Canine vasculature: A study of α_1-adrenoceptors and heart failure

by

Sally Anne Argyle M.V.B. M.R.C.V.S. Cert S.A.C.

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from

The Division of Neuroscience and Biomedical systems, Institute of Biomedical & Life Sciences and the Department of Veterinary Clinical Studies, University of Glasgow. Glasgow, G12 8QQ.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

SUMMARY

Heart disease is an important cause of morbidity and mortality in the canine population, with the two most common causes of acquired heart disease being dilated cardiomyopathy and endocardiosis.

In human patients with heart disease, it has been noted that the clinical signs and symptoms are often of greater severity than expected from the degree of left ventricular dysfunction, suggesting that additional factors contribute to the syndrome. It has now been shown in several human and experimental animal studies, that blood flow to exercising skeletal muscle is reduced in heart failure and that this is not due to an inability to increase cardiac output. This suggests an inability of the vasculature to accommodate the increased blood flow required by the exercising muscle, leading to an early switch over to anaerobic metabolism and premature fatigue.

While the neurohumoral aspects of cardiac failure have been well characterised at a systemic level, the local vascular effects have not.

To gain further knowledge of the local effects and their role in the pathophysiology of cardiac disease, it is necessary to characterise normal vessels, in addition to examining vessels from heart failure animals.

With these goals in mind, the primary aim of this project was to characterise both the dog saphenous vein and the dog subcutaneous resistance arteries, in relation to their functional α_1-adrenoceptor population. Findings are discussed in detail in Chapter 3 and Chapter 4. The functional effects of five competitive reversible antagonists and the irreversible alkylating agent CEC, on noradrenaline mediated contractions of dog saphenous vein and dog subcutaneous resistance arteries, were analyzed.

In both vessels the α_1-adrenoceptors appeared to have a low affinity for the α_1-adrenergic antagonist prazosin, necessitating their classification as α_{1L}-adrenoceptors. In addition, in both vessels, there was evidence for the involvement of another subtype in the noradrenaline response. This receptor, despite a low affinity for prazosin had some characteristics of the α_{1B}-adrenoceptor.

Chapter 5 describes the cloning and sequencing of an 891bp fragment of the canine α_{1a}-adrenergic receptor cDNA. This subtype was chosen because of the mounting evidence that it is responsible for the α_{1L}- pharmacology. The fragment was initially isolated using reverse-transcription polymerase chain reaction (RT-PCR) and primers designed from areas of high homology in the α_{1a}-adrenoceptor of the human and bovine α_{1a}-adrenergic sequence. This fragment, together with a canine partial α_{1b}- sequence was used to probe cell lines expressing the human α_{1a}- and α_{1b}-adrenergic receptors, as well as canine prostate and brain RNA. The canine α_{1a}- probe failed to detect message in any of the samples and while the α_{1b}- probe hybridized to the α_{1b}- expressing cell line RNA, there was evidence for a lack of subtype specificity of this probe.

Finally, Chapter 6 describes experiments comparing isolated femoral artery, saphenous vein and subcutaneous resistance arteries from dogs with naturally occurring heart failure, and controls. The findings suggest that in heart failure there is a decrease in sensitivity to exogenous noradrenaline in both the saphenous vein and the femoral artery, but not in the resistance arteries. In addition, vasorelaxations to acetylcholine were examined in all vessels and no significant differences were found between vessels from control and heart failure dogs. Interestingly, on examination of the case details from the heart failure dogs used, it was found that in the cohort of patients used for the large vessel studies, the majority of animals had received no treatment, (seven out of eight animals were not treated). This was in contrast to the cohort used in the resistance

artery group, where four out of five animals had received treatment for their cardiac disease. The relevance of this and possible effects are discussed in this chapter.

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DECLARATION

I declare that the all the work for this thesis was carried out by myself, with the exception of the histology and immunohistochemistry which was prepared by Dr. Ian Montgomery. The work was carried out at the Autonomic Physiology Unit, Neurosciences and Biomedical Systems, West Medical Building, University of Glasgow, Glasgow, G12 8QQ, U.K.

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Abbreviations

ACEI	Angiotensin converting enzyme inhibitor
Ach	Acetylcholine
AI	Angiotensin I
AII	Angiotensin II
Ca^{2+}	calcium
cAMP	adenosine 3'-5'-cyclic monophosphate
cDNA	single stranded DNA
CEC	chloroethylclonidine
cGMP	guanosine 3'-5'-cyclic monophosphate
CRC	concentration response curve
°C	degrees centrigrade
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
DSCRA	dog subcutaneous resistance artery
DSV	dog saphenous vein
EDRF	endothelium-derived relaxing factor
EDHF	endothelium-derived hyperpolarizing factor
G protein	GTP-dependent regulatory proteins
GTP	guanosine triphosphate
HR	heart rate
I (1,4,5)P_3	inositol (1,4,5) trisphosphate
KCl	potassium chloride
KS	Krebs' solution
L-NAME	N^{ω}-Nitro-L-Arginine Methyl ester hydrochloride
l, ml, µl	litre, millilitre, microlitre
$MgCl_2$	Magnesium chloride
mRNA	messenger RNA
M (mM, µM, nM, pM, fM.)	Molar (moles per litre; millimolar, micromolar, nanomolar, picomolar, femtomolar)
mnths	months
NA	noradrenaline
O_2	oxygen
O.D.	optical density
PE	phenylephrine
PIP_2	phosphatidylinositol (4,5) bisphosphate
RAAS	renin angiotensin aldosterone system
RNA	ribonucleic acid
SV	stroke volume
s.e. mean	standard error of the mean
Taq polymerase	*Thermus aquaticus* DNA polymerase
TPR	total peripheral resistance
µm	micrometre
yrs	years

This thesis is dedicated to David and Blythe who have been my inspiration

CHAPTER 1

General introduction

1.0 α_1-Adrenoceptors

1.0.1 Historical perspective

1.0.2 α_1-adrenoceptor subtypes

1.0.3 Structure and genomic organization of the α_1-adrenoceptors

1.0.4 Signal transduction

1.1 Heart failure

1.1.1 Definition

1.1.2 Aetiology

1.1.3 Pathophysiology

1.1.4 Neurohumoral systems

1.1.5 Treatment of cardiac failure

1.1.6 Role of the peripheral vasculature in heart failure

1.2 Aims of this thesis

1.0 α_1-ADRENOCEPTORS

1.0.1 Historical perspective

Oliver and Schaffer (1895) showed that adrenal gland extract increased blood pressure when administered in vivo, thereby introducing the concept of the adrenoceptor system. Subsequent to this it was thought that the reason for variation in effects brought about by adrenotropic receptors (in some cases depressor and in others pressor), was due to the release of different endogenous catecholamines called sympathins, (sympathin I for inhibitory and sympathin E for excitatory), each one causing a different effect at the same receptor, (Cannon and Rosenbleuth, 1933).

This theory became obsolete when Ahlquist (1948) used five different catecholamines on eight different systems, in order to examine their rank order of potency for contraction and relaxation responses. Rank orders differed between pressor and depressor responses, suggesting that the receptors involved in each of these activities were in fact different. He named these two types of receptors α and β, with α- receptors being involved in excitatory responses, with the exception of the gut, and β- receptors being responsible for inhibitory responses, with the exception of the myocardium. Ahlquist's theory was later confirmed with the advent of a β-adrenoceptor antagonist (Moran and Perkins, 1958; Powell and Slater, 1958). Until this time only an α-adrenoceptor antagonist had been available.

Langer (1974) then discovered the existence of pre-junctional α-adrenoceptors which modulated the release of endogenous noradrenaline. These receptors differed pharmacologically from post-junctional receptors. An example of this was demonstrated by the relative potency of phenoxybenzamine at both sites. Phenoxybenzamine was 30 times more potent at post-junctional adrenoceptors. Due to these differences he decided

to classify the pre-junctional receptors as α_2-, and the post-junctional as α_1-adrenoceptors, based on anatomical and pharmacological properties.

In 1979, Drew and Whiting found that hypertension induced by the administration of phenylephrine, was more readily antagonized by prazosin, than hypertension induced by noradrenaline. Conversely, yohimbine antagonized the effects of noradrenaline to a greater extent This was one of several papers showing evidence of post-junctional α_2-adrenoceptors (Timmermans et al. 1979; Docherty et al. 1979). From this time onward α_1- and α_2-adrenoceptors were classified purely on pharmacological properties (Starke and Langer, 1979; Berthelsen and Pettinger, 1977). The presence of post-junctional α_2- as well as α_1- adrenoceptors, which mediate vascular smooth muscle contraction, is now well documented in a variety of vessels (Simonsen et al. 1997; Elliott, 1997; Wright et al. 1995; Nielsen et al. 1989), including the dog saphenous vein (MacLennan et al. 1997; De Mey and Vanhoutte, 1981).

1.0.2 α_1-adrenergic subtypes

Role of the α_1-adrenoceptors and relevance of multiple subtypes

The α_1-adrenoceptors are mediators of the sympathetic nervous system and as such, these receptors interact with the endogenous ligands noradrenaline and adrenaline. Due to this interaction and because of their wide distribution, these receptors have many important functions.

Receptors on vascular smooth muscle and in myocardium have a key role in the control of peripheral resistance and myocardial contractility (Minneman and Esbenshade, 1994; Minneman, 1988; Graham et al. 1996). Not only do these receptors have an important physiological role, but they have been implicated in certain pathological processes. Alterations in both the α_1- and α_2-adrenoceptor population on vascular smooth muscle

have been reported in heart failure (Forster and Armstrong, 1990; Forster et al. 1989; Stassen et al. 1997a; Stassen et al. 1997b). More recently, certain subtypes have been implicated in myocardial and smooth muscle hypertrophy (Xin et al. 1997; Milano et al. 1994a; Yamazaki et al. 1997; Milano et al. 1994b), as well as being involved in the phenomenon of ischaemic preconditioning of the myocardium (Kariya et al. 1997; Li et al. 1997). Their role in the dynamic component of benign prostatic hypertrophy has been well documented (Chapple, 1997; Hieble and Ruffolo, 1996). The importance of α_1- adrenoceptors is also reflected in the fact that currently they are targets for the drug treatment of a variety of conditions such as hypertension, benign prostatic hypertrophy, nasal congestion and angina pectoris (Hieble and Ruffolo, 1996; Minneman et al. 1993). It is clear from this, that an understanding of the contribution of individual subtypes to both physiological and pathological processes, will not only extend our knowledge of the pathways involved, but will open up the possibility for the development of subtype selective antagonists and agonists, which can be used more effectively and with fewer side effects.

The evidence supporting the existence of more than one subtype of α_1-adrenoceptor began to surface in the early 1980s. Since then the evolving sub-classification of these receptors has followed a tortuous and often confusing path.

Evidence based on agonist studies

The initial proposal for the sub-classification of these receptors into two populations came from agonist studies. Ruffolo et al (1977), made the initial observation that the imidazoline agonists and the phenethylamine agonists seemed to either have different modes of action at the same receptor population, or they acted on different types of α_1-adrenoceptor, since if desensitization was induced with an agonist from one group, the

tissue was not subsequently desensitized to agonist from the other group, but responses were reduced if a different agonist from within the same group were used. Godfraind et al (1982), while working on rat thoracic aorta, showed that the responses to the phenethylamine agonists were relatively less sensitive to calcium channel blockers when compared to the imidazolines. Further evidence for heterogeneity of the α_1-adrenoceptor population came from work on a variety of tissues, the responses of which, on the basis of antagonist profiles, were α_1-mediated, but which showed different responses to different classes of agonists. For example, in the rat anoccocygeus muscle, phenethylamine agonists produced a dose response curve with a definite shoulder, with the lower component of the curve demonstrating a phasic response. In comparison, imidazoline agonists, produced a monophasic concentration response curve in the same tissue, and the response was also phasic except at the highest concentrations of agonist. This led McGrath (1982) to suggest that the adrenoceptors mediating these responses be classified as α_{1a}- and α_{1b}-. In this classification, the phenethylamine agonists activated both the subtypes, thus accounting for the biphasic response, whereas the imidazoline agonists activated only the α_{1a}- subtype which were contributing to the lower phasic part of the phenethylamine concentration response curve. In addition, it was thought that the two proposed subtypes could be further distinguished by their second messenger activation pathways, with the α_{1a}- utilizing extracellular calcium and the α_{1b}- utilizing intracellular calcium. Usage of this classification scheme did not continue mainly due to the failure to identify antagonists which could distinguish the two subtypes.

Evidence from radioligand binding and functional experiments

Throughout the course of this introduction, native receptors will be referred to with uppercase letters and recombinant receptors will be referred to with lowercase letters, as

recommended by the committee for the nomenclature of α_1-adrenoceptors (Hieble et al. 1995).

In the case of functional and radioligand binding studies, receptors are classified with the use of selective antagonists. In the case of radioligand binding, the receptors are labelled using a radioactive drug, which is then competed off the receptor using a non-radioactive competitive congener (Starke, 1981). In functional studies the concentration of the antagonist which causes the concentration response curve to shift to the right by a factor of two is the antagonist's receptor dissociation constant (pA_2), and if two receptors are the same, then a given antagonist should have the same pA_2 value at both sites (Flavahan and Vanhoutte, 1986a). A more detailed description of the derivation of pA_2 values is given in the materials and methods section of this thesis.

Morrow et al (1985) and Morrow and Creese (1986) showed evidence for receptor heterogeneity in rat brain. They found that the antagonists WB4101 and phentolamine competed for [^{3}H] prazosin labelled sites with high and low affinities, suggesting that the prazosin was labelling a heterogeneous population of receptors. The high affinity sites were termed α_{1A}-, while the low affinity sites were termed α_{1B}-.

In 1987, Han and colleagues published a number of papers which followed on from this. A Nature publication (Han et al. 1987a), also distinguished subtypes based on the affinity for WB4101 at rat hippocampus, vas deferens, liver and spleen. Their results showed that in rat liver and spleen, the affinity for WB 4101 was low and the pattern of antagonism monophasic, suggesting the presence of only the α_{1B}- subtype. In contrast, both the vas deferens and the hippocampus displayed biphasic inhibition curves for WB 4101, suggesting the presence of both the α_{1A}- and the α_{1B}- subtypes. From this study they also concluded that the contraction induced by the α_{1B}-adrenoceptor was independent of extracellular calcium while that induced by the α_{1A}-adrenoceptor relied

on the opening of dihydropyridone sensitive calcium channels. Interestingly this tied in with the proposed classification of McGrath (1982). However, by the authors' own admission, the ability to distinguish the two subtypes, by sensitivity to calcium channel blockers, while possible for the tissues which they examined, did not hold true for all tissues and thus while the division based on differing affinity for WB 4101 gained credence, the source of calcium as a subtype distinguishing feature, did not. In fact, in a later publication (Han et al. 1990), it was demonstrated from cells in culture that both α_{1A}- and α_{1B}- adrenergic receptors could stimulate inositol phosphate accumulation to a similar extent and it has since been shown that all the cloned receptors can induce the accumulation of inositol phosphate (Cotecchia et al. 1995; Schwinn et al. 1991; Theroux et al. 1996; Minneman and Esbenshade, 1994).

Han et al (1987b), went on to use both the alkylating agent chloroethylclonidine (CEC) and WB4101. In this instance, they found that again there were high and low affinity sites in rat brain for WB4101 competition against [^{125}I] BE 2254 binding, and that CEC seemed to inactivate only the low affinity WB4101 sites, although quantitatively the number of sites inactivated by CEC compared to the low affinity WB4101 sites did not correlate well. In a paper the following year they showed that this discrepancy was probably due to incomplete access of the CEC to sensitive sites, since when the experimental conditions were altered and the CEC inactivation carried out in hypotonic as opposed to isotonic solution, the proportion of sites inactivated increased and was more compatible with the proportion of WB4101 low affinity sites(Minneman et al. 1988).

In the late 1980s the advent of further subtype selective antagonists lent support to the proposed α_{1A}- and α_{1B}- classification. In particular, both a urapidil derivative, 5 methylurapidil and a 1,4-dihydropyridine, niguldipine, were found to be selective for

the α_{1A}- subtype (Graziadei et al. 1989; Hanft and Gross, 1989; Gross et al. 1988), with 5 methylurapidil having an ≈70 fold selectivity for the α_{1A}- subtype as opposed to only a 20-30 fold selectivity exhibited by WB 4101 in radioligand binding experiments (Hanft and Gross, 1989).

Evidence from molecular studies

Although the α_{1A}- and α_{1B}- classification had met with a certain amount of scepticism and uncertainty, the existence of these subtypes, in addition to a third, (α_{1D}-), was confirmed from 1988 onwards with the successive cloning and sequencing of these receptors, and their identification as different gene products (Smiley et al. 1998). Due, in the beginning, to a limited number of subtype-selective antagonists, there was some confusion as to the identity of the cloned receptors in relation to their native counterparts. The following paragraphs aim to summarise the sequence of events without, hopefully, causing too much additional confusion.

The first of these receptors to be cloned, sequenced and expressed was the hamster α_{1b}- (Cotecchia et al. 1988). This was isolated from DDT_1MF-2 cells, which are a hamster derived smooth muscle cell line. When this 2Kb cDNA clone was expressed in COS-7 cells, it was shown to have a low affinity for WB 4101 and phentolamine, and to be associated with the accumulation of inositol phosphate. From these findings and based on the information from functional and radioligand binding studies already discussed above, it was decided that this clone represented the hamster α_{1B}- adrenergic receptor. A second receptor was identified by the same group and the results published the following year (Schwinn et al. 1990). This time a clone was derived from a bovine brain cDNA library using a probe derived from the hamster α_{1b}- adrenoceptor. This receptor was ≈ 70% homologous to the hamster α_{1b}- sequence, supporting the fact that

this was an α_1- adrenoceptor. The new clone was mapped to a different human chromosome to the hamster α_{1b}- and when this clone was expressed in COS-7 cells, unlike the α_{1b}-, it showed a 10 fold higher affinity for the antagonists WB 4101 and phentolamine, together with a high affinity for the agonist oxymetazoline. Although this profile supported the classification of this receptor as an α_{1a}-, the sensitivity of the receptor to CEC, (generally associated with the α_{1b}-), together with an inability to detect expression of mRNA in rat tissues described as possessing α_{1A}- adrenoceptors from binding studies, led the authors to believe that this was a novel adrenoceptor not identified from radioligand or functional studies, and it was named the α_{1c}-adrenoceptor. A third clone was identified from a rat cerebral cortex library (Lomasney et al. 1991). Due to the high affinity of this clone for WB 4101 and a distribution in rat tissues corresponding to the native α_{1A}-adrenoceptor from radioligand binding studies, it was assumed that this clone represented the α_{1A}-adrenoceptor. At roughly the same time another group had identified an α_1-adrenoceptor using solution-phase library screening of a rat brain library (Perez et al. 1991). This clone did not convincingly fall into either the α_{1A}- or α_{1B}- classification, since despite a high affinity for WB 4101, the affinity for 5 methylurapidil and niguldipine was much lower than at α_{1A}- adrenoceptors and the sensitivity to CEC was lower than expected at α_{1B}- adrenoceptors. Based on this evidence, the clone was considered to be a novel α_1-adrenoceptor and was named the α_{1d}-. It soon became clear that the α_{1d}- was identical in sequence to the α_{1a} clone (Lomasney et al. 1991), with the exception of two codons, and that these were in fact encoding for the same receptor, generally now accepted to be the α_{1d}- (Minneman and Esbenshade, 1994).

At this point it seemed that while the cloned α_{1b}- corresponded to and encoded for the native α_{1B}-, the cloned α_{1d}- had no functionally defined counterpart, the native α_{1A}- had no cloned counterpart and the cloned α_{1c}- had no functional counterpart.

Despite this all was not lost. From about 1994 onwards it became generally accepted that the cloned α_{1c}- should be renamed the α_{1a}- and that this cloned receptor corresponded to and encoded for the native α_{1A}-adrenoceptor (Hieble et al. 1995). Evidence for this came from a number of observations. It was demonstrated that alterations in the experimental conditions under which sensitivity to CEC was examined, could influence the results, and subsequently sensitivities approximately 20% lower than expected were derived for the human and bovine α_{1c}- clones (Schwinn et al. 1995). A decrease in the sensitivity to CEC was also seen in the rat homologue (Laz et al. 1994). Another study reported a higher sensitivity to niguldipine in the rat homologue of the receptor compared to the bovine clone (Forray et al. 1994a), and with the use of more sensitive techniques such as RNase protection assays and *in situ* hybridization studies, the mRNA for this subtype had a wider tissue distribution than originally shown with Northern analysis (Price et al. 1994; Price et al. 1993). In addition, a functional study of the α_1-adrenoceptors mediating smooth muscle contraction, in the isolated perfused kidney of the rat, showed a high degree of correlation in the functional potency of the antagonists used with the affinity of the same ligands for the cloned α_{1c}- receptor (Blue et al. 1995). This type of correlation was mirrored in a number of other studies, for example, Testa et al (1995), Ford et al (1994) and Faure et al (1994a).

With the advent of the α_{1D}- selective ligand BMY 7378 (Goetz et al. 1995), it became apparent that the cloned α_{1d}-adrenoceptor did have a functional counterpart. Since then it has been identified as playing a role in a variety of tissue types including rat renal

artery (VillalobosMolina et al. 1997), rat aorta (Kenny et al. 1995), rat aorta and iliac artery (Piascik et al. 1995) and rat myocardium, aorta, vas deferens and spleen (Deng et al. 1996).

In summary therefore, there are currently three native subtypes of α_1-adrenoceptor, namely α_{1A}-, α_{1B}- and α_{1D}-. These would appear to correspond to, and be encoded by, the three cloned α_1-adrenoceptors namely, the α_{1a}-, α_{1b}- and α_{1d}- . The term α_{1c}- is no longer to be used. To date one or more of the three cloned receptors have been identified in a variety of species including human, rat, murine, bovine and rabbit. With particular relevance to this thesis, as far as I am aware the only canine sequence currently known is a partial sequence for the canine α_{1b}-adrenoceptor (Libert et al. 1989).

From functional, radioligand or mRNA expression studies, although often more than one subtype is expressed or appears to have a functional role, frequently a particular subtype seems to predominate in a particular tissue. Some examples are listed. The α_{1A}-adrenoceptor has been identified in rat resistance arteries (Kong et al. 1994; Ibarra et al. 1997), rat renal artery (Piascik et al. 1997; VillalobosMolina et al. 1997), vas deferens (Moriyama et al. 1997; Burt et al. 1995), rat renal vascular bed (Blue et al. 1995) and rabbit ear artery (Fagura et al. 1997), from functional studies. Expression of the α_{1a}- subtype mRNA has been demonstrated in human heart, liver, cerebellum and cerebral cortex (Faure et al. 1995; Hirasawa et al. 1995; Price et al. 1994), rat, monkey and human bladder and prostate (Nasu et al. 1996; Walden et al. 1997) and rabbit and guinea pig liver (GarciaSainz et al. 1995). The α_{1B}- subtype has been demonstrated in rat mesenteric resistance artery (Piascik et al. 1997), rat spleen (Burt et al. 1995) and rabbit cutaneous resistance arteries (Smith et al. 1997) from functional studies. Expression of the α_{1b}- subtype has been identified in human spleen, kidney and fetal

brain (Price et al. 1994), rat liver (GarciaSainz et al. 1995; Faure et al. 1995) and rat pineal (Sugden et al. 1996). As already mentioned the α_{1D}- subtype has been identified in rat aorta and rat iliac artery (Fagura et al. 1997; Kenny et al. 1995; Piascik et al. 1995; Deng et al. 1996) from functional studies. Expression studies have identified the α_{1d}- subtype in human aorta and cerebral cortex (Price et al. 1994), rabbit aorta, prostate, vas deferens and cerebral cortex (Suzuki et al. 1997) and guinea pig liver (Faure et al. 1995).

It is important to highlight at this point, that in functional studies, the classical α_{1A}-adrenoceptor subtype would appear to only have been identified in rat tissues. By classical α_{1A}-, I mean a functional receptor with a high affinity for prazosin (>9), as well as a high affinity for the α_{1A}- subtype-selective ligands. The relevance of this becomes clear in the next section, where the α_{1L}-adrenoceptor is discussed. Although in the previous paragraph I mentioned that the rabbit ear artery α_1-adrenoceptor was classified as α_{1A}-, it is interesting to note that values for the affinity of prazosin were not quoted in this study (Fagura et al. 1997).

α_{1L}-adrenoceptor pharmacology

While the sub-classification of the α_1-adrenoceptors into α_{1A}-, α_{1B}-, and α_{1D}- was being resolved, an additional purely functional classification was coming to light.

A characteristic of all the subtypes mentioned so far, is that in radioligand binding and functional experiments, these receptors all have a high affinity for the α_1- ligand prazosin.

From examination of functional experiments, it became clear that there was a considerable spread in the pA_2 values for prazosin at α_1-adrenoceptors (Drew, 1985). The variability in the potency of this antagonist was taken as evidence for receptor

heterogeneity. This was supported by a study of rabbit pulmonary artery which showed that prazosin antagonised the agonist clonidine in a non-competitive way, revealing both a high and a low affinity site (Holck et al.1983). Flavahan and Vanhoutte (1986a) reviewed the literature and suggested that receptors could be classified as α_{1H}- if the affinities for prazosin and yohimbine were greater than 9 and 6.4 respectively, and α_{1L}- if the affinities for prazosin and yohimbine were less than 9 and 6.2 respectively.

Muramatsu et al (1990b) evolved this suggested classification scheme, and based on functional experiments using prazosin, HV 723, WB 4101, yohimbine, phentolamine and CEC on a selection of blood vessels, they divided the α_1-adrenoceptors into three groups. The α_{1H}-receptors had a high affinity for prazosin ($pA_2 > 9.5$), that was greater than the affinity for either HV 723 or WB 4101, and they were sensitive to CEC. The α_{1L}- adrenoceptors had a similar pA_2 value for prazosin and HV 723 and the affinity for prazosin was generally <9. The α_{1N}-adrenoceptors had a low affinity for prazosin ($pA_2<9$) and a higher affinity for HV 723 ($pA_2>9$). Both the α_{1L}- and the α_{1N}- adrenoceptors were insensitive to CEC. Other ligands which appear to distinguish the low affinity prazosin sites by having a low affinity at these receptors relative to that for the α_{1A}-adrenoceptor are, RS-17053 (Leonardi et al. 1997; Testa et al. 1997; Marshall et al. 1996; Ford et al. 1996a), SNAP 5089 and REC 15/2627 (Testa et al. 1997; Leonardi et al. 1997).

It was unclear as to how this classification scheme could be reconciled with the α_{1A}-, α_{1B}- and α_{1D}- classification, which was evolving in parallel and whose subtypes could not be distinguished by prazosin. Oshita et al (1992) suggested that the α_{1A}-, α_{1B}- and α_{1D}- were subtypes of the α_{1H}- receptors, but this still meant that there was no cloned counterpart for the prazosin insensitive receptors ($\alpha_{1L/N}$-).

Although the identity of the gene encoding the α_{1L}-adrenoceptor is still not completely clear, there is now some convincing evidence that the α_{1A}-adrenoceptor is responsible for the α_{1L}- pharmacology. This idea initially came from experiments looking at the human α_{1a}-adrenoceptor expressed in cell lines (Ford et al. 1997a; Ford et al. 1996a). Radioligand binding experiments revealed a profile typical of the classical α_{1A}-pharmacology, with a high affinity for prazosin, WB 4101, 5 methylurapidil, niguldipine and RS-17053. When these results were compared to experiments measuring inositol phosphate accumulation, it was shown that the pA_2 values for these antagonists fell, and values were more typical of those seen at the α_{1L}-adrenoceptor in functional studies. The fall in the pA_2 values could not be explained by an overall lowering of the values, since affinity estimates for tamsulosin, indoramin and REC 15/2739 remained high and comparable to radioligand binding values. In addition, similar experiments carried out using the α_{1b}- and α_{1d}- cloned subtypes did not exhibit such changes in affinity estimates between the two types of experiment. It was also shown that if the conditions of radioligand binding were altered and whole cells rather than membranes used, together with a change in the media, results from radioligand binding at the cloned α_{1a}- could be altered to more closely mirror the α_{1L}-adrenoceptor pharmacology (Williams et al. 1996).

To date four isoforms of the human α_{1a}-adrenoceptor have been identified. These have been named $\alpha_{1a\text{-}1}$, $\alpha_{1a\text{-}2}$, $\alpha_{1a\text{-}3}$, $\alpha_{1a\text{-}4}$ and they all diverge in sequence at their carboxy termini (Chang et al. 1998; Hirasawa et al. 1995). It has been shown that when all four isoforms are expressed in cell lines and inositol phosphate accumulation measured, all the isoforms display the α_{1L}-adrenoceptor pharmacology (Ford et al. 1997b; Chang et al. 1998). Figure 1.1 shows a diagram summarising the current subdivisions of the α_1-adrenoceptors.

The α_{1L}-adrenoceptor is mainly associated with mediating noradrenaline induced contraction of the lower urinary tract tissue of man (Muramatsu et al. 1995; Muramatsu et al. 1994; Ford et al. 1996a) and animals, including the dog (Leonardi et al. 1997; Testa et al. 1997), and the rabbit (Leonardi et al. 1997; Shannon Kava et al. 1998; Deplanne and Galzin, 1996). In addition, a pharmacological profile consistent with this subtype has been identified in a variety of blood vessels including, rabbit cutaneous resistance arteries (Smith et al. 1997), rat small mesenteric artery (Van der Graaf et al. 1996a), dog saphenous vein, mesenteric vein and artery (Muramatsu et al. 1995), and rabbit thoracic aorta (Muramatsu et al. 1990a).

α_1-adrenoceptor classification

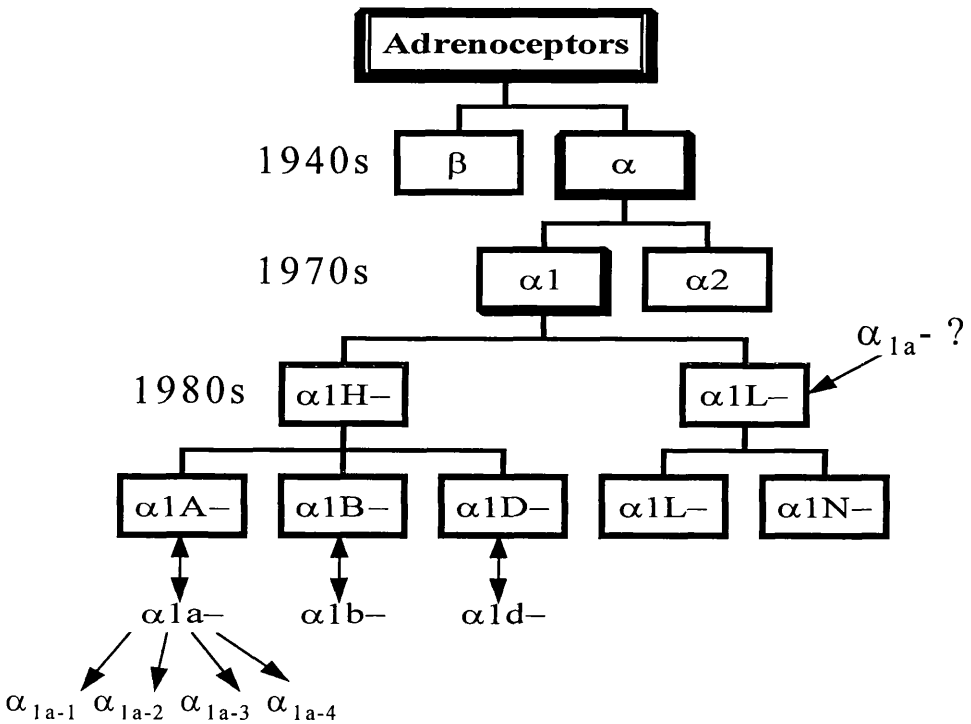

Figure 1.1. Chart summarising the classification of the α_1-adrenoceptors.

1.0.3 Structure and genomic organization of the α_1-adrenoceptors

α_1-adrenoceptors are part of a larger family of cell receptors that are coupled to guanine nucleotide regulatory proteins (G proteins) (Gilman, 1986). Hydropathicity analysis of the cloned receptors has shown a similarity to rhodopsin and bacteriorhodopsin and indicates a putative structure consisting of a single polypeptide chain ranging in length from 429-561 amino acid residues, comprising seven transmembrane spanning domains interspersed with alternate hydrophilic intra and extracellular loops, an extracellular amino terminus and a cytoplasmic carboxy terminus (Graham et al. 1996; Schwinn et al. 1990; Cotecchia et al. 1988). A theoretical diagram of this is illustrated in Figure 1.2. The exact three dimensional structure and orientation of the α_1-adrenoceptors is still being resolved.

Homology between the subtypes is highest in the transmembrane spanning domains with homology in the order of 65-75% at the amino acid level when the subtypes are compared (Cotecchia et al. 1995). Homologies are even higher between species for a particular subtype as can be seen from sequence comparisons in Chapter 6, where for example it is shown that homology between the α_{1a}-adrenoceptor sequences for different species is in the order of $\approx 90\%$.

The different subtypes are encoded by different genes with the α_{1b}- being located on human chromosome 5 and the α_{1a}- being located on human chromosome 8 (Smiley et al. 1998; Schwinn et al. 1990). Unlike most of the other G protein coupled receptors, the α_1-adrenoceptors have a single large intron located at the end of the putative VI transmembrane spanning domain (Cotecchia et al. 1995; Perez et al. 1994; Ramarao et al. 1992). A recent study also examined the genomic organization of the exon cassettes encoding the four isoforms of the human α_{1a}-adrenoceptor ($\alpha_{1a\text{-}1}$, $\alpha_{1a\text{-}2}$, $\alpha_{1a\text{-}3}$, $\alpha_{1a\text{-}4}$). In this study, workers were unable to locate the exon encoding for the $\alpha_{1a\text{-}2}$ isoform but

showed that the exon for the $\alpha_{1a\text{-}1}$ isoform lay beside the VII transmembrane domain region with no intron gap, the $\alpha_{1a\text{-}4}$ isoform lay 1.4Kb downstream and the $\alpha_{1a\text{-}3}$ exon lay approximately another 7Kb downstream (Chang et al. 1998).

A number of studies using mutated and chimeric α_1-adrenoceptors have been able to identify regions of the receptor responsible for G protein interactions, homologous desensitization and ligand binding.

Zhao et al (1996) found that three residues lying side by side on the extracellular loop of the fifth transmembrane spanning domain could alter the antagonist affinity of WB 4101 and phentolamine. Therefore if these sites on an α_{1b}-adrenoceptor were mutated to the residues found in the α_{1a}-adrenoceptor, this changed the antagonist profile from α_{1b}- to α_{1a}- in relation to WB 4101 and phentolamine. If the residues were mutated back the antagonist affinity could be reversed back to that for the α_{1b}-.

Lattion et al (1994), using truncated forms of the α_{1b}-adrenergic receptor, demonstrated that the carboxy terminal portion of the receptor was important in phosphorylation and homologous desensitization. Taking things a step further, (Diviani et al. 1997) it was shown that in fact 21 amino acids in the carboxy terminal portion of the receptor contained the phosphorylation sites associated with desensitization.

Using a chimeric receptor (β_2-/α_{1b}-), it was shown that the third intracellular loop of the α_1-adrenoceptor was important in the coupling of the receptor to the G protein. Further analysis revealed that 27 residues contained in the amino end of the third intracellular loop were sufficient to allow G protein coupling (Cotecchia et al. 1992). In addition, the same group were able to demonstrate that point mutations in the carboxy end of the third intracellular loop could produce constitutively active receptors (Kjelsberg et al. 1992).

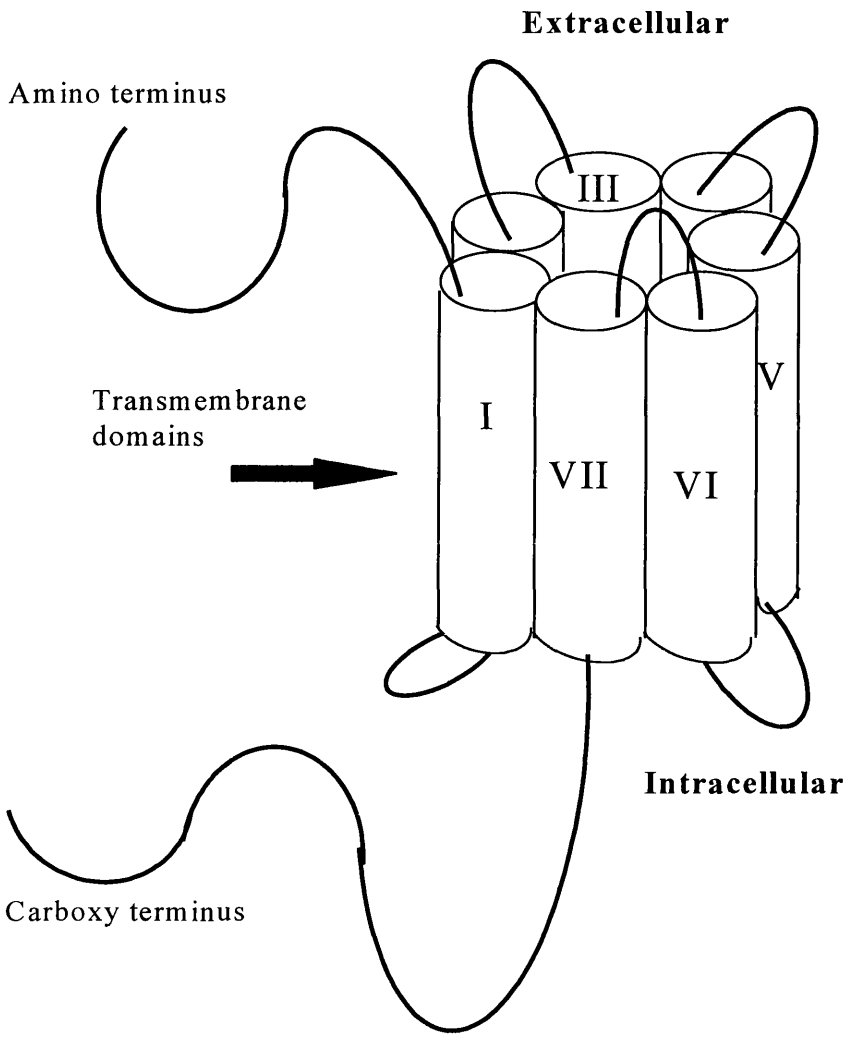

Figure 1.2. Schematic diagram of the proposed structure of an α_1-adrenoceptor. This diagram is a modified version of a diagram shown by Strosberg (1991). The diagram illustrates the seven transmembrane spanning domains lying within the cell membrane. These are connected by alternate intracellular and extracellular loops. An amino terminus lies extracellularly and a carboxy terminus lies within the cell.

1.0.4 Signal transduction

As already discussed above, it was previously thought that the α_{1A}- and α_{1B}- subtypes of adrenoceptor could be distinguished on the basis of their signal transduction mechanisms, with the α_{1B}- subtype depending on intracellular calcium and the α_{1A}- depending on extracellular calcium (Han et al. 1987a; McGrath, 1982). It has since been demonstrated that due to exceptions to the rule, this was not a reliable differentiating feature.

Although the pathways leading to an increase in intracellular calcium concentration are still not clear, it is generally accepted that α_1-adrenoceptors couple to G proteins usually of the pertussis toxin-insensitive family. G proteins are heterotrimeric structures made up of α, β and γ subunits. The α subunits are divided into several families. Examples of some of these families are, G_i and G_o, which are pertussis toxin-sensitive, G_s which are pertussis toxin-insensitive, G_{12} about which little is known and G_q which are pertussis toxin-insensitive. The G_q family is the one which is associated with α_1-adrenoceptor mediated phosphatidylinositol metabolism and the family comprises $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 16}$ (Wu et al. 1992, Guarino et al. 1996). Coupling to the G protein leads to activation of a membrane phospholipid, generally phospholipase C, which in turn catalyzes the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2), releasing diacylglycerol and Ins (1,4,5) P_3. Ins (1,4,5) P_3 induces the release of calcium from intracellular stores. Diacylglycerol activates Protein Kinase C which phosphorylates a number of intracellular proteins including calcium channels (Minneman and Esbenshade, 1994; Minneman, 1988).

A number of studies have aimed to identify differences in signal transduction pathways utilized by different subtypes of α_1-adrenoceptor, but again, if differences exist, they remain to be fully elucidated. Since the cloning of the α_1- subtypes, expression of the

receptors in cell lines has allowed the study of a particular subtype in isolation. Interpretation of the findings of these types of studies should, however, be treated with caution. Generally in transfection studies, the level of receptor expression is extremely high. Under these conditions, the amount of G protein and second messengers become limiting to the response. Under such circumstances, receptors may couple to and activate G proteins and second messenger systems that the native receptors would not. This can lead to errors in conclusions regarding signalling pathways and ligand affinities (Kenakin, 1997; Minneman and Esbenshade, 1994).

Bearing this in mind, from transfection studies it would seem that all the cloned receptors can induce inositol phosphate accumulation (Cotecchia et al. 1995; Schwinn et al. 1991; Theroux et al. 1996; Minneman and Esbenshade, 1994), although the α_{1a}-adrenoceptor seems to couple to phospholipase C with the greatest affinity (Theroux et al. 1996; Schwinn et al. 1991). These differences could be explained by the different subtypes interacting with different G proteins. Wu et al (1992), showed that all three subtypes of cloned receptor could couple to both $G_{\alpha q}$ and $G_{\alpha 11}$. In contrast only α_{1a}- and α_{1b}- coupled to $G_{\alpha 14}$, and only α_{1b}- coupled to $G_{\alpha 16}$.

While the above G_q family proteins are all pertussis toxin-insensitive, there is also evidence that α_1-adrenoceptors do interact with pertussis toxin-sensitive G proteins. Boonen and DeMey (1990), demonstrated that the contractile responses of α_1-agonists in the rat mesenteric resistance artery were abolished by treatment with pertussis toxin and Gurdal et al (1997), were able to demonstrate that in rat aorta, although part of the phenylephrine mediated response was pertussis toxin-insensitive and associated with $G_{\alpha q}$, a proportion of the response was sensitive to the application of pertussis toxin, and seemed to be associated with an interaction of the α_{1B}-adrenergic receptor with the pertussis toxin-sensitive $G_{\alpha o}$.

As well as activation of phospholipase C, α_1-adrenoceptors can activate phospholipase D and phospholipase A_2 (Balbao and Insel, 1998; Graham et al. 1996). In addition, the α_1- adrenoceptors can increase intracellular cAMP (Chang et al. 1998; Schwinn et al. 1991; Cotecchia et al. 1990). This latter effect has been most studied in the α_{1b}-adrenoceptor and it would seem that in transfected cells, this receptor can couple to G_s (Horie et al. 1995).

It has recently been shown that the α_{1d}-adrenoceptor can induce smooth muscle cell hypertrophy through activation of the mitogen-activated protein kinase (MAP kinase) cascade (Xin et al. 1997), which has been identified as an important pathway involved in regulating the growth and differentiation of cells. As this study examined native receptors in smooth muscle cells, the pathway allowing activation of the MAP kinase cascade is likely to be a real event. Another study using neonatal myocytes showed that both α_1- and β- adrenoceptors were involved in MAP kinase and raf-1 kinase activation and that the action of both receptor types appeared to be synergistic (Yamazaki et al. 1997).

1.1 Heart Failure

1.1.1 Definition

The aim of the body is to maintain cardiac output, which is defined as the volume of blood pumped out of the ventricles per minute. When for some reason the body fails to circulate enough blood to meet the metabolic demands of the body or the blood backs up within the venous or capillary bed, this is termed heart failure (Hamlin, 1988). The main factors controlling cardiac output are outlined in Figure 1.3.

1.1.2 Aetiology

Causes of heart failure may be primarily cardiac or vascular and aetiologies differ with the species involved. In man the main causes of heart failure are hypertension and coronary artery disease. In the domestic canine population there are two main causes of acquired cardiac disease, namely endocardiosis and idiopathic dilated cardiomyopathy.

Endocardiosis

This is the most common acquired cardiac disease in the canine population. One study suggested that 58% of dogs over the age of 9 years had evidence of severe valvular disease at postmortem (Whitney, 1974). Endocardiosis of the atrioventricular valves is the most common finding with the mitral valve being affected in about 60% of cases. Involvement of both mitral and tricuspid valves is found frequently but involvement of the tricuspid valve alone is much less likely. In general smaller breeds of dog are more commonly affected and males are 1.5 times more likely than females to develop the condition (Keene, 1988). The condition has also been reported in pigs (Guarda et al. 1993; Castagnaro et al. 1997).

The aetiology is still not understood. Certainly some breeds have a much higher incidence, for example the cavalier King Charles Spaniel (Haggstrom et al. 1992), suggesting a genetic component to the condition.

Grossly the valves become thickened and irregular in outline and this is thought to be due to defective or degenerative alterations in the collagen or glycosaminoglycan content of the valve leaflets. In effect the atrioventricular valve becomes leaky so that during ventricular systole, blood jets back into the atria instead of being ejected out into the aorta and pulmonary artery. Stroke volume is therefore reduced (Keene, 1988).

Dilated cardiomyopathy

The aetiology of dilated cardiomyopathy is also unknown. This is the most common myocardial disease of dogs. The mean age of affected animals is 4-6 years. Male animals and especially the "giant breeds" are more commonly affected (Tidholm and Jonsson, 1997). Common breeds involved are Great Danes, Wolfhounds, Dobermann Pinschers, Boxers, Springer Spaniels and Cocker Spaniels. On postmortem the heart is large and dilated. There may be concurrent endocardiosis but even if the valve leaflets are normal there has often been valvular regurgitation due to dilation of the valve annulus. Focal endocardial fibrosis is often present and histologically there is myocardial cellular degeneration (Tidholm and Jonsson, 1997; Tidholm, 1996; Cobb, 1992).

A number of less common conditions can also cause acquired cardiac disease in dogs such as, endocarditis, cardiac tamponade, intracardiac neoplasia and hypertrophic cardiomyopathy (although this latter condition is prevalent in the feline population (Luis Fuentes, 1992)).

Congenital cardiac anomalies can also precipitate the syndrome of cardiac failure and occur not uncommonly in the canine population. Some examples of these would be pulmonic stenosis, aortic stenosis, ventricular septal defects, atrioventricular valve dysplasias and patent ductus arteriosis (Olivier, 1988).

1.1.3 Pathophysiology of heart failure

In respect of the pathophysiology, the sequence of events resulting in the syndrome of cardiac failure are similar irrespective of the initiating cause.

When there is a fall in the cardiac output, this causes a fall in blood pressure, since blood pressure is the sum of cardiac output times total peripheral resistance (Levick,

1992). The body aims to maintain the blood pressure through a number of reflex arcs which involve baroreceptors, cardiopulmonary receptors, arterial chemoreceptors and muscle receptors. These receptors send messages to the nucleus tractus solitarus in the brain. This in turn leads to neurohumoral activation. In the short term these mechanisms may well be beneficial to the body, but in the longer term they can actually contribute to and worsen the syndrome of heart failure (Francis et al. 1984). Figure 1.3 summarises the factors which influence cardiac output and Figure 1.4 summarises the factors involved in the genesis of heart failure. In Figure 1.3, Starling's law describes the phenomenon whereby, within certain limits, an increase in preload (end diastolic volume), causes an increase in stroke volume. The relationship is not linear, since above a certain limit, further increases in preload are associated with a plateau and then a decline in stroke volume (Levick, 1992). Laplace's law S=Pr/2W, links the dimensions of the ventricle to wall stress. The main determinant of afterload (the force against which the ventricle contracts), is the total peripheral resistance and this is directly proportional to wall stress on the ventricle. However, chamber dimensions will also have an effect on wall stress and the force of contraction of the ventricle. In the equation S = Pr/2W, S is the wall stress or afterload, P is the pressure, r is the chamber radius and W is the wall thickness. From this it can be seen that in dilated cardiomyopathy for example, where the chamber is dilated and the wall is thin, S will be bigger leading to increased wall stress and a reduction in force production. (Hamlin, 1988).

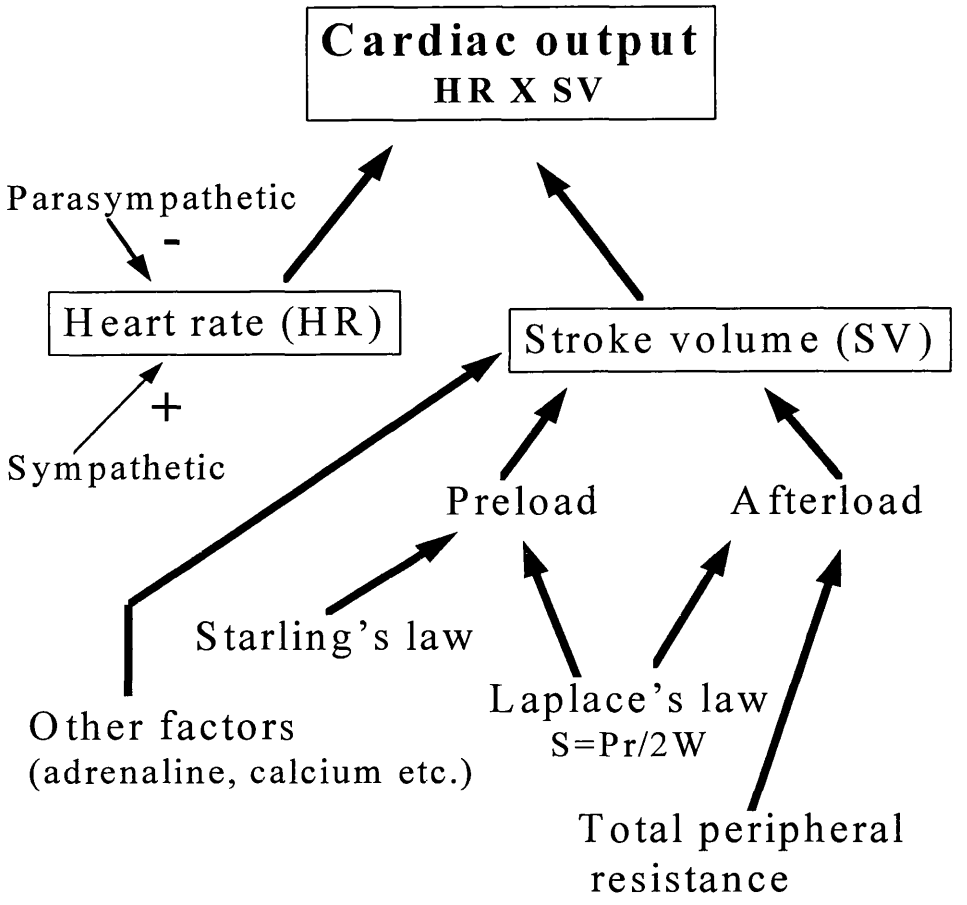

Figure 1.3. Flow diagram representing the main factors that contribute to the control of cardiac output.

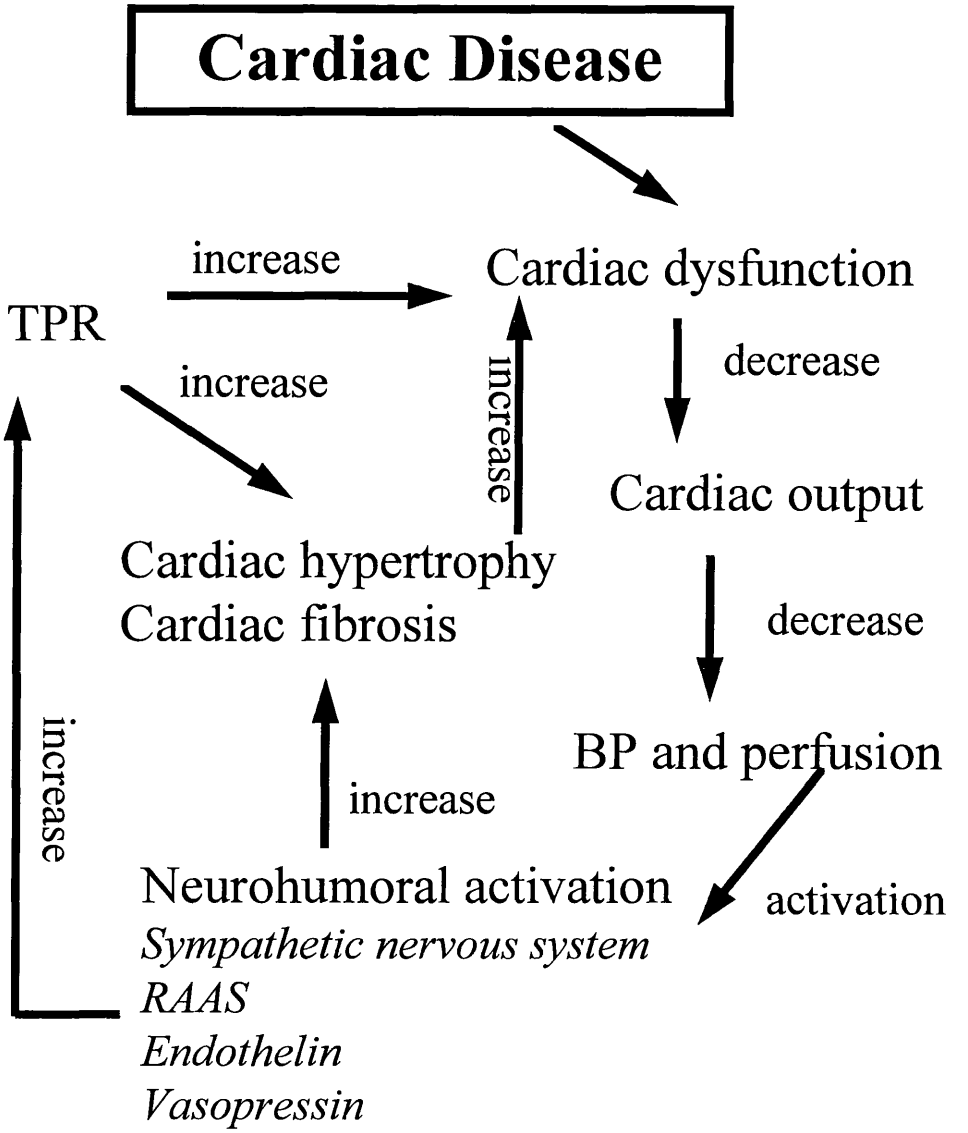

Figure 1.4. The heart failure cascade. TPR is total peripheral resistance, RAAS is the renin angiotensin aldosterone system and BP is blood pressure.

1.1.4 Neurohumoral systems

Neurohumoral activation comprises several important components. These include, the sympathetic nervous system, the renin angiotensin aldosterone system, endothelin, vasopressin and the natriuretic peptides.

Sympathetic nervous system

Activation of the sympathetic nervous system occurs very early in the course of disease and elevation in circulating catecholamines has been well documented. (Francis et al. 1984; Grassi et al. 1995; Hasking et al. 1986; Leimbach et al. 1986; Cohn et al. 1984; Thomas and Marks, 1978). Increased sympathetic activity leads to increases in heart rate through β-adrenoceptors in the myocardium and peripheral vasoconstriction primarily through α_1- but also α_2-adrenoceptors in vascular smooth muscle. The exact mechanism of elevated circulating catecholamines has been the subject of some debate. Several papers suggested that the increased levels are simply due to increased release from the nerve terminal and the adrenal gland (Meredith et al. 1993; Hasking et al. 1986). However, more recent publications suggest, not only that an increased release of catecholamine is involved, but also that there appears to be an impairment in the reuptake of noradrenaline by nerve terminals in the myocardium (Bohm et al. 1995; Beau and Saffitz, 1994), which contributes to local elevation and leads to spillover into the plasma.

The overall effect of sympathetic activation is to increase heart rate, force of contraction and increase total peripheral resistance. While in the short term these effects may be beneficial in maintaining the blood pressure, in the longer term they are almost certainly detrimental (Francis et al. 1984). An obvious adverse effect is the increased total peripheral resistance which increases the afterload on the myocardium. This in turn

increases myocardial wall stress which is directly proportional to myocardial oxygen consumption. In addition, it now seems clear that α_1-adrenoceptors can mediate vascular smooth muscle and myocyte growth and hypertrophy (Xin et al. 1997; Milano et al. 1994; Yamazaki et al. 1997; Milano et al. 1994). This will also contribute to the raised peripheral resistance and to ventricular remodelling. The ventricular remodelling is characterised by lengthening of the myocytes, slippage of the myocytes and interstitial growth. The net effect of all these changes is to alter the shape and lower the efficiency of the ventricle thereby having a deleterious effect on the patient (Cohn, 1995).

In fact, it has been shown that plasma levels of noradrenaline have an inverse relationship to prognosis, i.e. the higher the levels of circulating catecholamines, the poorer the prognosis for the patient (Cohn et al. 1984).

Another feature of increased sympathetic activity is a decrease in β-adrenoceptor sensitivity and receptor number, due to homologous desensitization and downregulation of these receptors. The effects can be seen in the myocardium and vascular smooth muscle. In the canine pacing induced model of heart failure it has been shown that there is a decrease in density of β-adrenoceptors in the myocardium, but this effect can be reversed on cessation of pacing (Larosa et al. 1993). Decreased β-adrenoceptor density can also be demonstrated in human myocardium from heart failure patients (Bristow et al. 1982; Bristow et al. 1986). Downregulation of peripheral vascular β-adrenoceptor function has also been demonstrated in dogs with pacing induced heart failure (Kiuchi et al. 1993). There would also appear to be a role for alteration in the G protein composition of cells contributing to the decreased β-adrenoceptor function. This can be demonstrated by a decrease in G(s) (stimulatory), without alteration in G(i) (inhibitory) content (Vatner et al. 1996; Lai et al. 1996).

Renin angiotensin aldosterone system (RAAS)

Renin is released from the juxtaglomerular apparatus in the kidney. The release of renin is triggered by a fall in local perfusion and sympathetic activity. Renin converts angiotensinogen into angiotensin I. Angiotensin I is in turn converted to angiotensin II by angiotensin converting enzyme (ACE) (Levick, 1992). Originally this conversion was thought to occur primarily in the lung, but it now seems clear that ACE activity is more widely distributed throughout the endothelium in the vascular system and that local renin release from tissues other than the kidney plays an important role (Timmermans et al. 1993; Diet et al. 1996). Angiotensin II has numerous effects. It causes the release of aldosterone from the adrenal gland which in turn is responsible for the retention of sodium and water (Hall, 1986). It has also been shown that chronic administration of aldosterone can induce a reduction in baroreceptor sensitivity in dogs (Wang, 1994), a phenomenon which is well described in heart failure (Dibnerdunlap and Thames, 1992; Wang et al. 1996). Angiotensin II is a potent vasoconstrictor by direct activity on AT_1 receptors on the vascular smooth muscle (Timmermans et al. 1993). Angiotensin II also modulates α_1-adrenoceptor function and has been shown to enhance noradrenergic neurotransmission (Minatoguchi and Majewski, 1994; Cox et al. 1996), as well as increasing the transcription and expression of α_1-adrenoceptors in vascular smooth muscle (Hu et al. 1995). Conversely, the use of angiotensin converting enzyme inhibitors has been shown to decrease α_1-adrenoceptor sensitivity in human vessels (Kimura et al. 1997). ACE enhances the breakdown of the vasodilator bradykinin, thereby having an indirect vasoconstrictor effect (Warren and Loi, 1995). Activation of the RAAS is associated with elevations in levels of endothelin (Clavell et al. 1996) and angiotensin II has been shown to have a direct depressant effect on myocytes which appears to be mediated through the AT_1 receptor. The growth effects of angiotensin II

have been well documented and angiotensin II can promote hypertrophy of cardiac myocytes through actions mediated primarily through AT_1 receptors (Booz and Baker, 1996), as well as hypertrophic (Berk et al. 1989; Geistefer et al. 1988), and hyperplastic (Dubey et al. 1992), responses in vascular smooth muscle.

Endothelin

Endothelin is a 21-amino acid peptide, and was first described in 1988 (Yanagisawa et al. 1988). It is synthesized by the endothelium. Three endothelins have been described but endothelin-1 is the most important. The actions of endothelin are mediated by ET_A and ET_B receptors (Cannan et al. 1996). ET_A receptors are located on vascular smooth muscle where they mediate vasoconstriction and ET_B receptors are located on the endothelium where they mediate vasodilation. ET_B receptors have also been localized to smooth muscle where they can mediate vasoconstriction (Rubanyi and Polokoff, 1994).

Endothelin can also mediate hypertrophic and mitogenic responses (Gwathmey and Paige, 1994; Booz and Baker, 1996). Levels of endothelin are elevated during cardiac failure in both experimental models (Margulies et al. 1990), and in human heart failure patients (Stewart et al. 1992). During experimental heart failure the inotropic response to endothelin is enhanced (Li and Rouleau, 1996), as is coronary artery contraction (Cannan et al. 1996). The pulmonary circulation is thought to be important in the clearance of endothelin, and ET_B receptors may be especially important in this role (Dupuis et al. 1996).

There are numerous interactions with endothelin and the other factors involved in neurohumoral activation. For example, in experimental heart failure the RAAS appears to increase the levels of endothelin (Clavell et al. 1996), atrial natriuretic peptide

decreases the release of endothelin-1 secretion (Wada et al. 1996), and α_1-adrenoceptors may be involved in the activity of endothelin (Todorov et al. 1995).

Vasopressin

Vasopressin is released from the posterior pituitary gland and has antidiuretic actions as well as being a powerful vasoconstrictor (Levick, 1992). During heart failure levels of this hormone are elevated (Francis et al. 1984).

Natriuretic peptides

There are three natriuretic peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (Winaver et al. 1995). Levels of BNP and ANP are elevated in heart failure.

Atrial nariuretic peptide is produced by atrial myocytes in response to stretch (Brenner et al. 1990). ANP is also produced by ventricular myocyctes, but only when ventricular hypertrophy is present (Lee et al. 1988). Levels of ANP are elevated during heart failure and have been shown to correlate positively with mortality in patients with cardiac disease (Swedberg et al. 1990). ANP induces diuresis and vasodilation (Swedberg et al. 1990). It also decreases the activity of the RAAS (Fett et al. 1993), endothelin-1 production (Wada et al. 1996) and, it can modulate the release of vasopressin (Winaver et al. 1995). ANP has also been shown to have anti-growth and anti-proliferative effects on vascular smooth muscle cells (Winaver et al. 1995).

BNP is also elevated in cardiac disease and is produced predominantly by ventricular myocytes (Yasue et al. 1994). There is evidence that BNP is a superior marker of ventricular dysfunction compared to ANP (Yamamoto et al. 1996).

1.1.5 Treatment of cardiac failure

The mainstays of treatment of cardiac disease in the canine population comprise the use of diuretics, vasodilators and cardiac glycosides.

