46 ± 0.08	6.69 ± 0.11	7.09 ± 0.06	7.32 ± 0.2
S.vein	6.86 ± 0.16	6.33 ± 0.17	6.44 ± 0.08	6.28 ± 0.15	7.24 ± 0.09	7.12 ± 0.17
Ear artery	7.04 ± 0.03	6.65 ± 0.2	6.34 ± 0.14	6.09 ± 0.13	6.99 ± 0.11	7.04 ± 0.1
Ear vein	7.8 ± 0.25	7.72 ± 0.13	7.89 ± 0.16	7.77 ± 0.14	7.79 ± 0.1	7.57 ± 0.12

Comparison of NA pD_2 expressed as the -log of the EC_{50} (concentration producing 50% of the maximum response) of NA in four pairs of arteries and veins of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2. Initially all tissues were exposed to cumulative concentration of NA (1 nM-300μM). Following washout, all preparations were contracted with KCl (125mM). Following washout, tissues were contracted again with KCl (125mM). Following washout, preparations exposed cumulatively to NA. Following washout, the preparations contracted with NA cumulatively in presence of cocaine (1μM) that added 10-15 before to inhibit neuronal uptake of NA.

Each point represents mean ± s.e.mean.(n=6).

Table 4.7

Effect of cocaine (1μM) on CCRC to NA in different preparations of the coronary ligated and sham operated rabbits that show significant difference.

preparation		pD_2 of CCRC to NA	pD_2 of CCRC to NA + Cocaine
ear artery	sham	6.34 ± 0.14	6.99 ± 0.11 **
ear artery	Ligated	6.09 ± 0.13	7.04 ± 0.1 **
saphenous vein	sham	6.44 ± 0.08	7.24 ± 0.09 ***
saphenous vein	Ligated	6.28 ± 0.15	7.12 ± 0.17 ***
saphenous artery	sham	6.46 ± 0.08	7.09 ± 0.06 *
saphenous artery	Ligated	6.69 ± 0.11	7.32 ± 0.2 *

Comparison of pD_2 that is expressed as the -log of the EC_{50} (concentration producing 50% of the maximum response) of NA in the presence and absence of cocaine (1μM) in ear artery and saphenous vein or artery of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2.

Data are expressed as mean ± s.e.mean (n=6). Statistical comparisons with controls were carried out using paired Student's t-test, * $p<0.05$, ** $0.01<p<0.001$, *** $p<0.001$.

Table 4.8

Vessel	Maximum response 1st CCRC to NA **sham**	Maximum rresponse 1st CCRC to NA **Ligated**	Maximum response 2ndCCRC to NA **sham**	Maximum response 2ndCCRC to NA **ligated**	Maximum response 3rd CCRC to NA +cocaine **sham**	Maximum response 3rd CCRC to NA +cocaine **Ligated**
Aorta	4.26 ± 0.36	5.75 ± 0.73 *	6.4 ± 0.54	6.44 ± 1.01	5.89 ± 0.57	7.05 ± 0.81 *
Vena cava	1.19 ± 0.33	1.56 ± 0.16	1.5 ± 0.3	1.74 ± 0.21	1.58 ± 0.24	1.82 ± 0.18
R. artery	6.71 ± 0.61	6.65 ± 0.32	7.61 ± 0.62	7.07 ± 0.49	6.25 ± 0.67	5.73 ± 0.32
R. vein	1.48 ± 0.19	1.6 ± 0.38	1.54 ± 0.26	2.08 ± 0.73	1.48 ± 0.23	1.75 ± 0.37
S. artery	2.34 ± 0.34	2.91 ± 0.19	2.53 ± 0.41	3.35 ± 0.3	2.34 ± 0.44	2.76 ± 0.24
S.vein	2.92 ± 0.34	3.1 ± 0.49	3.32 ± 0.43	3.63 ± 0.53	3.09 ± 0.41	3.53 ± 0.5
ear artery	1.79 ± 0.29	1.37 ± 0.19	2.2 ± 0.34	1.72 ± 0.31	2.05 ± 0.32	1.5 ± 0.33
ear vein	0.2 ± 0.04	0.31 ± 0.06	0.45 ± 0.07	0.46 ± 0.05	0.28 ± 0.03	0.41 ± 0.09

Comparison of maximum response expressed as tension (g) of NA in four pairs of arteries and veins of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2. Initially all tissues were exposed to cumulative concentration of NA (1nM-300μM). Following washout, then all preparations were contracted with KCl (125mM). Following washout, again, tissues were contracted with KCl (KCl, 125mM). Following washout, preparations exposed cumulatively to NA. Following washout, the preparations contracted with NA cumulatively in presence of cocaine (1μM) that added 10-15 before to inhibit neuronal uptake of NA. Maximum response is expressed as tension (g).

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired student's t-test.

Table 4.9

Vessel	Maximum response (g) First KCl sham	Maximum response (g) First KCl Ligated	Maximum response (g) Second KCl sham	Maximum response (g) Second KCl Ligated
Aorta	3.61 ± 0.37	4.76 ± 0.71	4.9 ± 0.4	5.8 ± 0.72
Vena cava	1.07 ± 0.26	1.32 ± 0.12	1.27 ± 0.18	1.18 ± 0.31
Renal artery	6.34 ± 0.7	5.86 ± 0.42	7.09 ± 0.59	7.06 ± 0.24
Renal vein	1.23 ± 0.29	1.25 ± 0.15	1.04 ± 0.17	1.09 ± 0.15
Saphenous artery	2.31 ± 0.37	2.79 ± 0.19	2.59 ± 0.35	3.15 ± 0.26
Saphenous vein	5.17 ± 0.3	5.93 ± 0.45	5.06 ± 0.31	5.48 ± 0.48
Ear artery	1.76 ± 0.26	1.29 ± 0.37	2.09 ± 0.27	1.55 ± 0.28
Ear vein	0.32 ± 0.06	0.47 ± 0.03	0.36 ± 0.06	0.5 ± 0.04

Comparison of contractions to KCl (125mM) in four pairs of arteries and veins of the coronary ligated and sham operated rabbits after 8 weeks operation in group 2. Initially all tissues were exposed to cumulative concentration of NA (1nM-300μM). Following washout, all preparations were contracted with KCl (125mM). Following washout, tissues were contracted again with KCl (125mM). Contraction to KCl is expressed as tension (g).

Data are expressed as mean ± s.e.mean (n=6).

4.4.7 Effect of cocaine treatment

Cocaine (1μM) was used to inhibit neuronal uptake of NA in the isolated arteries and veins. All the tissues tested from sham, only in ear artery and saphenous artery or vein was the 3rd CRC to NA i.e. in the presence of cocaine, shifted to the left indicating increased sensitivity to NA. In the saphenous vein and ear artery there occurred despite a fall in sensitivity between the first and second tests in the absence of cocaine. In the ear artery cocaine allowed recovery to a value not unexpectedly different from the first test, but in saphenous vein the test in cocaine exceeded even in value of the first test (This also occurred in arteries, although no change occurred between the first two tests). These effects (and non-effect) of cocaine were reduced similarly in both sham and 8 weeks ligated rabbits (Table 4.7).

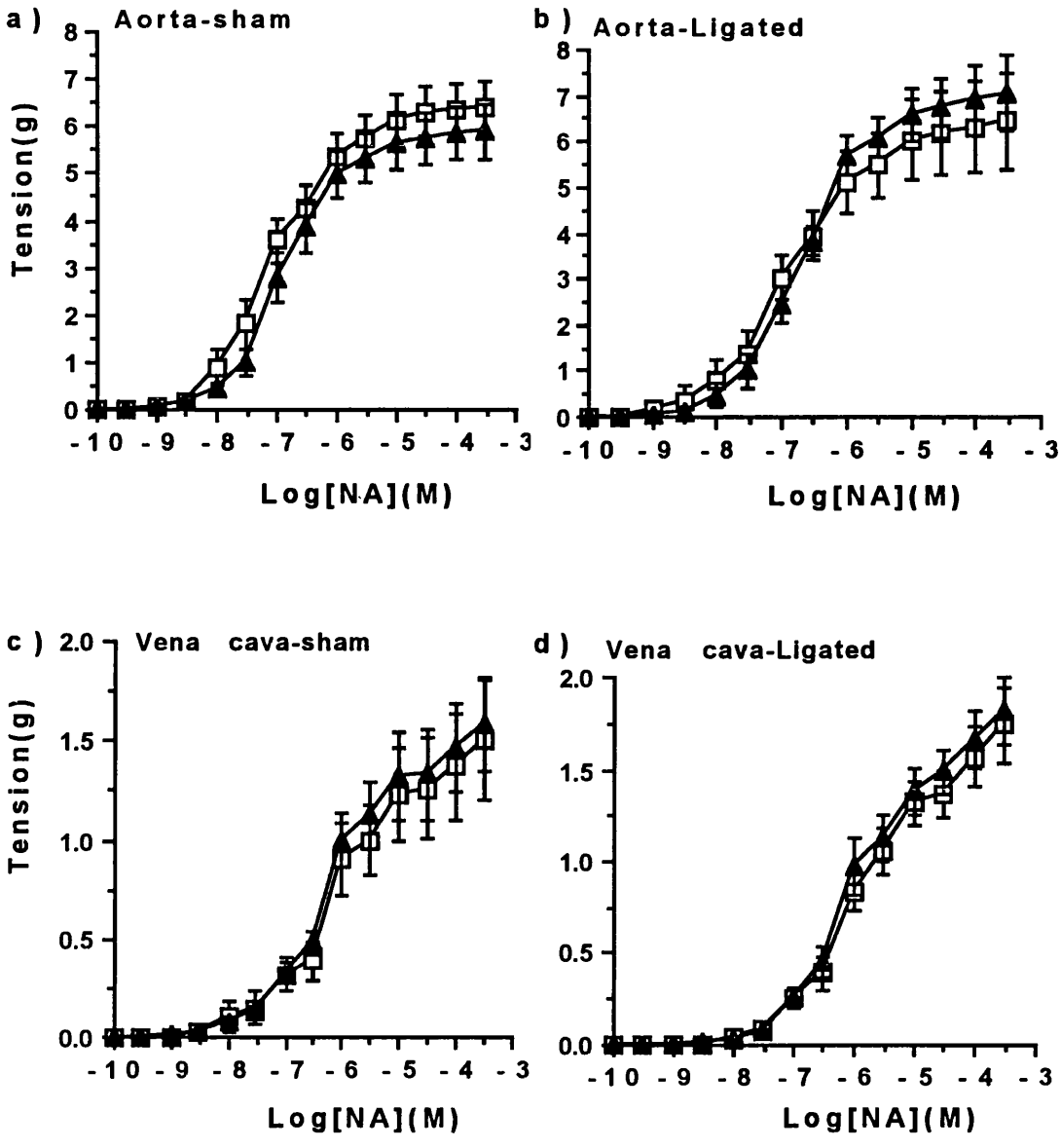


Figure 4.27

CCRC to NA and effect of cocaine (1μM) in the isolated aorta and vena cava of the sham operated rabbits with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. The CCRC to NA after 2 KCl (125mM) represented by (□) while (▲) represents CCRC to NA in presence of (1μM) cocaine.

Each point represents mean ± s.e.mean (n=6).

