# The study of responses mediated by $\alpha_2$ and $\alpha_1$-adrenoceptors in the tail and mesenteric resistance arteries from transgenic mice.

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**Contents Page**

**Abstract**

The work presented in this thesis describes the development of a method to investigate $\alpha_2$-mediated responses in the tail and first order mesenteric resistance arteries of the mouse. Furthermore, responses mediated by $\alpha_1$-adrenoceptors have been studied and, in part, subtyped in these vessels. These aims were achieved by using a combination of subtype selective ligands and transgenic technology.

$\alpha_2$-adrenoceptors, expressed in the tail artery, mediate contractile responses that at $37^0$C are susceptible to profound, persistent desensitisation. In the absence of a fully functional $\alpha_{2A/D}$ receptor pool, contractions are affected by the way in which an agonist is administered. At $22^0$C, the $\alpha_2$-mediated response is significantly potentiated, a response that does not rely on the $\alpha_{2A/D}$ subtype, but is critically dependent on previous exposure to an $\alpha_1$-selective agonist.

In mesenteric resistance arteries, $\alpha_2$-adrenoceptors, proposed to exist on the endothelium, mediate vasodilatations. Responses depend on more than one $\alpha_2$-subtype, and are mediated by nitric oxide, and possibly EDHF.

In the tail artery, $\alpha_{1A}$-adrenoceptors mediate contraction with little, if any, contribution from the $\alpha_{1B}$-subtype, and increasing age has little effect on the responses gained. Like the tail artery, the $\alpha_{1A}$-receptor subtype is the major receptor mediating contraction of mesenteric arteries. However, in the absence of the $\alpha_{1B}$-adrenoceptor, a small, but significant contraction, mediated by the $\alpha_{1D}$-adrenoceptor is uncovered.

The use of subtype selective ligands and transgenic mice, has clarified the function of $\alpha_2$ and $\alpha_1$-adrenoceptors in two mouse blood vessels, and allowed partial subtyping of the response. Alone, neither technique would have provided such clarity.

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**Declaration**

The work presented in this thesis is entirely my own, with the exception of figures 8.1 **A** and 8.1 **B** which were produced by Ann McGee and figure 2.1, and figure 2.2, which were kindly donated by Jude S. Morton.

This work has not been presented in part or alone for any other degree course. Some of the work contained herein has been published in part: a list follows.

1. **McBride M**, Daly C.J, Tsujimoto G, McGrath J.C (2003). Removal of the $\alpha_{1B}$-adrenoceptor uncovers an $\alpha_{1D}$ component in mesenteric resistance arteries. Tenerife abstract: P84.
2. **M McBride,** Daly C. J, McGrath J. C (2002). Contractile responses to UK14304 in the mouse tail artery differ when the drug is administered cumulatively or non-cumulatively. Hypertension **40** (**4**): 581 PD.02.

3. **McBride M**, Daly C.J, McGrath J.C (2002). Contractile responses to UK14304 are inhibited by low concentrations of rauwolscine but are unaffected by prazosin in the mouse tail artery. British Journal of Pharmacology **137** (Supplement): 91P.

4. **McBride M**, Daly C.J, McGrath J.C (2002). $\alpha_1$ and $\alpha_2$ adrenoceptors mediating resistance artery contraction as revealed by receptor "knockouts". The Pharmacologist **44**: No.2 (Supplement) A79, 59.4.

5. **McBride M**, Daly C.J, McGrath J.C (2002). Rauwolscine potentiates maximum contractions to UK14304 in tail arteries from mice. Vascular Neuroeffector Meeting (Meeting Abstract).

6. **McBride M**, Daly C.J., McGrath J.C. Tail arteries from D79N mice show reduced responses to UK14304 compared with wild type. British Journal of Pharmacology **135**: 304P, 2002.

7. McGrath JC, Pediani JD, Macmillan J, Mackenzie J, Deighan C, Woollhead A, McGrory SP, **McBride M**, Ali Z, Malekzadeh-Shafaroudi M, Cotecchia S, Arribas SM, Vila E, Briones A, Perez D, Mullins J, Tsujimoto G & Daly CJ. (2002). Adventitial cells are identified as the major location of vascular alpha1B-adrenoceptors and may drive vascular remodelling. British Journal of Pharmacology **137**: (Suppl); 21P (Abstract).

8. Daly C.J, Deighan C, McGee A, Mennie D, Ali Z, **McBride M**, McGrath J.C. A knockout approach indicates a minor vasoconstrictor role for vascular $\alpha_{1B}$-adrenoceptors in mouse. Physiological Genomics **9**: 85-91, 2002.

**List of abbreviations**

| | |
|---|---|
| BMY7378 | (dihydrochloride 8-[2-[4-(2-methoxyphenyl)-1-piperozyn]-8-azaspiro (4,5) deconc-7,9-dione |
| $Ca^{2+}$ | calcium |
| cAMP | adenosine 3'-5' cyclic monophosphate |
| CEC | chlorocthylclonidine |
| DAG | diacylglycerol |
| DR | dose ratio |
| $IP_3$ | inositol-1,4,5-triphosphate |
| K | potassium |
| KO | knockout (receptor subtype deleted from the murine germ line) |
| $\alpha_{1B}$ | denotes a receptor subtypc that has been classified pharmacologically |
| $\alpha_{1b}$ | denotes a receptor cloned, then expressed in a cell line |
| L-NAME | $N^{\omega}$-nitro-L-arginine methyl ester hydrochloride |
| 5HT | 5-hydroxytryptamine |
| 5MeU | 5-methylurapadil |
| mRNA | messenger RNA |
| NA | noradrenaline |
| $Na^{2+}$ | sodium |
| NOS | nitric oxide synthase |
| $pA_2$ | affinity estimate of an antagonist derived from a Schild plot |
| $pEC_{50}$ | negative log of agonist concentration producing fifty percent of the maximal response, alone, or in the presence of an antagonist |
| PE | phenylephrine |
| µm | micrometer (unit of measurement) |
| U19 | U46619 |
| UK | UK14304 |
| SEM | standard error of the mean |

**List of figures**

**Summary**

Chapter three

1. A method (shown to be effective in the rat tail artery) has been applied and adapted for successful study of UK14304-mediated contractions in the mouse tail artery, where a contractile function has been established, and in part, subtyped to $\alpha_{2A/D}$-adrenoceptors.
2. The effects of elevated vascular tone and nitric oxide synthase inhibition have been studied in detail, to determine suitable conditions for further pharmacological analysis of responses mediated by $\alpha_2$-adrenoceptors in the mouse tail artery.
3. Low levels of tone and/or inhibition of nitric oxide release enhance sensitivity and increase the size of the response gained. In terms of size and sensitivity the most advantageous conditions are high elevation of vascular tone with U46619 to levels comparable to 50 % of the noradrenaline response.
4. All of the protocols tested were carried out in the WT and D79N, for partial subtyping of the response. The sizes of responses gained were significantly smaller in the D79N. This indicates that at $37^0$C, and under these conditions, the $\alpha_{2A/D}$-adrenoceptor is involved in contraction of the murine tail artery.

Chapter four

1. UK-mediated contractions are susceptible to profound, persistent desensitisation and the study of responses in the tail artery is complicated by the development of rhythmic contractions. In the absence of a fully functional population of $\alpha_{2A/D}$-adrenoceptors the UK response is dependent on the method in which the agonist is administered, evidenced by enhanced responses in the D79N when UK14304 is administered non-cumulatively.

2. Rhythmic contractions develop in the mouse tail artery to a variety of exogenous agonists, an effect which is abolished by low concentrations (without causing a significant reduction in maximum) of nifedipine, an L type calcium channel antagonist
3. Desensitisation of the UK response is receptor specific and is alleviated by reducing the time that receptors are exposed to agonist or construction of non-cumulative response curves.
4. The desensitisation caused by UK is reversed by rauwolscine, representing a unique response that may reflect a "switching on" of a response, or the inhibition of receptor internalisation, therefore permitting a further response.
5. The non-cumulative UK response in the WT and D79N is competitively antagonised by rauwolscine, but unaffected by prazosin. This indicates that the UK-mediated contractions, results from selective stimulation of vascular $\alpha_2$-adrenoceptors (even at the highest agonist concentrations tested).

Chapter five

1. The contractile response to UK in the mouse tail artery has been assessed at two temperatures, $22^0$C and $37^0$C; responses are of comparable size and sensitivity in the WT and D79N at both temperatures. However, previous exposure to phenylephrine leads to a potentiation in contractility at $22^0$C. Partial subtyping of the response (with the D79N) indicates that the $\alpha_{2A/D}$-adrenoceptor is not involved in the enhanced response.
2. The phenylephrine response is unaffected by a reduction in temperature, but significantly potentiated in the D79N at $22^0$C. This represents a unique phenotype, which may be due to upregulation of $\alpha_1$-mediated contractions in the absence of a functional $\alpha_{2A/D}$ response.

3. Contractions to the agonists, phenylephrine, noradrenaline and 5HT were studied in WT and D79N mice. Phenylephrine (i.e. $\alpha_1$) and 5HT-mediated contractions are comparable in both strains, indicating no change in function of these receptors at $37^0$C. The noradrenaline response at $37^0$C is significantly smaller in the D79N tail artery than the WT. This suggests that at $37^0$C the $\alpha_{2A/D}$-adrenoceptor is involved in mediating contractions to this non-selective agonist.

Chapter six

1. UK causes concentration-related vasodilatations in first order mesenteric resistance arteries from WT and D79N mice, which appear to involve two $\alpha_2$-receptor subtypes, and depend on the release of nitric oxide, and possibly EDHF.
2. Rauwolscine antagonises the UK-mediated response in the WT, but at high agonist concentrations the effect is overcome. This data provides evidence that the vasodilator response is receptor specific and is most probably mediated by $\alpha_2$-adrenoceptors located on the endothelium.
3. The UK-response is inhibited by L-NAME ($1 \times 10^{-4}$M), but the effects are surmountable, indicating the involvement of another endothelial derived relaxing factor. Elevated levels of $K^+$ (15 and 30mM) cause concentration dependent reductions in UK-mediated relaxations, indicating the involvement of EDHF in the relaxant response (inconclusive without the use of prostaglandin inhibitors and nitric oxide scavengers).

Chapter seven

1. The effects of prazosin (at 4 and 16-months) and subtype selective antagonists were studied in the mouse tail artery. The $\alpha_{1A}$-adrenoceptor appears to be the

major subtype leading to contraction, while the $\alpha_{1B}$-adrenoceptor plays little, if any, role in the development of contractile responses, in young and old mice.

2. Nifedipine prevents the development of rhythmic contractions in the mouse tail artery, and causes a significant reduction in the phenylephrine response in the WT and $\alpha_{1B}$ KO at both age points. The reduction in maximum is more pronounced at four-months in the KO, an unexplained phenotype, which may indicate a greater dependency on extracellular calcium in the absence of functional $\alpha_{1B}$-adrenoceptors.
3. Prazosin competitively antagonises phenylephrine-induced contractions in the WT and $\alpha_{1B}$ KO tail artery and, at both age points, causes a significant reduction in maximal responses at high antagonist concentrations.
4. At both age points, the $pA_2$ value for prazosin is slightly lower in the $\alpha_{1B}$ KO than the WT, and at 16-months the slope of the Schild regression plots shifts from unity, which is indicative of non-competitive antagonism.
5. Alone, prazosin provides little information on the subtype (s) involved in the contractile response in the mouse tail artery, but combined with the use of subtype selective antagonists provides clarity on the role of each receptor in contraction.

Chapter eight

1. It has been established that like the tail artery, the $\alpha_{1A}$-adrenoceptor is the principal contractile receptor in first order mesenteric resistance arteries. However, in the absence of functional $\alpha_{1B}$ -adrenoceptors, a unique, contractile function, mediated by the $\alpha_{1D}$-subtype has been uncovered.
2. Responses have been studied at 4 and 16-months, and are clarified in arteries from older animals. In the $\alpha_{1B}$ KO a 5MeU resistant component of contractions

is obvious, and is notably absent in the $\alpha_{1D}$ KO. BMY7378 abolishes the 5MeU resistant component of the contractile response, leaving a response that is comparable to that of the $\alpha_{1D}$ KO.

3. This data provides evidence that, at least in the $\alpha_{1B}$ KO, the $\alpha_{1D}$-adrenoceptor plays a small, but significant, role in contraction of first order mesenteric arteries of the mouse.

# Chapter one

Introduction and literature review

**General Introduction**

Adrenoceptors were first described over fifty years ago. The function of each adrenoceptor has been studied extensively, and the role they play in regulating a diverse array of physiological functions has been highlighted. With the advent of transgenic technology in the last five years, physiological/pharmacological research has been provided with a new tool that enables the study of individual receptor subtypes. I have used a combination of classical pharmacology combined with the use of transgenic mice to study the function of adrenoceptors in two murine blood vessels, the tail and first order mesenteric resistance arteries. The murine strains that I have used include a normal 'wild type' control (C57/BL6c/129Sv), $\alpha_{1B}$ knockout, $\alpha_{1D}$ knockout, and the D79N ($\alpha_{2A/D}$ mutant).

Many of the recent advances in the treatment of cardiovascular disease have come from intensive research on animal models of human disease. Over the last few years there has been an explosion in the study of murine models of human disorders for a number of reasons. The availability and cost of mice makes them extremely desirable to work with. More importantly, advances in molecular biological techniques have provided us with mice harbouring mutated, upregulated, constitutively active or deleted receptors.

Transgenic mice are currently being used in the field of cardiovascular biology in an attempt to delineate the role of individual receptor subtypes and to determine what role, if any, adrenoceptors play in the development and progression of cardiovascular disease. The availability of these transgenic mice in part obviates the need for subtype selective antagonists, the lack of which has hampered the clarification of receptor-mediated responses for many years. However, the use of subtype selective antagonists is still desirable, and can have advantages over the use of knockout mice. The main

reason for this is that antagonists exclude the possibility that compensatory mechanisms lead to a change in phenotype, which cannot always be excluded when using transgenics.

On the basis of size the murine tail artery and first order mesenteric branch can be defined as resistance arteries; that is they have a diameter of less than 400μm. However, this definition arose from studies carried out on human resistance vessels, and there are a far greater number of vessels that therefore fall into this category in the mouse than in human species. Due to this, murine arteries having a diameter less than 400μm, are described as distributing vessels, as their role in the maintenance of peripheral blood pressure is as yet unclear.

The first objective of this work was to develop a protocol, suitable for the study of responses mediated by postjunctional $\alpha_2$-adrenoceptors in the mouse tail artery. Following successful completion of this objective, I then used the D79N ($\alpha_{2A/D}$ mutant) mouse to classify the responses gained. Having established the response in the murine tail artery, I then applied the same principal to the study of $\alpha_2$-mediated responses in first order mesenteric resistance arteries. Again, I had the intention of determining what role the $\alpha_{2A/D}$-adrenoceptor contributes to responses by use of the D79N mouse.

In relation to responses mediated by vascular $\alpha_1$-adrenoceptors, this work has two aims. Firstly to determine the contribution of the $\alpha_{1B}$-adrenoceptor to contractile responses in the mouse tail artery. Given the magnitude of this task, I studied the effect of prazosin on phenylephrine-induced contractions while colleagues in our laboratory determined the effect of subtype-selective ligands. In addition to responses in the tail artery I also

set out to study the responses mediated by $\alpha_1$-adrenoceptor subtypes, known not to be the major receptor leading to contractions of mesenteric resistance arteries. To achieve this aim, I took advantage of the availability of two strains of mice carrying deletions of the $\alpha_{1B}$ and $\alpha_{1D}$-receptor subtypes, combined with the use of subtype selective antagonists.

## 1.0 Transgenic Mice: A new pharmacological tool

### 1.0.1 Genetically altered mice

Transgenics can be defined as those animals carrying a segment of exogenous genetic information. To date, transgenic mice have provided us with considerable insight into the functions of a variety of receptors, including, adrenoceptors. One of the main advantages of using gene-targeted animals, is that it simplifies the interpretation of results, and makes the assignment of functions to a given receptor subtype(s) under complex physiological conditions far easier. In addition to this, gene targeting also overcomes the problems associated with the use of non-selective ligands.

Until the development of transgenic technology, the rat was the major rodent species used to delineate the role of adrenoceptor subtype (s) in vascular biology, in a variety of tissue types. Unfortunately, manipulation of genes is far easier in the mouse than the rat, so the focus of many research laboratories has now shifted. The disadvantage of changing the species, in which responses are studied, is that many experiments have to be repeated to determine if functional responses are similar in the mouse to those already elucidated in the rat.

Targeted gene disruption was first developed in the mid to late 1980s [Thompson et al, 1989]. The most common method for generating gene-targeted mice is to use 129Sv cell lines to knockout, overexpress, or mutate a gene of interest. Once the gene of choice has been successfully altered, embryonic 129Sv stem cells are injected into blastocysts from C57BL/6 mice [Picciotto & Wickman, 1998]. During production of the F2 generation, the genetic material of both species is mixed, segregated, and randomly selected. To counteract this, mice from the F2 generation are then

backcrossed seven times. In doing so, the progeny have a genetic makeup which is 99 % identical. This reduces the level of heterogeneity within the transgenic mouse colony. Backcrosses into the 129Sv murine strain are possible, but are rarely carried out because of the unreliable reproductive cycle of this mouse. Controlling the heterogeneity of transgenics is imperative, especially when interpreting experimental results. It has also been suggested that inbreeding to maintain the genetics within a strain should be avoided [Banbury Conference, 1997].

1.0.2 Overexpressing a gene of interest

Genes can be overexpressed in many different animal species, including the rat. However, in the majority of studies mice are favoured, because of the wealth of experience that now exists in manipulation the murine genome. Once a gene of interest has been identified, it is cloned, and inserted into the genome of a target animal, or, if preferred a cell line. When the inserted DNA segment has been stably transfected and incorporated into the recipient genome, animals can be genotyped and bred. The progeny of successful transfections are frequently used to determine the role of the overexpressed receptor/gene product and/or to determine the signalling pathways utilised when a receptor becomes active.

1.0.3 Deleting a germline receptor

Several murine models carrying gene directed knockouts of adrenoceptor subtypes have recently become available. One example of this is the $\alpha_{2A/D}$ knockout mouse. The $\alpha_{2A/D}$-adrenoceptor is removed from the murine germline by insertion of a premature stop codon. When Western blot analysis confirms that the gene has been successfully

removed, embryonic stem cell clones carrying the deletion are transferred into pseudopregnant females and mice are bred as described previously.

### 1.0.4 A cautionary note

Compensatory changes resulting from the up, or down regulation of other structurally related receptor subtypes or activation of alternative signalling pathways cannot always be excluded as the reasons for an altered response or unusual phenotype. Studies using transgenic mice most at risk of phenotypic changes appear to be behavioural studies, as contradictory results can occur, even within the same breeding batch [Crabbe et al, 1999]. To prevent inconsistencies, reproducibility of results is key. Data from different groups should be monitored very closely, so that real changes can be interpreted as receptor specific, and not be attributed to compensatory mechanisms that result from the lack of a functional receptor.

## 1.1.0 $\alpha_1$-adrenoceptor pharmacology

### 1.1.1 Discovery

Almost one hundred years ago, it became apparent that hormones and endogenous neurotransmitters caused more than one physiological response. Proposed to be the result of stimulating more than one receptor belonging to the same subclass. For example, adrenaline, isolated from the adrenal glands given *in vivo* caused vasoconstriction, followed by vasodilatation [Rang et al, 1995 (Churchill Livingstone)]. Some time later, Ahlquist first proposed the subdivision of adrenoceptors based on the rank order of potency for noradrenaline, adrenaline and the synthetic beta agonist, isoprenaline [Ahlquist, 1948]. Subdivisions of the β-adrenoceptor subclass were suggested some time later when differential responses resulting from the stimulation of β-adrenoceptors were described. Stimulation of β-adrenoceptors expressed in the heart causes positive inotropic effects, while activation of β-adrenoceptors expressed in the vasculature causes vasodilatation. These responses were attributed to the activation of different β-adrenoceptor subtypes.

McGrath and co workers first described heterogeneity within the $\alpha_1$-adrenoceptor subclass in 1982. Their findings were based on the discovery that phenylethanolamine agonists produce a biphasic response curve. They discovered that a portion of the contractile response in the rat anococcygeus was resistant to antagonism with prazosin. The prazosin resistant component of contraction persisted even when prazosin was combined with an $\alpha_2$-antagonist [McGrath et al, 1982]. *In vitro* experiments on the rabbit basilar artery and the rat anococcygeus then led McGrath and co workers to conclude that more than one $\alpha_1$-subtype existed, and that $\alpha_1$-adrenoceptors should be sub-divided into $\alpha_{1a}$ and $\alpha_{1b}$.

Non-phenylethanolamine agonists are unresponsive in the rabbit basilar artery, but produce a monophasic curve in the rat anococcygeus. Low concentrations of phenylephrine appear to stimulate $\alpha_{1a}$-adrenoceptors, while $\alpha_{1b}$-adrenoceptors mediate contractions at higher drug concentrations. At that time, a lack of suitable antagonists to distinguish between low and high affinity binding sites prevented further analysis.

Flavahan and Vanhoutte used an alternative method to classify $\alpha_1$-adrenoceptors, and described responses mediated by $\alpha_1$-adrenoceptors according to their potency for prazosin and yohimbine. They proposed that there were two $\alpha_1$-subtypes, each having different affinities for prazosin. Stimulation of the high affinity site, named the $\alpha_{1H}$, yields a $pA_2$ value greater than 9.0 for prazosin, and a $pA_2$ greater than 6.4 for yohimbine. Low affinity receptors, termed $\alpha_{1L}$-adrenoceptors, have reduced affinity for both prazosin and yohimbine [Flavahan & Vanhoutte, 1986].

Radioligand binding studies are a useful pharmacological tool, because they provide information on the number, molecular character and physiological function of receptors [Wood et al, 1979]. Due to the vast amount of information generated by these experiments, this technique has been used extensively to enhance our understanding of adrenoceptor functions and to define subtype selective responses.

Saturation binding experiments revealed two distinct binding sites based on the affinity of phentolamine and prazosin. Phentolamine and prazosin have different affinities for each binding site. Conclusive evidence that more than one $\alpha_1$ receptor exists, came from studies using the subtype selective antagonist WB4101 [$^3$H] in binding studies on

the rat brain. $\alpha_1$-adrenoceptor-mediated responses were then described on the basis of the potency of WB4101 to antagonise responses. Receptors were described as WB4101 sensitive $\alpha_{1A}$, and prazosin sensitive $\alpha_{1B}$-adrenoceptors [Morrow and Creese, 1986]. However, pharmacological subclassification was not widely accepted until 1994 when studies using selective $\alpha_1$ and $\alpha_2$ ligands were completed [Bylund et al, 1994].

CEC is an alkylating analogue of clonidine that has been used to distinguish between different $\alpha_1$-adrenoceptor subtypes. CEC reduces the number of available $\alpha_1$ binding sites by alkylating plasma membrane bound α-adrenoceptors. Given this, $\alpha_1$-adrenoceptors were then subdivided, on their susceptibility to antagonism with CEC, because studies suggested that this drug could distinguish responses mediated by the $\alpha_{1B}$-adrenoceptor from those resulting from stimulation of the $\alpha_{1A}$-receptor-subtype [Elhawary et al, 1992, Guarino et al, 1996].

To date, all of the $\alpha_1$-adrenoceptors cloned, have high affinity for prazosin and fit with the classification proposed by Flavahan and Vanhoutte, of the high affinity $\alpha_{1H}$ [Flavahan & Vanhoutte, 1986]. However, to date no receptor subtype has been cloned that fits the description of the low affinity-binding site, the $\alpha_{1L}$ receptor [Docherty, 1998]. Ford and colleagues explained these findings by proposing that the $\alpha_{1L}$-adrenoceptor may represent an energetically favourable conformation of the $\alpha_{1A}$-adrenoceptor [Ford et al, 1998]. In recent years evidence in support of this hypothesis has grown.

Current classifications have led to the identification of nine adrenoceptor subtypes. The α-subclass these include the $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, $\alpha_{2A/D}$, $\alpha_{2B}$, $\alpha_{2/C}$, in addition to the uncloned,

illusive $\alpha_{1L}$. β-adrenoceptors have been subdivided into $\beta_1$, $\beta_2$ and $\beta_3$ and a forth subtype, the $\beta_4$-adrenoceptor has been proposed to exist [Guimaraes & Moura, 2001].

### 1.1.2 Expression of $\alpha_1$-adrenoceptors

The three $\alpha_1$-adrenoceptor subtypes expressed in humans, are encoded by separate genes on chromosomes 8, 5 and 20, which correspond to the $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$ subtypes respectively [Hieble et al, 1995]. In humans and in animal species, alpha adrenoceptors control a plethora of physiological functions; including vasoconstriction, regulation of vascular growth, blood pressure regulation, heart rate, cognitive functions, metabolism, water and metabolite balance [Kunos & Ishac, 1987] and lipolysis. The physiological importance of $\alpha_1$-adrenoceptors becomes clear in potentially life threatening situations, such as shock and haemorrhage. In these situations, stimulation of $\alpha_1$-adrenoceptor-mediated contractions can function to save life, when the sympathetic nervous system regulates blood vessel contraction to maintain blood flow, and can maintain pressure even when a twenty percent loss of a patient's blood volume has occurred [Little & Kirkman, 1997].

The existence of multiple receptor subtypes in arteries, veins and tissues, makes the study of subtype specific responses complex. In addition to the complexity of the situation where more than one $\alpha_1$-adrenoceptor is expressed in a given tissue or blood vessel, the major adrenoceptor subtype responsible for vascular contractions varies depending on the species studied and the vessel type [Docherty et al, 1998]. Having said this, there are a few tissues that contain a pure population of $\alpha_1$-adrenoceptors. Two examples of this are the rat liver and spleen. Radioligand binding studies show that both of these tissues contain a pure population of $\alpha_{1B}$-adrenoceptors [Hieble et al,

1995]. In the liver $\alpha_1$-adrenoceptors control a range of essential physiological functions including gluconeogenesis, fatty acid metabolism and protein release [Garcia-Sainz et al, 1999]. Human livers express predominantly $\alpha_{1A}$-adrenoceptors [Price et al, 1994]; while the major subtype expressed in animal models varies between species.