Diuretics are used in an attempt to control the congestion seen in heart failure which is most often exhibited as pulmonary oedema. Frusemide is the diuretic of choice. This is a high ceiling loop diuretic which reduces the resorption of sodium and chloride in the loop of Henle (Kittleson, 1988). Often if the diuresis induced by frusemide is insufficient then it may be combined with another class of diuretic such as spironolactone. This latter drug is a potassium sparing diuretic and has been shown to be effective in the management of human patients refractory to frusemide alone (Vanvliet et al. 1993).

Digoxin is a cardiac glycoside which is used frequently in canine heart failure. It has a variety of actions which include positive inotropy, resetting of the baroreceptor reflex, reduction in the release of renin from the juxtaglomerular apparatus and vagomimetic activity, for example, slowing of conduction through the atrioventricular junction (Kittleson, 1988). One of the main indications of digoxin is in the management of atrial fibrillation. This dysrhythmia is commonly found in dogs with atrial enlargement often associated with the common cardiac diseases such as dilated cardiomyopathy and endocardiosis. In this case, digoxin helps to slow down the ventricular response to the atrial fibrillation (Keene, 1996).

One of the most successful treatments in recent years has been the angiotensin converting enzyme inhibitors (ACEI). These drugs prevent the formation of angiotensin II from angiotensin I, by inhibiting the angiotensin converting enzyme. This results in a blockade of the effects of both ACE and angiotensin II which have been described in the section titled renin angiotensin aldosterone system. They are now used extensively in

the management of heart disease in the human population. An increasing number of studies has shown that these drugs have significant benefits and increase longevity, quality of life and haemodynamic parameters both in the human population (CONSENSUS trial study group, 1987) and in the canine population (Woodfield et al. 1995; Hamlin et al. 1996; The IMPROVE study group, 1995).

1.1.6 The role of the vasculature in heart failure

For several decades it has been clear that exercise intolerance is an important component of cardiac disease. Not only this, but the degree of exercise intolerance exhibited by patients is often disproportionate to their degree of left ventricular dysfunction (Francis et al. 1984). This has led to the belief that there must be an additional factor contributing to the syndrome in addition to the myocardial dysfunction itself. One theory is that there is an alteration in skeletal muscle metabolism, preventing the exercising muscle from working efficiently (Coats, 1996).

The other factor which is considered to be important and which is relevant in this study, is that there is an alteration in the peripheral vasculature which contributes to increased total peripheral resistance and prevents normal dilation of the vasculature, which would normally increase blood flow to the exercising skeletal muscle. Indeed, a number of studies both on human patients and animal models of disease have confirmed that blood flow to exercising skeletal muscle is reduced in heart failure (Zelis et al. 1969; Musch and Terrell, 1992; Higgins et al. 1972). Wilson et al (1984), found a reduction in limb blood flow and an increased O_2 extraction, together with early lactate production, in heart failure patients when compared to normal controls. LeJemtel et al (1986) described similar findings, but this time compared single and two limb exercise. In normal patients, cardiac output reached a maximum and oxygen uptake reached a

plateau during two limb exercise. In contrast, during single limb exercise, maximal cardiac output was not achieved prior to fatigue, although vascular flow was greater in the limb during single leg exercise compared to two limb exercise. In the heart failure group, patients had a reduced limb blood flow and did not show an increased blood flow to the limb when only single leg exercise was performed, suggesting an impaired blood flow. Taking things a step further, Jondeau et al (1995), demonstrated that normal patients asked to perform leg exercise to exhaustion achieved maximal cardiac output, and oxygen consumption reached a plateau. In contrast, heart failure patients did not achieve maximal cardiac output during leg exercise, since additional arm exercise in the failure group induced a further increase in their cardiac output. This suggested that blood flow to the lower limb and not cardiac output was the limiting factor for the heart failure patients.

This led to an increasing interest in the peripheral vasculature, both in relation to characterisation of normal vessels and in relation to the identification of alterations in the heart failure state.

To date a number of studies have looked at the vasculature in vivo and in vitro. Results have often been conflicting but findings fall into two main categories. 1. Alteration in vasorelaxation. 2. Alteration in smooth muscle contraction.

Vasorelaxation

Two important pathways are involved in mediation of vascular smooth muscle relaxation, namely pathways involving the formation of guanosine 3'-5'-cyclic monophosphate (cGMP) or those involving adenosine 3'-5'-cyclic monophosphate (cAMP), (Vanhoutte, 1996; Nasa et al. 1996). The three main relaxing factors released from the endothelium are nitric oxide, prostacyclin and endothelium-derived

hyperpolarizing factor (EDHF). The release of these factors from the endothelium can be triggered by a number of substances. Acetylcholine, for example, induces the release of nitric oxide and also EDHF (Vanhoutte, 1996). Nitric oxide is also released under basal conditions and release can be triggered due to shear stress on the vascular wall (Hayoz et al. 1993). Adenosine can also stimulate or inhibit the formation of cAMP depending on the subtype of receptor involved. This substance is released from cells by facilitated diffusion and also from the degradation of released ATP. It has been shown that mice lacking the adenosine A_{2a} receptor demonstrate hypertension, suggesting that the action of adenosine at this receptor subtype is important in mediating a degree of basal vasorelaxation (Ledent et al. 1997).

A growing number of human and animal studies have now demonstrated alterations in relaxation responses in vessels from heart failure patients and animals.

A consistent finding is that acetylcholine mediated, endothelium-dependent relaxation responses, are attenuated in heart failure. This has been demonstrated in forearm blood flow studies (FBF) in human heart failure patients (Katz et al. 1992; Drexler et al. 1992; Kubo et al. 1991; Hirooka et al. 1994), in isolated resistance sized gluteal arteries from heart failure patients (Angus et al. 1993), in the femoral artery and coronary arteries of dogs with experimentally induced heart failure (Wang et al. 1994; Kaiser et al. 1989), and in rats with experimentally induced heart failure, both in isolated vessels and in intact hindquarter resistance vessels (Nasa et al. 1996; Drexler and Wenyan, 1992). This finding is unlikely to be due to an inability of the vascular smooth muscle to relax to the nitric oxide released from the endothelium, since most studies have shown, that unlike the relaxation to acetylcholine, the relaxation to sodium nitroprusside or nitroglycerine, which are endothelium-independent vasorelaxing agents, using the cGMP pathway, is not attenuated in heart failure, both in vivo, (Drexler et al. 1992;

Drexler and Wenyan, 1992; Wang et al. 1994; Kaiser et al. 1989; Hirooka et al. 1994), and in isolated vessels, (Nasa et al. 1996; Angus et al. 1993). It therefore seems more likely that the endothelium is defective in heart failure. Interestingly, from the point of view of this study, one group failed to show alteration in acetylcholine induced vasorelaxation, where isolated dorsal pedal artery and saphenous vein segments from dogs with experimentally induced heart failure were examined, (Forster et al. 1989).

Several studies have also reported a reduced cAMP mediated vasodilation in heart failure. For example, Mathew et al (1993), examined isolated canine pulmonary arteries from dogs with experimental heart failure and showed, that while vasorelaxations to acetylcholine and bradykinin were comparable to controls (cGMP mediated), vasorelaxations to isoproterenol, arachidonic acid and prostacyclin, were all attenuated in the failure group (cAMP mediated). Nasa et al (1996), also demonstrated a reduction in isoproterenol mediated vasorelaxation in thoracic artery and pulmonary artery in rats with experimental heart failure.

Results regarding alterations in basal nitric oxide production in heart failure are less conclusive. Some workers report no change in basal nitric oxide production, as estimated by vasoconstrictor response to nitric oxide synthase inhibitors (Drexler and Wenyan, 1992; Kubo et al. 1994). In contrast, Elsner et al (1991), found that there was a decreased vasoconstrictor response to N^G-nitro-L-arginine (NNA) in dogs with experimental heart failure, suggestive of reduced basal nitric oxide and Teerlink et al (1994), found that the enhanced response of intact isolated aortic rings from rats with heart failure was due to a reduced basal nitric oxide production in the heart failure versus the control animals. Finally, several studies report evidence for enhanced nitric oxide production in heart failure, the three studies cited all being in human heart failure patients (Drexler et al. 1992; Habib and Oakley, 1994; Winlaw et al. 1994).

Vasoconstrictor responses

Fewer studies have examined agonist responses in heart failure and the results from these studies are inconsistent. Obviously factors such as the species, the stage and severity of disease, the blood vessels examined, the experimental design and drug treatment will all have an influence and can contribute to the findings for individual studies.

A number of studies by Forster et al, have demonstrated an enhanced sensitivity and maximal response to noradrenaline in isolated dorsal pedal artery and saphenous vein segments from pacing induced heart failure dogs when compared to controls (Forster et al. 1989; Forster and Armstrong, 1990). On further examination, it appeared that the response to α_1-agonists was enhanced while response to α_2-agonists was decreased. Stassen et al (1997a; 1997b) also found an increased sensitivity to phenylephrine in isolated vessels from rats with experimental heart failure, although maximal response was reduced. The difference appeared to be related to calcium influx and not to protein kinase C or the IP_3 mediated release of intracellular calcium. Teerlink et al (1994), in endothelium denuded aortic rings from heart failure rats, found a reduction in maximal response to both noradrenaline and potassium chloride. This was similar to findings of Angus et al (1993), who also found a reduction in maximal response in gluteal resistance arteries from human heart failure patients, when a variety of agonists were used including noradrenaline, angiotensin I and angiotensin II.

In vivo studies have been similarly conflicting with Feng et al (1994), demonstrating reduced responses to both α_1- and α_2-adrenoceptor agonists in forearm blood flow studies. Meanwhile, Indolfi et al (1994), also in forearm blood flow studies in human patients, reported no change in α_1- and α_2- adrenoceptor agonist responses. Kubo et al (1989), showed a similar response to the α_2-antagonist yohimbine in control and heart

failure patients in forearm blood flow studies also supportive of the finding that there was no alteration in the α_2-adrenoceptor population.

1.2 Aims of this thesis

As outlined in the introduction, the resistance artery component of the peripheral vasculature plays an important role in maintenance of total peripheral resistance. There is now convincing evidence that vascular alterations contribute to the syndrome of heart failure, but as yet the exact mechanisms involved have not been fully elucidated. It is a prerequisite that before pathophysiological changes can be characterised, the "normal" characteristics of in this case, blood vessels, must be understood. On this basis, this thesis has three main aims.

1. Since α_1-adrenoceptors are important mediators of vascular smooth muscle contraction, the primary aim of this project was to characterise the dog saphenous vein and subcutaneous resistance arteries, in relation to α_1-adrenoceptors mediating contraction to exogenous noradrenaline. The dog saphenous vein has been used in pharmacological studies before, therefore some characterisitics of this vessel were already known. In contrast, to my knowledge, isolated canine subcutaneous resistance arteries have not been studied.

2. In addition to functional characterisation of the α_1-adrenoceptors, another aim of this project was to clone and sequence the canine α_{1a}- adrenoceptor. This subtype was selected because of the mounting evidence from the literature that the α_{1a}- subtype is responsible for the α_{1L}- adrenoceptor pharmacology. This latter subtype appears to play a functional role in both of the vessels that were examined.

3. Although studies on isolated vessels from experimental models of heart failure have been conducted, studies using isolated vessels from dogs with naturally occurring heart failure have not been carried out. For this reason, segments of femoral artery, saphenous vein, and subcutaneous resistance arteries were collected from clinical heart failure cases which were euthanased because of their heart disease. Experiments were designed to identify alterations in vasoconstriction and vasorelaxation responses between control and heart failure animals, taking into account the aetiology of the heart failure and treatment if any.

CHAPTER 2

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MATERIALS AND METHODS FOR FUNCTIONAL STUDIES

2.0 Materials

2.0.1 Solutions

Krebs-Henseleit solution (KS) was of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, $NaHPO_4$ 1.2 and glucose 11.5. Na_2EDTA 0.023mM was also included in the KS solution at all times to prevent degradative oxidation of noradrenaline.

For the antagonist studies, in both the saphenous vein and subcutaneous resistance arteries, certain drugs were included in the KS solution. This modified KS was termed blockers Krebs' (BKS) and had an identical composition as Krebs' solution but in addition, cocaine (3μM) (Aboud et al. 1993), corticosteroid (30μM) (Blue et al. 1995) and propranolol (1μM) (Forster, 1996) were added to block neuronal uptake, non-neuronal uptake and β-adrenoceptors respectively.

Potassium Chloride solution (KPSS), was a 125mM potassium chloride solution which had the same composition as KS except that the NaCl was replaced with KCl.

2.0.2 Drugs

The following compounds were used:

(R) A-61603 (Abbott laboratories); (-)-noradrenaline bitartrate (Sigma); delequamine (RS-15385-197, Roche Bioscience formerly Syntex, gift of Dr. Whiting); rauwolscine (Roth); WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride), Research Biochemicals International); HV723 (α-ethyl-3,4,5-trimethoxy-α-3-((2-(2-methoxyphenoxy)ethyl))-amino)propyl)benzeneacetonitrile fumarate, a gift from I. Muramatsu; 5 methylurapidil (Research Biochemical

International); chloroethylclonidine (Research Biochemicals International); prazosin HCl (Pfizer); cocaine HCl (MacCarthy's); propranolol HCl (Sigma); corticosterone-2 acetate (Sigma); BMY 7378 (dihydrochloride 8-[2-[4-(2-Methoxyphenyl)-1-piperozynl]ethyl]-8-azaspiro[4.5]decone-7,9-dione, Research Biochemicals International); (-)- phenylephrine HCl (Sigma); UK-14304 (Research Biochemicals International); L-NAME (N^{ω}-Nitro-L-Arginine Methyl ester hydrochloride, Sigma); Acetylcholine Chloride (Sigma).

All drugs were made up daily from salts in deionised water, with the exception of noradrenaline which was initially dissolved in 23μM Na_2EDTA. Further dilutions were then made using deionised water.

2.0.3 Animals used

Dogs were euthanased at the local dog and cat home using pentobarbitone sodium (Euthatal 200mg/ml Rhone Merieux) at a dose of 150mg/Kg bodyweight, administered by intravenous injection. Dogs of all breeds, ages, sexes and weights were utilised. Animals were given a clinical examination in order to exclude any with obvious clinical abnormalities. For the heart failure study, animals used were those diagnosed and treated at Glasgow University Veterinary School. These animals were euthanased because of failure to respond to treatment or because of worsening of their disease.

2.1 Vessel Removal

2.1.1 Saphenous vein removal

Vessels were removed immediately after euthanasia by careful dissection, ensuring as little connective tissue remained attached to the vessel as was possible. The

intermediate section of the lateral saphenous vein was consistently removed each time (Figure 2.1). Vessels were then placed in ice cold KS and used within 24 hours.

2.1.2 Femoral artery removal

The artery was dissected from the femoral triangle. This is located anatomically at the proximal medial aspect of the hindlimb and is bounded by the sartorius muscle cranially and the pectineus and adductor muscles laterally. The medial femoral fascia and adipose tissue were dissected to reveal the femoral artery and vein running together with the artery in a cranial location (Figure 2.1). After removal the artery was treated in an identical fashion to the saphenous vein.

2.1.3 Subcutaneous resistance artery removal

A patch of skin overlying the gluteal muscles was removed and placed directly into ice cold KS. This was transported back to the laboratory and resistance sized arteries were removed with the aid of a dissecting microscope. As for the larger vessels, resistance vessels were used within 24 hours of removal.

2.2 METHODS

2.2.1 Mulvany Halpern wire myograph

Functional experiments of resistance arteries were carried out using a four chamber Mulvany Halpern wire myograph (J.P. Trading. Aarhus, Denmark.) (Mulvany and Halpern, 1976).

Resistance arteries were cut into approximately 2mm lengths and mounted between two 40μm wires. One wire was attached to a fixed head while the other was attached to a head which in turn was connected to a force transducer. The force transducer was in

turn connected to a Linseis (TYP 2066) four channel pen recorder to allow recordings of force production (Figure 2.2).

2.2.2 Organ bath apparatus

Functional experiments of the saphenous vein (S.V.) and femoral artery (F.A.) were performed in 10ml organ baths. Vessels were cut into 5mm ring sections and mounted between two wires both 0.35mm in diameter. One wire was fixed to a perspex post while the other was suspended by cotton thread and attached to a Grass FT03 force transducer and micrometer (Figure 2.3.). Measurements were recorded on a Linseis (TYP 7208) eight channel pen recorder. Vessels were bathed in Krebs' solution (K.S.) and maintained at 37°C. KS was bubbled with 95%0_2/5%CO_2 at all times.

2.2.3 Normalisation procedure

The process of normalisation was first described by Mulvany and Halpern (1977), for small resistance sized arteries and since then has become an accepted procedure when studying resistance arteries using the Mulvany Halpern wire myograph.

The basis of the normalisation procedure is the generation of a circumference/tension relationship for each vessel, which when fitted to an exponential equation, allows the calculation of the diameter at which the vessel should be set in order to be equivalent to a given transmural pressure if that vessel were relaxed. Generally for resistance arteries, vessels are set at 0.9 of L100 which means 90% of the diameter due to a transmural pressure of 100mmHg.

The actual procedure is carried out by starting with the wires suspending the vessel touching and putting the vessel through a number of stretches at one minute intervals. Each time the vessel is stretched, the micrometer reading is recorded together with the

force at the end of the minute interval. This allows calculation of the vessel circumference by this:

$$L = (\pi + 2)\, d + 2f \qquad \text{EQUATION 1.}$$

where L is the circumference, d the diameter of the wires and f the distance between the two wires.

Tension produced by the vessel is calculated by this:

$$T = F/2g \qquad \text{EQUATION 2.}$$

where F = the force produced in mN, g is the length of the vessel and tension is defined as the circumferential wall force per unit length.

In this study the resistance arteries were normalised to 0.9 of L100 as described. The same procedure was adapted for the saphenous vein and femoral artery in a similar way to Angus et al (1986). The main reason for this decision was the wide variety of dogs involved in the study, leading to considerable variation in the size of vessels being used. It therefore seemed more desirable to adopt a procedure which would take vessel length and diameter into consideration rather than simply setting vessels at a given resting tension. In addition, looking at the literature there seemed to be a wide range of optimal resting tensions given for these particular vessels. For example Constantine et al (1982) quote a value of 0.6g for saphenous vein compared to a value of 3g given by Guan et al (1990). In the case of the saphenous vein the vessel was set at 0.9 of L20 which means 90% of the diameter if the transmural pressure were 20mmHg. This was a more appropriate pressure for a vein (Levick, 1992). The same setting was also used for the

femoral artery. While in vivo pressures in this artery would be considerably higher, it was not possible to achieve the force required to normalise these arteries to 0.9 of L100 and this method still took into account variations in vessel dimensions and vessels produced consistently good reproducible contractions.

All calculations were performed using a computer iterative fitting technique where the computer then gives the micrometer setting in order to achieve 0.9 of L20 or L100 and calculates the equivalent transmural pressure at this setting using the Laplace relationship, assuming that the vessel wall is sufficiently thin for the relationship to apply and that the curvature caused by the wires has no effect. The Laplace equation is as follows:

$$P = 2\pi T/L. \qquad \text{EQUATION 3.}$$

2.3 Starting procedure used prior to all protocols

After mounting in KS solution all vessels were allowed to equilibrate for a thirty minute period. Vessels were then normalised and again allowed a further thirty minute rest period. After normalisation, vessels were washed several times and if BKS solution was required for the experiment it would replace the KS from this point onwards. A siting concentration of noradrenaline (10μM) was added to the bath. When the contraction had reached a plateau, vessels were washed back down to baseline. Twenty minutes later the KS was replaced by a 125mM KCl solution. As before when the contraction reached a plateau, vessels were washed down to baseline. The KCl contraction was

repeated ten minutes later. This routine made up the starting protocol for all experiments, as it was found that subsequent to this protocol, there was good reproducibility of up to four concentration response curves for all vessels.

After a further thirty minutes the protocol would start. Concentration response curves were constructed in a cumulative manner using half log increments. All responses were allowed to plateau before the next concentration of agonist was added to the bath.

Details of the specific protocols used are discussed in the relevant sections.

2.4 Data analysis

2.4.1 Analysis of agonist concentration response curves

Responses to agonists were calculated as active effective pressure (KPa) and these responses were expressed as such, or as a percentage of maximum of the first curve for that particular ring unless otherwise stated.

All concentration response curve (CRC) data for individual experiments was curve fitted using the computer software GraphPad Prism 2.01 (Institute for Scientific Information, San Diego, California, U.S.A.). Where data could not be fitted to the models described below, data was analyzed on Microsoft excel spreadsheets and pEC_{50} values derived by interpolation, not from a model. The mean of the raw data was calculated and data presented as mean ± standard error of the mean (s.e.mean). This was then graphed in logarithmic space.

2.4.2 One Site model

The interaction between a ligand and a receptor is governed by the Law of Mass Action (Clark, 1937). The relationship between the ligand concentration and the ligand

receptor complex is rectangular hyperbolic when plotted in arithmetic space, and sigmoidal when plotted in logarithmic space. Concentration response curves when plotted in a similar fashion follow the same pattern, leading to the conclusion that tissue response must be a linear function of receptor occupancy (Clark, 1937). Since the concentration response curve conforms to a sigmoid it can be defined by a logistic equation which generally takes the form of the Hill equation used by GraphPad software and given in the equation below.

$$E = \frac{\alpha \cdot [A]^{nH}}{[A]_{50}^{nH} + [A]^{nH}}$$

Where E is the response, α is the maximal response, [A] is the agonist concentration, nH is the midpoint slope and $[A]_{50}$ is the concentration of agonist required to generate 50% of α, (Roberts et al. 1996; Black et al. 1985).

This allowed the derivation of a number of parameters for each curve namely, upper asymptote, Hill slope (midpoint slope) and pEC_{50} (negative log of $[A]_{50}$) values.

Curves could then be compared by performing Students' t test (two groups), or one way analysis of variance (ANOVA) (more than two groups), comparing these parameters. In all cases $P < 0.05$ was taken as indicating a significant difference. In the case of ANOVA a Bonferroni post test allowing multiple comparisons was employed to determine the origin of any significant differences (Wallenstein et al. 1980).

For illustrative purposes, mean curves were generated by meaning the curve fit parameters from individual experiments and generating a curve upon which the raw mean data ± s.e.mean could be superimposed.

Judgment of how well the data fitted the equations used in the curve fitting procedure was determined in a number of ways. Simply looking at the graphs and how well the data seemed to fit the curve played a large part. In addition to this, the program provided a number of parameters which were also taken into consideration. These were as follows.

(1). The Sum-of-Squares which is the sum of the vertical distances of the points from the curve and is expressed in the same units as the Y values.

(2). R^2 value which represents the goodness of fit. This is the fraction of the variability in the Y values which can be explained by the equation. Therefore if R^2 is 0 then the line fits the data no better than a horizontal line going through the mean Y values. If R^2 is equal to 1.0 then there is no scatter and all the data points lie exactly on the line.

(3) Runs test. This measures the number of runs which is a cluster of data points lying above or below the line. If the number of runs is lower than the program expects then this suggests that the fit is not good.

(4). 95% confidence intervals for the parameters defined by the curve fit (upper asymptote, EC_{50} and Hill slope). If the intervals are very wide this again may suggest that the fit is not good.

2.4.3 Two site model

In some instances the data did not appear to fit the one site model as judged by the points discussed above. In this case the data was fitted to a two site model described by the following equation:

$$E = \alpha 1/(1+10^{((LogEC_{50}1\text{-}X)*n_H1)})+\alpha 2/(1+10^{(LogEC_{50}2\text{-}X)*n_H2)})$$

where X is the logarithm of the agonist concentration, E is the response, n_H is the midpoint slope and α is the upper asymptote. Numbers 1 and 2 denote the two sites. The two site model was first described by Furchgott (1981), and further developed by Lemoine and Kaumann (1983) and Kenakin (1992). Dr Gillian Watt (James Black Institute), kindly assisted in the programming of the GraphPad Prism software with the two site model (personal communication). In order to assess which model fitted best (the one site Hill equation or the two site described above) an F test was performed. Simply comparing the Sum-of Squares for the two equations can be misleading. The more complex equation, because it has more parameters, will tend to appear as a better fit (Van der Graaf et al. 1996b). What the F test does, is to compare the relative increase in the Sum-of-Squares to the relative increase in the degrees of freedom when going from the complex (two site), to the simple (one site), model. If the complex model is the better fit, the relative increase in the Sum-of-Squares will be greater than the relative increase in the degrees of freedom. Alternatively, if the more simple model is correct, the relative increase in both parameters would be about equal. GraphPad automatically performs this test when asked to fit two equations simultaneously.

2.4.4 Analysis of antagonist studies

The aim of antagonist studies is to derive a value that is a measure of the potency of a particular antagonist. The two values used are pA_2 and pK_B values. The derivation of these is described shortly. In general, if two receptors are the same they should have the same pA_2 or pK_B values for a given antagonist regardless of the tissue or the agonist used (Kenakin, 1982).

For the antagonist studies, potency of the competitive antagonists used was calculated by performing Schild regression (Arunlakshana and Schild, 1959). A range of

concentrations was employed for each antagonist used. DR values were then calculated for individual experiments. The DR is the dose ratio and is the ratio of the EC_{50} in the presence and absence of the antagonist. The EC_{50} values used for the Schild analysis were derived from the curve fitting procedure for individual experiments. In the cases where curve fitting was not applicable then EC_{50} values were derived by interpolation from Microsoft Excel spreadsheets.

The log (DR-1) was then plotted against the concentration of antagonist (log [B]) and linear regression performed through the points. Certain requirements must be fulfilled for Schild analysis to be a realistic measure of antagonist potency, namely the measurements must be made under equilibrium conditions, a range of antagonist concentrations must be used and the shift in the concentration response curves caused by increasing concentrations of the antagonist must produce parallel rightward displacements with no significant decrease in the upper asymptotes (Kenakin, 1992; Kenakin et al. 1992; Arunlakshana and Schild, 1959). Where the linear regression line intercepts the X axis is termed the pA_2 value. This is the concentration of antagonist that causes a two fold shift in the pEC_{50} of the agonist CRC. If the slope of the linear regression line is not significantly different from unity, then the pA_2 value is a good measure of antagonist potency and will be close to the pK_B. A pK_B value is defined as the antagonist equilibrium dissociation constant (Roberts et al. 1996; Furchgott, 1981). It can be calculated from a single antagonist concentration using the Schild equation (Jenkinson, 1991; Kenakin, 1982; Arunlakshana and Schild, 1959).

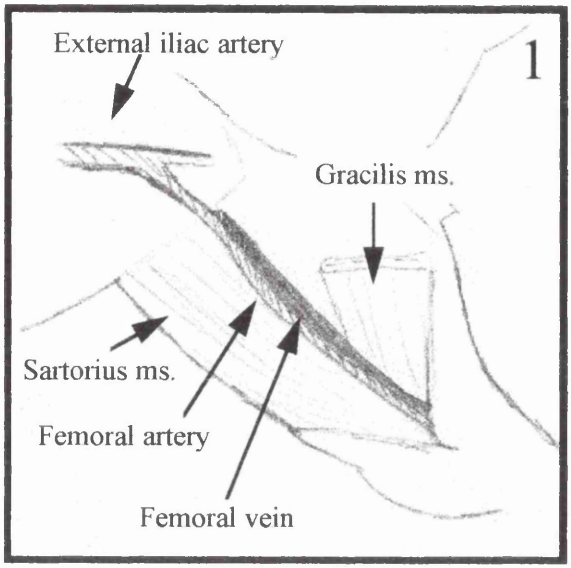
$$\log (DR-1) = \log[B] + \log K_B$$

This calculation assumes a slope of 1.

If Schild regression under equilibrium conditions, produces a slope not significantly different from unity, this suggests a competitive interaction with a single receptor population and it is reasonable to derive a pK_B value from a single concentration. If the Schild regression slope is significantly different from unity, then if pK_B values are calculated from each of the concentrations used, generally there is a significant difference in the pK_B values over the range of concentrations and thus it can be seen that calculation based on a single concentration would be unreliable. This finding suggests a non-competitive interaction possibly indicating a non-homogeneous population of receptors. In summary, the pA_2 value is derived from the Schild plot where the slope is not constrained. The pK_B value is derived from the Schild equation and assumes a slope of unity.

2.4.5 Analysis of relaxation studies

As for the agonist studies data was converted into active effective pressure and this in turn was expressed as a percentage relaxation.


External iliac artery
1
Gracilis ms.
Sartorius ms.
Femoral artery
Femoral vein

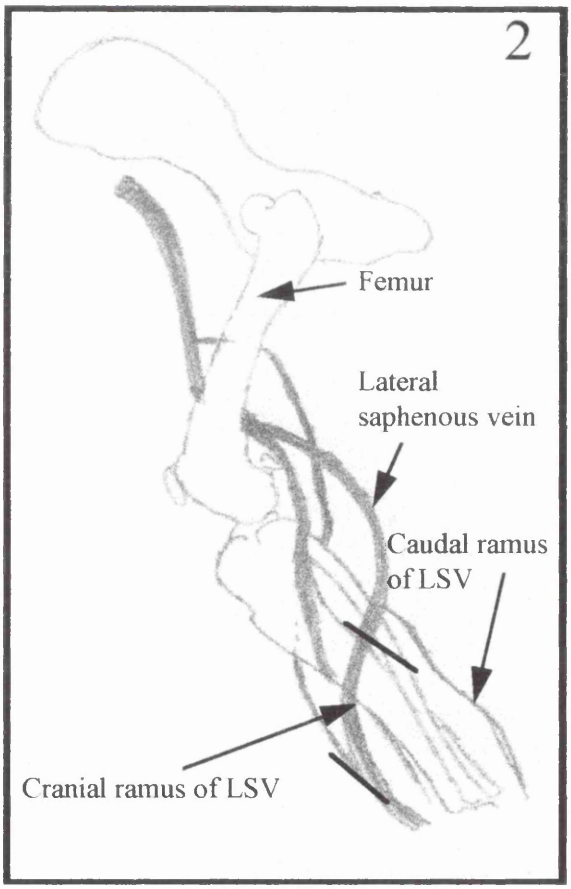

2
Femur
Lateral
saphenous vein
Caudal ramus
of LSV
Cranial ramus of LSV

Figure 2.1. Anatomical location of the femoral artery and the saphenous vein. Panel one illustrates the medial aspect of the pelvic limb, demonstrating the femoral triangle. The femoral artery can be seen lying caudal to the sartorius muscle after arising from the external iliac artery. Panel 2 illustrates the path followed by the saphenous vein. The diagram illustrates the lateral aspect of the pelvic limb. The section of vessel used lies in between the two bold black lines and comprises the cranial ramus of the vessel as it runs superficially over the lateral aspect of the hindlimb above the hock joint.

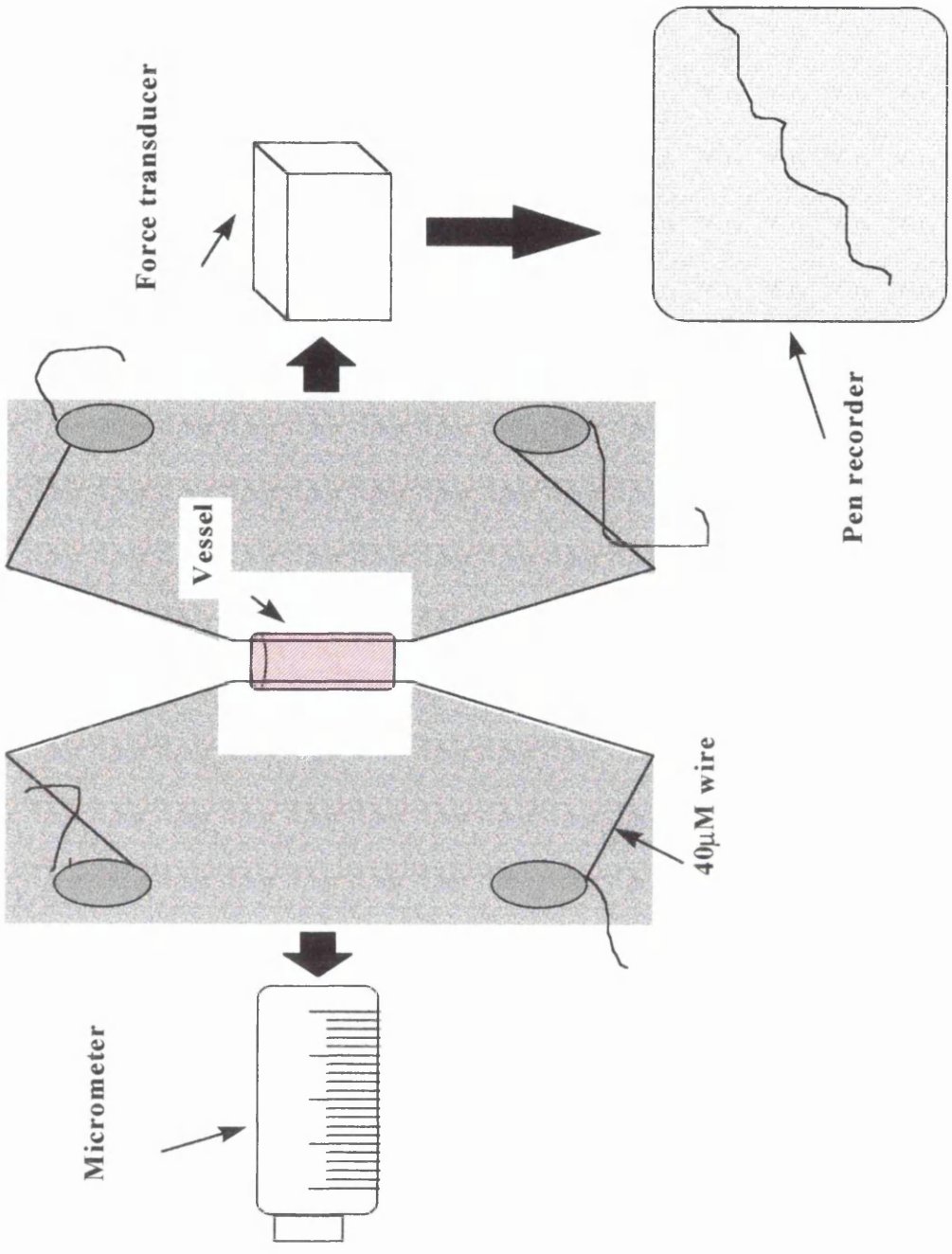


Figure 2.2 Schematic representation of a Mulvany Halpern wire myograph.

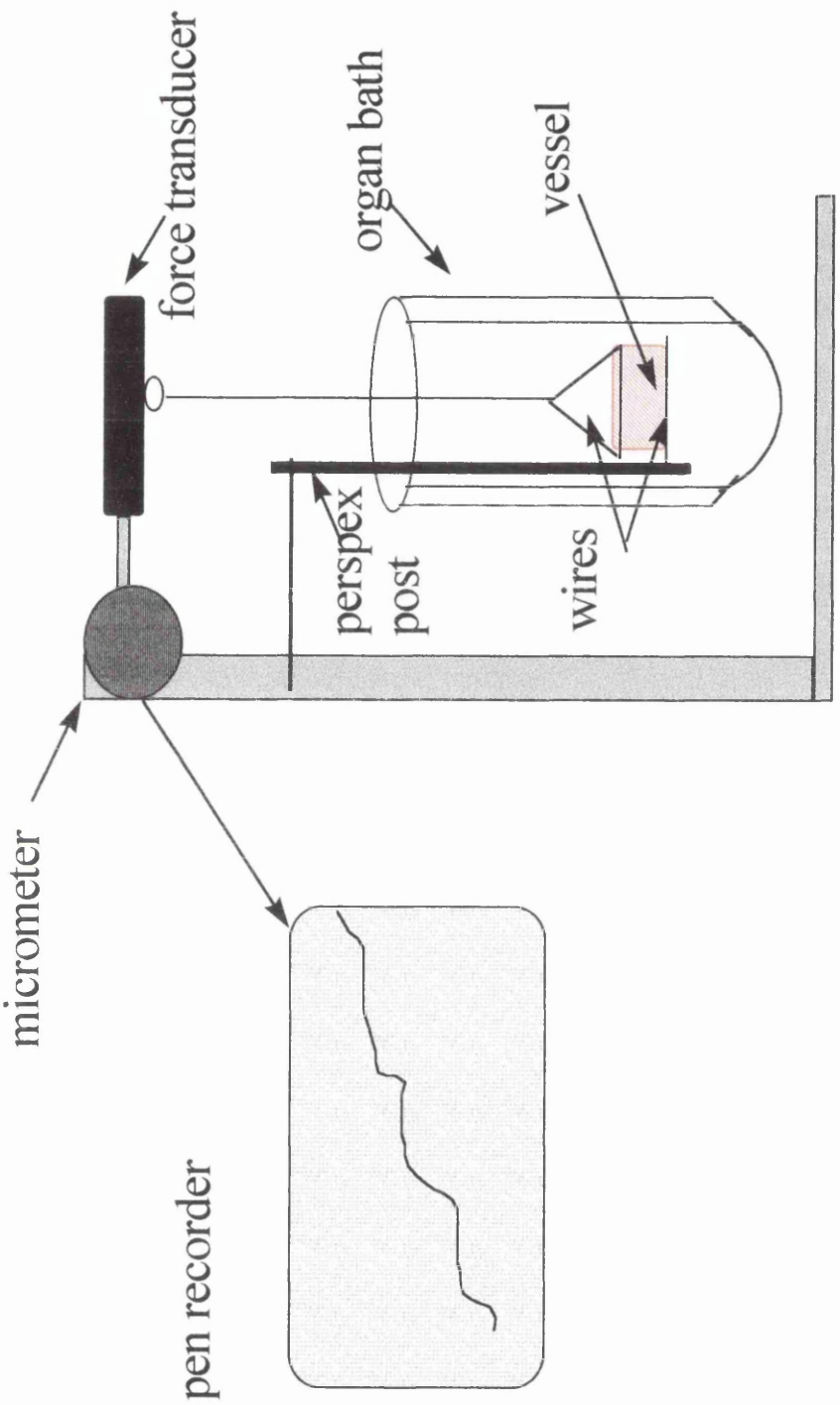


Figure 2.3. Schematic representation of an organ bath.

2.5 Microscopy

Specimens of saphenous vein, femoral artery and subcutaneous resistance artery, which had been fixed for 24 hours in 10% neutral buffered formalin and dehydrated with graded alcohol, were embedded in paraffin wax. Sections, cut at 5μm using a Leitz 1212 rotary microtome, and mounted on poly-l-lysine (Sigma) coated slides were dried for 12-18 hours at 36°C. The dried sections were then stained by Haemalum and Eosin, or immunocytochemically for the presence of neuropeptide Y and tyrosine hydroxylase. A Zeiss Axiophot microscope was used to examine the stained sections and Kodak Ektachrome 64 used for colour photography. Details of the staining techniques can be found in Appendix 1. All microscopy was performed by Dr. I. Montgomery.

MATERIALS AND METHODS FOR MOLECULAR BIOLOGY STUDIES

2.6 Handling of nucleotides

2.6.1 RNA Isolation

All glassware and solutions used in the handling of RNA were pre-treated with a 0.1% v/v solution of diethylamine pyrocarbonate (DEPC), to destroy RNAses and minimize degradation of RNA. The solutions and glassware were then autoclaved to remove traces of DEPC.

Gloves were worn at all times and all pipette tips and polypropylene tubes were autoclaved to minimize exposure of RNA to RNAses.

Tissue was collected immediately after euthanasia and snap frozen in liquid nitrogen. RNA isolation was carried out using RNAzolB (Biogenesis Ltd.) according to the manufacturer's instructions.

Briefly, frozen tissue was added to RNAzol B (2mls per 100mg of tissue) and homogenised using a polytron homogeniser. Homogenate was then transferred to a sterile eppendorf. In the case of cells in culture, culture media was removed and 1ml of RNAzolB added per 10^6 cells. Lysate was then transferred to a sterile eppendorf.

0.1mls of chloroform was added to the homogenate/lysate and tubes were shaken vigorously for 15 seconds before being placed on ice for 5 minutes. Tubes were then centrifuged at 12000g, for 15 minutes at 4°C.

The upper aqueous phase was removed to a fresh tube and an equal volume of isopropanol added. Tubes were incubated on ice for 15 minutes and then centrifuged at 12000g for 15 minutes at 4°C.

Supernatant was carefully removed and the RNA pellet washed in 800µl of 75% ethanol by vortexing and subsequent centrifugation at 7800g for 8 minutes at 4°C. Ethanol was carefully removed and the pellet allowed to air dry for several minutes before being resuspended in DEPC-treated water or TE buffer (10mM Tris pH 8.0, 1mM ethylenediamine tetracetic acid), made with DEPC-treated water.

Aliquots of RNA were removed for quantitation by spectrophotometry as described below. Aliquots of samples were also separated electrophoretically using agarose gel electrophoresis allowing visualisation of the 28S and 18S ribosomal bands indicative of good quality RNA (Dolnick and Pink, 1983). RNA was stored at -70°C until required.

2.6.2 RNA and DNA quantitation

RNA was diluted in DEPC-treated water or TE buffer made with DEPC-treated water. Usually a 1:500 dilution was used. The optical density was then measured at 260nm and 280nm. An RNA concentration of 40μg/ml has an absorbance at 260nm of 1. Therefore the concentration is 40 X O.D. 260nm X dilution factor = μg/ml. The quality of the RNA sample as regards degradation and protein contamination was determined by estimating the 260 : 280nm ratio. Pure RNA has a ratio of 2.0. (Sambrook et al. 1989; Chomczynski and Sacchi, 1987). In the case of DNA, the sample was again diluted in TE buffer or water and the optical density measured. A concentration of 50μg/ml gives a 260nm absorbance of 1. Pure DNA has a 260 : 280nm ratio of 1.8 (Sambrook et al. 1989).

2.6.3 Agarose gel electrophoresis

Unless otherwise stated 1% agarose gels were used. In general, the percentage agarose used will depend on the molecular weight of the fragments being separated. Small fragments (< 400bp) would require a higher percentage gel whereas larger fragments, (> 3Kb) would require a lower percentage gel. Agarose was dissolved in 0.5X TBE buffer and the gels were also run in this buffer at between 50 - 100Volts. 1 X TBE buffer is made up of 89mM Tris (pH 8.0), 89mM Boric acid and 2mM EDTA. Ethidium bromide (0.5μg/ml) was used to stain gels in order to visualise the DNA on a UV transilluminator. 1Kb size markers from Gibco-BRL were used at all times and samples were loaded with one tenth volume of 10X loading buffer (0.25% bromophenol blue and 40% v/v glycerol in water).

2.6.4 Isolation of DNA from agarose gels

Bands were cut out of agarose gels taking as little excess agarose as possible. Supelco GenElute™ spin columns or Supelco GenElute™ spin columns minus ethidium bromide, from Sigma, were used according to the manufacturer's instructions. The eluted DNA was either used directly or ethanol precipitated and resuspended in the appropriate buffer prior to use.

2.6.5 Ethanol precipitation

RNA and DNA were precipitated by the addition of one tenth the volume of 3M sodium acetate (pH 5.4) and 2 volumes of 100 % ethanol. Samples were then stored at -20°C for 30 minutes followed by centrifugation at 12,000g for 30 minutes at 4°C. The pellet was then washed in 70% ethanol by vortexing and subsequent centrifugation at 12,000g for 5 minutes. The ethanol was then removed and the pellet allowed to air dry for several minutes (on ice if RNA) before being resuspended in an appropriate volume of water or TE buffer.

2.7 Amplification of sequence of interest

2.7.1 cDNA synthesis

First strand cDNA synthesis from RNA was made using the Pharmacia Biotech First-Strand cDNA synthesis kit according to the manufacturer's instructions.

Briefly, 5μg of total RNA in a 20μl volume is denatured at 65°C for 10 minutes and then chilled on ice. This is then added to a reaction mix containing the Moloney Murine Leukaemia virus-reverse transcriptase enzyme, bovine serum albumin (0.08 mg/ml), dATP (1.8 mM), dCTP (1.8mM), dGTP (1.8mM) and dTTP (1.8mM), DTT solution (15 mM) and one of a choice of primers. In this study either a random hexadeoxynucleotide

primer (0.2μg) or a gene specific primer (20pm) was used. The final volume of the reaction was 33μl. This was incubated at 37°C for one hour. The cDNA was then used immediately for polymerase chain reaction or stored at -70°C until required.

2.7.2 Polymerase chain reaction

Experiments were carried out using a Hybaid "omnigene" thermal cycler.

Unless otherwise stated the reaction was carried out in a 50μl volume using the following: 1X PCR buffer (Gibco-BRL), 10pm of each primer, 200μM of each dATP, dTTP, dCTP, dGTP, 1.5mM $MgCl_2$, 5μl of cDNA from cDNA synthesis 33μl reaction and 0.5μl of *Taq* Polymerase enzyme (5 units/μl Gibco-BRL). This was made up to 50μl with sterile deionised water. For each primer set used, negative controls were run in parallel. These contained identical ingredients except that the cDNA template was replaced with sterile water.

Unless otherwise stated, the cycle programme used was as follows: 95°C for 3 minutes followed by 30 cycles comprising denaturation (95°C for 30 seconds), annealing (48°C for 30 seconds) and extension (72°C for 30 seconds). This was followed by a 5 minute extension period at 72°C.

At the end of the reaction aliquots of the PCR reactions were electrophoresed on 1% agarose gels as described in section 2.5.3, to allow visualisation of the PCR products.

2.7.3 Primers

With the exception of the primers included in the cDNA synthesis kit and the human GAPDH primer (Glyceraldehyde-3-phosphate dehydrogenase, Clontech) used as a control for the polymerase chain reaction, all primers were custom made by Cruachem and on arrival resuspended in sterile water to give 200mM stock solutions. These were

stored at -20°C until required, when they were further diluted for either polymerase chain reaction or sequencing.

2.8 Cloning of DNA fragments

Cloning of PCR products was carried out using Invitrogen's Original TA Cloning® Kit according to the manufacturer's instructions. This relies on the fact that *Taq* polymerase adds a single deoxyadenosine to the 3' end of PCR products. The vector used in the kit has 3' deoxythymidine overhangs which allows the PCR product to ligate into the vector. Figure 2.4 represents the pCR® 2.1 plasmid used in the kit.

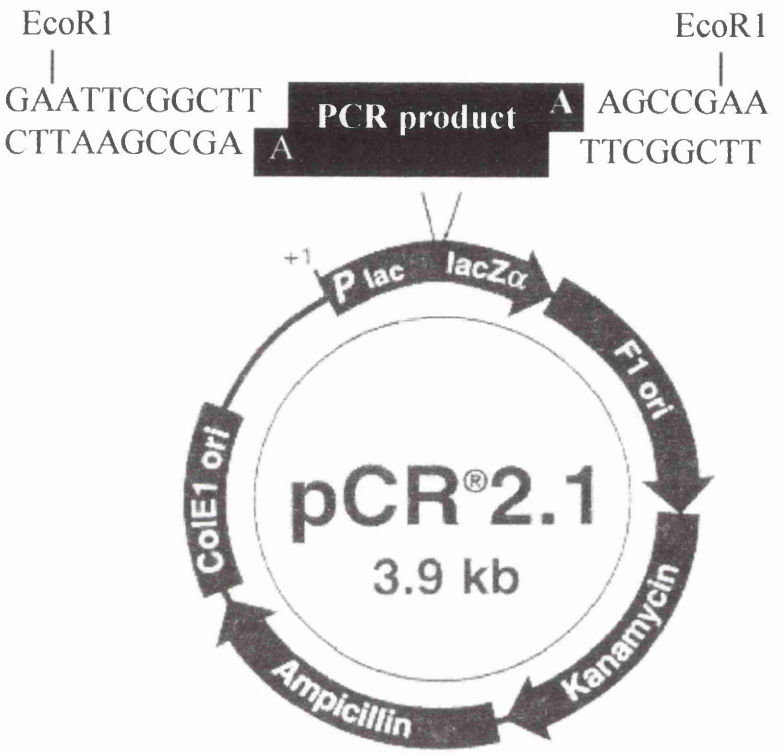


Figure 2.4. Diagram of the pCR® 2.1 plasmid. The PCR product can be inserted into the lac Zα fragment and is then flanked by EcoR1 restriction sites.

2.8.1 LB (Luria-Bertani) plates and LB broth

LB broth was made using the following ingredients. 1% w/v bacto-tryptone; 0.5% w/v bacto-yeast extract; 1% NaCl; 1.5% w/v bacto-agar. 950mls of deionised water was added and the pH adjusted to 7.0. The mixture was then made up to a final volume of 1litre, autoclaved and subsequently stored at room temperature until required. LB plates were prepared using the same recipe except that 15g/l of agar was added prior to autoclaving. The LB agar was cooled and ampicillin added to a concentration of 50μg/ml. Agar was then poured into 100mm plates and allowed to set. These plates were then used for the growth of bacteria containing plasmids carrying an ampicillin resistance gene. Plates were streaked with bacteria using an inoculating loop which was sterilised by flaming and cooling.

2.8.2 Ligations

A ligation reaction was set up using the following: 3-5μl of fresh PCR product (if kept too long the A overhangs can be degraded), 1μl of 10X ligation buffer, 2μl of pCR®2.1 vector (50ng), 1μl of T4 DNA ligase, made up to a total volume of 10μl. This was incubated overnight at 14°C.

2.8.3 Transformations

LB plates containing 50μg/ml of ampicillin were spread with 40μl of 40mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) dissolved in dimethylformamide, and allowed to dry.

Meanwhile the ligation mixture was centrifuged and stored on ice. One vial of INVαF' competent cells was carefully thawed on ice and 2μl of 0.5M β-Mercaptoethanol gently stirred into the cells with the pipette tip. 2μl of ligation mix was also stirred into the

thawed cells and the mixture then incubated for 30 minutes on ice. Cells were then heat-shocked at 42°C for 30 seconds and placed back on ice. 250µl of SOC media was added to each vial of cells and placed in a horizontal shaker at 37°C for 1 hour at 225rpm. Two previously prepared LB plates, streaked with X-gal, were used per transformation. One plate was spread with 50µl of the transformation mix while the other was spread with 200µl of the same transformation mix. Plates were left at room temperature for 15 minutes, then inverted and placed in an incubator overnight at 37°C. The following morning plates were removed from the incubator and placed in the cold room (4°C) for several hours after which colonies were selected for further growth and analysis.

2.8.4 Analysis of colonies

The pCR®2.1 vector contains the lac promoter and the lacZα cDNA which produces β-galactosidase and in the presence of X-gal, will give blue colonies. If the insert has been succesfully cloned into the plasmid, this will disrupt the lacZα cDNA and the colonies will be white.

Between 15 and 20 colonies were selected from each transformation. Each colony was inoculated into 3mls of LB broth containing 50µg/ml of ampicillin and grown up overnight at 37°C in a horizontal shaker at 225rpm. Plasmids were then isolated and restriction digested to screen for the presence of the insert.

Colonies which did contain plasmid with the desired insert were re-plated and stored in the cold room for up to 3 months. Glycerol stock solutions were also made and stored at -70°C.

2.9 Plasmid isolation

Plasmids were purified using the Wizard® *Plus* SV Minipreps DNA purification system from Promega.

2mls of bacterial culture were centrifuged at 10,000g for 1 minute to pellet the bacterial cells. Cells were then resuspended in 250µl of cell resuspension solution (50mM Tris-HCl and 10mM EDTA). 250µl of cell lysis solution was then added (0.2M NaOH and 1%SDS) and mixed by inversion. 5 minutes later 10µl of Alkaline protease solution was added and again mixed by inversion. 350µl of neutralisation solution was added (4.09M guanidine hydrochloride; 0.759M potassium acetate; 2.12M glacial acetic acid; pH 4.2.) and again mixed by inversion.

The lysate was then centrifuged at 14,000g for 10 minutes at room temperature. Clear lysate was then transferred by decanting into a spin column and centrifuged at 14,000g for 1 minute at room temperature. The column was then washed twice with column wash solution (60mM potassium acetate, 10mM Tris-HCl, pH 7.5; 60%ethanol) by spinning the wash through the column in the centrifuge.

The DNA was then eluted using 100µl of nuclease-free water. Aliquots of the plasmid prep could be used for restriction digest and the rest of the prep was stored at -20°C.

2.10 Restriction digests

Restriction digests were carried out using Gibco-BRL restriction enzymes in conjunction with the appropriate reaction buffer. Digests were usually carried out in 20µl volumes using about 1µg of DNA, 2µl of 10X reaction buffer and 5-10 units of

enzyme. Samples were mixed and incubated at 37°C for one hour. Amounts were scaled up for larger volume digests.

2.11 Sequencing

2.11.1 Cycle sequencing

Sequencing was performed using the Thermo Sequenase cycle sequencing kit from Amersham incorporating ^{33}P labelled dideoxynucleotides. A reaction mix was set up containing 2µl reaction buffer, 50-500ng of DNA, 2pm of primer and 8 units of thermo sequenase DNA polymerase. The reaction was made up to a final volume of 20µl with sterile water. Four termination reactions were set up for each sample containing 2µl of termination master mix and 0.5µl of each labelled ddNTP. 4.5µl of the reaction mix was added to each of the termination mixes and these were subjected to 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. 4µl of stop solution was then added to terminate the reaction.

2.11.2 Sequencing gel

A 6% acrylamide gel was used of the following composition. For 100mls of gel solution, 19:1 acrylamide : bis-acrylamide 20mls, 42g of Urea, 10mls of 10X glycerol-tolerant gel buffer made up to 100mls with water. Before pouring, 1ml of 10% ammonium persulphate was added together with 25µl of TEMED (N, N, N', N'-tetramethylethylenediamine). The glycerol-tolerant gel buffer (TTE buffer) was made up as follows. For 20X buffer, 216g Tris base, 72g Taurine, 4g $Na_2EDTA\bullet 2H_20$ made up to 1litre in water.

Gels were cast in vertical Bio-Rad sequencing gel kits, run in 1X TTE buffer and the temperature maintained at 50°C.

Once run, gels were transferred onto 3MM Whatman paper, covered in Saran wrap and dried on a vacuum operated gel drier. Once dry, gels were exposed overnight to radiographic film and subsequently film was developed on an X -OGRAPH Compact X2 film developer.

2.12 Northern analysis

2.12.1 Solutions used for northern analysis

DEPC-treated water: 50µl DEPC per 500mls of water, left for 12 hours and then autoclaved.

20 X SSC: 3M NaCl, 0.3M Na_3 citrate. DEPC treated and autoclaved.

10 X MOPS buffer: 0.2M 3-[N-Morpholino]-propane-sulphonic acid, 0.05M Na acetate pH 7.0, 0.01M Na_2EDTA. Made up with DEPC-treated water and filter sterilised. Stored at 4°C in a light-proof bottle.

100 X Denhardt's solution: 2% w/v bovine serum albumin, 2%w/v Ficoll™, 2% w/v polyvinylpyrolidone (PVP). Filter sterilised and stored at -20°C.

10% SDS: 10% w/v lauryl sulfate sodium salt made up with DEPC-treated water.

2.12.2 Formaldehyde gels

A 1% denaturing agarose gel was used. This was made by dissolving 3g of agarose in 219mls of DEPC-treated water and 30mls of 10 X MOPS. Once the agarose had dissolved the solution was cooled to 50°C and 51mls of 37% formaldehyde added. The gel was then immediately poured into a horizontal Bio-Rad gel casting kit. All this was performed in a fume hood to avoid inhalation of formaldehyde fumes. The gel was allowed to set for one hour. Gels were run in 1 X MOPS buffer at 100 Volts.

2.12.3 Sample preparation

20μg of total RNA was ethanol precipitated and resuspended in 6μl of DEPC-treated water. The RNA was then added to a solution containing 12.5μl of deionised formamide, 2.5μl of 10 X MOPS and 4μl of 37% formaldehyde. RNA was then heated to 65°C for several minutes and chilled on ice. 2.5μl of loading buffer was added (50% v/v glycerol and 0.1mg/ml bromophenol blue), and samples loaded into the gel.

Formamide was deionised using Bio-Rad analytical grade mixed bed resin AG 501-XB (D) resin 20-50 mesh. 100mls of formamide was added to the resin in the fume hood and stirred for one hour. The blue resin turned yellow once the process was complete. The formamide was then filtered twice through Whatman No1 paper, aliquoted and stored frozen until required.

2.12.4 Blotting procedure

Once the gel had been run, the ladder and any excess gel was trimmed. The ladder was stained with ethidium bromide and photographed with a fluorescent ruler to allow subsequent judging of RNA species sizes.

A thick glass plate was placed on a stand and covered with 3MM Whatman paper which dipped on all four sides into a tray containing 10 X SSC solution. The gel was inverted and placed directly on top of the paper. Hybond-N+ membrane (Amersham), was cut to fit the gel exactly and placed gently on top of the gel. A plastic pipette was gently rolled over the membrane to remove any air bubbles. Three layers of 3MM Whatman paper were placed over the membrane and finally several layers of paper towel were placed over this. Old radiographic film was placed around the gel to prevent the capillary action bypassing the gel itself. A weight of approximately 800g was placed on top of the paper towels ensuring even weight distribution, and the blot was left

overnight. The following morning the membrane was carefully peeled off the gel and fixed in an ultraviolet stratalinker. Membranes were wrapped in Saran wrap and stored in the fridge until required.

2.12.5 Preparation of probes

cDNA probes were labelled with [α-^{32}P] dCTP using Ready-To-Go® DNA labeling beads from Pharmacia Biotech. The principals of the labelling procedure are based on the use of random oligodeoxyribonucleotides which anneal to the DNA template. The Klenow fragment enzyme then allows incorporation of dNTPS which are present including the radiolabelled dCTP. Briefly, 25-50ng of linearized DNA dissolved in TE buffer, made up to a volume of 45µl with water and denatured at 95°C for 5 minutes. The DNA was then added to the tube containing the bead along with 5µl of [α-^{32}P] dCTP (3000Ci/mmol). The reaction tube was then incubated at 37°C for 30 minutes. Following this, the probe was run through a Nick™ column (Pharmacia Biotech), which is a sephadex column containing sephadex® G-50 DNA grade. This removed unincorporated isotope. Labelled DNA was then eluted from the column in TE buffer and was ready for use.

Oligonucleotide probes were end-labelled with [γ-^{32}P] ATP. 200ng of oligonucleotide were made up to 6µl in water. The following were then added to the tube: 1µl of 10X Kinase buffer (Promega); 1µl of T4 polynucleotide kinase (Promega); 2µl of [γ ^{32}P]ATP. The tube was then incubated at 37°C for 30-60 minutes. The reaction was stopped by the addition of 1µl of 0.2M EDTA. The following were then added in order to precipitate the labelled probe: 170µl of water; transfer RNA (1µl of 10mg/ml solution); 20µl of 3M sodium acetate, pH 5.7; 600µl of 100% ethanol. The tube was

placed on dry ice for 2 minutes followed by a 5 minute spin at 12,000g. The ethanol was removed and the pellet resuspended in 20 μl of TE buffer. 10μl was used for each hybridization.

All isotope handling was carried out with the use of protective screens, and everything carefully monitored for ^{32}P contamination.

2.12.6 Hybridization

Blots were placed inside hybridization tubes and prehybridization solution added. The tube was then placed in a revolving incubator at 65°C for one hour. The prehybridization solution was made up as follows: 2.5mls 20 X SSC, 0.5mls 100X Denhardt's, 0.5mls 10% SDS, 0.02mls salmon sperm DNA (heat denatured at 95°C first and then placed on ice) and 6.48mls of water.

After one hour the labelled probe was added to the prehybridization buffer and the blot hybridized overnight at 65°C.

2.12.7 Washing of membranes

Membranes were washed in 2 X SSC/ 0.1%SDS for 10 minutes at 65°C. This wash was repeated. If the blot was still hot then it was washed in 1 X SSC/ 0.1%SDS for 10 minutes at 65°C. A final high stringency wash of 0.1 X SSC / 0.1% SDS for 10 minutes at 65°C may be used, but is only recommended if the probe is specific and not for related sequences.

Membranes were then wrapped in Saran-wrap and exposed to radiographic film for 12 hours to one week (depending on the strength of the signal) at -70°C. Film was developed on an X -OGRAPH Compact X2 film developer.

CHAPTER 3

Functional classification of α_1-adrenoceptors mediating contraction to exogenous noradrenaline in the dog saphenous vein

3.0 Abstract

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3.0 Abstract

The aim of this study was to functionally classify the subtype(s) of α_1-adrenoceptor mediating contraction of the dog saphenous vein to exogenous noradrenaline.

Agonist profiles to noradrenaline, phenylephrine, (R) A-61603 and UK14304 confirmed the presence of post-junctional α_1- and α_2- adrenoceptors, with a rank order of potency of (R) A-61603 > noradrenaline > UK14304 > phenylephrine.

To assess antagonism of the α_1- mediated responses, the reversible competitive antagonists prazosin, WB 4101, HV 723, BMY 7378 and 5 methylurapidil were used in the presence of an α_2-antagonist (either 1μM rauwolscine or 0.1μM delequamine). The low potency of prazosin and HV723 suggested the presence of the α_{1L}-adrenoceptor subtype. The non-competitive nature of 5 methylurapidil and BMY 7378, and the baseline contraction and rightward shift in the concentration response curves in the presence of the highest concentration of the irreversible antagonist chloroethylclonidine, are highly suggestive of the presence of a second receptor subtype. Despite the low affinity for prazosin, the additional receptor subtype most closely resembles the α_{1D}-adrenoceptor.

3.1 Methods

5mm sections of saphenous vein were set up in 10ml organ baths, normalised and the starting protocol completed, as described in the materials and methods section of this thesis. After normalisation vessels were maintained in blockers Krebs' solution. The experimental protocols employed in this section fell into two categories outlined below. All comparisons were made using one way analysis of variance (ANOVA), followed by a Bonferroni post test, unless stated otherwise. A P value < 0.05 was deemed statistically significant unless stated otherwise.

3.1.1 Agonist profiles

An agonist profile was performed in order to ascertain the contribution of both α_1- and α_2-adrenoceptors in mediating contraction in this vessel. Hence, noradrenaline was used since it is the endogenous agonist and will act via both α_1- and α_2-adrenoceptors, phenylephrine was used as an α_1-agonist and UK14304 was used as an α_2-agonist. In addition the Abbott compound was used since it is an α_{1A}-selective agonist which has a potency of α_{1A}->α_{1B}->α_{1D}-, (Knepper et al. 1995). Vessels were allowed a forty minute recovery period after the starting protocol (section 2.3 materials and methods) and between subsequent concentration response curves. Cumulative concentration response curves were then constructed to the following agonists, using half log increments. Noradrenaline (NA) and phenylephrine (PE), starting at a concentration of 1nM and increasing up to a maximum of 1mM if required. (R) A-61603 (Abbott compound) starting with a concentration of 0.3nM and increasing up to a concentration of 30μM if required. UK14304 starting with a concentration of 1nM and increasing up to a concentration of 0.1μM if required. Adequate time was allowed between additions of agonist for the previous response to have reached a plateau. Three concentration

response curves (CRC) were carried out on each ring using a different agonist each time. Results were expressed as a percentage of the response to the 10μM siting concentration of noradrenaline, and plotted in logarithmic space. Experiments were curve fitted using GraphPad prism software and fitted to a one site model unless otherwise stated as described in section 2.4 of materials and methods.