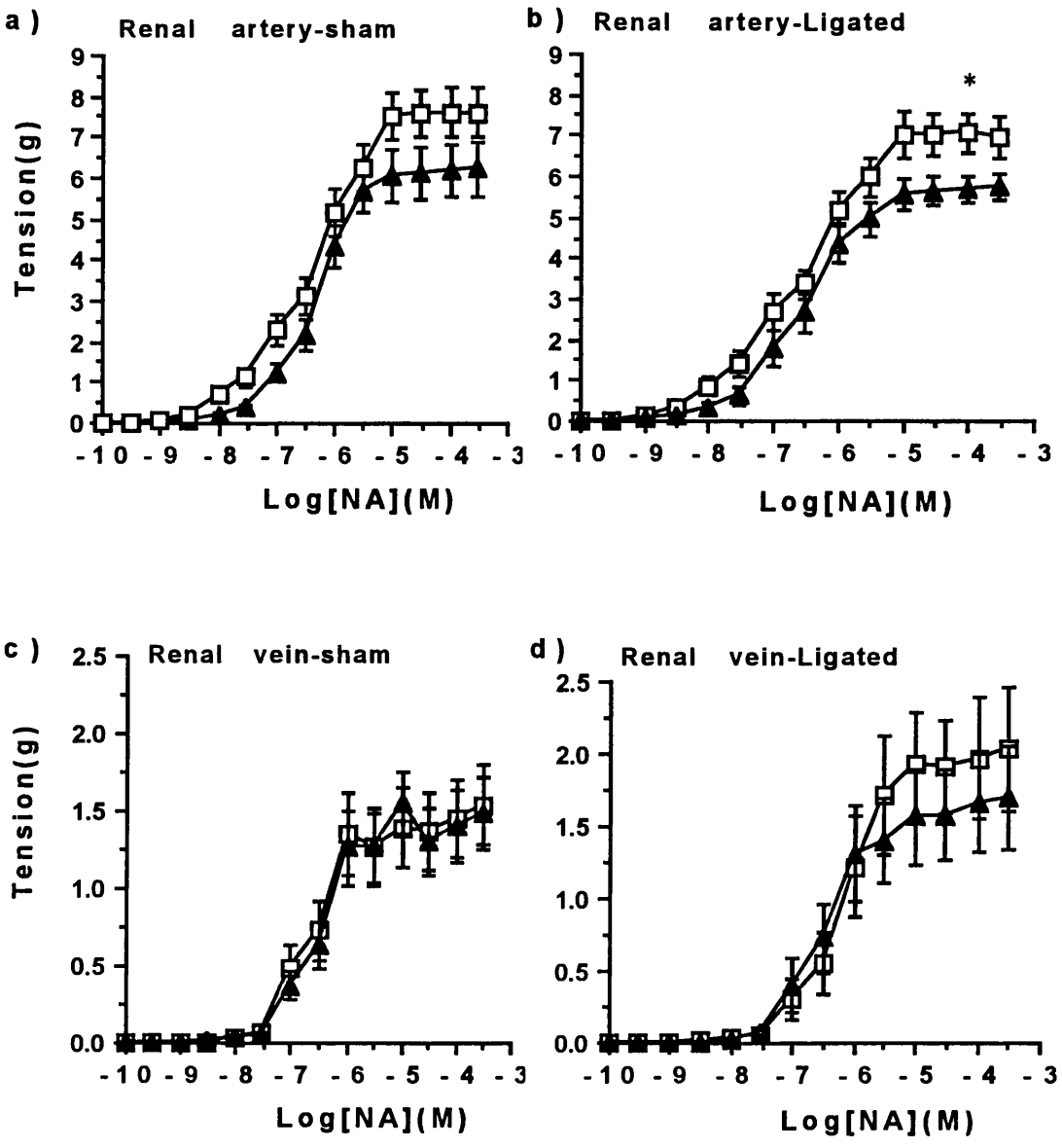


Figure 4.28

CCRC to NA and effect of cocaine (1μM) in the isolated renal artery and renal vein of the sham operated rabbits with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. The CCRC to NA after 2 KCl (125mM) represented by (□) while (▲) represents CCRC to NA in presence of (1μM) cocaine.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, paired Student's t-test.

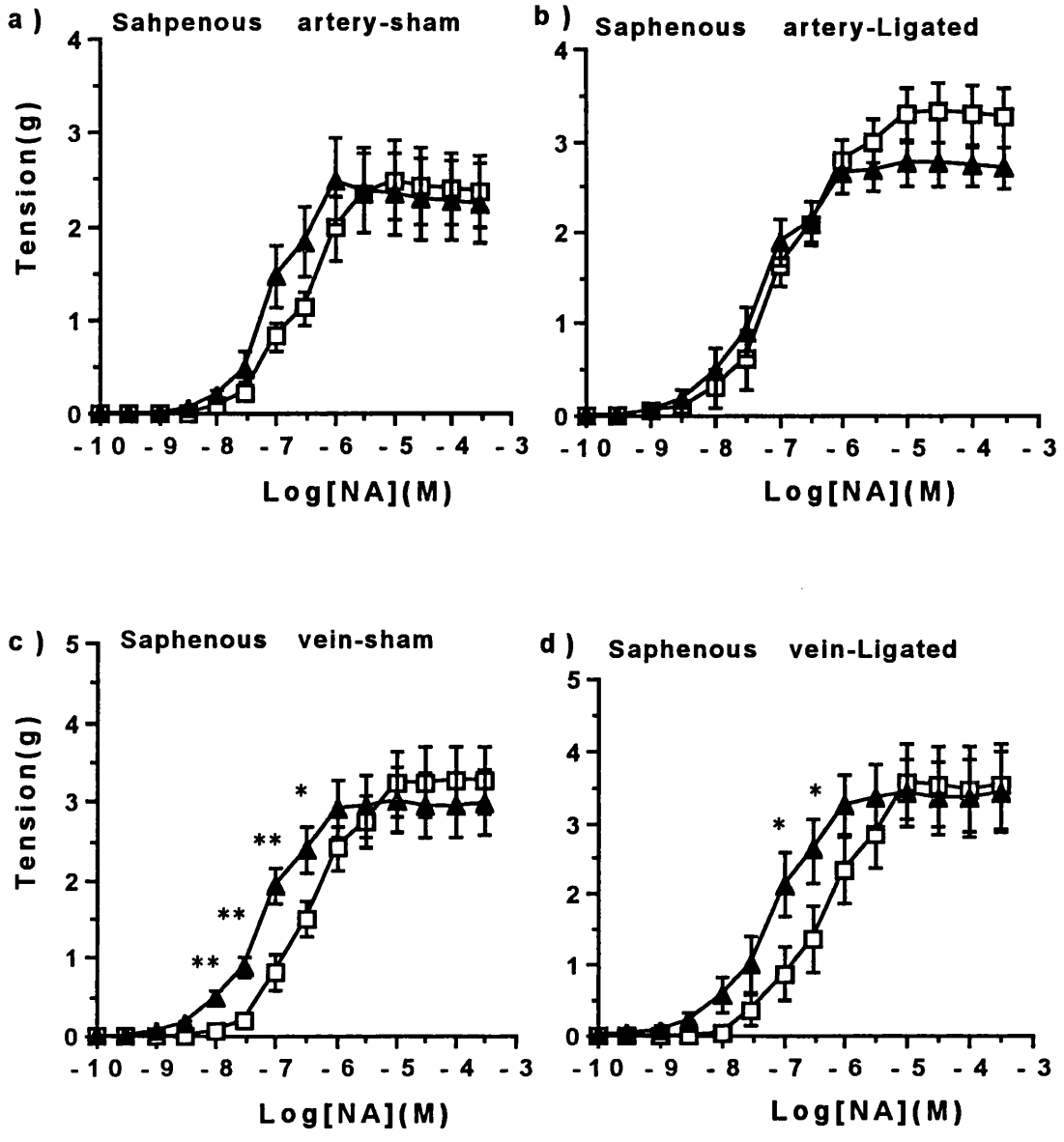


Figure 4.29

CCRC to NA and effect of cocaine (1μM) in the isolated saphenous artery and saphenous vein of the sham operated rabbits with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. The CCRC to NA after 2 KCl (125mM) represented by (□) while (▲) represents CCRC to NA in presence of (1μM) cocaine.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, ** $0.001<p<0.01$, paired Student's t-test.

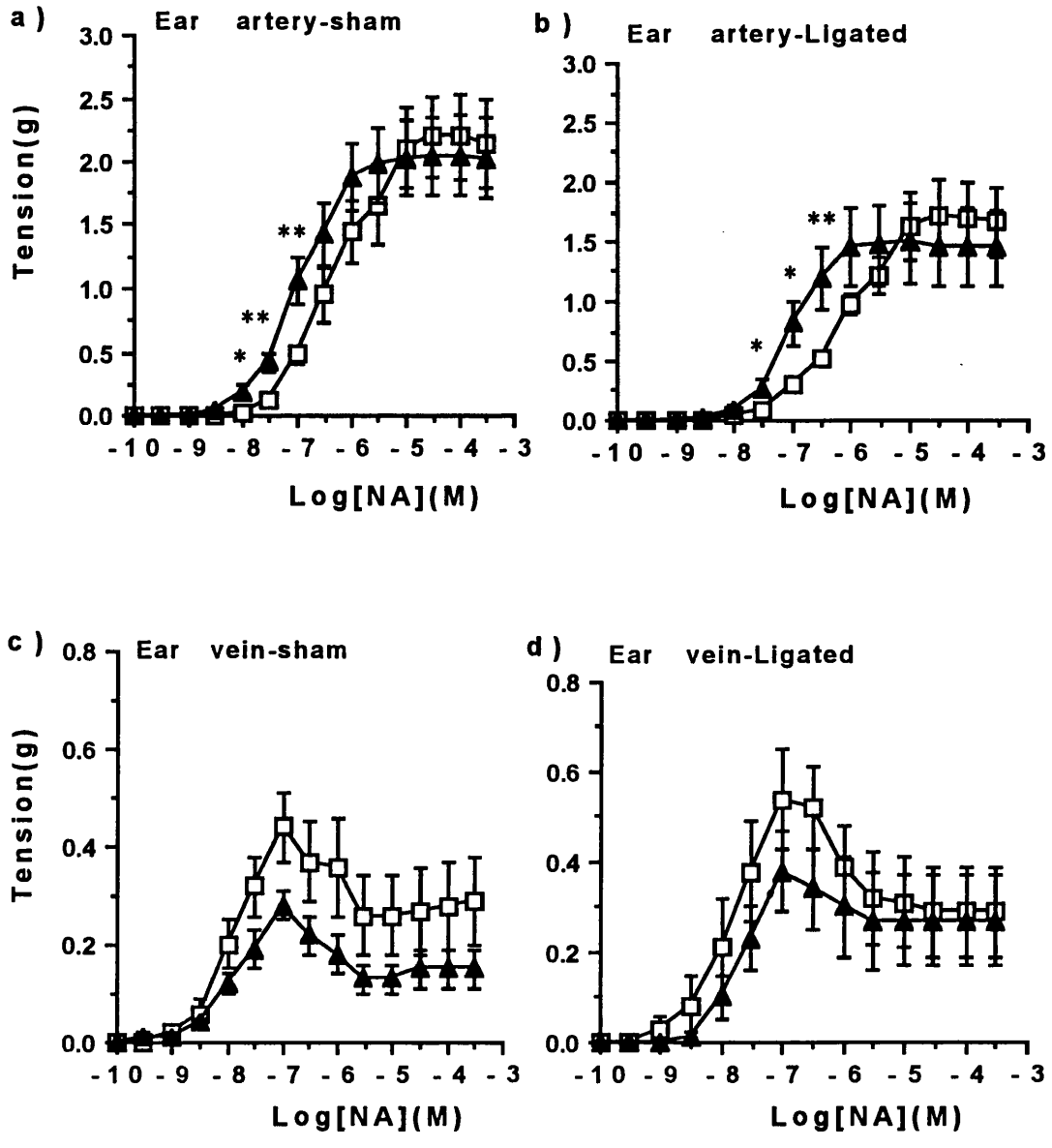


Figure 4.30

CCRC to NA and effect of cocaine (1μM) in the isolated ear artery and ear vein of the sham operated rabbits with mean ejection fraction of (70.5 ± 2.13) and coronary ligated rabbits with mean ejection fraction of (46.5 ± 4.4) after 8 weeks operation in group 2. The CCRC to NA after 2 KCl (125mM) represented by (□) while (▲) represents CCRC to NA in presence of (1μM) cocaine.

Each point represents mean ± s.e.mean. (n=6). Statistically significant differences are represented by * $p<0.05$, ** $0.001<p<0.01$, paired Student's t-test.

Staining with glyoxylic acid technique (Figure 1-8)

Sympathetic nerves axons and varicoses demonstrated using the glyoxylic acid technique (Bjorklund *et al.*, 1972 and Dr. Ian Montgomery as personal communication), were observed on the four pairs of arteries and veins of rabbit as a network of bright blue fluorescence.

Figure 1

Aorta: sparse innervation at media/adventitia junction, occassionally in the outer media.

Figure 2

Renal artery: less sparse innervation with only a few varicosities at the media/adventitia junction.

Figure 3

Saphenous artery: dense innervation at adventitia/media junction.

Figure 4

Ear artery: dense innervation at adventitia/media junction and also fine axons and varicosities between smooth muscle cells of the outer media.

Figure 5

vena cava: no nerves.

Figure 6

Renal vein: sparse innervation, only 8-10 axons entering the media from the adventitia.