The major $\alpha_1$-adrenoceptor subtype mediating catecholamine-induced responses in human arteries, depends on the vascular bed studied and on the age of a patient [Rudner et al, 1999]. Contractions in human resistance arteries result from dual activation of $\alpha_1$ and $\alpha_2$-adrenoceptors [Parkinson et al, 1992]. When the $\alpha_1$-adrenoceptor is the dominant contractile receptor, analysis has been carried out to determine which $\alpha_1$ subtype predominates (in essence, the response has been subtyped). Measuring mRNA/protein levels have shown that the $\alpha_{1a}$-adrenoceptor is the most widely expressed $\alpha_1$-receptor subtype. The $\alpha_{1a}$ subtype is found in vascular, pulmonary, renal, splanchnic and coronary arteries. In the venous circulation all three $\alpha_1$-adrenoceptor subtypes are expressed to comparable levels [Rudner et al, 1999].

Agonist-induced changes in $\alpha_1$-adrenoceptor expression levels

Human embryonic kidney (HEK 293) cells have been used to study agonist-mediated changes in the expression of $\alpha_1$-adrenoceptors. Noradrenaline-induced changes in receptor expression have been studied in this cell line. Prolonged exposure to noradrenaline has no effect on the expression of the $\alpha_{1A}$ subtype, but increases expression of the $\alpha_{1B}$, while causing downregulation of $\alpha_{1D}$-adrenoceptors. The effect of noradrenaline on the expression of all three $\alpha_1$-adrenoceptor subtypes is time-dependent. Inhibiting protein kinase C with calphostin C prevents $\alpha_{1D}$ downregulation,

but is ineffective in halting increased expression of $\alpha_{1B}$-adrenoceptors. The $Ca^{2+}$-ATPase inhibitor thapsigargin, blocks enhanced $\alpha_{1B}$ expression [Lei et al, 2002]. To conclude, in HEK 293 cells prolonged exposure to noradrenaline has differential effects on the expression of $\alpha_1$-adrenoceptors. Noradrenaline-induced changes in the expression of $\alpha_{1D}$ and $\alpha_{1B}$-adrenoceptor appear to result from utilisation of different signalling cascades [Lei et al, 2002].

### 1.1.3 Subtype specific responses

#### $\alpha_{1A}$ Adrenoceptors

The $\alpha_{1A}$-adrenoceptor was originally termed the $\alpha_{1C}$ subtype, but was later renamed. The initial confusion in nomenclature arose from studies where the cloned $\alpha_{1C}$ receptor failed to localise its mRNA in tissues already subtyped as $\alpha_{1A}$ [Piascik & Perez, 2001]. Subtype selective agonists that bind $\alpha_{1A}$-adrenoceptors with high affinity are oxymetazoline and methoxamine. Antagonists that selectively bind to the $\alpha_{1A}$-adrenoceptor are 5-methylurapadil, WB4101 and niguldipine. The $\alpha_{1B}$-adrenoceptor is distinguished from the $\alpha_{1A}$ by having a lower affinity for all of these compounds. [Piascik et al, 1997]

$\alpha_{1A}$ knockout mice are slightly hypotensive, having a ten percent reduction in resting blood pressure; in addition agonists selective for the $\alpha_{1A}$ receptor do not cause pressor responses in the $\alpha_{1A}$ knockout mouse. However, phenylephrine still causes an elevation in blood pressure, which is slightly attenuated compared with controls [Rokosh & Simpson, 2001]. This confirms the hypothesis that the $\alpha_{1A}$-adrenoceptor mediates

pressor responses in the mouse, and that other $\alpha_1$-receptor subtypes are involved in the control of blood pressure regulation.

The gene promoter for the rat $\alpha_{1a}$-adrenergic receptor has recently been cloned and characterised, and found to contain multiple transcription initiation sites. Gel shift analysis confirms that hypoxic conditions can cause a shift in the promoter sequence and directly activate transcription [Michelotti et al, 2003], illustrating that pathophysiological conditions can affect expression of the $\alpha_{1A}$-adrenoceptor at the transcriptional level.

Responses mediated by $\alpha_{1A}$ and/or $\alpha_{1L}$-adrenoceptors

Stimulation of $\alpha_{1A}$-adrenoceptors causes contraction of smooth muscle cells in the lower urinary tract [Marshall et al, 1995]. Contrary to this, other studies suggest that the $\alpha_{1L}$-adrenoceptor is the major contractile subtype in the urinary tract [Ford et al, 1996a]. The existence of the $\alpha_{1L}$-adrenoceptor relies upon pharmacological analysis, and it has been suggested that the $\alpha_{1L}$ represents a low affinity state of the $\alpha_{1A}$-adrenoceptor. If this were the case, it would account for the variability in experimental data, and will help explain why attempts to isolate the $\alpha_{1L}$ receptor using molecular techniques have been unsuccessful. The identification of a number of cDNA splice variants of the $\alpha_{1A}$-adrenoceptor supports the hypothesis that the $\alpha_{1L}$-adrenoceptor represents an energetically favourable conformation of the $\alpha_{1A}$-subtype. These variants have been termed the $\alpha_{1A-1}$, $\alpha_{1A-2}$ and $\alpha_{1A-3}$. Each splice variant has a different amino acid sequence and a variable carboxy terminal chain length [Chang et al, 1998]. The function of each splice variant is currently under investigation.

The rank order of potency of three $\alpha_1$-agonists has been determined in canine subcutaneous resistance arteries, and found to be A61603> noradrenaline> phenylephrine. This is consistent with the predominance of the $\alpha_{1A}$ subtype-mediating contraction of cutaneous arteries in the dog [Argyle & McGrath, 2000]. However, the potency of the antagonist's prazosin and HV723 are low, indicating the presence of the $\alpha_{1L}$-adrenoceptor.

Noradrenaline-induced contractions of cutaneous canine vessels are insensitive to antagonism with low concentrations of CEC, while high concentrations, reduce the contractile maximum without affecting sensitivity. Taken together the affinity and potency order of ligands in canine cutaneous arteries suggest that the $\alpha_{1A}/\alpha_{1L}$-adrenoceptors mediate contractile responses [Argyle & McGrath, 2000].

$\alpha_{1B}$ Adrenoceptors

The role of the $\alpha_{1B}$-adrenoceptor in the control of arterial blood pressure and blood flow has been poorly defined. This is partly due to a lack of suitable subtype selective ligands. A limitation, which has now been overcome by the creation of transgenic mice harbouring mutated, overexpressed and deleted $\alpha_{1B}$-adrenoceptors.

*In vitro* studies on rabbit resistance arteries suggest that the $\alpha_{1B}$-adrenoceptor may be involved in the development of vascular contractions. Cutaneous resistance arteries contract in response to phenylephrine, noradrenaline and the $\alpha_{1A}$-selective agonist, A61603. The higher binding affinity of A61603 indicates that the $\alpha_{1A}$-adrenoceptor is the dominant subtype mediating contraction in this artery, while the involvement of the

$\alpha_{1B}$-adrenoceptor is secondary. However antagonist affinities refute the data gained with the three agonists [Smith et al, 1997].

Incubating cutaneous rabbit arteries with CEC prior to constructing a noradrenaline response curve leads of a significant reduction in the maximum response, without affecting tissue sensitivity. Prazosin and WB4101 are both potent antagonists of receptor-mediated contractions in rabbit cutaneous arteries [Smith et al, 1997]. The $pA_2$ values determined suggest a prazosin-sensitive receptor-binding site. However Schild regression analysis for both of these antagonists have slopes that are different from unity, indicative of two site binding. Detailed analysis of the agonist affinities and antagonist potency suggests that the $\alpha_{1B}$-adrenoceptor mediates contractile responses in addition to the $\alpha_{1L}$, which plays a secondary contractile role in contraction of rabbit cutaneous resistance arteries [Smith et al, 1997].

Mice carrying a homozygous deletion of the $\alpha_{1B}$-adrenoceptor have normal baseline blood pressure, and contractile responses to non-selective $\alpha_1$-agonists are only slightly attenuated [Cavalli et al, 1997]. This led to the conclusion that the contribution the $\alpha_{1B}$-adrenoceptor makes to the control of peripheral blood pressure can be considered as minor. This hypothesis is supported by other literature [Chen et al, 1995, Hrometz et al, 1999].

Contrary to this, mice lacking functional $\alpha_{1B}$-adrenoceptors have been found to be hypotensive at rest [Cavalli et al, 1997]. So one would expect that mice overexpressing the $\alpha_{1B}$-adrenoceptor would be hypertensive at rest. However this is not the case, in fact

they have a slight, but significant reduction in systemic blood pressure [Zuscik et al, 2001].

In an attempt to clarify what, if any, cardiovascular functions are controlled by the $\alpha_{1B}$-adrenoceptor, Zuscik and co-workers produced a mouse overexpressing the $\alpha_{1B}$-adrenoceptor. The $\alpha_{1B}$ receptor was overexpressed with its isogenic promoter, so that overactivity of the $\alpha_{1B}$-adrenoceptor only occurs in tissues that normally express this receptor subtype. Two $\alpha_{1B}$ mutations were employed; they were single and triple mutants of the hamster $\alpha_{1B}$-adrenoceptor. Both of these mutations cause $\alpha_{1B}$-adrenoceptors to couple constitutively to the Gq/phospholipase C pathway, which leads to protein kinase C and $IP_3$ release [Zuscik et al, 2001]. By definition, these receptors can be referred to as constitutively active.

Transgenics with altered $\alpha_{1B}$ receptors: effects on cardiovascular function

Overexpressing $\alpha_1$-adrenoceptors causes hypertrophy of cultured cardiac myocytes, an effect that has been attributed to the $\alpha_{1A}$-adrenoceptor [Knowlton et al, 1991]. *In vivo*, overexpression of the $\alpha_{1B}$-adrenoceptor causes cardiac hypertrophy; confirmed by echocardiographic analysis and an increased heart to body weight ratio [Zuscik et al, 2001], while other vital organs are unaffected. Basal heart rate is decreased in mice overexpressing the $\alpha_{1B}$-adrenoceptor, which may result from altered electrical conductance in purkinje fibers of the murine heart. This seems likely, because $\alpha_{1B}$-adrenoceptors regulate contractility of purkinje fibers [Balzo et al, 1990].

Phenylephrine causes concentration-related contractions of myograph mounted murine blood vessels. Overexpressing the $\alpha_{1B}$-adrenoceptor has no effect on the phenylephrine response when compared with control blood vessels. This poses the question of how then, does overexpression of the $\alpha_{1B}$-adrenoceptor lead to hypotension? It may be that overexpressing the $\alpha_{1B}$-adrenoceptor causes a decrease in sympathetic nerve activity. This hypothesis is supported by experiments that show a fifty percent reduction in circulating catecholamines in mice overexpressing the $\alpha_{1B}$-adrenoceptor. Therefore, hypotension that occurs in mice overexpressing the $\alpha_{1B}$ receptor subtype probably results from autonomic dysfunction, caused by the neurodegeneration that occurs when expression of the $\alpha_{1B}$-adrenoceptor is altered [Zuscik et al, 2001]. This data indicates that the reduction in resting blood pressure that occurs when the $\alpha_{1B}$-adrenoceptor is overexpressed is not attributable to direct effects on peripheral blood vessels. Again, this suggests that the $\alpha_{1B}$-adrenoceptor is not a major contributor to blood pressure regulation in the mouse.

Cardiac myocytes overexpressing $\alpha_{1b}$-adrenoceptors have been studied in order to elucidate the second messenger cascades activated by this adrenoceptor subtype. Stimulation of $\alpha_{1b}$-receptors, expressed in cardiac myocytes, leads to the activation of SRE/c-fos luciferase genes and MAPK signalling cascades [McWhinney et al, 2000]. It appears that the activation of both $\alpha_{1b}$ and $\alpha_{1a}$-adrenoceptors in these cells may be required for the development of cardiac hypertrophy, as each subtype appears to lead to a different physiological response by activating alternative signalling pathways [McWhinney et al, 2000].

Mice overexpressing $\alpha_{1B}$-adrenoceptors develop myocardial hypertrophy, evidenced by an increase in the size of the ventricular septum and thickening of the ventricular wall. In addition, other more generalised abnormalities appear, such as a reduction in heart rate and reduced cardiac output. *In vivo*, intravenous administration of phenylephrine causes a reduction in pressor responses; and at rest, mice overexpressing the $\alpha_{1B}$-adrenoceptor are hypotensive [Zuscik et al, 2001]. *In vivo*, experiments in pithed mice confirm that phenylephrine-induced pressor effects result from activation of either the $\alpha_{1B}$ or $\alpha_{1D}$-adrenoceptor [McCafferty et al, 1999].

Plasma levels of the catecholamines, adrenaline and noradrenaline are reduced in mice overexpressing the $\alpha_{1B}$-adrenoceptor. In addition to causing an alteration in catecholamine levels, cortisol levels are also significantly lower, indicative of a generalised reduction in sympathetic outflow [Zuscik et al, 2001].

Saturation curves constructed from radioligand binding experiments show that tamsulosin labels significantly fewer $\alpha_{1B}$-adrenoceptors than does prazosin in rat-1 fibroblasts and homogenised rat liver. However, noradrenaline displacement curves are similar for tamsulosin and prazosin, which indicates that the reduced receptor labelling is specific for the $\alpha_{1B}$-adrenoceptor.

Tamsulosin is used clinically because it does not have dramatic effects on blood pressure, unlike most other $\alpha_1$-antagonists. It has been proposed that this may be explained by the lower affinity of tamsulosin for the $\alpha_{1B}$-adrenoceptor. However, this seems unlikely given that the $\alpha_{1B}$-adrenoceptor seems to play only a minor role in the

control of blood pressure regulation, and given that mice lacking functional $\alpha_{1B}$ receptors are only slightly hypotensive [Cavalli et al, 1997].

Role of the $\alpha_{1B}$-adrenoceptor in normal brain functions

Overexpressing the $\alpha_{1B}$-adrenoceptor, *in vivo*, causes a progressive neurodegenerative condition that has been likened to Shy-Drager syndrome [Zuscik et al, 2000]. Neurodegeneration caused by overexpression and constitutive activity of the $\alpha_{1B}$-adrenoceptor is granulovascular in nature. In early development the pathology is restricted to areas of the brain expressing native $\alpha_{1B}$-adrenoceptors. However, increasing age causes the neuronal damage to spread and encompass the entire murine brain. Physiologically, these neuronal abnormalities cause a Parkinsonian like dysfunction of the hindlimbs. Mice harbouring these mutated receptors also develop severe grand mal seizures and dysplasia of the cerebral cortex [Zuscik et al, 2000].

Mice have a dense sympathetic innervation in the cerebral circulation, but in humans innervation patterns are much more diffuse; reflected by a reduction in the number of $\alpha_1$-adrenoceptors found in the human brain [Bevan et al, 1998a]. Given this, it may be somewhat hasty to suggest that dysfunction of $\alpha_1$-adrenoceptors in the CNS of the mouse will manifest in similar disorders in humans.

$\alpha_{1D}$ Adrenoceptors

When the $\alpha_{1D}$-adrenoceptor was first cloned, it was termed the $\alpha_{1A}$, but after further pharmacological analysis, the nomenclature of $\alpha_1$-adrenoceptor subtypes was revised to bring them into line with functional studies [Hieble et al, 1995]. The presence of a functional population of $\alpha_{1D}$-adrenoceptors has been shown, *in vitro*, in the rat aorta.

Vasoconstrictor responses in the rat aorta are antagonised by BMY7378; a selective $\alpha_{1D}$ antagonist [Saussy et al, 1994].

Although all three $\alpha_1$-adrenoceptor subtypes are expressed in a wide variety of vascular beds, one subtype usually dominates vascular contraction. In rodent animal models, the $\alpha_{1D}$-adrenoceptor has been found to mediate contractions of large, conduit arteries [Piascik et al, 1997]. In addition to the large calibre blood vessels, the $\alpha_{1D}$ subtype is also responsible for contracting femoral, iliac and superior mesenteric arteries in the rat [Hrometz et al, 1999]. $\alpha_{1D}$ knockout mice have normal baseline resting blood pressure, but phenylephrine-induced pressor responses are attenuated [Chalothorn et al, 2003].

In the absence of extracellular calcium, noradrenaline causes small transient contractions of the rat aorta, by stimulating $\alpha_{1D}$-adrenoceptors located on vascular smooth muscle cells. However, potassium chloride (KCl)-induced contractions are completely abolished in calcium free conditions. This illustrates that noradrenaline-mediated contractions of the rat aorta depend on intra and extracellular calcium, unlike KCl-mediated responses, which rely solely on extracellular calcium. Hypoxia has no effect on transient contractions induced by noradrenaline in calcium free conditions [Marriott & Marshall, 1989]. This may reflect a protective mechanism, whereby large conduit arteries can maintain some level of contraction when oxygen and calcium levels are low.

### 1.1.4 Cellular location of $\alpha_1$-adrenoceptors

Molecular cloning techniques have permitted the $\alpha_{1A}$-adrenoceptor to be removed and replaced with the LacZ gene. This gene encodes $\beta$ galactosidase production, and

permits visualisation of $\alpha_{1A}$-receptor sites *in situ*. The presence of β galactosidase confirms that the $\alpha_{1A}$-adrenoceptor is expressed widely in resistance arteries and arterioles and that the $\alpha_{1A}$-adrenoceptor is required for the maintenance of arterial blood pressure. Furthermore, that functional $\alpha_{1A}$-adrenoceptors are not expressed in the mouse aorta [Rokosh & Simpson, 2002].

Confocal microscopy combined with the use of vital cellular dyes, allows $Ca^{2+}$ waves to be recorded, monitored, and visualised within a living cell after receptor stimulation. Irrespective of their pre or postjunctional location, $\alpha_1$-adrenoceptors can be found on the surface of cells, or bound to intracellular organelles. In unstimulated, stably transfected rat-1 fibroblasts, $\alpha_{1B}$-adrenoceptors are found in close association with the plasma membrane. Stimulation of these plasma membrane-bound receptors with selective agonists causes internalisation and association of the agonist-bound receptors with arrestin molecules [Chalothorn et al, 2002]. In sharp contrast to the cellular location of $\alpha_{1B}$-adrenoceptors, which are found primarily on the cell surface, unstimulated $\alpha_{1D}$-adrenoceptors are bound to intracellular organelles.

Exposing rat-1 fibroblasts to prazosin, now known to be an inverse agonist, causes a redistribution of intracellular $\alpha_{1D}$-adrenoceptors. The agonist bound $\alpha_{1D}$-adrenoceptors translocate, and can then be found in close association with the plasma membrane. These findings suggest that unstimulated $\alpha_{1D}$-receptors expressed in rat-1 fibroblasts may be constitutively active [McCune et al, 2000]. *In vitro* experiments support this hypothesis, and suggest that constitutively active $\alpha_{1D}$-receptors may exist in the rat tail artery [Gisbert et al, 2000]. Recently data has been presented that suggests native $\alpha_{1D}$ receptors may be constitutively active, *in vivo*, in the rat aorta [Gisbert et al, 2000], a

finding that may explain their intracellular location, as activated receptors are recycled in a cell.

$\alpha_1$-adrenoceptor antagonists inhibit receptor activity because they compete with endogenous agonists for access to receptor-binding sites. The access of a drug to a receptor-binding site is critical in determining what effect agonist/antagonists can produce. If a receptor is sequestered within the intracellular milieu, the lipophilicity of a drug determines whether binding occurs, and not the ligand's affinity for the receptor. These additional considerations have only been highlighted in recent years and should be given serious consideration when new subtype-selective compounds are being developed.

QAPB has been used in flow cytometry experiments to determine the cellular distribution of $\alpha_1$-adrenoceptor subtypes within a cell [Sugawara et al, 2002]. $\alpha_{1b}$-adrenoceptors are exclusively located on the cell surface, while the $\alpha_{1a}$ subtype is more widely distributed, but predominantly found on intracellular organelles. When a receptor is localised to intracellular sites, the affinity that a drug has for this receptor becomes secondary and the antagonist's lipophilicity determines whether binding occurs [Sugawara et al, 2002]. This may explain the inconsistencies observed when CEC has been used to study functional responses-mediated by the $\alpha_{1B}$-adrenoceptor, because it appears that the drug only alkylates membrane-bound receptors.

The $\alpha_{1D}$-adrenoceptor is the principal $\alpha_1$-subtype causing contraction of the murine carotid artery, where BMY7378 abolishes phenylephrine-induced contractions [Deighan et al, 2000]. The presence of functional $\alpha_{1D}$-adrenoceptors has also been

shown in the rat iliac artery and aorta, where BMY7378 antagonises (with high affinity) phenylephrine-induced contractions of these arteries. Under normal physiological conditions, agonist-induced stimulation of $\alpha_{1D}$-adrenoceptors fails to contract caudal and renal arteries. Although expression of the $\alpha_{1D}$-adrenoceptor has been confirmed in both of these arteries, the low affinity of BMY7378 for phenylephrine-induced contractions in rat caudal and renal arteries suggests little, if any, contractile function for the $\alpha_{1D}$-subtype [Piascik et al, 1995]. This indicates that the major $\alpha_1$-subtype contributing to contraction of these vessels is a subtype other than the $\alpha_{1D}$-adrenoceptor.

The location of $\alpha_1$-adrenoceptors within a cell has been studied in HEK 293 cells, and the majority of unstimulated $\alpha_{1D}$-adrenoceptors are sequestered on intracellular binding sites [Chalothorn et al, 2002]. Phenylephrine has no effect on the cellular distribution of $\alpha_{1D}$-adrenoceptors expressed in cultured cells, but does lead to the phosphorylation of ERK1 and ERK2, as does $\alpha_{1A}$ and $\alpha_{1B}$ receptor stimulation [Chalothorn et al, 2002].

Agonist-mediated internalization of $\alpha_{1A}$-adrenoceptors is significantly slower than that of the $\alpha_{1B}$-adrenoceptor [Chalothorn et al, 2002]. Once activated, stimulation of $\alpha_{1A}$-adrenoceptors with selective agonists causes rapid internalisation of agonist bound receptors, which is preceded by an increase in ERK1 and ERK2 activity. Internalisation of $\alpha_{1A}$ and $\alpha_{1B}$-adrenoceptors depends upon the availability of arrestin and dynamin molecules within the cell [Chalothorn et al, 2002].

Fluorescent, lipophilic ligands negate the need for specific antibodies for G protein coupled receptors, and overcome the problems associated with access to receptor binding sites. Fluorescent ligands can be used in live cell preparations as well as fixed

blood vessels segments. This makes them useful tools for the study of receptor activity [MacKenzie et al, 2000]. Approximately forty percent of the $\alpha_1$-adrenoceptors expressed in cultured human smooth muscle cells, are located on intracellular binding sites [MacKenzie et al, 2000]. QAPB, also know as BODIPY-prazosin is a fluorescent form of the classical $\alpha_1$-antagonist, prazosin. QAPB has high affinity for $\alpha_1$-adrenoceptors expressed in human smooth muscle cells, and competes with prazosin in radioligand binding experiments. Given this, the cellular location of $\alpha_1$-adrenoceptors should be considered when designing ligands for these receptors, as a high proportion of functional receptors are sequestered on internal surfaces in vascular cells.

1.1.5 $\alpha_1$-adrenoceptors: a role in development

$\alpha_1$-adrenoceptors may be important in the development and maturation of sexual function in the rat. Male rats aged 40, 60 and 120 days, were studied to investigate the role of $\alpha_1$-adrenoceptors in sexual development. This study has shown that expression levels of $\alpha_1$-adrenoceptors change with increasing age, and altered expression levels are accompanied by an increase in testosterone levels. Increased levels of reproductive hormones are accompanied by an overall increase in body and epididymal weight. The presence of all three $\alpha_1$-adrenoceptor subtypes was confirmed in the reproductive system of rats at each age point [Queiroz et al, 2002].

Displacement curves carried out on homogenised caput and cauda epididymis, show that although all three $\alpha_1$-adrenoceptor subtypes are expressed, the $\alpha_{1A}$-adrenoceptor is the major subtype expressed in the epididymis of 40 day old, sexually immature rats [Queiroz et al, 2002]. The predominance of this $\alpha_1$-subtype in the epididymis of

sexually immature rats, suggests that the $\alpha_{1A}$-adrenoceptor may play a role in sexual development.

Furthermore, *in vitro* experiments have shown that $\alpha_1$-antagonists decrease ejaculatory capacity in the rat, which leads to a reduction in sperm quality. So $\alpha_1$-adrenoceptors are expressed in, and mediate a variety of functional responses in the rat reproductive system, and appear to contribute to the development and maintenance of fertility [Ratnasooriya & Wadsworth, 1990]. In addition to the rat, the guinea pig epididymis also contains a mixed population of $\alpha_1$ and $\alpha_2$-adrenoceptors, which when stimulated with selective ligands participate in vasoconstrictor responses [Haynes & Hill, 1996].

Studies carried out to determine where adrenoceptors are expressed, provide valuable information. However, caution should be exercised when interpreting data, as the presence of mRNA for a given subtype is not always indicative of functional receptors [Hrometz et al, 1999].

1.1.6 $\alpha_1$ adrenoceptors and vascular contraction

$\alpha_1$-selective agonists cause contractions by stimulating receptors that are located on vascular smooth muscle cells, in a wide variety of vascular beds. Stimulation of $\alpha_{1A}$ and $\alpha_{1D}$-adrenoceptors with selective agonists, leads to contractile responses in a number of murine arteries. The $\alpha_{1D}$-adrenoceptor is the major subtype leading to contraction of the murine aorta, carotid and superior mesenteric artery [Daly et al, 2002]. While the $\alpha_{1A}$-adrenoceptor mediates contraction of the caudal, and first order mesenteric blood vessels [Daly et al, 2002]. The $\alpha_{1B}$-adrenoceptor is expressed in a

number of peripheral vessels, but the use of subtype selective ligands and transgenic mice has shown that the $\alpha_{1B}$-adrenoceptor plays only a minor role in contraction.