3.1.2 Antagonist studies

Four to seven saphenous vein rings were set up in parallel. For each experiment one ring was designated as a time control and no antagonist used. Each other ring was assigned one of five competitive reversible antagonists, or the irreversible alkylating agent chloroethylclonidine (CEC). In addition to the blocking agents already present in the blockers Krebs' solution, either 0.1μM of the α_2-adrenoceptor antagonist RS-15385-197 (delequamine), or 1μM of the α_2-antagonist rauwolscine, were present in the baths at all times. An initial cumulative control CRC to noradrenaline was performed in each ring. After this, vessels were washed until they returned to baseline and a concentration of antagonist added to the bath. A forty minute period for equilibration was allowed before a second CRC to noradrenaline was performed. Up to four curves, but more often two, were performed on each ring, each time using an increasing concentration of the antagonist that had been assigned to that ring. Experiments where there was a significant change in the maximum, pEC_{50} or Hill slope of the time control curve, were excluded from the study. Comparisons of these parameters were made using one way ANOVA followed by a Bonferroni post test. Time control data is shown in Figure 3.6. The antagonists used according to this protocol were prazosin, 5 methylurapidil, BMY7378, WB4101 and HV723. Analysis of antagonist action was performed by Schild analysis (pA_2) as described in the materials and methods section 2.4 of this

thesis. Estimation of antagonist affinity was also calculated using single antagonist concentrations again as described in section 2.4.

The protocol used for CEC was different. After the control curve, a concentration of CEC was added to the bath and left for one hour. The vessel was then washed ten times over a forty minute period. A second curve to NA was then performed, (Williams and Clarke, 1995; O'Rourke et al. 1995). Due to the irreversible nature of this antagonist only one concentration of CEC was used on each ring.

3.2 Results

All results are given as mean ± s.e. mean and n = the number of experiments unless otherwise stated.

3.2.1 Agonist profile

Values were derived from at least six different animals. Experiments were performed in the absence of an α_2-adrenoceptor antagonist. All the agonists used in this study caused concentration-dependent increases in tension of the dog saphenous vein. The pEC_{50} values of the agonists used were as follows: NA 6.7 ± 0.08, n = 7; PE 5.94 ± 0.07, n = 11; Abbott compound 7.57 ± 0.50, n = 6; UK14304 6.35 ± 0.28, n = 5. This resulted in a rank order of potency of Abbott > NA > UK14304 > PE, with the Abbott compound being 7.39X more potent than NA, 42.8X more potent than PE and 16X more potent than UK14304. The results are summarised in Table 3.1. All curves fitted to the one site model (Figure 3.1) with the exception of a single UK14304 experiment, which fitted better to the two site model of receptor activation (Figure 3.2). This two site curve was not included in the calculation of agonist potency. One way ANOVA revealed a significant difference in the maximum values for the agonists used. A Bonferroni post

test showed that this was due to the maximum achieved by UK14304 being significantly lower than the other three agonists used. In fact, the upper asymptote for UK14304 was approximately 50% of that for the other agonists. There was also a significant difference in the Hill slope parameters for the different agonists, most noticeably due to the shallowness of the curves to UK14304.

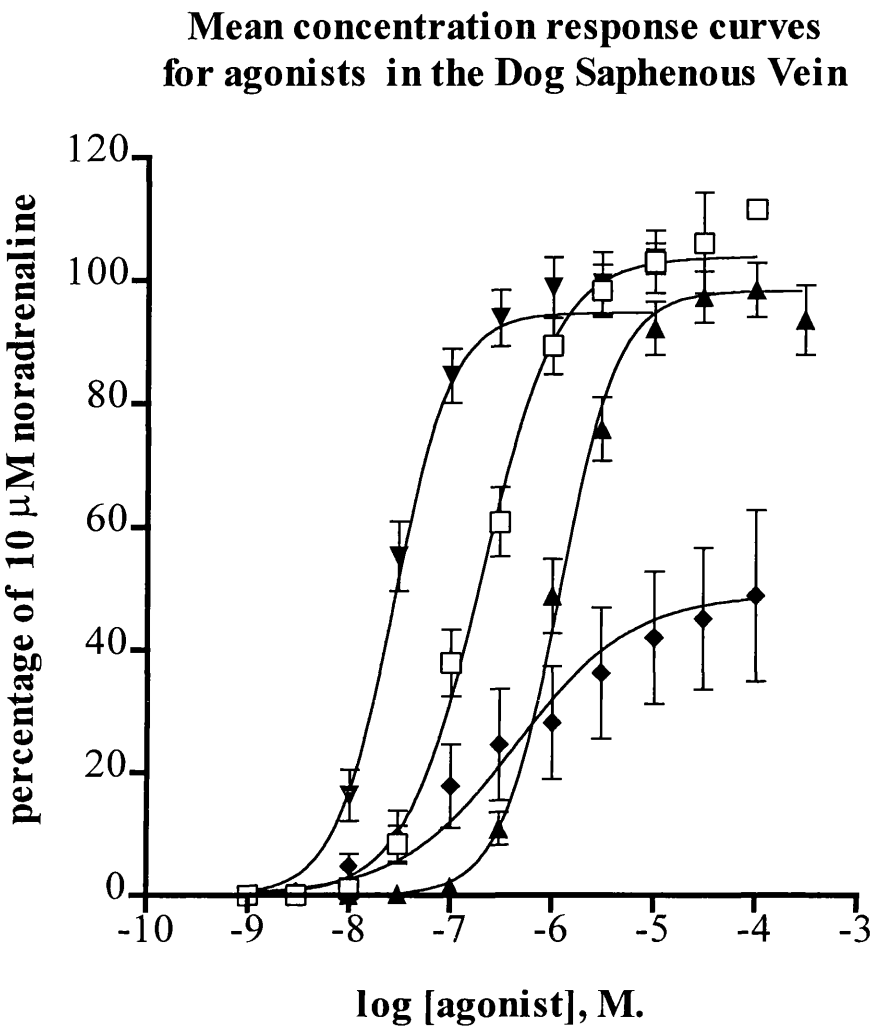


Figure 3.1. Mean concentration response curve data for the agonist profiles in the dog saphenous vein. Noradrenaline (□), *n* = 7; phenylephrine (▲), *n* = 11; Abbott compound (▼), *n* = 6; UK14304 (◆), *n* = 5. Curves were generated by meaning the curve parameters obtained from the individual curve fits. The raw mean data ± s.e. mean was superimposed on the mean curves.

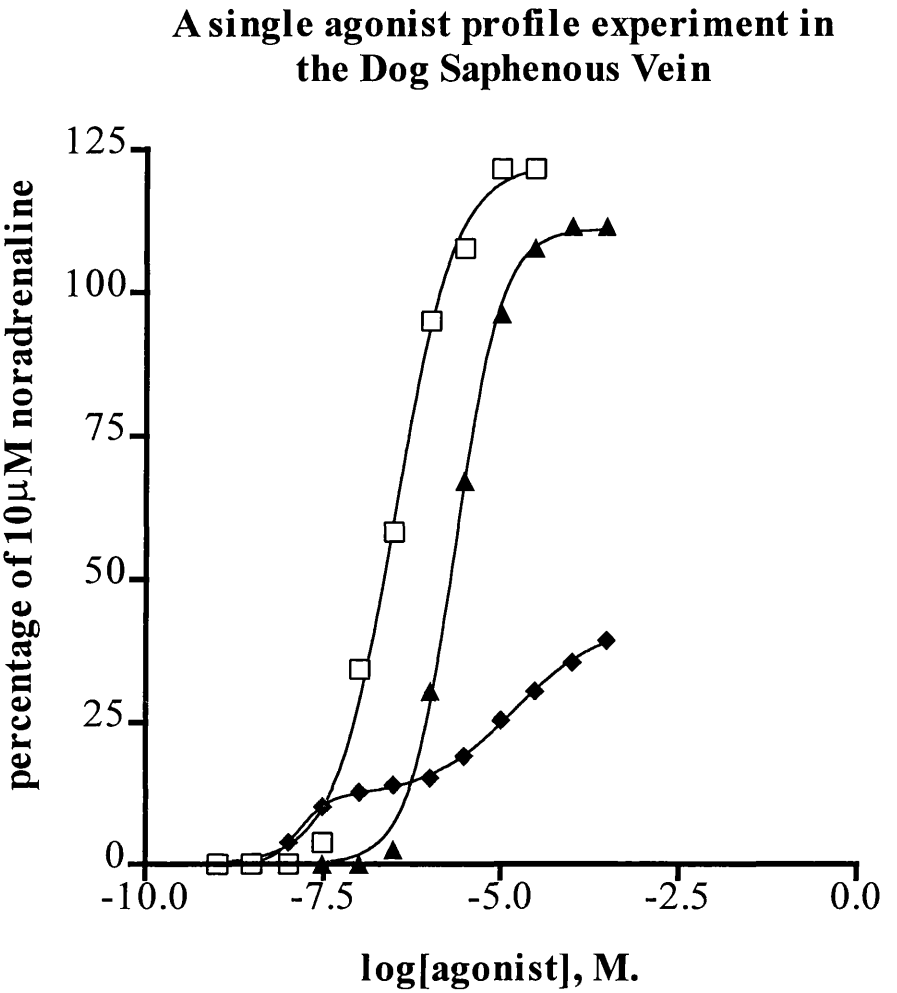


Figure 3.2. Agonist profile for a single experiment demonstrating the two site fit for the UK14304 curve in dog saphenous vein. Noradrenaline (□), phenylephrine (▲) and UK14304 (◆). The other two agonist curves fit to a one site model.

3.2.2 Competitive antagonists

A range of five competitive reversible antagonists was used. Values were derived from experiments carried out using at least four different animals. pA_2 values were derived as described in materials and methods in Chapter 2. The first set of antagonist studies was conducted in the presence of 1μM of the putative α_2-antagonist rauwolscine. The same experiments were then repeated in the presence of 0.1μM of the α_2-antagonist delequamine. The reasons for repeating the experiments in the presence of delequamine are explained as follows.

The presence of α_2-adrenoceptors necessitated the use of an α_2-antagonist. Unfortunately there is no satisfactory irreversible blocking agent for these receptors. Phenoxybenzamine is an irreversible antagonist at α-adrenoceptors. It has been used successfully to isolate α_2-adrenoceptors in a mixed population of α_1- and α_2-adrenoceptors in both the rabbit saphenous vein (Daly et al. 1988b) and the dog saphenous vein (MacLennan et al. 1997). However, in the rabbit saphenous vein it was not possible to satisfactorily isolate the α_1-adrenoceptor population using receptor protection with prazosin (Daly et al. 1988b), and in the dog saphenous vein, phenoxybenzamine selectively blocked the α_1-adrenoceptor population in the absence of receptor protection (Constantine et al. 1982), tying in with the findings of Dubovich and Langer (1974), that phenoxybenzamine has a much higher affinity for α_1- versus α_2-adrenoceptors. The only other option was to use a selective reversible α_2-adrenoceptor antagonist. Initially, the putative α_2- antagonist rauwolscine (1μM) was used, based on a study by Leech and Faber (1996), who used this antagonist for the same purpose. In another study, when used in rabbit blood vessels, it appeared to be α_2-selective up to a concentration of 2.5μM (Daly et al. 1988a). However, on analysis of the data, the profound effect that this antagonist had on the concentration response curves to

noradrenaline (Figure 3.3), suggested the possibility that rauwolscine was also affecting the α_1-adrenoceptor population. Fishing experiments were then performed using 0.1μM of the α_2-antagonist RS-15385-197 (delequamine) for comparison (Figure 3.3). This latter antagonist is thought to be highly α_2-selective, and up to a concentration of 10μM has been reported to have no effect on α_1-adrenoceptor agonist curves in the DSV (Brown et al. 1993). The present study showed that 1μM rauwolscine shifted the CRC of phenylephrine to the right and suppressed the maximum, unlike 0.1μM delequamine which had no effect (Figure 3.4). On the other hand, delequamine at concentrations of both 0.1μM and 1μM, shifted the concentration response curve of the α_2-agonist UK 14304, as can be seen in Figure 3.5. Incidentally, in this particular experiment as in one other, UK 14304 concentration response curves fitted to the two site model. The addition of delequamine seemed to shift only the lower part of the curve which, as previously mentioned, may indicate that UK 14304 is acting at the α_1-adrenoceptor population also, and that while the lower part of the curve is α_2-mediated, the upper part of the curve may be α_1-mediated. This finding was not addressed in the present study but it would be of interest to investigate this further.

Observations of the effect of rauwolscine were in agreement with a paper by Daniel et al (1996), which suggested that rauwolscine antagonized an unusual subtype of α_1-adrenoceptor in the DSV when phenylephrine was the agonist. At this point, all the antagonist studies were repeated in the presence of 0.1μM delequamine. This was done in order to prevent the problem outlined by Kenakin (1982), of "frame shifting" pA_2 values derived when an antagonist has been used that also affects the population of interest in addition to the population of receptors which the experimenter wishes to exclude.

Mean concentration response curve data for the antagonists used both in the presence of rauwolscine and delequamine are shown in Figures 3.7 to 3.17. Schild regressions are shown in Figures 3.18 to 3.19. Table 3.2 summarises slope and pA_2 values for the competitive antagonists. The pA_2 values calculated from individual antagonist concentrations in the presence of rauwolscine or delequamine are listed in Tables 3.3 and 3.4 respectively. Each antagonist is discussed separately giving results in the presence of rauwolscine first and then comparing this to results in the presence of delequamine.

The α_1-antagonist prazosin, was used at concentrations of 1nM, 10nM and 0.1μM ($n = 4$ for each concentration). In the presence of rauwolscine, increasing concentrations of the antagonist caused parallel rightward displacements of the concentration response curves to noradrenaline, with no significant alteration in maximum values (P of .05). One way ANOVA of the Hill slopes showed a significant difference (P of 0.0059), with a Bonferroni post test revealing that this was due to a decrease in the slope in the presence of 0.1μM prazosin. pEC_{50} values in the presence of prazosin were also significantly different from controls ($P< 0.0001$), but from the post test this was due only to the highest concentration of the antagonist. Schild regression yielded a pA_2 value of 7.36, with a slope significantly different from negative unity (-0.8 to -0.30, *95% confidence intervals (C.I.)*). When pA_2 values were calculated for each antagonist concentration, they were found to be significantly different due to a higher value with the 1nM concentration of prazosin. A pA_2 value of 7.35 ± 0.03 ($n = 4$) was obtained with 0.1μM prazosin.

In contrast, in the presence of delequamine, prazosin, while also causing concentration-dependent rightward shifts in the concentration response curves, caused no significant change in either the maximum or Hill slope parameters (P of 0.17 and 0.10

respectively). pEC_{50} values were significantly changed ($P<0.0001$), this time due to both the 10nM and 0.1μM concentrations of prazosin. Schild regression yielded a higher pA_2 value of 8.31, with a slope not significantly different from negative unity (-1.01 to -0.64, *95% C.I.*). pA_2 values calculated from single antagonist concentrations were not significantly different over the range of antagonist used and a pA_2 value of 8.09 ± 0.07 ($n = 4$) was calculated in the presence of 0.1μM prazosin.

The α_{1D}- selective antagonist, BMY 7378 was used at concentrations of 1nM, 10nM and 0.1μM ($n = 4$ for each concentration). In the presence of rauwolscine, none of the concentrations used caused a significant change in the pEC_{50}, or Hill slopes of the curves to NA (P of 0.25 and 0.40 respectively). Maximum values were significantly altered (P of 0.045). Although not large, the difference was due to a reduction in the maximum in the presence of 0.1μM BMY 7378. As none of the concentrations of antagonist caused a significant shift in the concentration response curves and the effect was not concentration-dependent (Figure 3.9), a meaningful Schild regression was not possible, since the regression line would have been almost horizontal. PA_2 values calculated from each antagonist concentration were significantly different, with a value of 6.54 ± 0.17 ($n = 4$) in the presence of 0.1μM BMY 7378. This suggested that the interaction was not competitive.

Results in the presence of delequamine were similar. This time maximum values as well as pEC_{50}, and Hill slopes were not significantly different (P of 0.15, 0.19 and 0.75 respectively). When Schild regression was performed, a slope significantly different from negative unity was obtained (-0.52 to -0.09, *95% C.I.*) with an X axis intercept of 7.2 (pA_2). Again, pA_2 values calculated from single antagonist concentrations were significantly different. A pA_2 of 7.04 ± 0.12 ($n = 4$) was calculated from 0.1μM BMY 7378.

The $\alpha_{1A/D}$-selective antagonist WB4101 was used at concentrations of 1nM, 10nM and 0.1μM (*n* = 4-5 for each concentration). In the presence of rauwolscine, WB 4101 produced parallel dextral displacements of the NA concentration response curves, with no significant alterations in maximum or Hill slope values (P of 0.12 and 0.28 respectively). pEC_{50} values were significantly different (P<0.0001), but from the post test this appeared to be due only to the highest concentration of the antagonist. Schild regression yielded a pA_2 value of 8.06 and a slope not significantly different from negative unity (-1 to -0.23, *95% C.I*). PA_2 values calculated for 10nM and 0.1μM WB 4101 were not significantly different as judged by a t test. The 1nM concentration was not included since only one pA_2 value was obtained. This was due to negative values being derived from the other three experiments because of the low potency of WB 4101 at this concentration. A pA_2 value of 7.7 ± 0.11 (*n* = 4), was derived from 0.1μM WB 4101.

In the presence of delequamine, WB 4101 also produced parallel rightward displacements of the concentration response curves to NA, with the exception of the highest concentration used. This concentration of WB4101 caused a significant decrease in the maximum value and a significant lowering of the Hill slope parameter (P of 0.0012 and 0.0006 respectively). This time both 10nM and 0.1μM WB 4101 contributed to the significant change in the pEC_{50} values (P < 0.0001). Schild regression analysis gave a slope not significantly different from negative unity (-1.22 to -0.57, *95% C.I.*) and a higher pA_2 value of 8.86. pA_2 values calculated over the range of antagonist concentrations used were not significantly different and a pA_2 value calculated using the intermediate concentration of WB4101, which caused no slope or maximum value change, yielded a value of 8.88 ± 0.15 (*n* = 4).

HV723 was used at concentrations of 1nM, 10nM and 0.1μM (*n* = 4-5 for each concentration). In the presence of rauwolscine, increasing concentrations of the antagonist caused parallel rightward displacements in the concentration response curves to NA, with no significant change in maximum or Hill slope parameters (P of 0.41 and 0.82 respectively). Both 10nM and 0.1μM HV 723 contributed to the significant change in the pEC_{50} values (P< 0.0001). Schild regression yielded a pA_2 value of 8.16 with a slope not significantly different from negative unity (-1.15 to -0.35, *95% C.I.*). pA_2 values were not significantly different over the range of antagonist concentrations and 0.1μM HV 723 gave a pA_2 value of 7.8 ± 0.02 (*n* = 4).

In the presence of delequamine, HV 723 in increasing concentrations caused rightward shifts in the concentration response curves to noradrenaline with no significant alteration in maximum values (P of 0.35). There was however a significant lowering in the Hill slope parameter for all concentrations of the antagonist used (P < 0.0001). Schild regression for this antagonist yielded a slope not significantly different from negative unity (-1.18 to -0.66, *95% C.I.*), and a higher pA_2 value of 8.98. pA_2 values were not significantly different over the range of antagonist concentrations used and a pA_2 estimation from the highest concentration of antagonist gave a value of 8.77 ± 0.25 (*n* = 4).

The α_{1A}-selective antagonist, 5 methylurapidil, was used at concentrations of 10nM, 0.1μM and 1μM (*n* = 4-7 for each concentration used). In the presence of rauwolscine, increasing concentrations of 5 methylurapidil caused no significant alterations in either the maximum, Hill slope or pEC_{50} parameters (P of 0.15, 0.82 and 0.05 respectively). Schild regression, as for BMY 7378, in the presence of rauwolscine, was not possible because of the lack of effect of the antagonist. A pA_2 of 6.00 ± 0.77 was derived from 1μM 5 methylurapidil.

Results for 5 methylurapidil in the presence of delequamine were quite dissimilar. The two highest concentrations of the antagonist caused rightward shifts in the concentration response curves to noradrenaline with no significant alteration in the maximum value (P of 0.06). 0.1μM did however cause a significant decrease in the Hill slope parameter. The effect of 1μM 5 methylurapidil was more complex. Three out of five experiments fitted better to a two site model. The two out of five experiments that fitted to a one site model caused a significant decrease in the maximum from control, (Figures 3.16 and 3.17).

Schild analysis performed using only the experiments that fitted to the one site model in the calculation, gave a slope not significantly different from negative unity (-1.34 to -0.83, *95% C.I.*) and a pA_2 value of 8.35. A Schild regression using the high affinity site in the two site fits for the 1μM concentration, produced an almost identical Schild regression with a slope of -1.42 to -0.89 *(95% C.I.)* and a pA_2 of 8.31.

It was not possible to produce a Schild plot using the low affinity site from the 1μM concentration as the plot deviated significantly from the model. Instead a pA_2 value calculated from this low affinity site gave a value of 6.29 ± 0.28 ($n = 3$).

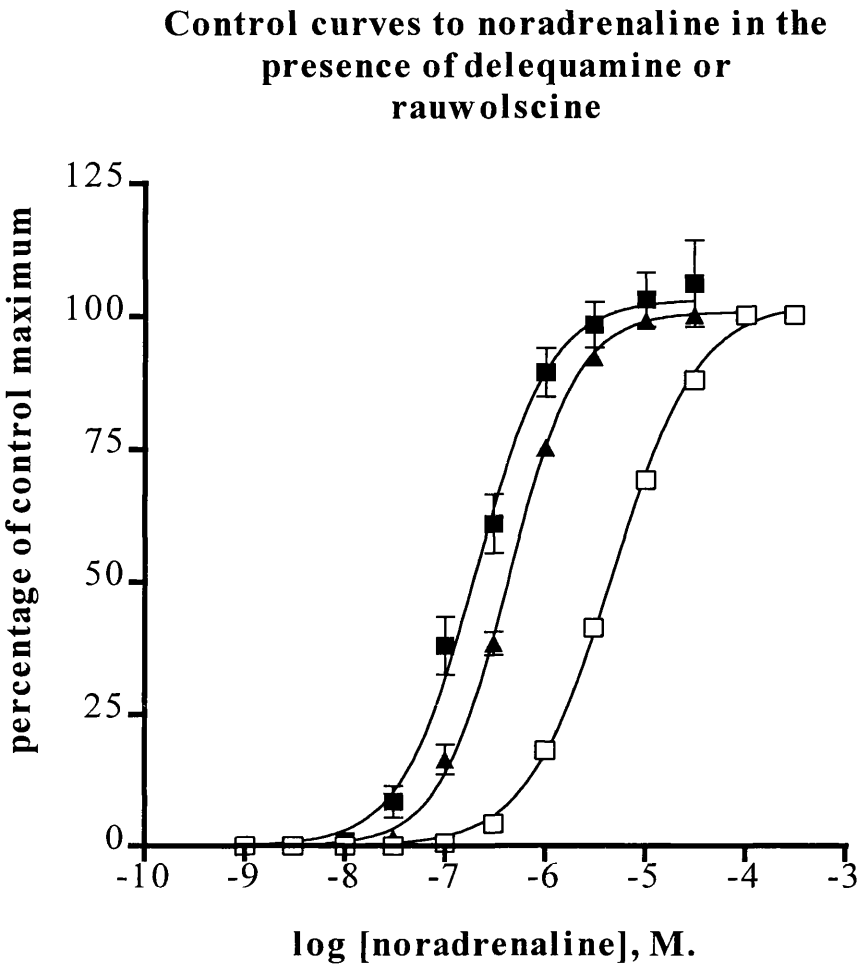


Figure 3.3. Graph illustrating the effect of either 1μM rauwolscine or 0.1μM delequamine on NA concentration response curves in DSV. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Noradrenaline (■), n = 6; noradrenaline plus 1μM rauwolscine (□), n = 4; noradrenaline plus 0.1μM delequamine (▲), n = 4.

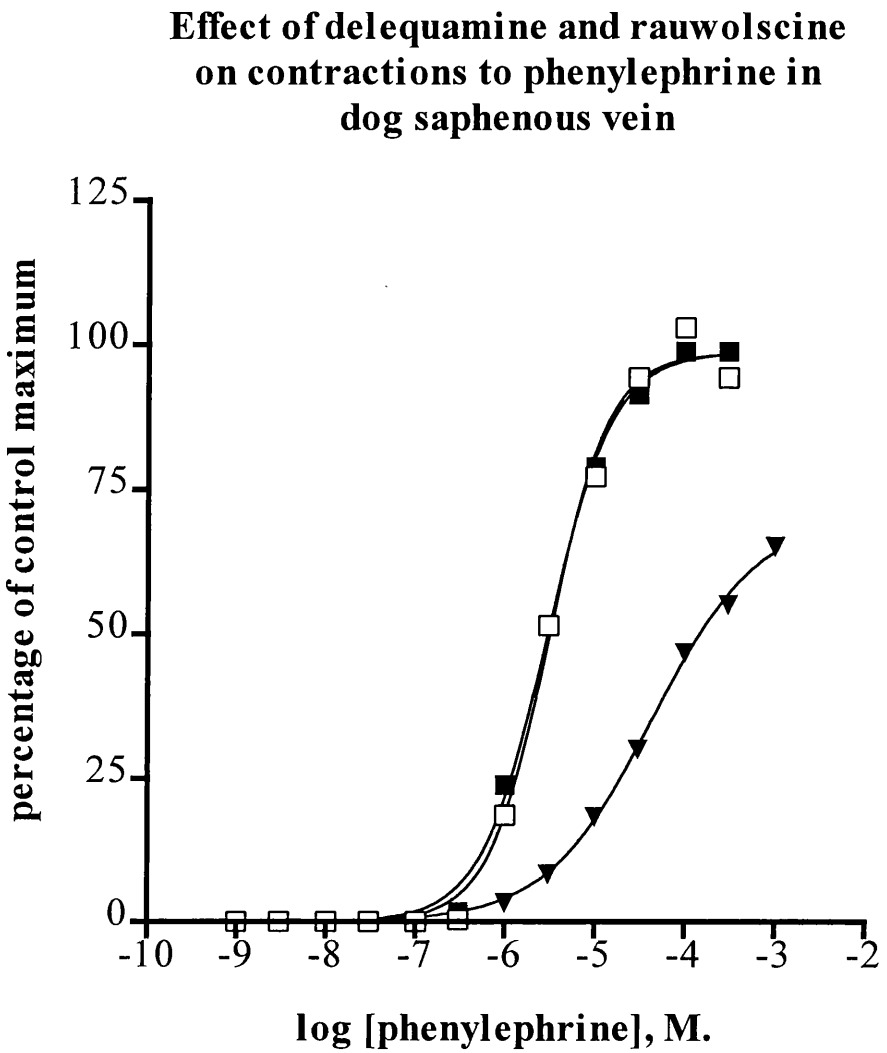


Figure 3.4. Graph showing data from a single experiment in the DSV, comparing the effect of 0.1μM delequamine and 1μM rauwolscine on a response curve to phenylephrine. PE (□); PE plus 0.1μM delequamine (■), PE plus 1μM rauwolscine (▼).

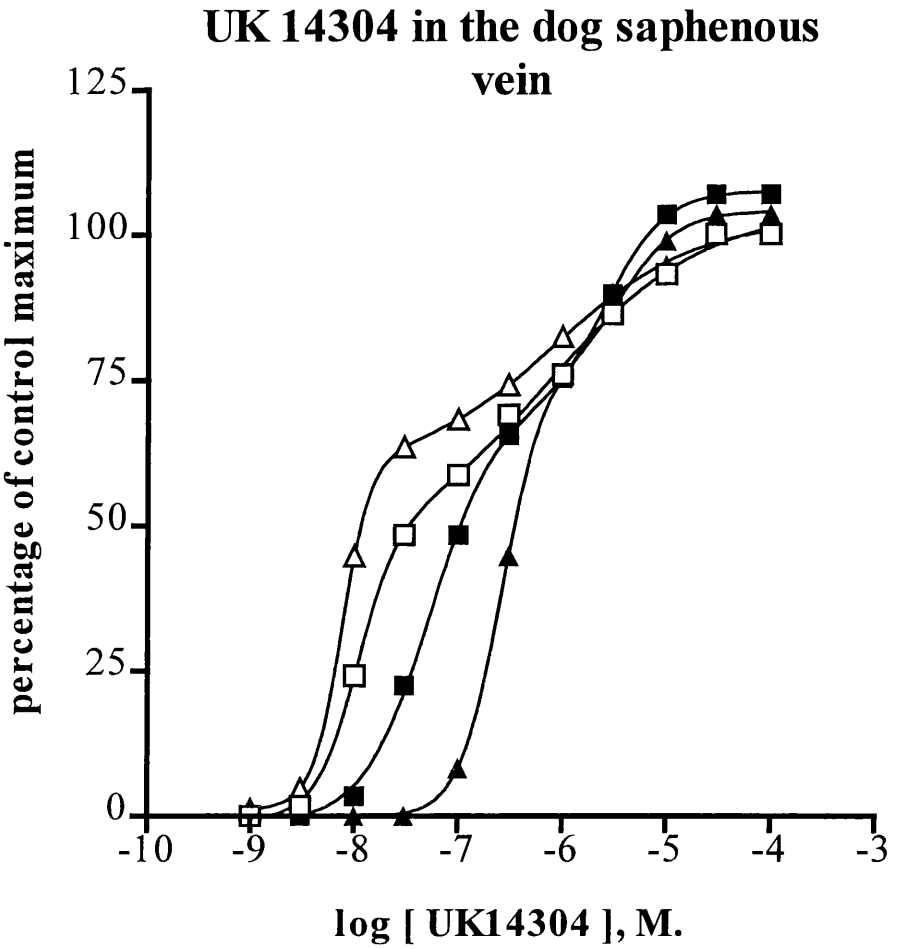


Figure 3.5. Graph showing data from a single experiment in the DSV, comparing the effect of 0.1μM delequamine and 1μM delequamine on a response curve to UK 14304. All curves are fitted to the two site model for receptor activation. Control UK 14304 for 0.1μM delequamine (□); UK 14304 plus 0.1μM delequamine (■), Control UK 14304 for 1μM delequamine (△); UK 14304 plus 1μM delequamine (▲).

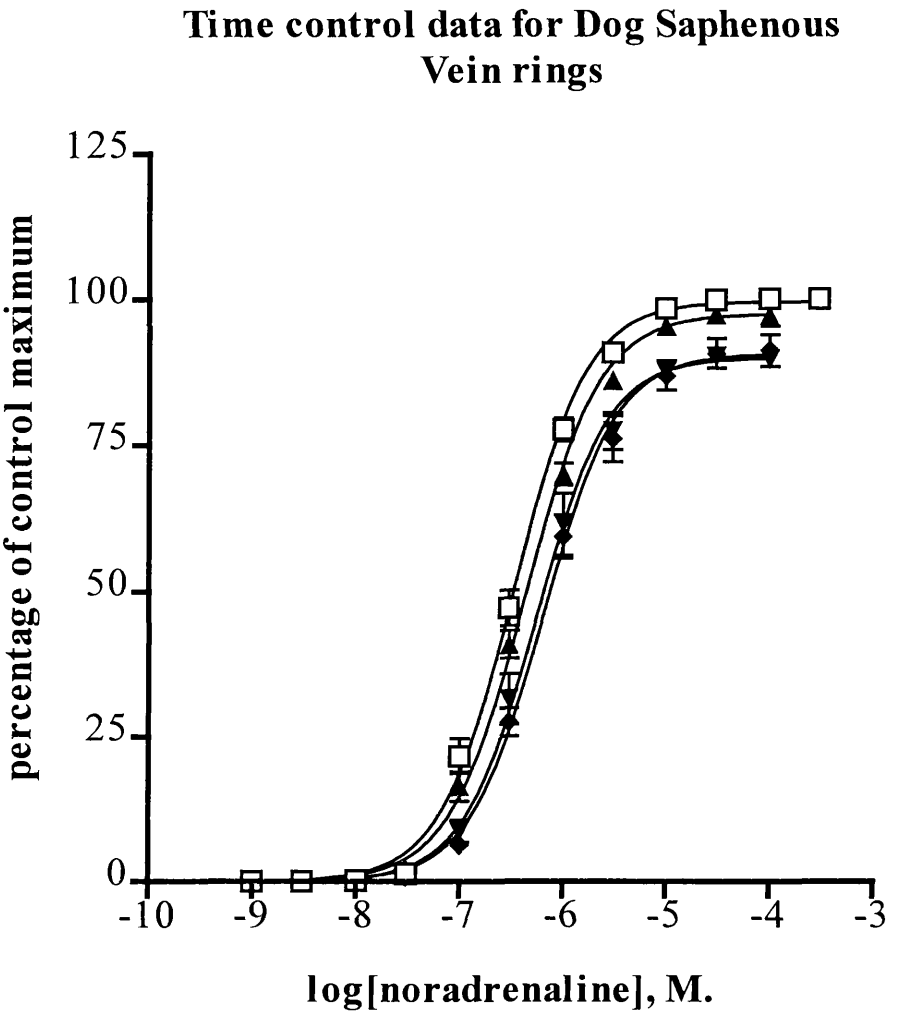


Figure 3.6. Mean time control concentration response curves for dog saphenous vein. Curves were created by meaning the parameters derived from the curve fitting and generating mean curves. Mean raw data ± s.e. mean, was then superimposed on the mean curves. Curve 1 (□), n = 13; curve 2 (▲), n = 13; curve 3 (▼), n = 4; curve 4 (◆), n = 3.

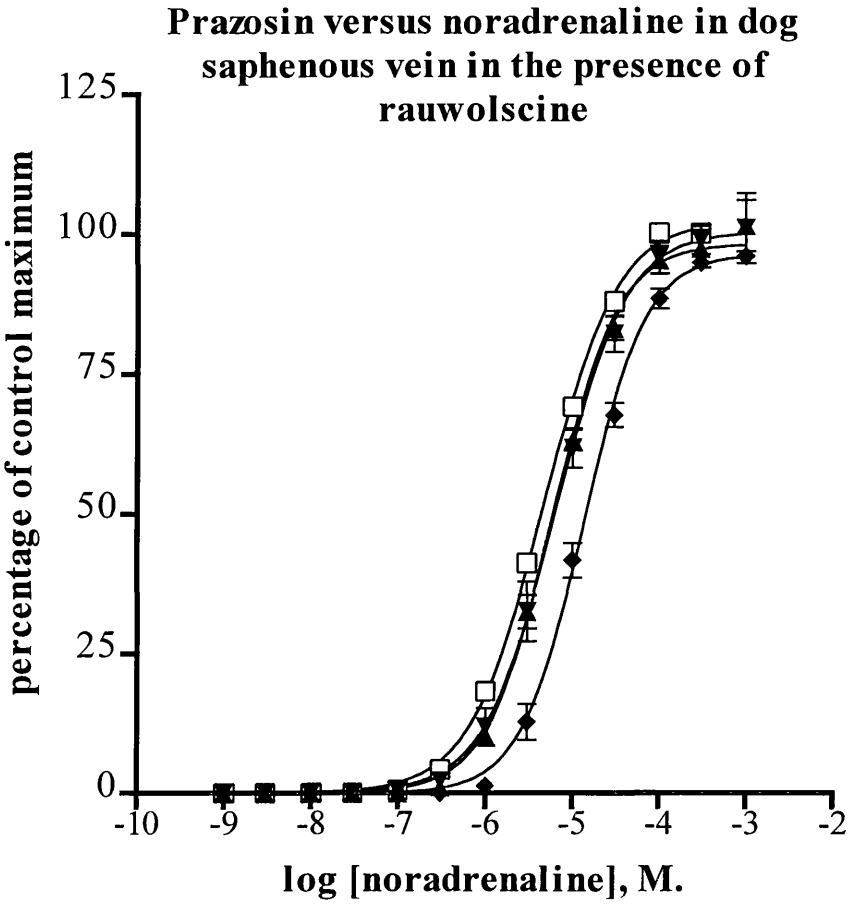


Figure 3.7. Mean concentration response data for prazosin in DSV in the presence of rauwolscine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 12; 1nM prazosin (▼), n = 4; 10nM prazosin (▲), n = 4 ; 0.1μM prazosin (◆), n = 4.

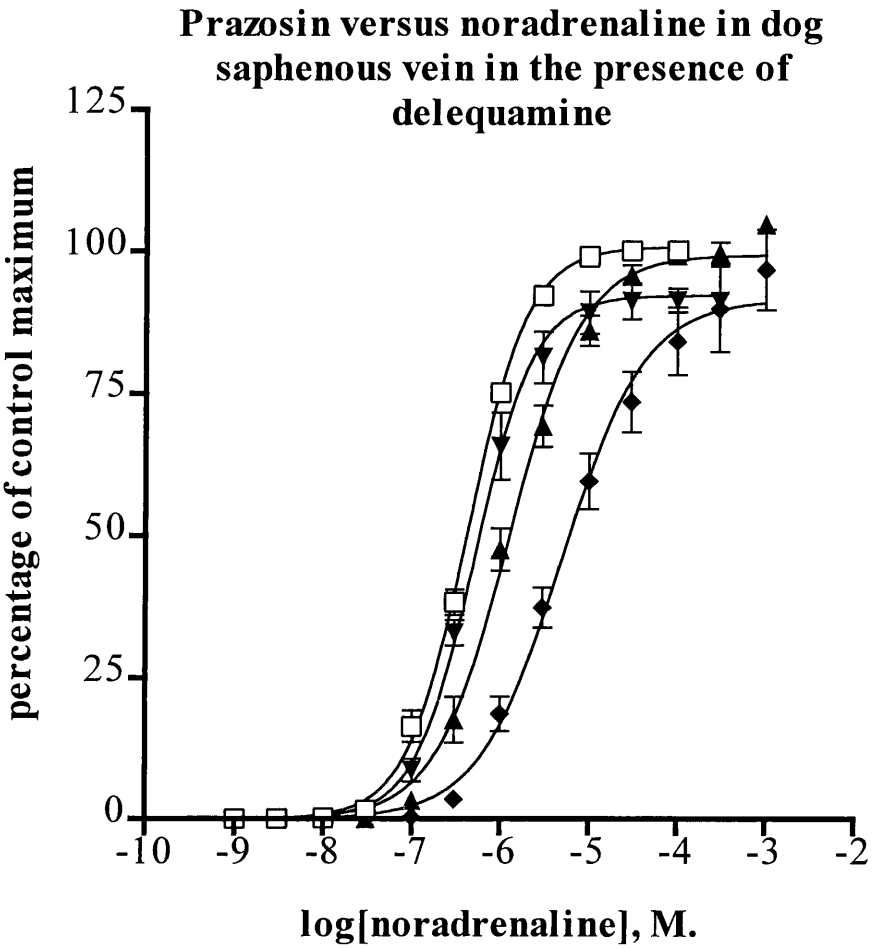


Figure 3.8. Mean concentration response data for prazosin in DSV in the presence of delequamine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 12; 1nM prazosin (▼), n = 4; 10nM prazosin (▲), n = 4; 0.1μM prazosin (◆), n = 4.

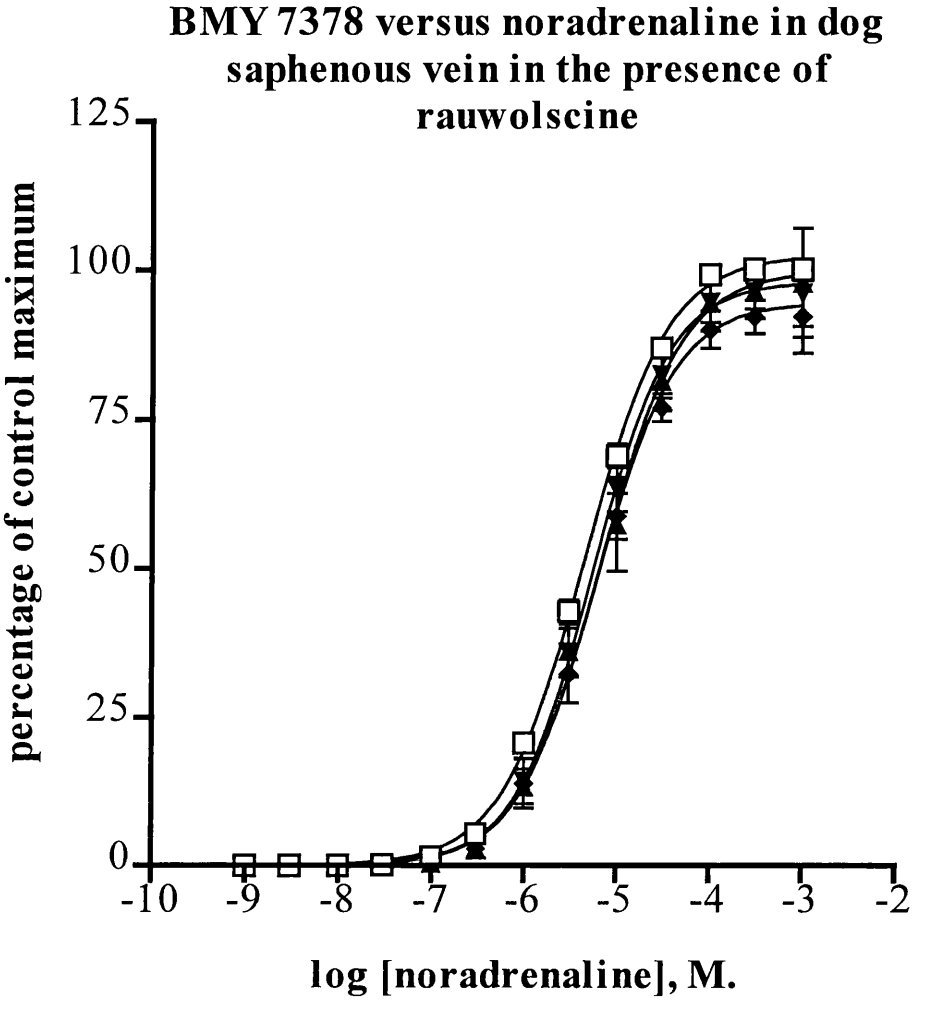


Figure 3.9. Mean concentration response data for BMY 7378 in DSV in the presence of rauwolscine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 12; 1nM BMY 7378 (▼), n = 4; 10nM BMY 7378 (▲), n = 4 ; 0.1μM BMY 7378 (◆) , n = 4.

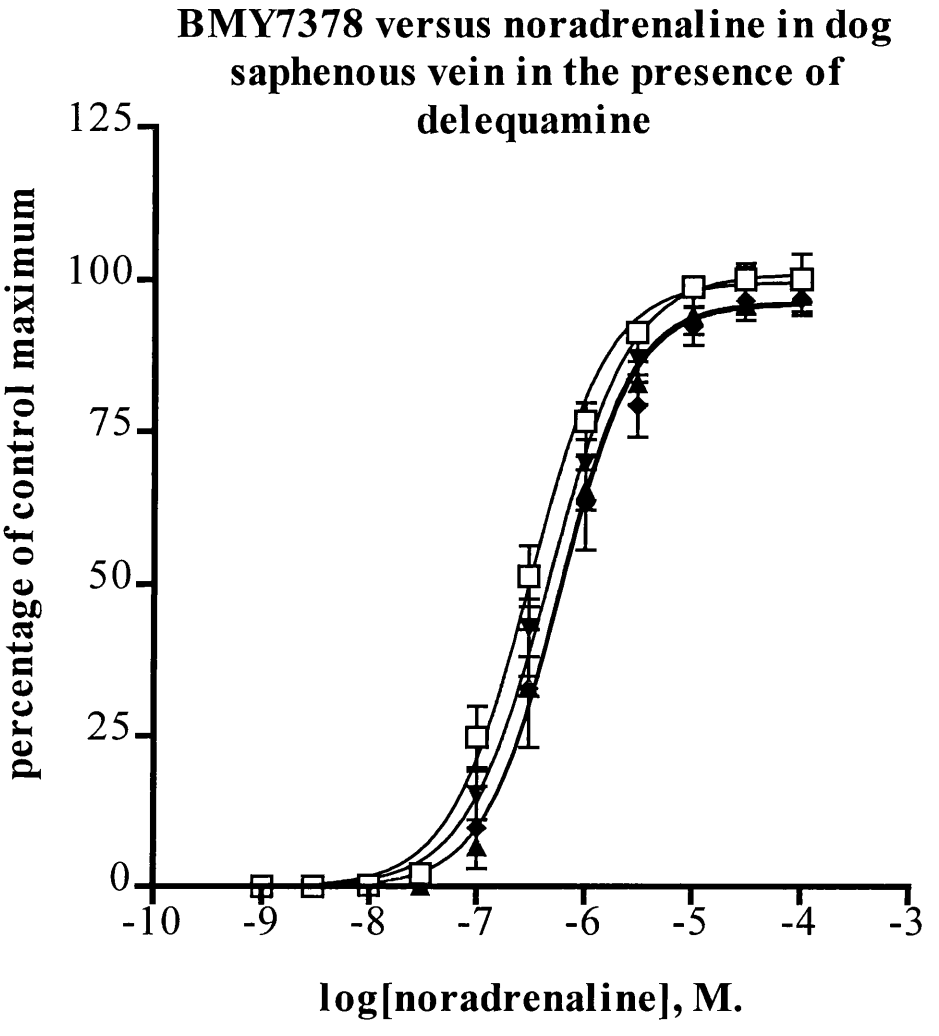


Figure 3.10. Mean concentration response data for BMY 7378 in DSV in the presence of delequamine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), $n = 6$; 1nM BMY 7378 (▼), $n = 4$; 10nM BMY 7378 (▲), $n = 4$; 0.1μM BMY 7378 (◆), $n = 4$.

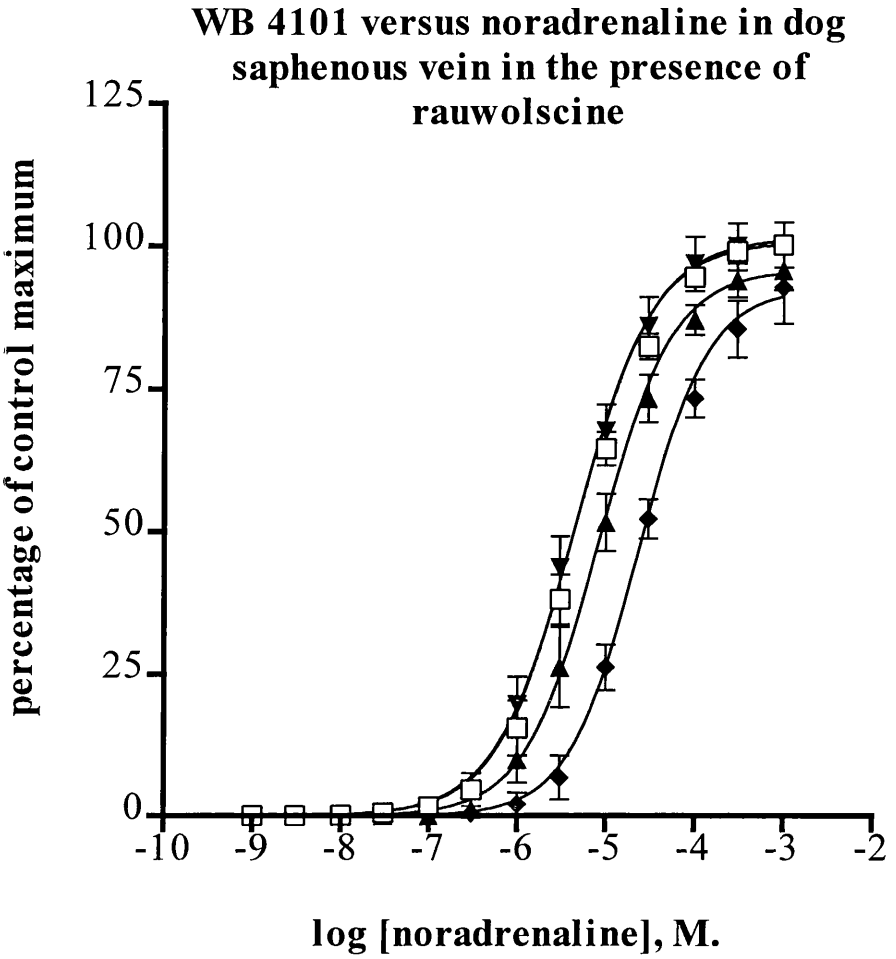


Figure 3.11. Mean concentration response data for WB4101 in DSV in the presence of rauwolscine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), $n = 12$; 1nM WB 4101 (▼), $n = 4$; 10nM WB 4101 (▲), $n = 4$; 0.1μM WB 4101 (◆), $n = 5$.

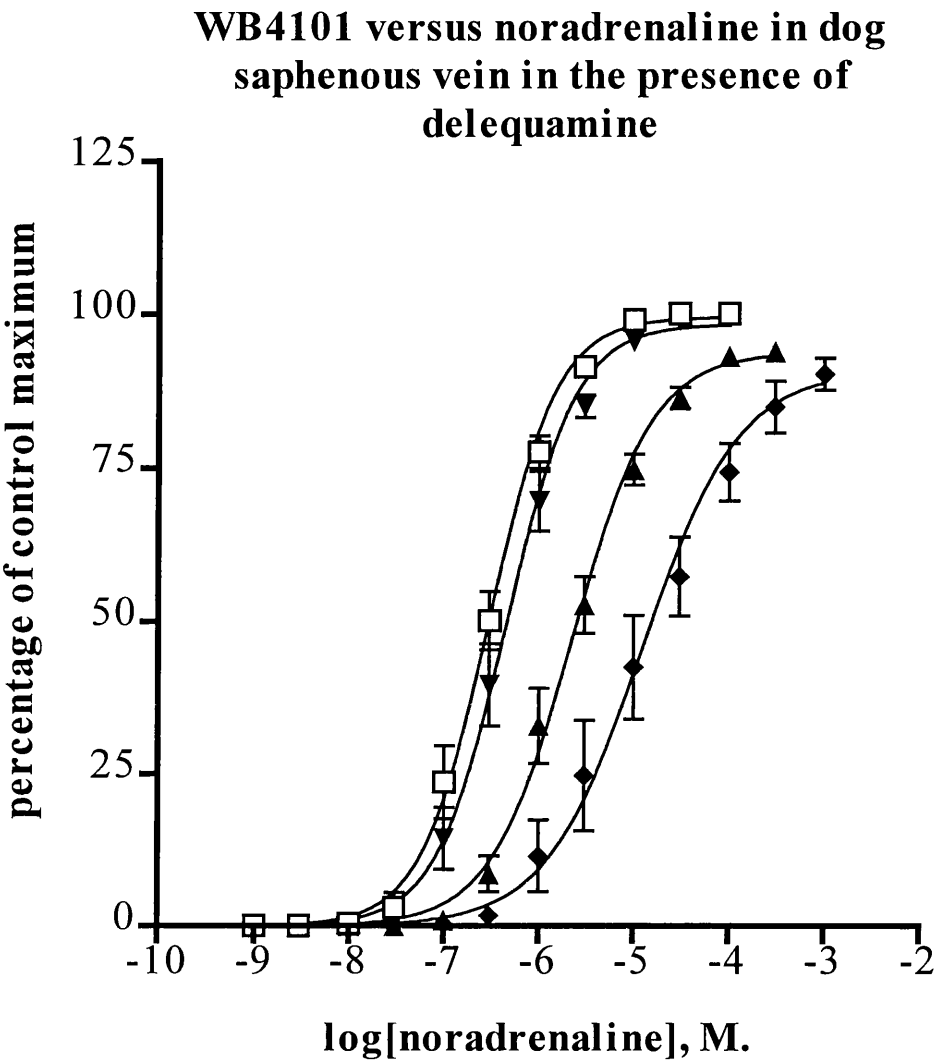


Figure 3.12. Mean concentration response data for WB4101 in DSV in the presence of delequamine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□) , $n = 7$; 1nM WB 4101 (▼), $n = 4$; 10nM WB 4101 (▲), $n = 4$; 0.1μM WB 4101 (◆), $n = 5$.

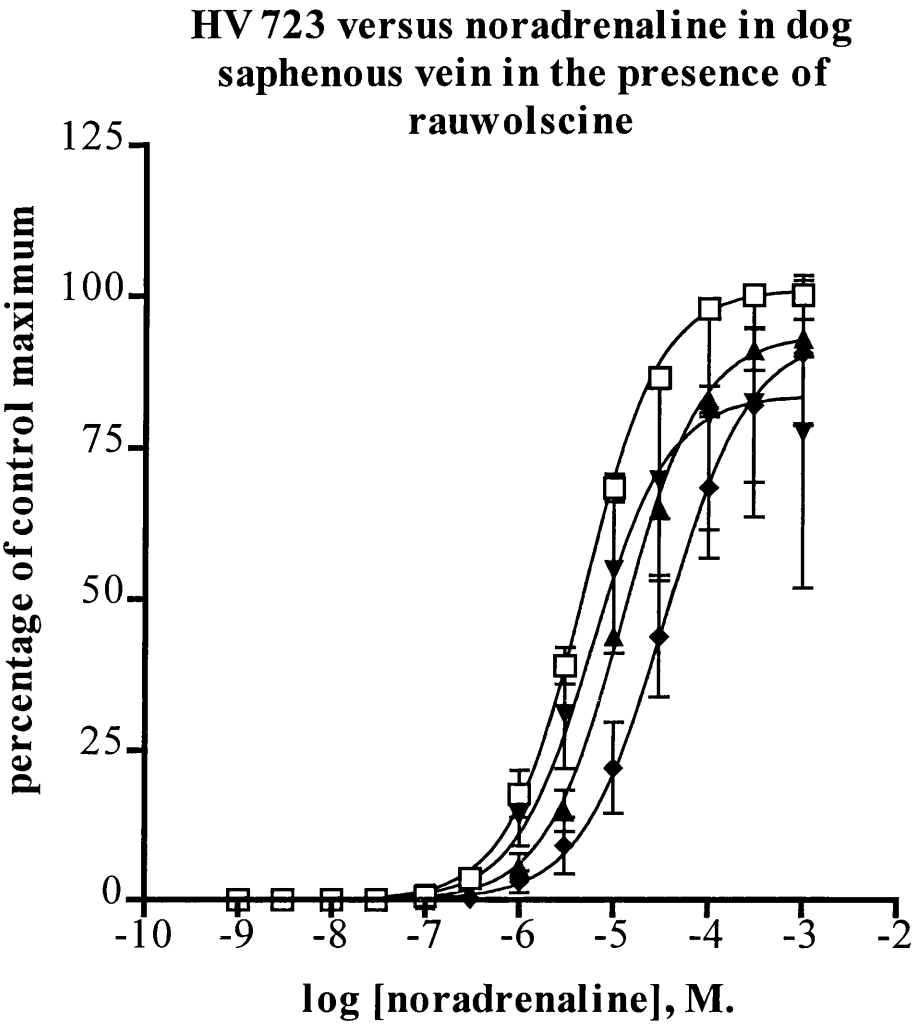


Figure 3.13. Mean concentration response data for HV723 in DSV in the presence of rauwolscine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 12; 1nM HV 723 (▼), n = 4; 10nM HV 723 (▲), n = 4 ; 0.1μM HV 723 (◆), n = 4.

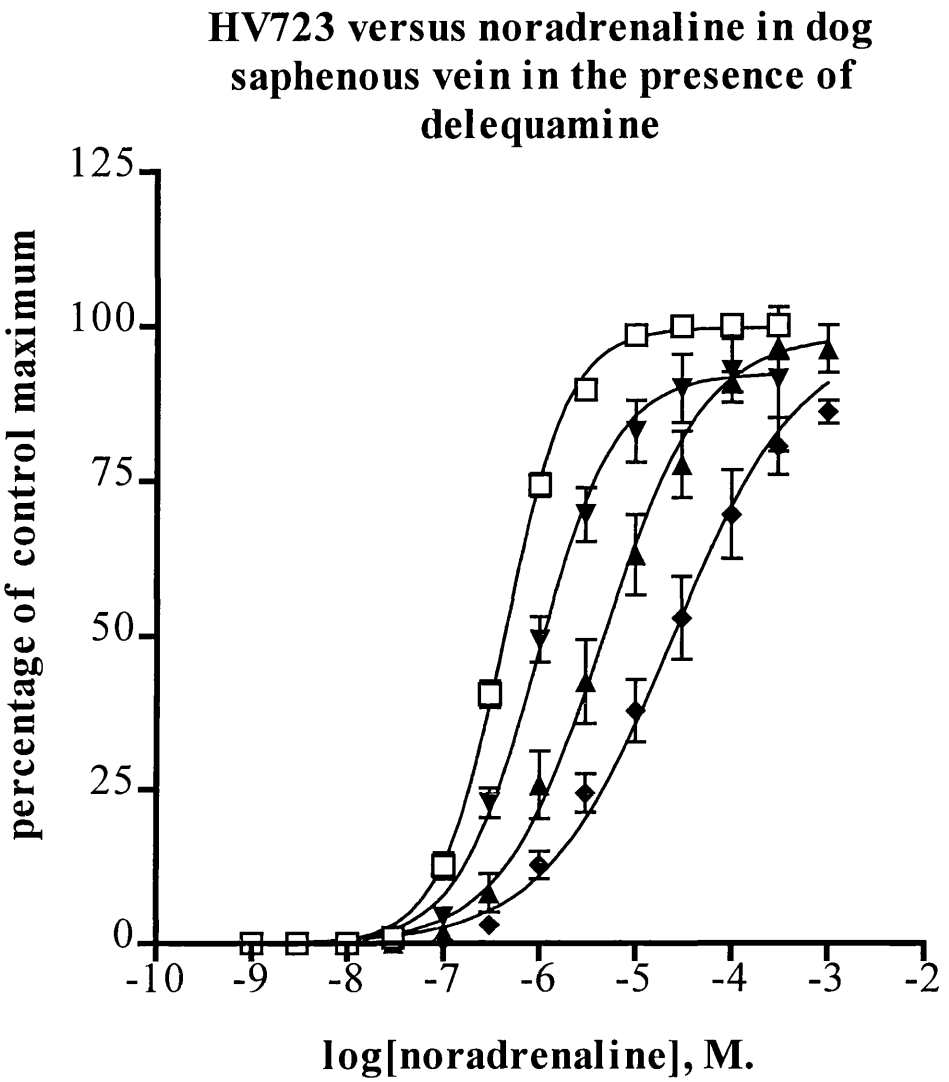


Figure 3.14. Mean concentration response data for HV723 in DSV in the presence of delequamine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 12; 1nM HV 723 (▼), n = 4; 10nM HV 723 (▲), n = 4 ; 0.1μM HV 723 (◆), n = 4

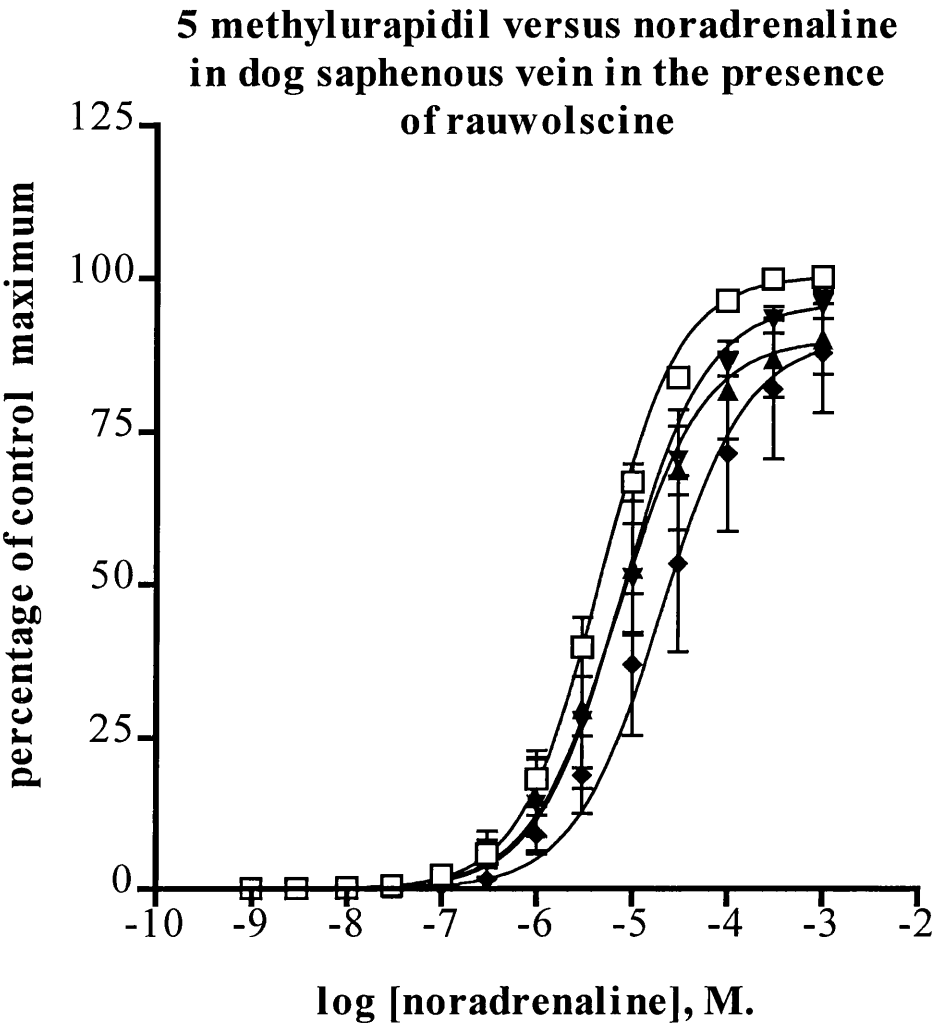


Figure 3.15. Mean concentration response data for 5 methylurapidil (5 MeU) in DSV in the presence of rauwolscine. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), $n = 12$; 10nM 5 MeU (▼), $n = 4$; 0.1μM 5 MeU (▲), $n = 4$; 1μM 5 MeU (◆), $n = 4$.