Figure 7

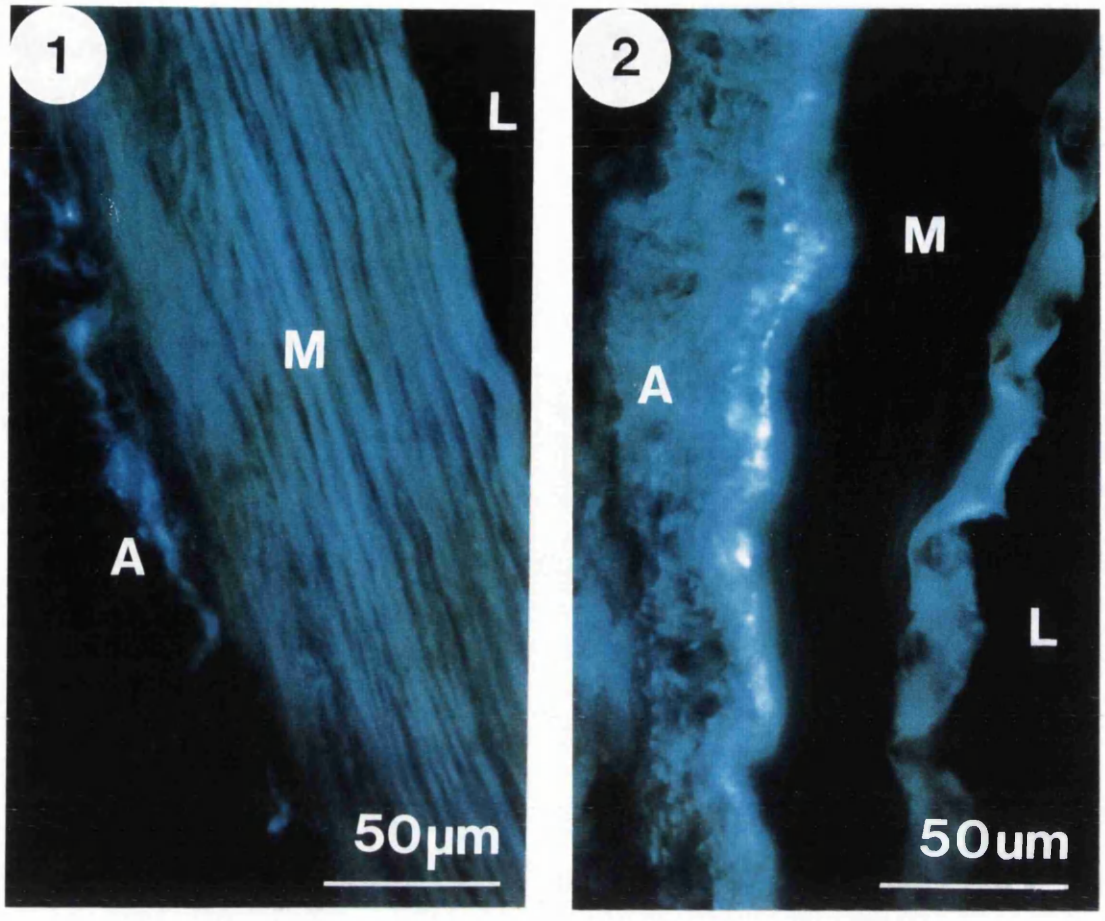
Saphenous vein: dense innrevation in media and adventitia.

Figure 8

Ear vein: very little evidence of innervation.

Key to figures

L= lumen A= adventitia M= Media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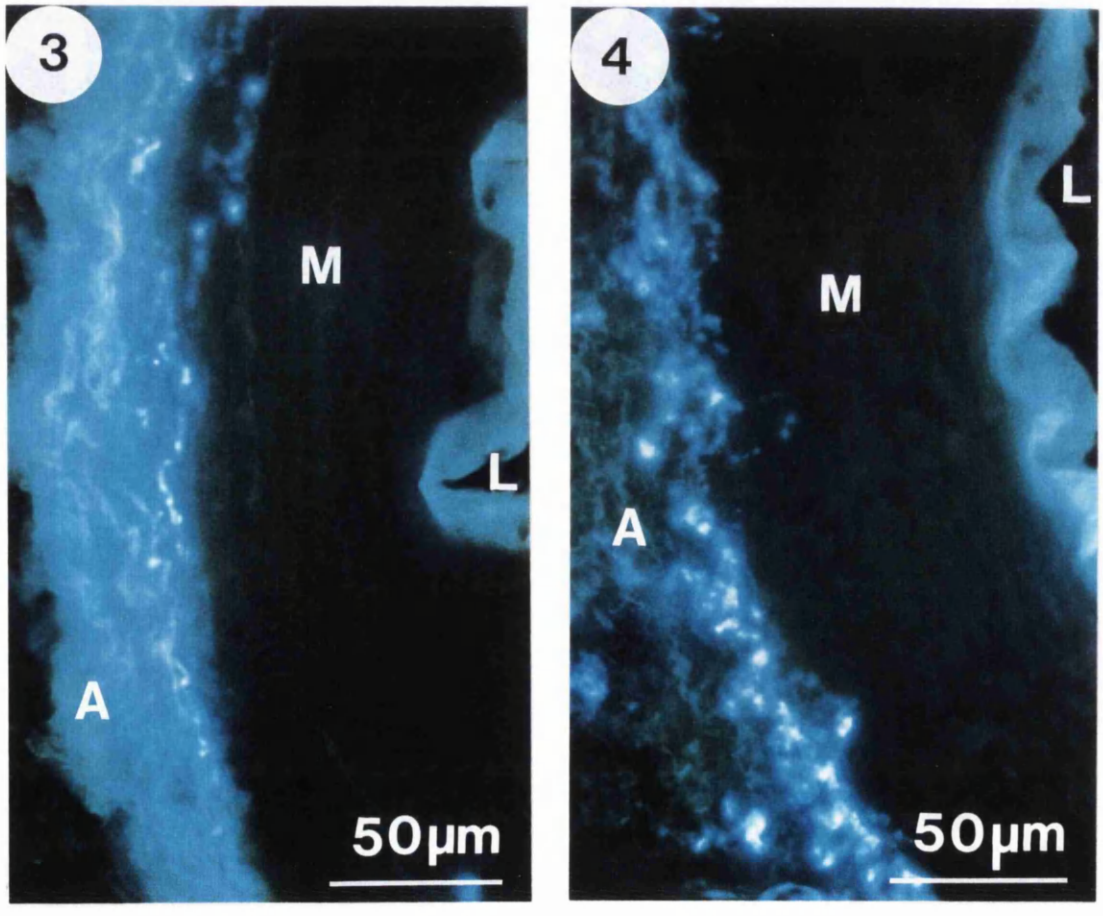
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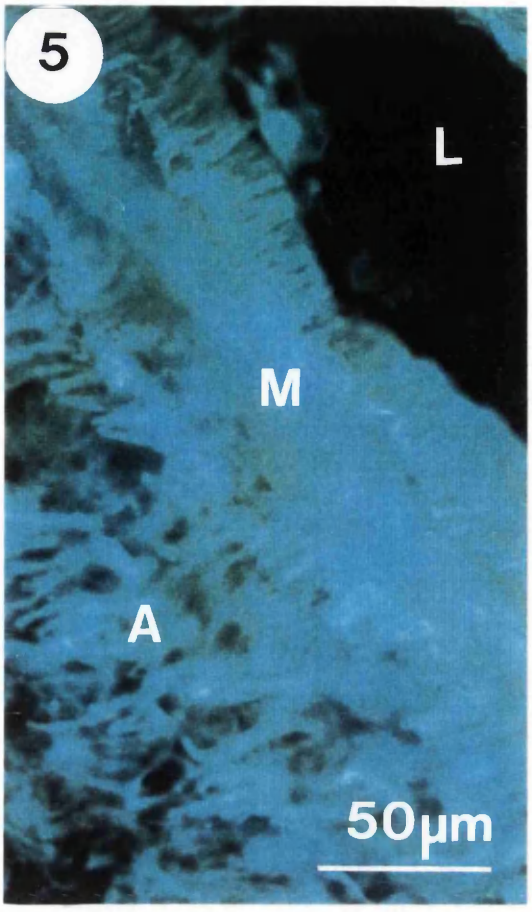
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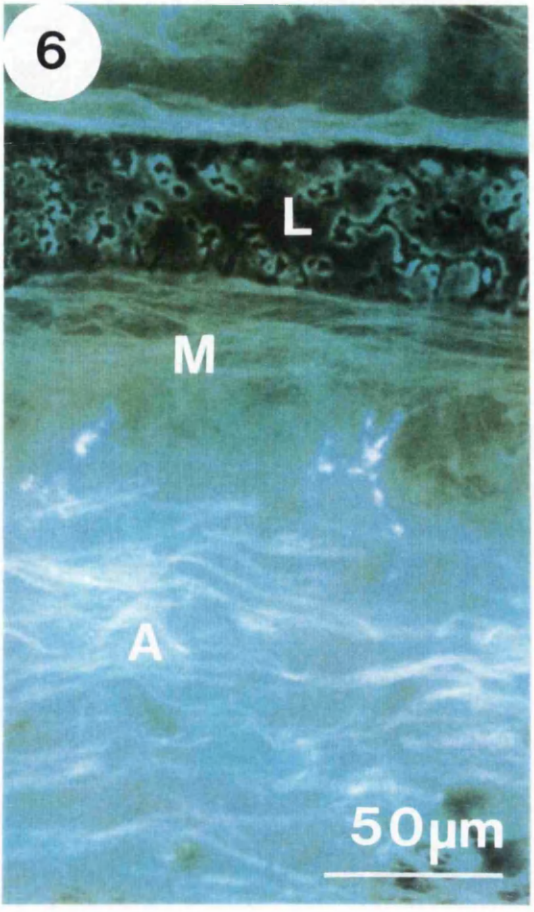
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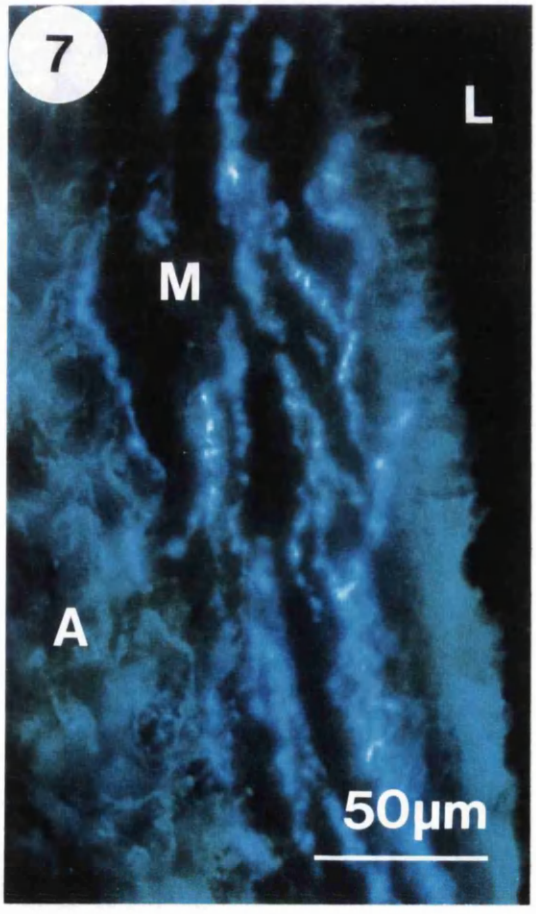
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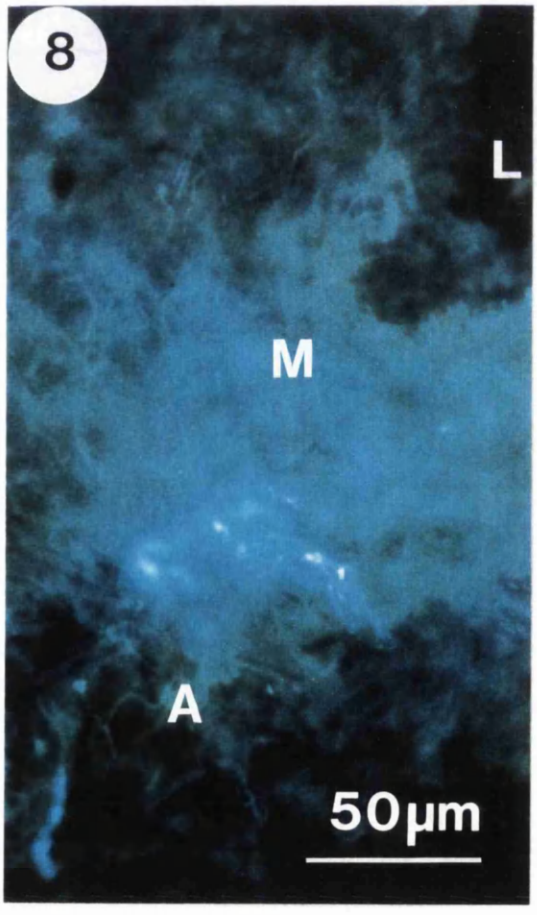
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Section 5: Results of 16 weeks coronary ligated rabbits

In sixteen weeks coronary ligated rabbits one group was studied with the same protocols as in eight weeks coronary ligated rabbits. In this group four isolated arteries and veins (aorta, vena cava, renal artery and renal vein) were investigated.