Stimulation of adrenergic receptors with selective ligands causes an increase in calcium wave activity in vascular tissues [Ruehlmann et al, 2000]. Mitochondrial inhibitors decrease the contractile force generated by stimulation of vascular $\alpha_1$-adrenoceptors in the rat tail artery by 50-80%, but calcium concentrations are unaffected [Sward et al, 2002]. Confocal microscopy has shown that mitochondrial inhibitors reduce $\alpha_1$-adrenoceptor-mediated calcium wave activity [Sward et al, 2002]. This is not surprising, given that it has already been shown that stimulation of adrenergic receptors causes an increase in calcium currents in vascular tissue [Peng et al, 2001]. In addition to a reduction in $\alpha_1$-mediated contractions, inhibition of myosin phosphatase with calyculin decreases relaxant responses in the rat tail artery by thirty percent [Sward et al, 2002].

In a number of tissues, stimulation of the $\alpha_{1A}$-adrenoceptor leads to smooth muscle cell contraction. In the rat, preparations that contract following stimulation of the $\alpha_{1A}$-adrenoceptor include the renal and tail artery [Villalobos-Molina & Ibarra, 1996] and vas deferens. In the rat vas deferens, stimulation of the $\alpha_{1A}$-adrenoceptor activates two calcium pathways, firstly, by releasing $Ca^{2+}$ from intracellular stores and secondly, by opening voltage operated $Ca^{2+}$ channels on the plasma membrane of stimulated cells [Burt et al, 1998].

A61603 is a potent selective agonist at $\alpha_{1A}$-adrenoceptors. Ligand binding studies show that this compound has a thirty-five fold greater potency for the $\alpha_{1a}$ compared with $\alpha_{1b}$

and $\alpha_{1d}$ binding sites. The greater affinity of A61603 for the $\alpha_{1A}$-adrenoceptor has also been demonstrated *in vitro* in the rat vas deferens and in canine prostate strips, both of which have previously been subtyped as $\alpha_{1A}$. Enhanced potency for one adrenoceptor subtype provides a useful method for studying subtype specific responses in arterial and venous beds in a variety of different animal species [Knepper et al, 1995].

WB4101 and 5MeU selectively antagonise agonist-mediated and electrically evoked changes in pupil diameter in the rat eye [Yu & Koss, 2002]. Selective antagonism with WB4101 and 5MeU confirms that the major adrenoceptor subtype leading to contraction of smooth muscle cells surrounding the rat eye results from stimulation of the $\alpha_{1A}$ subtype.

Major and minor contractile roles for $\alpha_1$-adrenoceptors

Contractions of the rat caudal artery appear to be mediated by more than one $\alpha_1$-adrenoceptor subtype. Noradrenaline-induced contractions in this artery are concentration-dependent, and are antagonised by prazosin in a competitive manner, indicated by a Schild regression slope of 1. The $\alpha_1$-selective antagonist RS17053 shifts noradrenaline-mediated contractions to give a biphasic response curve. Following CEC treatment and RS17053, noradrenaline concentration response curves are monophasic. BMY7378 is an $\alpha_{1D}$-selective antagonist, and in spite of the presence of mRNA for the $\alpha_{1D}$ receptor in the rat tail artery, BMY7378 has little effect on noradrenaline-mediated contractions [Piascik et al, 1995]. Therefore, it appears that the major contractile receptor in the rat caudal artery is the $\alpha_{1A}$-adrenoceptor, with a minor, but significant contractile response being mediated by CEC sensitive $\alpha_{1B}$-adrenoceptors [Lachnit et al,

1997]. The potency order of agonists in the rat caudal artery is similar to that found in the isolated rat kidney, which is predominantly $\alpha_{1A}$ [Blue et al, 1995].

Contractions of the guinea pig cauda epididymis are mediated by α-adrenoceptors. Phenylephrine-induced contractions are concentration-dependent, and are antagonised by CEC, but only in the presence of the $\alpha_2$-antagonist, idazoxan [Haynes & Hill, 1996]. Interestingly, contractile responses to phenylephrine are enhanced by the presence of the $\alpha_2$-selective agonist, xylazine. When mounted tissues are incubated with xylazine prior to construction of a phenylephrine response, the concentration curve is shifted to the left. This indicates that sensitivity has been enhanced. Analysis of functional responses in the cauda epididymis reveals that a mixed population of $\alpha_1$ and $\alpha_2$-adrenoceptors exist, and stimulation of both subclasses leads to contractile responses. Activation of $\alpha_2$-adrenoceptors enhances calcium influx, which explains the potentiating effect of xylazine on phenylephrine-induced responses [Haynes & Hill, 1996]. Due to the obvious problems associated with gaining tissue samples from healthy human subjects, the literature documenting the functional responses in reproductive organs is limited.

All three $\alpha_1$-adrenoceptor subtypes are expressed in human prostate tissue [Walden et al, 1999], where they mediate smooth muscle cell contraction. Phenylephrine causes contraction of cultured human prostatic stromal cells, which results in a significant shortening in cell length. Phenylephrine-induced contractions of cultured stromal cells are inhibited by L-type calcium channel antagonists, and PKC inhibitors [Preston & Haynes, 2003].

1.1.7 Relaxations mediated by $\alpha_1$-agonists

$\alpha_2$-adrenoceptors mediate relaxant responses in a number of arteries, the mechanisms of which have been studied widely. Until recently, $\alpha_1$-adrenoceptors were generally considered to carry out only contractile functions. However, research now suggests that stimulation of $\alpha_1$-adrenoceptors can lead to relaxant responses in rat pulmonary arteries by causing the release of nitric oxide [Boer et al, 1999].

Furthermore, phenylephrine and noradrenaline appear to mediate two distinct vascular responses in pressurised rat mesenteric beds. The first is a small but significant relaxation. The second is a sustained concentration-dependent contraction. Relaxant responses occur at low agonist concentrations, and lead to a significant drop in perfusion pressure. To determine if this response depends on a functional endothelium denuded and normal vessels were studied. Removal of the endothelium was achieved by perfusing the vessel lumen with distilled water, a method proposed to mimic mechanical damage caused by rubbing [Bolton et al, 1984]. The lack of a functional endothelial surface prevents phenylephrine and noradrenaline-induced relaxant effects, while contractile responses are unaffected. Therefore, the relaxant responses mediated by $\alpha_1$-adrenoceptors appear to result from stimulation of endothelial receptors.

The cellular signalling pathways leading to $\alpha_1$-adrenoceptor-mediated relaxations of rat mesenteric arteries involve calcium, evidenced by the antagonising effect of $Ca^{2+}$-ATPase inhibitors and phospholipase C blocking drugs. Furthermore, blockade of $\alpha_{1D}$ and $\alpha_{1B}$-mediated responses with BMY7378 and CEC attenuates the relaxant response, while inhibiting the $\alpha_{1A}$-adrenoceptor with selective ligands has no effect on noradrenaline and phenylephrine-induced relaxations [Filippi et al, 2001].

Cultured bovine endothelial cells were used to study the mechanisms involved in $\alpha_1$-mediated relaxant responses in greater detail. Phenylephrine causes a significant rise in inositol monophosphate levels in cultured endothelial cells and BMY7378 antagonises phenylephrine-induced increases in cNOS activity [Filippi et al, 2001]. Taken together, this data indicates that postjunctional $\alpha_1$-adrenoceptors can be stimulated, and cause small, but significant relaxant responses, which are followed by sustained contractile responses in resistance arteries of the rat. Relaxations depend on a functional endothelium and have been proposed to result from stimulation of $\alpha_{1D}$-adrenoceptors.

1.1.8 Smooth muscle cell hypertrophy

Circulating levels of catecholamines are elevated in hypertensive patients and in individuals under extreme stress. Hypertension and stress are known risk factors for the development of cardiovascular disease. In addition to contractile responses, the catecholamines noradrenaline and adrenaline have effects on cell growth and proliferation via stimulation of $\alpha_1$-adrenoceptors [Chen et al, 1995]. In cultured rat cardiac myocytes, the regulation of transcription factors that control cell growth and size, are affected by stimulation of $\alpha_{1A}$-adrenoceptors [Knowlton et al, 1993]. In contrast, prolonged stimulation of $\alpha_{1D}$ and $\alpha_{1B}$-adrenoceptors with selective agonists, leads to an increase in $\alpha$ actin; which is an important component involved in the development of vascular contraction [Chen et al, 1995]. Furthermore, overexpressing the $\alpha_{1B}$-adrenoceptor *in vivo* leads to cardiac hypertrophy. This data supports the hypothesis that $\alpha_{1B}$-adrenoceptors regulate growth of cardiac and vascular tissue, both of which are altered in hypertension.

Treating isolated cardiac myocytes, from neonatal rats with $\alpha_1$-selective agonists leads to an elevation in $\alpha_{1A}$ mRNA/protein levels. Enhanced levels of the $\alpha_{1A}$-adrenoceptor are accompanied by a decrease in the expression of $\alpha_{1B}$ and $\alpha_{1D}$-adrenoceptors [Rudner et al, 1999]. Altered α-adrenoceptor expression in cardiac myocytes has been proposed to contribute to the development of myocardial hypertrophy [Rokosh et al, 1996]. In addition to catecholamines, hormones can also affect the expression of $\alpha_1$-adrenoceptors. Insulin causes an upregulation of $\alpha_{1d}$-adrenoceptors expressed in vascular smooth muscle cells isolated from the rat [Hu et al, 1996]. Whether these processes are at work *in vivo*, will be more difficult to prove.

1.1.9 Regulation of cellular growth

In addition to mediating vascular contractions, $\alpha_1$-adrenoceptors also regulate the growth of vascular smooth muscle cells. In $\alpha_{1B}$ knockout mice there is a reduction in the total number of adventitial cells in the mouse tail artery. This suggests that the $\alpha_{1B}$-adrenoceptor plays an active role in the maintenance of vascular growth [Daly et al, Unpublished observation]. $\alpha_{1D}$ and $\alpha_{1B}$-adrenoceptors are proposed to play an active role in the control of vascular growth, and are known regulators of MAPK activity. By acting as regulators of ERK activity the $\alpha_{1D}$-adrenoceptor can regulate the growth of aortic smooth muscle cells in the rat [Xin et al, 1997]. By activating different signalling pathways in smooth muscle cells the $\alpha_{1B}$ receptor subtype functions primarily, to control vascular growth [McWhinney et al, 2000].

So what other mechanisms control growth? The presence of caveolin has been used as a marker for cell growth. Cholesterol and glycosphingolipids are found in caveloae which form small invaginations in the plasma membrane of cells. Caveolin is a marker

for the presence of caveolae, which inhibits the release of vascular growth factors [Couet et al, 1997]. Caveolin expression levels are down regulated in rapidly dividing cells and are significantly attenuated in developing tumours [Fujita et al, 2001].

Isolated cardiac cells become hypertrophic after prolonged exposure to catecholamines; an effect that is proposed to result from the activation of $\alpha_1$-adrenoceptor signalling cascades [Ishikawa & Homey, 1997]. So how do signalling molecules do this? One possibility is that they interact with, and alter caveolin levels; allowing vascular growth to proceed unchecked.

Caveolin and $\alpha_1$ signalling molecules are found in close association in rat cardiac tissue. To determine if these factors contribute to the development of cardiac hypertrophy, their expression levels were studied in cardiac tissue from the rat [Fujita et al, 2001]. The distribution of $\alpha_1$-adrenoceptors in the plasma membrane of cells has previously been considered as random. That is until now. Fujita and co workers have shown that $\alpha_1$-adrenoceptors can be found in discrete areas of the cell membrane, closely associated with caveolin.

Caveolin-3 controls cellular growth; expression of caveolin-3 is significantly lower in cardiac tissue from spontaneously hypertensive rats compared with normotensive controls at one and three months of age. However, the expression of caveolin-3 is unaltered at three months of age compared with that at one month. This suggests that caveolin is not involved in the progression of hypertension in developing rats, but may be involved in the early stages of the disease [Fujita et al, 2001]. The interaction

between caveolin and $\alpha_1$-adrenoceptor expression levels is poorly understood and requires further investigation.

1.1.10 Age related changes in vascular structure and function

The structure and function of blood vessels is altered with increasing age. Vascular changes occurring with age may be the result of altered receptor expression or be due to functional changes in the existing receptor population. For example, it is well documented that β-adrenoceptor function is altered with age. Relaxant responses mediated by β-adrenoceptors are attenuated with increasing age, while noradrenaline mediated contractions are enhanced [Marin et al, 1991]. In addition to changes occurring in β and $\alpha_1$-adrenoceptors, $\alpha_2$-mediated contractions are also significantly attenuated in old animals [Marin, 1995].

Age has been established as an independent risk factor for the development of cardiovascular disease. In developed countries the population has a greatly increased life expectancy; so this area of research generates a tremendous amount of interest, and funding. The phenotype of vascular smooth muscle cells changes with age, from a contractile to a synthetic phenotype. Such changes probably result from altered signalling pathways and or reduced responsiveness to smooth muscle cell inhibitors [Lundberg & Crow, 1999].

Age-related changes in $\alpha_1$-adrenoceptor expression levels are vessel specific. Indeed arteries from patients over sixty-five years old have a two-fold increased expression of adrenoceptors in mammary arteries. The principal $\alpha_1$-adrenoceptor subtype mediating contraction of mammary arteries from young adults has been found to be the $\alpha_{1A}$

subtype. In later life, $\alpha_1$-adrenoceptor expression levels shift from $\alpha_{1A}$ toward the $\alpha_{1B}$ [Rudner et al, 1999]. Whether similar shifts occur in other vascular beds is as yet unknown.

Resistance arteries are important regulators of peripheral blood pressure. Therefore, any change in the expression levels and or the function of adrenoceptors in these arterial beds could have profound affects on blood pressure regulation. $\alpha_{1D}$-mediated contractions of resistance arteries are significantly enhanced with increasing age [Rudner et al, 1999]. If the increase in the expression of the $\alpha_{1D}$-adrenoceptor reflects a functional population of receptors involved in contraction this may, in part, account for the rise in blood pressure that accompanies advancing age.

High levels of fat and a cholesterol rich diet cause lipid rich plaques to form in large calibre arteries in humans. Plaque formation is unaffected by age, but progression of the condition is age-dependent. A developing plaque becomes fibrous, ulcerated, and calcium rich. Calcification makes the plaque unstable and it is at this stage that a fatal thrombotic lesion can occur [Robert, 1999]. Ageing is associated with a breakdown in regulatory functions at the receptor level [Xia et al, 1998]. Altered adrenoceptor expression may contribute to plaque instability. The mechanisms whereby this occurs are unclear.

1.1.11 Excitatory responses in brain motorneurons

Noradrenergic neurons project into all areas of the central nervous system and are instrumental in the control of motor, sensory, cognitive, emotional and autonomic functions [Aston-Jones et al, 1988, Friedman et al, 1991]. Stimulation of postjunctional

$\alpha_1$-adrenoceptors regulates spinal motorneurone activity. The subtypes involved in these responses have only recently been defined [Wada et al, 1997].

$\alpha_{1B}$ mRNA has been detected in eighty-five percent of rat motorneurons. The remaining $\alpha_1$-adrenoceptor subtypes namely, $\alpha_{1A}$ and $\alpha_{1D}$ are also expressed, but their expression levels are lower than the $\alpha_{1B}$ and appear to be more random [Volgin et al, 2001]. Thus, data indicates that the major $\alpha_1$-adrenoceptor subtype regulating the function of spinal motorneurones appears to be the $\alpha_{1B}$-adrenoceptor.

1.1.12 Regulation of receptor function

Binding of tritiated prazosin to cultured cells is significantly reduced when cells are pre-treated with adrenaline and noradrenaline. The reduction in available binding sites suggests catecholamine-induced receptor internalisation; a process that requires protein kinase C. When receptors are sequestered to the internal environment of a cell, prazosin, a hydrophobic ligand has no access, but lipophilic ligands are free to cross the plasma membrane and bind receptors [Cowlen & Toews, 1988].

Receptor desensitisation, which manifests as reduced responsiveness to applied agonists, occurs when susceptible receptors are phosphorylation by G protein receptor kinases (GRK'S) [Ferguson et al, 1997]. The role of protein kinases C in receptor desensitisation is poorly defined. It appears that the GRK activated by protein kinase C is critical in determining whether desensitisation occurs and that GRK2 and GRK3 are involved, while GRK4 and GRK5 do not participate in agonist-induced receptor desensitisation [Diviani et al, 1995].

1.1.13 Quantitative pharmacology in single cell preparations

Detailed pharmacological experiments have been carried out in single cells; to determine if quantitative pharmacology can be performed in cell lines and/or dissociated cells [Pediani et al, 2000].

In cultured cells, phenylephrine mobilises intracellular calcium stores and causes voltage-gated calcium channels to open, recruiting extracellular calcium for contraction. In rat-1 fibroblasts the $\alpha_{1A}$-selective agonist, A61603 was found to be 141 times more potent than phenylephrine at inducing the mobilisation of calcium currents. The antagonist potency order in this cell line was determined as WB4101>prazosin>BMY7378, and the antagonism produced by WB4101 and prazosin was insurmountable. The selectivity of A61603 and the antagonist potency order confirm that the use of this cell line provides an alternative method whereby $\alpha_{1A}$-mediated responses can be analysed pharmacologically. In addition to potency and affinity estimates the responses gained were reproducible, quantifiable, and could be measured over real time [Pediani et al, 2000]. The data gained from these experiments provides evidence that single cell systems are useful tools for the assessment of adrenoceptor-mediated responses.

1.1.14 Pharmacology of constitutively active $\alpha_1$adrenoceptors

Before a receptor can be defined as constitutively active it must fulfil three criteria. These are an increased affinity for agonist, no change in antagonist potency, and enhanced basal levels of second messengers. Mutations of the third cytoplasmic loop of the $\alpha_{1a}$ and the $\alpha_{1b}$-subtypes cause these receptors to become constitutively active [McWhinney et al, 2000].

Constitutively active receptors induce signal transduction in the absence of exogenous agonists. This feature distinguishes constitutively active from wild type receptors. Experimentally, constitutive activity can be induced, by overexpressing the native receptor to ninety percent of normal expression levels. The advantage of this is that the signalling pathways activated by a constitutively active receptor can be studied in greater detail than normal physiological conditions permit [McWhinney et al, 2000].

Constitutively active receptors and inverse agonists are relatively new concepts, and have only recently been introduced to the study of receptor activity. Constitutive receptor/G protein coupling leads to persistent receptor cycling from the membrane to intracellular organelles, then back to the plasma membrane. Recent studies using a variety of antagonists have shown that these blocking drugs can shift the equilibrium of constitutively active receptors. Surprisingly, this shows that not only do antagonists have affinity for receptors, but they also have efficacy, and do not as previously assumed, act merely as blocking agents that prevent endogenous and exogenous agonist from binding. Prazosin acts as an inverse agonist in CHO cells expressing constitutively active $\alpha_{1a}$-adrenoceptors [Zhu et al, 2000].

Overexpression the $\alpha_{1a}$ adrenoceptor in cardiac myocytes leads to the receptor becoming constitutively active; the net result of which is, phosphatidylinositol hydrolysis and ANF-luciferase gene expression, and recruitment of the germ line promoters SP1 and SRE. Therefore, by overexpressing the $\alpha_{1a}$-adrenoceptor in cardiac myocytes, pathways activated by the $\alpha_{1A}$-adrenoceptor have been identified, which may be involved in cardiac cell growth [McWhinney et al, 2000].

1.1.15 $\alpha_1$-adrenoceptors can induce rhythmic arterial contractions

Upon stimulation with agonists, susceptible arteries develop rhythmic activity *in vitro*, and may do so *in vivo*. The development of regular variations in tone is termed vasomotion [Peng et al, 2001]. Vasomotion is a common physiological phenomenon and is of pathophysiological importance [Griffith and Edwards, 1997]. Adrenoceptor agonists have been shown to induce rhythmic contractions in the rat mesenteric artery and in the mouse tail artery [McBride et al, unpublished observation].

Low concentration of the endogenous agonist, noradrenaline, cause $Ca^{2+}$ waves to develop, a process that requires an intact endothelium or a cGMP donor drug [Peng et al, 2001]. Contrary to this, in other studies removal of the endothelium can actually enhance vasomotion [Griffith and Edwards, 1997]. Therefore the role of the endothelium in the development of rhythmic contractility is unclear.

Regardless of endothelial function, vasomotion only develops when a sufficient number of smooth muscle cells are recruited and become active at the same time. This happens when intracellular calcium is released from the sarcoplasmic reticulum. In order for contractions to become rhythmic, a sufficient number of vascular smooth muscle cells must be recruited to contract. Cell to cell communication, probably occurs via gap junctions, which promote synchronised calcium release in a number of vascular smooth muscle cells. The flux of positive ions changes the membrane potential and smooth muscle cell contraction ensues, in a synchronised, rhythmic manner [Peng et al, 2001].

The development of vasomotion is not unique to arteries, and has also been studied in veins. Repetitive, asynchronous calcium waves can be induced by phenylephrine in the rabbit inferior vena cava. Stimulation of $\alpha_1$-adrenoceptors in this blood vessel recruits smooth muscle cells that contract in an all-or-nothing manner. The more cells participating in the contraction, the greater the force generated [Ruehlmann et al, 2000]. Although the development of rhythmic contractions is fascinating and attracts a great deal of interest, unwanted rhythmic activity can hamper the study of subtype specific responses in susceptible arterial beds, a factor which is highlighted in chapter four and seven of this thesis.

1.1.16 $\alpha_1$-adrenoceptors and hypertension

Patients with essential hypertension often have an increased vascular load and frequently have elevated levels of circulating catecholamines in their blood stream [Cottone et al, 1998]. Peripheral blood vessels from these patients and vessels isolated from hypertensive animals exhibit similar pathogenic alterations. A common functional alteration in blood vessels from hypertensives is that they develop supersensitivity to exogenous agonists. Increased sensitivity to contractile agents may reflect a generalised reduction in the number of available receptors, whereby the remaining population become supersensitive. Regardless of the way in which receptors become supersensitive to exogenous agonists, this represents a mechanism, which may contribute to the maintenance of high blood pressure *in vivo*.

Rats that spontaneously develop hypertension (SHR) and rodents with renal-induced hypertension are useful animal models for the study of the development and progression of hypertension. Both of these animal models exhibit altered adrenoceptor-mediated

responses. In the SHR, the aorta and carotid display a significant upregulation of $\alpha_{1D}$-adrenoceptors [Michael et al, 1993]. Hypersensitivity to exogenous agonists is common in hypertension, and may be mediated by the $\alpha_{1D}$-adrenoceptor, because this receptor subtype has been shown to be upregulated in blood vessels from hypertensive animals.

A variety of murine blood vessels contract in response to stimulation of $\alpha_{1D}$-adrenoceptors. These include, mesenteric and femoral arteries, and the carotid and aorta [Kenny et al, 1995, Daly et al, 2002], which all contract when $\alpha_{1D}$-adrenoceptors are stimulated. Contractions of downstream resistance arteries, such as rat mesenteric vessels have been attributed to stimulation of the $\alpha_{1L}$-adrenoceptor [Van der Graff et al, 1996a], but appear to be the result of stimulating $\alpha_{1A}$-adrenoceptors [Daly et al, 2002]. Considered together, data from the rat and mouse provides strong evidence that suggests the $\alpha_{1D}$ subtype is involved in the maintenance of peripheral resistance. In young rats the $\alpha_{1D}$-adrenoceptor plays only a minor role in vasoconstriction. However, *in vitro* experiments on mature animals show a shift toward $\alpha_{1D}$-mediated vasoconstrictions, indicative of a change in the major subtype leading to vascular contractions [Ibarra et al, 1997]. Furthermore, radioligand-binding studies confirmed that there is an alteration in the expression of $\alpha_1$-adrenoceptors in the kidneys of rats with renal-induced hypertension; and $\alpha_{1D}$-adrenoceptor subtypes mediate vasoconstriction in the rat myocardium, aorta, and vas deferens [Deng et al, 1996, Want et al, 1997] and mouse aorta.

The SHR animal model has been used to study the role of the $\alpha_{1D}$-adrenoceptor in hypertension. *In vitro* experiments on isolated blood vessels from the SHR have been

conducted before and after the onset of clinical symptoms. BMY7378, blocks $\alpha_1$-adrenoceptor-mediated contractions in the SHR but has no effect on control animals. Furthermore, in older animals BMY7378 shifts the phenylephrine response curve to the right. This data indicates that the $\alpha_{1D}$-adrenoceptor mediates vascular contractions before the onset of clinical symptoms, and in rats with established hypertension [Villalobos-Molina et al, 1999].

The $\alpha_{1D}$-adrenoceptor appears to play a dual role in hypertension, firstly, in the development of the disease, evidenced by the abundance of this receptor in young spontaneously hypertensive rats. Secondly, $\alpha_{1D}$-adrenoceptors appear to be involved in the maintenance of hypertension because mature rats show a significant upregulation in $\alpha_{1D}$-adrenoceptor-mediated responses.

1.1.17 Benign prostatic hyperplasia

Elderly men are commonly afflicted by a condition known as benign prostatic hyperplasia, a disorder of the lower urinary tract that leads to obstruction of urine outflow; which severely affects a patient's quality of life. Surgical intervention to rectify the obstruction is highly successful. However the stress of surgery, which involves general anaestheisa and transurethral resectioning often leads to morbidity [Garcia-Sainz et al, 1999].