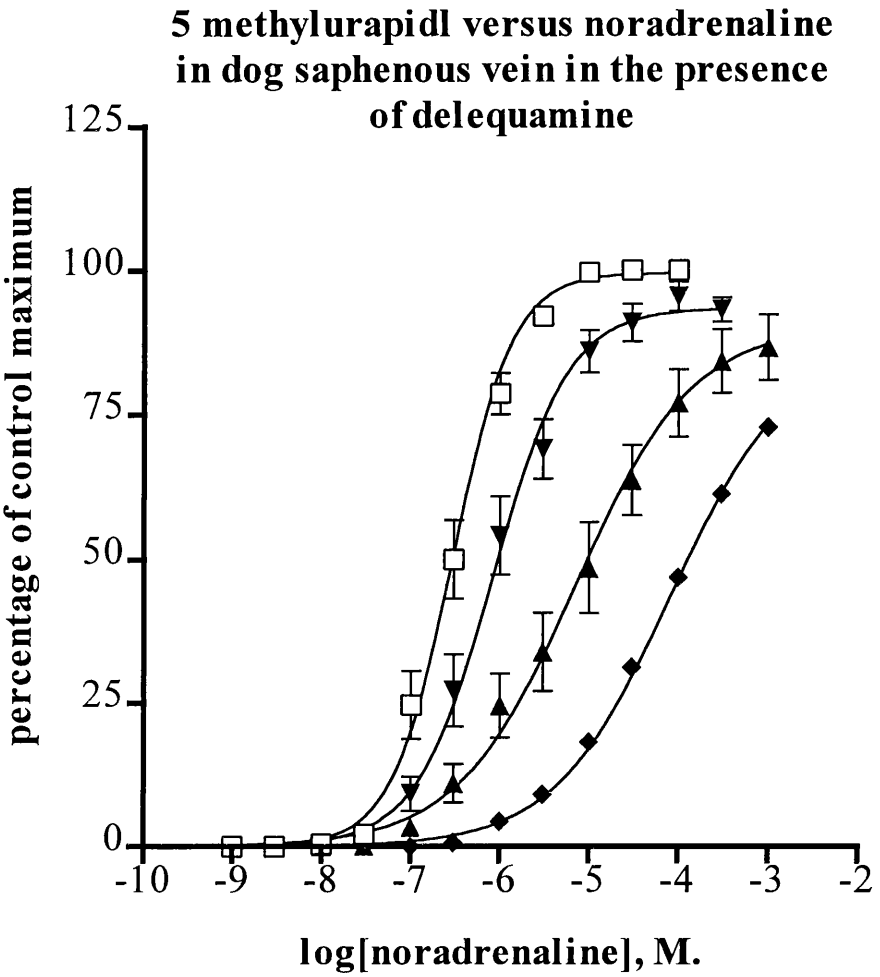


Figure 3.16. Mean concentration response data for 5 methylurapidil (5 MeU) in DSV in the presence of delequamine. Graph showing the one site curves only for 1μM 5MeU. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 7; 10nM 5 MeU (▼), n = 4; 0.1μM 5 MeU (▲), n = 4 ; 1μM 5 MeU (◆), n = 2 .

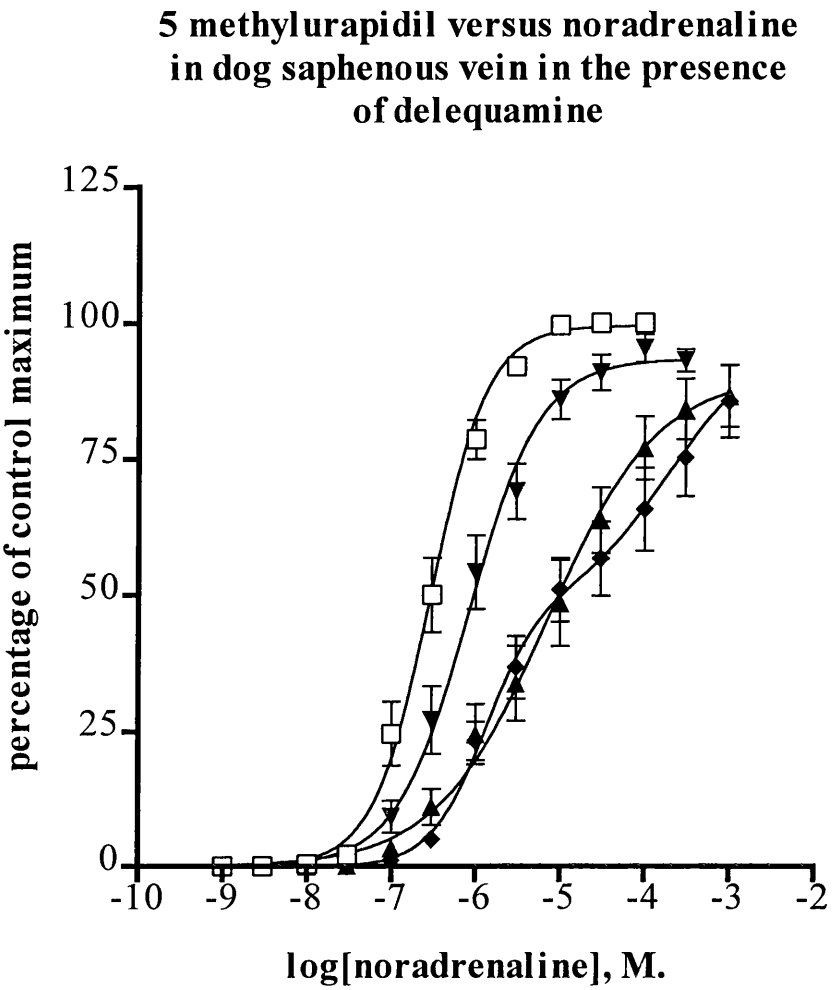


Figure 3.17. Mean concentration response data for 5 methylurapidil (5 MeU) in DSV in the presence of delequamine showing the two site curves only for 1μM 5MeU. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed. Control (□), n = 7; 10nM 5 MeU (▼), n = 4; 0.1μM 5 MeU (▲), n = 4 ; 1μM 5 MeU (◆), n = 3.

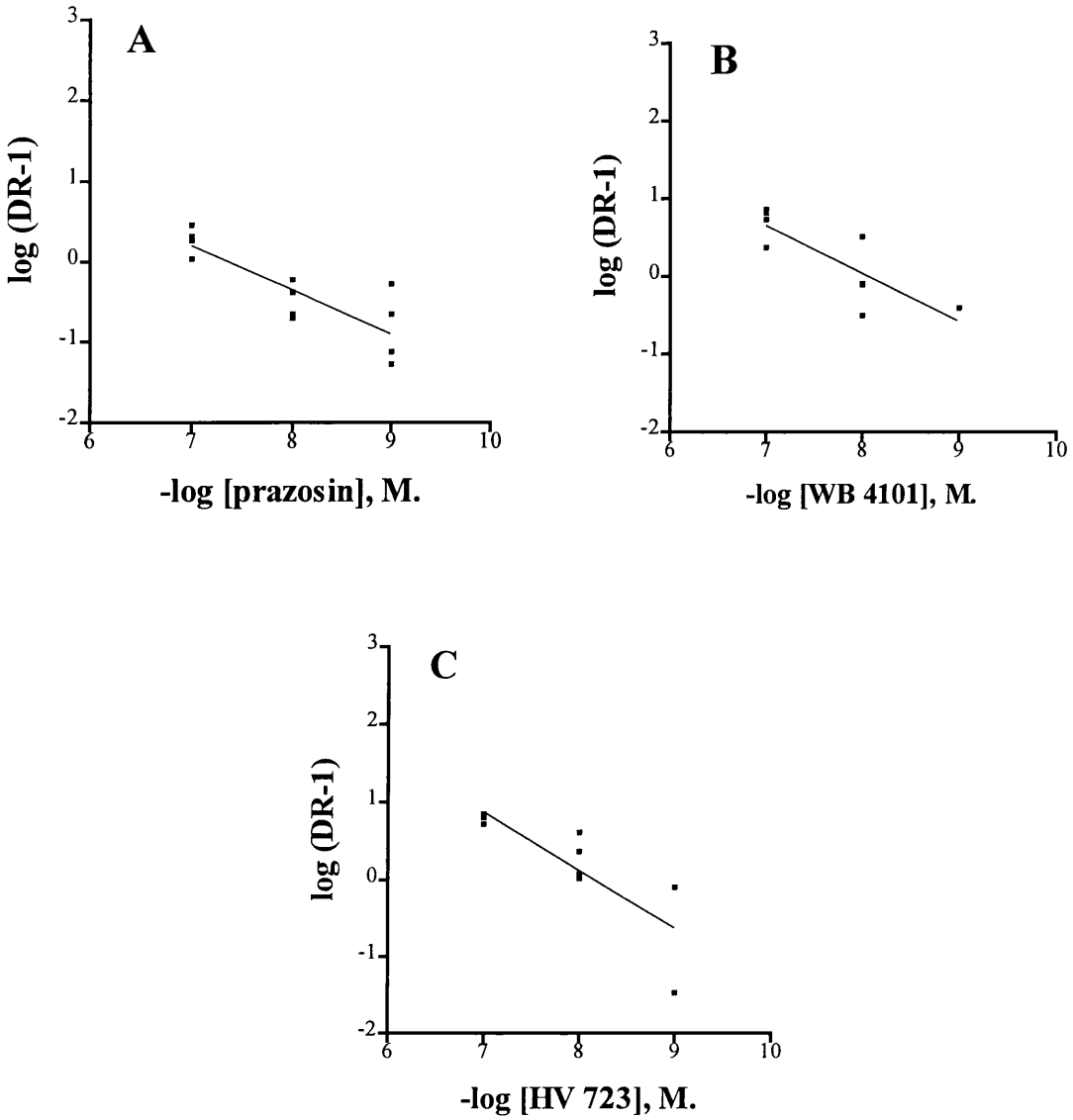


Figure 3.18. Schild plots for prazosin, WB4101 and HV723 in the dog saphenous vein in the presence of rauwolscine. Graph A, prazosin; graph B, WB 4101; graph C, HV 723.

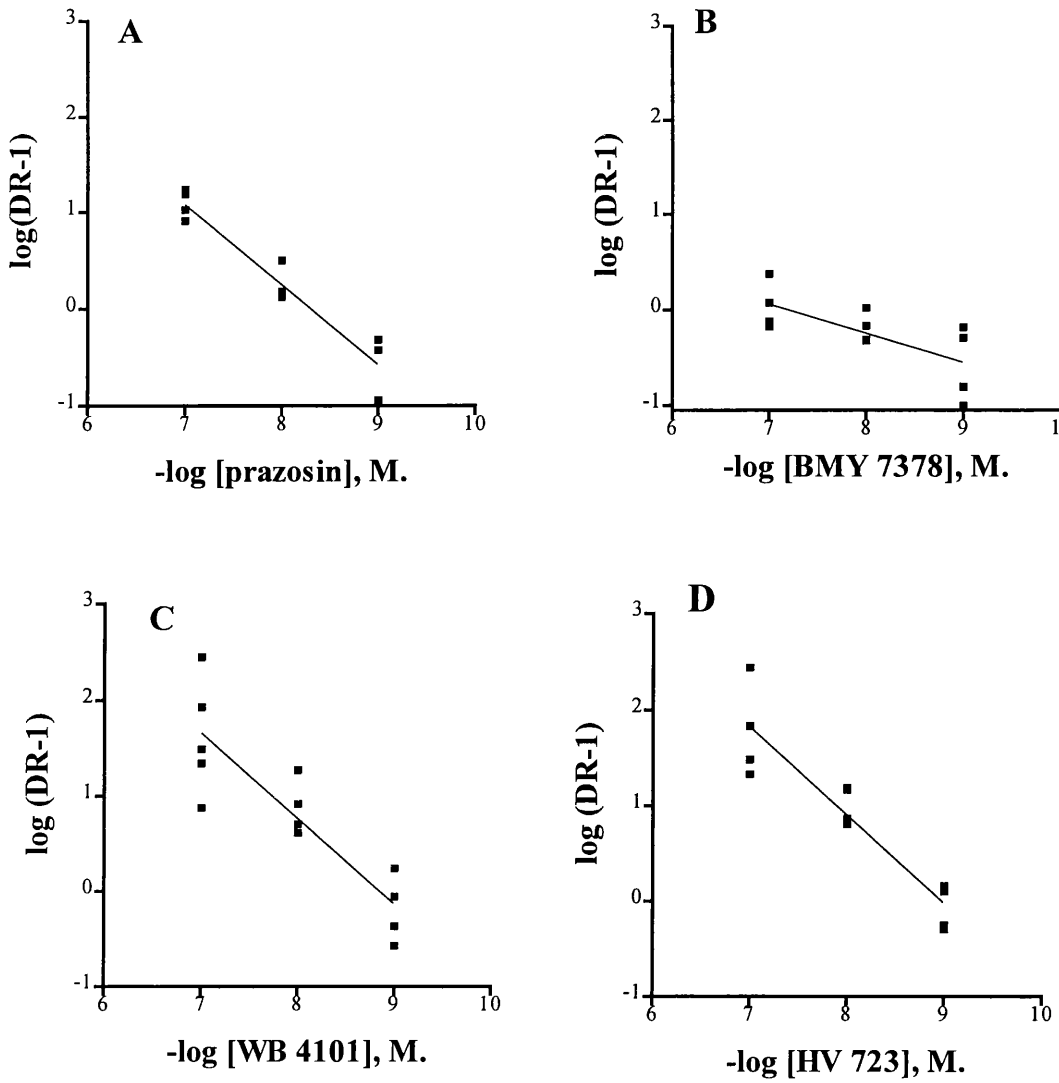


Figure 3.19. Schild plots for prazosin, BMY 7378, WB4101 and HV723 in the dog saphenous vein in the presence of delequamine. Graph A, prazosin; graph B, BMY 7378; graph C, WB 4101; graph D, HV 723.

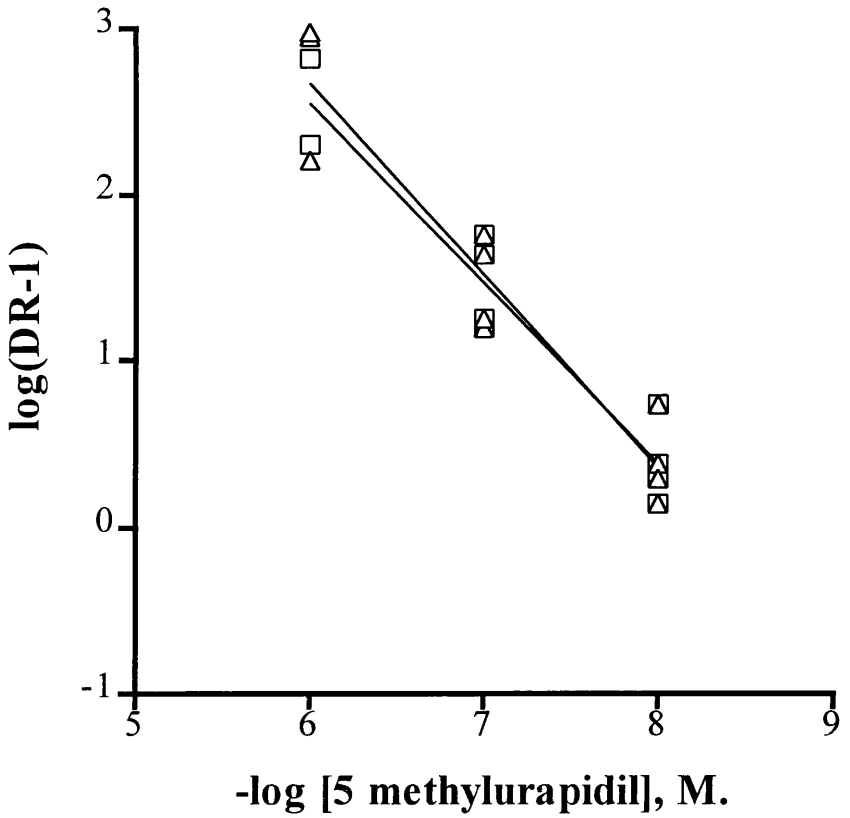


Figure 3.20. Schild plot for 5 methylurapidil in the dog saphenous vein in the presence of delequamine. DR-1 values for both the one site (□) and the high affinity two site fit (Δ) are displayed for 1uM 5 methylurapidil.

Agonist	pEC_{50}	maximum ± s.e. mean (% of 10μM NA)	Hill slope ± s.e. mean
Noradrenaline	6.7 ± 0.08 (n = 7)	103.8 ± 5.04 (n = 7)	1.17± 0.04 (n = 7)
Phenylephrine	5.94 ± 0.07 (n = 11)	98.23 ± 3.93 (n = 11)	1.53 ± 0.08 (n= 11)
UK14304	6.35 ± 0.28 (n = 5)	49.09 ± 10.14 (n = 5)	0.76 ± 0.14 (n = 5)
(R) A-61603	7.57 ± 0.05 (n = 6)	94.7 ± 5.22 (n = 6)	1.54 ± 0.09 (n = 6)

Table 3.1. Comparison of pEC_{50}, maximum and Hill slope parameters for agonists used in the dog saphenous vein.

ANTAGONIST	Slope with rauwolscine	Slope with delequamine	pA_2 with rauwolscine	pA_2 with delequamine
Prazosin	-0.8 to -0.3	-1.01 to -0.65	7.36	8.31
BMY 7378	NA	-0.52 to -0.09	NA	7.20
5 methylurapidil one site	NA	-1.34 to -0.83	NA	8.35
5 methylurapidil two site, high affinity	NA	-1.42 to -0.89	NA	8.31
WB 4101	-1 to -0.23	-1.22 to -0.57	8.06	8.85
HV723	-1.15 to -0.35	-1.18 to -0.66	8.16	8.98

Table 3.2. Slope parameters and pA_2 values derived from Schild regression of competitive antagonists versus noradrenaline, in the dog saphenous vein in the presence of delequamine and rauwolscine. Slopes are given as 95% confidence intervals. NA shows where it was not possible to perform Schild analysis.

ANTAGONIST	pA_2 from Schild	concentration of antagonist	$pA_2 \pm$ s.e.mean[a] (n)
prazosin	7.36	1nM	8.17 ± 0.23 (4)
		10nM	7.51 ± 0.11 (4)
		0.1μM	7.35 ± 0.04 (4)
BMY 7378	NA	1nM	8.54 ± 0.17 (4)
		10nM	7.26 ± 0.1 (4)
		0.1μM	6.54 ± 0.17 (4)
HV 723	8.16	1nM	8.21 (2)
		10nM	8.27 ± 0.14 (4)
		0.1μM	7.8 ± 0.03 (4)
WB 4101	8.059	1nM	8.59 (1)
		10nM	7.84 ± 0.12 (4)
		0.1μM	7.7 ± 0.11 (4)
5 MeU	NA	10nM	8 ± 0.33 (3)
		0.1μM	6.8 ± 0.42 (3)
		1μM	6 ± 0.77 (4)

Table 3.3. Comparison of pA_2 values from Schild analysis with values calculated from individual antagonist concentrations[a] in the dog saphenous vein in the presence of rauwolscine. Noradrenaline was the agonist used. NA shows where it was not possible to perform Schild analysis.

ANTAGONIST	pA_2 from Schild	concentration of antagonist	$pA_2 \pm$ s.e.mean[a] (n)
prazosin	8.31	1nM	8.44 ± 0.19 (4)
		10nM	8.23 ± 0.1 (4)
		0.1μM	8.09 ± 0.08 (4)
BMY 7378	7.20	1nM	8.43 ± 0.2 (4)
		10nM	7.80 ± 0.08 (4)
		0.1μM	7.04 ± 0.12 (4)
HV 723	8.98	1nM	8.93 ± 0.12 (4)
		10nM	9.01 ± 0.1 (4)
		0.1μM	8.77 ± 0.25 (4)
WB 4101	8.85	1nM	8.81 ± 0.18 (4)
		10nM	8.88 ± 0.15 (4)
		0.1μM	8.62 ± 0.34 (4)
5 MeU	8.35	10nM	8.39 ± 0.13
		0.1μM	8.46 ± 0.28
		1μM one site	8.56 ± 0.26
		1μM two site, low affinity	6.29 ± 0.28
		1μM two site, high affinity	8.71 ± 0.25

Table 3.4. Comparison of pA_2 values from Schild analysis with values calculated for each antagonist concentration[a] in the dog saphenous vein in the presence of delequamine (noradrenaline was the agonist used).

Antagonist	[a]α_{1a}-human clone ([^{3}H-InsPs]) pK_B	[b]α_{1L}- LUT pA_2	[c]DSV α_{1N}- pA_2	[d] α_{1D}- rat aorta pA_2	[e]α_{1B}-rat spleen pA_2	DSV present study pA_2
Prazosin	8.7 ± 0.1	8.7 ± 0.1	7.94 ± 0.07	9.6 ± 0.1	9.2	8.31
WB 4101	8.9 ± 0.1	8.9 ± 0.1	8.58 ± 0.10	9.0 ± 0.1	8.1	8.85
5-MeU	8.2 ± 0.1	8.2 ± 0.1	N.D.	7.6 ± 0.1	7.1	6.29 ± 0.28 (L) 8.56 ± 0.26 (H)
BMY 7378	N.D.	6.4 ± 0.1	N.D.	8.5 ± 0.1	7.4	7.04 ± 0.12
HV 723	N.D.	8.8 ± 0.1	9.09 ± 0.06	8.7 ± 0.1	N.D.	8.98

Table 3.5. Comparison of published pA_2 values with values obtained in this study of the DSV. Values for DSV are in the presence of delequamine. [a] human clone α_{1a} - (Daniels et al. 1996); [b] human lower urinary tract tissues α_{1L}- (Ford et al. 1996a) ; [c] DSV values from Muramatsu et al (1990) ; [d] rat aorta α_{1D}- (Ford et al. 1996a); [e] rat spleen α_{1B}- (Burt et al. 1995) ; [f] present study pA_2 values from Schild analysis are quoted with the exception of values given with ± s.e. mean. These latter are pA_2 values derived from a single antagonist concentration.

3.2.3 Irreversible antagonists

CEC in the presence of 1μM rauwolscine, was used at concentrations of 0.1μM, 1μM and 100μM. Values are the results of experiments derived from four different animals. The two highest concentrations used had no effect on the NA concentration response curves. 100μM CEC caused an irreversible baseline contraction of 12.92 ± 4.1% (n = 4). In addition, this concentration significantly depressed the maximum, lowered the Hill slope and shifted the pEC_{50} value compared to control. pEC_{50} values were as follows: Control 5.36 ± 0.04, (n = 12); 0.1μM CEC 5.35 ± 0.08, (n = 4); 1μM CEC 5.45 ± 0.08, (n = 4); 100μM CEC 4.83 ± 0.05 (n = 4).

CEC in the presence of delequamine, was used at concentrations of, 1μM, 10μM and 100μM (n = 4 for each concentration). The two lowest concentrations used caused no significant change in the maximum or pEC_{50} values. pEC_{50} values of 6.50 ± 0.07, 6.31 ± 0.09 and 6.19 ± 0.05 were obtained for the control curve, 1μM and 10μM respectively. 1μM and 10μM did however significantly lower the Hill slope parameter and 10μM CEC caused a baseline contraction. 100μM CEC did significantly reduce the curve maximum and significantly shifted the pEC_{50} value to 5.37 ± 0.12. This highest concentration also caused an irreversible baseline contraction in the vessels averaging 37.86 ± 6.38% (n = 4) of the maximum response in the control curve. This was significantly higher than that seen in the presence of rauwolscine. Mean concentration response curves in the presence of rauwolscine or delequamine are represented in Figures 3.21 and 3.22 respectively.

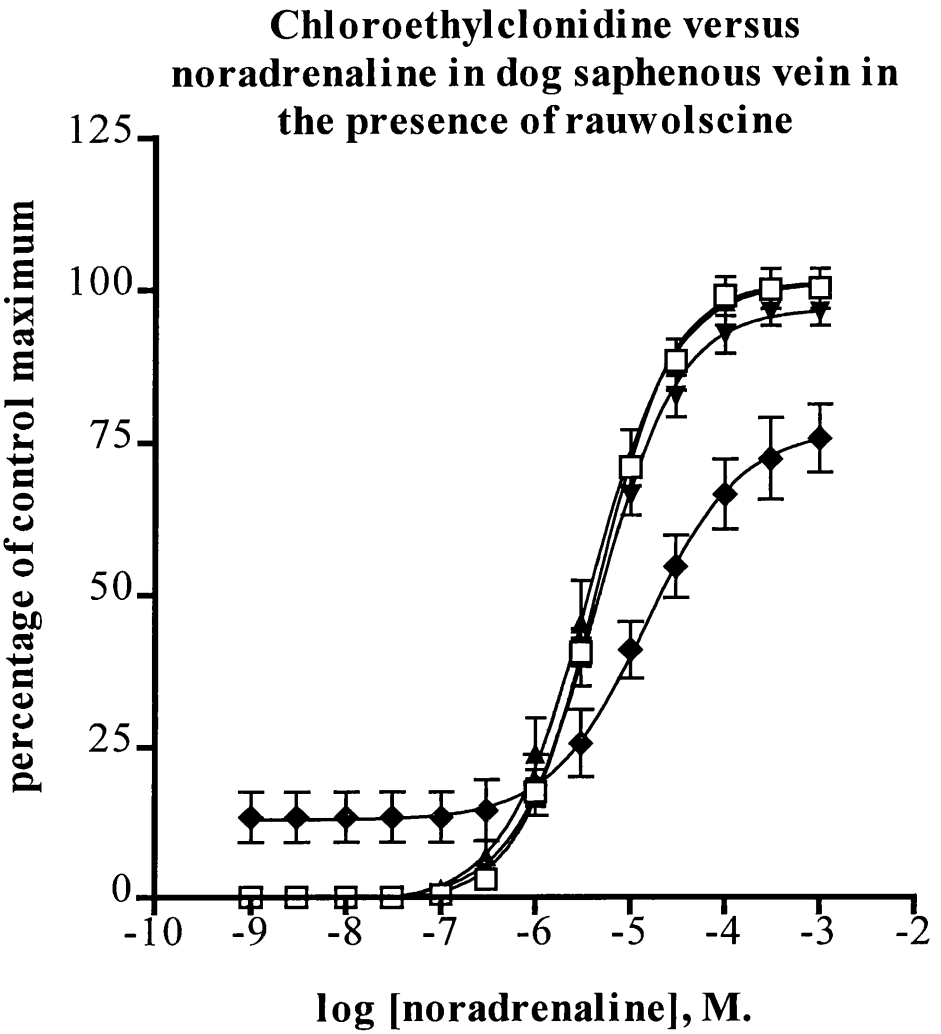


Figure 3.21. Mean concentration response curves in the DSV, to noradrenaline in the presence of CEC and rauwolscine. Control (□), n = 12; 0.1μM CEC (▼), n = 4; 1μM CEC (▲), n = 4; 100μM CEC (◆), n = 4. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed.

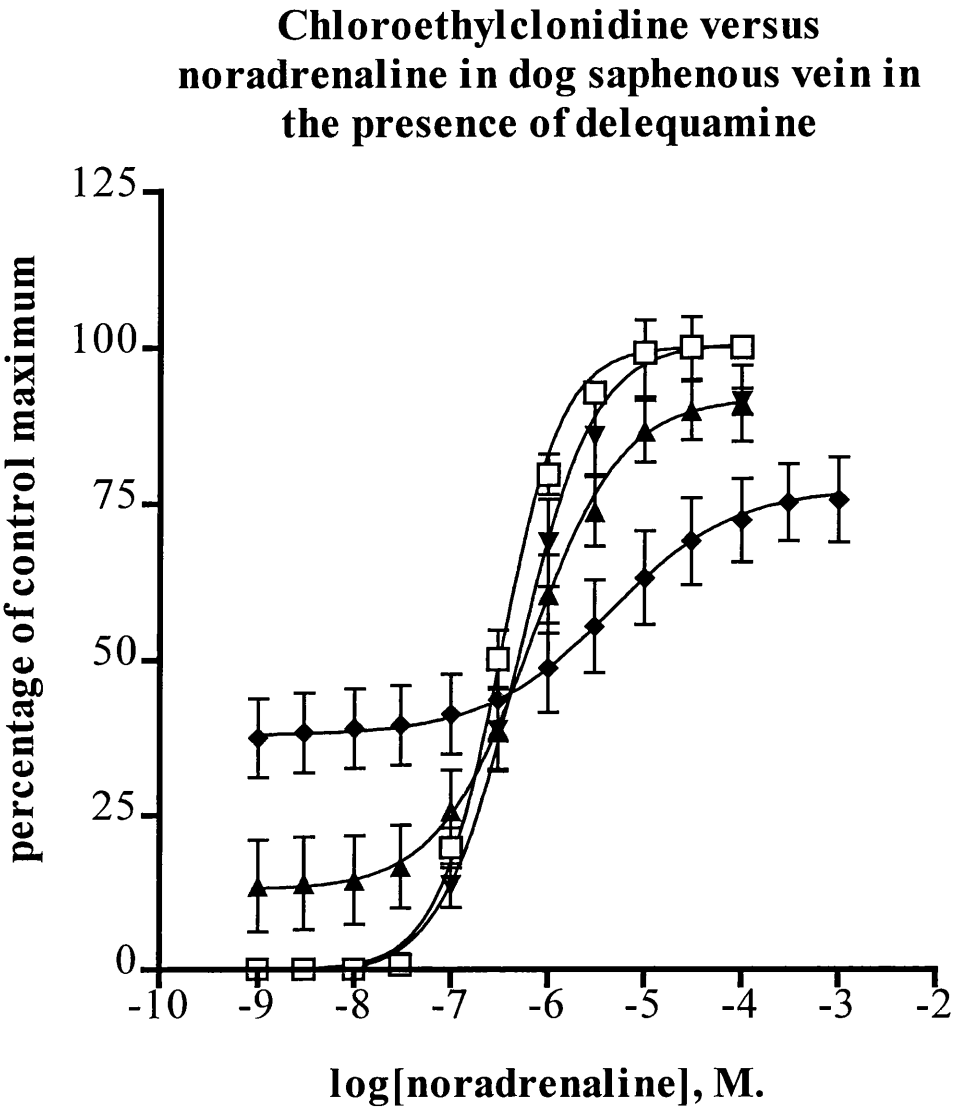


Figure 3.22. Mean concentration response curves in the DSV, to noradrenaline in the presence of CEC and delequamine. Control (□), n = 12; 1μM CEC (▼), n = 4; 10μM CEC (▲), n = 4; 100μM CEC (◆), n = 4. Mean curves were generated from mean parameters derived from curve fitting. The mean raw data ± s.e. mean has been superimposed.

3.3 Discussion

The dog saphenous vein (DSV) has been used extensively in a wide variety of vascular research studies and thus within certain limits it has been well characterised. This made it a good choice of vessel for this study.

It is now well accepted that the DSV has both α_1- and α_2- post-junctional adrenoceptors, which mediate contraction to exogenous noradrenaline. Evidence for this first came from in vivo studies by Drew and Whiting (1979). Later a number of studies looked at isolated rings of vein and compared responses to a number of agonists and antagonists (Flavahan and Vanhoutte, 1986b; Alabaster et al. 1985; Flavahan et al. 1984; Constantine et al. 1982; Sullivan and Drew, 1980; De Mey and Vanhoutte, 1981). These studies found that exogenous noradrenaline activated post-junctional α_1- and α_2-adrenoceptors and that the lower half of the curve was predominantly α_2- mediated while the upper portion of the curve was primarily α_1- mediated. In general, all these studies found that the α_1-agonist phenylephrine was less potent than noradrenaline, while there was some variation seen between studies in the rank order of the α_2-agonists used. De Mey and Vanhoutte (1981) used the α_2-agonist clonidine and found it only slightly less potent than noradrenaline. In contrast, Alabaster at al (1985) found the α_2-agonist they chose (UK14304) to be more potent than either noradrenaline or phenylephrine.

In agreement with the studies mentioned above, I also found noradrenaline to be more potent than phenylephrine. The α_2-agonist UK14304 was of intermediate potency to phenylephrine and noradrenaline, being more potent than the former and less potent than the latter. As for the other studies cited, phenylephrine and noradrenaline were full agonists in this tissue, while UK14304 only achieved about 50% of the response produced by these two agonists. Another interesting finding was the significant

difference in the Hill slope parameters between agonists. A Bonferroni post test showed that this was due to the shallowness of the UK14304 curve. The phenylephrine curve was also significantly steeper than the noradrenaline curve. This has previously been observed by Guimaraes and Paiva (1987). The steepness of the curves to both the Abbott compound and phenylephrine may indicate that these agonists are more likely to be activating a single population of receptors as opposed to either noradrenaline or UK 14304 (Table 3.1), since shallower curves can be due to an interaction of the agonist with more than one receptor population.

In addition to the agonists already mentioned, the Abbott compound (R) A-61603 was used. A-61603 was developed a number of years ago, but only since the recent discovery of α_1-adrenoceptor subtypes, has its potential as a subtype-selective agonist come to light.

Most currently available information on this substance has been published in a paper by Knepper (1995). Sensitivity (EC_{50} values) of this agonist was compared in a variety of tissue types (native subtypes) and cell lines, transfected with the three cloned α_1-subtypes (α_{1a}-, α_{1b}- and α_{1d}-). Included in the tissues selected were rat vas deferens and canine prostate. Although in the paper these were both classified as having α_{1A}-adrenoceptors, these tissues have both been described elsewhere in the literature as containing α_{1L}-adrenoceptors (Muramatsu et al. 1995). This is relevant to the vessels used in this study because of their low affinity to prazosin which is discussed later. Knepper shows A-61603 to be 35-150 fold more potent at α_{1A}-adrenoceptors compared to either α_{1B}- or α_{1D}-.

Comparisons were also made between A-61603 and different agonists at the three native subtypes. At canine prostate strips A-61603 was 165 fold more potent than phenylephrine and 128 fold more potent than noradrenaline. At the α_{1B}-adrenoceptor in

rat spleen, A-61603 was 40 fold more potent than phenylephrine. In contrast, at α_{1D}-adrenoceptor sites in rat aorta, the compound was less potent than phenylephrine. It should however be noted, that in the present study, the R-enantiomer rather than the racemic mix was used. The R-enantiomer appears to confer potency to the compound. Knepper also quotes figures for (R) A-61603 at the canine prostate, showing a 590 fold greater potency than phenylephrine and a 460 fold greater potency than noradrenaline. These figures are considerably higher than those obtained in this study. Unfortunately, no values are available for (R) A-61603 at the other α_1-subtypes.

To summarise the results of the agonist studies, the rank order of potency of the agonists used would support the presence of both post-junctional α_1- and α_2-adrenoceptors (Wright et al. 1995). The Abbott compound was the most potent of the agonists used. This would support the view that the $\alpha_{1A/L}$-adrenoceptor is involved. Its relative potency in relation to phenylephrine, while much lower when compared to Knepper's study, for $\alpha_{1A/L}$- adrenoceptors, could be explained by the coexistence of another receptor subtype where the compound does not show the same order of magnitude in the increased sensitivity over phenylephrine. The discrepancies between studies, of the potency of UK14304 compared to noradrenaline could be due to the relative proportion of α_1- and α_2- adrenoceptors. It has been demonstrated that the density of α_2-adrenoceptors increases from the distal to the proximal portion of the DSV (Guimaraes and Nunes, 1990; Guimaraes et al. 1991; Pereira et al. 1991). According to the diagrams drawn by Guimaraes and Nunes (1990), the section of DSV used in this study was intermediate. In many of the other studies the exact region of vein used is not clear, thus anatomical location of the segment of vessel used may explain some of the variations seen between studies.

Although not addressed in this study, it is worth mentioning, that it has been suggested that in some vessels, UK 14304 may act as an agonist at α_1-adrenoceptors (Nagadeh et al. 1994). This could explain the two site fit seen for UK 14304 in several of the DSV rings (Figures 3.2 and 3.4.) and the shallowness of the Hill slopes seen in this study (Table 3.1).

In addition to agonists, a selection of α_1-adrenoceptor antagonists was used in order to functionally classify the subtype of α_1-adrenoceptor responsible for contraction of this vessel to exogenous noradrenaline. The current concepts and nomenclature for classification of α_1-adrenoceptors have already been discussed in the introduction in Chapter 1. The α_1-antagonist prazosin, was used in addition to the following putative subtype-selective antagonists: The α_{1A}-selective 5 methylurapidil (Hanft and Gross, 1989; Graziadei et al. 1989; Gross et al. 1989; Gross et al. 1988); the α_{1D}-selective BMY 7378 (Goetz et al. 1995); HV 723 to distinguish α_{1L}- and α_{1N}- (Oshita et al. 1988; Muramatsu et al. 1995); the $\alpha_{1A/D}$-selective WB 4101 (Kenny et al. 1995; Morrow and Creese, 1986). In addition, the irreversible alkylating agent CEC was used to determine the presence of α_{1B}-adrenoceptors (Michel et al. 1993; Gross et al. 1989; Minneman et al. 1988; Minneman, 1988; Han et al. 1987b).

A number of studies have already tried to classify α_1-adrenoceptors in the DSV. From functional studies the one common finding has been the low affinity for prazosin (pK_B or pA_2 values less than 9), (Alabaster et al. 1985; Sullivan and Drew, 1980; De Mey and Vanhoutte, 1981; Guimaraes and Nunes, 1990; Flavahan and Vanhoutte, 1986b; Flavahan et al. 1984). Beyond this, the subtype(s) involved are still unresolved. Guimaraes et al (1991), suggested that there was more than one subtype, with the proximal part of the vessel being α_{1A}- due to the high potency of WB4101, and distally α_{1B}-, because WB4101 was less potent in this region but the effect of the agonist

phenylephrine was greater. Hicks et al (1991) concluded on the presence of the α_{1A}- in the DSV, again based on the high affinity to WB4101. They also felt that there was an additional subtype because of the non-competitive action of some of the antagonists on contractions to phenylephrine. Although it has been suggested that phenylephrine, especially at high agonist concentrations, may mediate its action through α_2-adrenoceptors in the DSV and human vessels (Hair et al. 1988; Hicks et al. 1991; Guimaraes et al. 1987), the relative insensitivity of phenylephrine contractions to calcium channel blockers compared to the α_2-agonist BHT 920 and the lower potency of rauwolscine against phenylephrine induced contractions compared to other α_2-adrenoceptor agonists, has been used to argue that this is unlikely. The lack of effect of the selective α_2-antagonist delequamine, on contractions to phenylephrine in this study would support this latter view.

Daniel et al (1996) also found a high potency for WB4101 (pK_B 8.3), but because of the low affinity for 5 methylurapidil (pK_B 6.2), concluded that this indicated the presence of an α_{1D}- rather than an α_{1A}- receptor subtype. Both these antagonists seemed to act competitively. However, only a narrow concentration range was explored. Potency of prazosin was also low and varied depending on the concentration used from a pK_B of 6.69 to 7.76. It was concluded that the subtype involved was similar to the α_{1D}-, but was atypical, because the sites seemed to be somewhat sensitive to CEC and rauwolscine.

Findings from this study are summarised in Tables 3.2 to 3.4 and were somewhat interesting when comparisons were made between experiments carried out in the presence of rauwolscine with those conducted in the presence of delequamine. In general, there appeared to be a lowering of antagonist affinities when rauwolscine rather than delequamine was used (as already mentioned, Kenakin (1982) describes this effect

when an antagonist interferes with the receptor population being studied). In addition to this, the antagonists most dramatically affected by the use of one or other α_2-antagonist were prazosin, 5 methylurapidil and WB 4101. Prazosin in the presence of rauwolscine had a very low affinity and Schild regression was suggestive of a non-competitive interaction due to a slope significantly different from negative unity. In contrast, the interaction became competitive and the affinity much higher when delequamine was used. It is not clear as to why the interaction should have been non-competitive with rauwolscine, but this is possibly just a reflection of the very low affinity of this ligand for the receptors remaining unaffected by rauwolscine. What is clear, is that rauwolscine is interacting at prazosin sensitive sites in DSV. 5 methylurapidil also had a very low affinity in the presence of rauwolscine. When delequamine was used, 5 methylurapidil became much more potent and the highest concentration used appeared to unmask a high and a low affinity site. Interestingly, the low affinity site yielded a pA_2 of 6.29 ± 0.28 which was similar to a pA_2 obtained with the same concentration in the presence of rauwolscine (6.00 ± 0.77). This would seem to suggest that rauwolscine, in addition to interacting with prazosin sensitive sites, also interacts with high affinity sites identified by 5 methylurapidil, but not with 5 methylurapidil low affinity sites in the DSV. Finally, WB 4101 in the presence of rauwolscine, had a low potency and fulfilled the criteria for a competitive interaction. In the presence of delequamine, the interaction was not strictly competitive due to a decrease in maximum with the highest concentration of antagonist. To summarise, rauwolscine would appear to interact with α_1-adrenoceptors which are prazosin-sensitive, 5 methylurapidil-sensitive and WB 4101-sensitive.

Focusing solely on results obtained in the presence of delequamine, prazosin interacted in a competitive fashion with a low affinity (pA_2 of 8.31) as seen in other studies. The

interaction with HV723 was not strictly competitive as the antagonist altered the slope of the concentration response curves. However, Schild analysis produced a pA_2 of 8.98. From these two antagonists alone, the subtype of adrenoceptor could be classified according to Muramatsu's scheme (Muramatsu et al. 1990b; Muramatsu et al. 1995) as an α_{1L}-. The interaction with WB4101 was not strictly competitive as the highest concentration caused a decrease in the maximum value. PA_2 values calculated from individual antagonist concentrations ranged from 8.81 ± 0.18 with the lowest concentration, to 8.62 ± 0.34 with the highest concentration but were not significantly different across the range of antagonist concentrations used. The lower figure was comparable to data from Muramatsu et al (1990b) (pA_2 8.58 ± 0.10) and the higher value similar to a pA_2 of 8.9 ± 0.1 from human lower urinary tract classified as α_{1L}- (Ford et al. 1996b).

The α_{1D}-antagonist BMY 7378 (Goetz et al. 1995) interacted non-competitively. A pA_2 value calculated from the lowest concentration used was 8.43 ± 0.2. This could support the presence of the α_{1D}-adrenoceptor since in rat aorta, now generally accepted to possess this subtype, values of 8.9 ± 0.1 have been obtained (Goetz et al. 1995). With increasing concentrations of this antagonist the pA_2 values fell significantly giving a pA_2 of 7.04 ± 0.12 with the antagonist concentration of 0.1μM, which would be more in line with the affinity of this antagonist at either the α_{1A}- or α_{1B}-adrenoceptor.

Finally, 5 methylurapidil had the most complex effect in this vessel. The interaction was non-competitive. Experiments using the highest concentration either caused a decrease in the maximum and fitted to a one site model, or fitted to a two site model. PA_2 values calculated from individual concentrations were not significantly different with the exception of the low affinity site identified with 1μM 5MeU. Interestingly the

pA_2 value of 6.29 ± 0.28 from this low affinity site was similar to the low affinity obtained in the DSV by Daniel et al. (1996).

Interpretation of the results from CEC was also complicated as this antagonist, at the highest concentrations used, caused an irreversible baseline contraction in the presence of rauwolscine and delequamine. CEC normally has been considered as an irreversible antagonist which has some affinity for all subtypes of α_1-adrenoceptor but most strongly irreversibly alkylates the α_{1B}- subtype (Muramatsu et al. 1995; Schwinn et al. 1995; Forray et al. 1994a; Perez et al. 1991) and has least effect on the α_{1A}- and α_{1L}- subtypes. It has also been suggested that in the rat aorta, CEC acts as an agonist at α_2-adrenoceptors, although in this case no direct contractile action of CEC is seen (Docherty and O'Rourke, 1997; O'Rourke et al. 1995). The contraction caused by CEC in the DSV has been well documented. Nunes and Guimaraes (1993) thought that this action was mediated by α_2-adrenoceptors because of the sensitivity of this action to rauwolscine. Low et al (1994) also studied this effect of CEC on the DSV. They too found that CEC acted through rauwolscine sensitive sites and to a lesser extent prazosin sensitive sites. Nunes and Mota (1994) showed that this action was mediated via a protein kinase C-dependent mechanism which would fit in with the findings of Low et al (1994) where rauwolscine blocked the release of internal stores of calcium and calcium influx induced by CEC. These latter findings were therefore interpreted as suggesting that the rauwolscine sensitive sites involved in the baseline contraction seen with CEC were α_1- rather than α_2-adrenoceptors, a conclusion that ties in well with the findings from the present study and that of Daniel (1996). The sensitivity of CEC's baseline contraction to rauwolscine is also supported in part by findings in the present study. When the antagonist studies were performed in the presence of 1μM rauwolscine, a baseline contraction of 12.92 ± 4.09% (n = 4) was observed. This was

significantly lower when compared to a baseline contraction of 37.86 ± 6.38% (n = 4), seen when delequamine was the α_2-antagonist used. This suggests that in effect, rauwolscine is protecting the receptor sites that contribute to the CEC induced contraction in this vessel. Only the highest concentration of CEC (100μM) caused a significant shift in the pEC_{50} value and a decrease in the maximum of the NA CRC. From the literature, interpretation of our findings with CEC is difficult because of the subjectivity involved and the wide variety of protocols used. My feeling is that the interaction of CEC with this vessel is two fold. The baseline contraction seems, in part at least, to be caused by rauwolscine sensitive α_1-adrenoceptors. Looking at the difference in the competitive antagonists when either rauwolscine or delequamine was used, these rauwolscine sensitive sites are α_{1A}- or α_{1L}- like receptors. Secondly, the shift to the right with depression of the maximum seen with the highest concentration of CEC would support the presence of α_{1B}-, or α_{1D}-adrenoceptors.

To summarise, the findings from this study confirm the presence of α_1- and α_2-adrenoceptors. The Abbott compound was the most potent of the agonists used. Although the Abbott compound's relative potency was lower than that stated for α_{1A}-\$\alpha_{1L}$-adrenoceptors in Knepper's study (1995), the possible existence of a heterogeneous population of α_1-adrenoceptors, which may include the α_{1A}-\$\alpha_{1L}$- adrenoceptor subtype, could account for a lowering of the potency of this agonist.

The potencies of prazosin and HV723 were compatible with the presence of the α_{1L}-subtype. The potencies of WB4101 and 5 methylurapidil were also comparable to values obtained at tissues thought to possess both the α_{1L}-subtype and the α_{1A}- subtype (Table 3.5). In a number of vessels, 1μM 5 methylurapidil seemed to uncover a low and a high affinity site. This together with the sensitivity to CEC and the non-competitive interaction of BMY 7378, may indicate the presence of a second α_1- subtype. The

additional subtype has similarities to the α_{1D}-adrenoceptor due to the relatively high affinity of 1nM BMY 7378 and due to the sensitivity to 100μM CEC.

It therefore seems fairly clear that the α_{1L}-adrenoceptor is involved in vascular smooth muscle contraction of this vessel. The results are also convincing with regards involvement of an additional subtype. Due to the low affinity of prazosin and since currently there are no further subdivisions of the α_{1L}-subtype, while it can be said that the additional subtype has some characteristics of the α_{1D}-adrenoceptor, at present it can not be satisfactorily classified. Since all the antagonist studies were performed in the presence of noradrenaline it would be of value to repeat these experiments using the subtype selective agonist (R) A-61603 in an attempt to obtain pA_2 values for these antagonists at a pure receptor population.

CHAPTER 4

Functional classification of α_1-adrenoceptors mediating contraction of canine subcutaneous resistance arteries to exogenous noradrenaline

4.0. Abstract

4.1 Methods

4.1.1 Agonist studies

4.1.2 Competitive antagonists

4.1.3 Non-competitive antagonists

4.2 Results

4.2.1 Agonist profile

4.2.2 Competitive antagonists

4.2.3 Non-competitive antagonists

4.3. Discussion

4.0 Abstract

The aim of this study was to functionally classify the subtype(s) of α_1-adrenoceptor mediating contraction of dog resistance arteries to exogenous noradrenaline.

Agonist profiles to noradrenaline, phenylephrine, (R) A-61603 and UK14304, suggested that the responses were predominantly mediated by a population of post-junctional α_1-adrenoceptors.

The reversible competitive antagonists prazosin, WB 4101, HV 723, BMY 7378 and 5 methylurapidil were used. As for the DSV, the low potency of prazosin and HV723 suggested the presence of the α_{1L}- adrenoceptor subtype.

The actions of chloroethylclonidine in the resistance arteries, were dissimilar to those in the dog saphenous vein, in that the baseline contraction caused by chloroethylclonidine was not a consistent feature in resistance vessels. However, the highest concentration used, although causing no significant shift in the pEC_{50} value compared to control, did cause a decrease in the maximum.

The sensitivity to 100μM chloroethylclonidine, together with significant decreases in the upper asymptotes of concentration response curve data, in the presence of all competitive antagonists, with the exception of BMY 7378, may indicate the presence of additional α_1-adrenoceptor subtypes. The degree of sensitivity to CEC could support the involvement of an α_{1B}- or an α_{1D}-like receptor. The declining pA_2 values for BMY 7378, seen with increasing antagonist concentration, would further support the involvement of the α_{1D}-adrenoceptor. However, the low potency of prazosin, within the current classification scheme, makes it impossible to classify additional subtypes involved in the response.

4.1 Methods

Canine subcutaneous resistance arteries, approximately 2mm in length and 255 ± 7 μm in diameter (n = 89) were dissected and mounted in Mulvany Halpern wire myographs and normalised to 0.9 of L100 as described in materials and methods, section 2.2.3. After normalisation, vessels were maintained in blockers Krebs' solution for the rest of the experiment. Following the normalisation procedure, vessels were allowed a forty minute equilibration period, followed by the starting protocol as outlined in materials and methods section 2.3. Agonist profiles and antagonist studies were performed in these vessels as for the dog saphenous vein. n = the number of experiments unless otherwise stated.

4.1.1 Agonist studies

Three consecutive concentration response curves (CRC), each to a different agonist, were performed on each ring. The agonists used were noradrenaline (NA), phenylephrine (PE) and either (R) A-61603 or UK14304. A forty minute recovery period was allowed between each concentration response curve. Cumulative concentration response curves were performed using half log increments with the following concentration ranges: Noradrenaline (NA) and phenylephrine (PE), starting at a concentration of 1nM and increasing up to a maximum of 1mM if required. (R) A-61603 (Abbott compound) starting with a concentration of 0.3nM and increasing up to a concentration of 30μM if required. UK14304 starting with a concentration of 1nM and increasing up to a concentration of 0.1μM if required. Responses were allowed to reach a plateau before subsequent concentrations of agonist were added to the bath. Responses were expressed as a percentage of the 10μM NA siting concentration and

plotted in logarithmic space. No α_2-antagonists were present during the agonist profile experiments.

4.1.2 Competitive antagonists

In addition to the blocking agents already present in the blockers Krebs', 0.1μM of the α_{2}-adrenoceptor antagonist, RS-15385-197 (delequamine) was present in the Krebs' solution at all times. For each experiment four vessel rings were set up in parallel. One ring was assigned as a time control and each other ring was assigned one of five antagonists. Up to four consecutive cumulative concentration response curves to NA were performed in each ring. For curve one, no antagonist was added to the bath. For each subsequent curve, an increasing concentration of the antagonist assigned to each ring was added to the bath, with the exception of the time control. Antagonists were allowed a forty minute incubation period with the vessel before the next concentration response curve was performed. Experiments where there was a significant change in the maximum or pEC_{50} of the time control, as judged by one way ANOVA, were excluded from the study. A P value < 0.05 was judged to be statistically significant. Time control data is shown in Figure 4.1 where data was expressed as a percentage of the maximum achieved in the first curve. The antagonists used were prazosin, BMY 7378, 5 methylurapidil, WB 4101 and HV 723.

4.1.3 Irreversible antagonists

Again four rings were mounted in parallel with one ring assigned as a time control. 0.1μM delequamine was present in the bath at all times. A CRC to noradrenaline was performed in all rings. With the exception of the time control, either 0.1μM, 1μM or 100μM of the irreversible antagonist chloroethylclonidine (CEC) was added to the bath.

CEC was incubated for one hour and then vessels were washed out 10 times over a forty minute period. A second CRC to NA was then performed in all rings.

4.2 Results

Initially, as for the dog saphenous vein, individual concentration response curve data was analysed using the graph fitting program GraphPad Prism 2.1. It soon became clear that much of the CRC curve data did not conform consistently to either the one or two site model described in Chapter 2, section 2.4. and that there seemed to be no pattern regarding the ability to place data into the category of one site fit, two site fit or no appropriate fit. One obvious problem seemed to be the lack of data points in the lower half of the curve. An attempt to solve this problem was made by using quarter log, instead of half log increments for the concentration response curves. This did not improve the fitting. A number of figures have been inserted to try and demonstrate the problem. Figure 4.2 illustrates two concentration response curves from single vessels, one to PE and the other to NA in the presence of blockers Krebs', without RS-15385-197. Both are fitted to the one site model and it can be seen that the points on the upper part of both curves do not fit satisfactorily. Figure 4.3 again illustrates data from a single experiment, this time with increasing concentrations of prazosin. Experiments were conducted in blockers Krebs' and RS-15385-197. As for Figure 4.2, it is clear that the curves are not described by the model. Figure 4.4 illustrates data from a single experiment this time in the presence of WB4101. The curve in the presence of 0.1 μM WB 4101 did fit to the two site model, but the control and the other concentrations of WB 4101 fitted to neither the one or two site models.

Data was therefore analysed on Microsoft Excel spreadsheets and the pEC_{50} values derived by interpolation rather than curve fitting. The result of this was, that while it was possible to obtain maximum and pEC_{50} data for each experiment, it was not possible to assess midpoint slope parameters.

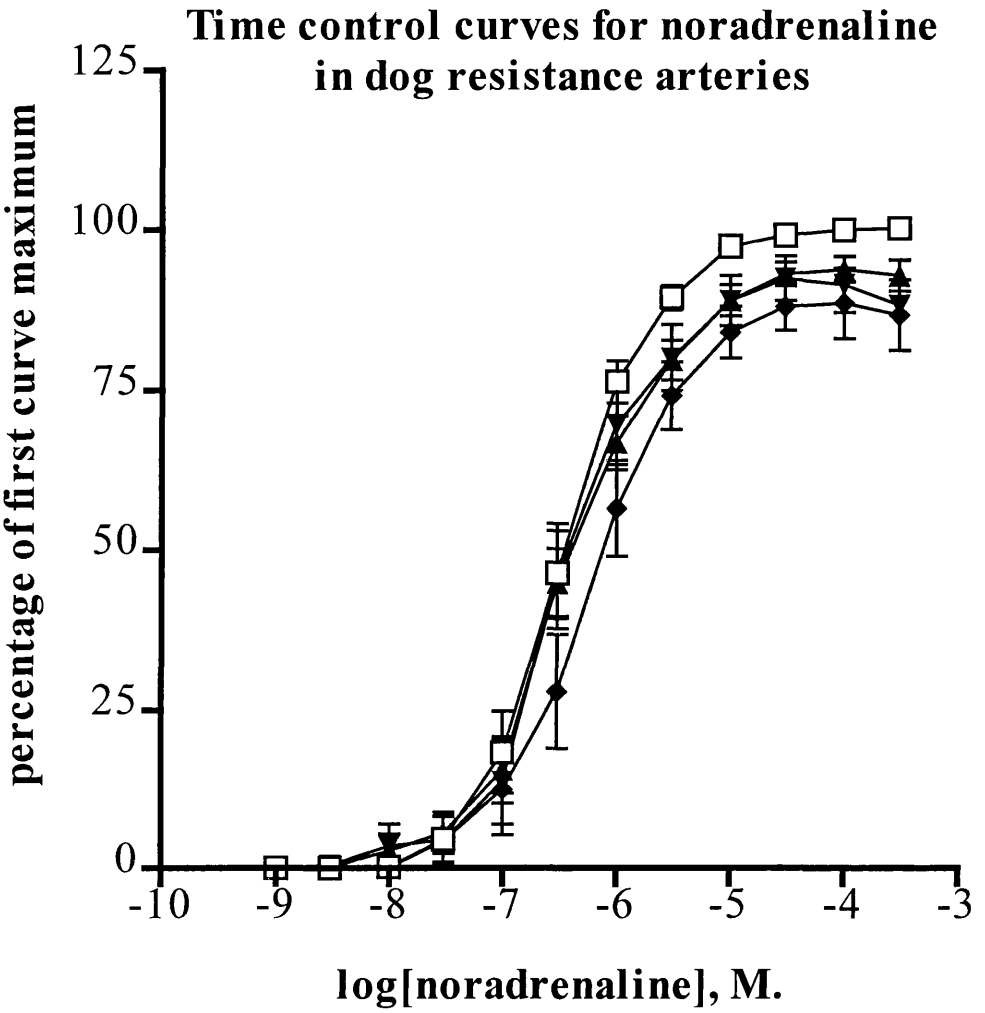


Figure 4.1. Time control concentration response curves to noradrenaline in canine resistance arteries. Data points represent mean ± s.e. mean and are expressed as a percentage of curve one. Curve 1 (□), $n = 21$; curve 2 (▲), $n = 21$; curve 3 (▼), $n = 12$; curve 4 (◆), $n = 10$.

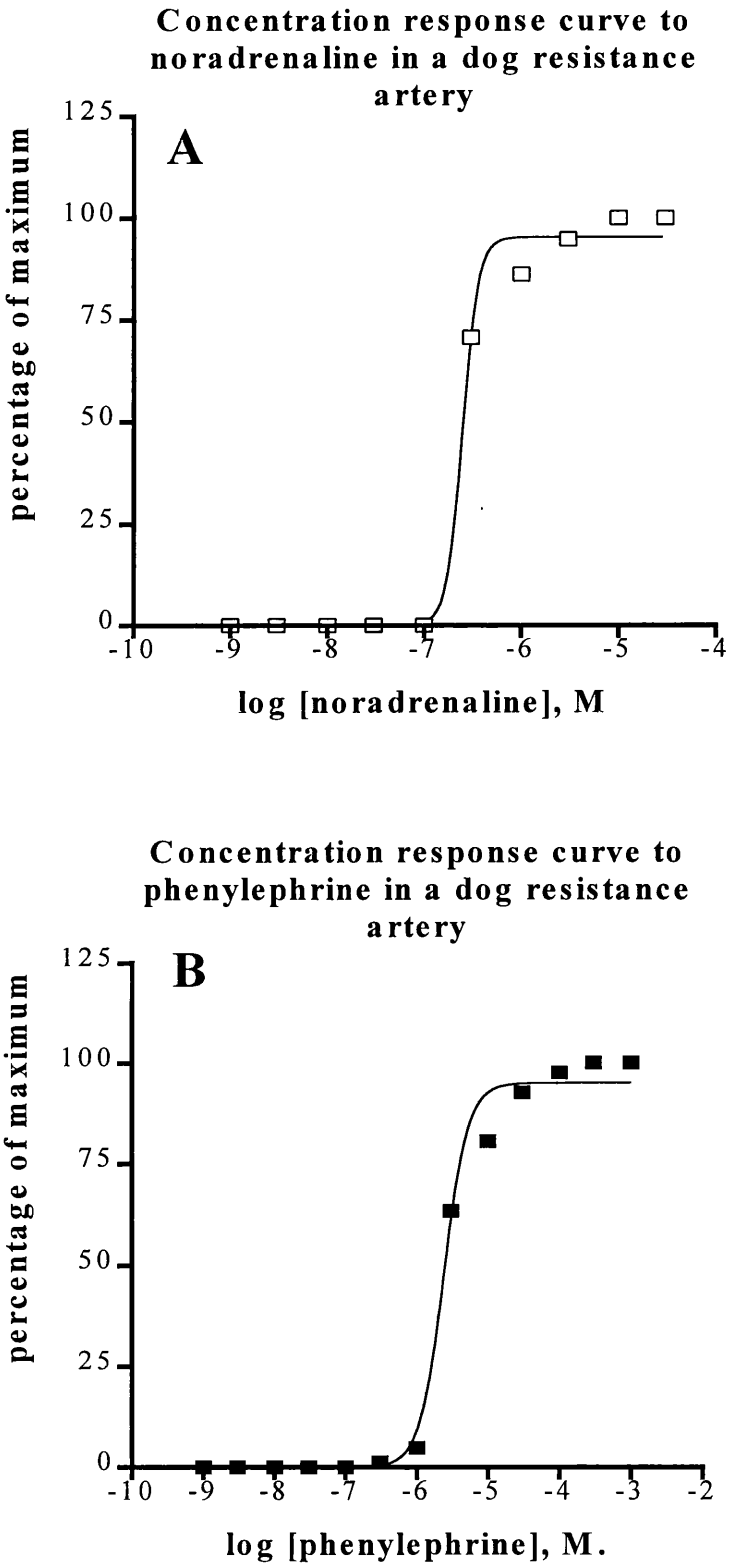


Figure 4.2. Graphs illustrating individual experiments in dog resistance arteries fitted to a one site model. Graph A shows a CRC curve to NA from a single experiment. Graph B shows a CRC curve to PE from a single experiment.

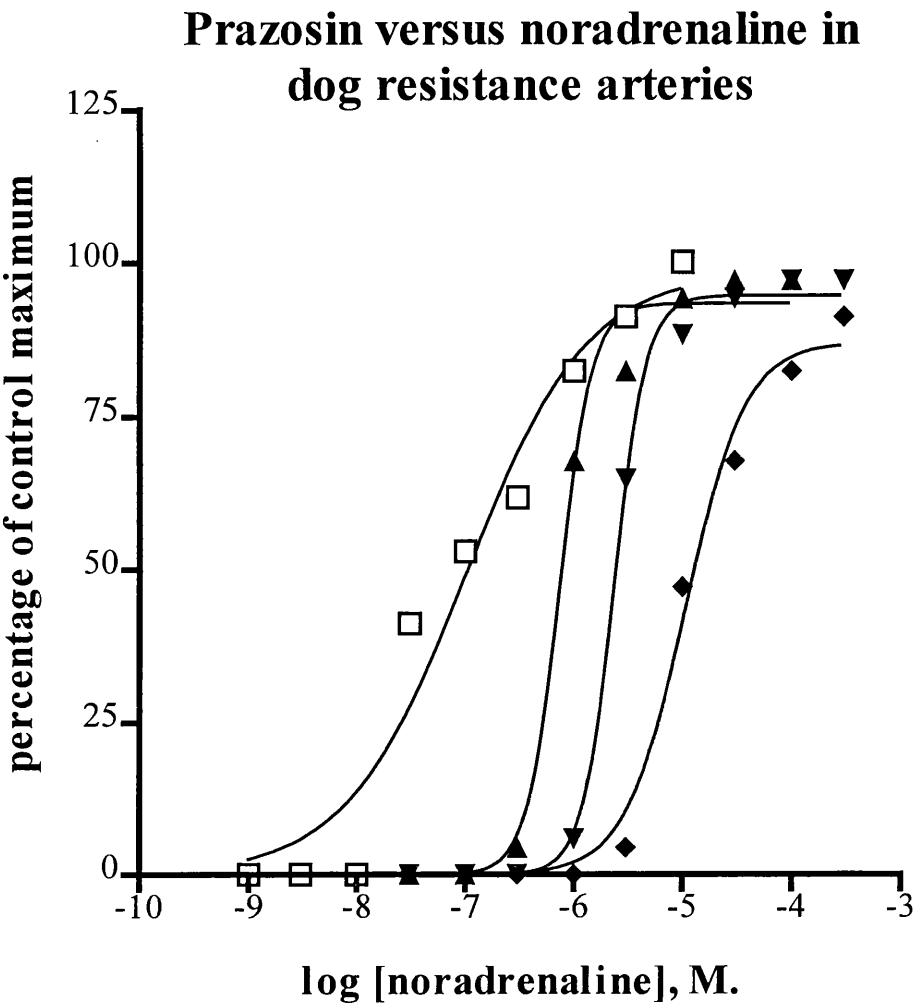


Figure 4.3. Increasing concentrations of prazosin versus noradrenaline from a single dog resistance artery fitted to the one site model. Control (□) ; 1nM prazosin (▲); 10nM prazosin (▼) ; 0.1μM prazosin (◆). Results are expressed as a percentage of the control curve maximum.