The data from this group is very similar to results obtained in 8 weeks groups. As in 8 weeks groups, we were not able to find differences in relaxation responses to ACh, SNP, ATP or adenosine and vasoconstriction responses to NA or KCl, between coronary ligated and sham operated rabbits after 16 weeks operation. Therefore, description of results of this group does not need more details (see results of 8 weeks groups).

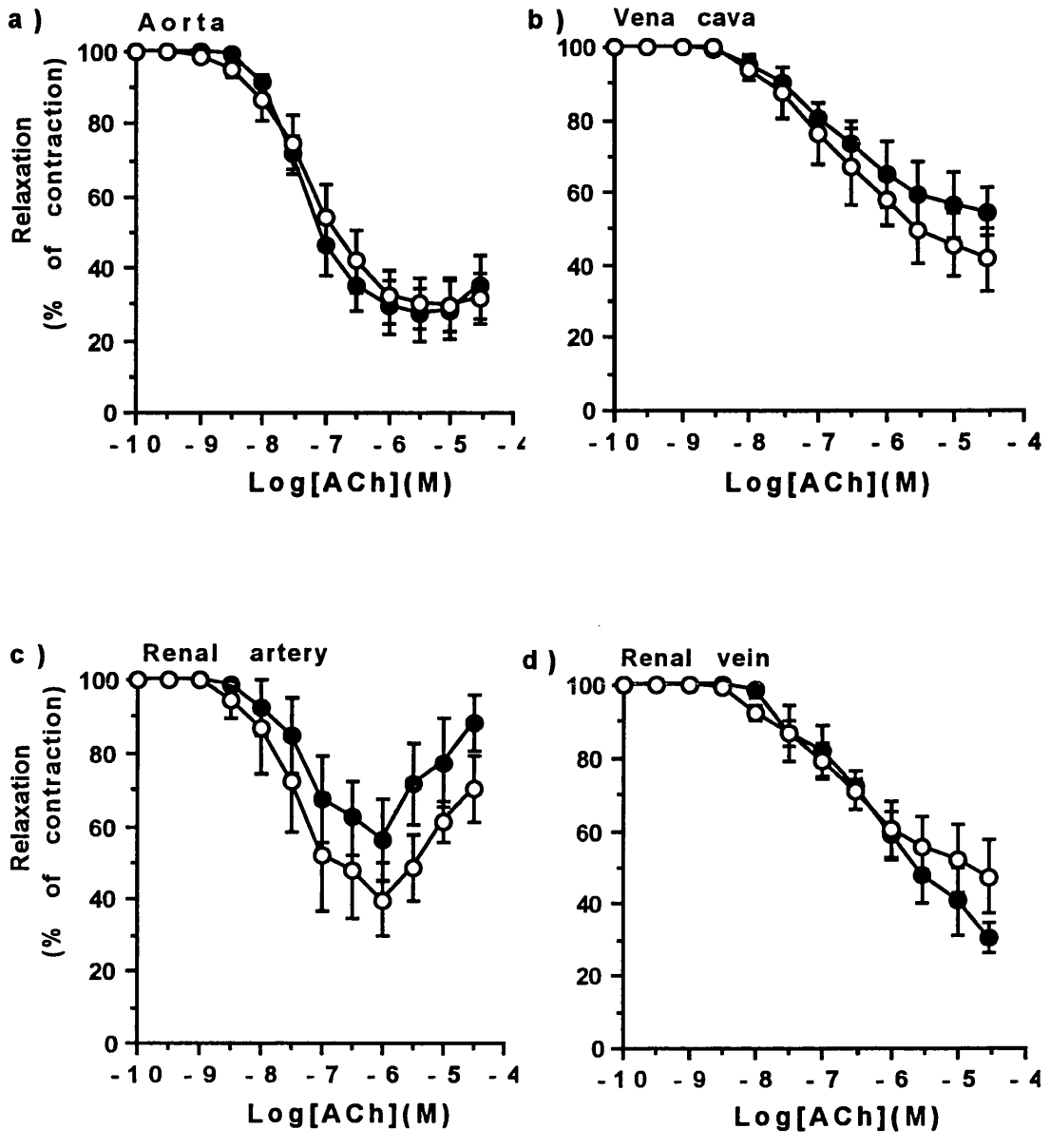

Figure 4.31

Relaxation to ACh in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (●) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

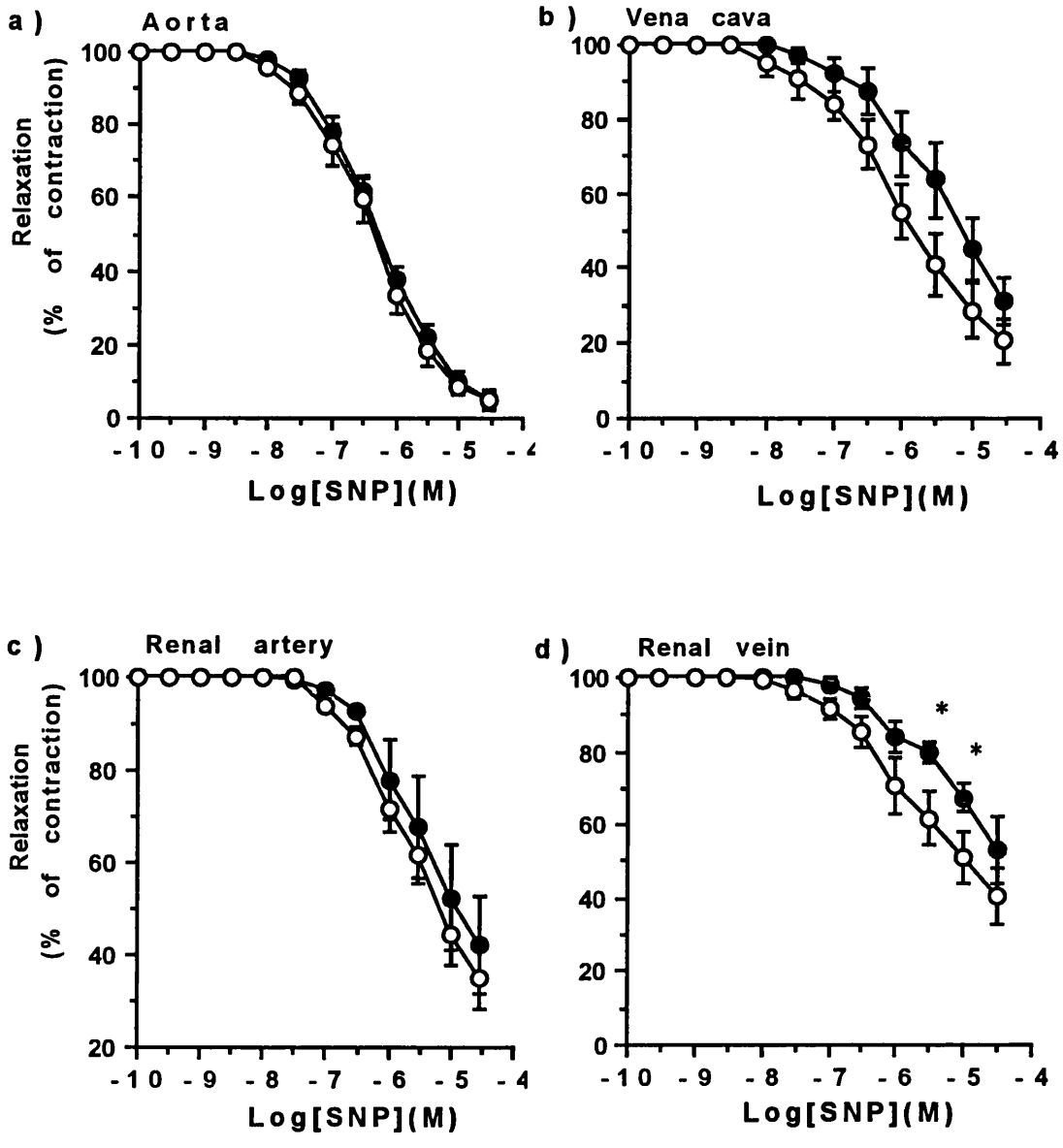

Figure 4.32

Relaxation to SNP in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (●) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. Results are expressed as % of maximum response to NA (1μM) that used for inducing tone.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired Student's t-test.

Table 4.10

Vessel	pIC_{50} (Ach) sham	pIC_{50} (Ach) Ligated	pIC_{50} (SNP) sham	pIC_{50} (SNP) Ligated
Aorta	7.24 ± 0.14	7.32 ± 0.05	6.41 ± 0.12	6.33 ± 0.07
Vena cava	6.95 ± 0.21	6.6 ± 0.19	5.84 ± 0.16	5.57 ± 0.17
Renal artery	7.33 ± 0.36	7.2 ± 0.18	5.73 ± 0.14	5.63 ± 0.15
Renal vein	6.51 ± 0.13	6.19 ± 0.11	5.64 ± 0.15	5.47 ± 0.16

Comparison of pIC_{50} expressed as the -log of the IC_{50} (concentration producing 50% of the maximum relaxation attained within the range tested) in sham and coronary ligated rabbits in response to ACh and SNP after 16 weeks operation.

Data are expressed as mean ± s.e.mean (n=6).

Table 4.11

Vessel	pIC_{25} (Ach) sham	pIC_{25} (Ach) Ligated	pIC_{25} (SNP) sham	pIC_{25} (SNP) Ligated
Aorta	7.53 ± 0.25	7.56 ± 0.08	6.99 ± 0.16	6.9 ± 0.11
Vena cava	6.93 ± 0.39	6.57 ± 0.29	6.63 ± 0.1	5.88 ± 0.3
Renal artery	7.54 ± 0.42	7.21 ± 0.33	6.05 ± 0.2	5.72 ± 0.2
Renal vein	6.7 ± 0.38	6.78 ± 0.3	6.12 ± 0.3	5.31 ± 0.14 *

Comparison of pIC_{25} expressed as the -log of the IC_{25} (concentration producing 25% of the maximum relaxation to NA (1μM) used for inducing tone) in sham and coronary ligated rabbits in response to ACh and SNP after 16 weeks operation.

Data are expressed as mean ± s.e.mean (n=6). Statistical comparisons with controls were carried out using unpaired Student's t-test * $p<0.05$.