$\alpha_1$-adrenoceptor expression levels are altered in prostatic hyperplasia. Given this, it has been proposed that drugs that selectively inhibit $\alpha_1$-mediated contractions in the lower urinary tract may obviate the need for surgical intervention. The $\alpha_{1A}$ and $\alpha_{1L}$-adrenoceptors are the major adrenoceptor subtypes-mediating contraction in the human

prostate. Therefore, ligands that selectively block $\alpha_{1A}$ and $\alpha_{1L}$-mediated responses offer potential benefits over non-selective compounds. Tamsulosin has been used as a successful treatment for benign prostatic hyperplasia for several years, not least because the drug-induced reductions in blood pressure are slight in comparison to those produced by other $\alpha_1$-blockers.

1.1.18 Drug treatments that target $\alpha_1$-adrenoceptors

A great deal of research has gone into the development of antagonists that are structurally related to prazosin, in an attempt to develop high affinity subtype-selective antagonists. Prazosin is a quinazoline-bearing compound. Substitution of the piperazine ring has shown that the quinazoline moiety is essential for the activity and selectivity of prazosin. The selectivity and activity of prazosin-like compounds depends on the length of their alkaline chain [Melchiorre et al, 2000]. If a cyclopentane ring is used to replace the carbon chain that separates the amine and phenoxy group, this enhances affinity for $\alpha_1$-adrenoceptors compared to $5HT_{1A}$ receptors.

Antagonism of $\alpha_1$-receptors causes vasodilatation of veins and arteries because endogenous $\alpha_1$-agonists are prevented from binding available receptors. Inhibiting the effects of endogenous $\alpha_1$-agonists can be advantageous, but chronic administration of $\alpha_1$-antagonists frequently leads to compensatory increases in catecholamine release [Izzo et al, 1981]. One side effect of an increase in sympathetic tone and elevated catecholamine release is postural hypertension, a condition that is characterised by dizziness upon standing. This often leaves patients agitated and doubtful about the consequences of using medication with such severe side effects.

Non-selective $\alpha_1$-antagonists are effective antihypertensives in clinical conditions. However, there is an increased incidence of heart failure with these drug treatments, which now precludes their use as antihypertensives [Guthrie & Siegel, 1999]. Subtype selective $\alpha_1$-antagonists may prove to be more suitable drugs for the treatment of hypertension, if unwanted side effects and increased mortality are to be avoided.

Urapadil was originally marketed as an alternative drug therapy to prazosin. However, urapadil causes peripheral vasodilatation and increases muscle sympathetic activity, like prazosin. It has since been hypothesised that all drugs causing peripheral vasodilation will eventually cause compensatory increases in sympathetic nerve activity, thus their use is still contraindicated [Grassie et al, 2000].

Tamsulosin has fewer side effects than other $\alpha_1$ antagonists because it does not cause hypotensive responses at therapeutic doses [Fogler et al, 1995]. The reduction in side effects has been proposed to be due to tamsulosin having low affinity for the $\alpha_{1B}$-adrenoceptor [Hein et al, 2000]. However, *in vitro* studies show that this drug has selectivity for $\alpha_{1A}/\alpha_{1L}$-adrenoceptors. Given these results, it can be concluded that tamsulosin does not cause a significant reduction in blood pressure because, at therapeutic doses, it does not antagonise $\alpha_{1D}$-mediated responses. This data also provides evidence that the $\alpha_{1D}$-adrenoceptor plays a more significant role in the control of blood pressure, than the $\alpha_{1A}$ and $\alpha_{1B}$-receptor subtypes.

1.1.19 Ligand mediated changes in adrenoceptor expression levels

Prazosin antagonises $\alpha_1$-mediated responses by blocking receptor-binding sites. However, prazosin can also lead to an upregulation in the expression of $\alpha_1$-

adrenoceptors. In contrast to the effects of prazosin, the $\alpha_{1A}$-selective antagonist, KMD3213 does not alter the expression levels of $\alpha_1$-adrenoceptors; furthermore reserpine, which leads to a depletion of cathecholamine stores has no effect on adrenoceptor expression levels *in vitro* [Zhang et al, 2002]. Intraperitoneal injections of prazosin cause an upregulation of $\alpha_{1B}$ and $\alpha_{1A}$-adrenoceptors in the rat atria and enhance expression of $\alpha_{1B}$-adrenoceptors in the rat spleen. The significance of this is as yet unclear, but suggests that the effects of prazosin treatment *in vitro* can be demonstrated *in vivo* [Zhang et al, 2002].

To test whether inverse agonism is specific to prazosin and what role, if any, the sympathetic nervous system plays in this response, KMD3213 and reserpine-induced responses were studied. KMD3213 has no effect on $\alpha_1$-receptor expression levels in all of the tissues studied. Reserpine attenuates increases in tissue noradrenaline levels, while prazosin and KMD3213 have no effect. Leading to the conclusion that prazosin-mediated upregulation of $\alpha_1$-adrenoceptors may result from increased sympathetic nerve activity. This is supported by the results gained with reserpine, which prevents this from occurring [Zhang et al, 2002].

Inverse agonists increase receptor density in cells expressing constitutively active receptors and decrease G protein coupled receptor activity [Shyrock et al, 1998]. Prazosin acts as an inverse agonist at all three $\alpha_1$-adrenoceptor subtypes when they are expressed in cultured cell lines [Rossier et al, 1999]. Responses to KMD3213 and prazosin have been studied in CHO cells expressing constitutively active $\alpha_{1a}$-adrenoceptors. In this cell line, prazosin acts as an inverse agonist. Upon stimulation, the expression of $\alpha_{1a}$-adrenoceptors is increased. This is coupled to decreased GTP

gamma S basal binding and an attenuation of $IP_3$ levels. KMD3213 causes a parallel, rightward shift of the phenylephrine-induced concentration response curve, indicative of competitive antagonism [Zhu et al, 2000]. KMD3213 also reverses the actions of prazosin, and therefore leads to an elevation in second messenger levels. These findings are important clinically because of the unpredictability of the side effect of inverse agonists *in vivo*.

Antagonists have previously been considered as having an efficacy value of zero (and therefore are unable to produce a measurable response), but this is not actually the case. Prolonged exposure to antagonists in cell lines can cause an upregulation of receptors. A phenomenon that has been explained by a receptor theory, where, for the sake of simplicity, receptors can be considered as existing in two conformational states [Milligan et al, 1995]. R is considered as being inactive, while R* is free to couple to G proteins and lead to down stream signalling. Overexpressing a given receptor subtype is proposed to increase the number of copies of the receptor that exist in the active state. Drugs proposed to act as inverse agonist (these drugs are frequently antagonists, but not exclusively) have been proposed to have higher affinity binding for the inactive (R) state of the receptor compared with the active form (R*). This makes cell lines overexpressing receptors suitable systems for the study of the activity of drugs proposed to act as inverse agonists.

The kidney is an important organ that functions to maintain and control blood pressure. The expression of adrenoceptors in the kidney has been studied in animal models of hypertension. It came as no great surprise when an upregulation of both $\alpha_1$- and $\alpha_2$-adrenoceptors was found in the kidney of the SHR [Sanchez et al, 1986].

Stimulation of postsynaptic $\alpha_1$-adrenoceptors leads to contraction of smooth muscle cells in a plethora of arterial beds. Experiments in the pithed rat have shown that prazosin can inhibit nerve-induced tachycardia, and prevent contraction of the vas deferens [Docherty et al, 1984].

1.1.20 Receptor Signalling

Bovine $\alpha_{1a}$ adrenoceptors have been expressed in rat-1 fibroblasts and used to study phenylephrine-induced responses. Studies in cell lines have shown that $\alpha_1$-adrenoceptors can activate a variety of signalling pathways. The pathway activated appears to be dependent on the extracellular conditions and the cell line used to investigate intracellular signal transduction.

Stimulation of $\alpha_1$-adrenoceptors can lead to the activation of multiple signalling pathways. The pathway activated and the physiological outcome appears to depend on the G protein recruited [Garcia-Sainz et al, 1999], and the vessel or tissue type studied. All three $\alpha_1$-adrenoceptor subtypes mobilise calcium and activate phosphoinositide signalling. However the degree of activation that occurs, depends on the subtype stimulated.

All adrenoceptors belong to the G protein coupled receptor superfamily, and produce their effects by binding guanine nucleotide binding proteins (G proteins). In addition to adrenoceptors, other receptors belonging to this family include inflammatory mediators, serine proteases, neurotransmitters and hormones [Grady et al, 1997].

Molecular cloning studies reveal that there is a high degree of sequence homology between G protein coupled receptors. Leading to the hypothesis that all G protein coupled receptors may have evolved from a common ancestral gene [Piascik & Perez, 2001].

The serpentine structure of a G protein results in the formation of three intra and three extracellular loops. The third extracellular loop is often the site of agonist/receptor interactions [Piascik & Perez, 2001]. G proteins consist of an $\alpha$, $\beta$ and $\gamma$ subunit and have a heterotrimeric structure. Receptor/G protein subunit interactions normally occur at the plasma membrane [Gilman et al, 1987]. The interaction of an adrenoceptor agonist with a G protein causes the release of bound GDP for GTP. GTP then associates with the larger $\alpha$ subunit of the G protein and the $\beta\gamma$ subunits dissociate from the G$\alpha$GTP complex. The active G$\alpha$GTP complex is now free to produce a response by activating downstream signalling pathways. Hydrolysis of GTP to GDP permits reassociation of the $\beta\gamma$ to the $\alpha$ subunit of the G protein, and terminates the response [Summers & McMartin, 1993].

There is a great deal of homology in the structure of G protein coupled receptors. They all have a serpentine membrane-spanning domains consisting of 20-25 hydrophobic residues that form $\alpha$ helices. These receptors possess an intracellular carboxy terminus and an extracellular amino terminal with three extracellular loops. Intracellular receptor domains contain sites that can be phosphorylated by protein kinases after receptor activation [Grady et al, 1997].

$\alpha_1$-adrenoceptors consist of a single chain of polypeptides composed of between 429-561 amino acids. These receptors are characterised by a long carboxy terminus and compared with other G protein coupled receptors have a short third cytoplasmic loop [Summers & McMartin, 1993]. A conserved site in the third cytoplasmic loop is responsible for the formation of a hydrophilic catecholamine-binding domain [Graham et al, 1996].

Once activated, $\alpha_1$-adrenoceptors predominantly bind to pertussis toxin-insensitive G proteins of the $G_q/_{11}$ family. Some selectivity exists among the $\alpha_1$ subtypes with the $\alpha_{1D}$ coupling via $G_q$ or $G_{11}$, and not the $G_{14}$ and $G_{16}$, unlike the $\alpha_{1B}$ subtype. The $\alpha_{1B}$-adrenoceptor can mediate responses by binding to the $G_{14}$ and $G_{16}$ when they are transfected in cos-7 cells, in addition to coupling to $G_q$ and $G_{11}$ [Wu et al, 1992].

$\alpha_1$-adrenoceptor stimulation leads to the activation of phospholipase C, D and $A_2$ [Exton, 1996]. Activation of these enzymes leads to the opening of $Ca^{2+}$ channels, activation and or inactivation of $K^+$ channels and activation of the Na- $Ca^{2+}$ exchanger [Graham et al, 1996], all of which are important determinants of membrane potential.

Interactions of $\alpha_1$-selective agonists with receptors result in an increase in the concentration of cytosolic calcium by activation of phospholipase C. An elevation in cytosolic $Ca^{2+}$ leads to the production of 1,4,5-triphosphate ($IP_3$) and diacylglycerol. Diacylglycerol in turn, activates protein kinase C, which phosphorylates intracellular sites and modulates the transcription of genes [Graham et al, 1996]. $IP_3$ formation promotes the release of $Ca^{2+}$ from intracellular stores [Cowlen & Toews, 1988].

Hydrolysis of $IP_3$ promotoes the release of $Ca^{2+}$ stored in the endoplasmic and sarcoplasmic reticulum [Minneman, 1988], facilitating contractile responses.

$\alpha_1$-stimulation can also cause an influx of $Ca^{2+}$ from the extracellular environment by opening voltage-dependent $Ca^{2+}$ channels [Graham et al, 1996]. This is evidenced by the fact that removal of extracellular $Ca^{2+}$ inhibits $\alpha_{1A}$-mediated contractions in isolated vascular smooth muscle [Han et al, 1987]. In 1996, Graham and co workers recorded differences in the way that $\alpha_{1A}$ and $\alpha_{1B}$-adrenoceptors mobilise $Ca^{2+}$. They proposed that the $\alpha_{1A}$-adrenoceptor acts to increase $Ca^{2+}$ levels by opening voltage-dependent $Ca^{2+}$ channels, while the $\alpha_{1B}$-adrenoceptor causes a rise in intracellular calcium via phospholipase C and $IP_3$ [Graham et al, 1996]. However in other tissues, such as the rat vena cava $\alpha_{1B}$-adrenoceptors can cause an influx of extracellular calcium [Perez et al, 1993].

Vascular contractions brought about by stimulation of $\alpha_1$-adrenoceptors leads to enhanced actin-myosin chain interactions. An increase in intracellular calcium causes activation and recruitment of calmodulin. Calmodulin/ $Ca^{2+}$ complexes activate myosin light chain kinase (MLCK), which promotes actin myosin interactions via phosphorylation and hence, causes contraction of vascular smooth muscle cells.

1.1.21 Inflammatory responses involving $\alpha_1$-adrenoceptors

Cytokines are the chemical messengers of the immune systems, and as such can regulate and alter the functioning of cells. Cytokines can also regulate the expression of mRNA for $\alpha_1$-adrenoceptors in human monocytic and endothelial cells [Heijnen et al,

2002]. For example, in patients with chronic juvenile arthritis, stimulation of $\alpha_1$-adrenoceptors leads to the release of the cytokine IL-6 from mononuclear cells.

Mononuclear white cells express $\alpha_{1b}$ and $\alpha_{1d}$ mRNA. The cytokines, TNF$\alpha$ and IL-1$\beta$ cause a decrease in $\alpha_{1d}$ mRNA in THP-1 monocytic cells, but $\alpha_{1b}$ mRNA levels are unchanged. In addition to the $\alpha_{1b}$ and $\alpha_{1d}$-adrenoceptors, the cytokines IL-1$\beta$ and TNF$\alpha$ lead to enhanced expression of $\alpha_{1a}$ mRNA on mononuclear cells. Therefore, cytokines can regulate expression levels of $\alpha_1$ mRNA, but the changes in receptor expression are dependent on the subtype studied and the tissue type used [Heijnen et al, 2002]. Surprisingly, cultured human endothelial cells have been shown to express $\alpha_1$-adrenoceptors [Heijnen et al, 2002], a novel finding that requires further investigation. This supports the finding that relaxant responses mediated by $\alpha_1$ agonists require an intact endothelial surface.

1.1.22 $\alpha_1$-adrenoceptors and hypoxic conditions

The abdominal aorta divides into two common iliac arteries. In the rat iliac artery the administration of noradrenaline and phenylephrine leads to vascular contraction. Iliac and femoral arteries appear to be more vulnerable to hypoxic conditions than other arterial beds [Marriot & Marshall, 1990]. In the iliac and femoral arteries there is no evidence to suggest that $\alpha_2$-adrenoceptors are involved in contractile responses. Therefore, contractions of these arteries appear to depend solely on the activation of $\alpha_1$-adrenoceptors. In the absence of a functional endothelium, increasing concentrations of noradrenaline produce a biphasic response curve, while the phenylephrine-induced responses are monophasic. Hypoxic conditions reduce the maximal response to noradrenaline and phenylephrine, without affecting sensitivity. This data suggests that

$\alpha_1$-mediated vasoconstriction of the rat iliac artery is affected by $PO_2$ levels [Bartlett & Marshall, 2002]. The extent to which low oxygen levels alter $\alpha_1$-mediated contractions is not discussed further here because it is outwith the scope of this thesis.

## 1.2 $\alpha_2$-adrenoceptors

### 1.2.1 Background

Cloning studies have identified three genes encoding $\alpha_2$-adrenoceptors. These genes correspond to the $\alpha_{2A}$, $\alpha_{2B}$ and the $\alpha_{2C}$ receptor subtypes [Bylund et al, 1994]. Since the discovery of these three $\alpha_2$-receptors, the presence of a fourth subtype has been suggested, namely the $\alpha_{2D}$. The consensus now is that the $\alpha_{2A}$ and $\alpha_{2D}$ represent species homologues, and mediate similar functions in different species. In humans' three separate genes, located on chromosomes 10, 2 and 4 encode the $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$-adrenoceptors respectively [Lomasney et al, 1990]. The $\alpha_{2A}$-adrenoceptor is found almost exclusively in humans, while the $\alpha_{2D}$ receptor is expressed in rodents [Harrison et al, 1991]. $\alpha_{2A}$-adrenoceptors cloned from humans [Starke et al, 1995] and porcine tissue, have a slight difference in amino acid sequence to those cloned from the rat, [Bylund et al 1992], mouse and the guinea pig.

Although $\alpha_2$ adrenoceptors were initially discovered prejunctionally, they have since been shown to exist on the postjunctional membrane [Docherty and McGrath, 1980]. However since their molecular identification a great deal of research has focused on identification of the prejunctional receptor subtype. Most of the data presented points towards the $\alpha_{2A/D}$-subtype, evidenced by $\alpha_{2A/D}$ knockout mice having a ninety percent reduction in presynaptic feedback control [Altman et al, 1999]. Radioligand binding experiments have confirmed that the remaining responses are mediated by the $\alpha_{2C}$-subtype [Trendelenberg et al, 1994]. Without transgenic mice, the identification of the two $\alpha_2$ receptor subtypes mediating autoinhibition of noradrenaline release would have been impossible [Hein et al, 1998].

Postjunctional $\alpha_2$-adrenoceptors mediate contractile responses in a wide variety of veins and these responses have been studied extensively. However the identification and determination of responses mediated by $\alpha_2$-adrenoceptors in arteries has proved more difficult. Contractions in the venous circulation have been subtyped and generally result from stimulation of $\alpha_{2A/D}$ receptors. One notable exception to this is the human saphenous vein. There, contractile responses are mediated by the $\alpha_{2C}$-receptor subtype and are not mediated by the $\alpha_{2A/D}$ [Gavin et al, 1997].

1.2.2 Presynaptic $\alpha_2$-adrenoceptors

$\alpha_2$-adrenoceptors are expressed in the nucleus tractus solitaris and nucleus reticularis lateralis. Both of these areas of the brain are actively involved in the control of blood pressure [Rosin et al, 1993], leading to the hypothesis that, presynaptic $\alpha_2$-adrenoceptors are involved in the control of blood pressure. Stimulation of $\alpha_2$-adrenoceptors in the nucleus tractus solitaris reduces vasomotor tone, and leads to hypotension [Zendberg et al, 1979].

Autoreceptors regulate the release of their own transmitter, while heteroreceptors modulate the release of transmitters acting upon other receptor subtypes [Starke et al, 1989]. $\alpha_2$-adrenoceptors can be defined as autoreceptors, because they inhibit the release of noradrenaline from presynaptic nerve terminals. Early work to elucidate the functional responses controlled by $\alpha_2$-adrenoceptors suggested that all $\alpha_2$-adrenoceptors were located on the presynaptic terminal, where they act solely to regulate the release of noradrenaline from nerve terminals by means of a negative feedback loop [Starke et al,

1975]. The regulatory role of $\alpha_2$-adrenoceptors in mediating autoinhibition of noradrenaline release is now widely accepted [Starke, 2001]. While $\alpha_2$-adrenoceptors oppose further noradrenaline release, it is interesting to note that β-adrenoceptors actually enhance noradrenaline release [Starke et al, 1989].

$\alpha_{2A/D}$ and $\alpha_{2C}$-adrenoceptors regulate the release of noradrenaline from sympathetic nerve terminals. Inhibitory actions of the $\alpha_{2A/D}$ receptor appear to occur more rapidly than those mediated by the $\alpha_{2C}$-adrenoceptor. An explanation for this may be that expression levels of each subtype are different in neonates. Indeed it has since been shown that $\alpha_{2A/D}$-mediated autoinhibition exists at birth, while $\alpha_{2C}$-mediated reductions in noradrenaline release are not established until much later in murine development [Scheib et al, 2001].

When $\alpha_{2A/D}$ and $\alpha_{2C}$ receptors are expressed in the same cell line, their ability to activate potassium and calcium channels is comparable, as are their expression levels [Bunemann et al, 2001]. However, when activated, $\alpha_{2C}$-receptors remain activated after $\alpha_{2A/D}$-mediated inhibition of noradrenaline release has ceased. $\alpha_{2C}$-adrenoceptors have a higher affinity for noradrenaline than the $\alpha_{2A/D}$ [Link et al, 1992]. This higher affinity suggests that the $\alpha_{2C}$-adrenoceptor may control transmitter release at low frequency stimulation, while the $\alpha_{2A/D}$ responds to, and regulates, noradrenaline release after maximum nerve activity. So the differences in noradrenaline sensitivity between the $\alpha_{2A/D}$ and $\alpha_{2C}$-adrenoceptors can be explained by altered receptor activation kinetics [Bunemann et al, 2001]. The $\alpha_{2B}$-adrenoceptor does not regulate noradrenaline release

from sympathetic nerve terminals [Bucheler et al, 2002], but does control noradrenaline release from the rat thalamic nuclei [MacDonald & Scheinin, 1995].

The levels of noradrenaline found in human tissues increase with advancing age, which has been proposed to be due to a decline in central $\alpha_2$-adrenoceptor inhibitory effects [Zeigler et al, 1976]. Alternatively, it may be a result of a decrease in neuronal uptake, or possibly a combination of both. This area has not been fully explored, and future work will be of great importance for the treatment of hypertension.

$\alpha_{2A/D}$-adrenoceptors regulate sympathetic nerve activity in a variety of species. Many of the experiments performed that led to this conclusion were carried out in mice lacking functional $\alpha_{2A/D}$, $\alpha_{2B}$ and $\alpha_{2C}$-adrenoceptors. Kable and co workers used these tools to confirm earlier findings, which suggested that $\alpha_{2A/D}$ and $\alpha_{2C}$-adrenoceptors regulate 'negative feedback' of neuronal noradrenaline [Kable et al, 2000].

The regulation of noradrenaline and dopamine levels in the murine brain appears to be controlled by two $\alpha_2$-adrenoceptor subtypes. This has since been confirmed by the use of mice harbouring gene-directed deletions of each $\alpha_2$-adrenoceptor subtype. The principal subtype responsible for the control of dopamine release is thought to be the $\alpha_{2A/D}$-subtype [Trendelenburg et al, 1994]. The $\alpha_{2C}$ subtype, which is expressed at much lower levels in the murine brain, plays a secondary role [Tavaret et al, 1996].

Therefore, central $\alpha_2$-adrenoceptors act not only as autoreceptors, by inhibiting the release of noradrenaline, they also act as heteroreceptors by regulating dopamine release. The subtype responsible for the control of dopamine release in the rodent brain

has been proposed to be the $\alpha_{2A/D}$-subtype [Trendelenburg et al, 1994]. Dopamine and noradrenaline release are regulated by $\alpha_2$-adrenoceptors, as is the control of acetylcholine from cholinergic motor neurons [Scheibner et al, 2002]. Non-selective stimulation of $\alpha_2$-receptors decreases noradrenaline release by 96% in the cortex of the murine brain and reduces dopamine release from the basal ganglia by 76%.

So how does stimulation of an $\alpha_2$-adrenoceptor cause a decrease in noradrenaline release from sympathetic nerve terminals? Two possible modes of action have been suggested. In the locus coeruleus, an increase in $K^+$ conductance mediates inhibition of noradrenaline release from adrenergic nerves, and has been proposed to participate in the response in other anatomical locations [Starke et al, 1989]. However, the hypothesis that $K^+$ is the major ion responsible for modulation of noradrenline release is debatable, because many studies have shown that an inhibition of $Ca^{2+}$ entry, following nerve depolarisation prevents autoinhibtion, thus preventing exocytosis of vesicles enriched with neurotransmitters [Starke et al, 1989]. $\alpha_2$-adrenoceptors located on neurones within the central nervous system inhibit P, Q and N type calcium channels upon activation [Delmas et al, 1999].

### 1.2.3 Desensitisation of $\alpha_2$-adrenoceptors

Desensitisation of presynaptic $\alpha_2$-adrenoceptors occurs in the mouse atria [Bucheler et al, 2002]. *In vitro* studies have shown that $\alpha_2$-adrenoceptors are prone to agonist-induced receptor desensitisation [Eason et al, 1992]. Furthermore, Bucheler and colleagues demonstrated that prolonged exposure to selective agonists attenuates $\alpha_{2C}$-mediated autoinhibition [Bucheler et al, 2002], and consecutive noradrenaline response curves have a progressive reduction in maximal responses, which is indicative of

receptor desensitisation [Rodriguez-Martinez et al, 1999]. This may be the result of desensitised $\alpha_2$-adrenoceptors. Taken together, this provides evidence that $\alpha_2$-adrenoceptors are susceptible to desensitisation.

1.2.4 Postsynaptic $\alpha_2$-adrenoceptors

Studying the responses mediated by prejunctional $\alpha_2$ receptors has been relatively easy compared with the problems associated with the identification of postjunctional $\alpha_2$-adrenoceptor-mediated responses, *in vitro*. *In vivo* $\alpha_2$-adrenoceptor-mediated responses play a key role in the regulation of cardiovascular function in the central nervous system, and in the periphery. In the pithed rat, postsynaptic $\alpha_2$-adrenoceptors mediate vasoconstrictor responses [Docherty & McGrath, 1980]. Vascular contractions of peripheral blood vessels following stimulation of $\alpha_2$ receptors have been attributed to activation of $\alpha_{2A/D}$-adrenoceptors. This hypothesis is supported by studies in the D79N $\alpha_{2A/D}$ mutant mouse, where vasoconstrictor responses to $\alpha_2$ agonists are absent *in vivo* [MacMillan et al, 1996].