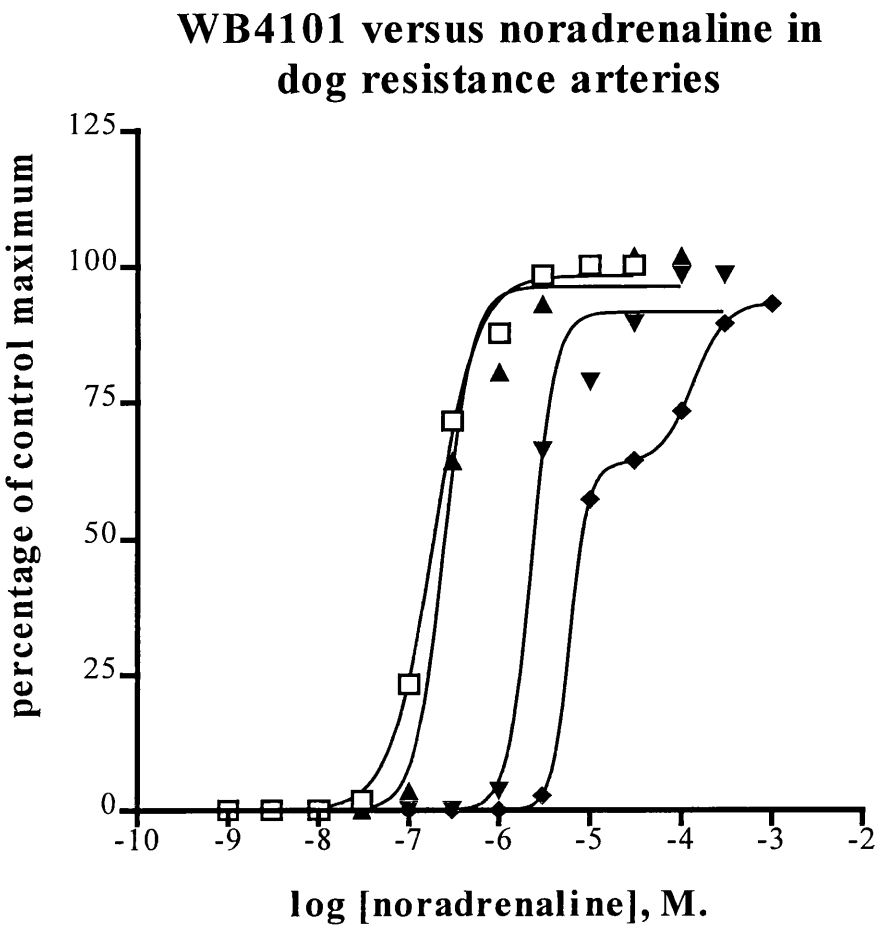


Figure 4.4. Increasing concentrations of WB 4101 versus noradrenaline from a single dog resistance artery. Control (□) ; 1nM WB 4101 one site fit (▲); 10nM WB 4101 one site fit (▼); 0.1μM WB 4101 two site fit (◆). Results are expressed as a percentage of the control curve maximum.

4.2.1 Agonist profile

Data for each agonist was derived from at least six different animals with the exception of UK14304 where four were used. Results are expressed as a percentage of the 10μM siting concentration of noradrenaline. PE, NA and the Abbott compound caused concentration-dependent increases in tension in the resistance arteries. The response to UK14304 was variable, with one vessel not responding to this agonist at all. This latter point should be noted since the results from UK 14304 are derived only from the other three vessels which responded within the range of agonist concentrations used. The pEC_{50} values of the agonists used were as follows: Noradrenaline 6.46 ± 0.1, n = 8; phenylephrine 5.83 ± 0.09, n = 10; Abbott 7.88 ± 0.11, n = 6 ; UK 14304 7.29 ± 0.22, n = 3. This gave a rank order of potency of Abbott > UK14304 > NA > PE, with the Abbott compound being 27X more potent than NA and 112X more potent than PE. One way ANOVA of the maximum values from individual experiments showed that they were significantly different ($P < 0.0001$). A Bonferroni post test showed that the difference was due to the maximum for UK14304 being significantly lower (42.33 ± 11.3%). In other words, NA, PE and the Abbott compound acted as full agonists, while UK 14304 was only a partial agonist. Results for the agonist studies are summarised in Figure 4.5 and Table 4.1.

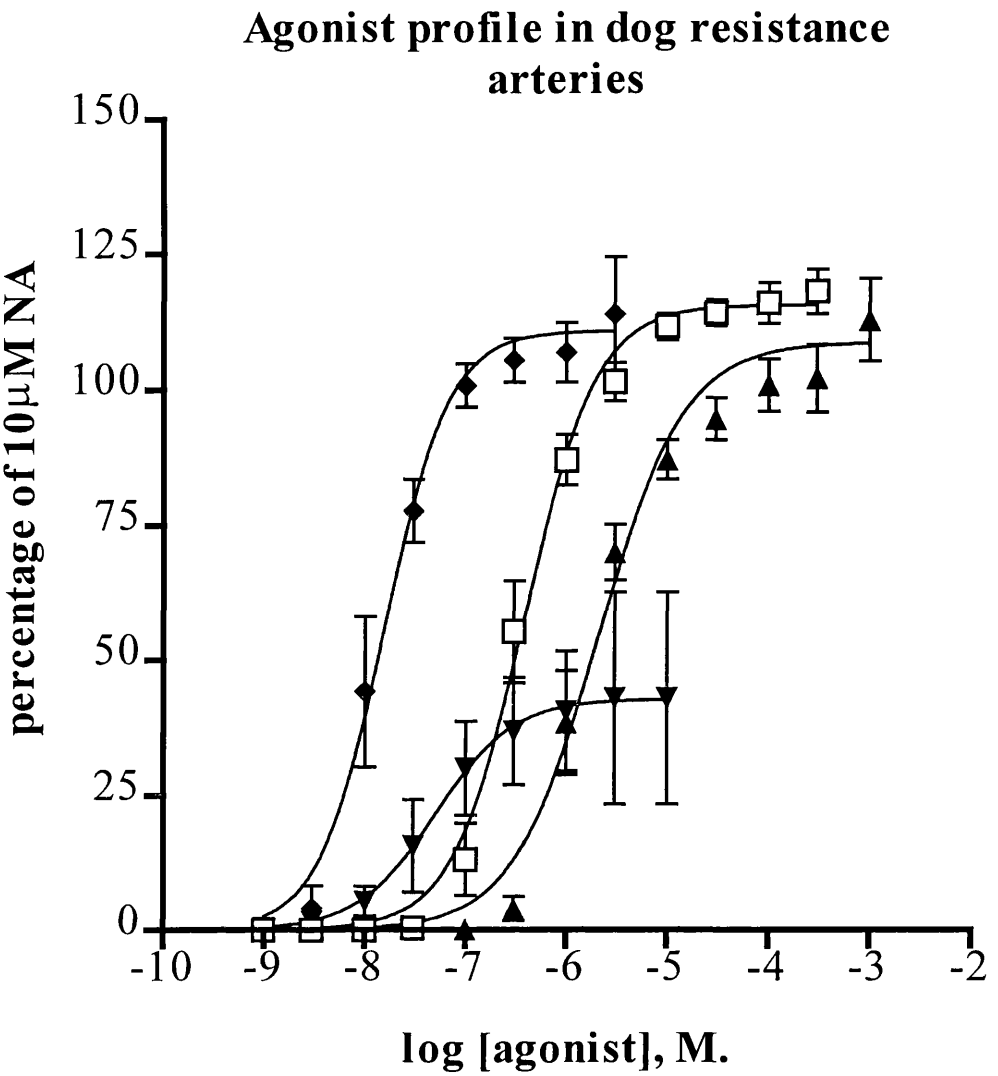


Figure 4.5. Agonist profile for canine resistance arteries. NA (□) $n = 8$; PE (▲) $n = 10$; Abbott (◆) $n = 6$; UK14304 (▼) $n = 3$. Points represent mean raw data ± s.e. mean. Raw data was derived by expressing the response as a percentage of the response to10μM noradrenaline.

Agonist	pEC_{50} ± s.e.mean (n)	maximum (%) ± s.e.mean
Noradrenaline	6.46 ± 0.1 (8)	114.9 ± 2.48 (8)
Abbott	7.88 ± 0.1 (6)	106.4 ± 4.52 (6)
Phenylephrine	5.83 ± 0.09 (10)	99.82 ± 4.71 (10)
UK14304	7.29 ± 0.22 (3)	42.33 ± 11.3 (3)

Table 4.1. Summary of agonist results in canine resistance arteries. Values are shown as means ± s.e. mean. Values in brackets represent the number of experiments. Maximum values are expressed as a percentage of the 10μM NA siting concentration response.

4.2.2 Competitive antagonists

Graphs illustrating the mean CRC data for the antagonists below are shown in Figures 4.6 to 4.10. Schild regressions are illustrated in Figures 4.11 to 4.14. Tables 4.2 and 4.3 summarise Schild regression parameters and pA_2 values.

The α_1-adrenoceptor antagonist prazosin, was used at concentrations of 1nM, 10nM and 0.1μM. Values for each concentration were derived from 6-8 different animals. This antagonist caused a concentration-dependent dextral shift in the noradrenaline concentration response curves. One way ANOVA showed that the pEC_{50} values were significantly different (P of 0.0005), while a Bonferroni post test showed that this was due only to the highest concentration of antagonist used. Maximum values were also (just) significantly different (P of 0.0419). The post test again showed that this was due to a significant decrease in the maximum only for the highest concentration of antagonist used. Schild regression yielded a slope not significantly different from negative unity and a pA_2 value of 8.36. pA_2 values calculated from single concentrations were not significantly different over the range of antagonist concentrations used. The pA_2 value calculated from the intermediate concentration was 8.21 ± 0.24 ($n = 8$).

The α_{1D}-selective antagonist BMY 7378, was used at concentrations of 1nM, 10nM and 0.1μM. Values were derived from 4-5 animals for each concentration. Increasing concentrations caused only marginal shifting of the concentration response curves to NA with no significant changes occurring in either the pEC_{50} or maximum values (P of 0.99 and 0.22 respectively). Due to the lack of effect of BMY 7378, many of the DR-1 values derived from the lowest concentration used were negative, not allowing log (DR-1) values to be derived for the Schild plot, and when Schild analysis was performed with the points obtained, the slope was significantly different from negative unity. PA_2

values derived over the range of antagonist concentrations used were significantly different, with a decline in the pA_2 value seen with increasing antagonist concentrations, supporting the findings from the Schild analysis. A pA_2 value derived from the highest concentration of antagonist used yielded a value of 6.51 ± 0.47 ($n = 4$).

The $\alpha_{1A/D}$- selective antagonist WB 4101 was used at concentrations of 1nM, 10nM and 0.1μM. Values for each concentration were derived from 5-6 animals. Increasing concentrations of antagonist caused concentration-dependent dextral shifts in the concentration response curves to NA. Maximum values were significantly different (P of 0.0018) and a post test showed that this was due to a significant decrease only in the highest concentration of antagonist used. pEC_{50} values were significantly different (P of <0.0001). A post test showed that this was due to a significant shift in the curves for 10nM and 0.1μM but not 1nM concentrations of antagonist. Schild regression yielded a slope not significantly different from negative unity, and a pA_2 value of 8.42. pA_2 values calculated for the three different antagonist concentrations were not significantly different and a pA_2 value of 8.82 ± 0.19 ($n = 6$) was derived from the 10nM concentration of WB 4101.

The antagonist HV 723, was used at concentrations of 1nM, 10nM and 1μM. Values were derived from 6-9 animals. The antagonist caused a concentration-dependent rightward displacement of the NA concentration response curves with a significant difference in the pEC_{50} values (P<0.0001). A post test showed that this difference was due to the two highest concentrations of antagonist only. Maximum values were also significantly different (P of 0.0051). This was due to a decrease in the maximum in the presence of 0.1μM HV 723 only. Schild regression produced a slope not significantly different from negative unity and a pA_2 value of 8.81. pA_2 values calculated for the

three concentrations used were not significantly different and a pA_2 value of 8.86 ± 0.29 (n = 9), was derived from the 10nM concentration of HV 723.

The α_{1A}-selective antagonist 5 methylurapidil (5 MeU), was used at concentrations of 10nM, 0.1μM and 1μM. Values were derived from 7-9 animals for each concentration. There was a concentration-dependent rightward displacement of the noradrenaline concentration response curves with a significant difference in the pEC_{50} values (P of < 0.0001) due to the two highest concentrations of antagonist used. Maximum values were also significantly different (P of < 0.0001), again due to a significant depression in the maximum in the presence of 1μM 5MeU. Schild regression yielded a slope not significantly different from negative unity and a pA_2 value of 8.08. pA_2 values calculated from each concentration used were not significantly different and a pA_2 value of 8.01 ± 0.18 (n = 9) was calculated from 0.1μM 5 methylurapidil.

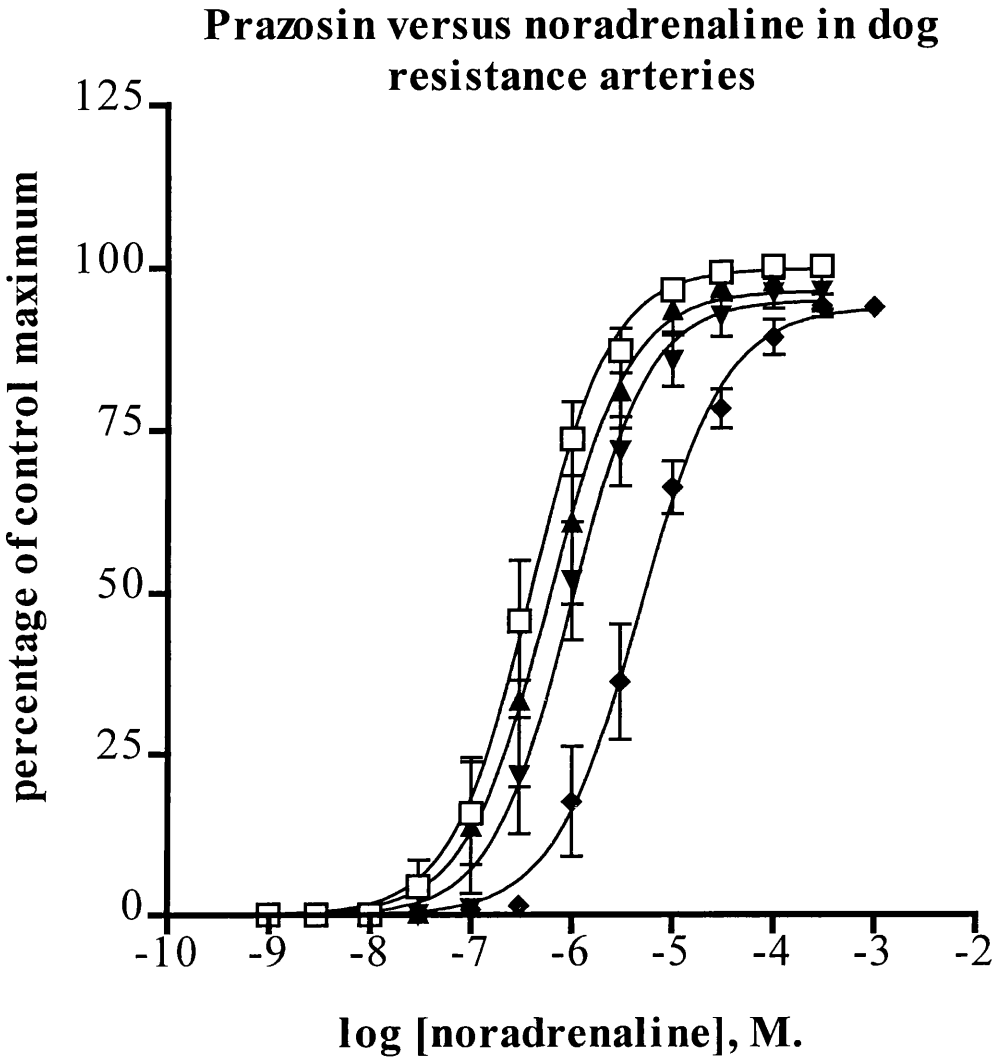


Figure 4.6. Mean CRC data for noradrenaline in the presence of prazosin in canine resistance arteries. Data points represent mean data ± s.e. mean. Responses are expressed as a percentage of the control curve maximum. Control (□), n = 10; 1nM prazosin (▲), n = 6; 10nM prazosin (▼), n = 8; 0.1μM prazosin (◆), n = 7.

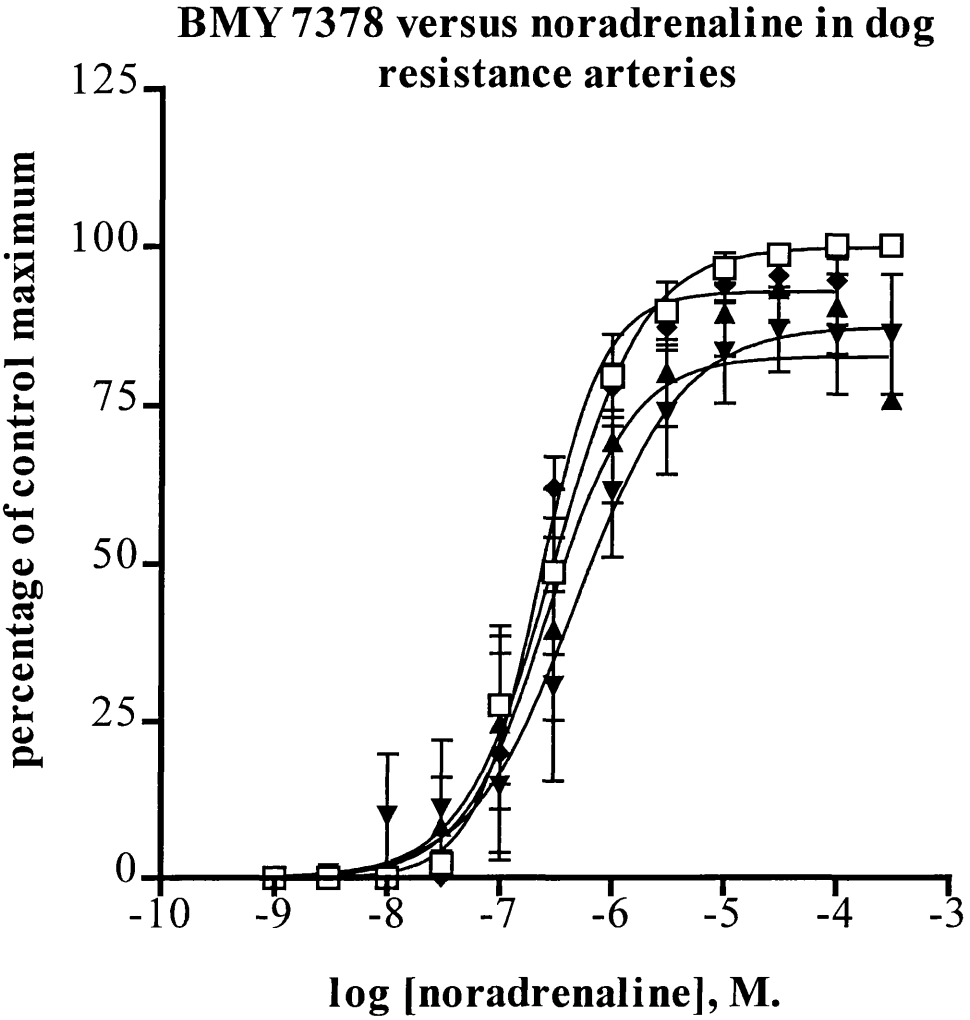


Figure 4.7. Mean CRC data for noradrenaline in the presence of BMY 7378 in canine resistance arteries. Data points represent mean data ± s.e. mean. Responses are expressed as a percentage of the control curve maximum. Control (□), *n* = 7; 1nM BMY 7378 (▲), *n* = 5; 10nM BMY 7378 (▼), *n* = 5; 0.1μM BMY 7378 (◆), *n* = 4.

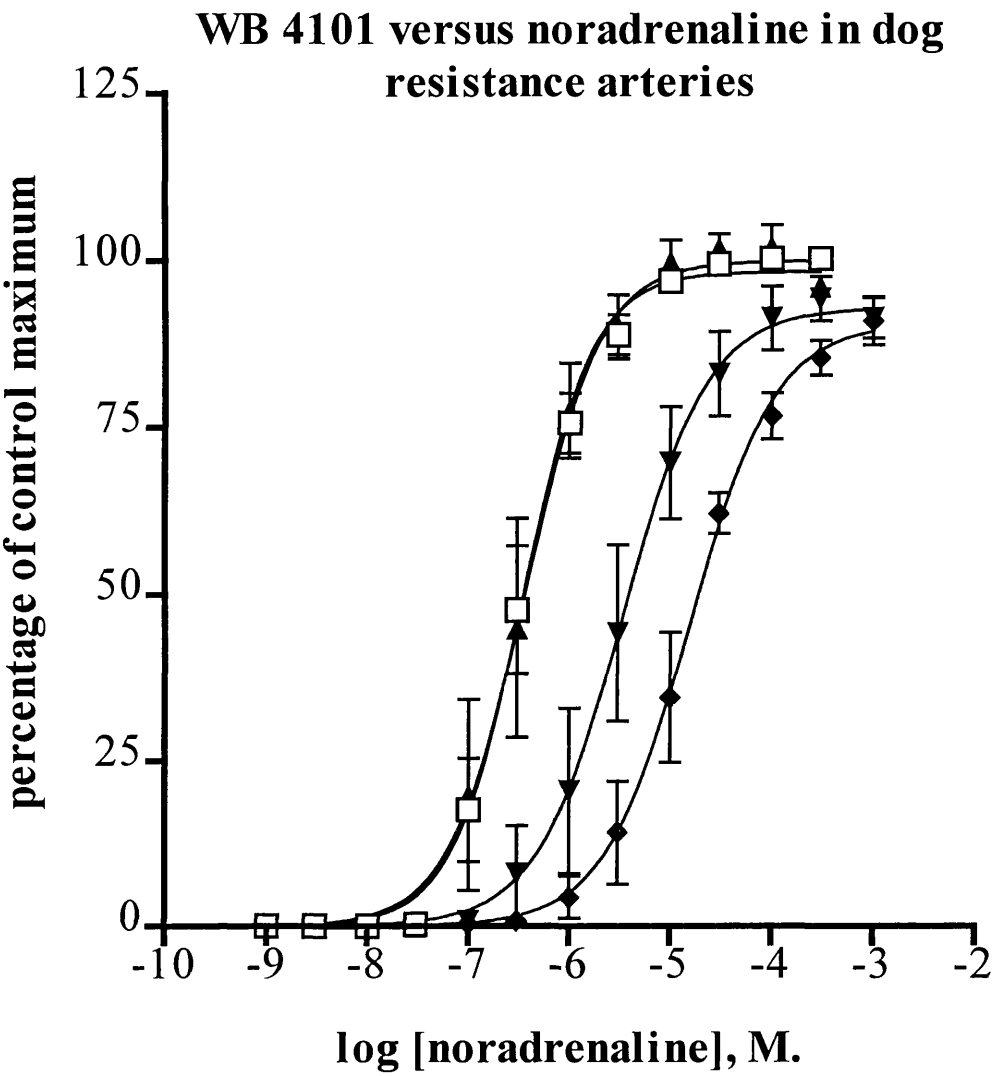


Figure 4.8. Mean CRC data for noradrenaline in the presence of WB 4101 in canine resistance arteries. Data points represent mean data ± s.e. mean. Responses are expressed as a percentage of the control curve maximum. Control (□), $n = 9$; 1nM WB 4101 (▲), $n = 5$; 10nM WB 4101 (▼), $n = 6$; 0.1μM WB 4101 (◆), $n = 6$.

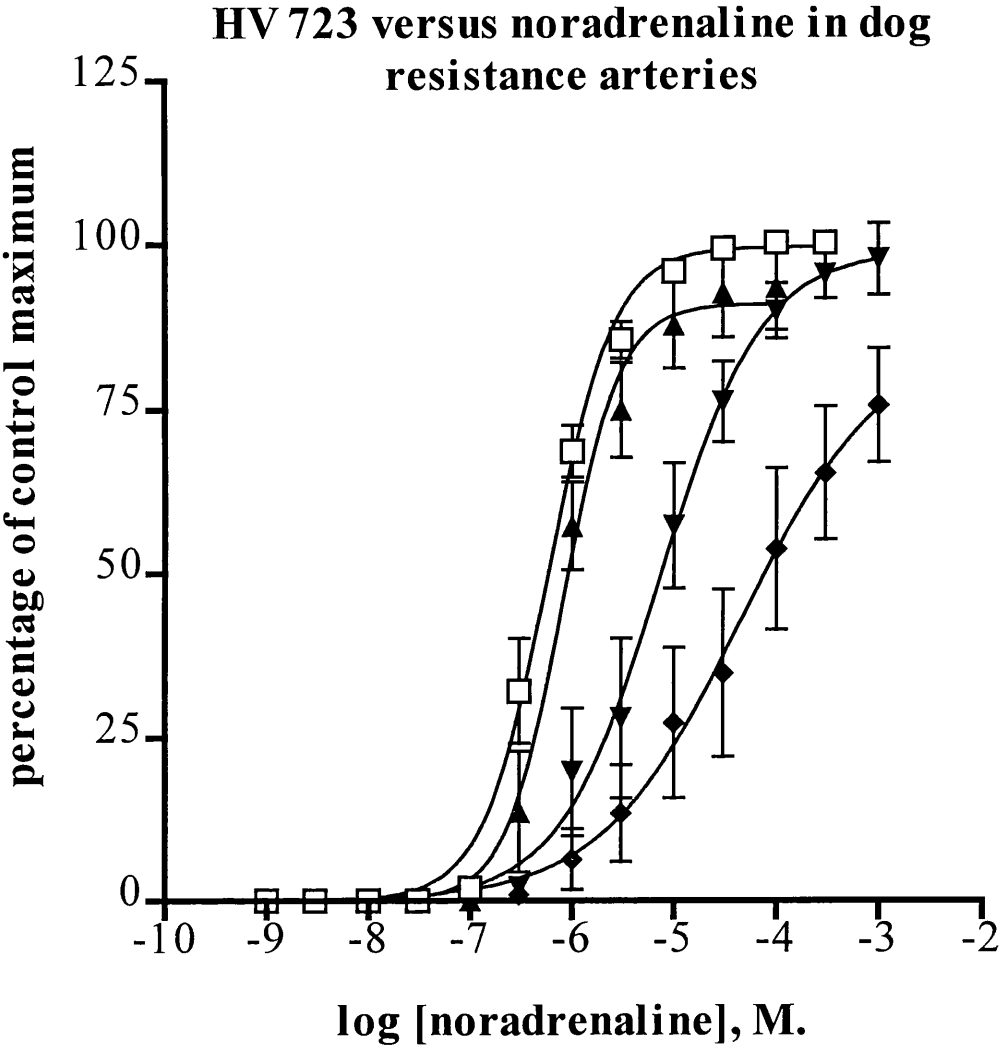


Figure 4.9. Mean CRC data for noradrenaline in the presence of HV 723 in canine resistance arteries. Data points represent mean data ± s.e. mean. Responses are expressed as a percentage of the control curve maximum. Control (□), $n = 11$; 1nM HV 723 (▲), $n = 6$; 10nM HV 723 (▼), $n = 9$; 0.1μM HV 723 (◆), $n = 7$.

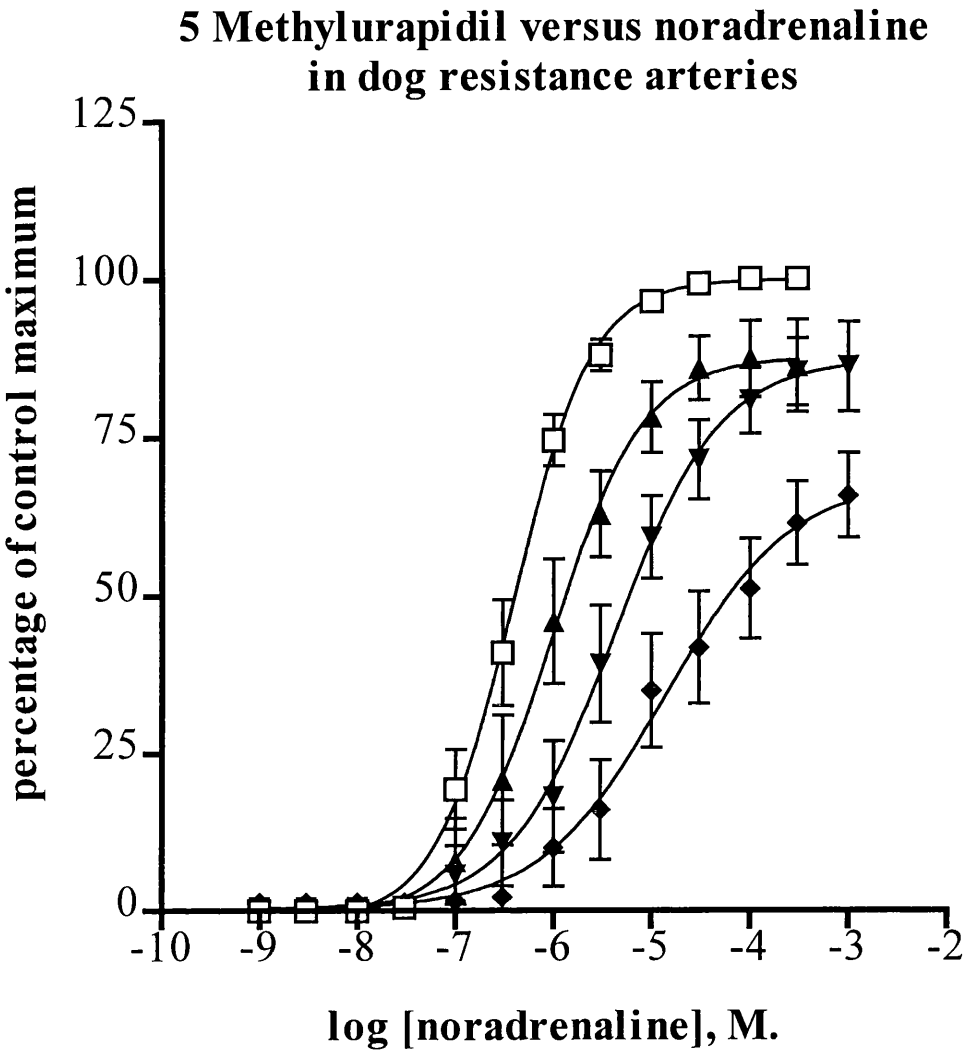


Figure 4.10. Mean CRC data for noradrenaline in the presence of 5 methylurapidil in canine resistance arteries. Data points represent mean data ± s.e. mean. Responses are expressed as a percentage of the control curve maximum. Control (□), n = 14; 1nM 5 MeU (▲), n = 7; 10nM 5 MeU (▼), n = 9; 0.1μM 5 MeU (◆), n = 9.

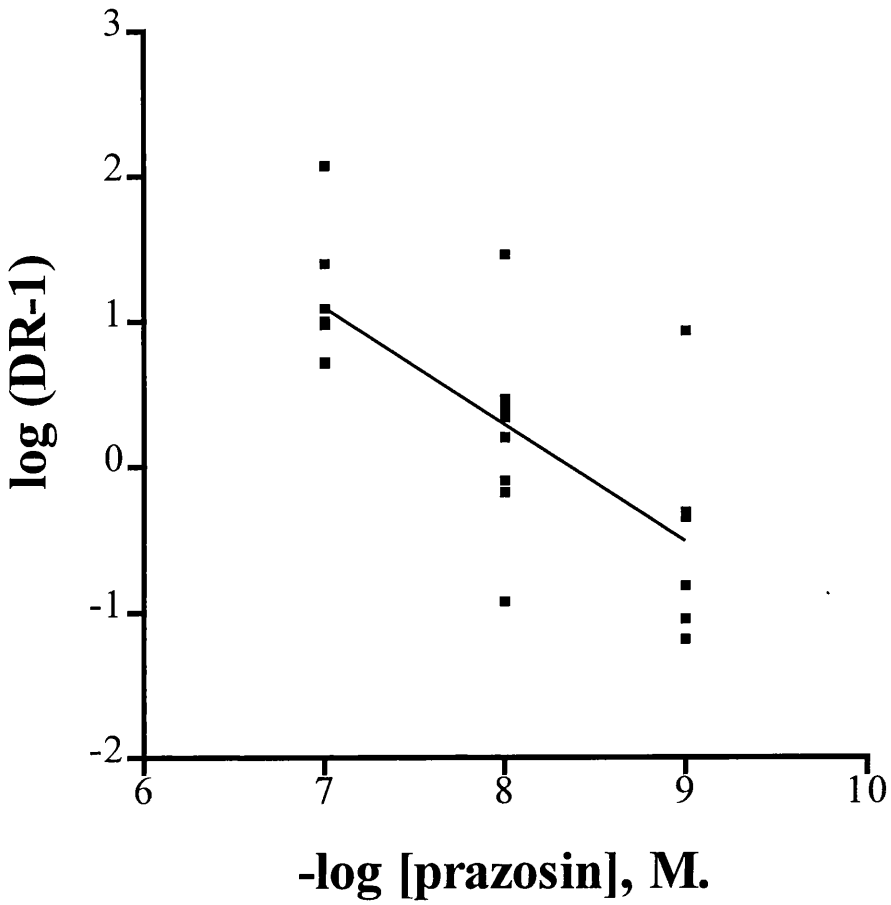


Figure 4.11 Schild plot for prazosin versus noradrenaline in canine resistance arteries. Points represent log (DR-1) values from individual experiments. The plot is composed of 21 data points in total.

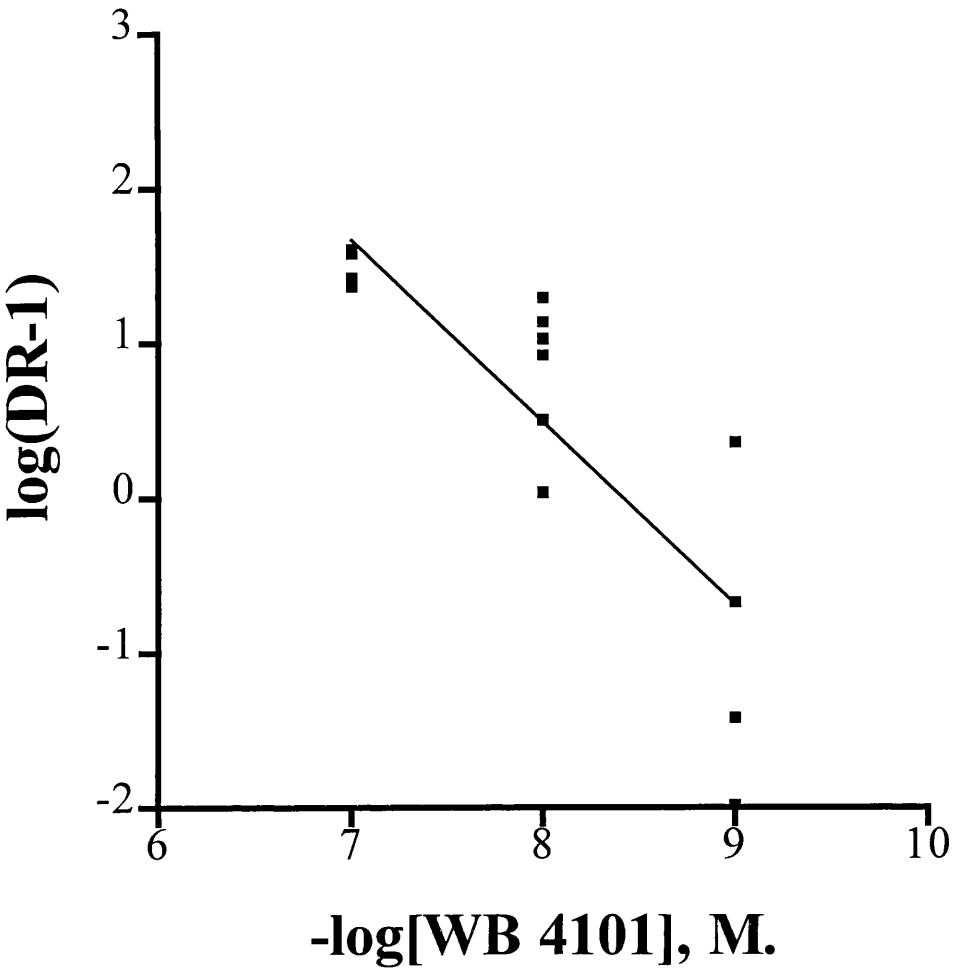


Figure 4.12. Schild plot for WB 4101 versus noradrenaline in canine resistance arteries. Points represent log (DR-1) values from individual experiments. The plot is composed of 15 data points in total.

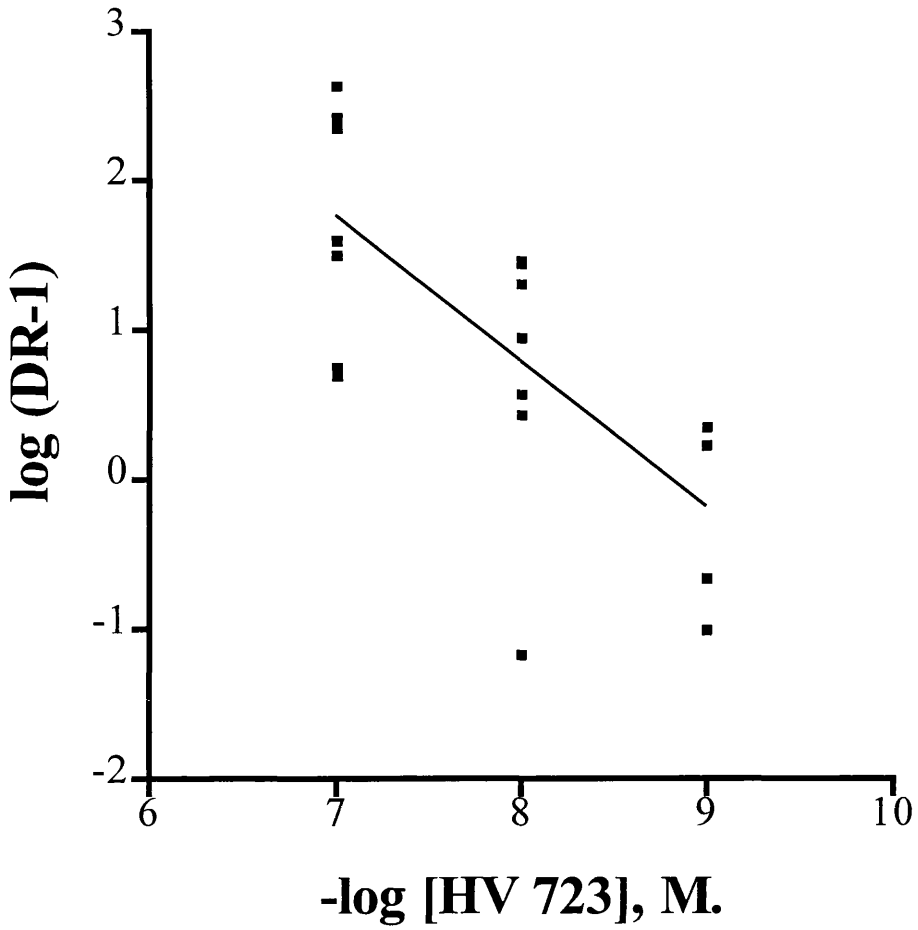


Figure 4.13. Schild plot for HV 723 versus noradrenaline in canine resistance arteries. Points represent log (DR-1) values from individual experiments. The plot is composed of 20 data points in total.

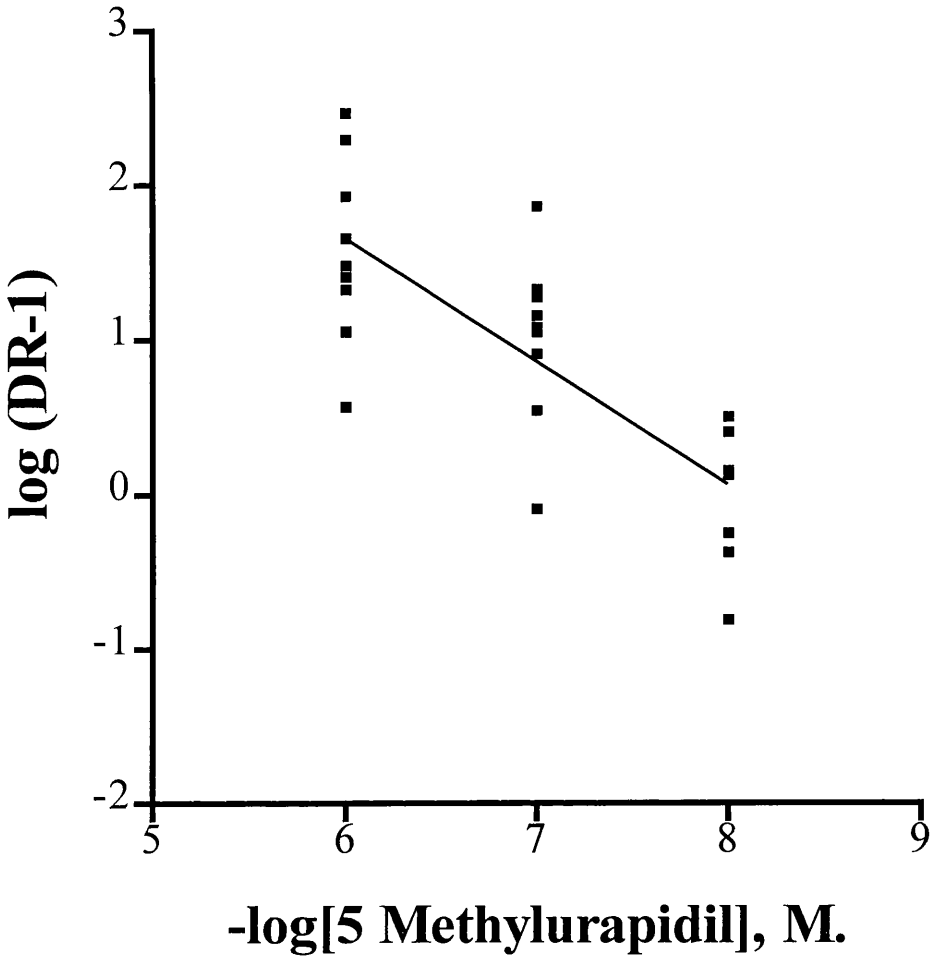


Figure 4.14. Schild plot for 5 methylurapidil versus noradrenaline in canine resistance arteries. Points represent log (DR-1) values from individual experiments. The plot is composed of 25 data points in total.

4.2.3 Irreversible antagonists

Chloroethylclonidine (CEC) was used at concentrations of 0.1μM, 1μM and 100μM. Values for each concentration were derived from 5-6 animals. None of the concentrations used caused a significant change in the pEC_{50} values for the NA concentration response curves (P of 0.975). 0.1μM and 1μM CEC caused no change in the maximum values, and in fact these curves were superimposible on the control curve. 100μM CEC caused a baseline contraction in only 2/5 experiments of 4% and 50%. A significant reduction in the maximum value was also seen with this concentration giving a mean maximum of 50.8 ± 8% (n = 5) of control. Figure 4.15 illustrates the mean CRC data for CEC.

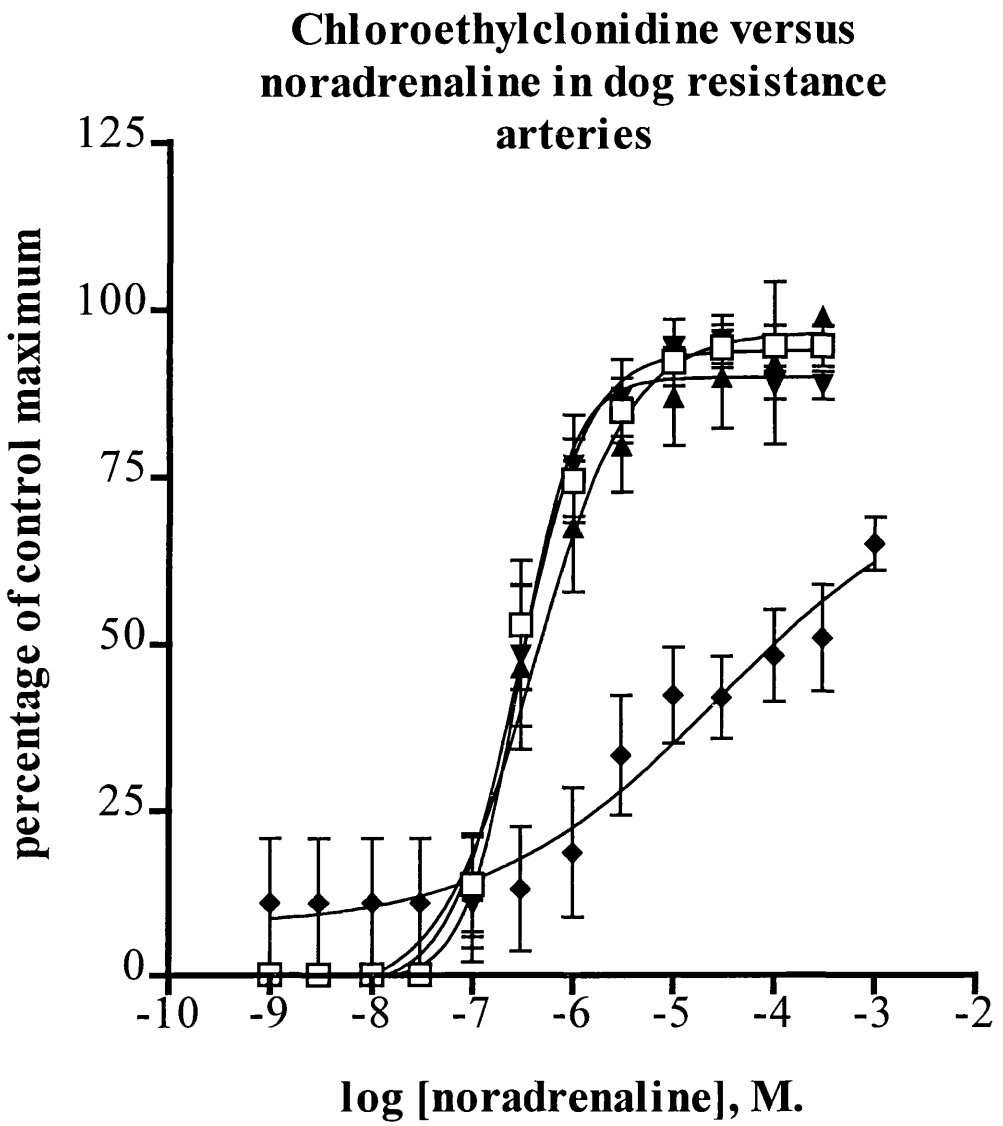


Figure 4.15. Mean CRC data for chloroethylclonidine in canine resistance arteries. Data points represent mean ± s.e. mean and are expressed as a percentage of the control curve maximum. Control (□), $n = 6$; 0.1μM CEC (▼), $n = 6$; 1μM (▲) CEC, $n = 6$; 100μM CEC (◆), $n = 5$.

ANTAGONIST	SLOPE (95% confidence intervals)	pA_2
Prazosin	-1.172 to -0.4365	8.36
BMY 7378	-0.8429 to 0.5064	4.82
5 Methylurapidil	-1.077 to -0.5106	8.08
WB 4101	-1.633 to -0.7132	8.42
HV723	-1.480 to -0.4608	8.81

Table 4.2. Table summarising Schild plot slope and pA_2 parameters for competitive antagonists in dog resistance arteries. Noradrenaline was the agonist used. Slope values are given as 95% confidence intervals.

ANTAGONIST	pA_2 from Schild	concentration of antagonist	$pA_2 \pm$ s.e.mean[a] (n)
prazosin	8.36	1nM	8.54 ± 0.31 (6)
		10nM	8.21 ± 0.24 (8)
		0.1μM	8.14 ± 0.18 (7)
BMY 7378	4.82	1nM	8.04 (2)
		10nM	7.72 ± 0.25 (4)
		0.1μM	6.51 ± 0.47 (4)
HV 723	8.81	1nM	8.72 ± 0.33 (4)
		10nM	8.86 ± 0.28 (9)
		0.1μM	8.70 ± 0.3 (7)
WB 4101	8.42	1nM	8.07 ± 0.50 (4)
		10nM	8.82 ± 0.19 (6)
		0.1μM	8.47 ± 0.05 (5)
5 MeU	8.08	10nM	7.96 ± 0.18 (7)
		0.1μM	8.01 ± 0.18 (9)
		1μM	7.57 ± 0.2 (9)

Table 4.3. Table summarising pA_2 values derived from Schild analysis and from individual antagonist concentrations, calculated for the competitive antagonists in the dog resistance arteries. PA_2 values derived from individual concentrations[a] are given as mean ± s.e. mean. Values in brackets represent the number of experiments. Noradrenaline was the antagonist used.

Antagonist	[a] α_{1a}-human clone ([^{3}H-InsPs]) pK_B	[b] α_{1L}-LUT pA_2	[c] DSV α_{1N}- pA_2	[d] α_{1D}- rat aorta pA_2	[e] α_{1B}-rat spleen pA_2	[f] DSV present study pA_2/ pK_B	[f] DSCRA present study pA_2/pK_B
Prazosin	8.7 ± 0.1	8.7 ± 0.1	7.94 ± 0.07	9.6 ± 0.1	9.2	8.31	8.36
WB 4101	8.9 ± 0.1	8.9 ± 0.1	8.58 ± 0.10	9.0 ± 0.1	8.1	8.85	8.42
5-MeU	8.2 ± 0.1	8.2 ± 0.1	N.D.	7.6 ± 0.1	7.1	6.29 ± 0.28 (L) 8.56 ± 0.26 (H)	8.08
BMY 7378	N.D.	6.4 ± 0.1	N.D.	8.5 ± 0.1	7.4	7.04 ± 0.12	6.51 ± 0.47
HV 723	N.D.	8.8 ± 0.1	9.09 ± 0.06	8.7 ± 0.1	N.D.	8.98	8.81

Table 4.4. Table comparing pA_2/pK_B values in dog resistance arteries to previously published data and to DSV values from this study. [a] human clone α_{1a} - (Daniels et al. 1996); [b] human lower urinary tract tissues α_{1L}- (Ford et al. 1996a) ; [c] DSV values from Muramatsu et al (1990) ; [d] rat aorta α_{1D}- (Ford et al. 1996a); [e] rat spleen α_{1B}- (Burt et al. 1995) ; [f] present study pA_2 values except those with standard errors which are pK_B values.

4.3 Discussion

Resistance arteries, because of their small size, and the relation of vessel radius to resistance to blood flow, (inversely related to the radius to the fourth power, according to Poiseuille's law (Levick, 1992)), play the most important role in regulation of total peripheral resistance. Despite the importance of resistance arteries, few studies have looked at these vessels in relation to adrenoceptor subtype and instead the majority of studies have focused on the large conduit vessels. One of the main reasons for this has been the comparatively recent advent of techniques such as perfusion and wire myography, allowing the study of isolated resistance sized vessels.

To my knowledge, at the time of writing this thesis, no studies have been published, examining α_1-adrenoceptors in canine peripheral resistance arteries and therefore comparisons can only be made with studies from other species and large vessel studies.

From the literature, α_1-adrenoceptors would seem to be the predominant receptor type involved in vascular smooth muscle contraction of resistance arteries to noradrenaline, although α_2-adrenoceptors have also been implicated. Studies by Nielsen et al (1989), Parkinson and Hughes (1995) and Stephens et al (1992), all involving human resistance sized arteries, confirm the presence of both α_1- and α_2- post-junctional adrenoceptors. Leech and Faber (1996) also found evidence for both α_1- and α_2-adrenoceptors in rat cremaster resistance artery. In contrast, Macmillan et al (1994) found a very low sensitivity for the α_2-agonist UK14304, compared to phenylephrine, in rabbit subcutaneous resistance arteries, suggesting that α_2-adrenoceptors are not involved in vascular smooth muscle contraction of this vessel.

In this study, the rank order of agonist potency was Abbott > UK14304 > NA > PE. Despite UK14304 appearing quite potent in this ranking, it should be noted that one out of the four vessels examined, failed to respond to this agonist, thus in reality UK 14304

was relatively less potent in this vessel than in the DSV, where it was more potent than phenylephrine. This would suggest that although there may be some α_2-adrenoceptor function, α_1-adrenoceptors are the main mediators of the response in this vessel. This conclusion would be supported by work in porcine vascular tissues (Wright et al. 1995), comparing agonist potencies to binding and antagonist studies, where relative potencies of NA, PE and UK14304 were correlated with the relative binding distribution of α_1- and α_2- adrenoceptors. In addition, it has also been reported in the literature that in certain vessels, UK 14304 may act as a partial agonist at α_1-adrenoceptors (Nagadeh et al. 1994). To rule this out in canine vessels, it would be of value to examine the effect of α_1-antagonists on the contractile responses to UK 14304. Despite this, to avoid complications which may arise from the activation of putative α_2-adrenoceptors, the α_2-antagonist delequamine was present at all times during the antagonist studies. The reasons for the use of delequamine, as opposed to the use of rauwolscine, have been discussed in Chapter 3.

As for the DSV, (R) A-61603 was the most potent of the agonists used (this is discussed in Chapter 3). In the case of the dog resistance artery, (R) A-61603 was 112X more potent than PE compared to only 43X more potent in the DSV. Although this does not completely support the presence of an α_{1A}-adrenoceptor subtype, (Knepper's paper (Knepper et al. 1995) quotes a 590 fold greater potency using the R- enantiomer of A-61603 in canine prostatic strips), it would be unreasonable to rule out the presence of this subtype based on a single previous study, as for example the presence of more than one subtype may alter the sensitivity to the agonist. In summary, the agonist profile would suggest that α_1-adrenoceptors predominantly mediate contraction to noradrenaline in dog resistance artery, although there may be some α_2-adrenoceptor

function. The relative potency of the Abbott compound may suggest the presence of the α_{1A}- subtype.

For all the competitive antagonists used, with the exception of BMY 7378, the highest antagonist concentration caused a significant decrease in the maximum, from the control curve. It could be assumed that this was a time-dependent change but the factors which support this being a true finding are as follows:

1. Time control curves were run in parallel with all experiments and maximums, as judged by one way ANOVA were not significantly different;

2. Although up to four consecutive curves were performed in some vessels, in the majority of experiments only two or three concentration response curves were performed, and for WB 4101, 5 methylurapidil and HV 723, experiments were performed using only the highest concentration of the antagonists (i.e. only two CRC curves were performed in total), specifically to exclude a time-dependent phenomenon.

3. Concentration response curves, in the presence of BMY 7378, showed no significant alterations in upper asymptotes over the range of antagonist concentrations used.

Concentration response curve data therefore, from WB 4101, HV 723 and 5 methylurapidil was not supportive of a strictly competitive interaction, as all three antagonists displayed insurmountable antagonism at the highest concentration. The significant difference for prazosin was marginal with P just less than 0.05 which was not as convincing of a non-competitive interaction.

Despite the fact that not all the concentration response curves strictly fulfilled the criteria for Schild analysis (i.e. parallel rightward displacements with no decrease in the upper asymptote), Schild regression and pA_2 value estimates from individual concentrations were performed for all antagonists and are summarised in Tables 4.3 and 4.4. With the exception of BMY 7378, all slope 95% confidence intervals encompassed

negative unity and none of the pA_2 values calculated for individual concentrations were significantly different over the range of antagonist concentrations studied. It would seem therefore, that while CRC data may suggest non-competitive interactions with some of the antagonists, this was not identified by subsequent Schild analysis. pA_2 values for prazosin and HV 723 are similar to those obtained in the DSV and would support the presence of the α_{1L}- receptor subtype. (Muramatsu et al. 1995; Muramatsu et al. 1990b). All the pA_2 values are shown in Table 4.4 where they can be compared to the results from the DSV in this study and different subtypes from other studies. It can clearly be seen that values are similar to the DSV and that both of these compare most closely with the α_{1a}- clone and the α_{1L}- of lower urinary tract.

The actions of chloroethylclonidine were dissimilar in some respects, to the effects of this antagonist in the DSV. The main difference was that only 2/5 dog resistance artery vessels developed a baseline contraction to 100μM CEC, whereas the baseline contraction was a consistent finding in all the DSV rings. In Chapter 3, it was discussed that a population of rauwolscine-sensitive receptors contributed to the baseline contraction to CEC seen in the DSV. In addition, these rauwolscine-sensitive receptors appeared to be α_1-adrenoceptors, which were $\alpha_{1A/L}$- like, in that they were sensitive to 5 methylurapidil, WB 4101 and relatively sensitive to prazosin, although the affinity for the latter antagonist was still < 9.

100μM CEC caused a significant decrease in the maximum in both vessels, but only a significant shift in the pEC_{50} in the DSV. Due to the decrease in maximum it would not be possible to rule out the presence of α_{1B}-adrenoceptors.

Comparing the findings of the present study with those of other studies which have examined resistance arteries, it would appear that the rat mesenteric resistance artery has received most attention. Van der Graaf (1996a) found similar results in this vessel to

results from this study and concluded on the presence of the α_{1L}-adrenoceptor. Smith and McGrath also investigated rat mesenteric resistance artery and concluded on the presence of the α_{1A}- subtype and /or the α_{1B}- subtype. Interestingly, this study found that the affinity for prazosin significantly decreased from 5 to 52 weeks of age so that at 52 weeks the receptors could have been classified as α_{1L}- on the basis of affinity for prazosin and HV 723 (Smith and McGrath, 1996). A study by Simonsen et al (1997) in horse penile resistance arteries also found that prazosin had a low affinity (pA_2 8.03 ± 0.09) and rabbit subcutaneous resistance arteries, (Smith et al. 1997) have been shown to have a low affinity prazosin site classified as α_{1L}-, but in addition seem to possess a high affinity prazosin site most closely resembling the α_{1B}-adrenoceptor.

To summarise, the antagonist findings support the presence of an α_{1A}- or α_{1L}-adrenoceptor. Due to the low affinity for prazosin, within the current classification scheme, this would strictly be referred to as an α_{1L}-adrenceptor. If in the DSV, the baseline contraction caused by CEC is genuinely contributed to by the receptors that also appeared to be α_{1L}- adrenoceptors in this vessel, then although on the basis of the antagonist affinities, the receptors in both vessels would be classified as the same subtype, the lack of ability to consistently cause a contraction in the presence of CEC, could suggest that the receptors in the dog subcutaneous resistance arteries are somehow different to those in the DSV.

The insurmountable nature of the antagonism to the highest concentrations of 5 methylurapidil, HV 723 and WB 4101, provide some evidence for the presence of more than one α_1-adrenoceptor subtype. If another subtype were present it could be similar to the α_{1B}-, because of the depression in maximum seen with CEC. There is also some evidence supporting the role of the α_{1D}-, since the interaction with BMY 7378 was not strictly competitive, evidenced by the declining pA_2 values seen with increasing

concentrations of the antagonist. A pA_2 of 8.04 derived in the presence of 1nM BMY 7378 would be supportive of involvement of the α_{1D}- subtype. The sensitivity to CEC could also be accounted for by the presence of the α_{1D}-adrenoceptor since this subtype is reported to have an intermediate susceptibility to alkylinization by this compound (Michel et al. 1993).

Any other subtype which is involved must also have a low affinity for prazosin. At this time no satisfactory classification scheme exists for the discrimination of low affinity prazosin sites other than their division into α_{1L}- or α_{1N}- (Muramatsu et al. 1995), However, evidence from this study, in both the DSV and the subcutaneous resistance arteries suggests that a further subdivision of α_1-adrenoceptors with a low affinity for prazosin must exist.

CHAPTER 5

Cloning and sequencing of the canine α_{1a}-adrenoceptor

5.0 Abstract

5.1 Results

5.1.1 RT-PCR

5.1.2 Cloning

5.1.3 Sequencing

5.1.4 Sequence analysis

5.1.5 Northern blotting

5.2 Discussion

5.0 Abstract

The aim of this study was to amplify the canine α_{1a}-adrenoceptor cDNA from dog tissue RNA, using reverse-transcription polymerase chain reaction (RT-PCR), and primers based on areas of high homology between α_{1a}-adrenoceptors from human and bovine published sequences. Although it was not possible to amplify the full length coding region, an 891bp fragment was amplified, which encoded the putative amino terminus through to the sixth transmembrane spanning domain of the receptor. This sequence was found to be over 90% homologous to published human and bovine α_{1a}- sequences. The sequence was submitted directly to the GenBank and given Accession No AF068283.

A clone encoding part of the canine α_{1b}-adrenoceptor, together with the α_{1a}- clone from this study, were used to probe two cell lines each over-expressing either the human α_{1a}- or α_{1b}- adrenoceptor. With equal loading of RNA from both cell lines, the α_{1b}- probe, while showing some cross-reactivity, produced a much stronger signal with the α_{1b}- cell line. In contrast, the α_{1a}- probe showed no reactivity with either cell line. Northern analysis was repeated using RNA extracted from canine prostate and brain tissue. The α_{1b}- probe identified message in both tissues, but again the α_{1a}- probe did not identify transcripts. Several possibilities exist that could explain the failure of the α_{1a}- probe to identify transcripts. These are discussed in section 5.2.

5.1 Results

All materials and methods have been discussed in the materials and methods.

5.1.1 RT-PCR

In order to amplify the canine α_{1a}-adrenoceptor, a number of primers were designed based on areas of high homology existing between the human and bovine published sequences.

Figure 5.1 is a schematic diagram representing the proposed structure of the α_1-adrenoceptor. The picture illustrates the presence of the seven putative transmembrane spanning domains and the amino and carboxy termini of the receptor.