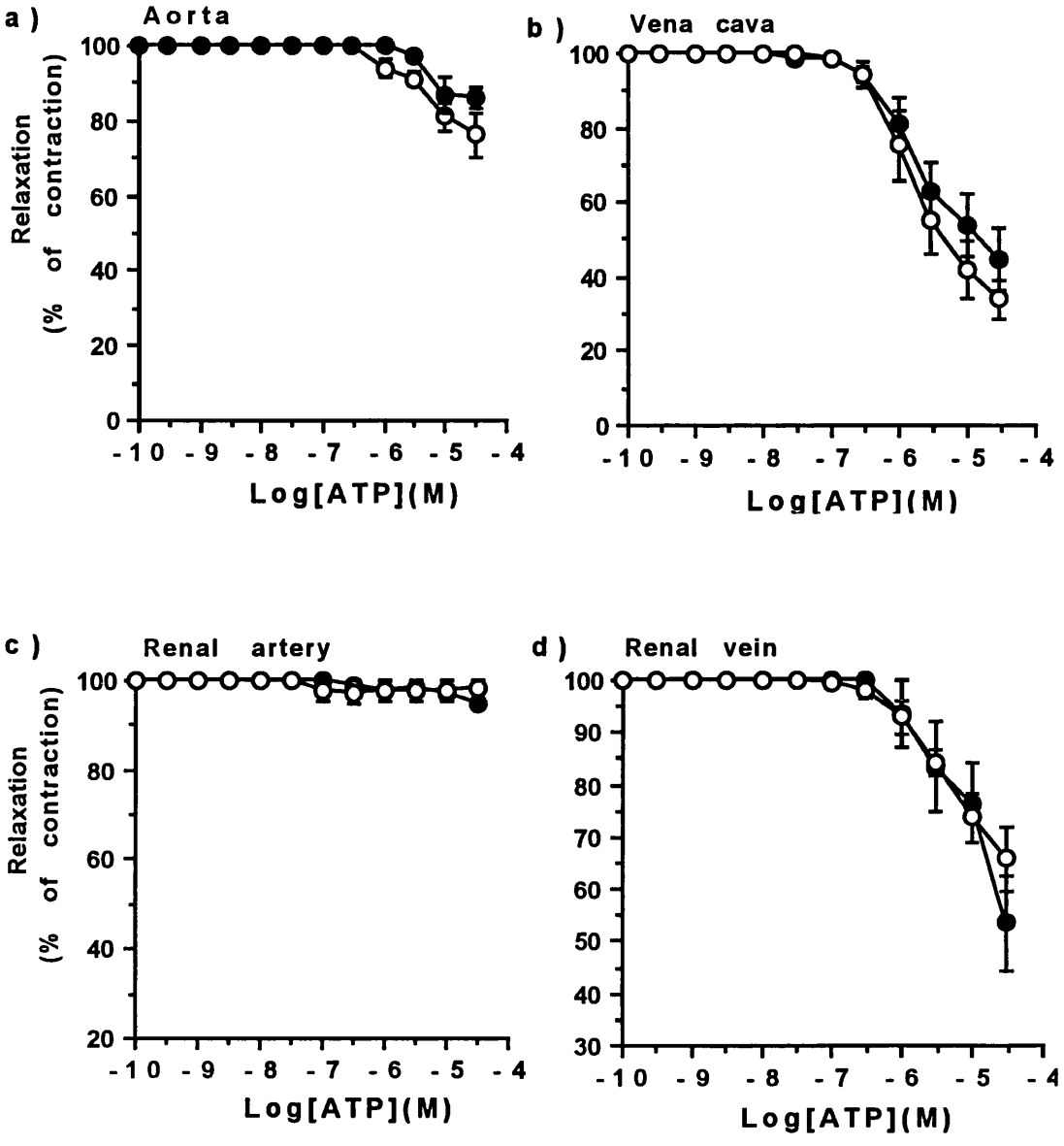

Figure 4.33

Relaxation to ATP in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (●) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

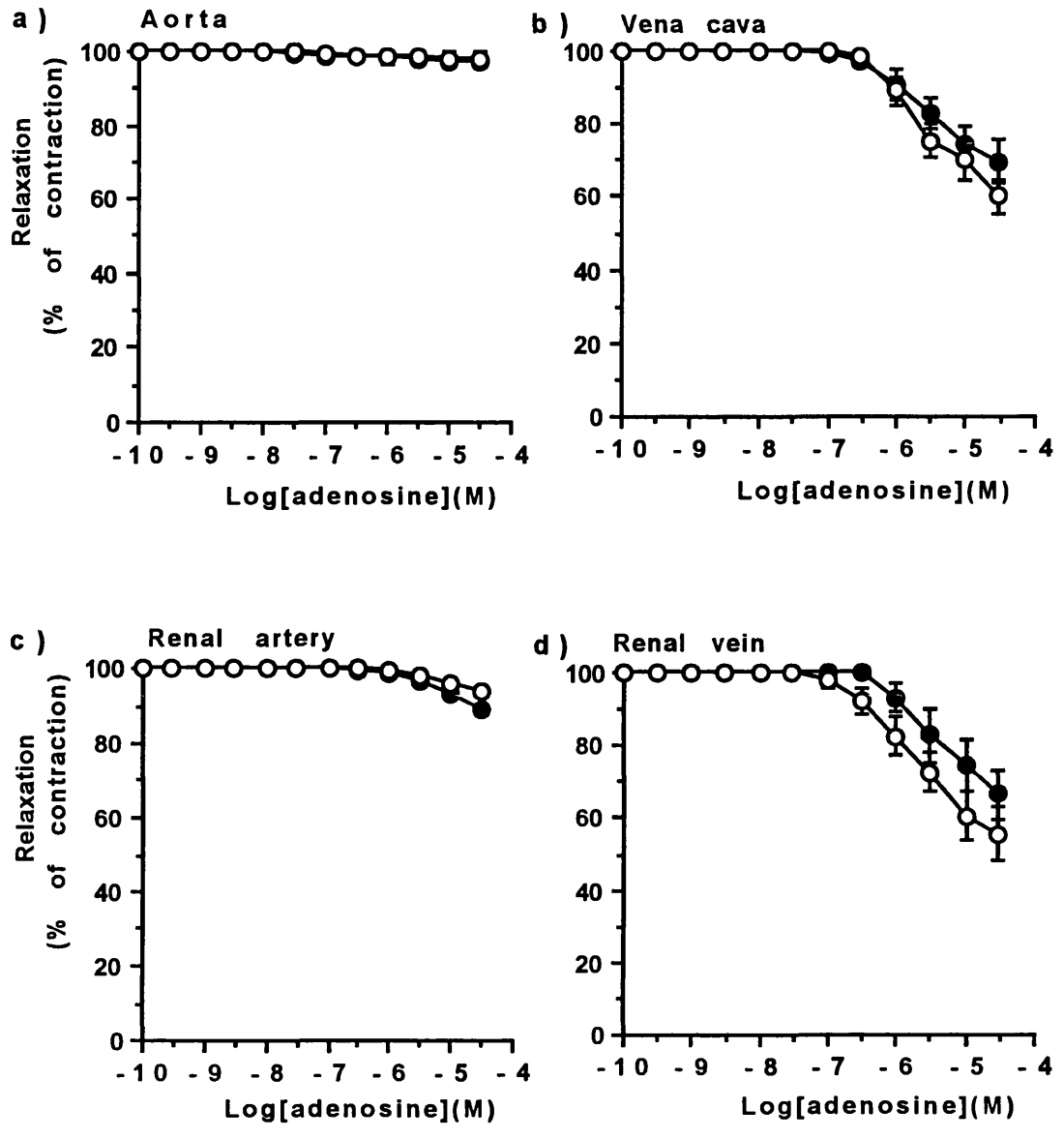

Figure 4.34

Relaxation to adenosine in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (●) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. Results are expressed as % of maximum response to NA (1μM) used for inducing tone.

Each point represents mean ± s.e.mean (n=6).

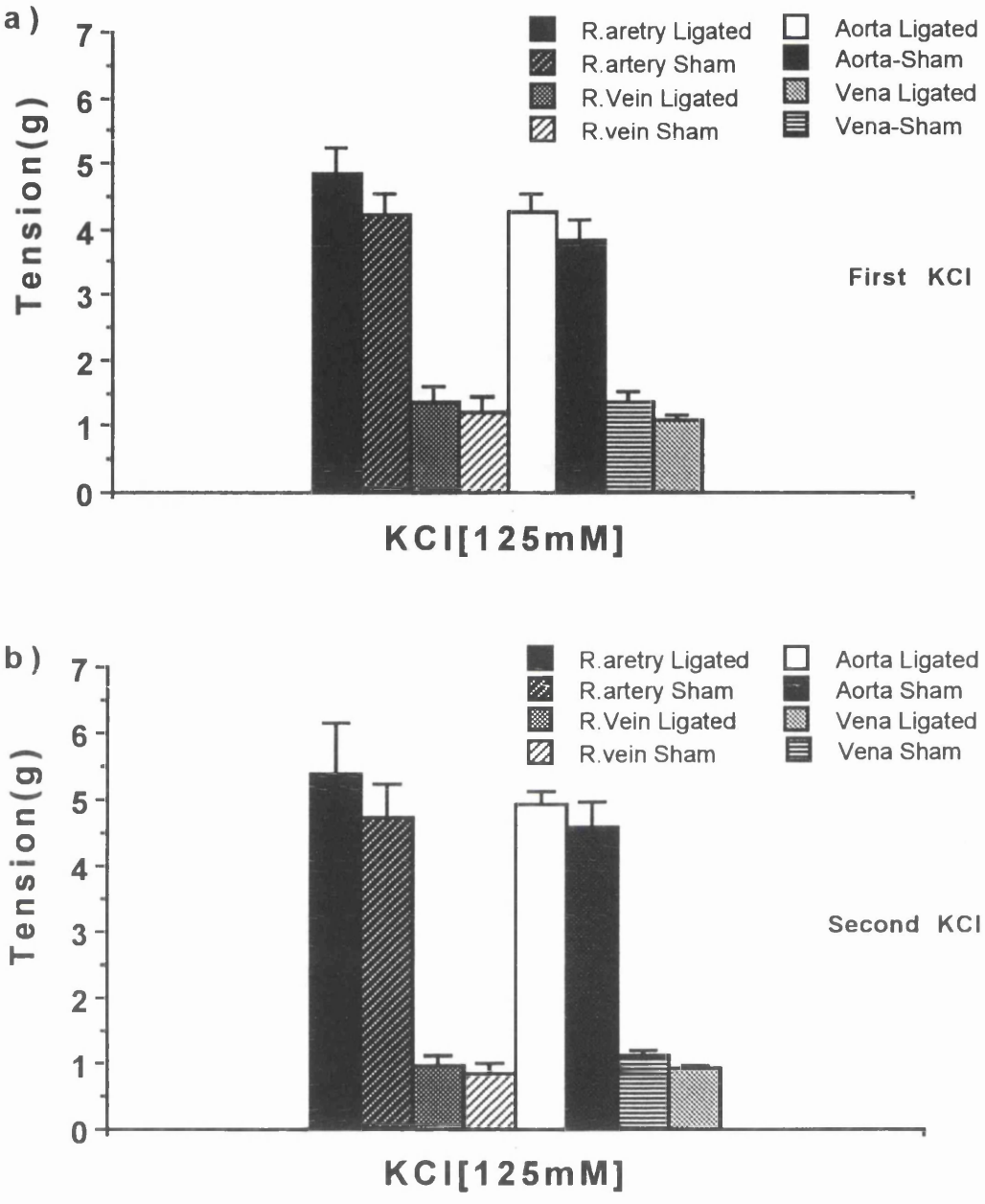

Figure 4.35

Responses to KCl in the four isolated arteries and veins of the sham operated with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (●) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. **a**) response to KCl after CCRC to NA **b**) response to KCl after first KCl.

Each point represents mean ± s.e.mean (n=6).

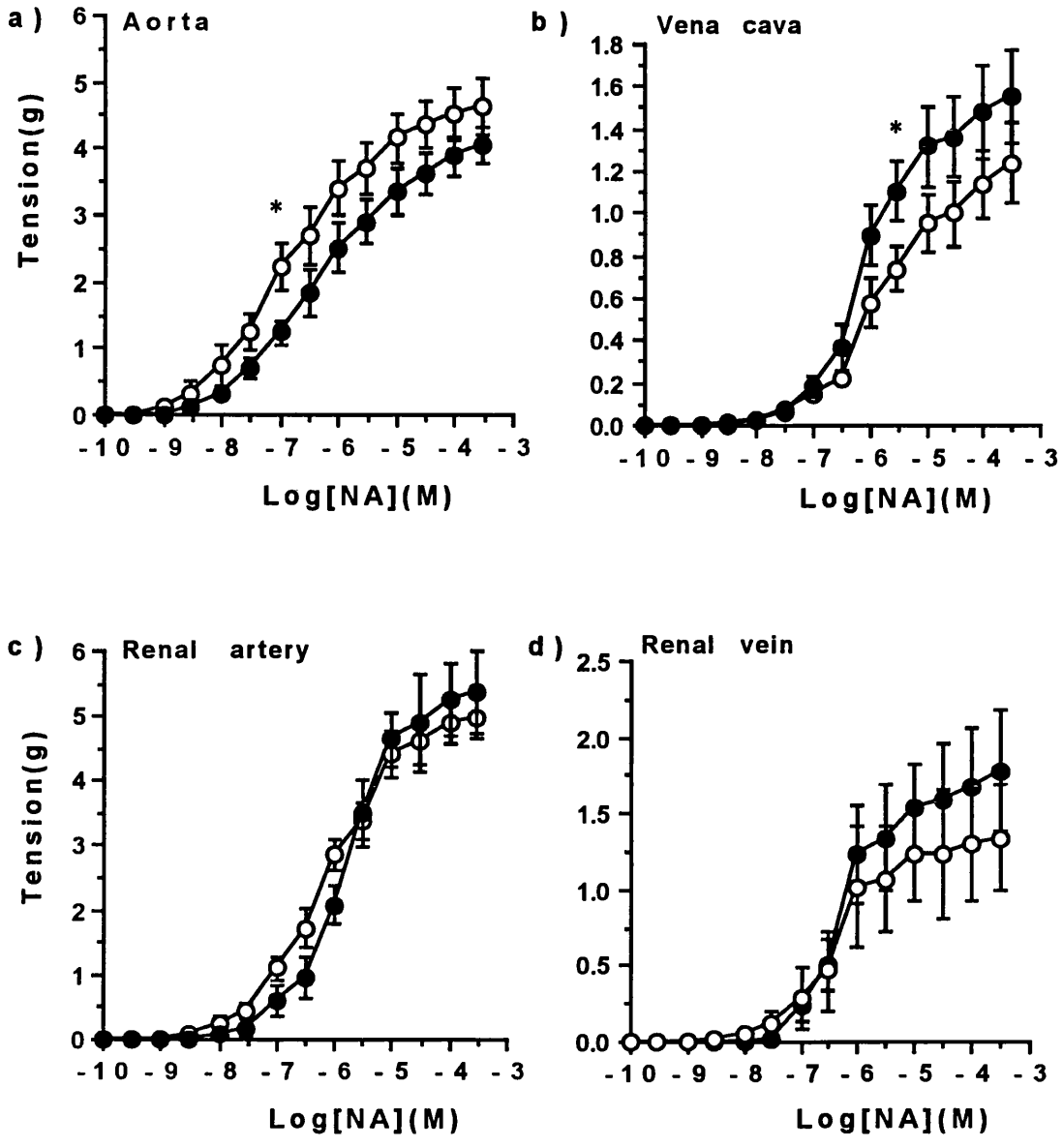

Figure 4.36

First CCRC to NA in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (●) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. Responses are expressed as tension (g).