Agonist-induced contractions of isolated aortic rings from female $\alpha_{2A/D}$ knockout mice are significantly smaller in size than responses gained in control arteries [Vandeputte & Docherty, 2001]. Yohimbine is a relatively non-selective $\alpha_2$-adrenoceptor antagonist, but has slightly higher affinity for the $\alpha_{2B}$ and $\alpha_{2C}$-adrenoceptors over the $\alpha_{2A}$ subtype [Link et al, 1992]. Yohimbine enhances noradrenaline-mediated contractions in aorta from control but not from $\alpha_{2A/D}$ knockout mice. Surprisingly, this suggests that the $\alpha_{2A/D}$ receptor may act to inhibit noradrenaline-evoked contractions in the aorta.

In $\alpha_{2A/D}$ knockout mice noradrenaline administered in the presence of an $\alpha_1$ antagonist has no effect on resting blood pressure [Duka et al, 2000]. However in $\alpha_{2B}$ and $\alpha_{2C}$ knockouts an alteration in blood pressure responses is observed [Duka et al, 2000]. This data suggests that the $\alpha_{2A/D}$ receptor is responsible for peripheral vasoconstrictor responses. Yet, the initial pressor response occurring after intravenous administration of an $\alpha_2$ agonist is lost in $\alpha_{2B}$ knockout mice [Link et al, 1996]. In addition to peripheral effects on arteries, $\alpha_2$-adrenoceptors cause contraction of veins, in particular the saphenous vein of the rabbit [Daly, 1993] and human [Gavin et al, 1997].

### 1.2.5 Cellular location of $\alpha_2$-adrenoceptors

The location of $\alpha_2$-adrenoceptors within a cell may dictate their function. In order to ascertain if this is the case, a number of studies have been carried out to determine where each $\alpha_2$-subtype resides in a cell. In cultured, transfected fibroblasts $\alpha_{2A/D}$ and $\alpha_{2B}$-adrenoceptors are located on the plasma membrane after receptor activation, but only the $\alpha_{2B}$ exhibits reversible endocytosis [Daunt et al, 1997]. Quiescent $\alpha_{2C}$-receptors are found in close association with the golgi and endoplasmic reticulum. Upon activation and/or exposure to cold temperatures, these receptors translocate to the plasma membrane [Jeyaraj et al, 2001], where they mediated enhanced contractile responses [Chotani et al, 2000].

### 1.2.6 $\alpha_2$ selective ligands

Clonidine has been used as an $\alpha_2$-selective agonist, but has affinity for both $\alpha_1$ and $\alpha_2$-adrenoceptors [Millan et al, 2000]. Chloroethylclonidine (CEC) is an alkylating analogue of clonidine and has affinity for all $\alpha_1$ and $\alpha_2$ adrenoceptor subtypes [Michel

et al, 1993]. Unlike clonidine, CEC is an irreversible agonist of $\alpha_2$-adrenoceptors, but is frequently used to distinguish responses mediated by the $\alpha_{1B}$-adrenoceptor, because it alkylates plasma membrane-bound receptors [Nunes & Guimares, 1993].

Xylazine, is an $\alpha_2$-receptor agonist, which causes concentration-dependent contractions of the guinea pig epididymis, when this tissue has been precontracted with phenylephrine [Haynes et al, 1999]. Incubation of the Gi$\alpha$2 mRNA antisense oligonucleotide attenuates xylazine-mediated contractions, without affecting tissue sensitivity. It appears that in the guinea pig epididymis $\alpha_2$-adrenoceptors cause contractions in preconstricted tissues by coupling to the Gi$\alpha$2 receptor subunit [Haynes et al, 1999].

In the human saphenous vein, stimulation of postjunctional $\alpha_2$ receptors leads to the development of contractile responses. Surprisingly, in this blood vessel, $\alpha_1$-mediated vasoconstrictions are essentially absent [Docherty, 1998]. The $\alpha_2$ adrenoceptor responsible for contraction in this blood vessel is proposed to be the $\alpha_{2C}$-subtype [Gavin et al, 1997]. $\alpha_2$-mediated contractile responses have also been shown in blood vessels from the pig and the dog [Hicks et al, 1991].

1.2.7 Responses mediated by $\alpha_2$-adrenoceptors are affected by increasing age

Cerebral arteries from young and old sheep contract in response to $\alpha_2$-selective agonists [Bishai et al, 2002]. The expression levels and density of $\alpha_2$-adrenoceptors is greater in foetal arteries when compared with cerebral arteries from mature sheep [Bishai et al, 2002]. Cerebral arteries from sheep in utero express both pre and postsynaptic $\alpha_2$-

adrenoceptors. However, in cerebral blood vessels from mature sheep, contractile responses resulting from activation of postjunctional $\alpha_2$-adrenoceptors cannot be detected [Bishani et al, 2002]. This suggests that the expression of $\alpha_2$-adrenoceptors in cerebral arteries decreases with advancing age, the process whereby this occurs, and the physiological significance has yet to be determined.

1.2.8 $\alpha_2$-mediated relaxant responses

An intact, functional endothelial surface is essential for the maintenance of physiological functioning of a blood vessel [Busse et al, 1998]. $\alpha_2$-adrenoceptor-mediated relaxations require an intact endothelium and have been studied in coronary, renal and mesenteric arteries [Cocks & Angus, 1983]. In porcine coronary arteries the $\alpha_{2A/D}$ and $\alpha_{2C}$-adrenoceptor subtypes are both expressed. Although the $\alpha_{2C}$-adrenoceptors is expressed at a much greater level, it is the $\alpha_{2A/D}$-adrenoceptor that mediates endothelium-derived relaxations in this artery. $\alpha_2$-mediated responses were characterized with [$^3$H] rauwolscine in endothelial membrane preparations. The competition binding curves and the use of ligands, proposed to distinguish between the two subtypes suggests that the $\alpha_{2A/D}$-adrenoceptor mediates relaxant responses [Bockman et al, 1993]. $\alpha_2$-mediated vasodilatations have also been studied in the rat. Stimulation of endothelial $\alpha_{2A/D}$-adrenoceptors, in the rat mesenteric resistance arteries causes relaxation, which is due to altered cAMP levels [Bockman et al, 1996].

1.2.9 Tail artery

Activation of postjunctional $\alpha_{2C}$-adrenoceptors in the rat tail leads to vasoconstrictor responses [Craig et al, 1995]. The rat and mouse tail artery have a dense sympathetic innervation and contain a functional population of $\alpha_2$-adrenoceptors [Medgett &

Langer, 1984], which are most probably both pre and postjunctional in location. Catecholamine-mediated contractions of this artery tend to increase in size in older animals. The increase in maximal contractions and changes in agonist sensitivity may be a result of altered oxidative stress [Diaz-Velez et al, 1996].

Malondialdehyde (MDA) is used as a marker for increased oxidative stress in an artery; high MDA levels are indicative of elevated oxygen radicals, which lead to vascular injury [Janero et al, 1990]. Enhanced plasma MDA levels have been reported in humans showing early signs of vascular disease [Sanderson et al, 1995].

In the rat tail artery plasma oxidative stress is elevated with age, evidenced by an increase in MDA and decreased levels of glutathione peroxidase in older animals [Rodriguez-Martinez et al, 1999]. MDA potentiates noradrenaline-evoked contractions in tail arteries from young, old and very old rats, but has no effect on catecholamine sensitivity.

Superoxide dismutase scavenges oxygen radicals, and in the rat tail artery has been shown to reverse age related changes in agonist responses, while hydrogen peroxide and scavengers of hydroxyl radicals were ineffective [Tsai et al, 1993]. Scavenging oxygen and hydroxyl radicals may reduce the supersensitivity to exogenous and endogenous agonists that often occurs in arteries from elderly patients. In doing so it may help to reverse functional alterations that can contribute to the development of pathological conditions.

1.2.10 Development of contractile responses in the rodent tail artery

$\alpha_1$-mediated contraction of the rat and mouse tail artery are predominantly mediated by the $\alpha_{1A}$-subtype, with little, if any, contribution for the $\alpha_{1B}$ and $\alpha_{1D}$-adrenoceptors Piascik et al, 1996, Daly et al, 2002]. The rat tail artery receives a rich blood supply via three arteries and veins which run the entire length of the tail, making this an important organ involved in thermoregulatory control [Rand et al, 1965].

Mibefradil is a non-selective calcium channel antagonist that blocks L, T and N type calcium channels [Bezprozvanny & Tsien, 1995]. Contractions mediated by endogenous catecholamines all depend on calcium, evidenced by the inhibiting effects of calcium antagonists such as mibefradil. Given our knowledge of this drug, it seems likely that transmitter release is inhibited by blocking N type calcium channels located on the prejunctional sympathetic nerve [van der Lee et al, 2000].

Xiao and colleagues have shown that $\alpha_2$-mediated vasoconstriction can enhance responses to $\alpha_1$ agonists in the rat tail artery, by altering calcium levels [Xiao et al, 1989]. Noradrenaline causes contractions in proximal sections of the rat tail artery in a concentration-dependent manner [Medgett et al, 1984]. In hypertension noradrenaline contractions are enhanced [Medgett et al, 1984]. Enhanced $\alpha_2$-adrenoceptors-mediated responses can be affected by prostaglandin release, which often complicates the interpretation of data. To prevent this, a number of blocking agents need to be used during experiments.

The development and study of responses mediated by postjuctional $\alpha_2$-adrenoceptors have proven difficult to study *in vitro*. One possible explanation for this is that *in vitro*

conditions do not mimic those *in vivo*. In the rat tail artery increasing the level of tone with arginine vasopressin allows $\alpha_2$-mediated vasoconstrictor responses to be studied [Templeton et al, 1989]. This was a major breakthrough in the study of $\alpha_2$-mediated responses. However, the responses gained display a high degree of variability and depend on the synergist used to raise tone, and the species used to study vascular responses. In proximal arterioles of the rat intestine sympathetic-induced elevations in vascular tone, did not reveal $\alpha_2$-mediated contractile or relaxant responses. This data indicates that, at least in proximal arterioles, postjunctional $\alpha_2$-adrenoceptors are essentially absent [Nase & Boegehold, 1998].

1.2.11 Thermoregulation

The tail artery is an important organ involved in thermoregulatory control in the rodent. Due to this, vascular responses of the tail artery from mice and rats have been studied at varying experimental temperatures. Maintaining low temperatures of $28^0$C enhances $\alpha_2$-mediated vasoconstrictions in cutaneous arteries of the mouse. The effect of temperature on contractile responses is proposed to result from the activation of quiescent $\alpha_{2C}$ adrenoceptors, which then participate in vascular contractility [Chotani et al, 2000].

In addition to the local effects of temperature, stimulation of central $\alpha_2$-adrenoceptors causes a reduction in core body temperature. These hypothermic responses are absent in D79N mice, but dose-dependent reductions in core body temperature occur in WT, $\alpha_{2B}$ and $\alpha_{2C}$ knockout mice [Hunter et al, 1997]. In human and animal species, the $\alpha_{2C}$-adrenoceptor has been implicated in the development of responses to cooling in limb and saphenous veins [Vanhoutte et al, 1985, Vanhoutte & Flavahan 1986, Chotani et al,

2000]. Central $\alpha_{2C}$-adrenoceptors have since been proposed to play a minor role in the control of hypothermic responses, because in the $\alpha_{2C}$ knockout mouse, hypothermic responses that normally result from stimulation of central $\alpha_2$-adrenoceptors are abolished [Sallinen et al, 1997].

Reducing the experimental temperature to $28^0$C causes a significant enhancement of UK14304-mediated contractions, in pressurised, myograph mounted arterial segments. The potentiation in responses is confined to the distal end of the murine tail, where $\alpha_1$-mediated contractions are attenuated [Chotani et al, 2000]. In HEK 293 cells stimulation of expressed $\alpha_{2C}$-adrenoceptors at $37^0$C (with an $\alpha_2$-selective agonist) has no effect on forskolin-induced cAMP accumulation. However, at $28^0$C, activation of $\alpha_{2C}$-adrenoceptors causes a concentration-related reduction in cAMP production. Subcellular fractioning of HEK 293 cells shows that at $37^0$C the $\alpha_{2A}$-subtype is primarily located on the plasma membrane, while $\alpha_{2C}$-adrenoceptors are sequestered on intracellular organelles, such as the golgi. [Jeyaraj et al, 2001]. However, at $28^0$C, the $\alpha_{2C}$-adrenoceptor translocates to the plasma membrane, where its expression becomes greater than that of the $\alpha_{2A}$ subtype. Evidence in support of this hypothesis is provided by the use of the drug, MK912. MK912 is an antagonist proposed to be selective for the $\alpha_{2C}$-adrenoceptor, and at $28^0$C, MK912 competitively antagonises $\alpha_2$-adrenoceptor mediated contractile responses of the pressurised tail artery [Chotani et al, 2000].

Patients suffering from Raynaud's phenomenon experience involuntary, vasospastic contractions of the fingers and toes. This is usually triggered by exposure to extremely cold temperatures. $\alpha_{2C}$-mediated contractions are enhanced in Raynaud's [Freedman et

al, 1989]. Given this, drugs that selectively antagonise contractile responses-mediated by this receptor subtype may have therapeutic benefits over non-selective antagonists.

Noradrenaline and phenylephrine cause concentration-dependent contractions of human hand veins, with noradrenaline being twenty four times more potent that phenylephrine. Enhanced sensitivity to noradrenaline indicates the presence of $\alpha_2$-adrenoceptors in human hand veins, that when stimulated participate in contractile responses. Prazosin and rauwolscine, given alone, failed to attenuate noradrenaline-mediated contractions but a combination of both drugs abolishes catecholamine-induced contractions.

In addition to the $\alpha_2$-adrenoceptor-mediated response, stimulation of 5HT receptors has also been shown to lead to contraction of human hand veins. Ketanserin and methergoline are antagonists at 5HT receptors. Ketanserin competitively inhibits 5HT-mediated contractions in human hand veins, while methergoline causes a significant reduction in maximal contractions. From this data it appears that contractions of human hand veins results from activation of a mixed population of receptors that include $\alpha_1$, $\alpha_2$ and 5HT receptors [Arner & Hogestatt, 1986].

1.2.12 Effect of $\alpha_2$-adrenoceptor stimulation on gastrointestinal motility and gastric sections

*In vivo*, the $\alpha_2$-agonist clonidine causes a significant decrease in gastrointestinal motility, and abolishes cholinergic mediated contractions. Histamine is known to inhibit gastrointestinal motility. Surprisingly, $\alpha_2$-mediated inhibition of gut motility is significantly greater than the inhibitory effects of histamine at comparable doses [Pozzoli et al, 2002].

Functional $\alpha_2$-adrenoceptors are expressed on cholinergic nerve terminals, and are involved in the regulation of gastric acid secretion [Tazi-Saad et al, 1992]. Furthermore, studies in pylorus-ligated rats have shown that intracerebroventricular (i.c.v) injections of clonidine or oxymetazoline cause a dose-dependent reduction in gastric acid secretion [Mullner et al, 2002]. The inhibitory effect of clonidine on gastric acid secretion is due in part, to stimulation of $\alpha_2$-adrenoceptors located on presynaptic fibres in the stomach lining [Mullner et al, 2002]. In the pylorus-ligated rat the major antisecretory effect of $\alpha_2$-agonists can be attributed to central $\alpha_{2A/D}$-adrenoceptors. Direct, gastroprotective effects of stimulating $\alpha_2$-adrenoceptors in the stomach appear to be mediated by the $\alpha_{2B}$-receptor subtype [Gyires et al, 2000b].

Mullner and co-workers found that the antisecretory actions of clonidine and oxymetazoline are inhibited by the opioid-selective antagonists naloxone and matrindole. In addition to this the $\mu/\delta$ opioid agonist $\beta$-endorphin causes comparable decreases in gastric acid secretion to those produced by $\alpha_2$-adrenoceptor ligands. This led to the hypothesis, that the antisecretory actions of clonidine cannot be attributed to direct stimulation of $\alpha_2$-adrenoceptors. Indeed, the antihypertensive and gastroprotective effects of clonidine have been proposed to result from opioid receptor activation [Gyires et al, 2000c].

Agonists that stimulate $\alpha_2$ and opioid receptors cause hyperpolarisation of neurones by increasing potassium conductance [North et al, 1987]. In the pylori-ligated rat i.c.v injections of glibenclamide, a $K_{ATP}$ channel antagonist, prevent the antisecretory effects of clonidine, oxymetazoline and $\beta$-endorphin. Whether $\alpha_2$-adrenoceptors indirectly

cause $K_{ATP}$ channel activation, or promote the release of endogenous agonists such as β-endorphin that act upon opioid receptors to stimulate $K_{ATP}$ channels, requires further investigation [Mullner et al, 2002].

$\alpha_{2A/D}$ adrenoceptors found within the gastrointestinal tract act solely to inhibit further transmitter release, unlike the inhibitory functions of $\alpha_2$-adrenoceptors located in the central nervous system, which require assistance from the $\alpha_{2C}$-receptor subtype [Scheibner et al, 2002].

1.2.13 $\alpha_{2A/D}$-adrenoceptors

Transgenic mice with mutated or deleted receptors are useful tools in elucidating the functions of individual adrenoceptor subtypes. Gene targeting causes a point mutation of an asparagine for an aspartate at position 79 of the second transmembrane spanning domain of the $\alpha_{2A/D}$-adrenoceptor; a mutation which is expressed in the D79N mouse. This substitution mutation selectively uncouples the $\alpha_{2A/D}$-adrenceptor from its $K^+$ channel, but activation of voltage gated $Ca^{2+}$ channels is unaffected [Suprenant et al, 1992]. Remarkably, although this is not a receptor knockout, binding analysis in isolated brain membranes reveals an eighty percent reduction in available receptor binding sites. So in essence the D79N mutation acts as a functional knockout. The remaining $\alpha_{2A/D}$-adrenoceptors in the D79N mouse are functional, and bind ligands normally [MacMillan et al, 1996].

In the D79N mouse, baseline blood pressure is unchanged compared with WT controls. Upon administration of UK14304 or dexmedetomidine (proposed to be selective agonists for $\alpha_2$-adrenoceptors), there is an attenuated decrease in blood pressure and

heart rate in the D79N, compared with controls. This indicates that the blood pressure lowering effects of central $\alpha_2$-adrenoceptors are lost in this mouse. This supports the hypothesis that the $\alpha_{2A/D}$ subtype is responsible for the hypotensive effects of sleetive $\alpha_2$-agonists. Although devoid of central $\alpha_{2A/D}$ mediated effects; in the periphery, the D79N mouse has a sufficient receptor reserve, capable of mediating contractions that result from activation of $\alpha_2$-adrenoceptors.

Expression levels of $\alpha_2$-adrenoceptors have been measured to determine if removal of one receptor leads to upregulation of the other $\alpha_2$-adrenoceptors-subtypes. In mice harbouring knockouts of the $\alpha_{2A/D}$, $\alpha_{2B}$ or $\alpha_{2C}$-adrenoceptor the expression levels of the remaining $\alpha_2$-adrenoceptor subtypes are unchanged [Link et al, 1995 & Altman et al, 1999]. Functional studies carried out in the D79N and $\alpha_{2A/D}$ knockout mouse, confirm that central $\alpha_{2A/D}$-adrenoceptors mediate hypotensive responses to $\alpha_2$ agonists. While in the $\alpha_{2B}$ knockout mouse $\alpha_2$-mediated pressor responses are absent. This led the authors to conclude that $\alpha_{2B}$-adrenoceptors mediate vasoconstrictor responses to $\alpha_2$-selective ligands [MacMillan et al, 1996]. Although previously used as successful antihypertensives, stimulation of $\alpha_2$-adrenoceptors, with selective agonists, produces a number of unwanted side effects. These include a dry mouth and sedation; both of which have been attributed to the $\alpha_{2A/D}$-receptor subtype. This precludes the use of such compounds, as their beneficial effects cannot be achieved without causing unwanted side effects.

Deletion of the $\alpha_{2A/D}$-adrenoceptor causes an increase in sympathetic outflow. The $\alpha_{2A/D}$ knockout mouse is tachycardic at rest and has depleted noradrenaline tissue levels.

These changes are accompanied by a down regulation of β-adrenoceptors in cardiac tissue [Altman et al, 1999].

Analysis of functional responses in the $\alpha_{2A/D}$, $\alpha_{2B}$ and $\alpha_{2C}$ knockouts and in the D79N mouse, have shown that the major adrenoceptor subtype controlling sympathetic outflow is the $\alpha_{2A/D}$. Electrical stimulation of adrenergic nerves in the mouse vas deferens promotes the release of endogenous noradrenaline, and leads to contractile responses. The presence of an $\alpha_2$-agonist prevents these contractions from developing. In the D79N, the inhibitory effect of $\alpha_2$-agonists is significantly reduced, but not abolished, suggesting the involvement of another receptor in the regulation of noradrenaline release [Altman et al, 1999]. In the rat atria $\alpha_{2A/D}$-mediated autoinhibition of noradrenaline release also appears to be assisted by another $\alpha_2$-receptor subtype.

D79N mice and $\alpha_{2A/D}$ knockouts have different resting heart rates, with the $\alpha_{2A/D}$ knockout having a higher rate per minute than the D79N. An explanation for the different resting heart rates in these animal models may be that the combined removal of central and peripheral $\alpha_{2A/D}$-mediated responses in the knockout leads to an increase in heart rate. In the D79N mutant, there may be a sufficient receptor reserve in the peripheral circulation to prevent an elevation in resting heart rate [Altman et al, 1999].

Chronic exposure to noradrenaline leads to a downregulation of β-adrenoceptors in cardiac tissue [Chang et al, 1982]. In mice lacking functional $\alpha_{2A/D}$-adrenoceptors there is a twenty-five percent reduction in functional β-adrenoceptors compared with control

animals [Altman et al, 1999]. The downregulation of β-adrenoceptors probably results from overexposure to noradrenaline. In the absence of functional $\alpha_{2A/D}$-receptors, noradrenaline release from adjacent sympathetic nerve terminals can occur unchecked, and persistent stimulation of β-adrenoceptors leads to a downregulation in the number of receptors expressed.

$\alpha_2$ agonists stimulate central $\alpha_2$-adrenoceptors, which causes a decrease in sympathetic outflow (following pressor effects), which ultimately leads to a drop in blood pressure, while stimulation of peripheral $\alpha_{2B}$-adrenoceptors, mediates pressor effects by activating peripheral receptors, which leads to vascular contraction [Gavras et al, 2001]. In conscious, unrestrained heterozygous $\alpha_{2B}$ knockout mice, the pressor effects of $\alpha_2$-adrenoceptor agonists are absent, while $\alpha_{2A/D}$-mediated hypotensive responses remain, and are potentiated in size, but the bradycardia normally observed when central $\alpha_2$-adrenoceptor are stimulated, is unaffected [Link et al, 1996]. The $\alpha_{2C}$-adrenoceptor has not been implicated in the resgulation of blood pressure, but stimulation of this subtype appears to be responsible for enhanced vasoconstrictor responses at $28^0$C, a function that may be important in controlling blood flow to exposed tissues, such as the digits [Chotani et al, 2000].

1.2.14 $\alpha_{2B}$-adrenoceptors

Generally, expression levels of the $\alpha_{2B}$-adrenoceptor are much lower than the $\alpha_{2A/D}$ in the central nervous system. $\alpha_{2B}$ expression is concentrated in two main areas of the brain and these are the thalamus and nucleus tractus solitaris area of the brain stem [Tavares et al, 1996]. In the peripheral circulation, there is more diffuse expression of

the $\alpha_{2B}$-receptor than in central areas. $\alpha_{2B}$-adrenoceptors located on vascular smooth muscle are thought to mediate peripheral vasoconstrictor responses to $\alpha_2$-agonists [Link et al, 1996]. The majority of results that support this hypothesis have come from experiments on transgenic mice. In $\alpha_{2B}$ knockout mice, the pressor responses that normally precede blood pressure lowering effects of $\alpha_2$ agonists are absent [Hein et al, 1998]. In addition to the effects of central $\alpha_2$-adrenoceptors, studies in the rat have revealed that the $\alpha_{2B}$-adrenoceptor-subtype is the dominant adrenoceptor found in the kidney [Pettinger et al, 1987].

1.2.15 Fertility and sexual development

Transgenic mice harbouring a gene directed deletion of the $\alpha_{2B}$-adrenoceptor have been produced by manipulation of murine genes using molecular techniques. However homozygous $\alpha_{2B}$ knockouts do not breed well, and therefore only heterozygous knockouts have been used for experimentation [Makaritsis et al, 1999]. The fact that homozygous knockouts have reduced fertility, suggests that the $\alpha_{2B}$-adrenoceptor is important in sexual development and/or may be essential for the maintenance of fertility [Altman et al, 1999], both of these areas of study require further investigation.

Heterozygous $\alpha_{2B}$ knockouts show a significant reduction in $\alpha_{2B}$-receptor expression levels [Link et al, 1996]. They have lower end point blood pressure than WT controls, and are unable to increase blood pressure in response to salt loading and subtotal nephrectomy [Makaritsis et al, 1999]. Given this they have been deemed suitable models for studying the responses mediated by $\alpha_{2B}$-adrenoceptors.

1.2.16 $\alpha_{2C}$ Adrenoceptors

Stimulation of $\alpha_2$-adrenoceptors with selective agonists such as clonidine and xylazine cause a number of clinically important cardiovascular effects, none of which have been attributed to stimulation of the $\alpha_{2C}$-receptor subtype. The majority of responses mediated by central $\alpha_{2C}$-adrenoceptors appear to be secondary to the dominant role of the $\alpha_{2A/D}$ subtype in regulating the release of noradrenaline from presynaptic nerve terminals.