The primer design proved particularly problematic and in fact, three different sets of primers were tested before a product of the right size and sequence was obtained. Initially, a selection of oligonucleotide primers were designed, based on the human α_{1a}-adrenoceptor sequence. Three isoforms had been described for this subtype (Hirasawa et al. 1995), each one diverging in the sequence of the putative carboxy terminus. The isoforms are named $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2}$ and $\alpha_{1A\text{-}3}$. Primers were designed such that the upstream (sense or 5') primer was common to all isoforms and overlapped the start codon (ATG). A different downstream (antisense or 3') primer was designed for each of the three different isoforms, each one overlapping the stop codon for that particular isoform. In addition, a downstream primer common to all three isoforms was designed.

The primers were as follows: Upstream primer, 5' gaccatggtgtttctctc 3' (bases 433-450 (Hirasawa et al. 1995)); $\alpha_{1A\text{-}1}$ downstream primer 5' ctagacttcctccccgttc 3' (bases 1383-1401 (Hirasawa et al. 1995)); $\alpha_{1A\text{-}2}$ downstream primer 5' caggcagatcatgaggtc 3' (bases 1927-1940 (Hirasawa et al. 1995)); $\alpha_{1A\text{-}3}$ downstream primer 5' gctggcttcatgtcatgg

3'(bases 1716-1733 (Hirasawa et al. 1995)); Common downstream primer 5' ctgtggtacaggaggattg 3' (bases 1682-1700 of α_{1A-3} (Hirasawa et al. 1995)).

Dog tissue was collected immediately after euthanasia, snap frozen, and stored in liquid nitrogen until required. Total RNA was isolated using the RNazolB method as described in materials and methods. cDNA was synthesised from total RNA (as described in materials and methods), from dog brain and heart using both the random hexamer primers and oligo(dt) primers. Polymerase chain reactions were set up using the following: 1X PCR buffer, 10pm of each primer, 200μM of each dNTP (dATP, dTTP, dCTP, dGTP), 3mM $MgCl_2$, 5μl of cDNA and 2.5 units of *Taq* polymerase. The reaction was made up to 50μl with distilled autoclaved water in 0.5ml polypropylene tubes. The conditions used for the PCR were, 95°C for 45 seconds (denaturation), 55°C for 45 seconds (annealing), 72°C for 3 minutes (extension) for a total of 35 cycles. This was followed by a 10 minute extension period at 72°C. The upstream primer was used in conjunction with one of each of the downstream primers giving rise to four primer pairs in total. The primer pair consisting of the upstream primer and the α_{1A-3} downstream primer, produced a band of the expected size (≈1.3Kb), when used with cDNA made from dog heart generated with random hexamer primers (Figure 5.2). Unfortunately, when this was subsequently sequenced, it was found to have little similarity to any of the published α_1-adrenoceptor sequences, but was highly homologous to a myocardial structural protein called titin.

A second set of primers was then designed, again based on both the human α_{1a}-sequence and the bovine α_{1a}- sequence. These were as follows: α_{1a}- upstream primer 5' ggaccatggtgtttctctccggaaatgc 3', corresponding to bases 92-119 of the bovine clone (Schwinn et al. 1990) and bases 432-459 of the human isoform 3 clone (Hirasawa et al. 1995), where there was 100% homology between the two species; α_{1a}- bovine

downstream primer 5' cctttagacttcctccccattttcactgaggg 3' corresponding to bases 1469-1500 of the bovine clone (Schwinn et al. 1990); α_{1a}- human downstream primer 5' ctagacttcctccccgttctcactgaggg 3' corresponding to bases 1373-1401 of the human clone (Hirasawa et al. 1995). On this occasion no products were produced with any combination of the primer pairs. During this time annealing temperature was varied between 50°C and 60°C, the length of each step in the cycle was altered between 45 and 60 seconds, between 10 and 50pm of primer was used and a variety of tissue cDNAs were used including, canine heart, brain and prostate and human prostate.

A slightly different approach was then adopted. This time a primer was designed for the synthesis of cDNA. 20pm of this was used in place of the random hexamer or oligo(dt) primer. The sequence of this primer was 5' atggggttgatgcagctgtt 3'. In addition a new downstream primer was used in conjunction with the previous α_{1a}- upstream primer. The sequence of the downstream primer was 5' acccaatgggcatcactaaga 3'. The sequence of the three primers and the bases that they correspond to from published sequences is shown in Figure 5.3. The new primer combination did produce a band of the expected size (891 base pairs), when used with cDNA made from dog prostate RNA, where the cDNA had been synthesised using the gene-specific primer. Figure 5.4 shows a photograph of a representative PCR reaction, which has been run out on a 1% agarose gel and stained with ethidium bromide to visualize the DNA. Optimum concentrations of the components of the PCR reaction were as follows: 200μM of each dNTP ; 1X PCR buffer; 1.5mM $MgCl_2$; 10pm of each primer; 2.5U *Taq* polymerase; 5μl of cDNA from a 33 μl reaction.

Optimum PCR cycle conditions were, a denaturation step for 3 minutes at 95°C, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (48°C for 30

seconds) and extension (72°C for 30 seconds). The process was concluded with a final 5 minute extension at 72°C. Samples were then stored at -20°C until required.

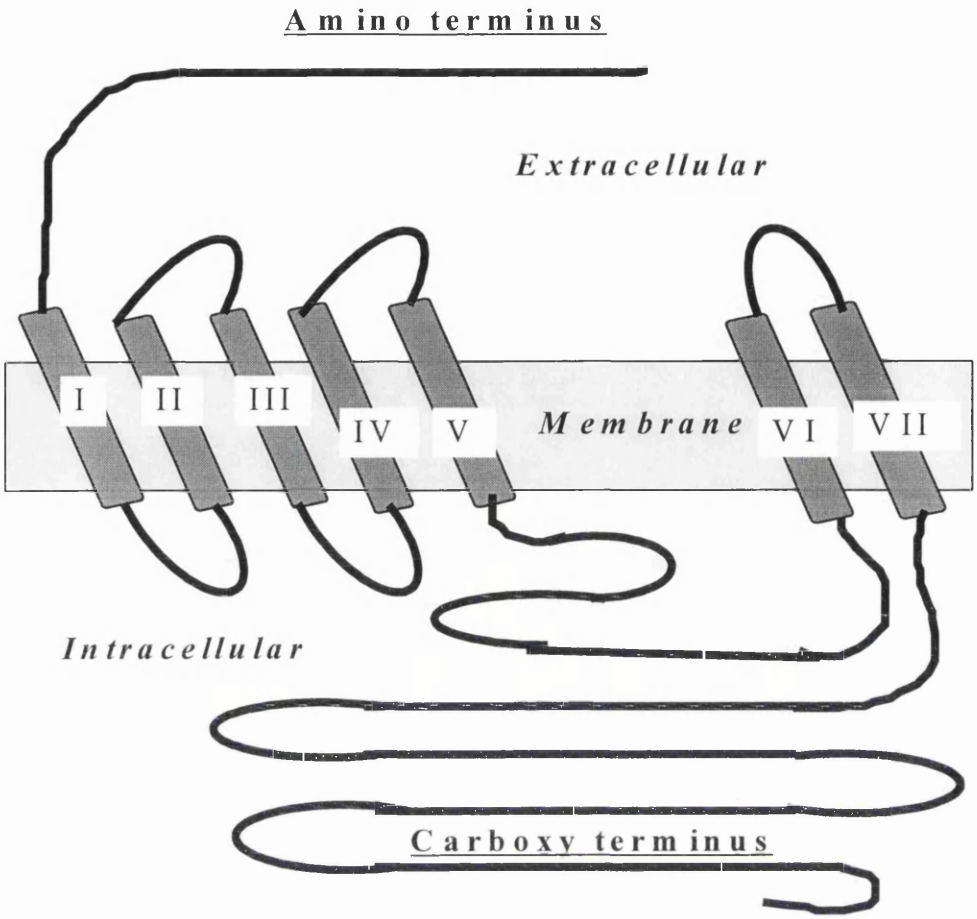


Figure 5.1. Theoretical two dimensional representation of an α_1-adrenoceptor. The putative transmembrane spanning domains are numbered I, II, III, IV, V, VI, VII.

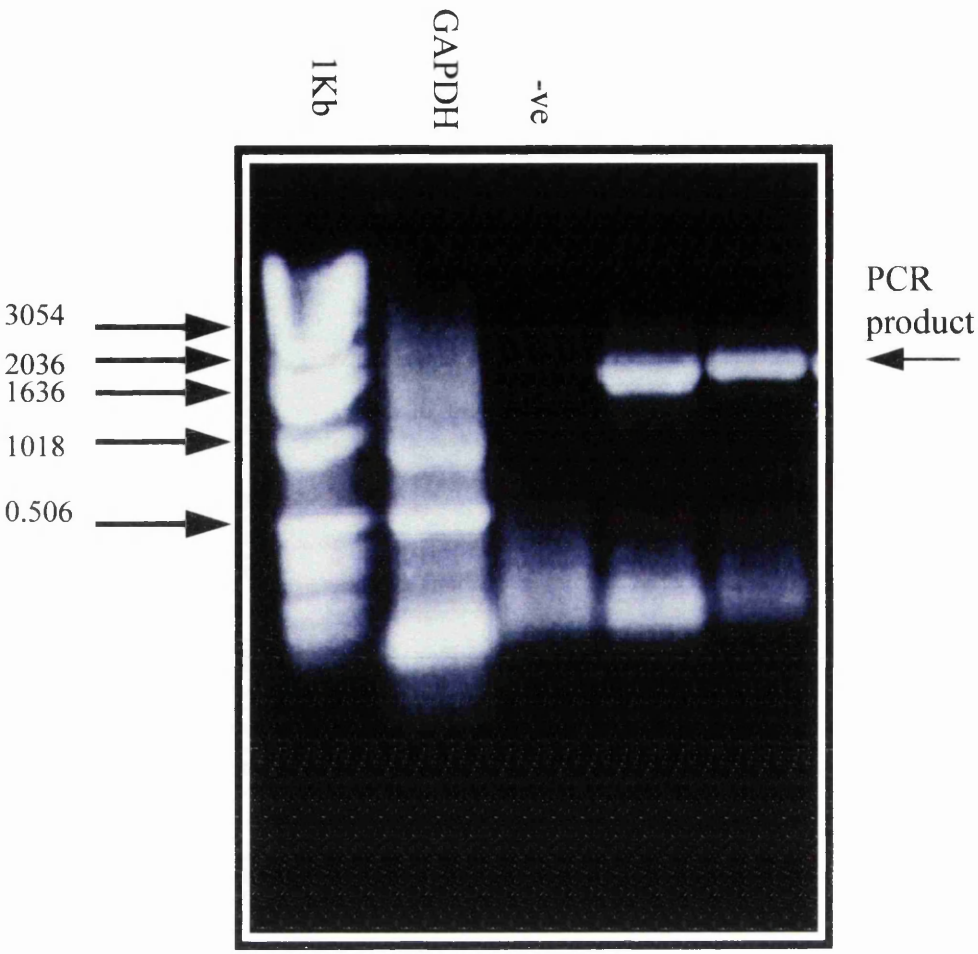


Figure 5.2. Representative gel showing PCR product with high homology to titin. Lane 1, 1kb ladder; lane 2, GAPDH; lane 3, negative control; lanes 4 and 5, PCR product from canine heart. The figures on the left hand side show the size markers in Kb.

5' ATG GGG TTG ATG CAG CTG TT 3' bases 951-972 human $\alpha_{1a\text{-}1}$ [a]
5' ATG GGG TTG ATG CAG CTG TT 3' bases 1387-1407 human $\alpha_{1a\text{-}2}$ [b]
5' ATG GGG TTG ATG CAG CTG TT 3' bases 1387-1407 human $\alpha_{1a\text{-}3}$ [c]
5' ATG GGG TTG ATG CAG CTG TT 3' bases 1048-1067 bovine α_{1a} [d]
5' ATG GGG TTG AGG CAG CTG TT 3' bases 729-748 canine α_{1b} [e]

5' ATG GGG TTG ATG CAG CTG TT 3' primer for reverse transcription

5' ~~~~~~ A TGG TGT TTC TCT CGG GAA ATG C 3' human $\alpha_{1a\text{-}1}$ [a] base 1-23
5' GGA CCA TGG TGT TTC TCT CGG GAA ATG C 3' human $\alpha_{1a\text{-}2}$ [b] base 432-459
5' GGA CCA TGG TGT TTC TCT CGG GAA ATG C 3' human $\alpha_{1a\text{-}3}$ [c] base 432-459
5' GGA CCA TGG TGT TTC TCT CCG GAA ATG C 3' bovine α_{1a} [d] base 92-119

5' GGA CCA TGG TGT TTC TCT CCG GAA ATG C 3' Upstream primer

5' ACC CAA TGG GCA TCA CTA AGA 3' human $\alpha_{1a\text{-}1}$ base 866-886[a]
5' ACC CAA TGG GCA TCA CTA AGA 3' human $\alpha_{1a\text{-}2}$ base 1302-1322[b]
5' ACC CAA TGG GCA TCA CTA AGA 3' human $\alpha_{1a\text{-}3}$ base 1302-1322[c]
5' ACC CAA TGG GCA TCA CTA AGA 3' bovine α_{1a}-clone base 962-982[d]
5' AGC CAA GCG GTA GAG CAA TGA 3' canine α_{1b}- clone base 643-663[e]

5' ACC CAA TGG GCA TCA CTA AGA 3' downstream primer

Figure 5.3. The three primers used to amplify the canine α_{1a}-sequence, lined up with the sequences from the bovine and human α_{1a}- and the canine α_{1b}- sequence. [a] (Hirasawa et al. 1995) Accession no L31774; [b] (Hirasawa et al. 1995) Accession no D32202; [c] (Hirasawa et al. 1995) Accession no D32201; [d] (Schwinn et al. 1990) Accession no J05426; [e] (Libert et al. 1989) Accession no X14050. Bases that are not homologous to the primer are underlined and ~ denotes where the sequence is not known.

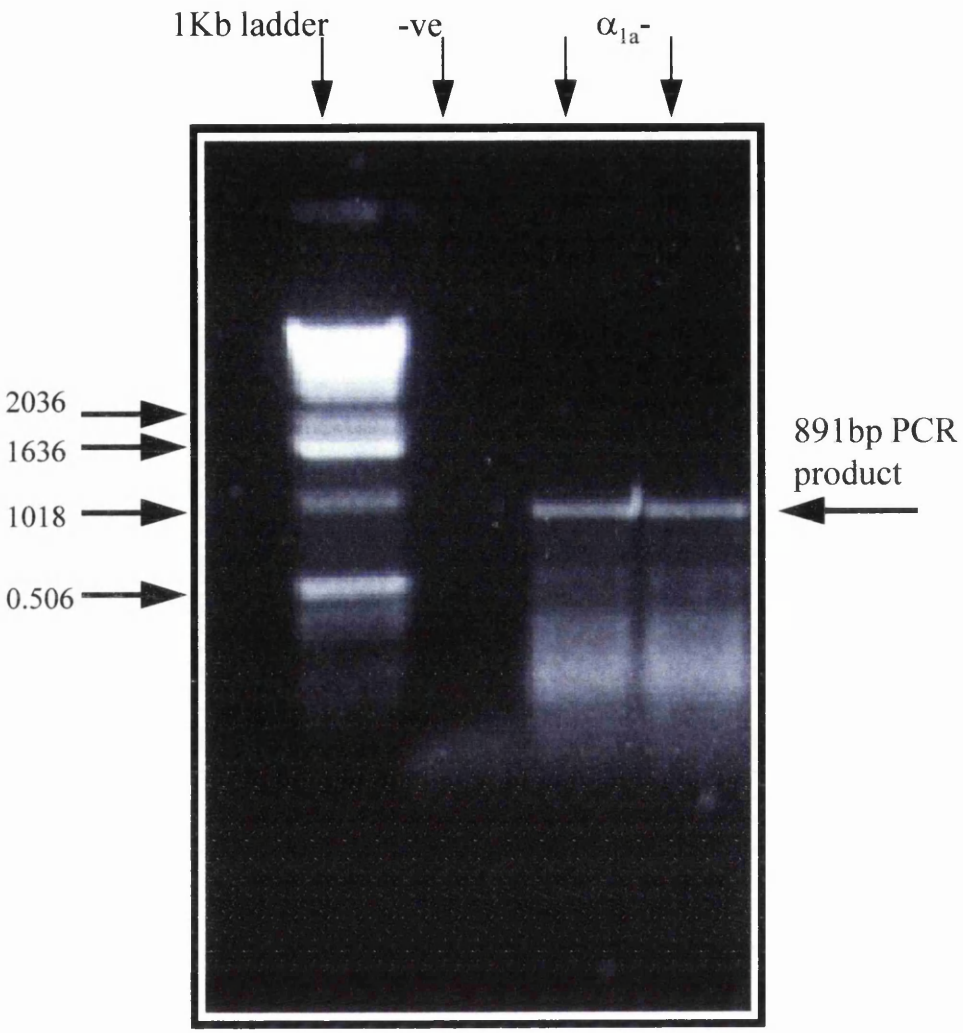


Figure 5.4. Representative gel showing the PCR product corresponding to the canine α_{1a}- partial sequence. Lane 1, 1KB ladder ; lane 2, negative control; lanes 3 and 4, canine α_{1a}- PCR product. Figures on the left show size markers in Kb.

5.1.2 Cloning

The PCR product was cloned using Invitrogen's Original TA Cloning® Kit as described in the materials and methods. Ligations were carried out using fresh PCR product to prevent loss of the A' overhangs which enable the product to insert into the plasmid. Thirty white colonies were selected from the transformation and grown up overnight in 3ml volumes of LB broth. Wizard® *Plus* SV Minipreps DNA purification system from Promega, was used to purify the plasmids from the cultures grown.

Minipreps were digested using the restriction enzyme EcoR1, as this site flanks the insertion site in the Invitrogen plasmid (pCR® 2.1). Digests were run out on a 1% agarose gel. Figure 5.5 shows a photograph of the gel identifying three of the selected colonies containing an insert of the correct size. The positive colonies were re-plated on ampicillin-containing agarose plates and stored at 4°C. In addition, the remainder of the cultures from the positive colonies were placed in vials containing 40% glycerol and 2% peptone, and stored at -70°C. The purified plasmid DNA from the three colonies was then used for sequencing.

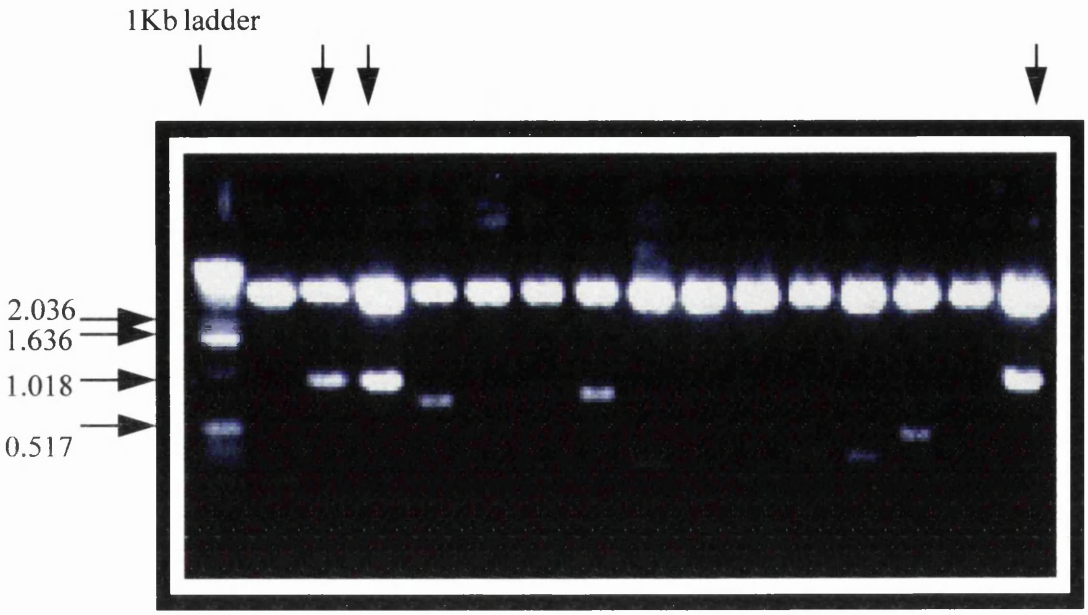


Figure 5.5. Photograph of gel showing the three colonies containing the canine α_{1a}- insert. Plasmid was purified from bacterial colonies and the purified plasmid digested with EcoR1. The resultant digest was run out on an agarose gel. The three plasmid preps containing an insert of the correct size (891bp), are indicated by arrows. Figures on the left are size markers in Kb.

5.1.3 Sequencing

The miniprep DNA, corresponding to one of the positive colonies, was sequenced using the Thermo Sequenase cycle sequencing kit from Amersham, incorporating ^{33}P labelled dideoxynucleotides as described in the materials and methods. 5µl of miniprep DNA was used in the sequencing reactions, which was equivalent to approximately 500ng of DNA. For the first sequencing reaction, the M13 reverse primer from Invitrogen was used (5'caggaaacagctatgac 3'). This primer is homologous to the pCR®2.1 plasmid sequence upstream of the insert and allowed the sequence to be read from the plasmid into the insert. For the subsequent sequencing reactions, primers were custom made (Figure 5.6). Conditions for sequencing were as follows: Denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds), extension (72°C for 30 seconds), for a total of 40 cycles. Reactions were run on a 6% polyacrylamide gel, exposed to radiographic film and developed as described in materials and methods .

The entire 891 bases of the upstream strand of the insert were sequenced. In addition, in order to obtain a consensus sequence, direct sequencing of PCR products was performed for both strands. This was done by excising the PCR product from an agarose gel and spinning the block of agarose through a Supelco GenElute™ spin column, as described in materials and methods. The DNA, when recovered was ethanol precipitated and resuspended in 10µl of TE buffer. For sequencing, the entire 10µl was used for a single reaction. This meant that several different PCR reactions were required to sequence the full length of the product. For the first reaction, the upstream and downstream primers that had been used for the RT-PCR were employed (Figure 5.3). Subsequent to this, custom primers were designed based on the sequence obtained from the previous sequencing, (Figure 5.6). The sequence of the clone was identical to the sequence obtained from the PCR sequencing, with the exception of a single base, where the clone

contained a guanine residue instead of an adenosine residue at base number 272 counting from the beginning of the ATG start codon. This was found to alter the amino acid encoded in this region from a tyrosine, in the case of the clone, to a cystine. At this point, another two clones were sequenced in this region and also found to have an adenosine residue, therefore adenosine was considered the consensus base and was used when determining the predicted amino acid sequence of the clone.

Primers for sequencing of the clone.

M13 reverse primer 5' CAGGAAACAGCTATGAC 3'

Upstream 1 5' GCAACATCCTGGTGATCCTC 3'

Upstream 2 5' ATAGGTGTGAGCTACCCGCT 3'

Upstream 3 5' TCATCCTGGTCATGTCTG 3'

Upstream 4 5' TCTGCGGCTGCCTTTTCTTA 3'

Primers for direct sequencing of the PCR product

Upstream 1 5' TCGAGATCCTGGGCTACT 3'

Upstream 2 5' TCGTCATCTCCATCGGGCCT 3'

Upstream 3 5' ATCGGAAGAACGCCCCAGTA 3'

Downstream 1 5' AGAAGTGCGTCTTGTTCTTG 3'

Downstream 2 5' AGCGCCGAGAAGAGCACGTA 3'

Downstream 3 5' ATGATGGACGCGGTGCAGCA 3'

Downstream 4 5' AAGATCACCCCGAGCAGAAT 3'

Figure 5.6. Primers used for sequencing from the clone and for direct PCR sequencing.

5.1.4 Sequence analysis

The nucleotide and predicted amino acid sequence of the clone are given in Figure 5.7. Sequence analysis was carried out using the University of Wisconsin Genetics Computer Group (G.C.G.) sequence analysis software programmes.

Using the BESTFIT programme (which employs the local homology algorithm of Smith and Waterman (1981)), it was determined that the canine clone had the following homologies at the nucleotide level with existing clones: 93% homology to the bovine α_{1a}- clone (Schwinn et al. 1990), accession number J05426; 93% homology to the three isoforms of the human α_{1a}- clone (Hirasawa et al. 1995); 93% homology to a novel fourth human α_{1a}- clone (Chang et al. 1998), accession number AF013261; 78% homology to the human α_{1b}- clone (Ramarao et al. 1992), accession number L31773; 85% homology to the canine partial α_{1b}- sequence (Libert et al. 1989), accession number X14050; 81% homology to the human and rat α_{1d}- clones (Schwinn et al. 1995), accession numbers L31772 and L31771. Figure 5.8 illustrates the amino acid sequence of the clone determined in this study and compares it to the amino acid sequence of both the bovine and human α_{1a}-adrenoceptor sequences.

```
          M   V   F   L   S   G   N   A   S   D   S   S   N   C
  ggacc atg gtg ttt ctc tcc gga aat gcc tcc gac agt tcc aac tgc
 T   H   P   P   A   P   V   N   I   S   K   A   I   L   L   G
acc cac ccg ccg gca ccg gtg aac ata tcc aag gcc att ctg ctc ggg
                                TM1
 V   I   L   G   G   L   I   I   F   G   V   L   G   N   I   L
gtg atc ttg ggg ggc ctc atc att ttt ggt gtg ctg ggc aac atc ctg
 V   I   L   S   V   A   C   H   R   H   L   H   S   V   T   H
gtg atc ctc tcc gtg gcc tgc cac cgg cat ctg cac tcg gtc act cac
                                TM2
 Y   Y   I   V   N   L   A   V   A   D   L   L   L   T   S   T
tac tac atc gtc aac ctg gcg gtg gcc gac ctc ctg ctc acc tcc acc
V   L   P   F   S   A   I   F   E   I   L   G   Y   W   A   F
gtg ctg ccc ttc tcg gct atc ttc gag atc ctg ggc tac tgg gcc ttt
 G   R   V   F   C   N   I   W   A   A   V   D   V   L   C   C
ggc agg gtc ttc tgc aat atc tgg gcg gcg gtg gac gtc ctg tgc tgc
                                TM3
 T   A   S   I   M   G   L   C   I   I   S   I   D   R   Y   I
acc gcg tcc atc atg gga ctc tgc atc atc tcc atc gac cgc tac ata
 G   V   S   Y   P   L   R   Y   P   T   I   V   T   Q   K   R
ggt gtg agc tac ccg ctg cgc tac ccc acc atc gtc acc cag aag agg
                                TM4
G   L   M   A   L   L   C   V   W   A   L   S   L   V   I   S
ggt ctc atg gct ctg ctc tgt gtc tgg gcg ctg tcc ctc gtc atc tcc
 I   G   P   L   F   G   W   R   Q   P   A   P   E   D   E   T
atc ggg cct ctc ttt ggc tgg agg cag ccg gcc ccc gag gac gag acc
I   C   Q   I   T   E   E   P   G   Y   V   L   F   S   A   L
atc tgt cag atc acc gag gag ccg ggc tac gtg ctc ttc tcg gcg ctg
                                TM5
 G   S   F   Y   V   P   L   T   I   I   L   V   M   Y   C   R
ggc tcc ttc tac gtg cca ctg acc atc atc ctg gtc atg tac tgc cgg
 V   Y   V   V   A   K   R   E   S   R   G   L   K   S   G   L
gtc tac gtg gtg gcc aag agg gag agc agg ggc ctc aag tct ggc ctc
 K   T   D   K   S   D   S   E   Q   V   T   L   R   I   H   R
aag act gac aag tcg gac tcg gag cag gtg acg ctc cgc atc cat cgg
 K   N   A   P   V   G   G   T   G   V   S   S   A   K   N   K
aag aac gcc cca gta gga ggc acc ggg gtg tcc agc gcc aag aac aag
 T   H   F   S   V   R   L   L   K   F   S   R   E   K   K   A
acg cac ttc tcg gtg agg ctc ctc aag ttc tcc cgg gag aag aaa gcg
                                TM6
A   K   T   L   G   I   V   V   G   C   F   V   L   C   W   L
gcc aaa acg ctg ggc atc gtg gtc ggc tgc ttc gtc ctc tgc tgg ctg
 P   F   F   L   V   M   P   I   G
cct ttt ttc tta gtg atg ccc att ggg t
```

Figure 5.7. Nucleotide sequence of the canine α_{1a}- clone together with the predicted amino acid sequence. The amino acid sequence is shown to start at the first methionine. Underlined sequence represents the putative transmembrane spanning domains which are designated TM1 through to TM6, and primer sequence is shown in italics.

```
                                          *     *
D1   M V F L S G N A S D S S N C T H P P A P V N I S K A I L L G
B1   M V F L S G N A S D S S N C T H P P P P V N I S K A I L L G
H1   M V F L S G N A S D S S N C T Q P P A P V N I S K A I L L G

D31  V I L G G L I I F G V L G N I L V I L S V A C H R H L H S V
B31  V I L G G L I L F G V L G N I L V I L S V A C H R H L H S V
H31  V I L G G L I L F G V L G N I L V I L S V A C H R H L H S V

                                                        *
D61  T H Y Y I V N L A V A D L L L T S T V L P F S A I F E I L G
B61  T H Y Y I V N L A V A D L L L T S T V L P F S A I F E I L G
H61  T H Y Y I V N L A V A D L L L T S T V L P F S A I F E V L G

                          *
D91  Y W A F G R V F C N I W A A V D V L C C T A S I M G L C I
B91  Y W A F G R V F C N V W A A V D V L C C T A S I M G L C I
H91  Y W A F G R V F C N I W A A V D V L C C T A S I M G L C I

D120 I S I D R Y I G V S Y P L R Y P T I V T Q K R G L M A L L C
B120 I S I D R Y I G V S Y P L R Y P T I V T Q K R G L M A L L C
H120 I S I D R Y I G V S Y P L R Y P T I V T Q R R G L M A L L C

                                                          *
D150 V W A L S L V I S I G P L F G W R Q P A P E D E T I C Q I T
B150 V W A L S L V I S I G P L F G W R Q P A P E D E T I C Q I N
H150 V W A L S L V I S I G P L F G W R Q P A P E D E T I C Q I N

                                          *     *
D180 E E P G Y V L F S A L G S F Y V P L T I I L V M Y C R V Y V
B180 E E P G Y V L F S A L G S F Y V P L T I I L V M Y C R V Y V
H180 E E P G Y V L F S A L G S F Y L P L A I I L V M Y C R V Y V

D210 V A K R E S R G L K S G L K T D K S D S E Q V T L R I H R K
B210 V A K R E S R G L K S G L K T D K S D S E Q V T L R I H R K
H210 V A K R E S R G L K S G L K T D K S D S E Q V T L R I H R K

        * *     *   * *       *
D240 N A P V G G T G V S S A K N K T H F S V R L L K F S R E K K
B240 N A Q V G G S G V T S A K N K T H F S V R L L K F S R E K K
H240 N A P A G G S G M A S A K T K T H F S V R L L K F S R E K K

D270 A A K T L G I V V G C F V L C W L P F F L V M P I G
B270 A A K T L G I V V G C F V L C W L P F F L V M P I G
H270 A A K T L G I V V G C F V L C W L P F F L V M P I G
```

Figure 5.8. Comparison of predicted amino acid sequence from the canine, bovine and human α_{1a}- adrenoceptors. Non-homologous areas are underlined and marked with *. D = dog, B = bovine and H = human.

5.1.5 Northern blotting

Two probes were generated for Northern analysis. The first was termed the α_{1a}- probe and this was the partial canine α_{1a}-adrenoceptor sequence (Figure 5.7) that had been cloned and sequenced in this study (891bp). The second probe was termed the α_{1b}-probe. This was kindly donated by Libert et al (1989), and comprised a clone encoding a partial sequence for the canine α_{1b}-adrenoceptor (accession number X14050). This clone was received as a 1.8Kb fragment inserted into the EcoR1 restriction site of the pBlueskript SK+ plasmid (2958bp plasmid from Stratagene). On receipt, INVαF' competent cells from the TA cloning kit, were transformed with the pBlueskript SK+ plasmid. Twelve colonies were selected and grown up in 3ml volumes of LB broth overnight. Plasmid DNA was purified from the colonies using the Wizard® *Plus* SV Minipreps DNA purification system from Promega. 8µl of each miniprep was digested with EcoR1 and run out on a 1% agarose gel. All of the colonies contained the insert (Figure 5.9). The inserts were excised from the gel and the DNA purified from the agarose using Supelco GenElute™ spin column minus ethidium bromide. Plasmid containing the α_{1a}- clone was also digested with EcoR1, and the insert band purified in the same way.

Total RNA was prepared from two cell lines of rat-1 fibroblasts, each stably expressing either the human α_{1a}-adrenoceptor or the human α_{1b}-adrenoceptor, (accession numbers L31774 and L31773 respectively), at high levels. These cells were kindly donated by Dr Janet MacKenzie. From radioligand binding experiments using [^{3}H]-prazosin, she had shown a B_{max} of 912 ± 38 fmol mg^{-1} protein and a K_d of 0.477 ± 0.11nM for the α_{1a}-cell line, and a B_{max} of 954 ± 55 fmol mg^{-1} protein and a K_d of 0.433 ± 0.14 nM for the α_{1b}- cell line (all values represent mean ± s.e. mean). B_{max} is the maximum density of binding sites and K_d is the equilibrium dissociation constant.

20μg of RNA from each cell line was run on a formaldehyde gel and subsequently transferred to a Hybond-N+ membrane (Amersham), as described in the materials and methods. The α_{1b}- probe was random labelled with [α-^{32}P] dCTP using Ready-To-Go® DNA labeling beads from Pharmacia Biotech, using ≈50ng of the probe DNA. This was hybridized overnight with the membrane. The following morning, the membrane was washed at low stringency using two washes with 2X SSC/0.1% SDS. The membrane was then exposed to radiographic film for 24 hours and Figure 5.10 illustrates the hybridization of the probe. The probe had hybridized to both cell lines to a single size RNA species of ≈ 5 Kb. The intensity of labelling was much stronger for the α_{1b}- cell line.

The filter was then stripped by placing the membrane in boiling 0.1% SDS. The hybridization was repeated using the α_{1a}- probe labelled in the same way as the α_{1b}- probe. The filter was again washed at low stringency, but this time no hybridization of the probe was seen despite exposure to film for 72 hours. In order to assess loading of the RNA, an 18S human ribosomal 49mer oligonucleotide probe corresponding to bases 364 to 413, was end-labelled with [γ-^{32}P] ATP as described in materials and methods. The sequence of the probe was 5' cgtggtcaccatggtaggcacggcgactaccatcgaaagttgatagggc 3'. This probe was hybridized overnight and washed the following morning at high stringency using two washes of 2X SSC/0.1%SDS, one wash of 1XSSC/0.1% SDS and one wash of 0.1XSSC/0.1%SDS. The filter was exposed to radiographic film for 6 hours and the resultant hybridization pattern is shown in Figure 5.10. This indicated that there was equal loading of RNA from both cell lines and therefore, that the α_{1b}- probe hybridized more strongly to the α_{1b}- expressing cell line rather than the α_{1a}- cell line, although some cross-reactivity was demonstrated.

A second membrane was prepared, this time using 20μg of RNA from both dog brain and dog prostate. Again the α_{1a}- probe did not hybridize to either lane. Figure 5.11 shows the results after a 72 hour exposure, when the α_{1b}- probe was used. In this case, the blots were washed to moderate stringency using two washes of 2X SSC/0.1%SDS and one wash of 1XSSC/0.1% SDS. The α_{1b}- probe hybridized to both lanes but with greater intensity for the dog prostate RNA. This time two different size transcripts of ≈5Kb and ≈3Kb, were seen on the film. The membrane was stripped using boiling 0.1%SDS, and this time a GAPDH cDNA was random-labelled and hybridized overnight to assess loading of RNA. Results of a 72 hour exposure are illustrated in Figure 5.11. In this case, the general intensity of the hybridization is low but the band for the dog prostate does appear stronger which may account for the increased intensity of labelling seen with the α_{1b}- probe at the prostate tissue.

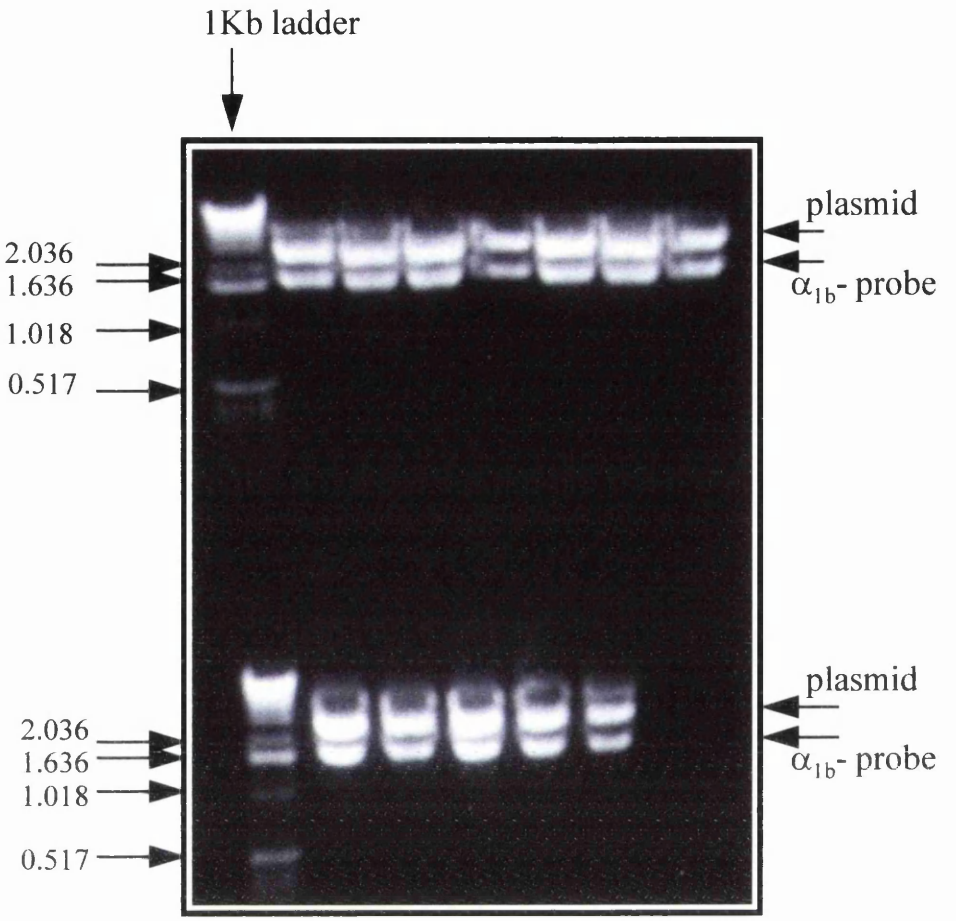


Figure 5.9. Photograph of gel showing the colonies containing the α_{1b}- insert. Plasmid was purified from the colonies and subsequently digested with Eco R1. Digests were then run on a gel and it can be seen that all plasmid preps contain the insert. The 1Kb ladder has been run in lane 1 of both the top and bottom row, and figures on the left show size markers in Kb.

Figure 5.10. Northern blot of human α_{1a}- and α_{1b}- expressing cell line RNA, hybridized to the α_{1b}-, and ribosomal probes. Panel 1 shows the pattern of hybridization of the α_{1b}- probe after a 24 hour exposure. Panel 2 shows the subsequent hybridization pattern of an 18S ribosomal oligonucleotide probe after a 6 hour exposure.

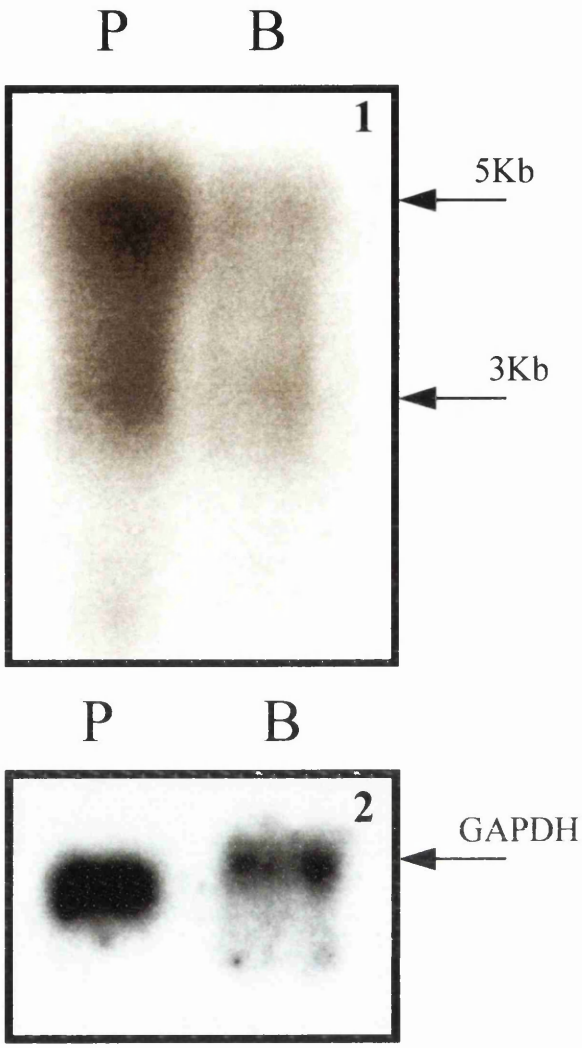


Figure 5.11. Northern blot of canine RNA hybridized to the α_{1b}- and GAPDH probes. P = prostate, B = brain. Panel 1 shows the pattern of hybridization with the α_{1b}- probe after a 72 hour exsposure. Panel 2 shows the pattern of hybridization with the GAPDH probe after a 72 hour exposure.

5.2 Discussion

In this study, native receptors are identified with uppercase letters and cloned receptors with lower case letters in accordance with the suggestions of the α_1-adrenoceptor nomenclature committee (Hieble et al. 1995). To reiterate from Chapter 1, three subtypes of native α_1-adrenoceptor have been identified by functional and radioligand binding studies. These are named α_{1A}-, α_{1B}-, and α_{1D}- (Hieble et al. 1995). These correspond to the three cloned α_1- adrenoceptors, α_{1a}-, α_{1b}-, and α_{1d}-. To date, the cloned receptors have been identified and sequenced in a number of species. However, with the exception of the partial α_{1b}- adrenergic sequence isolated by Libert et al (1989), none of the canine α_1- adrenergic sequences have been determined.

From the functional studies carried out on both the DSV and the subcutaneous resistance arteries in this study, there appeared to be a role for the α_{1L}-adrenoceptor in functional responses of both these vessels. The α_{1L}-adrenoceptor (Muramatsu et al. 1995; Flavahan and Vanhoutte, 1986a) does not fall neatly into the classification outlined above and although it has been identified functionally in a number of tissues, its relation to the cloned receptors is not clear. Despite this, there is convincing evidence to suggest that the α_{1a}-adrenoceptor is responsible for the functional α_{1L}-. Ford et al (1997a; 1996b), showed that while binding experiments on CHO-K1 cells expressing the human α_{1a}-adrenoceptor, gave a binding pattern characteristic of the classical α_{1A}-adrenoceptor (high affinity for prazosin, RS 17053, WB 4101, niguldipine and 5 methylurapidil), assays measuring inositol phosphate accumulation, with the same range of antagonists in the same cell line, revealed that the profile changed to that more characteristic of the α_{1L}-adrenoceptor, (low affinity for prazosin, RS 17053, niguldipine and WB 4101). In addition, it has been shown that by altering the media and using whole cells rather than membranes, it is possible to alter the binding characteristics of

CHO-K1 cells expressing the human α_{1a}-adrenoceptor, to give a profile more closely resembling that seen for functional studies at the so called α_{1L}-adrenoceptor (Williams et al. 1996). This alteration could not simply be explained by a lowering of all values since the effect was not seen with other subtypes of α_1-adrenoceptors and only certain antagonists showed lower values under the changed conditions. In addition, a novel fourth isoform of the human α_{1a}-adrenoceptor ($\alpha_{1A\text{-}4}$), has recently been identified (Chang et al. 1998). This isoform together with the other three isoforms previously described, (Hirasawa et al. 1995), have been transfected into cell lines. When antagonist experiments were carried out measuring inositol phosphate accumulation, all isoforms displayed the α_{1L}-adrenoceptor profile, with a low affinity for prazosin, RS-17053 and WB 4101 (Ford et al. 1997b; Chang et al. 1998).

With this in mind and with the results of the functional experiments, it seemed logical to investigate expression and functional characteristics of the canine α_{1A}-adrenoceptor in more detail. The first step was to identify and determine the sequence of this receptor from canine tissue.

The sequence homology, even at the nucleotide level, is very highly conserved among the α_1-adrenoceptor subtypes for different species (Graham et al. 1996). Based on this fact, it seemed reasonable to attempt to amplify the α_{1A}-adrenoceptor from canine tissue cDNA, using primers based on human and bovine sequences.

Unfortunately, when primers were designed to amplify the entire coding region for the receptor, a product corresponding to an adrenergic receptor sequence was not obtained. The reason for this is not clear but several possibilities exist.

1. The fact that the initial primer sets amplified titin, and the second set of primers failed to amplify anything, may indicate very low levels of expression or complex secondary structures preventing primer annealing and extension. With the second sets of primers,

in addition to altering many of the parameters such as cycle number, time and temperature as already detailed, reactions were carried out in the presence of varying concentrations of dimethylsulphoxide (DMSO), which should have helped to disrupt secondary structures if present (personal communication from Dr Robert Heeley).

2. Hirasawa et al (1995) and Chang et al (1998) have identified isoforms of the human α_{1a}-adrenergic receptor. These isoforms are identical apart from their carboxy termini, where the sequence diverges. If a similar situation exists for the canine receptor, then this could have contributed to the failure to amplify the entire sequence and may explain the fact that it was possible to amplify a partial sequence, once a new downstream primer had been designed, which corresponded to an area within the putative VI transmembrane spanning domain, rather than one where the downstream primer lay within the carboxy terminal region.

3. Another reason for failure to amplify the full length clone may have been in the primer design. In polymerase chain reactions the most important aspect contributing to the success of the reaction is the design and choice of the primers. In the design of primers for this study, several recommendations were followed (Sharrocks, 1994). Primers were designed from areas of high homology, length of the primers was kept between 18 and 30 bases, the AT to GC ratio was kept roughly 1:1 to maintain a similar melting temperature (T_m) for all the primers and where possible, primers were designed such that there was a "GC clamp" on the 3' end of the primer. In addition, all primers were checked for the absence of self homology and homology with their downstream counterpart, using a primer checking programme.

When the partial receptor was sequenced it was found to be over 90% homologous, at the nucleotide level, to the human and bovine α_{1a}-adrenergic sequences, compared to only approximately 70-80% homologous to either published α_{1b}- or α_{1d}- adrenergic

receptor sequences. This provided strong evidence that the sequence amplified did represent the canine α_{1a}-adrenergic receptor.

Rat-1 fibroblasts stably expressing the human α_{1a}- and α_{1b}-adrenergic receptors were available, and because of the high interspecies homology between the subtypes of adrenergic receptor, it was decided to use these cell lines to test the suitability of the α_{1a}-clone and the α_{1b}- clone, as probes for the identification and quantification of these two subtypes in tissue, since the cell lines could act as positive and negative controls.

Unfortunately, the α_{1a}-probe failed to hybridize to either cell line and also failed to hybridize to RNA prepared from canine prostate and canine brain, despite the fact that the clone had originated from RNA prepared from canine prostatic tissue. On the other hand, the α_{1b}- probe hybridized to both canine prostatic and brain tissue. However, despite the fact that hybridization was markedly greater at the α_{1b}- cell line versus the α_{1a}- cell line, the fact that the probe did hybridize to the α_{1a}- cell line would indicate that this probe is not subtype specific.

Some possible explanations for the failure of the α_{1a}- probe to identify transcripts are:

1. Northern blotting has been used to examine expression of α_1-adrenoceptor subtypes in other studies (Stewart et al. 1994; Schwinn et al. 1991; McGehee et al. 1990; Lomasney et al. 1991; Laz et al. 1994; Deng and Cornett, 1998; Alonzo-Llamazares et al. 1995; Beaulieu et al. 1997). Despite this, the levels of expression of these receptors is known to be low. Graham et al (1996) and Schwinn et al (1990), failed to identify α_{1A}- expression in tissues with functional α_{1A}- characteristics using the same technique. More sensitive methods are available and have been used, such as RNAse protection assays (Nasu et al. 1996; Moriyama et al. 1997; Price et al. 1994) and competitive RT-PCR (Chang et al. 1998; Faure et al. 1995). Even the use of polyA+ enriched mRNA

rather than total RNA may be useful. Although these techniques were out with the scope of this study, they may be investigated in the future.

2. From verbal communication with Dr Janet MacKenzie, problems were encountered with the rat-1 fibroblast cell lines as regards loss of expression of the adrenergic receptors, subsequent to the Northern blotting experiments. If the levels of expression in the cell lines had been falling this may have explained the inability of the α_{1a}- probe to identify transcripts in the RNA from these cells.

3. The α_{1a}- probe itself may have been the problem. Lack of homology would be the most likely reason for a probe to fail to work. This is an unlikely explanation in this case due to the homology of > 90% between the probe sequence and the human α_{1a}-sequence.

4. Expression of the α_{1a}-adrenoceptor may be very low in the canine tissues that were used for the Northern analysis. Prostate was used because this was the tissue from which the clone was derived and from binding and functional studies, the canine prostate has been characterised as having primarily either α_{1A}- or α_{1L}-adrenergic receptors (Testa et al. 1997; Knepper et al. 1995; Goetz et al. 1994). Brain was also included since human brain tissue has been shown to have α_{1A}-adrenoceptors (Price et al. 1994; Faure et al. 1995; Hirasawa et al. 1995), and while species differences do exist in distribution of receptor subtypes, often there are similarities.

In the case of the α_{1b}- probe, in the cell lines the probe only identified a single size of mRNA transcript (≈5Kb), whereas in the canine tissue the probe hybridized to two different sized mRNAs, namely a band ≈5Kb and a second band ≈3Kb. Different sizes of mRNA transcripts for α_1-adrenoceptors have been reported previously. In a study by Beaulieu et al (1997), four transcript sizes were observed for the α_{1D}-adrenergic receptor

in hamster hearts and Stewart et al (1994) identified three transcript sizes for the α_{1A}-adrenoceptor in rat heart and brain.

Libert et al (1989) used the same canine α_{1b}- probe that was used in this study, in a variety of canine tissues. They observed a tissue specific variation in the size of the transcripts identified with two transcripts of 4.8 and 2.6Kb in canine lung, two transcripts of 4.1 and 2.6Kb in canine heart and only a single transcript of 4.8Kb in canine stomach. As suggested by Libert et al, in the paper cited, and as already suggested in this discussion, this pattern may be due to lack of subtype specificity of the probe and thus, the probe may be identifying the presence of more than one subtype in the canine tissues. This could explain why only a single band was seen in the cell lines where only a single subtype is being expressed. Another explanation is, that it has been shown that in the 5' untranslated region of the α_{1B}-adrenoceptor, there are a number of translation initiation sites. In addition, an intron in the 5' untranslated region of the rat α_{1b}-adrenergic receptor also gives rise to alternative splicing (Jones et al. 1997; Graham et al. 1996). This would give rise to α_{1B}- transcripts of varying size.

In conclusion, from this study, part of the canine α_{1a}- adrenergic receptor has been identified and sequenced. The homology to both the human and bovine α_{1a}-adrenergic receptors is high (> 90%). Using Northern analysis, this probe was unsuccessful in identifying expression of α_{1A}-adrenergic receptor transcripts. The most likely explanation for this is probably due to low expression levels and lack of sensitivity of the technique. While hybridization with the α_{1b}- probe was achieved, the subtype selectivity of this probe was unconvincing due to cross-reactivity with the α_{1a}- cell line. With high homology even between different subtypes this will always be a potential problem.

For the future it will be desirable to isolate the full receptor. The partial sequence may be used as a probe to screen a canine cDNA library. Using this method it will be interesting to see if isoforms of this receptor exist as they do for the human α_{1a}-. In addition, a more sensitive method of detection will be adopted to examine tissue expression of the subtypes, such as semi-quantitative RT-PCR or RNase protection. The former may be particularly useful where small amounts of tissue (particularly blood vessels) are being used. With the full length sequence information it will be possible to generate more subtype-selective primers and probes in an attempt eliminate the problem of non-subtype selectivity.

CHAPTER 6

Functional characteristics of dog saphenous vein, dog femoral artery and dog subcutaneous resistance artery, and the effects of naturally occurring heart failure on these characteristics

6.0 Abstract

6.1 Method

6.1.1 Saphenous vein and femoral artery

6.1.2 Subcutaneous resistance arteries

6.1.3 Histology and immunohistochemistry

6.2 Results

6.2.1 Saphenous vein

6.2.2 Femoral artery

6.2.3 Subcutaneous resistance arteries

6.3 Discussion

6.0 Abstract

The objective of this study was to carry out a preliminary investigation into the functional characteristics of several isolated blood vessels from control and heart failure dogs. The literature concerning changes occurring in heart failure is conflicting probably due to species variation, drug treatment in some of the human studies and variation in the severity and duration of the heart failure.

To my knowledge, this is the first study to have examined isolated blood vessels from dogs with naturally occurring heart failure. The main findings of this study were as follows. There was a decreased sensitivity to noradrenaline in both the saphenous vein and femoral artery, but not the subcutaneous resistance artery, in heart failure animals. There was a significant increase in maximal response to potassium chloride in femoral arteries and resistance arteries from failure animals, while the maximal response to noradrenaline was only significantly greater in the femoral artery. There was no impairment of acetylcholine mediated relaxations in heart failure in any of the vessels studied, and neuronal reuptake of noradrenaline was most marked in the saphenous vein where it was not impaired in heart failure.

6.1 Methods

6.1.1 Saphenous vein (DSV) and femoral artery (FA)

5mm sections of vessel were taken from control and heart failure animals, mounted in 10ml organ baths, normalised and the starting protocol completed as described in materials and methods. Vessels were maintained in Krebs' solution and no blocking agents used unless otherwise stated.

Two rings were mounted from each vessel. After a forty minute equilibration period, a cumulative concentration response curve to noradrenaline was performed in all rings, starting with a concentration of 1nM and increasing up to a concentration of 1mM if required. Once a maximal contraction had been achieved, vessels were washed until they returned to baseline. At this point 1μM of the uptake-1 blocking agent cocaine, was added to the bath of one of the rings. Forty minutes later a second concentration response curve to noradrenaline was performed in this ring. The second ring was also allowed a forty minute recovery period. Subsequent to this, the vessel was precontracted with 1μM noradrenaline. Once the contraction had reached a steady state, a cumulative concentration response curve was performed to acetylcholine using a concentration range of 1nM to 10μM acetylcholine.

6.1.2 Subcutaneous resistance arteries (DSCRA)

Sections of resistance artery approximately 2mm in length and approximately 260 ± 11 microns in diameter (n = 40), were mounted in Mulvany Halpern wire myographs, normalised to 0.9 of L100 and the starting protocol completed as described in the materials and methods. From this time on vessels were maintained in Krebs' solution and no blocking agents used unless stated otherwise. Four vessels were set up in parallel from each animal. An initial concentration response curve to noradrenaline was

performed in all vessels starting with a concentration of 1nM and increasing up to a concentration of 1mM if required. Once a maximal contraction had been achieved, vessels were washed until they returned to baseline. At this point, vessel one was assigned as a time control, 1μM cocaine was added to the bath of vessel two, 100μM L-NAME (N^{ω}-Nitro-L-Arginine Methyl ester hydrochloride) was added to the bath of vessel three, and a combination of 1μM cocaine and 100μM L-NAME was added to the bath of vessel four. After a forty minute period a second concentration response curve to noradrenaline was performed. Vessels were again washed until baseline was reached. Finally, forty minutes after the second curve, the time control and the cocaine assigned vessel were precontracted with 1μM noradrenaline and once a steady state had been attained, a concentration response curve to acetylcholine was performed as for the DSV and FA.

In the DSV and the FA, concentration response curves to noradrenaline were curve fitted using GraphPad Prism as described in Chapter 2, allowing the derivation of maximum, pEC_{50} and Hill slope parameters from the curve fitting. Results were expressed as a percentage of the first concentration response curve maximum.

As discussed in Chapter 4, DSCRA were not amenable to curve fitting and therefore all data were analysed on Microsoft Excel spreadsheets and parameters derived by interpolation. Results were again expressed as a percentage of the first curve maximum.

Concentration response curves to acetylcholine for all vessels were analysed on Microsoft Excel spreadsheets and pEC_{10}, pEC_{25} and pEC_{50} values derived from interpolation. Results were expressed as a percentage of the contraction to 1μM noradrenaline.

All parameters were compared using paired or unpaired Students t test. A $P< 0.05$ was taken to be statistically significant and n = the number of experiments unless otherwise stated.

6.1.3 Histology and Immunohistochemistry

In order to assess the sympathetic innervation of the vessels being studied, immunohistochemistry was performed using anti-neuropeptide Y (NPY) and anti-tyrosine hydroxylase antibodies. The materials and methods for this are given in section 2.5 of materials and methods.

6.2 Results

6.2.1 Saphenous Vein

Values were derived from eight control animals and seven heart failure animals. Firstly, in the control group, noradrenaline caused a concentration-dependent increase in tone with a pEC_{50} of 5.93 ± 0.07 (n = 8). In the presence of 1μM cocaine, there was a left shift and a lowering of the maximum of the concentration response curve (Figure 6.1). This resulted in a significant shift in the pEC_{50} value (P of < 0.001) to 6.35 ± 0.07 (n = 8) and a significant decrease in the maximum (P of 0.0025) from 102.6 ± 0.56% to 87.56 ± 3.4%. Hill slope parameters did not alter significantly (0.94 ± 0.03, n = 8 with and without cocaine).

In the case of the heart failure group, a mean pEC_{50} value of 5.69 ± 0.12 (n = 7) was obtained for noradrenaline. As in the control group, cocaine caused a left shift in the concentration response curve, giving a mean pEC_{50} of 6.03 ± 0.12 (n = 7) which was significantly different (P of 0.015). From the mean graphs (Figure 6.2), there also appeared to be a depression in the maximum, but this was not statistically significant.

When the control and heart failure group were compared, the curves from the failure group appeared to be shifted to the right when compared to the controls (Figure 6.3). Curves were compared at the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values. The difference between the two groups was only significant at the pEC_{10} and pEC_{25} levels (P of 0.03 and 0.04 respectively). When the two groups were again compared after the addition of cocaine (Figure 6.4), their relative relationship had not changed except that the difference between the two groups was now only significant at the pEC_{10} value and no longer significant at the pEC_{25} value (P of 0.049 and 0.068 respectively).

Maximum responses to noradrenaline and 125mM potassium chloride were not significantly different between the two groups (P of 0.473 and 0.598 respectively).

Responses to acetylcholine were quite variable in both groups but overall acetylcholine only induced slight relaxations in the saphenous vein, with tone on maximal relaxation still comprising 69.9 ± 10.5 % (n = 7) of the tone produced by 1μM noradrenaline in the control group and 71.3 ± 11.4 % (n = 6) in the failure group. These were not significantly different. The two groups were also compared at the pEC_{10} value and again there was no significant difference. Due to the poor response to acetylcholine in this vessel, pEC_{25} and pEC_{50} values could not be derived for the majority of vessels. Graphs for acetylcholine concentration response curves are shown in Figure 6.5.

Cross sections of the saphenous vein were stained for the presence of the noradrenergic co-transmitter, neuropeptide Y (NPY), and the enzyme tyrosine hydroxylase (TH), which is involved in the enzymatic synthesis of noradrenaline from tyrosine. From the panels in Figure 6.6, it can be seen that the staining for NPY and TH is abundant and extends right into the media of the vessel. This indicates that the saphenous vein has a rich sympathetic innervation.

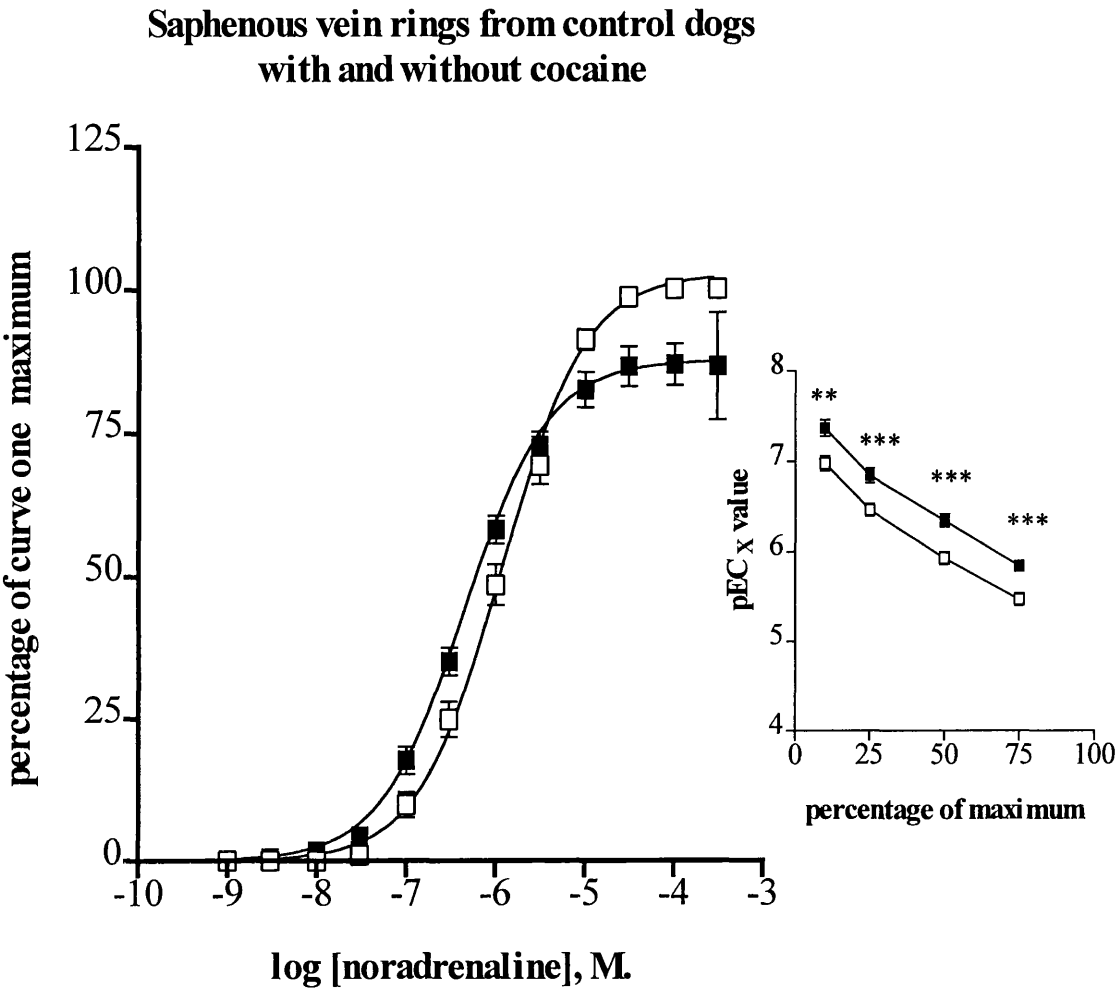


Figure 6.1. Concentration response curve data to noradrenaline in the dog saphenous vein from control animals. Graphs were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed on mean curves. Noradrenaline (□), n = 8; noradrenaline + 1μM cocaine (■), n = 8. The inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison before and after the addition of cocaine. The symbol *** denotes a P value of < 0.001 and the symbol ** denotes a P value < 0.01.