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired Student's t-test.

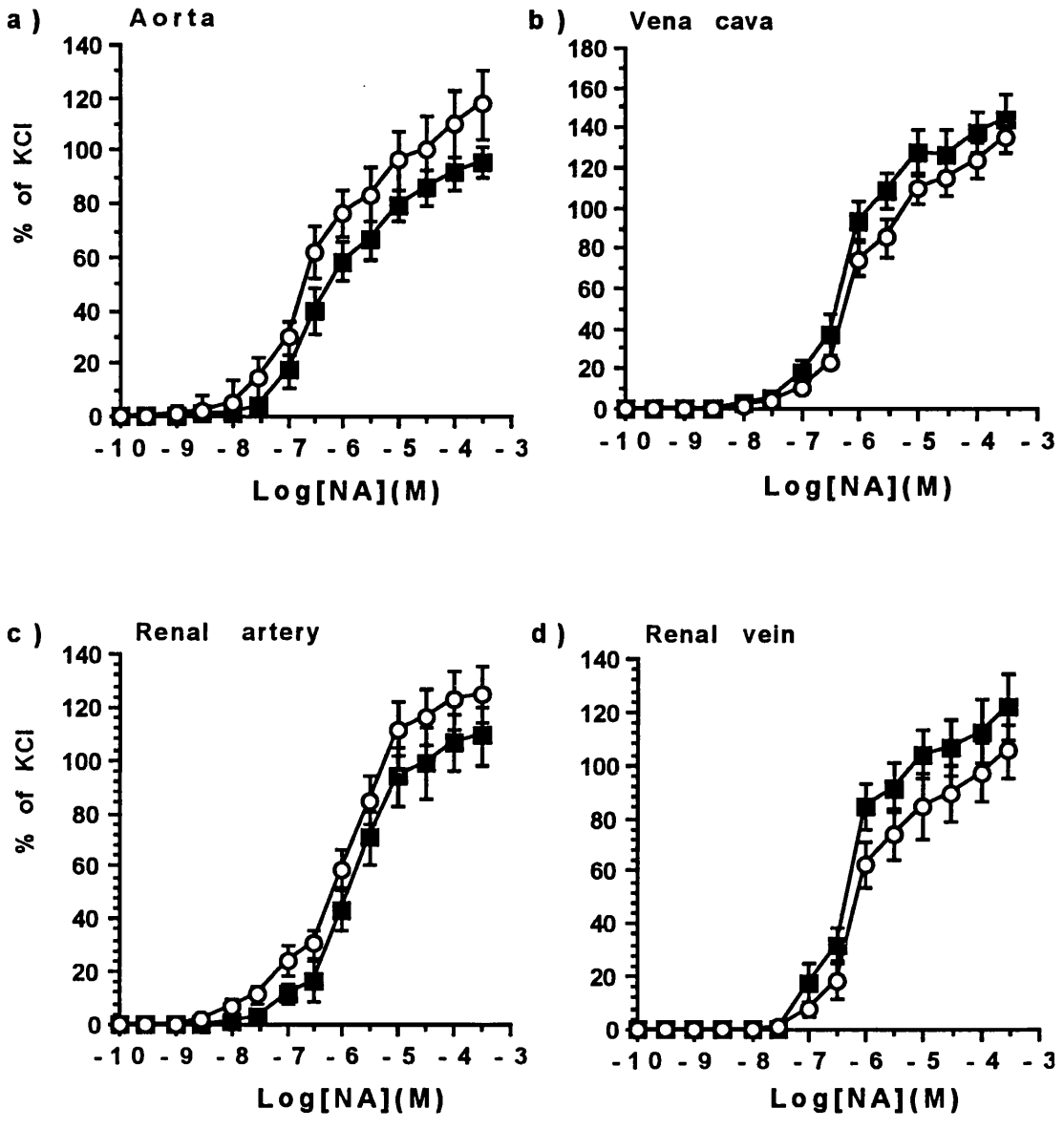

Figure 4.37

First CCRC to NA in the four isolated arteries and veins of the sham operated (○) with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits (■) with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. Results are expressed % of the maximum response to KCl (125mM) after CCRC to NA in each preparation.

Each point represents mean ± s.e.mean (n=6).

Table 4.12

Vessel	pD_2 1st CCRC to NA **sham**	pD_2 1st CCRC to NA **Ligated**	pD_2 2nd CCRC to NA **sham**	pD_2 2nd CCRC to NA **Ligated**	pD_2 3rd CCRC to NA (+cocaine) **sham**	pD_2 3rd CCRC to NA (+cocaine) **Ligated**
Aorta	6.78 ± 0.27	6.36 ± 0.22	6.85 ± 0.19	6.5 ± 0.18	6.61 ± 0.13	6.43 ± 0.12
Vena cava	5.86 ± 0.16	6.18 ± 0.14	6.06 ± 0.24	6.23 ± 0.03	5.97 ± 0.31	6.15 ± 0.14
R. artery	6.15 ± 0.11	5.69 ± 0.12	6.2 ± 0.11	6.05 ± 0.12	6.33 ± 0.12	6.23 ± 0.17
R. vein	6.06 ± 0.18	6.23 ± 0.02	6.17 ± 0.33	6.16 ± 0.06	6.08 ± 0.15	6.21 ± 0.08

Comparison of NA pD_2 expressed as the -log of the EC_{50} (concentration producing 50% of the maximum response) of NA in four arteries and veins of the coronary ligated and sham operated rabbits after 16 weeks operation. Initially all tissues were exposed to cumulative concentration of NA (1nM-300μM). Following washout, all preparations were contracted with KCl (125mM). Following washout, tissues were contracted again with KCl (125mM). Following washout, preparations exposed cumulatively to NA. Following washout, the preparations contracted with NA cumulatively in presence of cocaine (1μM) that added 10-15 before to inhibit neuronal uptake of NA.

Each point represents mean ± s.e.mean (n=6).

Table 4.13

Vessel	Maximum response 1st CCRC to NA sham	Maximum response 1st CCRC to NA Ligated	Maximum response 2nd CCRC to NA sham	Maximum response 2ndCCRC to NA Ligated	Maximum response 3rd CCRC to NA +cocaine sham	Maximum response 3rd CCRC to NA +cocaine Ligated
Aorta	4.62 ± 0.43	4.05 ± 0.29	5.64 ± 0.41	6.78 ± 0.35 *	5.08 ± 0.4	6.54 ± 0.34
Vena cava	1.24 ± 0.19	1.56 ± 0.22	1.76 ± 0.32	1.75 ± 0.33	1.66 ± 0.18	1.77 ± 0.29
R. artery	4.98 ± 0.33	5.37 ± 0.43	5.28 ± 0.46	6.1 ± 0.55	4.61 ± 0.44	4.83 ± 0.41
R. vein	1.4 ± 0.38	1.78 ± 0.31	1.72 ± 0.41	2.15 ± 0.37	1.44 ± 0.29	2.02 ± 0.49

Comparison of maximum response expressed as tension (g) of NA in four arteries and veins of the coronary ligated and sham operated rabbits after 16 weeks operation. Initially all tissues were exposed to cumulative concentration of NA (1nM-300μM). Following washout, all preparations were contracted with KCl (125mM). Following washout, tissues were contracted again with KCl (125mM). Following washout, preparations exposed cumulatively to NA. Following washout, the preparations contracted with NA cumulatively in presence of cocaine (1μM) that added 10-15 before to inhibit neuronal uptake of NA.

Each point represents mean ± s.e.mean (n=6). Statistically significant differences are represented by * $p<0.05$, unpaired student's t-test.

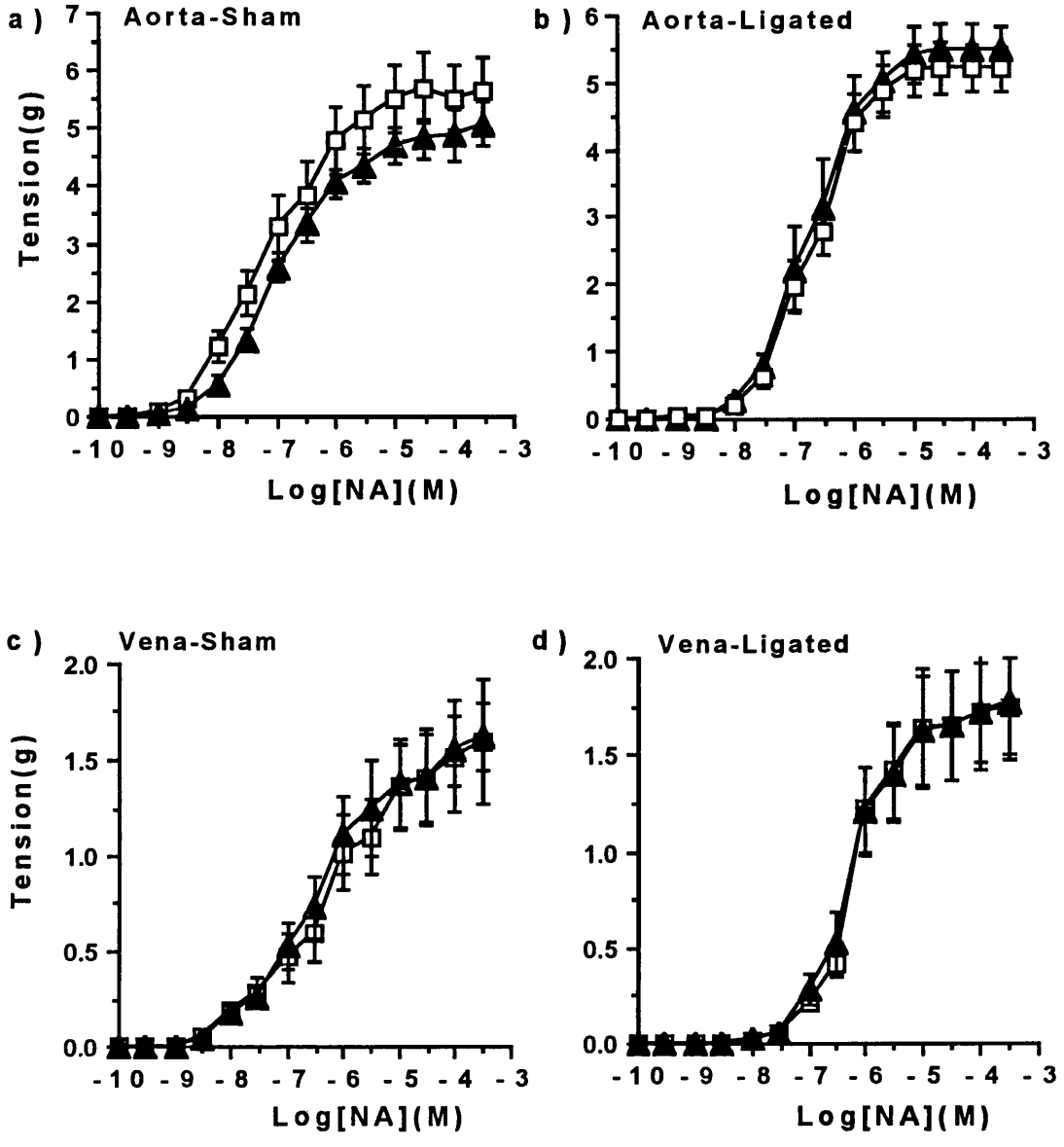

Figure 4.38

CCRC to NA and effect of cocaine (1μM) in the isolated aorta and vena cava of the sham operated rabbits with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. The CCRC to NA after 2 KCl (125mM) represented by (□) while (▲) represents CCRC to NA in presence of (1μM) cocaine.