Mice overexpressing the $\alpha_{2C}$-adrenoceptor are healthy, and sexually viable receptor [Sallinen et al, 1997]. Overexpressing the $\alpha_{2C}$-adrenoceptor leads to enhanced dopamine turnover, while $\alpha_{2C}$ knockouts exhibit diminished dopamine levels compared with controls [Sallinen et al, 1997]. This suggests that the $\alpha_{2C}$-adrenoceptor regulates dopamine release, in addition to noradrenaline, and therefore acts not only as an autoreceptor, but also as a heteroreceptor. These finding are extremely important because dopamine levels are an important determinant of mood, and behaviour. Furthermore, stress-dependent depression is altered by genetic mutations of the $\alpha_{2C}$-adrenoceptor [Sallinen et al, 1997].

1.2.17 Blood pressure regulation

Hypertension is diagnosed following a period of elevated blood pressure, which normally is the result of a persistent increase in resistance to blood flow. High blood pressure is regarded as a major risk factor for the development of other cardiovascular diseases, such as atherosclerosis and heart failure. The incidence of hypertension in the developed world has now reached epidemic proportions, hence the need for intensive research in this field.

$\alpha_2$-adrenoceptors are important regulators of cerebrovascular tone in a variety of animal species, including humans [Usui et al, 1985]. Under hypoxic conditions and in hypertension adrenergic nerves appear to maintain vascular tone in cerebral arteries [Busija & Heistad, 1984], but this depends on the species studied. For example, in canine cerebral arteries, $\alpha_2$-adrenoceptors play only a minor role in mediating vasoconstrictor responses [McPherson et al, 1994].

Patients at risk of developing hypertension participated in a series of haemodynamic and biochemical studies. A significant number of these patients had abnormal responses to $\alpha_2$-selective ligands. These findings suggest that the $\alpha_2$-adrenoceptor is involved in the development of hypertension before the onset of clinical symptoms [Dao et al, 1998]. The $\alpha_{2A/D}$ receptor is a potential target for subtype selective antihypertensives, as this receptor is responsible for the prolonged hypotensive effects of oral and intravenous $\alpha_2$ agonists [Altman et al, 1999]. However, the side effects associated with stimulation of peripheral receptors preclude their use in a clinical setting.

1.2.18 Development of Salt sensitivity in hypertension

Salt sensitivity is a frequent complication that develops in patients with essential hypertension. Experimentally, salt sensitivity can be induced in animal models by giving high doses of salt in drinking water with normal feeding. In addition to other risk factors, increased age and impaired renal function can enhance salt sensitivity in patients with essential hypertension [Oparil et al, 1988].

The development of salt sensitivity appears to depend on the expression of functional $\alpha_2$-adrenoceptors. Therefore it has been suggested that genetic background may predispose certain ethnic groups to develop salt sensitivity with hypertension. One example of this is the prevalence of the development of salt sensitivity with hypertension in the African American community [Oparil et al, 1988]. Whether individuals within this race have altered expression levels of $\alpha_2$-adrenoceptors has yet to be confirmed. All that is known is that both the sympathetic nervous system and $\alpha_2$-adrenoceptors play a role in the development of salt sensitivity in hypertension [Tsai & Lefkowitz, 1978].

$\alpha_{2B}$-adrenoceptors mediate contractile responses in peripheral arteries, and have been implicated in the development of salt-sensitivity with hypertension, because following subtotal nephrectomy $\alpha_{2B}$ knockout mice are resistance to salt loading [Gavras et al, 2001]. Nephrectomy and salt infusion, causes a slight decrease in systemic blood pressure in the $\alpha_{2B}$ knockout, while $\alpha_{2A/D}$ and $\alpha_{2C}$-knockouts and WT controls have elevated blood pressure [Makaritsis et al, 1999]. The elevation in blood pressure caused by salt loading has since been attributed to central $\alpha_{2B}$-receptors, and appears to be independent of peripheral receptors [Kintsurashili et al, 2002].

1.2.19 Sympathetic nervous system: A role in hypertension?

In addition to transgenic mice, two non-genomic models of human hypertension have been developed in the mouse; these are the renovascular two-kidney models, and the mineralocorticoid deoxycorticosterone-salt induced model of hypertension [Conrado et al, 1996].

It is well known, and documented that the sympathetic nervous system is involved in the control of blood pressure. Hence, this has become an area of great interest in relation to the development, and maintenance of hypertension. Research has focused on studying the changes that occur within the sympathetic nervous system in animal models with established hypertension [Mark et al, 1996].

Sympathetic nerve endings release noradrenaline as their primary transmitter in addition to other vasoactive substances such as ATP [Vanhoutte & Luscher, 1986]. Enhanced noradrenaline release often results from an elevation in sympathetic tone. Elevated sympathetic tone can, in turn, lead to an increase in blood pressure and, hence, will ultimately lead to a hypertensive state in humans and animal models [Ferrier et al, 1993].

Inhibition of nitric oxide synthase causes hypertension to develop in the rat. *In vitro*, contractile responses mediated by $\alpha_2$-adrenoceptors have been studied in arteries isolated from hypertensive animals when the activity of nitric oxide synthase has been prevented *in vivo*. Contractile responses are significantly enhanced in arteries from animals treated with L-NAME. The enhancement in contractility appears to be caused by the activation of two pathways, both of which involve calcium. One is a $Ca^{2+}$ pathway independent of tyrosine kinase; the second is tyrosine kinase-dependent, and controls intracellular $Ca^{2+}$ concentrations [Carter & Kanagy, 2002].

A technique used to determine the activity of the sympathetic nervous system in humans in microneurography; where an electrode takes electrical recordings from patient's arteries [Delius et al, 1972]. This gives an indication of muscle sympathetic activity

(MSA), and reflects the output of the sympathetic nervous system as a whole. A great deal of research has focused on this area because MSA is a major contributor to peripheral resistance, and measurements of MSA offer a non-invasive technique whereby alterations in sympathetic discharge patterns can be studied in pathophysiological situations. In young and borderline hypertensives, increases in MSA are common [Anderson et al, 1989, Lawton et al, 1990]. These patients also show alterations in sympathetic discharge patterns following exposure to elevated external stress factors.

1.2.20 Structural changes occurring in hypertension

Structural changes in the architecture of the vascular wall from patients with hypertension were first recorded over one hundred and fifty years ago. Yet the mechanisms leading to these structural alterations, and the order in which they develop, are still unknown. In fact, the distribution of cells within a healthy blood vessel wall is still poorly defined. But advances in confocal microscopy and the availability of fluorescent ligands have advanced our knowledge greatly.

Essential hypertension may be the result of endothelial dysfunction, possibly due to a lack of $\alpha_2$-mediated relaxant responses. This seems unlikely because it is well established that correction of hypercholesterolaemia can reverse endothelial dysfunction. Furthermore, blocking the release of oxygen radicals reverses hyperhomocysteinemia, which enhances flow-induced dilatations of coronary arteries [Ungvari et al, 2003]. Hypertension may also develop when receptors, located on the vascular wall, become supersensitive to endogenous agonists. Although increased contractile responses are common in blood vessels from hypertensives, data in support

of this hypothesis is often contradictory. It seems more likely that uncontrolled cell growth leads to vascular changes that increase pressure levels within blood vessels. As yet, the triggers for uncontrolled growth have not been established [Cooper, 1997].

The most common structural alteration that occurs in resistance arteries, isolated from hypertensive humans and animals is a reduced lumen size and an increase in the media:lumen ratio [Arribas et al, 1997]. An altered media to lumen ratio is indicative of vascular remodelling, and can be defined as a rearrangement of cells already present within the vascular wall. Vascular remodelling is quite distinct from vascular hypertrophy, which is caused by an excessive amount of vascular growth within a blood vessel [Mulvany, 1992]. Until recently, the study of functional responses and structural alterations in resistance arteries, have proved difficult to study because of the size of vessels, but the development of wire and pressure myographs has overcome this problem. Both of these systems have provided a great deal of information on structural and functional changes that affect resistance arteries in pathophysiological conditions [Aalkjaer et al, 1987].

1.2.21 Inflammatory responses involving $\alpha_2$-adrenoceptors

Atherosclerosis can be defined as an anti-inflammatory disorder, because the cells involved in the development and progression of symptoms belong to the immune system, and under normal circumstances play an active role in immunity. $\alpha_2$-adrenoceptors have been shown to affect the responses of cells that are active in the maintenance of immunity. Given this, it has been proposed that antagonism of $\alpha_2$-mediated responses may be a potential treatment to control the progression of atherosclerotic lesions in human coronary arteries [Bumgart et al, 1999].

The symptoms of many vascular diseases are localised in larger arteries, such as the carotid artery and the aorta. One such vascular disease is atherosclerosis. Mice lacking the apoE protein fed a normal diet develop hyperlipidemia and atherosclerosis [Smith & Breslow, 1997]. It is thought that apoE knockout mice develop vascular disease in large conduit arteries because the absence of apoE decreases LDL and VLDL clearance from the circulation [Smith & Breslow, 1997]. The klotho protein appears to protect against the development of atherosclerotic lesions and age related changes associated with vascular disease. The study of this protein may be useful for atherosclerosis research, in addition to the study of premature ageing conditions, such as progeria [Kuro-o et al, 1997]. The role of the $\alpha_2$-adrenoceptor in the maintenance and development of atherosclerosis requires clarification.

In addition to atherosclerosis, $\alpha_2$-adrenoceptors have also been implicated in the development of a number of other inflammatory conditions. Inflammed joints of the rat develop an increased responsiveness to $\alpha_2$ receptor agonists, while $\alpha_1$-mediated responses become attenuated [McDougall, 2001]. This is indicative of a shift in adrenoceptor expression levels, which may be triggered by inflammatory mediators.

1.2.22 Drug-induced responses mediated by $\alpha_2$-adrenoceptors

Drugs that stimulate $\alpha_2$-adrenoceptors are used clinically as adjuncts to anaesthesia, to treat glaucoma and attention deficit disorder, in addition to their classical antihypertensive effects [Kable et al, 2000].

*In vivo*, activation of postjunctional $\alpha_2$ receptors causes vasoconstriction, but this is followed by profound peripheral vasodilatation, which can lead to postural hypotension [Isaac, 1980]. As a result of this, drugs such as clonidine are rarely used clinically. The quest to find drugs that stimulate beneficial responses mediated by $\alpha_2$-adrenoceptors without causing persistent side effects continues.

Imidazoline receptors are located in the central nervous system and in the periphery, and it has been suggested that $\alpha_2$-agonists produce antihypertensive responses by non-selectively stimulating central imidazoline receptors. However, data from studies carried out on the D79N and on knockout mice suggests otherwise. In fact, drugs that selectively stimulate imidazoline receptors, namely rilmenidine and moxonidine are less effective than clonidine as antihypertensive treatments.

Stimulation of central imidazoline receptors leads to a reduction in blood pressure, in the absence of $\alpha_2$-mediated effects. However, when both imidazoline and $\alpha_2$-adrenoceptors are stimulated simultaneously, blood pressure lowering effects are enhanced [Bruban et al, 2002]. This is interesting, given that it has previously been proposed that drugs acting at imidazoline receptors produced hypotension, without the side effects associated with $\alpha_2$-receptor stimulation [Van Zwieten, 1999].

1.2.23 Drug treatments for hypertension

A wide variety of compounds that do not stimulate adrenoceptors are used clinically to treat hypertension. These include calcium channel antagonists, ACE inhibitors, Angiotensin II antagonists [Johansson et al, 2000] and nitrates. For simplicity sake, the

actions of these compounds will not be discussed further here, but for completeness I felt it necessary to record their use and availability.

Current drug therapies for the treatment of hypertension are successful, in as much as they decrease blood pressure. However, no treatment has yet been developed that fully reverses the structural and functional changes that have developed in blood vessels isolated from hypertensive patients.

1.2.24 Future drug therapies

Bivalent ligands that bind as dimers may increase drug/receptor selectivity. This method has already been used successfully to enhance the selectivity of drugs that stimulate opioid and 5HT receptors, both of which are G protein coupled receptors, as are all subclasses of adrenoceptors.

Monovalent yohimbine has selectivity for $\alpha_2$-adrenoceptors, but fails to distinguish between the three subtypes [Zheng et al, 2000]. In an attempt to overcome the lack of subtype selectivity, yohimbine dimers have been developed. Dimers are produced by reacting yohimbinic acid with aliphatic $\alpha$, $\omega$-diamines under peptide coupling conditions. All of the yohimbine dimers produced have a higher selectivity for transfected human $\alpha_{2b}$ and $\alpha_{2c}$ adrenoceptors expressed in CHO cells.

These studies identified that the extracellular binding loop of the human $\alpha_{2A/D}$-adrenoceptor has a greater number of acidic residues than the $\alpha_{2b}$. So at physiological pH, the ligand-binding pocket of the human $\alpha_{2b}$-adrenoceptor has a greater positive

charge than the other $\alpha_2$ subtypes. In the future, these binding properties may be exploited to develop subtype selective ligands [Zheng et al, 2000].

1.2.25 Analgesia, sedation and behaviour

In addition to the classical cardiovascular effects caused by stimulation of $\alpha_2$-adrenoceptors, $\alpha_2$ ligands can also be used to treat behavioural disorders such as attention deficit hyperactivity disorder [Hunt et al, 1995]. The $\alpha_2$-receptor subtype responsible for enhancing cognitive functions in behavioural disorders is unknown.

Compounds that act as agonists at $\alpha_2$-receptors have strong analgesic effects alone, or in combination with other drugs [Sullivan et al, 1987], and early indications are that all three $\alpha_2$-adrenoceptor subtypes mediate analgesic responses. In mice lacking functional $\alpha_{2B}$-receptors, the analgesic effects caused by nitrous oxide are completely abolished, suggesting a dominant role for the $\alpha_{2B}$ subtype in mediating this response [Sawamura et al, 2000].

Intrathecal administration of moxonidine inhibits substance P-induced behaviour in WT and D79N mice. These responses are antagonised by SK&F86466 and efaroxan showing that the anticociceptive effects are receptor specific. Clonidine increases tail-flick latency in WT, but not in D79N mice. This suggests that these responses are not mediated by the $\alpha_{2A/D}$-adrenoceptor, and are a result of stimulating another $\alpha_2$-adrenoceptor subtype [Fairbanks & Wilcox, 1999]. Antinociception achieved by stimulating the remaining $\alpha_2$ subtypes, namely $\alpha_{2B}$ and or $\alpha_{2C}$, is more desirable because $\alpha_{2A/D}$-adrenoceptor-mediated side effects can be avoided.

The tail flick latency test has been used to assess the antinociceptive effects of $N_2O$ in WT and D79N mice [Guo et al, 1999]. $\alpha_2$-adrenoceptors located in the spinal cord of the rat are proposed to mediate the antinociceptive effects of nitrous oxide ($N_20$). $N_20$ causes antinociception in WT mice, but responses are slightly attenuated in the $\alpha_{2A/D}$ mutant, the D79N. Antinociception responses mediated by dexmedetomidine are antagonised by yohimbine, but unaffected by prazosin. This provides evidence that the response obtained does not result from stimulation of $\alpha_1$-adrenoceptors, and is mediated solely by $\alpha_2$-adrenoceptors. In addition to the effect of dexmedetomidine, opiate antagonists also prevent the antinociceptive effect of $N_20$. Suggesting that $\alpha_2$ agonists may cause antinociception by an indirect mechanism as well as receptor-mediated effects.

$\alpha_2$-selective agonists do not cause antinociception in the D79N mouse, but $N_20$ causes the release of an agent with activity for the $\alpha_2$-adrenoceptor that mediates analgesic responses. In the absence of functional $\alpha_{2A/D}$-adrenoceptors the $\alpha_{2B}$ and $\alpha_{2C}$ receptor subtypes mediate antinociceptive responses [Guo et al, 1999]. In addition to analgesic, vasoconstrictor and antihypertensive actions, $\alpha_2$-agonists are used postoperatively for their sedative and hypnotic effects. Experiments on transgenic mice have proven that the sedative effects produced by $\alpha_2$-agonists are mediated solely by the $\alpha_{2A/D}$-adrenoceptor [Lakhlani et al, 1997]. The development of drugs with selectivity for the $\alpha_{2B}$ and $\alpha_{2C}$ receptors over the $\alpha_{2A/D}$ subtype may produce analgesia without the unwanted side effect of sedation or hypotension.

Stimulation of central $\alpha_2$-adrenoceptors has a profound effect on behaviour [Bjorklund et al, 2000] and memory [Tanilla et al, 1999]. Drugs selective for $\alpha_{2A/D}$ and $\alpha_{2B}$-receptors have huge potential uses for the treatment of disorders where cognitive functions are affected. While selectivity for the $\alpha_{2C}$-adrenoceptor may be a future therapy for schizophrenia, and other conditions where there is an altered startle response [Sallinen et al, 1998]. Mirtazepine is used clinically as an antidepressant, and is a known antagonist of $\alpha_2$-mediated responses, so in addition to other uses, antagonists for $\alpha_2$-adrenoceptors may be potential therapies for depression, obesity [Hieble & Ruffalo, 1991] and erectile dysfunction [Monoz et al, 1994].

Gene targeted mutations of the $\alpha_{2A/D}$-receptor have shown that this subtype plays an important antiepileptogenic role in the murine brain [Janumpalli et al, 1998], making $\alpha_{2A/D}$-selective compounds a potential therapy for epilepsy.

1.2.26 $\alpha_2$-adrenoceptors and motor control

Parkinson's disease affects one person in every hundred aged over sixty-years. This condition is diagnosed following an alteration in gait, and is thought to result from degeneration of dopamine neurones, but can spread to affect other areas of the brain [Javoy-Agid, 1984]. The $\alpha_2$-antagonist idazoxan given at a dose of 2.9mg/kg causes a marked improvement in motor abnormalities (abnormalities that are similar to those occurring with Parkinson's disease) in MPTP-treated monkeys. Improvements in motor skills are marked when compared to L-DOPA treatment, which is used to treat motor dysfunction. Idazoxan leads to a decreased in muscle rigidity and improved smoothness of movement. This study highlights the potential benefits of mainpulating $\alpha_2$-adrenoceptors to treat disorders that exhibit Parkinson-like motor dysfunction.

Dexmedetomidine causes dose-dependent reductions in locomotion in $\alpha_{2B}$ and $\alpha_{2C}$ knockout mice, but has no effect on locomotion in the D79N mouse [Hunter et al, 1997]. $\alpha_2$ agonists cause sedation in the mouse by stimulating central $\alpha_{2A/D}$-adrenoceptors in the locus coeruleus of the murine brain [Nacif-Coelho et al, 1994]. Furthermore, a lack of functional $\alpha_{2A/D}$-adrenoceptors abolishes antinociceptive responses to dexmedetomidine, while $\alpha_{2B}$ and $\alpha_{2C}$ knockouts are unaffected and respond like WT controls [Hunter et al, 1997].

1.2.27 Regulation of lipolysis

In addition to all of the physiological responses affected by stimulation of $\alpha_2$-adrenoceptors, it has now been hypothesised that $\alpha_2$-adrenoceptors are involved in the regulation of fatty acid metabolism and lipolysis. When functional $\alpha_2$ receptors are expressed in the absence of functional $\beta_3$-adrenoceptors, mice develop diet-induced obesity. The subtype(s) involved in the development and/or maintenance of this condition are unknown [Valet et al, 2000].

1.2.28 Signalling pathways activated by $\alpha_2$-adrenoceptors

Cellular signalling pathways utilized by $\alpha_2$-adrenoceptors have been studied extensively, but the G protein coupled to prejunctional receptors has not yet been determined. It has been proposed that activation of $\alpha_2$-adrenoceptors leads to an inhibition of further noradrenaline release from sympathetic neurones. Experiments in rodents have shown that the G proteins involved in autoinhibitory functions are pertussis toxin-insensitive [Allgaier et al, 1996]. Other work has shown that the prejunctional autoreceptors can signal via a pertussis sensitive G protein in the rat heart

and vas deferens [Docherty, 1988]. Stimulation of $\alpha_2$-adrenoceptors activates tyrosine kinases, which regulates calcium levels during contraction of the rat aorta [Carter & Kanagy, 2002].

Prejunctional $\alpha_2$-adrenoceptors mediate their responses by negative coupling to adenylate cyclase. However, the signalling pathways activated by postjunctional receptors have been more difficult to elucidate. In isolated guinea pig smooth muscle cells $\alpha_2$ receptor coupling prevents the accumulation of cAMP [Gupta et al, 1998]. In contrast, in human resistance arteries voltage operated $Ca^{2+}$ channels are activated following agonist/receptor interactions [Parkinson & Hughes, 1995]. Unlike $\alpha_1$-adrenoceptors, stimulation of $\alpha_2$-adrenoceptors has not been shown to cause phospholipase C production. The signalling pathways activated by $\alpha_2$-adrenoceptors vary between blood vessels and species, as is often the case for G protein coupled receptors.

**Statement of aims**

In this thesis $\alpha_2$ and $\alpha_1$-adrenoceptor-mediated responses have been studied in two murine arteries, the tail and first order mesenteric resistance arteries. In the tail artery, a series of protocols were investigated to determine suitable conditions to study $\alpha_2$-adrenoceptor-mediated responses in this artery, in an attempt to increase our understanding of the effect of stimulating peripheral $\alpha_2$-adrenoceptors expressed in cutaneous blood vessels. Furthermore, a mouse carrying a functional knockout of the $\alpha_{2A/D}$-adrenoceptor was employed to investigate what role this receptor subtype plays in responses in the tail artery and in mesenteric resistance arteries.

The $\alpha_{1A}$-adrenoceptor is the major $\alpha_1$-receptor subtype leading to contraction of the murine tail, and first order mesenteric resistance arteries [Daly et al, 2002]. However, $\alpha_{1B}$ and $\alpha_{1D}$-adrenoceptors are also expressed in these blood vessels. To determine if these receptors are involved in vasoconstrictor responses in these arteries, $\alpha_{1B}$ and $\alpha_{1D}$ receptor knockout mice were used in combination with subtype selective ligands.

The focus of my research has been to delineate the role of adrenoceptors in two resistance arteries, namely the tail artery, also referred to as the caudal artery and first order mesenteric resistance arteries. In these arteries, I have studied the function of both $\alpha_1$- and $\alpha_2$-adrenoceptors.

# Chapter two

## Materials and General Methods

## 2.1 Method used to study functional responses in isolated blood vessels

### 2.1.1 Wire myography

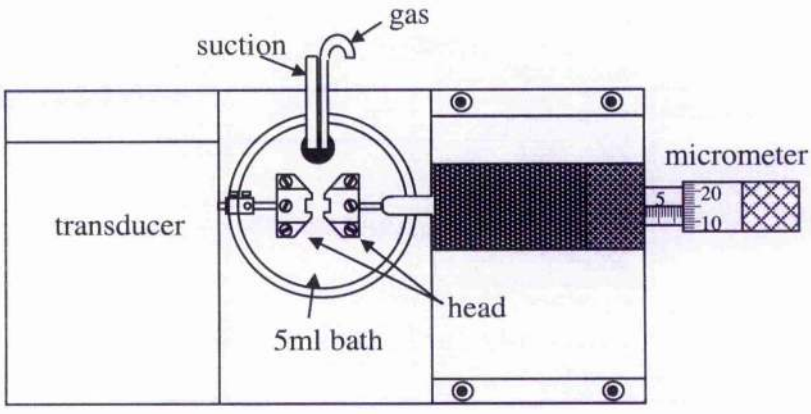
The wire myograph was first described in 1976 [Mulvany & Halpern, 1976]. The development of this system has revolutionised the study of small blood vessels. Before 1976, the majority of *in vitro* studies of vascular function were carried out in an organ bath set up. Although this technique proved reliable, and gave reproducible responses, it had one major limitation i.e., which was the size of the vessels that could be studied. Therefore, until the development of the wire myograph, larger diameter blood vessels were the focus of *in vitro* studies. The wire myograph provides a system that allows functional responses in resistance arteries to be investigated. Resistance arteries have a diameter of between 100 and 400μm, and are thought to contribute to peripheral resistance because they are primarily located at sights where there is a dramatic change in the pressure that blood flows against (for example when blood vessels branch, and the resistance to flow is greatly increased). Both of the vessels studied in the work presented here fall into that category on the basis of size, but the location of the tail artery questions this definition and only first order mesenteric arteries (because of their anatomical location), can be regarded as resistance vessels. The middle section of the mouse tail artery measures 314.5 ± 1.5μm in diameter. While first order mesenteric resistance arteries are smaller, having a diameter of 216.6 ± 3.5μm.

### 2.1.2 Myographs: description of equipment

The myographs used in all of the work within this thesis were purchased from Danish Myotech and were model type 600A or 610A. The myograph has a stainless steel bath containing two detachable stainless steel heads. The stainless steel heads can be replaced with perspex mounting heads containing a fixed electrode allowing the nerves

within the vessel wall to be electrically stimulated. Although this technique was not used in this work, I have included this description for completeness. The head on the far side of the myograph is mounted on a stainless steel arm that is connected to a micrometer. This arm can be moved backward and forward with the micrometer to form a vice like structure in the centre of the bath. On the near side of the myograph the mounted head is attached to a transducer, which measures force generation. These measurements are sent to an interface that gives a force reading in mN. The myograph interface sends an output to a Linseis 4 channel chart recorder (L6514-11), there upon producing a trace recording of force generated by a contracting vessel. A number of traces are shown throughout the results chapters. These were recorded from experiments using Adinstruments PowerLab software. A diagram showing an overview of a myograph bath is shown below in figure 2.1.

**Figure 2.1**



## 2.2 Vessel dissection

### 2.2.1 Dissection of mouse tail artery

There are three arteries and three veins, which run the entire length of the mouse tail artery. The artery most frequently used experimentally is the one running down the base of the tail, which is facing upward when the animal is in the supine position (face forward). The artery to which I refer was used for the experiments contained within this thesis.

All mice were killed by schedule one method, in accordance with Home Office guidelines. Mice were asphyxiated with $CO_2$, followed by cervical dislocation. Immediately after death, the mouse was laid on its back, belly facing toward researcher. Then an ink mark was made on the base of the tail to allow identification, after the tail has been detached from the body. Tails were stored in cold Krebs until the artery was removed. Before any further dissection, the tail arteries were measured and a 1.5cm segment beginning 2.5cms from the base of the tail was marked with ink. The average length of the murine tail artery was determined as $8.09 \pm 0.02$cms.