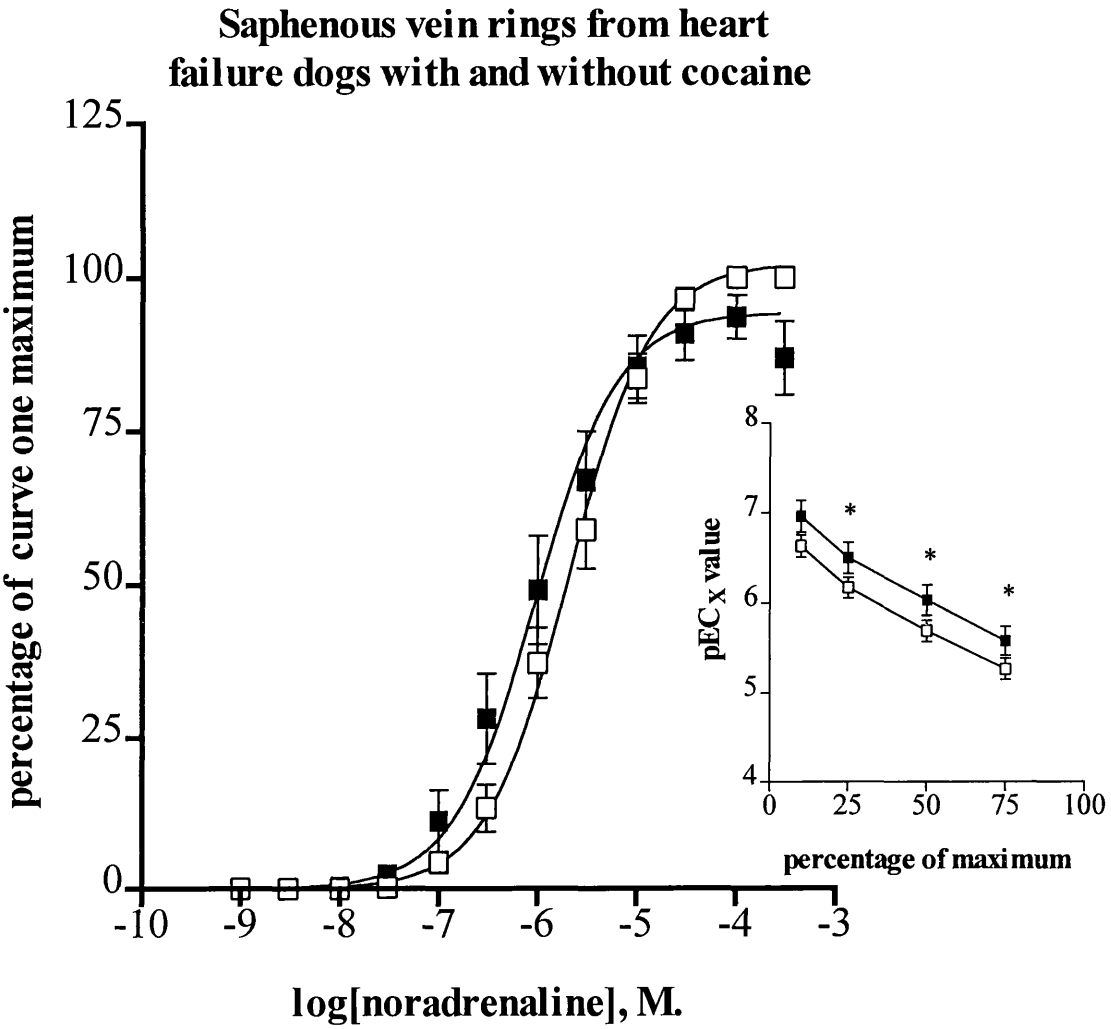


Figure 6.2. Concentration response curve data to noradrenaline in the dog saphenous vein from heart failure animals. Graphs were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed on mean curves. Noradrenaline (□), n = 7; noradrenaline + 1μM cocaine (■), n = 7. The inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison before and after the addition of cocaine. The symbol * denotes a P value < 0.05.

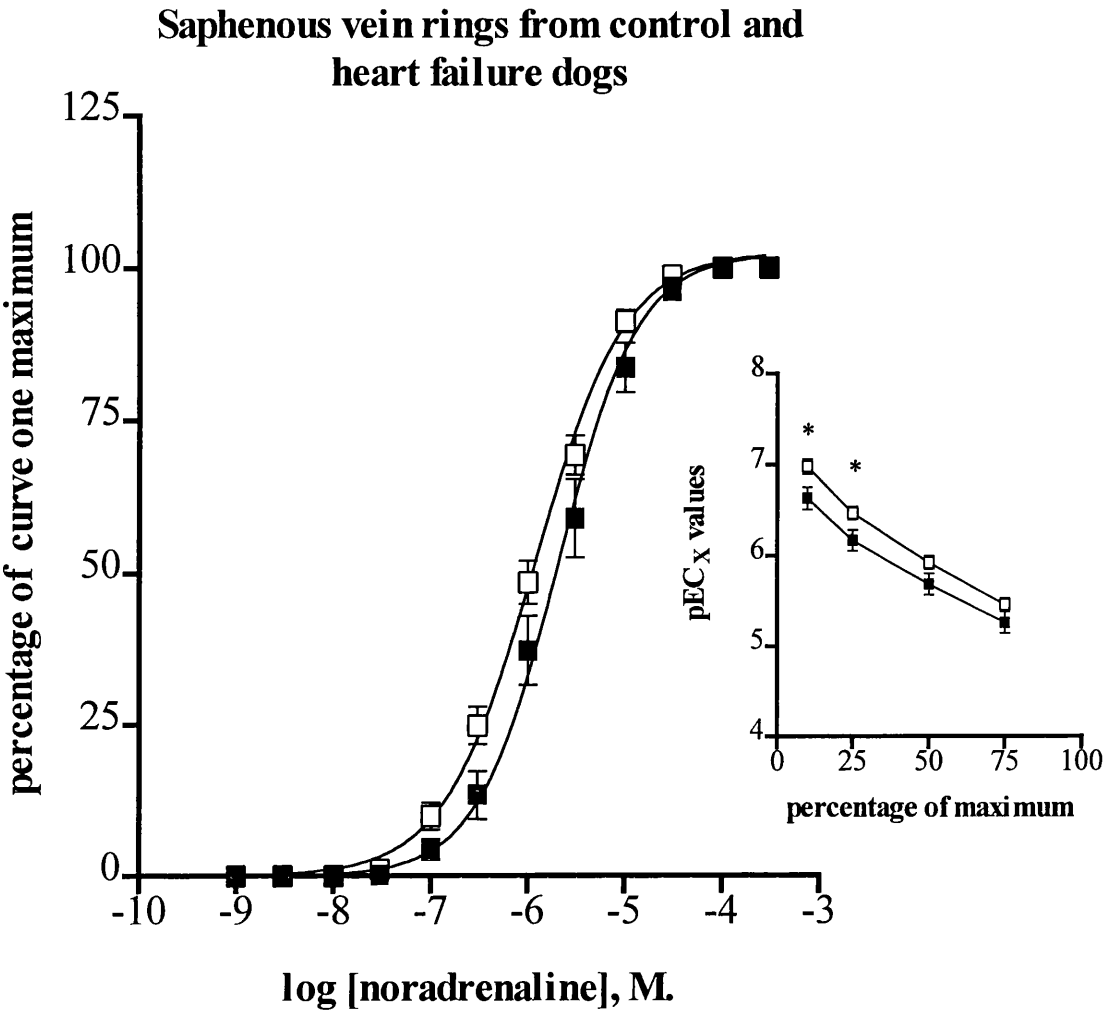


Figure 6.3. Concentration response curve data to noradrenaline in saphenous vein rings from control and heart failure animals. Curves were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed onto the mean curves. Control animals (□), $n = 8$; heart failure animals (■), $n = 7$. The inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison between the two groups. The symbol * denotes a P value < 0.05.

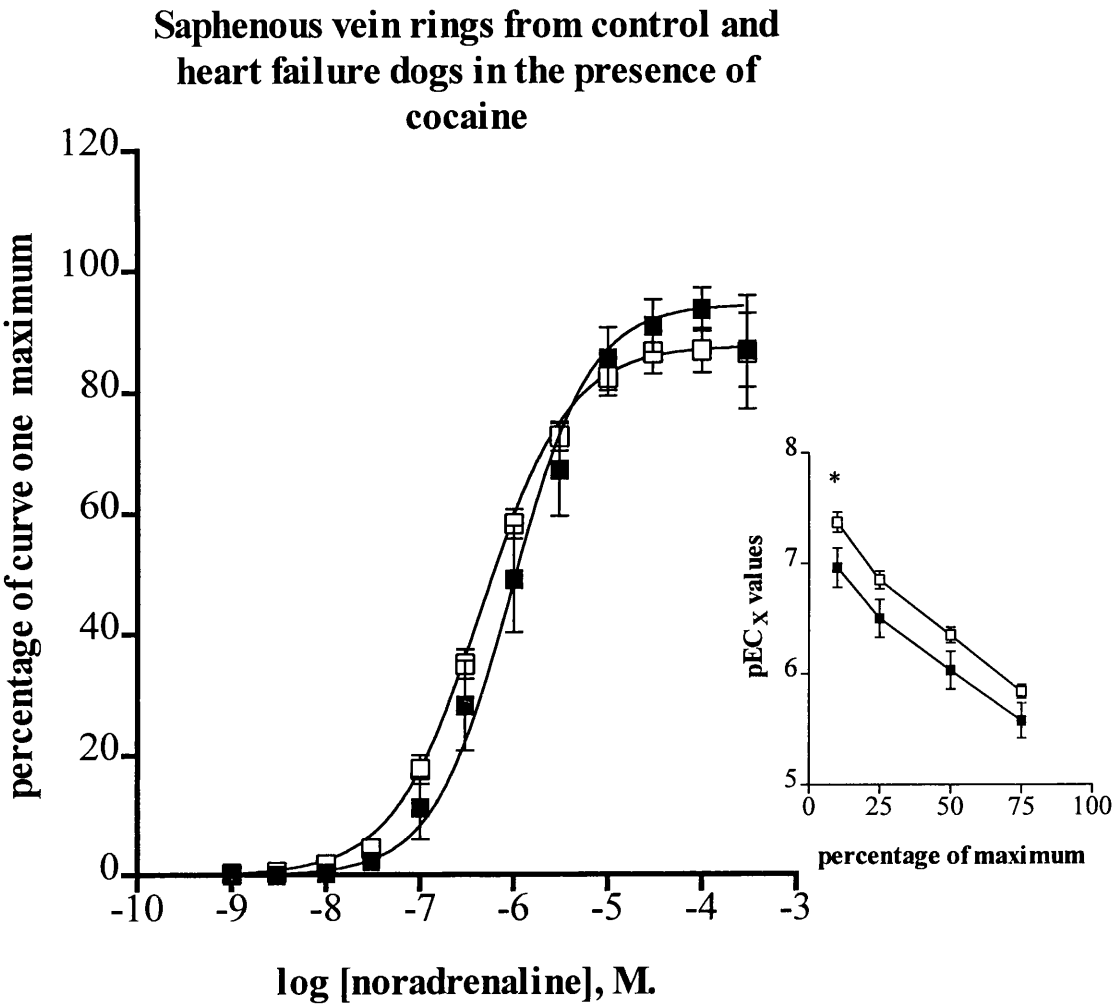


Figure 6.4. Concentration response curve data to noradrenaline in saphenous vein rings from control and heart failure animals after the addition of 1μM cocaine. Curves were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed onto the mean curves. Control animals (□), n = 8; heart failure animals (■), n = 7. The inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison between the two groups. The symbol * denotes a P value < 0.05.

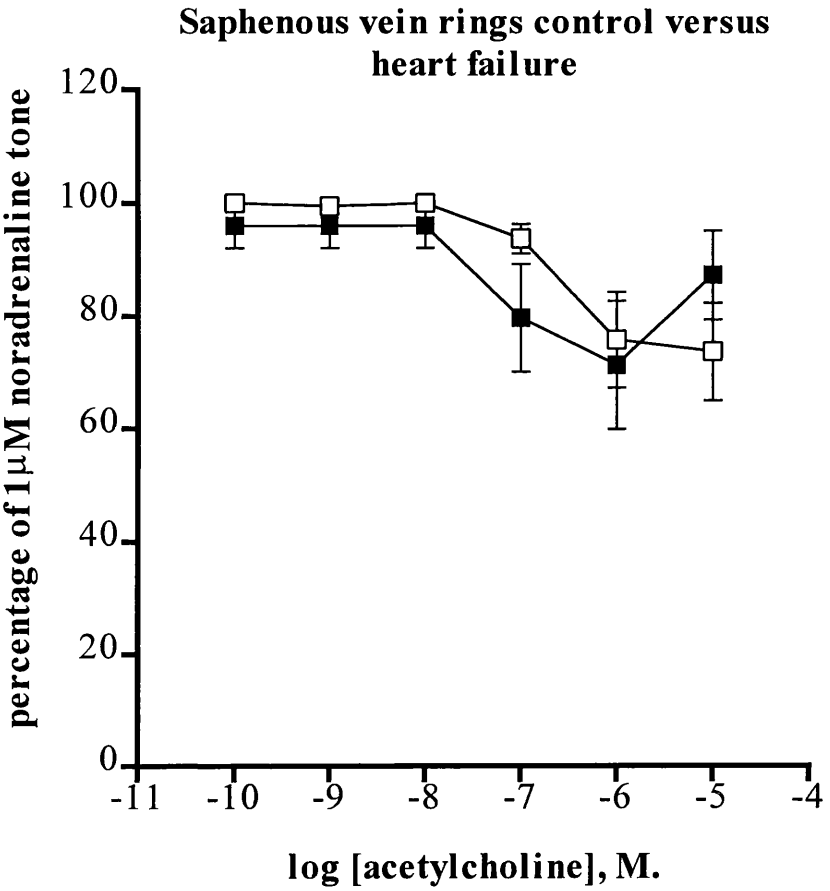


Figure 6.5. Concentration response curve data to acetylcholine from control and heart failure dogs. Results are expressed as a percentage of the tone produced by 1μM noradrenaline. Data points represent mean ± s.e. mean. Control animals (□), $n = 7$; heart failure animals (■), $n = 6$.

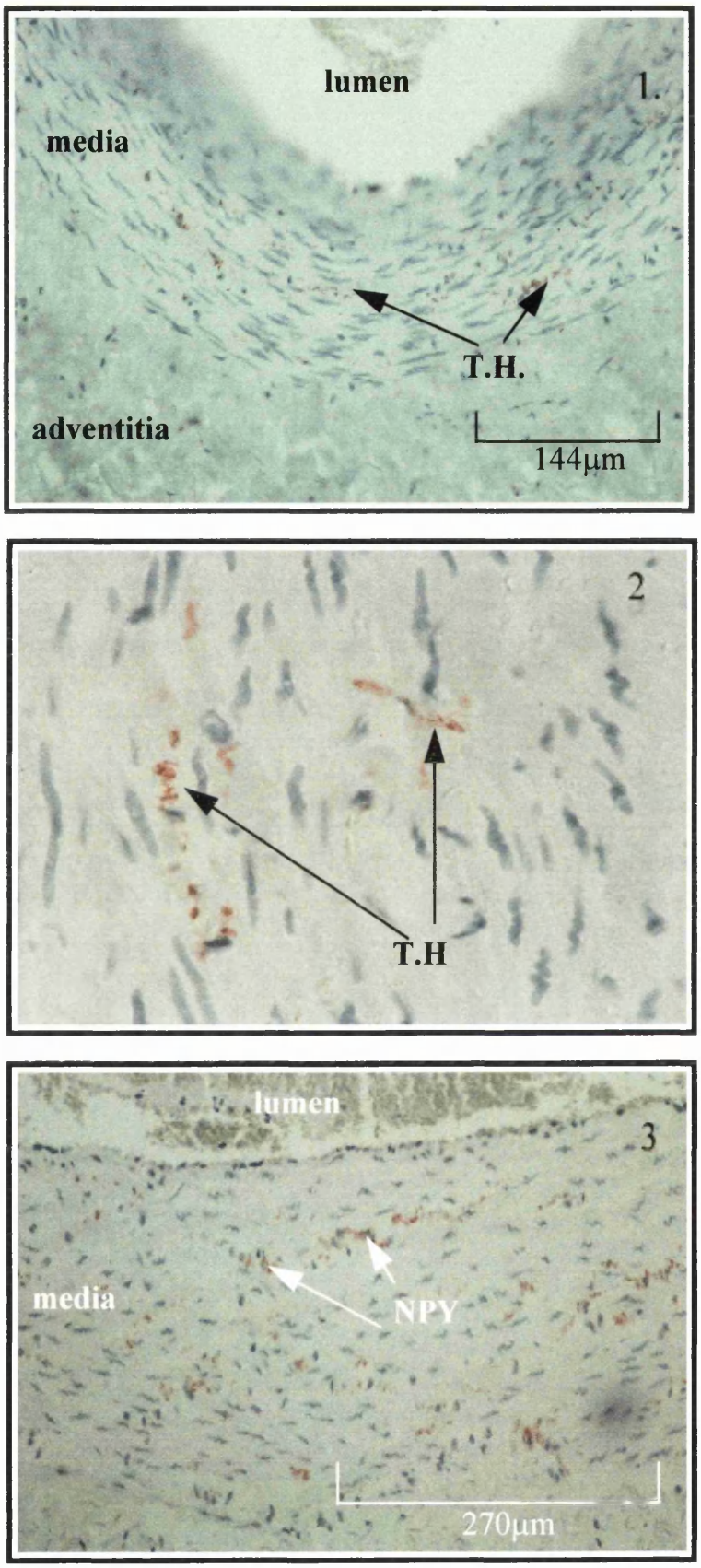


Figure 6.6. See next page for text ⟶

Figure 6.6. Saphenous vein immunohistochemistry. Panel 1 shows a cross section of the saphenous vein, showing areas of tyrosine hydroxylase (TH) staining seen as red. Panel 2 also shows tyrosine hydroxylase staining but under higher magnification . The area shown in this section comprises the media of the vessel. Panel 3 shows a cross sectional area of the saphenous vein showing areas containing NPY, again seen as areas of red staining which occur right throughout the media of the vessel. These panels illustrate that the saphenous vein is richly innervated. The distribution of nerves in this vessel is also unusual in that generally innervation only extends as far as the adventitial/medial border.

6.2.2 Femoral artery

Values were derived from eight control and seven heart failure animals.

In the femoral artery from the control group, the mean pEC_{50} for the noradrenaline concentration response curves was 6.05 ± 0.07 (n = 10). After the addition of 1μM cocaine the concentration response curve shifted to the right with a pEC_{50} of 5.82 ± 0.04 (n = 10) and there was a reduction in the maximum from 100.4 ± 0.3 % to 82.94 ± 4.35 % (n = 10). Both parameters were significantly different before, versus after the addition of cocaine, with P values of 0.02 and 0.003 for pEC_{50} and maximum values respectively. Mean Hill slope values before and after the addition of cocaine were also significantly different (P of 0.0001), with a Hill slope of 1.02 ± 0.03 without cocaine and 1.28 ± 0.02 with cocaine (n = 10). Mean data are illustrated in Figure 6.7.

In the heart failure group, noradrenaline caused contraction of the femoral artery with a pEC_{50} value of 5.54 ± 0.13 (n = 8). The addition of 1μM cocaine had no significant effect on the maximum, pEC_{50} or Hill slope of the noradrenaline curve (Figure 6.8).

When a comparison was made of the noradrenaline CRC data between the control and heart failure groups, the curve from the heart failure animals appeared to be noticeably shifted to the right. The difference between the two curves was significant at the level of the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values with P values in all cases of 0.002. When active effective pressures were compared for maximal response to both noradrenaline and 125mM potassium chloride, these were found to be significantly different in both groups (P of 0.0042 and < 0.001 respectively), with the following values: Maximum noradrenaline response in the control group of 3.64 ± 0.31 KPa, (n =10); maximum noradrenaline response in the failure group of 5.11 ± 0.35 KPa, (n = 8); maximum potassium chloride response in the control group of 2.18 ± 0.26 KPa, (n = 10); maximum potassium chloride response in the failure group of 4.46 ± 0.25 KPa, (n = 8).

Graphs are illustrated in Figure 6.9. Maximum values only appear different in the lower graph (B), since these values are expressed as active effective pressure, whereas the upper graph results (A) are expressed as a percentage of the noradrenaline CRC maximum.

After the addition of cocaine, there was no longer a significant difference between the groups at the pEC_{10}, pEC_{25}, pEC_{50} or pEC_{75} values. However, the difference between the maximum values was accentuated because of the reduction in maximum seen only in the control group in the presence of cocaine (Figure 6.10). Again the bottom graph (B) illustrates results expressed as active effective pressure, while the top graph illustrates results expressed as a percentage of the noradrenaline CRC maximum (A).

In the femoral artery, acetylcholine caused a maximal relaxation of 70.93 ± 6.6% (n = 9) of the response to 1μM noradrenaline in the control group, and 68.14 ± 13.13% (n = 6) in the failure group. These values were not significantly different when values from individual experiments were compared by Students t test. In addition, there was no significant difference for pEC_{10}, pEC_{25} and pEC_{50} values between the two groups. Acetylcholine had a pEC_{50} value of 6.58 ± 0.4 (n = 6) in the control group, and 6.71 ± 0.56 (n = 4) in the failure group. Mean CRC data is illustrated in Figure 6.11.

In contrast to the DSV, when immunohistochemistry was performed on sections of the femoral artery, for the presence of tyrosine hydroxylase and NPY, little staining was seen. This is illustrated by panels 1 and 2 in Figure 6.12. This indicates that the femoral artery has poor sympathetic innnervation.

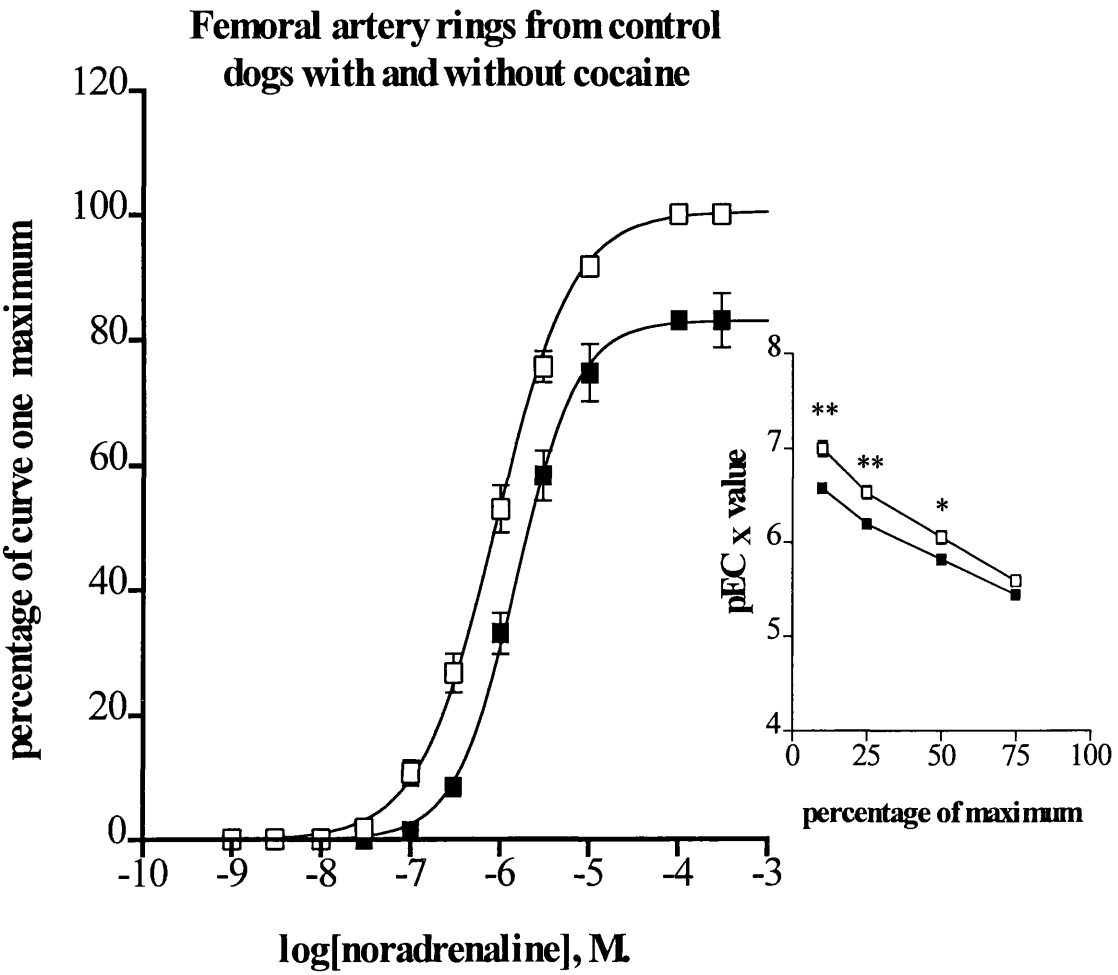


Figure 6.7. Concentration response curve data to noradrenaline in the dog femoral artery from control animals. Graphs were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed on mean curves. Noradrenaline (□), n = 10; noradrenaline + 1μM cocaine (■), n =10. The inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison before and after the addition of cocaine. The symbol ** denotes a P value < 0.01 and the symbol * denotes a P value < 0.5.

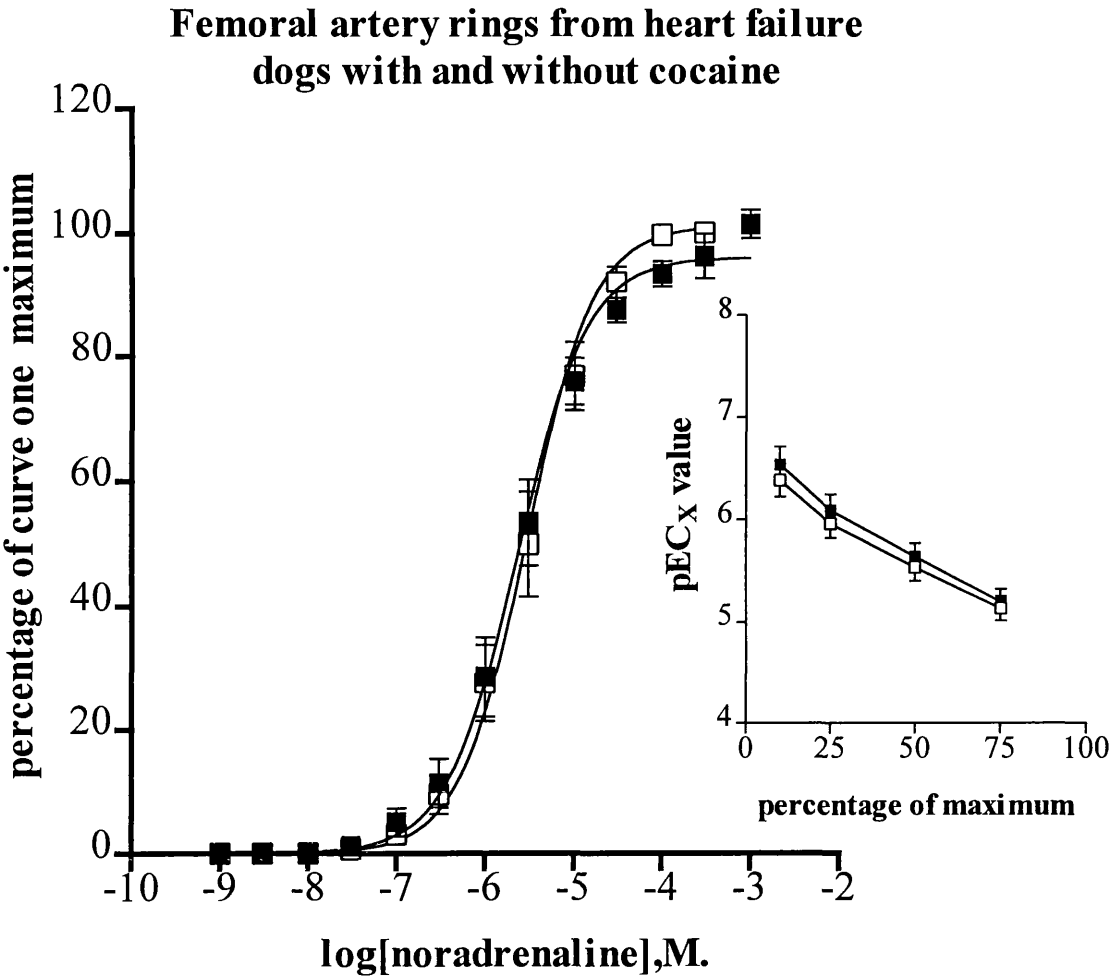


Figure 6.8. Concentration response curve data to noradrenaline in the dog femoral artery from heart failure animals. Graphs were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed on mean curves. Noradrenaline (□), n = 8; noradrenaline + 1μM cocaine (■), n =8. The inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison before and after the addition of cocaine.

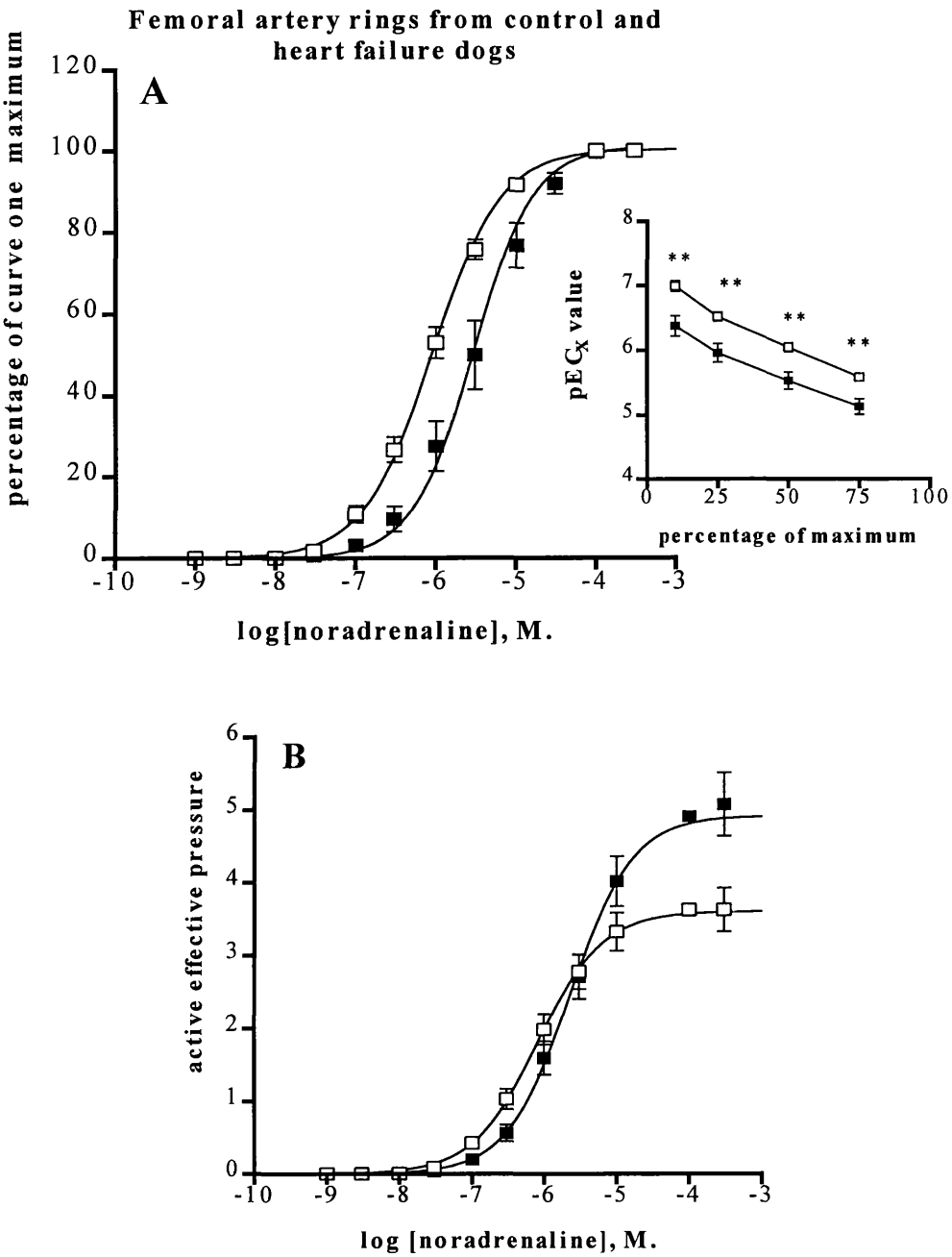


Figure 6.9. Concentration response curve data to noradrenaline in the dog femoral artery from control and heart failure animals. Graphs were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed on mean curves. Control animals (□), n = 10; heart failure animals (■), n =8. In graph A, the inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison between the two groups. The symbol ** denotes a P value < 0.01. Graph B shows the same CRC data expressed as active effective pressure (KPa).

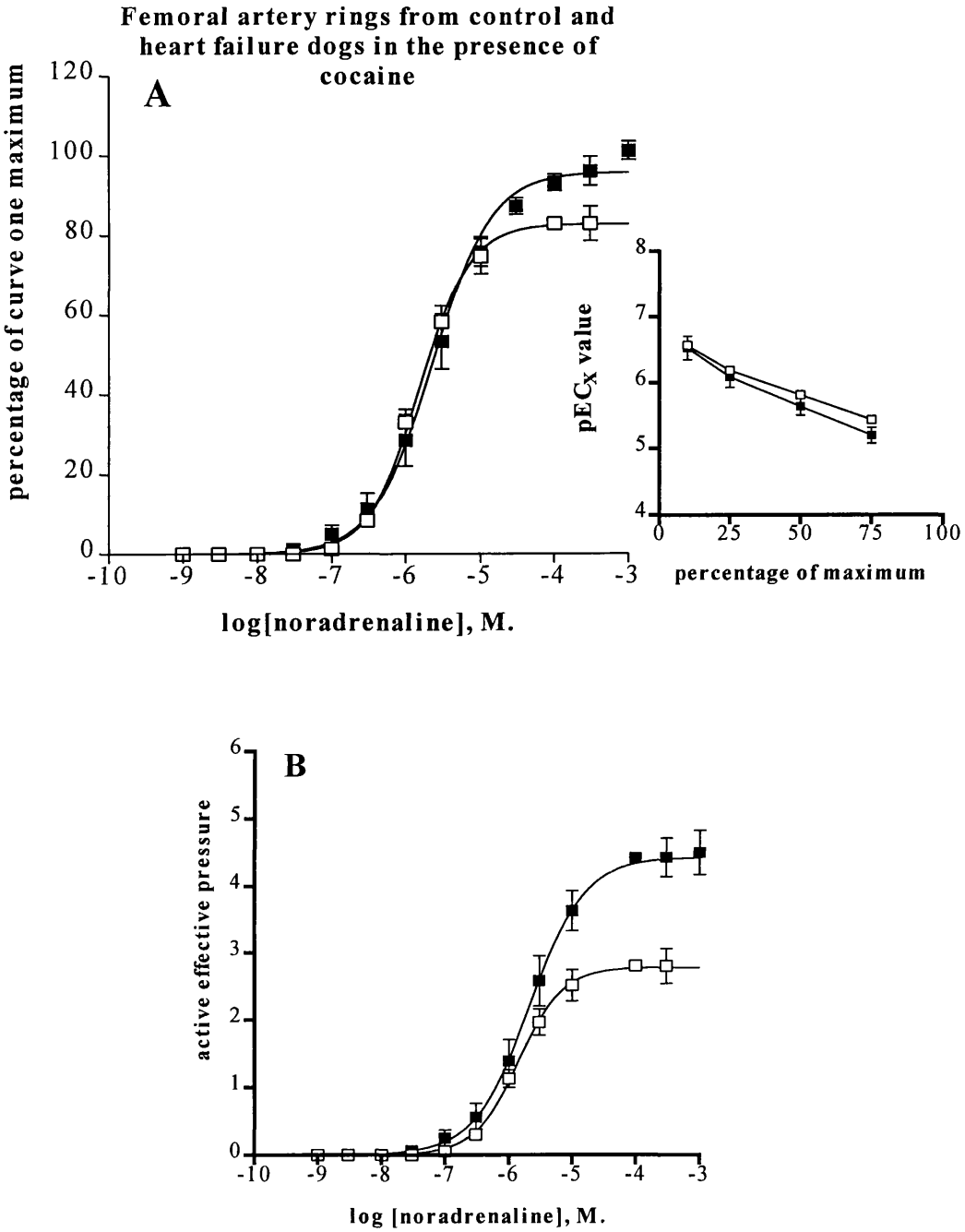


Figure 6.10. Concentration response curve data to noradrenaline in the dog femoral artery from control and heart failure animals after the addition of 1μM cocaine. Graphs were generated from mean parameters derived from curve fitting. Mean raw data ± s.e. mean was superimposed on mean curves. Control animals (□), n = 10; heart failure animals (■), n =8. In graph A, the inset graph serves to highlight the pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values and the comparison between the two groups. Graph B shows the same CRC data expressed as active effective pressure (KPa).

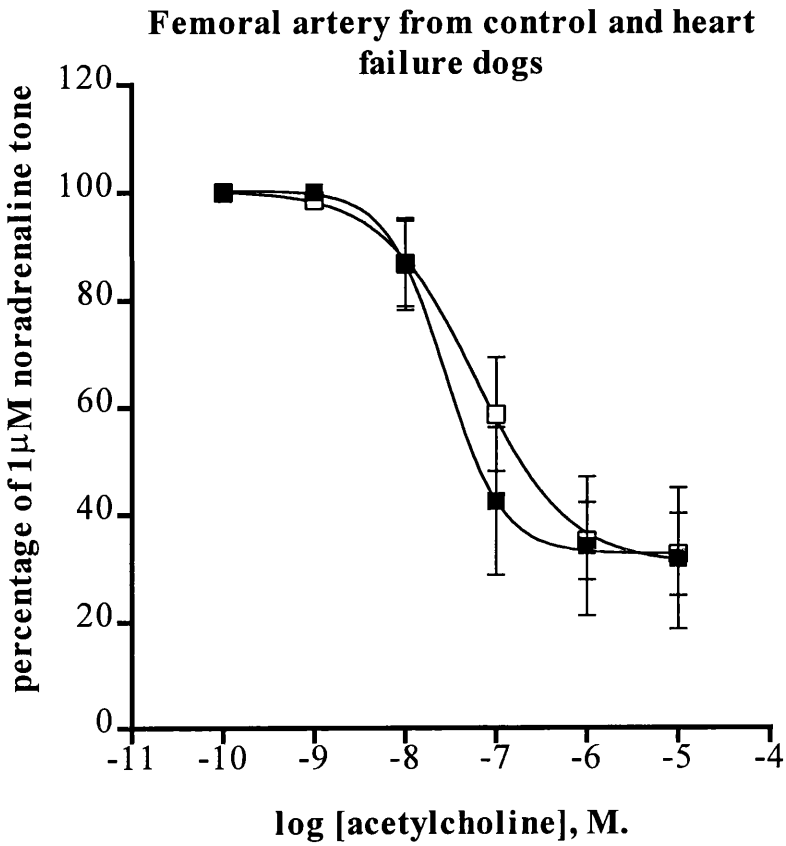
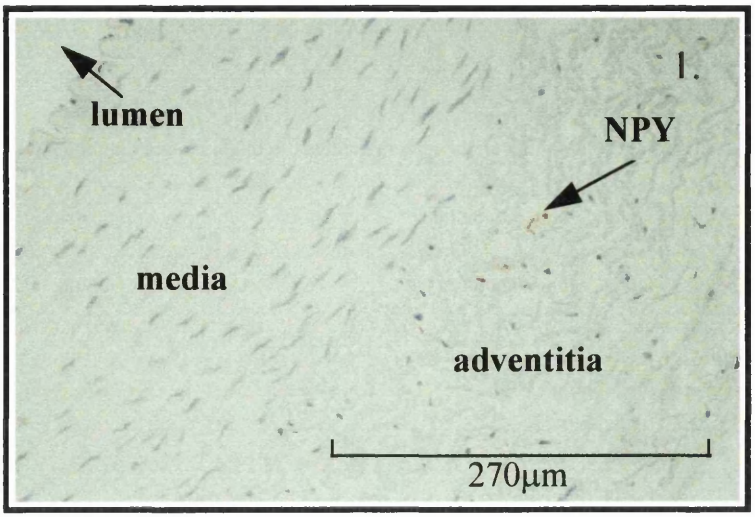


Figure 6.11. Concentration response curve data to acetylcholine from control and heart failure dogs in the femoral artery. Results are expressed as a percentage of the tone produced by 1μM noradrenaline. Data points represent mean ± s.e. mean. Control animals (□), $n = 9$; heart failure animals (■), $n = 6$.



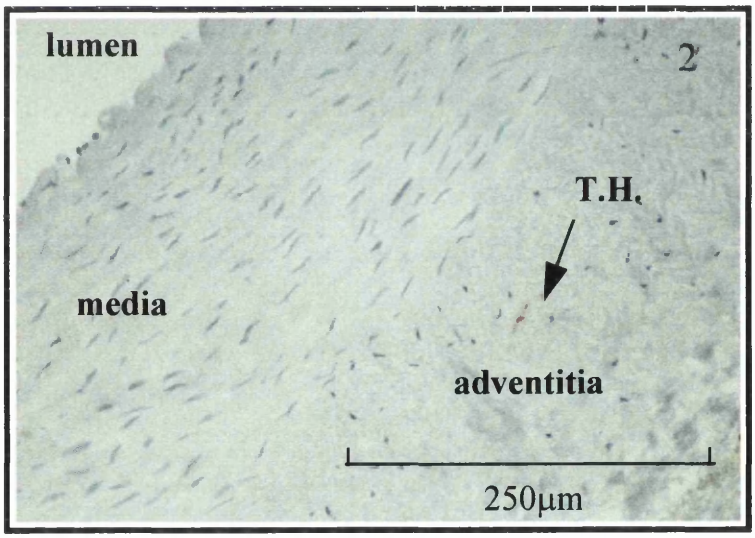


Figure 6.12. See next page for text 

Figure 6.12. Femoral artery immunohistochemistry. Both NPY and tyrosine hydroxylase appear as areas of red staining. Panel 1 shows scant red staining for NPY in a cross section of femoral artery. Panel 2 also demonstrates the low level of staining for tyrosine hydroxylase (TH). In both panel 1 and 2, any staining for either NPY or TH is limited to the adventitia of the vessel.

6.2.3 Subcutaneous resistance arteries

Values were derived from 6 control animals and 4-5 heart failure animals.

Time control vessels were run in parallel with experiments and it was found that there were no significant differences between maximum or pEC_{50} values between two consecutive noradrenaline concentration response curves. Mean time control data is illustrated in Figure 6.13.

In the control group, noradrenaline contracted the vessels with a mean pEC_{50} value of 6.4 ± 0.2 (n = 6). Although cocaine appeared to shift the curve slightly to the left there was no significant difference in the presence of 1μM cocaine to the maximum, pEC_{10} pEC_{25} or pEC_{50} values (P of 0.19, 0.19, 0.22, 0.76 respectively), (Figure 6.12). 100μM L-NAME had no significant effect on the pEC_{10}, pEC_{25}, pEC_{50} or maximum values of the noradrenaline CRC as illustrated in Figure 6.14, with P values of 0.84, 0.94, 0.46 and 0.58 respectively. The combination of L-NAME (100μM) and cocaine (1μM) caused a marked left shift in the CRC to noradrenaline. The difference between the two curves was significant at the level of the pEC_{25} value (P of 0.023) and the pEC_{50} value (P of 0.045) (Figure 6.14).

In the failure group, noradrenaline contracted the artery with a pEC_{50} value of 6.64 ± 0.33 (n = 5). 1μM cocaine had no significant effect on pEC_{10}, pEC_{25} pEC_{50} or maximum parameters (Figure 6.15). Although both 100μM L-NAME and the combination of 1μM cocaine and 100μM L-NAME appeared to shift the curve to noradrenaline to the left, the differences in the pEC_{10}, pEC_{25}, pEC_{50} and maximum parameters were not significant (Figures 6.15).

When comparisons were made between the control and failure groups, no significant differences could be found when maximum, pEC_{10}, pEC_{25} and pEC_{50} parameters were compared. This was true for responses to noradrenaline alone, and in the presence of

1μM cocaine, 100μM L-NAME and both cocaine and L-NAME (Figure 6.16). However, when responses to 125mM KCl were compared, it was found that the maximal response in the heart failure group was significantly higher than that in the control group with a maximal response in the failure group of 28.49 ± 2.45KPa (n =14) compared to 16.81 ± 1.8KPa (n = 24), in the control group ($P<0.004$).

Responses to acetylcholine were variable. The maximum relaxation in the control group was 43.39 ± 10.66% (n = 11) and in the failure group 75.59 ± 11.79% (n = 6). A pEC_{50} value of 6.32 ± 0.3 (n = 6), was obtained for the control group and a pEC_{50} value of 6.77 ± 0.22 (n = 5) for the failure group. Data from the control and failure animals is illustrated graphically in Figure 6.17. Although it appears that the failure animals showed an increased relaxation, when individual values were compared by t test, no significant difference was found between maximum values (P of 0.07). In addition no significant differences were found between the pEC_{10}, pEC_{25} and pEC_{50} values (P values of 0.79, 0.69 and 0.29 respectively).

Subcutaneous resistance arteries appeared to be well innervated as can be seen from the NPY staining of a whole-mounted vessel in Figure 6.18. The nerves form an intricate lace work like pattern over the entire surface of the vessel.

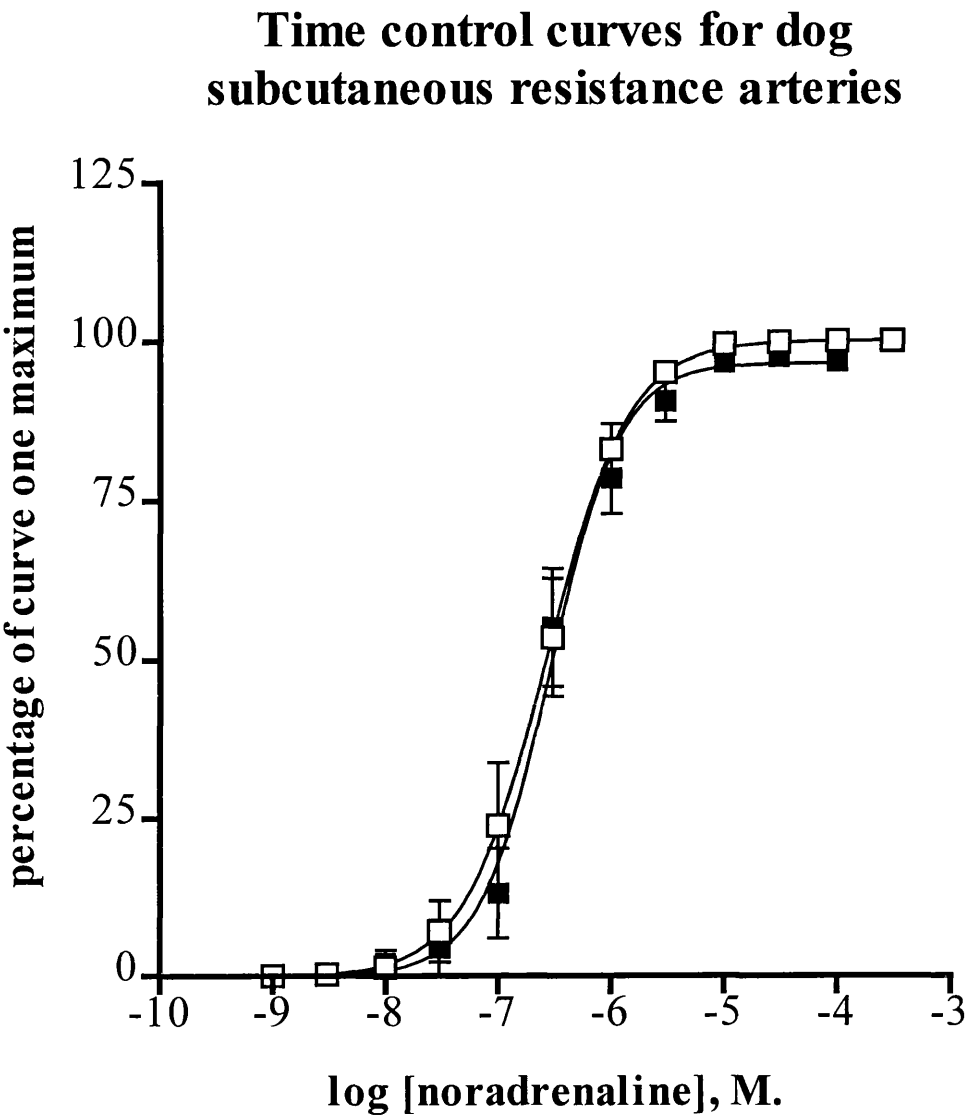


Figure 6.13. Mean time control data for dog subcutaneous resistance arteries. Data points represent mean data ± s.e. mean. Data is expressed as a percentage of maximum of the first CRC. Curve 1 (□), $n = 9$; curve two (■), $n = 9$.

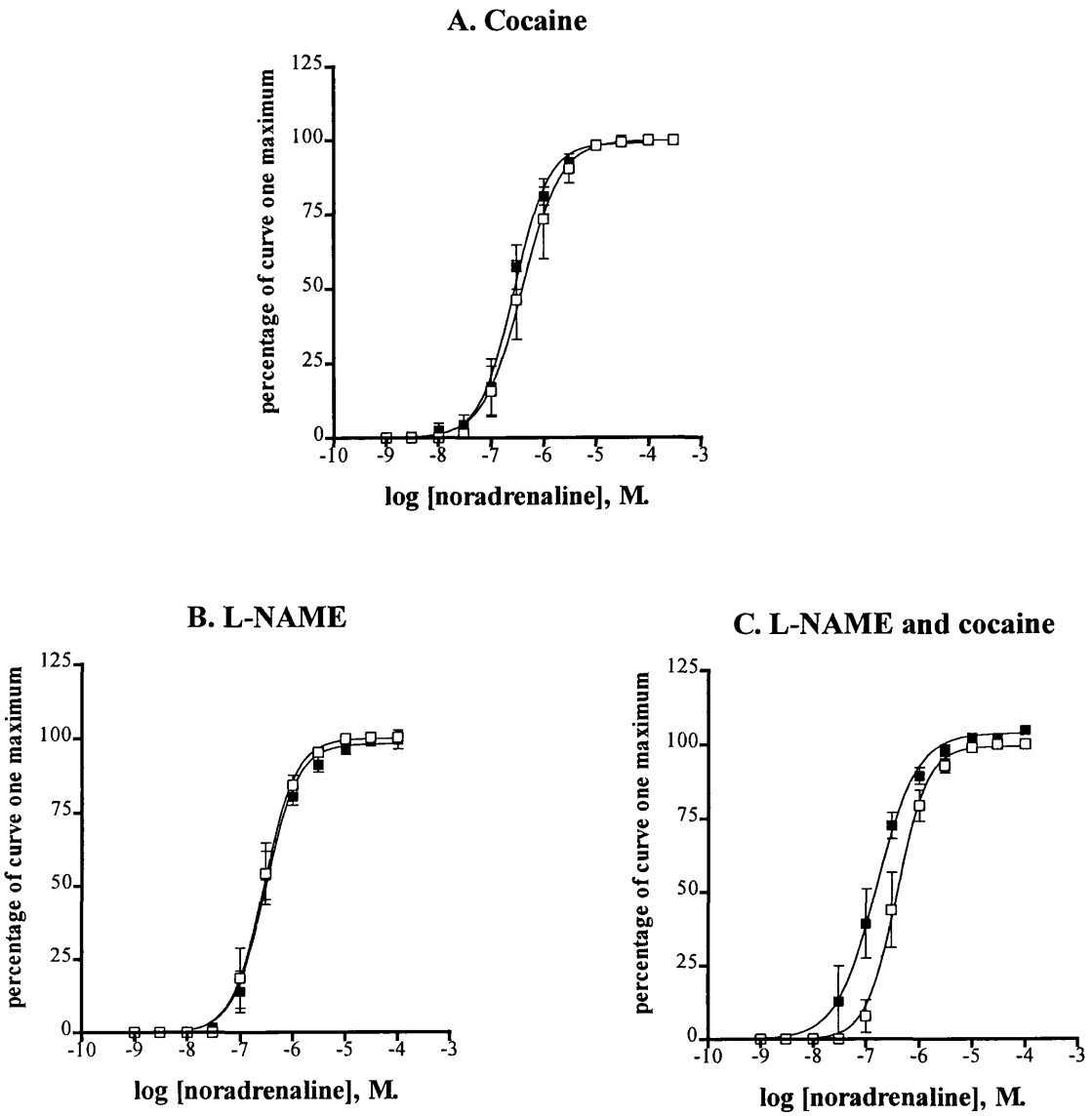


Figure 6.14. Graphs illustrating mean concentration response curve data from subcutaneous resistance arteries of control dogs. Responses are expressed as a percentage of the control curve. Graph A, NA (□) $n = 6$; NA and 1μM cocaine (■) $n = 6$. Graph B, NA (□) $n = 6$; NA and 100μM L-NAME (■) $n = 6$. Graph C, NA (□) $n = 6$; NA and 100μM L-NAME + 1μM cocaine (■) $n = 6$.

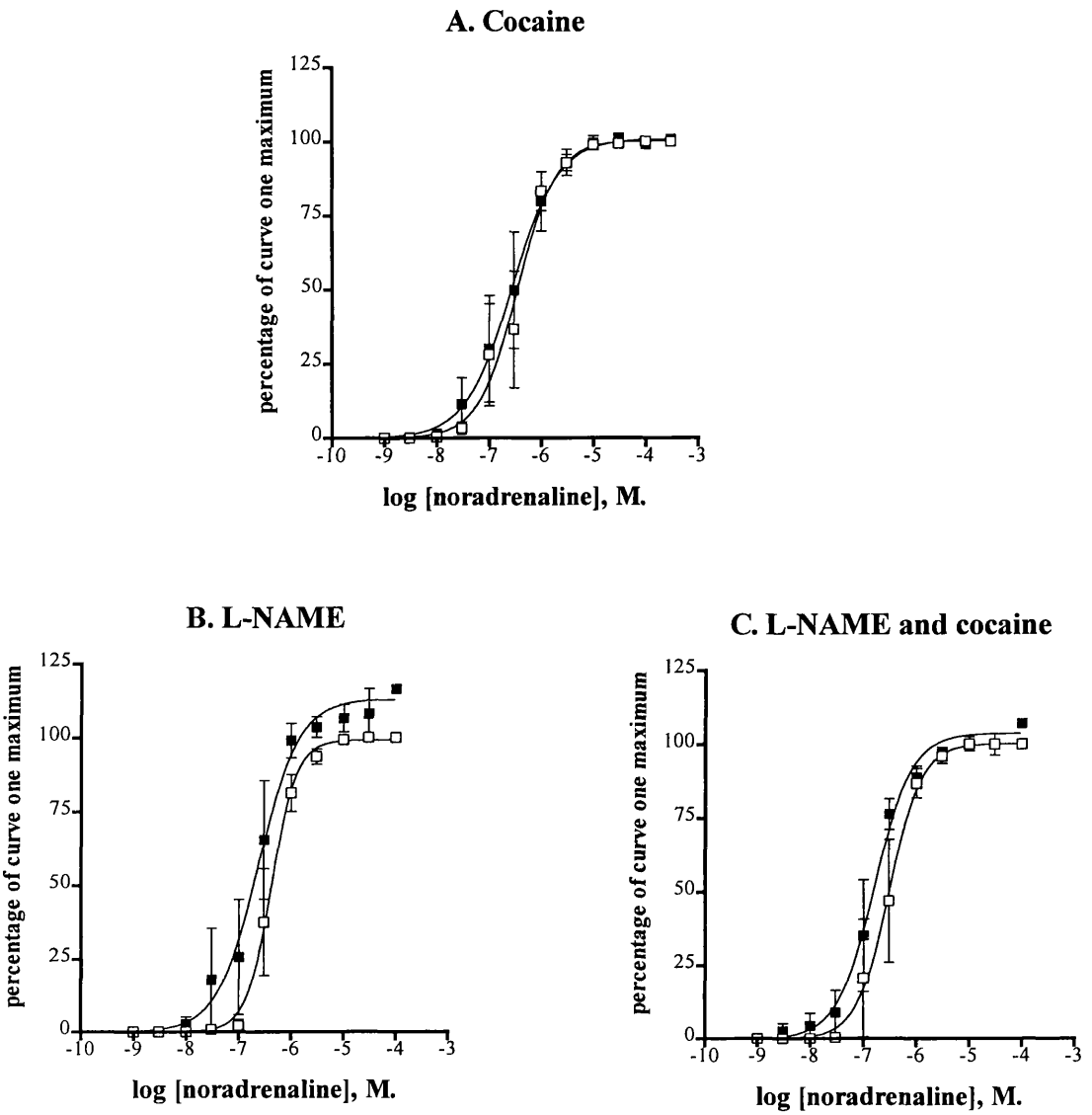


Figure 6.15. Graphs illustrating mean concentration response curve data from subcutaneous resistance arteries of heart failure dogs. Responses are expressed as a percentage of control maximum. Graph A, NA (□) $n = 5$; NA and 1μM cocaine (■) $n = 5$. Graph B, NA (□) $n = 4$; NA and 100μM L-NAME (■) $n = 4$. Graph C, NA (□) $n = 4$; NA and 100μM L-NAME + 1μM cocaine (■) $n = 4$.

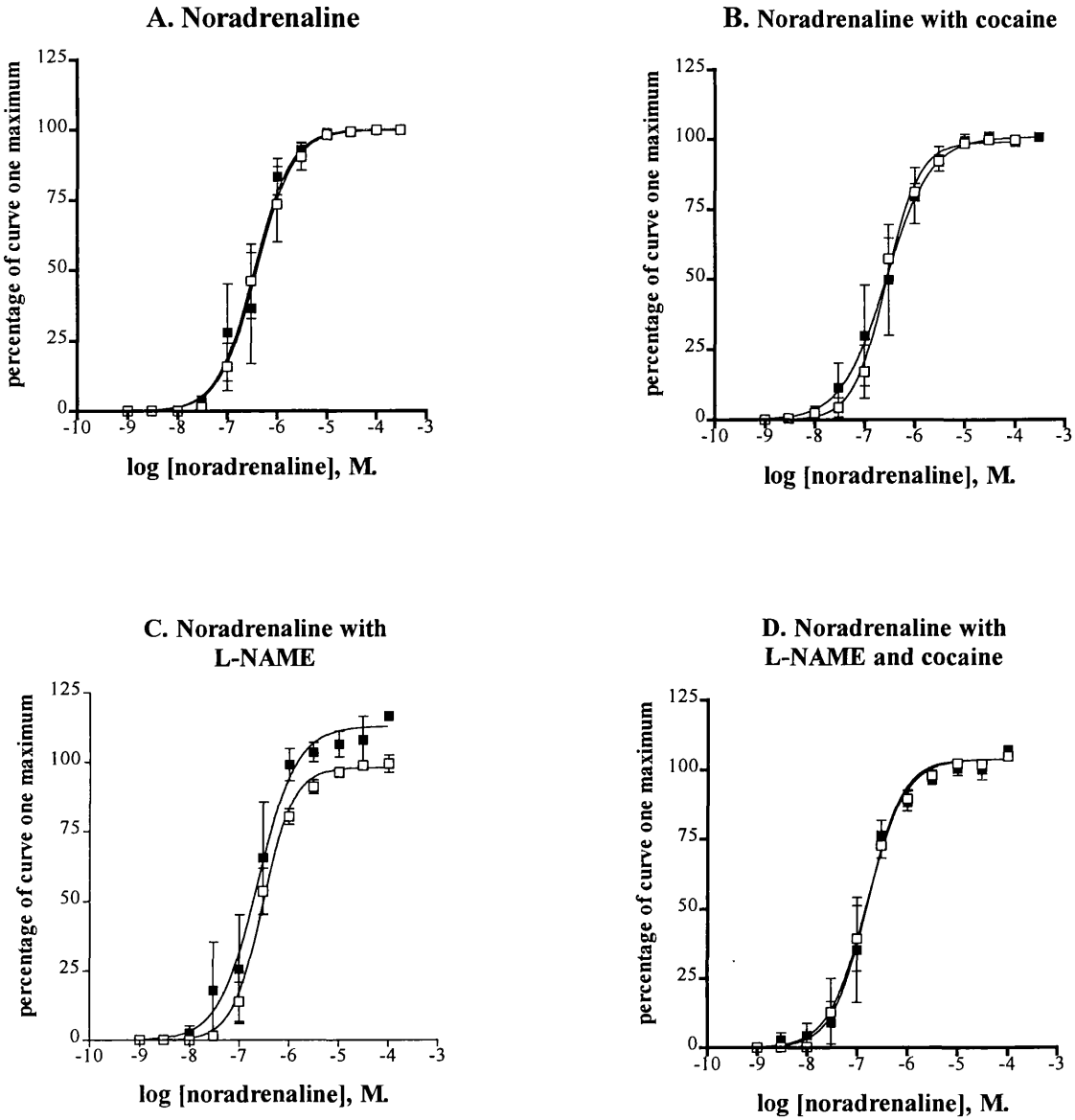


Figure 6.16. Mean concentration response curve data comparing control and heart failure dogs. Graph A (NA), control (□) n = 6; failure (■) n = 5. Graph B (NA in the presence of 1μM cocaine), control (□) n = 6 ; failure (■) n = 5. Graph C (NA in the presence of 100μM L-NAME), control (□) n = 6; failure (■) n = 4. Graph D (NA in the presence of 100μM L-NAME and 1μM cocaine), control (□) n = 6; failure n = 4.