Each point represents mean ± s.e.mean (n=6).

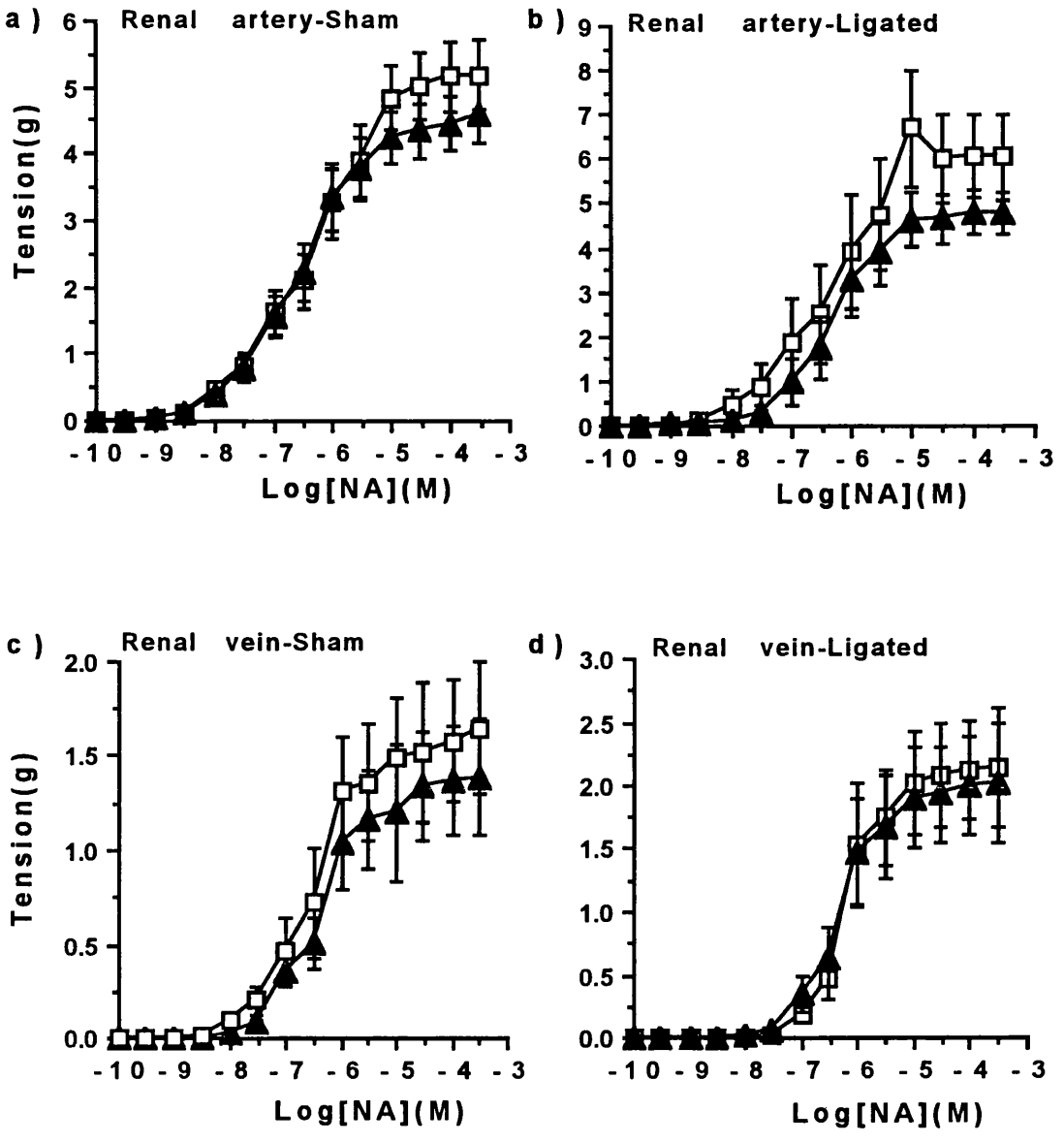

Figure 4.39

CCRC to NA and effect of cocaine (1μM) in the isolated renal artery and renal vein of the sham operated rabbits with mean ejection fraction of (74.57 ± 1.62) and coronary ligated rabbits with mean ejection fraction of (39.17 ± 2.91) after 16 weeks operation. The CCRC to NA after 2 KCl (125mM) represented by (□) while (▲) represents CCRC to NA in presence of (1μM) cocaine.

Each point represents mean ± s.e.mean (n=6).

Section 6: Discussion

The results of our present investigation led to 3 major conclusions with respect to the model. First the relaxation responses to acetylcholine, sodium nitroprusside, ATP and adenosine were not impaired. Second vasoconstrictions to noradrenaline were unaltered. Third contractions to KCl (125mM) were preserved in large vessels (arteries and veins) in coronary ligated rabbits after 8 or 16 weeks compared with a normal control population. This investigation is the first to examine endothelium function, smooth muscle responsiveness and α-adrenoceptor function in large vessels in this model of heart failure.

4.6.1 Endothelial function

It is known that the endothelium is important in the control of tone in both large and small vessels (Griffith *et al.*, 1988). Our finding in relaxations to acetylcholine are comparable to those observed in other models of heart failure in large vessels in isolated rings of dorsal pedal artery from dogs with pacing-induced heart failure (Forster et al., 1989b) and in the coronary and peripheral vessels in the same model of heart failure (Forster *et al.*, 1990; Main *et al.*, 1991). Recently O'Murchu and colleagues (1994) reported a preserved vasorelaxation response to acetylcholine in isolated vascular rings from the coronary, renal, and femoral arteries from dogs with heart failure induced by rapid ventricular pacing. In another model very similar to our model, in thoracic aorta acetylcholine-induced relaxation was identical in control and rats with coronary ligation after 1 week (Teerlink *et al.*, 1993).

In contrast to reports indicating normal endothelial function, there are many reports that show endothelial dysfunction in resistance arteries in different animal models of heart failure (Kaiser *et al.*, 1989; Lindsay *et al.*, 1992; Drexler & Lu,

1992) and patients with CHF (Kubo *et al.*,1991; Drexler *et al.*, 1992; Nakamura *et al.*, 1994; Hirooka, *et al.*, 1994).

Since much of the experimental work in different models of heart failure and human has concentrated on the smaller arteries and arterioles, the question arises to the functional significance and applicability of the changes in microvascular function that are observed in subjects with heart failure. It is possible that differences in the size of the vessels obtained from normal or heart failure subjects may influence response. In human studies caution should be in the influence of treatment or residual minor effects after treatment withdrawal on endothelium function or various receptor population in patients with heart failure. Since abnormalities of endothelium dependent vasodilation have been shown in several cardiovascular diseases, such as hypertension (Tesfamariam & Halpern, 1988)) or atherosclerosis (Harrison, *et al.*, 1987), other conditions associated with endothelial dysfunction such as hypertension and atherosclerosis should be considered in patients with CHF.

One possible explanation for reduced NO production is that in heart failure states cardiac output is reduced and the stimulus for NO production by the peripheral circulation would be reduced and this depression represents one local mechanism for abnormal control of the vasculature in CHF. But CHF is associated with activation of the sympathetic nervous and renin-angiotensin systems. Sustained increase of local and circulating noradrenaline concentrations therefore apparently is associated with an increase in endothelial NO release, which could reflect a compensatory mechanism to counterbalance excessive peripheral catecholamine-dependent vasoconstriction. In agreement with this possibility Noll and co-workers (1994) found that in aorta of cardiomyopathic hamsters relaxation response to acetylcholine was enhanced. Increased basal production of NO in heart failure consistent with reports in patients with CHF (Drexler *et al.*, 1992).

Reports of endothelium function in heart failure yield conflicting results. One reason could be attributed to differences between use of different beds of vascular system. As reported by Noll and colleagues (1994) in cardiomyopathic hamsters endothelium-dependent relaxation to acetylcholine was altered in aorta, whereas in mesenteric resistance arteries the response was not affected.

It is possible that these discrepancies can be attributed to differences among different models of heart failure. In cardiomyopathic hamsters, indirect signs of haemodynamic changes such as lung weight were assessed and exact quantification of the stage of heart failure is not known. In chronic rapid ventricular pacing, haemodynamic responses, endocrine changes, and the clinical and pathological picture of heart failure are all consistent with naturally occurring heart failure. Left arterial pressure was abnormally increased and dogs developed clinical evidence of heart failure, including ascites, muscular wasting, regurgitant murmurs, pulmonary congestive, tachypnea, and biventricular hypertrophy (Armstrong *et al.*, 1986; Kaiser *et al.*, 1989). The heart to body weight ratio was, however significantly increased in rats with chronic heart failure induced by coronary ligation, as were plasma noradrenaline concentrations (Lindsay *et al.*, 1992). In the rabbit coronary ligated model we have found no significant neuroendocrine changes at 8 weeks but have found a small but significant elevation of ANP levels at 16 weeks. Thus pathophysiology of heart failure differ with the model used and caution should be used in comparing the different models in response to acetylcholine and other agents.

Another possibility may be time that heart failure develop through as suggested by Teerlink and colleagues (1993). They found endothelial dysfunction develops through time in rats with heart failure induced by coronary ligation. Acetylcholine-induced relaxation was normal at 1 week, but severe dysfunction was evident at 4 weeks. We allowed the model to develop over either eight or sixteen weeks. It is unlikely that the duration of heart failure is responsible for the

observed acetylcholine response in our study since sham-operated and coronary ligated rabbits also produced similar responses after 16 weeks.

Since acetylcholine is the classic endothelium-dependent vasodilator, it was chosen as the reference endothelium-dependent vasodilator in our study and other models of heart failure. Defective release/production of NO from endothelium, reduced responsiveness of vascular smooth muscle to NO, impaired diffusion to the underlying smooth muscle, changes in receptor mediated responses, and facilitated breakdown of NO may be factors in the attenuation of endothelium-dependent relaxation to acetylcholine. Since in many reports in spite of impaired relaxation to acetylcholine, relaxation to sodium nitroprusside preserved, it is unlikely that the reduced response of vascular smooth muscles to NO and facilitated destruction of NO might be involved in the depressed response seen with acetylcholine.

The caution here is that the receptors on endothelium mediating the release of nitric oxide vary greatly between beds, species and within one branch order of the same bed (Angus & Michael, 1992). A test of acetylcholine-mediated release of nitric oxide may be affected by a change in the endothelial or by a change in the responsiveness of the smooth muscle cells to NO that is not necessarily associated with a change in nitric oxide release. Acetylcholine is destroyed by cholinesterase and nitric oxide can be inactivated by free radicals. Thus change in activity of this enzyme (cholinesterase) or free radicals can affect relaxation to acetylcholine. In rat pulmonary artery and thoracic aorta, vasodilation evoked with the calcium inophoreA23187 did not differ between rats with heart failure and normal rats although ACh-induced vasodilation was impaired in rats with heart failure (Ontkean *et al.,* 1991). Since A23187 causes nitric oxide release that is not mediated by receptors, it was concluded that receptor-mediated NO release was impaired.

Impaired relaxation response to acetylcholine may be related to a specific defect in muscarinic-mediated vasodilation rather than endothelium dysfunction as reported by Hirooka and co-workers (1992). In their investigation, the vasodilation responses to administration of graded concentrations of acetylcholine in the brachial artery, as determined with strain-gauge venous occlusion plethysmography, were decreased in the forearm circulation of patients with CHF when compared with those of normal control subjects. In contrast vasodilation responses to endothelium-dependent vasodilator substance P was similar in patients with CHF and in normal subjects. The finding of this study suggest that the decreased response to acetylcholine is a specific defect in muscarinic-mediated vasodilation (muscarinic receptors and/or postreceptor mechanisms coupled with muscarinic) rather than a generalised defect in endothelial production of nitric oxide or a change in the responsiveness of smooth muscle cells to NO.