From this point on, all further dissection of blood vessels was carried out with the aid of a Zeiss dissecting microscope (Stemi 2000). The thick, hairy skin on the tail was removed with dissecting scissors, exposing an artery and vein under a sheath of white connective tissue. The artery was removed from the tail, cleaned of any excess connective or fatty tissue and placed in fresh cold Krebs.

The freshly dissected artery was then placed in a petri dish containing fresh Krebs. Sections of artery measuring approximately 2mm were then cut, and each segment then

had a 40μm wire inserted into the vessel lumen. When the first guide wire was in place, the vessels were then ready to be transferred to the myograph bath, which contained cold, gassed Krebs solution.

2.2.2 Mesenteric artery dissection and vessel mounting

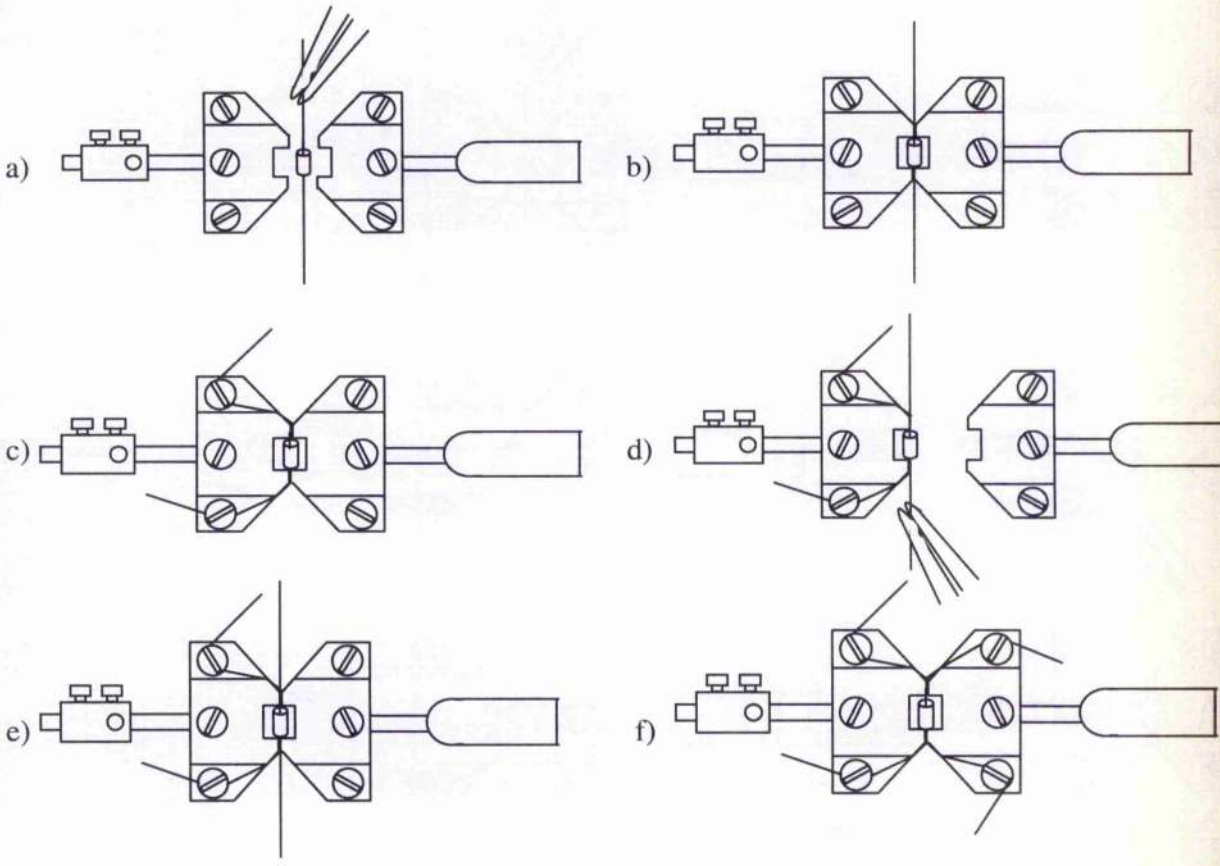
WT, D79N, $\alpha_{1B}$ and $\alpha_{1D}$ knockout mice were killed by $CO_2$ asphyxiation followed by cervical dislocation. The mesenteric arcade from these animals was removed from the abdominal cavity, and placed in fresh Krebs at room temperature. The mesentery was then pinned out on a petri dish, and with the aid of a dissecting microscope, the superior mesenteric artery was located. First order mesenteric resistance arteries are the first branch off the superior mesenteric artery; hence the nomenclature. Once located, several of these arteries were cleared of excess tissue and fat and stored in Krebs for *in vitro* studies.

2.3 Procedure for mounting vessels

Each stainless steel bath contained 5mls of fresh cold Krebs. The wire, with the vessel already mounted, was held within the stainless steel jaws to permit the wire to be secured to the near side arm using small metal screws. Once secure, heads were separated to allow insertion of a second 40μm wire. Insertion of a second wire was a technical challenge, and requires considerable expertise, if one is to avoid damaging the thin layer of endothelial cells, located on the vessel wall. To minimise damage, the second wire was slid along the primary mounting wire, which had already been secured. Once inserted and secured, the wires were lined up so that they became parallel, and just touching. Each stage of the mounting procedure has been summarised diagrammatically in figure 2.2, below.

After successful mounting of a blood vessel a perspex lid was fitted to the Krebs filled bath with a funnel attachment for delivery of fresh Krebs. The tight fitting lid helped to control the temperature within the bath. Each bath had an oxygen supply and heating was achieved by switching on a heat pad that spans the length of the entire mounting block. Tail and mesenteric arteries were set a tension of 0.25 and 0.17gms Force respectively. This was determined as a suitable resting tension after completion of a series of length tension experiments.

**Figure 2.2**



2.4 Resting tension: normalisation

Although this procedure was rarely used in experiments carried out in this thesis, I feel it necessary to provide a brief description of the normalisation procedure because it is commonly used in myography. Mulvany and Halpern first described this method in 1977. In brief, a rat mesenteric arterial ring was subjected to a series of stretches to determine the required tension the vessel should be set at to mimic *in vivo* conditions. The procedure was based on the law of Laplace, which described the relationship between wall tension, pressure and vessel radius.

Pi = Wall tension/(internal circumference/2π)

Pi is defined as the effective pressure. This is not the actual pressure within the vessel, but an estimate of that pressure which was required to stretch the vessel wall to cause a change in internal circumference. Wall tension is the force divided by the length of the vessel wall.

Wall tension = Force (F)/(Length (L) x 2).

Caution is required here, because the wall length is actually twice the length of the mounted vessel segment. Repetitive stretching gave a series of Pi values that can be recorded on a hand held Hewlett Packard computer. This machine can calculate the internal circumference of the vessel by use of the following equation.

IC1 = (micrometer reading at 1-micrometer reading at point B) x 2 + (ICB)

Point B is the point at which the two mounting wires were barely touching. If the wires used were both 40μm in diameter, the ICB was always equal to 205.6um. The micrometer readings were equivalent to the distance between the two wires.

The equation can be rearranged to give

Pi = (2π) x wall tension/ IC

The values for internal circumference and wall tension were then plugged into the equation to give:

$Pi = (2\pi) \times F / 2 \times L (205.6 + (2 \times \text{distance between the wires}))$

This method was used to calculate a micrometer reading that sets the vessel at a tension that mimics transmural tension at ninety percent of the tension required to reflect a pressure of 100mmHg.

So why was this technique not utilised in my work? There are several reasons; this method was developed for the study of third order mesenteric resistance arteries from the rat. As I have used two different vessels types in this study, neither of which are third order mesenteric, this was the first rationale for not succumbing to the pressure to normalise. Although the technique of normalisation provides several pieces of useful information about the vessel being studied, the procedure of repeated stretching has been shown to be extremely damaging. During the course of my training procedure, I did normalise a number of vessels, for practice, and to aid my understanding of this method. A highly elastic vessel, such as the carotid, aorta, or even the tail artery, had to be stretched to such a degree that it was almost impossible to successfully complete an experiment after normalisation.

## 2.5 Calibrating equipment

The myograph and Linseis pen recorder were calibrated at the beginning of each set of new experiments, because the procedure is labour intensive and time consuming. Each time the equipment was calibrated little variation was observed.

## 2.6 Experimental Protocols

### 2.6.1 Wake-up protocol for mesenteric and tail artery

Following an equilibration period of 30-35 minutes, each segment of tail artery was set at a resting tension of 0.25gms, and allowed to equilibrate for 20-30 minutes. At this stage, if required, tension was adjusted to reach 0.25gms. For all experiments involving UK14304, two sighting concentrations of the appropriate agonist ($1 \times 10^{-5}$M) were administered to each tissue, followed by four staggered washes with fresh Krebs over a fifteen minute period. A third concentration of agonist ($1 \times 10^{-6}$M) was added and the contractile response was allowed to reach a plateau. When the agonist-induced response was stable, acetylcholine ($3 \times 10^{-6}$M) was added to test viability of the endothelium. Acetylcholine-induced relaxations were determined in all vessels tested, if the relaxant response failed to reach thirty percent of the base contraction, vessels were excluded from further analysis. Acetylcholine can induce contractile responses, but only in the absence of endothelial derived relaxing factors [Chauhan et al, 2003], a phenomenon that did not occur in the vessels I studied. After completion of the wake up protocol, each tissue was washed four times, and allowed to rest for thirty minutes before commencing experimentation. Details of the drugs used during the experimental wake-up have been described in more detail in each appropriate results chapter.

### 2.6.2 Elevation of vascular tone

U46619 is a commercially available thromboxane mimetic. It is frequently used to elevate tone in functional experiments because it has a long duration of action, and provides a stable contraction, unlike thromboxane, which is rapidly broken down. In experiments investigating the relaxant effect of UK14304 in mesenteric arteries, U46619 ($1 \times 10^{-7}$M) was used to constrict each vessel segment. U46619 was also the

synergist of choice for experiments on tail artery, where an elevation in vascular tone was required to investigate contractile responses to the $\alpha_2$-selective agonist UK14304. In these experiments, the synergist was added in increasing concentrations until a level of tone approximately fifty percent of that gained to noradrenaline ($1 \times 10^{-5}$M) was achieved.

2.6.3 Assessment of the effects of antagonists

Each antagonist drug used was incubated with a tissue segment for 30 minutes prior to experimentation. In most of the protocols involving UK14304, desensitisation of contractile responses occurred. To overcome this problem, the effect of the antagonist drugs rauwolscine and prazosin were determined against first curves. With the exception of these experiments, unless otherwise stated, the antagonist was added after a first curve to the appropriate agonist, and allowed to equilibrate for 30 minutes.

2.6.4 Combined use of two antagonist drugs

A series of experiments were carried out in first order mesenteric resistance arteries where two antagonist drugs were used to block responses in the same tissue. In these experiments, an initial curve to phenylephrine was constructed. After a wash out and rest period, 5MeU was added to the bath. After a 30 minute equilibration period, a second cumulative curve to phenylephrine was carried out. This was followed by four consecutive washes, carried out over a 30 minute rest period. To prevent any drug interactions when more than one antagonist was used on a given vessel, a lag period was allowed between drug additions. So the first antagonist was added and allowed to equilibrate for 10 minutes, and then the second antagonist (namely BMY7378) was added to the bath. When the last drug was added, a 30 minute equilibration period was allowed before the construction of another phenylephrine curve.

## 2.7 Maintenance of Animals

All animals were housed in the University animal holding unit, where temperature is maintained at approximately $21^0$C. Humidity levels were approximately sixty percent and the air was filtered every hour while animals were kept in an artificial twelve-hour light/dark cycle. Animals used in the experiments were bred locally from ancestors gifted from other laboratories. All of the mice used were male and had been caged in batches, unless required for breeding. Mice were fed on a standard chow diet and provided with distilled drinking water (ad libitium).

## 2.8 Data analysis

### 2.8.1 Results

Data from each set of *in vitro* experiments was grouped together for the calculation of means, standard deviation and standard error values. The results gained were expressed in several ways. Contractile responses have been shown as gms Force. This data was not normalised or manipulated in any way. To assess the sensitivity of each tissue to a given agonist before and after treatment with an antagonist, data was expressed as a percentage of its own maximum, or a control curve maximum. For control data, results were expressed as a percentage of their own maximum. When an antagonist drug was used, the data was expressed as a percentage of the appropriate control curve. By expressing data in this way, $pEC_{50}$ values could be determined. In studies carried out in the mouse tail artery where only one concentration response curve was constructed, responses were expressed as a percentage of their own maximum, or where appropriate, as a percentage of the noradrenaline response, gained during the wake-up protocol.

2.8.2 Agonist Potency

$pEC_{50}$ values are standard measurements that indicate how sensitive a given tissue is to an applied agonist. The $pEC_{50}$ can be defined as the - log of the $EC_{50}$ (the concentration of agonist needed to produce fifty percent of the maximum response). These values were either calculated using a template spreadsheet, or by the use of Graph Pad Prism which can extrapolate data to estimate such values. For studies in the $\alpha_{1B}$ knockout mice, it was necessary to calculate $pEC_{25}$ values. The same principals applied for these calculations.

2.8.3 Antagonist potency

Antagonist potency can be estimated by calculating a $pK_B$ and or a $pA_2$ value. Where applicable, these values were determined. A $pK_B$ is the - log of the $K_B$, which is the dissociation equilibrium constant for an antagonist, and can be defined as the concentration of drug that occupies fifty percent of available receptors. $pK_B$ values are generally calculated when a single concentration of antagonist has been used in an experiment. The $pK_B$ values determined in this work were calculated as follows:

$pK_B = - \log K_B$ where the $K_B \approx$ [Antagonist] / r-1

r is equal to the antagonist concentration ratio, which is [A'] / [A], that is the concentration of agonist needed to produce an equal effect in the presence of [A'] and absence of antagonist [A]. This is where the $EC_{50}$ value is utilised.

When several concentrations of antagonist have been used in an experiment (normally a minimum of three), a $pA_2$ value can be calculated. The $pA_2$ is the log of the concentration of antagonist required to shift the response curve to an agonist by two fold compared to the control response. Where $pA_2$ values were calculated in this thesis,

they have been done in the manner previously described by Arunlakshana & Schild (1959). In order to do this, one must plot values obtained for log (dose ratio-1) (see previous section) against the concentration of antagonist used. When the antagonism was competitive in nature, the graph gave a straight line that had a slope that was not significantly different from unity. The point where the line intercepts the x-axis is the log $K_D$ and the $pA_2 = -\log K_B$.

It should be noted that although such values provide a considerable amount of information on receptor/drug interactions, they are only estimates and will be treated as such. However the validity of their use has been well established over the last fifty years, and they provide an invaluable tool for pharmacological studies.

## 2.9 Statistical analysis

Statistical comparisons between groups of two or more data were assessed using one-way analysis of variance (ANOVA), followed by a (Bonferroni) post-test, to determine points of significant difference. Comparisons between two experimental groups were made using a Student's t-test for paired and unpaired data sets where appropriate. It has become standard to regard a p value $< 0.05$ as statistically significant. In keeping with this I used the same criteria throughout this work. Graph Pad Prism, versions 2 and 3 were used for all statistical analysis.

## 2.10 Drugs and Solutions

### 2.10.1 Solutions

Krebs-Heinslets solution was prepared on a daily basis and had the following composition: NaCl 118.4mM, KCl 4.7mM, $CaCl_2$ 2.5mM, $KH_2PO_4$ 1.2mM, $MgSO_4$

1.2mM, $NaHCO_3$ 25mM and glucose 11.1mM, bubbled with 95% $O_2$ 5% $O_2$ to pH 7.4 at 37$^0$C. In chapter five, experiments in mounted tail arteries were carried out at room temperature. During the course of these experiments the laboratory temperature was monitored and recorded and found to be 21.5-23.5$^0$C. Krebs used to wash bathing arterial rings, was kept at room temperature, while gassed, and the pH was monitored throughout to confirm that it did not deviate from physiologically acceptable levels.

2.10.2 Drugs

The table below provides a comprehensive guide to the drugs used in this work, their supplier and the method used to dissolve the compound. Where water has been used this indicates the use of distilled water. DMSO is an abbreviation for dimethylsulphoxide, which was used to dissolve UK14304, according to manufacturers guidelines. Ethanol used to dissolve certain compounds was 100%. Where possible drugs were dissolved in distilled water, but some compounds required sonication. Compounds dissolved in solvents were made at concentrations higher than that required for experimentation and diluted down, to minimise solvent effects on responses. With the exception of Nifedipine and L-NAME, all other compounds were made fresh, stored in the freezer and used for five consecutive experiments. Nifedipine stock was stored in -20$^0$C freezer, and diluted daily, while L-NAME was made fresh for each use.

Table 2.1

| **Drug/Compound** | **Supplied by** | **Solvent for stock** |
|---|---|---|
| Noradrenaline hydrochloride | Sigma | 23μm EDTA, diluted in water |
| Phenylephrine hydrochloride | Sigma | Water (1M stock) |
| UK14303 (5-Bromo-6(2-imidazolin-2yamino)quinoxaline) | Tocris | DMSO (1 x $10^{-2}$M)/Water |
| U46619 (9, 11-Dideoxy-11α-epoxymethanoprostaglandin $F_2\alpha$ | | Ethanol (1 x $10^{-2}$M)/Water |
| L-NAME ($N^{\omega}$-nitro-L-arginine methylester) | Sigma | Water (1M Stock) |
| Rauwolscine hydrochloride | Research Biochemicals | Water (1 x $10^{-4}$M)/60min sonication |
| Prazosin hydrochloride | Tocris | Water (1 x $10^{-4}$M)/60min sonication |
| 5-methylurapadil (5-Methyl-6(3-[4-(2-methoxyphenyl)-1-piperazinyl]propyl)amino]1-3dimethyluracil | Research Biochemicals | Water (1 x $10^{-3}$M)/30mins sonication |
| BMY7378 (dihydrochloride 8-[2-[4-(2-methoxyphenyl)-1-piperozynl]ethyl]-8-azaspiro(4,5}decone-7,9-dione) | Research Biochemicals | Water (1 x $10^{-3}$M)/60mins sonication |
| 5-Hydroxytryptamine | Sigma | Water (1 x $10^{-2}$M)/20mins sonication |
| Nifedipine | Sigma | Ethanol (1 x $10^{-2}$M)/Water |

# Chapter three

## Development of a protocol to investigate $\alpha_2$-adrenoceptor-mediated responses in the murine tail artery *in vitro*

## 3.1 Introduction

*In vivo*, the study of $\alpha_2$-adrenoceptor-mediated responses has been relatively straightforward. However, *in vitro*, responses mediated by $\alpha_2$-adrenoceptors are often difficult to obtain, probably because *in vitro* conditions are unsuitable. The main objective of the work contained within this chapter was to develop a number of protocols, in an attempt to provide conditions that permit the study of consistent, reproducible $\alpha_2$-adrenoceptor-mediated responses.

In the pithed rat, pressor effects of exogenous α-adrenoceptor agonists are the result of dual activation of postjunctional $\alpha_1$-and $\alpha_2$-adrenoceptors [Docherty & McGrath, 1980]. $\alpha_2$-adrenoceptor-mediated increases in blood pressure have been shown to be inhibited by the $\alpha_2$-selective antagonists rauwolscine and idazoxan, but are unaffected by treatment with the $\alpha_1$-selective antagonist, prazosin.

The rat tail artery contains a functional population of pre and postjunctional $\alpha_2$-adrenoceptors [Medgett & Langer, 1984]. *In vitro*, the existence of a mixed population of functional $\alpha_1$ and $\alpha_2$-adrenoceptors in peripheral arteries was first suggested following experiments that utilised $\alpha_1$ and $\alpha_2$-selective antagonists. Prazosin competitively antagonised noradrenaline-induced contractions, but the combination of prazosin and idazoxan caused a significantly greater reduction in the noradrenaline-induced response, greater than the effect of prazosin alone [Rajanayagam & Medgett, 1987]. This indicated that in addition to the $\alpha_1$, $\alpha_2$-adrenoceptors also mediate contractile responses *in vitro*.

UK14304 and noradrenaline cause contraction of the isolated perfused rat tail by stimulation of $\alpha_2$-adrenoceptors. However, the development of these contractile responses only occurs in the presence of elevated vascular tone [Templeton et al, 1989]. Templeton and co workers increased the perfusion pressure within the isolated tail with a low concentration of arginine vasopression (AVP). In the absence of AVP-induced tone, UK14304 had no effect on perfusion pressure. However, when the tone was raised, UK14304 caused concentration-dependent increases in pressure that had a maximum effect that was comparable to thirty percent of the increase in perfusion pressure caused by noradrenaline. In the presence of elevated tone UK14304-induced contractile responses were selectively antagonised by rauwolscine, and were insensitive to prazosin.

Therefore, it can be concluded that the rat tail artery contracts by stimulation of a mixed population of $\alpha_1$ and $\alpha_2$-adrenoceptors. The $\alpha_1$-adrenoceptor appears to be the major contractile receptor, which is readily activated by noradrenaline or phenylephrine in the absence of tone [Templeton et al, 1989]; but $\alpha_2$-adrenoceptors play a small, but significant contractile function under specific physiological conditions (for example when vascular tone has been elevated). Since the discovery of $\alpha_2$-adrenoceptor-mediated contractile responses, much work has been carried out to determine which receptor subtypes contribute to responses in different arterial beds, and animal species. It has been reported that contractile responses of the rat tail artery results from activation of postjunctional $\alpha_{2C}$-adrenoceptors [Craig et al, 1995]. To determine what $\alpha_2$-subtypes were involved in mediating responses in the murine tail artery the D79N mouse has been used here.

## 3.2 Methods

WT (C57BL/6c/129Sv) and D79N ($\alpha_{2A/D}$ mutant) mice aged four-months were obtained from the University animal house for this study. Males of between 29.6-34.2g were killed by asphyxiation with carbon dioxide and the tail arteries were removed.

### 3.2.1 Vessel dissection and mounting

The main artery running down the base of the tail was located and a section 2.5cm from the base, measuring 1.5cm in length was marked. The skin was then removed to expose the artery, cleared of excess connective tissue and fat, was mounted in 5ml myograph baths (Described in detail in chapter two). Once mounted, resting tension of 0.25g was placed on each vessel (determined to be the optimal tension, from preliminary experiments). Each arterial ring was placed in Krebs, heated to $37^0$C, bubbled with a gas mixture of 95% $O_2$, 5% $CO_2$ and allowed to equilibrate for 20-30 minutes.

### 3.2.2 Experimental Protocols

A variety of different protocols were performed throughout the course of the experiments contained within this chapter. The aim of each protocol was to determine the influence of a particular aspect of the experimental conditions provided for the study of $\alpha_2$-adrenoceptor-mediated responses. Each protocol has been described in detail below.

### 3.2.3 Control Experiments

In control experiments arterial segments were set up, as described previously. After a resting period, a cumulative concentration response curve to the $\alpha_2$-selective agonist

UK14304 was constructed in half log units ($1 \times 10^{-9} - 3 \times 10^{-5}$M). No other drugs were present in the myograph bath during this protocol.

3.2.4 Elevation of vascular tone with U46619

U46619 is a thromboxane mimetic, this was used to raise vascular tone in mounted tail artery rings. Two different protocols were employed that made use of this compound. In the first, a predetermined low concentration of U46619 ($3 \times 10^{-9}$M) was added to each vessel segment to raise tone. When U46619-induced contractions reached a contractile plateau, a UK14304 curve was then constructed in each artery. In a second protocol, U46619 was added in half log units to give a contractile response comparable to fifty percent of the contraction obtained with noradrenaline ($1 \times 10^{-5}$M). Responses varied from tissue to tissue so the concentration of U46619 used ranged from $3 \times 10^{-8}$M to $1 \times 10^{-7}$M, dependent on the responsiveness of each mounted vessel.

3.2.5 Blockade of nitric oxide synthase with L-NAME

L-NAME covalently bonds to the enzyme nitric oxide synthase, and in doing so, prevents the release of the endothelial derived relaxing factor, nitric oxide. L-NAME, at a concentration of $1 \times 10^{-4}$M was used in three protocols. In the first, L-NAME was incubated with mounted arterial rings 20-25 minutes before construction of a cumulative UK14304 response curve. In the second, L-NAME was used, prior to elevation of vascular tone with the synergist, U46619. In the final protocol, L-NAME was administered to each myograph bath before raising vascular tone to fifty percent of the noradrenaline maximum, with increasing concentration of U46619. At the end of each protocol where L-NAME was used, the relaxant effect of acetylcholine was tested to confirm that L-NAME had successfully prevented the release of nitric oxide.

## 3.3 Results

### 3.3.1 Responses to cumulative UK14304

Figure 3.1 shows the responses gained to cumulative concentration response curve to UK14304, constructed in tail arteries from four-month old WT (figure 3.1 **A**) and D79N (figure 3.1 **B**) mice. In arteries from both strains, contractions at lower concentrations were absent or extremely small. At higher UK14304 concentrations small but significant contractile response was achieved. The maximum contraction gained in arteries from WT mice was 0.11 ± 0.05gms Force ($p = 0.007^{**}$). In D79N arteries the maximum change in tone was a mere 0.04 ± 0.02gms Force, which was not significantly different from zero ($p = 0.051$).

### 3.3.9 Comparison of all the protocols tested

Figure 3.2 summarises the results gained in all of the experimental protocols tested in WT (figure 3.2 **A**) and D79N (figure 3.2 **B**) tail arteries. In terms of size and sensitivity, high levels of vascular tone provided the most advantageous experimental conditions for the study of $\alpha_2$-adrenoceptor-mediated contractions, in both murine strains.