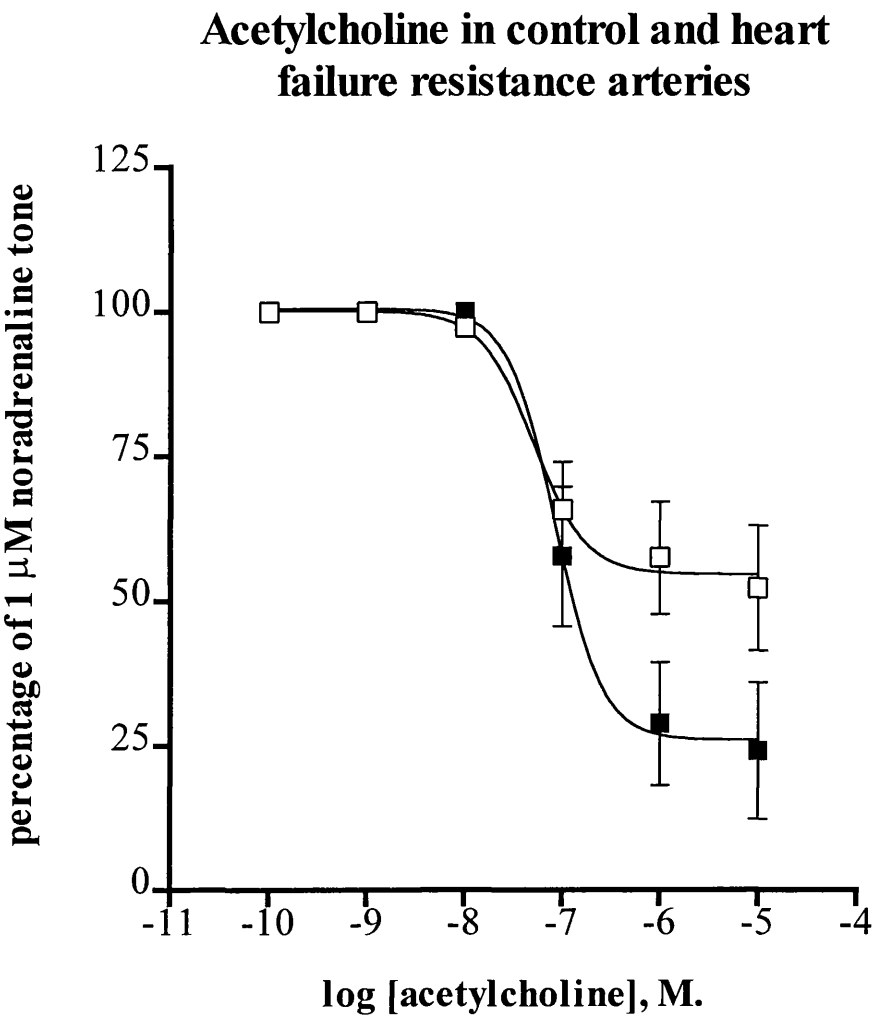
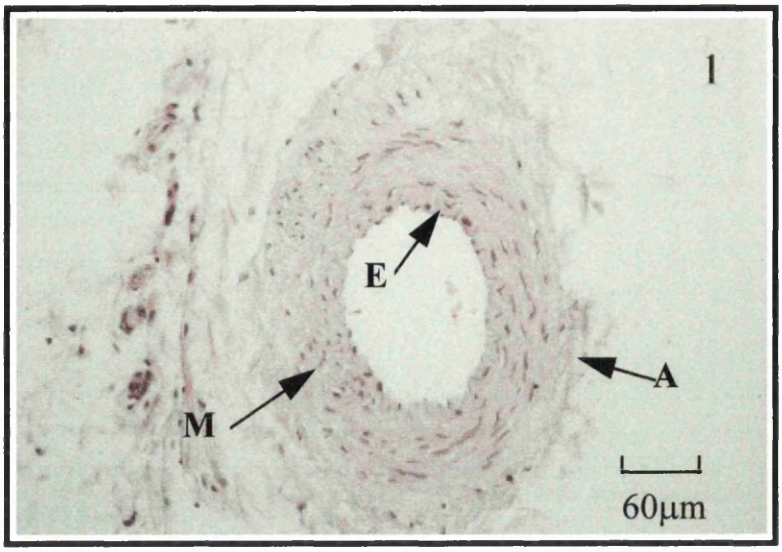


Figure 6.17. Mean concentration response curve data for acetylcholine in subcutaneous resistance arteries from control and heart failure dogs. Data points represent mean data ± s.e. mean. Results are expressed as a percentage of the tone produced by 1μM noradrenaline. Control (□) $n = 11$; Heart failure (■) $n = 6$.



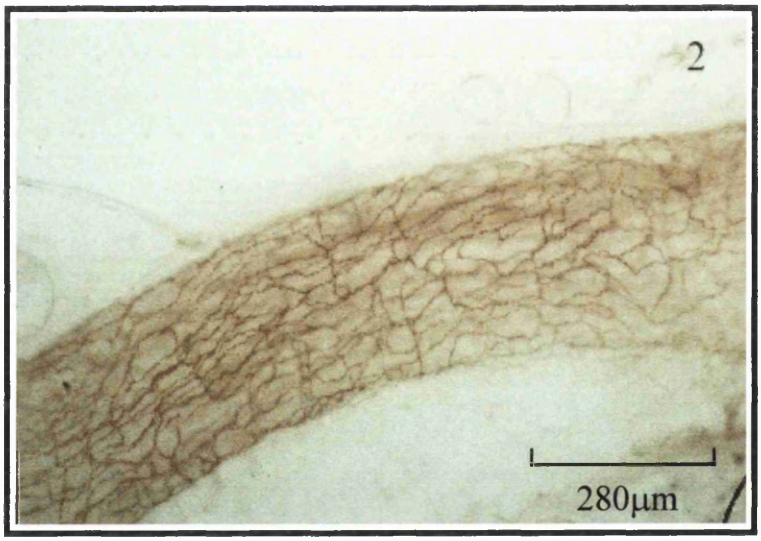


Figure 6.18. See next page for text 

Figure 6.18. Dog subcutaneous resistance artery histology and immunohistochemistry. Panel 1 shows a haemalum and eosin stained cross section of a representative DSCRA used in this study. In this section the endothelium (E) can be clearly seen, together with approximately five layers of smooth muscle making up the media (M) and the connective tissue of the adventitia (A). Panel 2 shows a whole-mounted preparation stained for NPY. There is abundant bronze coloured NPY staining forming a lacey pattern over the entire surface of the vessel.

VESSEL	pEC_{50} NA ± s.e. mean	pEC_{50} NA + 1μM cocaine ± s.e. mean
Saphenous vein	5.93 ± .07 (n = 8)	6.35 ± 0.07 (n = 8)
Femoral artery	6.05 ± .07 (n = 10)	5.82 ± 0.04 (n = 10)
Resistance artery	6.4 ± 0.2 (n = 6).	6.61 ± 0.12 (n = 6)

Table 6.1. Summary of pEC_{50} values from vessels of control animals before and after the addition of cocaine.

VESSEL	pEC_{50} NA ± s.e. mean	pEC_{50} NA + 1μM cocaine ± s.e. mean
Saphenous vein	5.69 ± 0.12 (n = 7)	6.03 ± 0.12 (n = 7)
Femoral artery	5.54 ± 0.13 (n = 8)	5.64 ± 0.13 (n = 8)
Resistance artery	6.64 ± 0.33 (n = 5)	6.63 ± 0.3 (n = 5)

Table 6.2. Summary of pEC_{50} values from vessels of heart failure dogs before and after the addition of cocaine.

case number	signalment	diagnosis	treatment
129293	8mnths, (M), Flat coated retriever.	Tricuspid and pulmonic valve dysplasia	none
129574	9yrs, (FN), Boxer. 2 year history of syncope. Presented with ventricular dysrhythmia	Cardiomyopathy	none (died suddenly)
126443	10yrs, (MN), King Charles Spaniel. Treated for 2 years.	Endocardiosis	frusemide enalapril digoxin
130395	12 weeks, (F), Golden Retriever .	Pulmonic stenosis, aortic stenosis, mitral and tricuspid dysplasia.	none
130469	6yrs, (M), St Bernard.	Dilated cardiomyopathy	none
130659	3.5yrs (M) Whippet	Dilated cardiomyopathy secondary to septic myocarditis	antibiotics
128870	11.7yrs, (M), Collie.	Endocardiosis	enalapril frusemide
10796	Aged, (M), King Charles Spaniel.	Endocardiosis	none

Table 6.3. Synopsis of heart failure cases used in the femoral artery and saphenous vein experiments. M = male, F = female, MN = male neutered, FN = female neutered.

case number	signalment	diagnosis	treatment
125322	10.9yrs, (M), Standard Poodle.	Dilated cardiomyopathy	frusemide spironolactone enalapril digoxin
130174	8yrs, (M), St Bernard. Recurrent disease over 12mnths.	Idiopathic pericardial effusion causing right sided failure	Drainage. No drugs used
131736	8.5yrs, (M), Great Dane. Treatment over a five month period	Dilated cardiomyopathy	frusemide digoxin enalapril topical nitroglycerine
132710	7yrs, (M), Doberman.	Dilated cardiomyopathy and endocardiosis	frusemide enalapril propranolol
128870	11.7yrs, (M), Collie.	Endocardiosis	enalapril frusemide

Table 6.4. Synopsis of heart failure cases used in the subcutaneous resistance artery experiments. M = male, F = female, MN = male neutered, FN = female neutered.

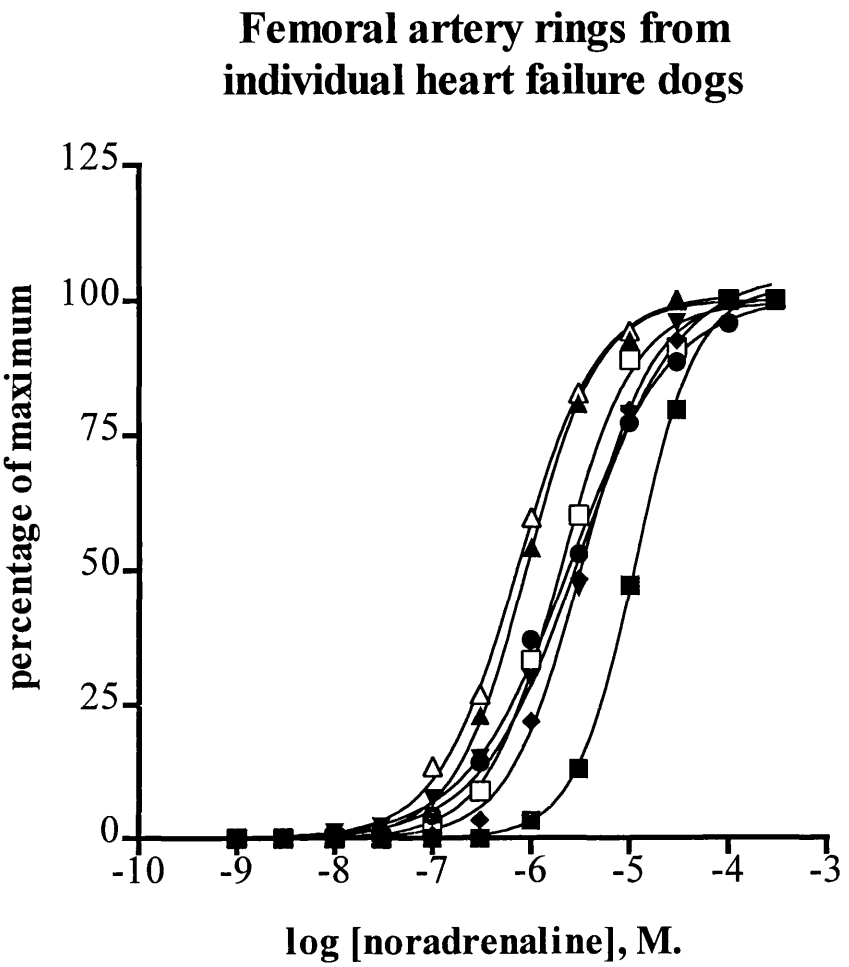


Figure 6.19. Individual concentration response curves from femoral artery rings of heart failure dogs. As can be seen from the graphs, two of the femoral artery rings appear to be more sensitive to noradrenaline than their counterparts. These are as follows: (△) case number 126443; (▲) case number 128870. In addition one vessel is much less sensitive to noradrenaline (■) case number 129293. Table 6.2 gives case details.

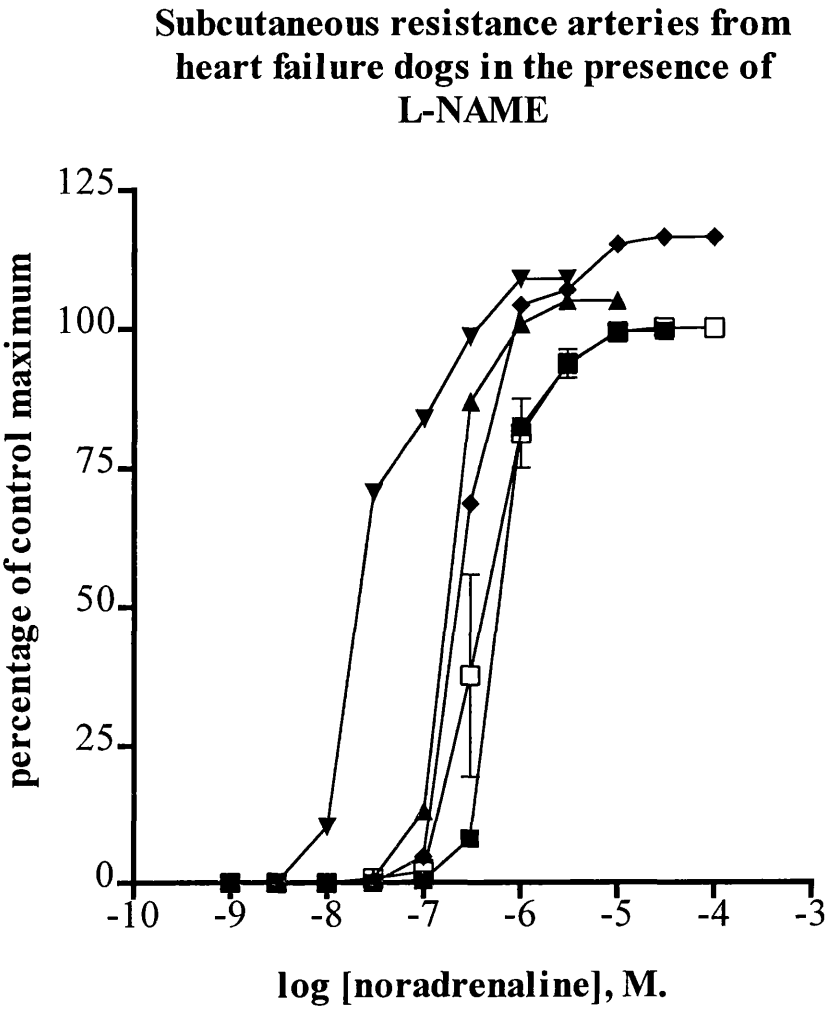


Figure 6.20. Individual concentration response curves from subcutaneous resistance arteries in the presence of L- NAME (from heart failure dogs). Individual experiments from subcutaneous resistance arteries are compared to the mean control curve. Control (□), n = 4; case number 125322 (■); case number 130174 (▲); case number 131736 (▼); case number 132710 (◆). As can be seen from the graph, the curve from case number 131736 is more sensitive to noradrenaline than the others. Case details are listed in Table 6.3.

6.3 Discussion

Heart failure and the importance of the vasculature in the pathophysiology of this condition has been discussed in the introduction in Chapter one, together with the relevance of cardiac disease in the canine population and the main aetiologies and treatment options currently available.

To date, a number of studies have examined the effects of congestive cardiac failure in isolated vessels from the dog and also vessels in the intact animal (Mathew et al. 1993; LeTran and Forster, 1997; Ianuzzo et al. 1996; Forster et al. 1989; Forster and Armstrong, 1990; Forster et al. 1991; Forster, 1995; Larosa et al. 1994; Forster et al. 1994; Forster and Campbell, 1993; Forster, 1996; Forster, 1995; Kaiser et al. 1989; Elsner et al. 1991; Kiuchi et al. 1993; Katz, 1995). All these studies used animals whose heart failure had been induced experimentally, with the pacing induced model being the most used and best characterised (Armstrong et al. 1986). In this study, vessels were harvested from animals in the canine population who had developed naturally occurring heart failure and who were seen at Glasgow University Veterinary hospital referral clinic. Animals had been referred to the hospital because of heart disease. On referral dogs were examined by a Veterinary cardiologist and underwent an examination consisting of clinical examination, thoracic radiography, electrocardiography and in most cases, diagnostic echocardiography and post mortem examination. This meant that each case had a definitive diagnosis of a specific cardiac disease. Ideally control animals should have been subjected to a similar clinical investigation to that of their heart failure counterparts. This unfortunately was not a feasible option. Control animals were however given a clinical examination. In the majority of cardiac diseases seen in the canine population, clinical examination will reveal some abnormality such as a murmur, poor pulses, reduced capillary refill time,

cough and inspiratory crackles on auscultation of the lung field, increased heart rate, jugular distension and or pulsation and abdominal distension. Animals exhibiting any of these signs were not included as controls.

Vessels were taken from dogs with heart failure, who were euthanased at their owner's request, because the cardiac disease had progressed and was no longer responsive to treatment, or because there was no appropriate treatment available and in one case due to the aggressive nature of the dog which prevented examination and administration of treatment. Tables 6.3 and 6.4, summarise the heart failure cases used in this study, giving information on the diagnosis and treatment if any. Several points need to be borne in mind when interpreting findings and making comparisons with the literature. Obviously in comparison with experimental models of heart failure, there is a non-uniformity in the aetiology of failure, breed of animal, age of animal, treatment and duration of the condition.

Responses to the catecholamine, noradrenaline and the endothelium-dependent vasodilator, acetylcholine were compared in control and heart failure groups. In addition, the effect of the agents cocaine and L-NAME were also examined. The rationale for using these latter two agents is discussed below.

Increased sympathetic activity is well documented as playing a part in the pathophysiology of cardiac failure (Leimbach et al. 1986; Grassi et al. 1995; Hasking et al. 1986; Cohn et al. 1984; Thomas and Marks, 1978). The assumption that increased plasma noradrenaline may simply be due to increased release from sympathetic nerve terminals may not necessarily be true. Noradrenaline in the neuroeffector junction is taken up by the nerve terminal (termed uptake-1) and by non-neuronal tissue (uptake-2). Uptake-1 is the most important and specific of the two uptake processes. Cocaine blocks uptake-1, thereby effectively increasing the amount of noradrenaline in the

neuroeffector junction. In the myocardium several studies have identified a decrease in noradrenaline uptake which contributes to the increased sympathetic stimulation of the post-junctional adrenoceptors (Liang et al. 1989; Himura et al. 1993; Beau and Saffitz, 1994; Eisenhofer et al. 1996). Cocaine was therefore employed in this study to see if this phenomenon also played a role in the peripheral sympathetic nerve terminals.

Furchgott and Zawadzki (1980) first highlighted the requirement of an intact vascular endothelium in order for acetylcholine to mediate vasorelaxation. Palmer et al (1987) identified the endothelium-derived relaxing factor released by the acetylcholine to be nitric oxide. Nitric oxide is released under both basal conditions and upon stimulation (Vanhoutte and Shimokawa, 1989). L-NAME is an inhibitor of nitric oxide synthase, the enzyme responsible for nitric oxide formation. The aim of using L-NAME in this study was to compare basal release of nitric oxide in control and heart failure animals.

Conduit vessels (Saphenous vein and femoral artery)

In the saphenous vein segments from control animals, sensitivity to noradrenaline before and after the addition of cocaine was comparable to values from the literature (Flavahan and Vanhoutte, 1986b; Alabaster et al. 1985; De Mey and Vanhoutte, 1981). 1μM cocaine significantly enhanced the sensitivity of the saphenous vein and decreased the maximal response. As is clear from Figure 6.6, the saphenous vein is well innervated, indicated by abundant staining for both tyrosine hydroxylase and NPY. The distribution of the innervation in this vessel was also interesting in that the nerves appeared to penetrate the wall of the vessel right up to the intimal layer rather than being limited to the adventitial/medial border. It is therefore logical that in this vessel cocaine would have the effect of increasing the sensitivity to noradrenaline, especially at low concentrations of the agonist. Similarly, the shift seen with cocaine in the controls

was still present in saphenous vein segments taken from heart failure animals. The shift in both groups was comparable, as when the differences in pEC_{10}, pEC_{25}, pEC_{50} and pEC_{75} values seen before and after cocaine, were compared by t test for both groups, there were no significant differences (P of 0.73, 0.41, 0.32 and 0.54 respectively). This would suggest that uptake-1 is not impaired in heart failure in the saphenous vein.

In control animals, the femoral artery was more sensitive to noradrenaline than the saphenous vein (pEC_{50} 6.05 ± 0.07 (n = 10)). This is in agreement with DeMey and Vanhoutte (1981), but in contrast to Forster and Armstrong (1990) who found the saphenous vein to be most sensitive to noradrenaline.

In contrast to the saphenous vein, the canine femoral artery is poorly innervated. This can clearly be seen in Figure 6.12, where staining with tyrosine hydroxylase and NPY is sparse and limited to the adventitia of the vessel. It would be logical therefore, to expect little effect from cocaine in this vessel. In the failure group this is certainly the case, with no significant differences in any of the parameters after the addition of 1μM cocaine. Surprisingly, in the control group, the same dose of cocaine caused a marked decrease in sensitivity and maximal response of the femoral artery rings. Cocaine does have local anaesthetic properties and therefore slows the rate of depolarisation by affecting sodium channels. It is also reported to have a depressant effect on smooth muscle contraction in a variety of smooth muscle types, for example intestinal and bronchial smooth muscle (Catterall and Mackie, 1996). It would appear for whatever reason, that the control vessels are more sensitive than the failure vessels to the depressant effects of cocaine. Interestingly in the study by Forster and Armstrong (1990), a reuptake blocker (desipramine 1μM) was used at all times in the Krebs' solution. Desipramine, pharmacologically, is a different class of drug to cocaine, being a tricyclic antidepressant (Baldessarini, 1996). It would however be interesting to see if

desipramine also has a depressant effect on the femoral artery, as this may explain the lower sensitivity to noradrenaline in Forster and Armstrong's study compared to this one.

In both the saphenous vein and femoral artery there was a rightward shift in the concentration response curves to noradrenaline in the heart failure animals compared to controls. This would indicate that in heart failure both femoral artery and saphenous vein become less sensitive to noradrenaline. The difference in the femoral artery was more marked with a significant difference existing between all parameters, whereas in the saphenous vein only the parameters compared in the lower part of the curve were significantly different. Working on the basis that some of the symptoms of heart failure are due to reduced blood flow to exercising muscle (Chapter 1), then it would be expected that vessels would be more sensitive to agonists. What may be important in this study is that the animals used would have been in end stage heart failure and it is perfectly possible that the decreased sensitivity is only a feature of severe disease.

In addition, when active effective pressures were compared for maximal response to noradrenaline and to 125mM KCl, values for the heart failure group were significantly higher for the femoral artery only. No difference was observed in the saphenous vein. This latter finding is probably the most important. Since the femoral artery is the main vessel supplying arterial blood to the hindlimb and hence to the skeletal muscle mass in this limb, it is possible that enhanced maximal contractile force exhibited by this vessel in the heart failure animals, plays a role in limiting blood supply to this region. This would tie in with the concept of reduced blood flow to exercising muscle being important in the pathophysiology of heart failure.

In the presence of cocaine the relationship between the two groups for the saphenous vein remained the same. Due to the disparate activity of cocaine in the femoral artery,

after the addition of cocaine, there was no longer a significant difference in the sensitivity of the two groups. Hence, it would seem that cocaine distinguishes between control and heart failure femoral artery rings. The reasons for this are not immediately clear and require further investigation.

In the saphenous vein, acetylcholine-induced vasorelaxation was poor and no significant difference was seen in heart failure. Acetylcholine-induced relaxations can be mediated by both nitric oxide and endothelium-derived hyperpolarizing factor (Vanhoutte, 1996), and these would not seem to be an important feature in the saphenous vein. Poor responses to vasorelaxing agents have been reported previously in this vessel (Hicks et al. 1991; Nunes and Mota, 1994; Forster and Armstrong, 1990).

Relaxations to acetylcholine in the femoral artery were much more marked with maximal relaxations of 70.9 ± 6.6% ($n = 9$) in controls and 68.1 ± 13.1% ($n = 6$) in the failure group. As for the saphenous vein, there was no significant difference in the response to acetylcholine between the control and failure animals. Numerous studies in both humans (Drexler et al. 1992; Ramsey et al. 1995; Katz et al. 1992; Hirooka et al. 1994) and animal models (Kaiser et al. 1989; Wang et al. 1994; Drexler and Wenyan, 1992; Kiuchi et al. 1993; Nasa et al. 1996; Mathew et al. 1993) have shown a reduction in acetylcholine induced vasorelaxation in a variety of vessels. However, in support of this study Forster et al (1990; 1989) found no differences in dorsal pedal artery or saphenous vein relaxations to acetylcholine before versus after heart failure in the dog pacing induced model, and Mathew et al (1993), found no differences in acetylcholine mediated relaxations in pulmonary arteries from dogs with pacing induced heart failure.

Subcutaneous resistance arteries

In the dog subcutaneous resistance arteries, noradrenaline contracted the vessels with a pEC_{50} of 6.4 ± 0.2 (n = 6). Hence, the resistance vessels were more sensitive to noradrenaline than either the saphenous vein or femoral artery (Table 6.1). Although the DSCRA appears to be well innervated, cocaine had less effect than in the DSV. In the presence of cocaine, in both the control and failure group, the noradrenaline curve is marginally shifted to the left and the maximum decreased. However, none of the differences reach statistical significance in either group, suggesting that neuronal uptake of noradrenaline does not play a large role in the control of local levels of noradrenaline in the resistance vessels.

In control animals, L-NAME had no significant effect on the noradrenaline concentration response curve, indicating that basal release of nitric oxide does not seem to be a dominant feature in these vessels. In contrast, in the failure group, L-NAME seemed to shift the noradrenaline response curve to the left. The difference in the two curves did not reach statistical significance, but the findings suggested that basal release of nitric oxide may have been elevated in the heart failure animals in this study. Increased levels of basal nitric oxide in human patients has also been suggested (Drexler et al. 1992; Habib and Oakley, 1994; Winlaw et al. 1994). However, when the individual curves from the heart failure dogs, in the presence of L-NAME, were plotted against the mean control curve, it was clear that one curve was much more sensitive than the others (Figure 6.20). This curve is from case number 131736. From Table 6.4, it can be seen that this is the only dog in the cohort which was receiving nitroglycerine ointment. This drug induces the formation of nitric oxide which can then act directly on the vascular smooth muscle. While this should not be a surprising finding, it is

exhilarating to observe that the drug would appear to be having the desired effect, at least in this animal.

In the control animals when cocaine and L-NAME were combined, there was a significant shift in the concentration response curve to the left indicating a significant increase in sensitivity in the presence of the combined drugs which was not seen with either substance on its own, indicating a synergistic effect. In contrast, in the heart failure group, the combination of the two drugs had no greater effect than L-NAME used in isolation. The numbers of heart failure animals are too low to draw any concrete conclusions from this difference, due to the variability in responses in the heart failure cohort exemplified in Figure 6.20. It could be hypothesised that the synergistic effect in the control situation may be caused by small increases in local noradrenaline, caused by the presence of cocaine, which in turn may stimulate the production of basal nitric oxide so that an effect with L-NAME is only seen when the two drugs are combined. Noradrenaline has been shown to stimulate nitric oxide production in the isolated mesentery of the rat (Yamamoto et al. 1994), and the desensitization to methoxamine seen in the rat mesentery would appear to be associated with nitric oxide production (Kamata and Makino, 1997).

When comparisons were made between the control and heart failure group, there was no alteration in sensitivity or maximal response to noradrenaline on its own or in the presence of cocaine, L-NAME, or cocaine and L-NAME combined. In contrast, maximal response to 125mM potassium chloride was significantly elevated in the failure group. Again as for the femoral artery, this is an important finding and may play a role in limiting blood flow.

Responses to acetylcholine, as for the larger vessels were not significantly different between the two groups.

It is well established that increased total peripheral resistance is a feature of congestive heart failure (Parmley, 1985), so that while it seems reasonable that maximal responses in the femoral artery are enhanced, it is somewhat surprising to find that there seemed to be a decrease rather than an increase in agonist sensitivity in saphenous vein and femoral artery from heart failure animals. Also surprising, was the finding that there was no alteration in sensitivity of the resistance arteries, since these vessels are the most important in the control of total peripheral resistance.

Desensitization and homologous downregulation of β-adrenoceptors in heart failure, has now been well documented in the myocardium and would also appear to occur in the peripheral vasculature, (Kiuchi et al. 1993; Bristow et al. 1982; Bristow et al. 1986). For the β-adrenoceptors, desensitization is due to stimulation with catecholamines, is associated with phosphorylation of the receptor, and is mediated by protein kinase A and a specific β-adrenergic receptor kinase. Desensitization of the receptor is followed by downregulation which is characterised by a loss of binding sites (Morris et al. 1991). In the case of α_1-adrenoceptors, desensitization of the response on exposure to catecholamines is also well documented and has been best characterised for the α_{1b}-adrenergic receptor, where it has been shown that phosphorylation of sites in the carboxy terminus are involved (Diviani et al. 1997; Lattion et al. 1994). In contrast, loss of receptor number would not appear to occur (Seasholtz et al. 1997a; Seasholtz et al. 1997b). Counterbalancing this are the findings that angiotensin II increases transcription of α_1-adrenergic mRNA in vascular smooth muscle cells (Hu et al. 1995), and that adrenaline can also increase α_1-adrenergic receptor expression mediated through β_2-adrenergic receptors (Morris et al. 1991).

The net effect of these interactions is unclear and will probably vary from one situation to the next.

A number of other studies have examined agonist sensitivity in heart failure in a variety of vessels and species. Results have been conflicting. The most logical comparison to make would be with the dog pacing induced model. Forster et al (1987), in contrast to this study, found increases in sensitivity to noradrenaline in both dog saphenous vein and dorsal pedal artery. In agreement with this study they also found increases in maximal tension in the artery after heart failure, but they also saw increases in tension in the saphenous vein. As already mentioned, their study used desipramine. If desipramine had the same effect as cocaine in the present study, then the contrasting effect of the drug on control versus heart failure artery could account for the apparent increasing sensitivity seen in the dorsal pedal artery. In addition, in a subsequent study by the same group, they were no longer able to demonstrate differences in the dorsal pedal artery (Forster, 1995), although findings for the saphenous vein were consistent with the previous report (Forster et al. 1987). In a separate study by the same group (Forster and Armstrong, 1990), α_1- and α_2- agonists were examined and the study showed that there was an enhanced responsiveness to α_1- agonists, but that the response to α_2- agonists was diminished in heart failure. Depression of vascular α_2-adrenoceptor function has also been shown in rat vasculature following heart failure (Feng et al. 1996). Saphenous vein does have post-junctional α_2-adrenoceptors (Drew and Whiting, 1979), which are thought to contribute mainly to the lower part of the noradrenaline curve (Flavahan and Vanhoutte, 1986b; Alabaster et al. 1985; Flavahan et al. 1984; Constantine et al. 1982; Sullivan and Drew, 1980; De Mey and Vanhoutte, 1981). This may explain findings for the saphenous vein in the present study, where the difference between the failure and control group was only significant for the lower part of the concentration response curve. In support of findings for both vessels, an in vivo study examining dorsal hand vein blood flow in human patients with congestive cardiac

failure (Feng et al. 1994), showed a decreased responsiveness to both phenylephrine and clonidine in severe cardiac failure, suggesting decreased responsiveness involving both α_1- and α_2-adrenoceptors. Slightly different findings again were reported by Angus et al (1993). In human resistance arteries taken from gluteal biopsies, no differences were found in sensitivity to a variety of agonists, but for all the agonists used there was a significant decrease in maximal response in the failure versus the control patients. The agonists used were noradrenaline, angiotensin I and angiotensin II, suggesting that the effect was not solely due to alterations involving the α-adrenoceptors. Two recent studies by Stassen et al (1997a; 1997b), examined resistance arteries and thoracic aorta from rats with experimentally induced heart failure. In contrast to the present study, they found an increased sensitivity to the agonist phenylephrine but a decreased maximal response in heart failure in the resistance arteries. The difference appeared to be associated with calcium influx induced by phenylephrine but not potassium chloride. There was also some suggestion of increased α_1-adrenoceptor density after heart failure, although when this was normalised for DNA content the difference was not significant. To confound matters further, Kaiser et al (1989) in an in vivo study found no alteration in response to noradrenaline in femoral arteries of dogs with pacing induced heart failure.

Several possible reasons exist for the discrepancies between studies.

(1) The stage and severity of the cardiac failure. In this study the majority of animals had severe cardiac disease often of several years duration and in most cases were euthanased because of their disease. This contrasts with the experimental models where rapid pacing may have only been of 4-5 weeks duration prior to vessel isolation (Forster and Armstrong, 1990).

(2). The use of drug therapy. In the case of the experimental animal models, treatment has generally not been used. In contrast, patients in the human studies may be on a variety of drug treatments. Interestingly in this study, of the cohort of eight animals with heart failure who were used in the femoral artery and saphenous vein experiments, only two were on treatment with drugs (Table 6.3). Another one was treated with antibiotic, but this would not be expected to alter vascular reactivity. If individual concentration response curves for the heart failure animals are graphed, there are no particular outliers in the saphenous vein experiments (not shown). In the femoral artery experiments this is not the case. Two of the animals seem to have curves which are shifted to the left compared to the others. It may be significant that these two were the only animals receiving treatment in the group (Figure 6.19). When this observation is considered along with the fact that 4/5 of the cohort used for study of the resistance arteries in failure, were on treatment and did not show alterations in sensitivity to noradrenaline, it should be considered that treatment may mask changes in the reactivity of vessels. Obviously at this stage this is only a preliminary observation. Increased numbers of heart failure animals are required, such that the heart failure group can be further subdivided on the basis of aetiology and treatment modality.

(3). Experimental populations tend to be more uniform with regard to age and breed. The studies carried out on experimental dogs state the use of adult dogs but do not elaborate on this. In this study because of the source of the control population, accurate ages were not available for these animals. It would be reasonable to say however, that there was a fairly even distribution of age bands among the control group. In contrast, in the heart failure cohort, with the exception of two dogs < 1 year of age and a 3.5 year old whippet, all the dogs would be considered old. A number of studies have examined changes in noradrenaline sensitivity and endothelial function with age (Haidet et al.

1995; Toda and Miyazaki, 1987; Shimizu and Toda, 1986; Kaiser and Ptu, 1992; Vila et al. 1997; Carvajal et al. 1995; Tabernero and Vila, 1995; Gurdal et al. 1995). On examining the literature, the main observation is that alterations depend very much on the species and the blood vessel studied. Gurdal et al (1995) and Carvajal (1995), both report a decrease in sensitivity to noradrenaline in rat aorta with age, which could explain the findings of this study. In contrast, two in vivo studies of beagle hindlimb blood flow did not identify changes in either vasorelaxant or vasoconstrictor activity in the femoral artery (Kaiser and Ptu, 1992; Haidet et al. 1995), but vasoconstriction in isolated beagle coronary arteries showed enhanced vasoconstriction to catecholamines (Toda and Miyazaki, 1987). These latter three studies, because they relate to canine vasculature, are probably more relevant. They would support the argument that the decreased sensitivity seen in the conduit vessels in heart failure, from this study, is not age related, although the increased maximal response observed in the femoral artery from failure animals may be.

To summarise the findings of this study. In the heart failure animals there is a decreased sensitivity to noradrenaline in both femoral artery and saphenous vein but not in the subcutaneous resistance artery. The difference is most marked in the femoral artery where in addition, maximal responses to both noradrenaline and 125mM KCl are increased. Since maximal response is increased to both noradrenaline and KCl, this is unlikely to be solely an adrenergic-mediated phenomenon, but may involve a general enhancement of vasoconstriction. The reason for the enhancement of only the potassium mediated response in the resistance arteries is not clear. In the large vessels the alteration in sensitivity to noradrenaline could be due to endothelial modulation of the response. Inducible nitric oxide and/or endothelium-derived hyperpolarizing factor (EDHF) are unlikely to play a role because of the similarity in response to acetylcholine

in the two groups of animals, although in the future it would be interesting to look at the relative contribution of EDHF, if any, in the acetylcholine mediated response, and if there is a contribution, to examine if the relative contribution of EDHF and nitric oxide change in the disease state. Differences in basal nitric oxide production or adenosine 3',5'-cyclic monophosphate (cAMP) vasorelaxant pathways could be responsible. Alternatively, the alterations may be due to changes in adrenergic receptor density and number (although you may expect to see a decrease in maximal response if this was the case), or in the efficiency of receptor coupling and signal transduction.

Finally, the alteration may be a more general alteration in contractile ability of vascular smooth muscle, in which case alterations with other non-adrenergic agonists would be expected.

There was no alteration in sensitivity to noradrenaline in the resistance arteries. Masking of alterations due to treatment must be considered. Although it appears as if there may be differences in basal nitric oxide production in the resistance artery vessels between control and failure animals, this was mainly due to a single animal who had been treated with nitroglycerine ointment. This requires further elucidation with increased numbers of animals.

Future studies require increased numbers of animals, making it possible to divide heart failure animals into groups depending on aetiology and treatment modalities. A logical progression would be to determine if the apparent decreased sensitivity in heart failure in the large vessels is agonist-dependent and therefore, to look at other agonists such as vasopressin, angiotensin and endothelin. Although the α_1-adrenoceptor population may not solely be involved in the changes seen, on the bases of studies such as those carried out by Stassen et al (1997a; 1997b), where there appears to be an increase in α_1-adrenoceptor density and alterations in α_1-mediated calcium influx, it would be of

interest to focus on the α_1-adrenoceptor population in relation to α_1-adrenoceptor subtypes, receptor density and levels of receptor expression at the mRNA level.

CHAPTER 7

General discussion

There are several reasons for studying receptors. These are, the understanding of normal physiological processes, the understanding of pathophysiological processes, the understanding of how drugs currently in use work and the development of new more effective drugs.

The reasons for examining the canine α_1-adrenoceptors include all these categories, but in particular, the long term aim, from my perspective, is to understand pathophysiological changes, especially in heart failure and to aid in the development of new drug treatments for this and other conditions. With regard to the potential for drug development, it is often difficult to identify possibilities until the groundwork has been covered. However if, for example, the α_1-adrenoceptor subtypes involved in a variety of vascular beds can be identified, then it may be possible to use subtype-selective α_1-adrenoceptor antagonists which will selectively enhance blood flow to a particular region, without having a significant effect on, say for example, systemic blood pressure.

Results from the studies on functional classification of the α_1-adrenoceptors, mediating vascular smooth muscle contraction in the saphenous vein and the subcutaneous resistance arteries, exemplify the limitations and problems encountered with α_1-adrenoceptor classification at present.

One obvious problem in the saphenous vein, is the coexistence of an α_2-adrenoceptor population. In an ideal situation an irreversible α_2-antagonist should be used to exclude this population of receptors from the analysis. No such antagonist is available, although unsuccessful attempts have been made using phenoxybenzamine, which has a much higher affinity for α_1- rather than α_2-adrenoceptors (Daly et al. 1988b; Dubovich and Langer, 1974; Constantine et al. 1982). The only other option is to use a reversible antagonist. The limitations of this are, that once equilibrium has been achieved, the

antagonist can freely dissociate and reassociate with the receptors. This is potentially problematic especially at high agonist concentrations, where the agonist may break through the α_2-antagonist effect, leading to an underestimation of the α_1-antagonist's potency. In addition, when an irreversible antagonist such as CEC is being used, this antagonist is inevitably going to access some of the α_2-adrenoceptor population as the reversible antagonist dissociates from the receptors.

The lack of reliable subtype-selective antagonists is also the cause of considerable frustration. This is best illustrated by the lack of antagonists which have a high affinity for the α_{1B}-adrenoceptor subtype and can distinguish it from both α_{1A}- and α_{1D}-. Chloroethylclonidine is one of the few antagonists currently available, but it is far from ideal for several reasons. In addition to its antagonist effects, it also acts as an agonist. This has been demonstrated in this study and others, by the contraction seen in the DSV (Nunes and Mota, 1994; Low et al. 1994; Nunes and Guimaraes, 1993). O'Rourke et al (1995), also suggested that CEC acted as an agonist at adrenoceptors in the rat aorta.

Although CEC alkylates α_{1B}-adrenergic receptors preferentially, it will bind to the other receptor subtypes (Michel et al. 1993), and the degree of alkylation is often influenced by experimental conditions and the species involved. In a recent study, using flow cytometry and confocal imaging techniques, it was shown that in cell lines expressing either the α_{1a}- or α_{1b}- adrenoceptors, that the α_{1b}-adrenoceptors localised on the cell membrane, whereas α_{1a}-adrenoceptors seemed to predominantly localise internally (Hirasawa et al. 1997). If this is really the case, then this may explain why the hydrophilic CEC preferentially alkylates the α_{1B}- subtype, since access to these sites would be greater. This also means that as exposure time increases, or for example membranes are used rather than whole cells in binding experiments, CEC will gain access to and inactivate other receptor subtypes, explaining the influence of

experimental conditions. Some of the confusion caused by the unreliability of CEC can be seen by the difficulties which were encountered in the classification of the adrenergic receptor cloned by Schwinn et al, (1990). This receptor was initially classified as the α_{1c}-, since although the antagonist profile was similar to that for the native α_{1A}-, it appeared more sensitive to CEC than would have been expected for an α_{1A}- adrenergic receptor. It was only later, when the rat homologue of the receptor was shown to have a lower sensitivity to CEC (Laz et al. 1994), that it became apparent that the comparative sensitivity of the bovine clone was due to a species variation (Forray et al. 1994a), and that in fact the bovine clone represented the α_{1A}- adrenergic receptor.

The current classification scheme also imposes limitations. In both the vessels examined in this study, the affinity for prazosin was low ($pA_2 < 9$). This is indicative of the α_{1L}- subtype, which encompasses the α_{1L}- and α_{1N}- subdivisions distinguished by HV 723 (Muramatsu et al. 1995). These receptors with low affinity for prazosin are considered to be insensitive to CEC.

However, in both cases there was evidence from the pattern of interaction of the other antagonists used, that more than one subtype was involved in the responses of the saphenous vein and the resistance arteries. In the saphenous vein this was based on the antagonist profile of 5 methylurapidil, which at the highest concentration used seemed to distinguish a low and a high affinity site, and the non-competitive interaction of BMY 7378. In the case of the resistance arteries, evidence for the involvement of more than one subtype came from the reduction in upper asymptote seen with some of the antagonists used, in addition to the non-competitive interaction of BMY 7378 based on Schild analysis.

Although the pitfalls associated with the use of CEC have been highlighted, both vessels seemed to display a certain amount of sensitivity to this antagonist, which would lend support to the evidence for a heterogeneous population of receptors.

These findings have given rise to two main issues. Firstly, although the α_{1L}-adrenoceptor has been demonstrated in both vessels, based on response to CEC, there would appear to be some differences in this subtype when the two vessel types are compared. Secondly, if additional receptors are involved, since they too have a low affinity for prazosin, how are they to be classified ?.

In the saphenous vein it was concluded that rauwolscine could interact with a population of α_1-adrenoceptors, which also contributed to the baseline contraction caused by CEC. This population of adrenoceptors seemed to correspond to the α_{1L}- subtype already mentioned. In the subcutaneous resistance arteries, the antagonist profile also necessitated classification of the receptors as α_{1L}-, although the baseline contraction to CEC was only seen in two of the vessels used. Three reasons could exist for this difference.

1. The α_1-adrenoceptors in the two vessels, although both classified as α_{1L}- are in fact different, supporting the suggestion that further subdivision within the low affinity prazosin sites does exist. If the α_{1L}-adrenoceptor is in fact a conformational variation of the α_{1A}-, then it would not be terribly surprising that there would be more than one conformational variant, leading to functional alterations in the receptor with regard to antagonist profiles.

2. The conclusion as regards the receptors responsible for the contraction to CEC in the DSV are wrong.

3. Despite the use of delequamine, the α_2-adrenoceptor population in the DSV may have interfered with the antagonist profile in this vessel and could be the explanation for the evidence of involvement of other receptor subtypes in this vessel.

The α_2-adrenoceptor in the DSV has previously been classified as the α_{2A}- subtype (MacLennan et al. 1997). It could be argued that the potency of some of the antagonists used in this study would be consistent with their activity at the α_2-adrenoceptors. For example, the affinity for 5 methylurapidil, in the presence of delequamine, at the low affinity site in the DSV (pA_2 6.29 ± 0.28), and its affinity in the presence of rauwolscine at the highest concentration used (pA_2 6.00 ± 0.77), could be consistent with an action at α_2-adrenoceptors, since a pA_2 potency range of 4 to 6 has been given for this antagonist at α_2-adrenoceptors (Wilson et al. 1991). In addition, it has been shown that CEC can inactivate the α_{2A}- and α_{2C}- adrenoceptors as well as the α_{1B}- (Michel et al. 1993).

Evidence which would support the theory that the subtypes identified were all of the α_1-adrenoceptor type would be the affinity of the other antagonists. Firstly, in the DSV, prazosin in the presence of delequamine acted in a competitive manner, with a pA_2 value of 8.31. This is much higher than would be expected at the α_{2A}-adrenoceptors, since of the three α_2-subtypes, prazosin has the lowest affinity at the α_{2A}-, and a pK_B value of 5.19 has been obtained for this antagonist at the α_2-adrenoceptors in the DSV (MacLennan et al. 1997). Also, in the same study, a pK_B value of 7.42 was obtained for WB 4101, at α_2-adrenoceptors in this vessel. This is lower than the value of 8.85 obtained in this study, and although there may be considerable overlap in the potency ranges for this antagonist at both α_1- and α_2- adrenoceptors, a pA_2 value of 8.85, falls within the potency range at α_1-adrenoceptors of 8-10, given by Wilson et al (1991).

In conclusion, in the DSV, there is evidence for the involvement of more than one receptor subtype. One of the subtypes involved seems to have all the characteristics of

the α_{1L}-adrenoceptor when compared with the literature. The other subtype or subtypes involved also have a low affinity for prazosin and are sensitive to CEC. At this time, the possibility that the coexisting population of α_2-adrenoceptors has contributed to these findings cannot be excluded. In the resistance arteries, prazosin also has a low affinity. Again there is evidence for the involvement of more than one subtype of receptor and again there is a degree of sensitivity to CEC. Unlike the DSV, α_2-adrenoceptors would appear to have little part to play in functional responses, supporting the existence of further divisions of the low affinity prazosin receptors in this vessel.

The discussion so far raises questions regarding the usefulness of functional studies in α_1-adrenoceptor classification, especially in this case, where it seems to be difficult to reach a satisfactory conclusion.

The answer lies in the reasons for wishing to establish the subtypes involved in the first place. Radioligand binding studies or molecular studies examining expression of receptor subtypes, may give more satisfactory and clear cut answers, but when the long term aim is to understand possible pathophysiological alterations in the α_1-adrenoceptors mediating the functional response of a vessel, and to develop antagonists that will affect this functional response, then other methods of study may bear little relation to the functional reality. Examples of the importance of functional studies can be taken from the literature. As already discussed, benign prostatic hypertrophy (BPH) is an important disease in the human and canine population. In man, the α_1-adrenoceptors play an important role in the dynamic component of urinary tract obstruction (Hieble and Ruffolo, 1996). Although non-selective α_1-antagonists, such as prazosin, are effective, the hunt has been on going to develop a subtype-selective antagonist that will have fewer systemic side effects. If only radioligand binding studies

and molecular studies on prostatic tissue were taken into consideration, it would seem clear from binding, that the subtype of α_1-adrenoceptor in the prostate is the α_{1A}- (Goetz et al. 1994). This would be supported by expression levels of mRNA, where although all three cloned subtypes are expressed, expression of the α_{1a}- subtype is highest (Moriyama et al. 1996; Nasu et al. 1996; Faure et al. 1994b). However, from functional studies, this is not quite the case, and indeed the α_{1L}-adrenoceptor seems to be important in mediating contraction in prostatic tissue from a number of species (Shannon Kava et al. 1998; Deplanne and Galzin, 1996; Leonardi et al. 1997; Testa et al. 1997; Ford et al. 1996a; Kenny et al. 1996). Therefore, based solely on binding studies, α_{1A}- selective antagonists such as, RS 17053, SNAP 5089 and Rec 15/2627, would be expected to be effective in the treatment of BPH. In fact, all these antagonists distinguish the α_{1A}-adrenoceptor from the α_{1L}- due to the fact that they have a lower affinity at the latter subtype in functional studies (Leonardi et al. 1997).

Although I have underlined the importance of the functional approach in studying the α_1-adrenoceptors, the molecular approach has proved an important and valuable tool and encompasses a plethora of techniques and approaches.

The cloning and sequencing of the receptor subtypes, although initially confusing, did help in clarifying the idea that subtypes do exist and that they are the result of different gene products. The expression of individual subtypes in cell lines has provided insights into the structure function relationships of these G protein coupled receptors, although the assumption that receptors expressed at high levels in cell lines behave in a similar fashion to native receptors must be treated with caution. In particular, the use of mutant and truncated forms of receptor have identified regions of the receptor important in ligand binding, G protein coupling and phosphorylation/desensitization.

With knowledge of the sequence of the specific subtypes, it has been possible to use techniques to identify the levels of expression of the three cloned subtypes in tissues (Price et al. 1994; Nasu et al. 1996; Piascik et al. 1994) and monitor alterations in levels of expression in disease states (Beaulieu et al. 1997).

In Chapter 5, I describe the isolation of a partial sequence of the canine α_{1a}-adrenoceptor. The reasons for this have been discussed, but to reiterate, were due to the findings of the functional studies demonstrating involvement of the α_{1L}-adrenoceptor. Due to recent evidence suggesting the α_{1a}-adrenoceptor as being responsible for the functional α_{1L}-, it seemed logical to isolate this subtype first. Although the full length receptor was not isolated, the fragment which has been cloned and sequenced, can now be used as a probe to screen a cDNA library. This should allow the full length receptor to be identified and it will be interesting to see if, like the human α_{1a}-adrenoceptor (Chang et al. 1998), isoforms of the canine α_{1a}-adrenoceptor exist. With knowledge of the sequence of the receptor, it will be possible to use a variety of techniques to measure levels of expression in canine tissues, including blood vessels. As has been demonstrated in Chapter 5, and from other studies (Schwinn et al. 1990), Northern blotting is not ideal. One reason for this may be the relatively low level of expression of the adrenergic transcripts, which could have contributed to the failure of the canine α_{1a}-probe to identify transcripts in this study (Graham et al. 1996). Other more sensitive techniques such as PCR, semi-quantitative PCR and RNAse protection assays have already been used successfully in other studies, and the sensitivity of techniques such as PCR would enable examination of relatively small amounts of tissue, such as the blood vessels used in this study.

It would also be of interest to express the full length receptor in cell lines and examine the antagonist profile in relation to inositol phosphate accumulation, to see if, like the

human, the canine α_{1a}-adrenoceptor can exhibit the α_{1L}- profile (Ford et al. 1997b; Ford et al. 1996b).

More recently, subtype-specific antibodies have been used to measure levels of expressed receptor protein on the cell surface using flow cytometry (Hirasawa et al. 1998). This will allow the relationship between not only expression of mRNA and function to be made, but also allow exploration of the relationship between mRNA expression, expression of the receptor on the cell surface and functional contribution of the expressed receptor. The use of flow cytometry in this way is exciting in that it allows the localisation of the receptors on different cell types by the use of cell type specific antibodies in conjunction with the α_1-adrenoceptor specific antibody. This is not possible with radioligand binding. In a recent study (Piascik et al. 1997), it was shown, again using an α_{1B}-adrenoceptor antibody, that receptor protein was expressed in a variety of blood vessels which had previously been shown to express the α_{1B}- subtype at the mRNA level. When functional studies were carried out on these blood vessels, it appeared that the α_{1B}-adrenergic receptor did not have a functional role in mediating contraction of all the vessels in which it was expressed. This raises the question as regards the role of receptors expressed in this way, that do not appear to partake in a functional role. Of course, as already described in the introduction, α_1-adrenoceptors would seem to be involved in other functions apart from vascular smooth muscle contraction. Their role in mediating cell hypertrophy is the source of current research interest (Xin et al. 1997) and possibly certain subtypes may play a more important role in mediating these effects, than others.

A recent novel approach to the study of the adrenoceptors has been the use of antisense oligonucleotides (Gonzalez-Cabrera et al. 1998; Piascik et al. 1997). Antisense technology is treated with a degree of well deserved scepticism and it could be imagined

from the high degree of homology seen between the α_1-adrenoceptor subtypes, that generation of subtype-selective antisense oligonucleotides would be problematic. Despite this, a publication by Piascik et al (1997), describes the use of antisense oligonucleotides for each of the three cloned α_1-adrenoceptors, where the oligonucleotide targets the translation initiation site for the receptor. The antisense oligonucleotides did seem to have the effect of blocking the required subtype and findings were backed up by functional and antibody studies on a variety of vessels. Although the concept of the use of antisense oligonucleotides as therapeutic tools is a long way off, these may well prove useful as an additional means of targeting individual subtypes or removing additional receptor populations, for example a coexisting population of α_2-adrenoceptors such as occurs in the DSV, enabling analysis of the remaining α_1-adrenoceptor population. Information could also be obtained on parameters such as turn over time of receptors, i.e. the length of time between blocking expression of the mRNA and the loss of receptor on the cell surface. Some information on this has already been shown, (Gonzalez-Cabrera et al. 1998) with the use of an α_{1B}-antisense oligonucleotide in DDT_1 MF2 cells, where the density of receptors was reduced by 28% within 24 hours, as ascertained by radioligand binding.

As already mentioned, one of the problems associated with the study of receptors in native tissues has been the presence of multiple receptor subtypes, making dissection of the effects created by a single subtype difficult. One way of overcoming this has been the expression of individual recombinant receptors in cell lines. This too creates potential problems as the cell line may often not contain the same intracellular signalling materials as the native tissue. One potential way around this is the current development of lines of transgenic knockout mice, which are lacking a particular

subtype (Smiley et al. 1998). α_{1B}- knockout mice are currently being studied by other workers in this laboratory.

In Chapter 6 I have discussed experiments comparing vessels from control and heart failure dogs. The main findings from these experiments were, that in heart failure in the saphenous vein and femoral artery, there appeared to be a reduction in sensitivity to exogenous noradrenaline. In addition, there was no reduction in acetylcholine mediated relaxation. In the resistance arteries there was no change in noradrenaline sensitivity or acetylcholine mediated relaxation, although it was hypothesised that treatment in the cohort of heart failure animals used for the resistance artery studies (4/5 on treatment), may have contributed to this finding. Another important finding was that in the femoral artery, maximal responses to both noradrenaline and potassium chloride were significantly greater in the vessels from heart failure animals, while in the resistance arteries the maximal response to potassium chloride only, was significantly enhanced in the vessels from the heart failure cohort.

The main limitation to this part of the study was the supply of clinical material. Nevertheless, the findings from the conduit vessels are convincing and provide a good basis for future work.

A logical progression would be to examine the effect of endothelial denudation on the reduction in sensitivity to noradrenaline seen in heart failure, and also to establish if the alteration in sensitivity is observed with other types of agonist such as angiotensin, endothelin and vasopressin.

It would also be valuable to examine the α_1-adrenoceptor population in more detail in heart failure animals, especially if α_1-adrenoceptors appear to be contributing to the alteration in sensitivity to noradrenaline identified in the femoral artery and saphenous vein. It is possible that there could be changes in receptor number, receptor subtype or

receptor coupling and signal transduction. Reports from the literature in humans and experimental models of heart failure are inconclusive reporting both increases, (Forster et al. 1989; Forster and Armstrong, 1990; Stassen et al. 1997a; Stassen et al. 1997b), decreases, (Feng et al. 1994) or no change (Indolfi et al. 1994; Kubo et al. 1989) in the responsiveness of α_1- and α_2-adrenoceptors.

Further studies would take the form of experiments designed to address each of these possibilities. Functional and radioligand binding experiments would help to address the issues of receptor number and receptor subtype. In addition, vessels from some of the heart failure animals already used have been stored in liquid nitrogen for the purpose of examining expression of the α_1-adrenergic subtypes once the full length receptors have been identified and the technique established in control tissue.

In addition to a more detailed look at the α_1-adrenoceptor population, it would be of value to examine the role of the endothelium more closely.

Although no differences were found in any of the vessels to acetylcholine mediated relaxations, it would be of interest to look at the relative roles of nitric oxide and endothelium-derived hyperpolarizing factor in this response, as it is now clear that both of these can be released by acetylcholine, depending on the vessels examined (Vanhoutte, 1996).

It would also be of interest to examine cAMP-mediated vasorelaxations, for example prostacyclin mediated vasorelaxation, since one study has demonstrated impairment of cAMP-mediated vasorelaxations in canine pulmonary arteries from experimental heart failure dogs, despite no alteration in the cGMP-mediated vasorelaxations (Mathew et al. 1993).

Finally, with increased numbers of clinical cases, it will be possible to subdivide the heart failure animals into groups based on aetiology and treatment, to see if any of these parameters influence findings.

To summarise, functional classification of the canine vascular α_1-adrenoceptors has provided a useful and revealing insight into the complexities of receptor classification, and while answering some question, it has raised others. Although not complete, good progress has been made towards isolation of the full length canine α_{1a}-adrenoceptor, which will allow expression of this subtype to be studied in canine tissues. Both the functional and molecular approach can then be used to make a comparison between control dogs and animals with naturally occurring heart failure, since it has already been established that in both the saphenous vein and femoral artery there appears to be a loss of sensitivity to noradrenaline.

This ground work and the questions it raises, together with the exciting possibilities arising from novel approaches such as the use of subtype-specific antibodies and antisense oligonucleotides, ensures a bright and exciting future in this area of research.

Appendix I

HAEMALUM & EOSIN (H&E.)

Solutions.

GILL II HAEMATOXYLIN (Gill et al. 1974).

1% EOSIN.

Method.

1.) Xylene or substitute. = 2 minutes.
2.) Xylene or substitute. = 2 minutes.
3.) Absolute alcohol. = 2 minutes.
4.) Absolute alcohol. = 2 minutes.
5.) 90% Alcohol. = 2 minutes.
6.) 90% Alcohol. = 2 minutes.
7.) 70% Alcohol. = 2 minutes.
8.) Tap water. = Rinse.
9.) Haematoxylin. = 5 minutes.
10.) Blue in running tap water. = 5 minutes.
11.) Eosin. = 2 minutes.
12.) Running tap water. = Quick rinse.
13.) 70% Alcohol. = Quick rinse.
14.) 90% Alcohol. = 1 minute.
15.) 90% Alcohol. = 1 minute.
16.) Absolute alcohol. = 2 minutes.
17.) Absolute alcohol. = 2 minutes.
18.) Xylene or substitute. = 2 minutes.
19.) Xylene or substitute. = 2 minutes.
20.) Mount in synthetic mounting medium.

Result.

Nuclei. = Blue/purple/black.

All other structures. = Shades of pink/red.

IMMUNOPEROXIDASE.

Immunostaining by the indirect method.

Wax sections.

Method.

1.) Bring sections to water (and treat if necessary).

2.) Inhibit endogenous peroxidase. = 30 minutes.

3.) Wash in gently running tap water for 15 minutes.

4.) Wipe slide, **leaving section moist,** and circle section with Pap Pen, **allow circle to dry.**

5.) Rinse in Buffer (PBS or TBS).

6.) Options. (Wax sections normally require both.)

A. Antigen retrieval.

The one of choice for the antibody.

B. Block non-specific sites.

Normal serum from species supplying second antibody diluted 1:30 with sera diluent. Add to slide and leave for 15 minutes. **Do not rinse sections,** but draw off excess serum with a tissue and proceed with IHC.

7.) Apply the primary antibody at a predetermined optimal dilution in sera diluent. (NPY 1:12000 and TH 1:4000).

8.) Place slides in moist chamber and incubate, **in the dark,** at room temperature (or 4°C) for 1-48 hours depending on the dilution of the antibody.

9.) Buffer. = 3 x 5 minutes.

Either:

10.) Apply peroxidase-conjugated second antibody diluted in sera diluent for 30 minutes to 1 hour at room temperature.

11.) Buffer. = 3 x 5 minutes.

Or:

10.) Finish with an ABC kit.

11.) Buffer. = 3 x 5 minutes.

12.) Develop peroxidase using the intensified Graham and Karnovsky DAB method or Sigma *Fast* DAB tablets. = 5 minutes.

Or:

Sigma AEC kit, or equivalent.

13.) Wash in running tap water for 10 minutes.

14.) Counterstain nuclei as and if required.

15.) AEC, mount in aqueous mountant, peroxidase can be DCM

Result.

Antigenic sites = black or red.

Nuclei as counterstain.

IMMUNOPEROXIDASE.

Immunostaining by the indirect method.

Whole mounts.

Method.

Ian Montogomery uses needle dishes for this method.

1.) Fixation as sections.

2.) PBS/TBS + 0.2% Triton X-100. = 2 x 20 minutes. **Use for all buffer rinses.**

3.) Inhibit endogenous peroxidase. = 30 minutes.

4.) Wash in tap water for 15 minutes with several changes.

5.) Options. (Normally both)

A. Antigen retrieval.

The one of choice for the antibody. These specimens normally require a little longer.

B. Block non-specific sites.

Normal serum from species supplying second antibody diluted 1:30 with sera diluent. Add to specimen and leave for 30 minutes. **Do not rinse specimens and proceed with IHC.**

6.) Incubate in the primary antibody, at a predetermined optimal dilution in sera diluent, overnight at room temperature (or 4°C) **in the dark.** (NPY 1:12000, TH 1:4000)

7.) Buffer. = 3 x 20 minutes.

Either:

8.) Apply peroxidase-conjugated second antibody diluted in sera diluent for 30 minutes to 1 hour at room temperature.

9.) Buffer. = 3 x 5 minutes.

Or:

8.) Finish with an ABC kit.

9.) Buffer. = 3 x 5 minutes.

10.) Develop peroxidase using the intensified Graham and Karnovsky DAB method or Sigma *Fast* DAB tablets. = 5 minutes.

Or:

Sigma AEC kit, or equivalent.

11.) Wash in several changes of tap water for 10 minutes.

12.) Counterstain nuclei as and if required.

13.) AEC, mount in aqueous mountant, peroxidase can be DCM

Result.

Antigenic sites, black or red.

Nuclei as counterstain.

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