To further investigate this we chose ATP and adenosine as vasodilators to study the function of endothelium in this model of heart failure independent of muscarinic receptors. Adenosine is a vasodilator in almost all vascular beds, and endogenous adenosine regulates blood flow in many organ systems. Both endothelium-dependent and endothelium-independent responses to ATP and adenosine have been reported (De Mey & Vanhoutte, 1981; Headrick & Berne 1990; Koga *et al.,* 1992; Fredholm *et al.,* 1994). As in relaxations to acetylcholine, relaxations to ATP and adenosine were identical in sham operated and coronary ligated rabbits.

According to De Mey and Vanhoutte (1982); Seidel and LaRochelle (1987); Luscher and co-workers (1988), arteries generally relax more in response to acetylcholine compared to veins. They suggested that the endothelium has a much lower modulatory role in veins as compared with the arteries. Their study is not consistent with the current findings in some pairs of veins and arteries (saphenous vein and ear vein compared with corresponding arteries). Also, the

ability of veins to relax was confirmed by the observation of their responses to the endothelium-independent vasodilator sodium nitroprusside, which like acetylcholine evokes relaxation by increasing intracellular concentrations of cyclic GMP in smooth muscle cells (Moncada *et al.*, 1991). The poor and low levels of relaxation to ATP and adenosine in arteries compared with the veins may be related to the low population of purinoceptors or difference in vascular reactivity between arteries and veins.

Our data suggest normal stimulated nitric oxide release in systemic larger vessels (arteries and veins) in coronary ligated rabbits after 8 or 16 weeks. We failed to assess the basal NO activity by measuring the increase in force that can occur when arterial and venous segments were exposed to L-NAME. The basal release of nitric oxide may be negligible in conduit rabbit vessels, or alternatively, its effect on vasomotor tone in large vessels is overridden by other factors. It is possible that basal NO release is also unaltered or changed.

4.6.2 Response to sodium nitroprusside

Nitrovasodilators were used as endothelium-independent vasodilators to investigate function of smooth muscle cells since like nitric oxide, it directly activates smooth muscle guanylate cyclase (Moncada et al., 1991).

Our findings agree with (Kaiser *et al.*, 1989 and Main *et al.*, 1991) in large vessels in dogs with pacing-induced heart failure, in aorta of rat with coronary liagation (Lindsay *et al.*, 1992) and human with CHF (Kubo *et al.*, 1991; Creager *et al.*, 1991; Hirooka, *et al.*, 1994) endothelium-independent relaxations to sodium nitroprusside were unaltered. In an *in vivo* study, recently it was reported that vasodilator sodium nitroprusside reduced mean arterial blood pressure to a similar

extent in vehicle-and adriamycin-treated rabbits that were either anaesthetised or pithed (Minatoguchi & Majewski, 1994).

Our current investigation is confirmed by the lack of any difference between ligated and sham-operated rabbits in the relaxation response to sodium nitroprusside. Thus, a generalised abnormality in function of smooth muscle cells is not supported.

4.6.3 Response to KCl

Abnormal function of the contractile apparatus of vessels, can be assessed by contraction to potassium chloride. In our study, high potassium chloride (125mM) was used as contractile agent. It causes direct smooth muscle depolarisation leading to smooth muscle contraction via calcium entry through voltage sensitive calcium channels.

We observed that contraction to KCl was not different from control at either 8 or 16 weeks. Our results agree with other reports (Forster *et al.*, 1989b; Forster & Armstrong, 1990; Forster *et al.*, 1990) in dogs with pacing induced heart failure indicate contraction to potassium chloride (125mM) was unchanged. We suggest that there is no general change in contractile apparatus or smooth muscle responsiveness in this model of heart failure.

4.6.4 α-adrenoceptor activity

This study demonstrates that the physiological mixed α_1/α_2-agonist noradrenaline produced concentration-dependent contractions in arteries and veins from controls and rabbits with coronary ligation. Our study agree with some of the reports, in pacing-induced CHF in canine coronary arteries (Main *et al.*, 1991), in canine

femoral artery (Kaiser *et al.*, 1989) and in human with CHF (Kubo *et al.*, 1989; Indolfi *et al.*, 1994) showing vasoconstrictions to noradrenaline were unaltered in this model of heart failure.

It has been known that plasma noradrenaline levels are elevated in CHF patients and increased circulating catecholamines in chronic heart failure might be expected to lead to adrenergic down regulation. However in sharp contrast to β_1-adrenoceptors down regulation in failing hearts, the α_1-adrenoceptor population is maintained at normal levels in the failing human heart (Bristow *et al.*, 1988). In peripheral vessels some investigators found increased tissue sensitivity to adrenergic agents during CHF (Forster *et al.*, 1989a; Forster & Armstrong, 1990). Contrary to Forster and co-workers, Main and colleagues (1991) found that in pacing-induced CHF in canine coronary arteries, the maximal contractile response to methoxamine was attenuated. The data suggest α_1-adrenoceptor-mediated constriction is diminished.

Our results indicate that the vascular responsiveness to noradrenaline was preserved in this model of heart failure.

4.6.5 β-adrenoceptor activity

Since circulating catecholamines exert a tonic vasodilatory effect on peripheral vessels via β-adrenoceptors, we investigated the role of β-adrenoceptors in coronary ligated rabbits. It is now well established that β-adrenoceptor function is abnormal in the myocardium of patients with CHF. In failing ventricles, there are decreased numbers of β-adrenoceptors, reduced isoprenaline-stimulated adenylate cyclase activity, and depressed inotropic and chronotropic responsiveness to β-adrenoceptor agonists (Bristow *et al.*, 1986). Bristow and colleagues (1993), examined isolated cardiac tissue using radioligand-binding techniques and

reported that β_1-adrenoceptor down regulation occurred in the failing left ventricle, whereas there was no change in β_2-adrenoceptors. The fact that noradrenaline has greater β_1-than β_2-adrenoceptor effects may explain the selective down-regulation of β_1-adrenoceptors. In spite of the known down-regulation of β_1-adrenoceptors in the failing left ventricle, few data in peripheral vessels are available.

Since the trigger for β-adrenoceptor desensitisation is thought to be chronically elevated circulating catecholamines (Bristow, 1993), this condition should also induce desensitisation of peripheral β-adrenoceptors as well as those in the heart.

In the present study we found no change in isoprenaline-induced relaxations in coronary ligated rabbits compared with a normal control population. Our results are consistent with prior studies (Frey *et al.,* 1988; Creager *et al.*, 1991) investigating this problem which find no peripheral β-adrenoceptor desensitisation.

4.6.6 Effect of neuronal uptake blockade

Arterial plasma noradrenaline is 163% higher in patients with heart failure than in control patients. High plasma noradrenaline correlates directly with the haemodynamic severity of the disease and inversely with survival (Jenning & Esler, 1990). Elevated levels of circulating noradrenaline in CHF may result from impaired peripheral reuptake of this catecholamine.

Our results in the present experiment showed that neuronal uptake of noradrenaline plays no significant role in aorta, renal artery , renal vein, vena cava and ear vein of the rabbit. As reported by Nilsson (1984) neuronal uptake of noradrenaline plays a minor role in rat aorta. In spite of clear evidence for a substantial effect of cocaine on noradrenaline uptake in a variety of tissues

(Furchgott *et al.,* 1963), cocaine treatment (1μM) had only a very modest effect on development of mechanical responses to exogenous noradrenaline in ear artery, saphenous vein or artery.

Cocaine has generally been used as the prototype drug for inhibition of neuronal uptake of catecholamines. Furchgott and co-workers (1963) showed that a maximum potentiation of the response to noradrenaline in rabbit aorta and guinea pig and cat left atria could be obtained with 10-30 μM cocaine. Since catecholamine neuronal uptake is the major means of terminating sympathetic neural transmission, the adrenergic response is potentiated by cocaine. Inhibition of neuronal uptake by cocaine can have minor effects on responses to exogenous noradrenaline in blood vessels, particularly when those blood vessels have a network of sympathetic neuroeffector complexes that is small in relation to the muscular layer (Billman, 1990). Since cocaine has both a sympathomimetic effect (inhibition of neuronal uptake of noradrenaline) and, at higher concentration, a local anaesthetic property (Na^+ channel blockade), caution should exercised in relation to the concentration of cocaine. We used 1μM cocaine to inhibit neuronal reuptake of noradrenaline and avoid the local anaesthetic effect.

These results suggest that the density of sympathetic innervation of mentioned preparations are significantly less than that of ear artery and saphenous vein or artery. It appears that among arteries and veins that were studied, ear artery and saphenous artery or vein have innervation which can influence sensitivity to NA. To further investigate this we studied the network of sympathetic nerves stained by glyoxylic acid. In agreement with the functional results, it was shown that ear artery and saphenous artery or vein have dense innervation but in other preparations sparse innervation or in vena cava no nerves were observed.

In the present experiment, effects of cocaine on noradrenaline responses were identical in sham operated compared with coronary ligated rabbits. This finding

may indicate no impairment of noradrenaline neuronal reuptake in this model of heart failure.

4.6.7 Conclusion

Models of heart failure usually have a complex pathophysiology so that results are difficult to interpret. Furthermore, reports of changes in endothelium function, smooth muscle responsiveness, activity of receptors etc. are conflicting. These discrepancies could be attributed to differences between *in vitro* versus *in vivo* experiments, the use of conscious versus anaesthetised animals, the use of different beds of vascular system and, potentially, to species differences. Results obtained in any model of heart failure have to be interpreted and extrapolated to humans with caution. One difficulty in studying the development of heart failure has been the lack of a well-characterised animal model that clearly mimics heart failure in human. Difficulties in using man in the study of heart failure are the usual confounding influence of concomitant pharmacologic therapy and the uncertainty associated with identifying the time at which heart failure begins.

Animal models of heart failure have been ones of major surgical trauma, myocardial ischemia, and pharmacologic or toxic depression of cardiac function. However, all of these models may directly affect neurohormonal factors independent of the primary influence of heart failure. A combination of neural, humoral, and local factors appears to be involved in the alteration of vascular responses seen in heart failure. In the current investigation we have evaluated the effects of coronary ligation on rabbits as a model for heart failure. However, the syndrome of heart failure eventually involves multiple physiological and pathophysiological pathways and different stages. The degree of heart failure as well as the aetiology may also play a role in response to agents we used.

Although the exact degree of heart failure in this model is difficult to determine, it clinically corresponds to left ventricular dysfunction.

The results of the haemodynamic measurements suggest that short-term (8 week) and long-term (16 week) coronary ligation in the rabbits each corresponds to a model of experimental asymptomatic left ventricular dysfunction or minimally symptomatic LVD rather than of clinical heart failure. This model of heart failure used in this study differs from chronic heart failure models and offers several advantages over other models. Technically, it is relatively easy to produce and the most important advantage is that we can study very early stage of heart failure. Clinical heart failure, as currently recognised by doctors, is an end-stage condition that treatment has a limited effect. Since heart failure develops from left ventricular dysfunction, study of this stage of heart failure can help to prevent left ventricular dysfunction progressing to heart failure. Thus we can investigate factors leading to progression of left ventricular dysfunction to heart failure. Furthermore, this model produces a similar situation to coronary heart disease, which is a very common cause of death in patients with heart failure (Maurice & Cobbe, 1992).

There is evidence that the resistance vasculature is altered to some degree in this model at both 8 and 16 weeks ligation (unpublished observations). There was abnormal function of endothelium-derived vasodilation post-ligation: subcutaneous arteries had reduced function, pulmonary arteries had increased function. Noradrenaline contraction in rabbit subcutaneous arteries was influenced by ligation: threshold was lowered.

In this regard, the results of our study suggest that vasoconstriction to noradrenaline and normal stimulation of endothelial NO are preserved in larger peripheral conduit vessels. This study provides evidence that large vessel physiology is unaltered in this particular model of experimental left ventricular

dysfunction. This difference between the resistance vessels and large vessels may be important since it suggest that experimental heart failure affects that part of the circulation which is involved with the control of the peripheral vascular resistance. The changes demonstrated here provide a useful model for studying the progression of Left ventricular dysfunction to full blown heart failure. These findings have highlighted which aspects of vascular function are most abnormal and which should be targeted for the therapeutic intervention. Correction of these abnormalities can be a useful way in preventing of heart failure.

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