### 3.3.2 Effect of elevated tone alone and with L-NAME on the maximum size of UK14304-induced contractions

Contractile responses to UK14304 were investigated when vascular tone had been elevated with a low concentration of the synergist, U46619 ($3 \times 10^{-9}$M). Cumulative concentration response curves to UK14304 were constructed in tail arteries from WT (figure 3.2 **A**) and D79N (figure 3.2 **B**) mice. At a concentration of $3 \times 10^{-9}$M U46619 caused small, but stable, contractions. In WT arteries, the size of U46619-induced contractions was 0.13 ± 0.04gms Force, while in arteries from D79N mice the response

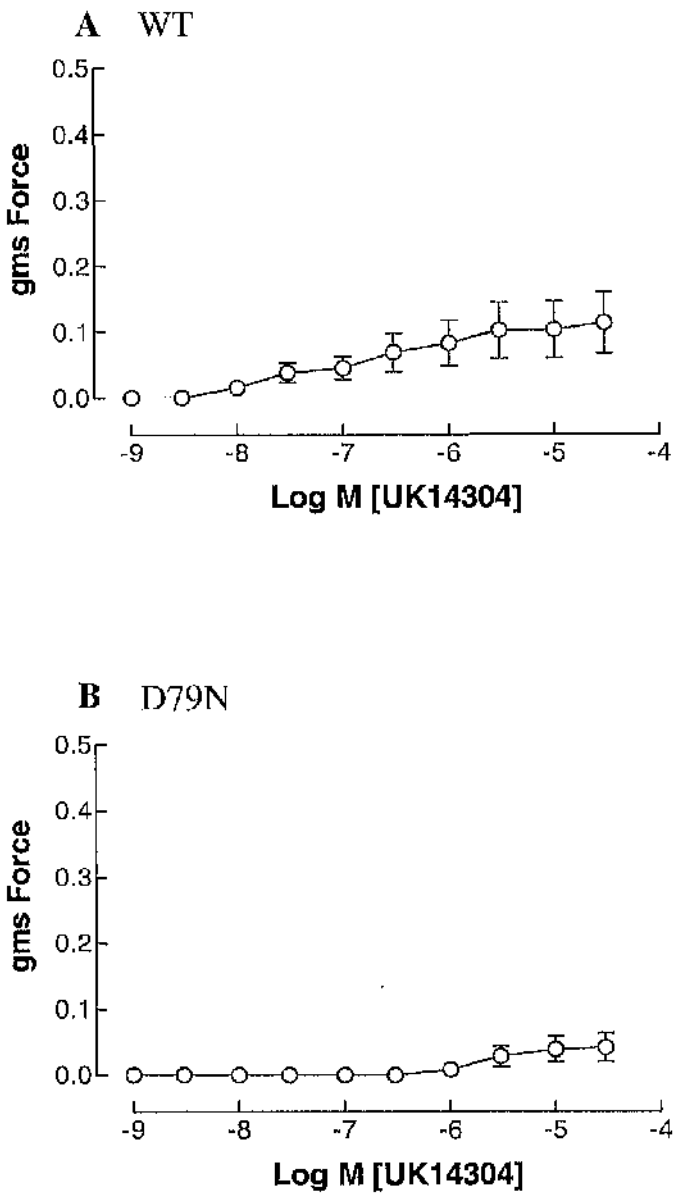



Figure 3.1: The UK14304 response in tail arteries from 4-month old WT and D79N mice. **A** Cumulative response curve to increasing concentrations of UK14304 (○, n = 7 ) in WT arteries. **B** Cumulative response curve to increasing concentrations of UK14304 (○, n = 10 ) in D79N arteries. Each point represents mean ± standard error.

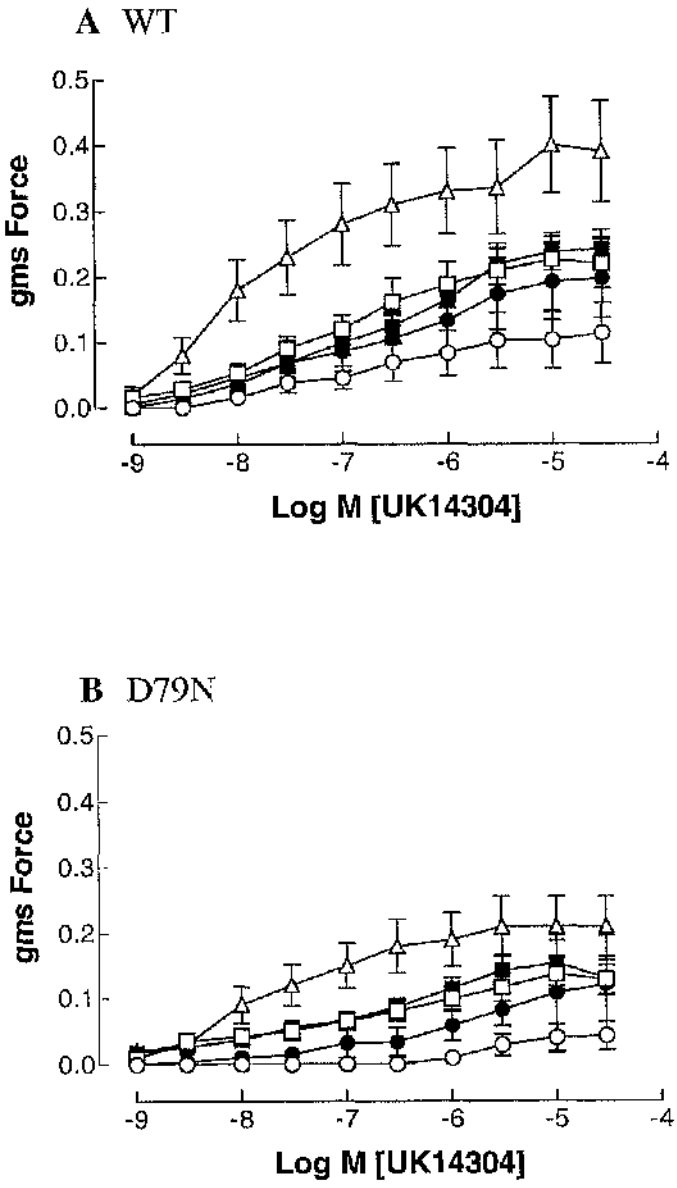



Figure 3.2: Responses in tail arteries from 4-month old WT and D79N mice. To UK only (○), with U19 (●, 3 x $10^{-9}$M), U19 with L-NAME (■, 1 x $10^{-4}$M), L-NAME only (□), and U19 (△, 50 % of NA max), respectively. **A** UK curves in WT arteries, n = 7, 6, 8, 7, and 12. **B** UK curves in D79N arteries, n = 10, 10, 9, 10, and 13. Each point represents mean ± standard error.

tended to be smaller at 0.05 ± 0.01gms Force. Statistical analysis showed that U46619-induced contractions in WT were no different from the D79N, probably because of the high variability of responses.

In the presence of low levels of vascular tone contractile responses to UK14304, although still small, were significantly greater than those achieved in a control curve. In WT arteries, U46619, at a concentration of $3 \times 10^{-9}$M significantly enhanced UK14304-induced contractions. In the presence of low concentration of U46619 the maximum UK14304 contraction was 0.25 ± 0.06gms Force, compared with the control curve maximum of 0.11 ± 0.05gms Force ($p = 0.02$*).

In tail arteries from four-month old D79N mice, elevation of vascular tone with a low concentration of U46619 also enhanced contractile responses. In the presence of U46619-induced tone, the maximum UK14304-mediated contraction was 0.12 ± 0.07gms Force, which was significantly greater than the control curve maximum of 0.04 ± 0.02gms Force ($p = 0.04$*)

Incubating mounted tail arteries with L-NAME prior to elevation of vascular tone, and construction of a UK14304 response curve provided no advantage over using each agent separately ($p = 0.22$). In the presence of L-NAME the contractile response to a low concentration of U46619 ($3 \times 10^{-9}$M) was 0.18 ± 0.06gms Force in WT arteries, and 0.10 ± 0.02gms Force in arteries from D79N mice, again analysis of these responses gained showed that they were not significantly different ($p>0.05$).

In WT and D79N mice, in the presence of U46619 ($3 \times 10^{-9}$M) and L-NAME, UK14304 caused concentration-dependent contractions that were significantly greater than UK14304 control curves. The maximum contraction in arteries from WT mice was $0.24 \pm 0.04$gms Force, compared with that gained in the presence of low U46619 alone $0.25 \pm 0.05$gms Force.

In tail arteries from D79N mice, incubation with L-NAME prior to elevation of tone with U46619 ($3 \times 10^{-9}$M) gave a contractile maximum of $0.13 \pm 0.03$gms Force, which was significantly greater than the control curve maximum of $0.04 \pm 0.02$gms Force ($p = 0.01$*). However, the maximum response gained with U46619-induced tone and L-NAME was not significantly different from the maximum with U46619 ($3 \times 10^{-9}$M) alone, which was $0.12 \pm 0.07$ ($p = 0.8163$).

3.3.3 Sensitivity to UK14304 is enhanced in the presence of U46619-induced tone

In the presence of low levels of U46619-induced ($3 \times 10^{-9}$M) tone the sensitivity to UK14304-mediated contractions was enhanced in the mouse tail artery. In WT arteries the $pEC_{50}$ value calculated is $6.5 \pm 0.2$ compared with a value of $5.9 \pm 1.0$ for the control protocol ($p = 0.029$*). In tail arteries from D79N mice, U46619-induced tone shifts the $pEC_{50}$ value from $5.7 \pm 0.01$ in the control curve to $7.0 \pm 0.2$ ($p = 0.003$**). Therefore, it appears that the presence of the synergist, U46619, even at low levels, increased the sensitivity of the murine tail artery to UK14304-mediated contractions, in WT and D79N strains.

3.3.4 Effect of L-NAME on UK14304-induced contractions alone and with elevated tone

In this series of experiments L-NAME was used in two ways. Firstly, L-NAME (1 x $10^{-4}$M) was incubated with mounted arterial segments prior to construction of a concentration response curve to UK14304. Secondly, L-NAME incubation preceded elevation of vascular tone with low concentrations of the synergist, U46619.

The contractile responses to UK14304 in tail arteries from four-month old WT and D79N mice are shown in figure 3.2. In arteries from WT mice (figure 3.2 **A**), incubation with L-NAME significantly increased the maximum contraction from 0.11 ± 0.05gms Force in a control curve, to 0.22 ± 0.04gms Force with L-NAME ($p = 0.04$*). The combination of L-NAME, and U46619-induced tone gave a UK14304-mediated contraction with a maximum response of 0.24 ± 0.03gms Force. The size of contractions gained with L-NAME, and U46619-induced tone (3 x $10^{-9}$M) was no greater than when L-NAME was given alone ($p>0.05$).

Figure 3.2 **B** also shows UK14304-mediated contractions in tail arteries from four-month old D79N mice that have been incubated with L-NAME alone, and L-NAME before U46619-induced elevation of vascular tone. The size of the responses gained were generally smaller than those obtained in WT arteries. Response curves with L-NAME (1 x $10^{-4}$M) had a maximum contraction to UK14304 of 0.12 ± 0.02gms Force, which was significantly greater than the control curve maximum of 0.04 ± 0.02gms Force ($p = 0.04$*). In the presence of elevated vascular tone, the maximum response gained in D79N tail arteries was 0.13 ± 0.03gms Force. Considering the results gained in WT and D79N arteries, it appears that the combination of elevating vascular tone and

blockade of nitric oxide release provide no greater benefit than using U46619 to elevate tone, or L-NAME independently of each other.

3.3.5 Tail arteries are more sensitive to UK14304 in the presence of L-NAME

In tail arteries from WT mice, the $pEC_{50}$ shifts from 5.9 ± 1.0 in a control curve, to 6.4 ± 0.2 in the presence of L-NAME ($p = 0.0003$***). In arteries from D79N mice, the $pEC_{50}$ was shifted from 5.7 ± 0.01 in a control curve, to 6.8 ± 0.2 in the presence of L-NAME ($p = 0.0015$**). These findings show that incubating tail arteries with L-NAME increased the sensitivity to UK14304 in the WT and D79N. A table containing the maximum responses and the $pEC_{50}$ values for the protocols tested is shown below.

| | WT | | D79N | |
|---|---|---|---|---|
| | $pEC_{50}$ | Max. | $pEC_{50}$ | Max. |
| Control | 5.9 ± 1.0 | 0.11 ± 0.05, n = 7 | 5.7 ± 0.01 | 0.04 ± 0.02, n = 10 |
| [Low] U46619 | 6.5 ± 0.2* | 0.25 ± 0.06, n = 6* | 7.0 ± 0.20* | 0.12 ± 0.07, n = 10* |
| L-NAME | 6.4 ± 0.2* | 0.22 ± 0.04, n = 7* | 6.8 ± 0.20* | 0.12 ± 0.02, n = 10* |
| [High] U46619 | 8.0 ± 0.2* | 0.39 ± 0.07, n = 12** | 8.1 ± 0.20* | 0.21 ± 0.04, n = 13** |

Table 3.1 Maximum response and $pEC_{50}$ values in WT and D79N tail arteries

3.3.6 Effect of elevating tone to fifty percent of the noradrenaline maximum

Figure 3.3 shows the contractile responses to UK14304 in a control curve, and when U46619 has been used to achieve a high level of vascular tone in tail arteries from WT (figure 3.3 **A**) and D79N (figure 3.3 **B**) mice. U46619 was added in half log units ($3 \times 10^{-8} - 1 \times 10^{-7}$M) to achieve a contraction comparable to fifty percent of the response to noradrenaline at a concentration of $1 \times 10^{-5}$M. In the WT, U46619-induced tone

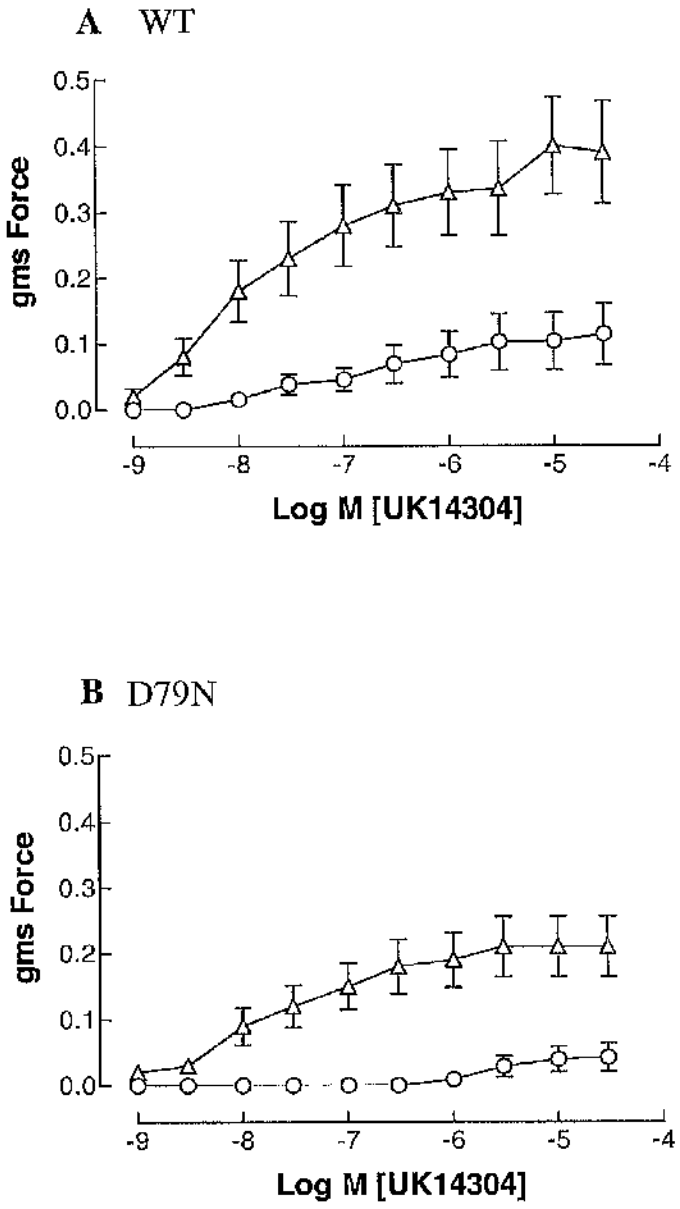



Figure 3.3: Responses in tail arteries from 4-month old WT and D79N mice to UK only, and with U19 ($3 \times 10^{-7} - 1 \times 10^{-7}$M) to elevate tone to 50% of the NA maximum. **A** UK control curves (○, n = 7 ), and with high U19-tone ( △, n = 12), in the WT. **B** UK control curves (○, n = 10), and with high U19-tone ( △, n =13) in the D79N. Each point represents mean ± standard error.

reached a maximum of 0.34 ± 0.06gms Force, compared with 0.28 ± 0.04gms Force in the D79N arteries. Comparison of these values showed that the size of the response gained is not statistically different ($p = 0.21$).

In both murine strains, contractions to UK14304 were significantly greater than those obtained in the control protocol. In tail arteries from WT mice the control curve maximum was 0.11 ± 0.05, whilst in the presence of high levels of U46619-induced tone the maximum response was significantly greater at 0.39 ± 0.07gms Force ($p = 0.008$**). In arterial rings from D79N mice the maximum force generated in a UK14304 control curve was 0.04 ± 0.02gms Force. In the presence of high levels of U46619-induced tone the maximum contraction in the D79N was significantly greater that controls, at 0.21 ± 0.04gms Force ($p = 0.0018$ **).

Contractile responses in WT and D79N mice in the presence of high levels of vascular tone are significantly greater than the contractions gained in a control curve. In addition to being significantly greater than controls, contractile responses were also significantly larger than all of the other protocols tested. These include, U46619 at $3 \times 10^{-9}$M, L-NAME ($1 \times 10^{-4}$M) alone, and L-NAME with [low] U46619 ($p<0.05$). When the maximum responses gained in WT and D79N tail arteries were compared, it was found that contractions in the WT were significantly greater than those obtained in the D79N ($p = 0.0210$ *).

3.3.7 Sensitivity to UK14304 is enhanced in the presence of high levels of U46619-induced tone

In figure 3.4 the contractile responses to UK14304 in a control curve, and in the presence of high levels of U46619-induced tone are shown for the WT (figure 3.4 **A**) and D79N (figure 3.4 **B**) mice, and have been expressed as a percentage of their own maximum response. In WT arteries the control curve $pEC_{50}$ was 5.9 ± 1.0, while in the presence of the high levels of U46619-induced tone the $pEC_{50}$ was shifted to 8.0 ± 0.2 ($p = 0.0002$***). Similarly, $pEC_{50}$ values are also shifted in tail arteries from D79N mice. For the control curve a $pEC_{50}$ value of 5.7 ± 0.01 has been determined, while in the presence U46619 the value was shifted to 8.1 ± 0.2 ($p = 0.0007$***).

3.3.8 Combined contractile response to high levels of vascular tone and increasing concentrations of UK14304

Figure 3.5 shows the combined contractile response to high levels of U46619-induced tone, and increasing concentrations of UK14304, in tail arteries from four-month old WT (figure 3.5 **A**) and D79N (figure 3.5 **B**) mice. In WT arteries the combination of UK14304 and U46619 gave a contractile maximum of 0.73 ± 0.08gms Force, while in D79N arteries the maximum was 0.42 ± 0.06gms Force. Comparison of the results gained in WT and D79N arteries revealed that, again, the contractile responses were significantly greater in arteries from WT mice ($p = 0.02$*).

3.3.10 Combined contractile responses approach an adrenergic maximum in WT but not in D79N

Figure 3.6 shows the combined contractile response to high levels of U46619-induced tone and UK14304 in tail arteries from the WT (figure 3.6 **A**) and D79N (figure 3.6 **B**),

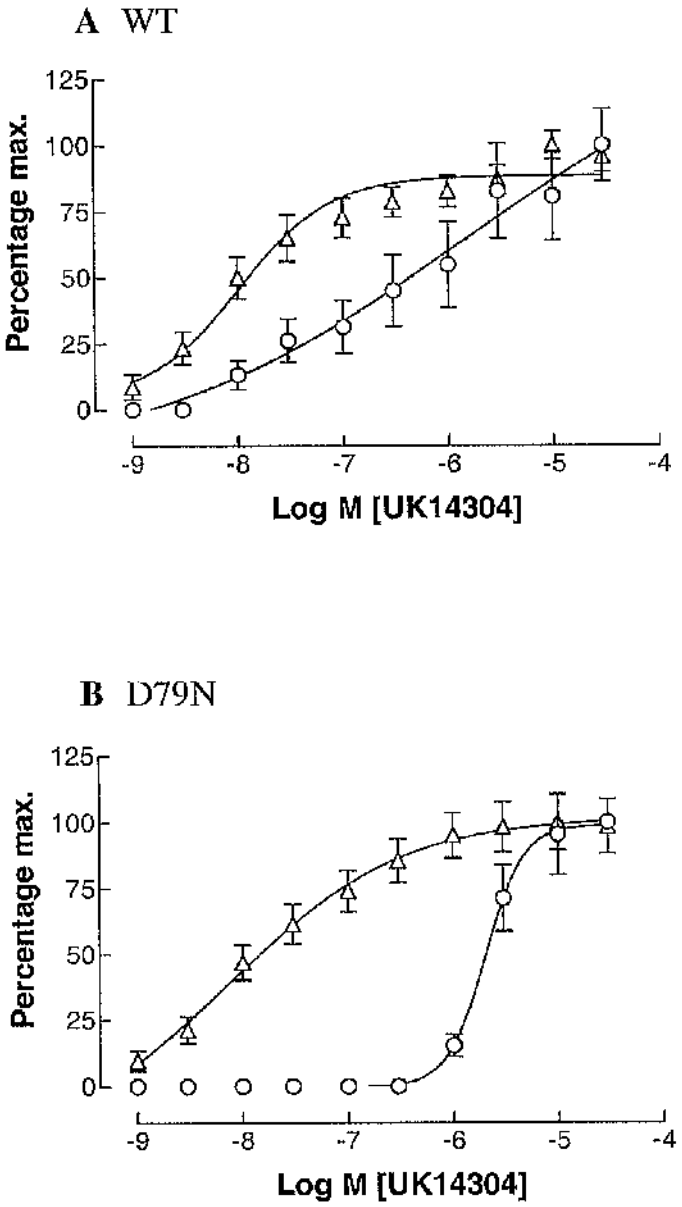



Figure 3.4: The UK14304 response in tail arteries from 4-month old WT and D79N mice, expressed as a percentage of the max. response. **A** UK control curves (○, n = 7 ), and in the presence of high U19-tone ( △, n = 12) in the WT. **B** UK control curves (○, n = 10 ), and in the presence of high U19-tone ( △, n = 13) in the D79N. Each point represents mean ± standard error.

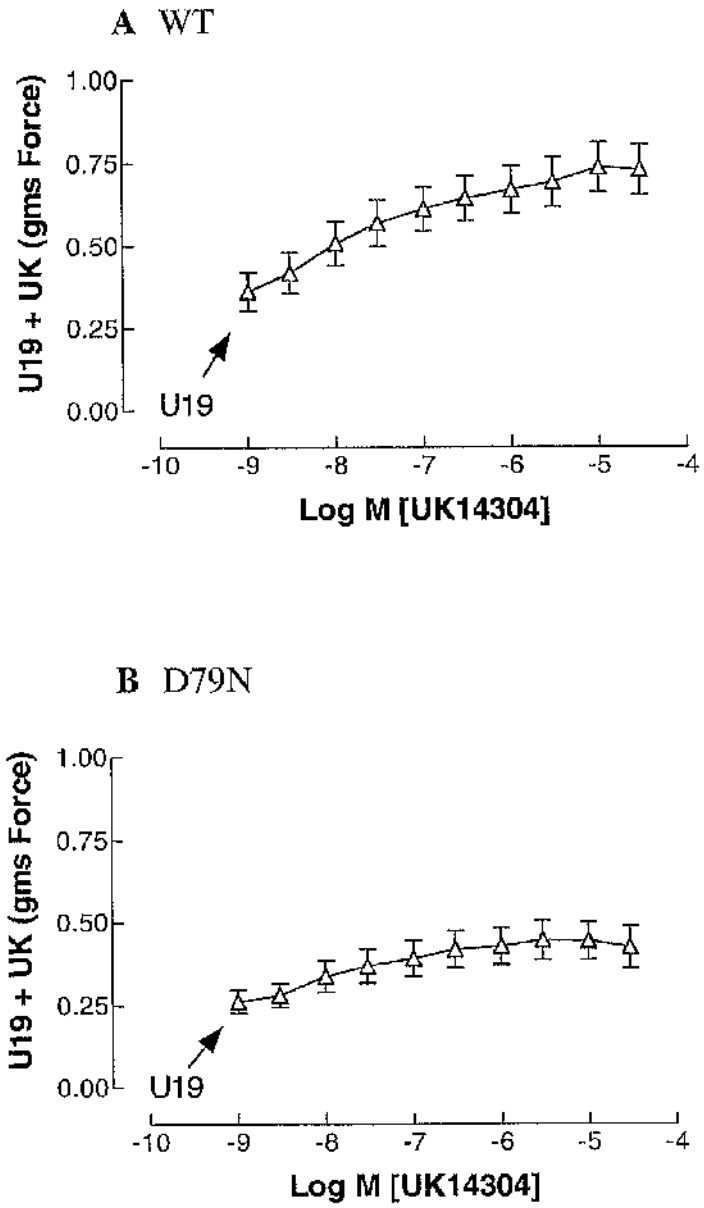



Figure 3.5: Combined contractile response to UK and U19 (to 50% of the NA max) in tail arteries from 4-month old WT and D79N mice. **A** A UK curve in WT arteries (△, n = 12). **B** A UK curve in D79N arteries (△, n = 13). Arrows are indicative of the U19-induced increase in basal tone. Each point represents mean ± standard error.

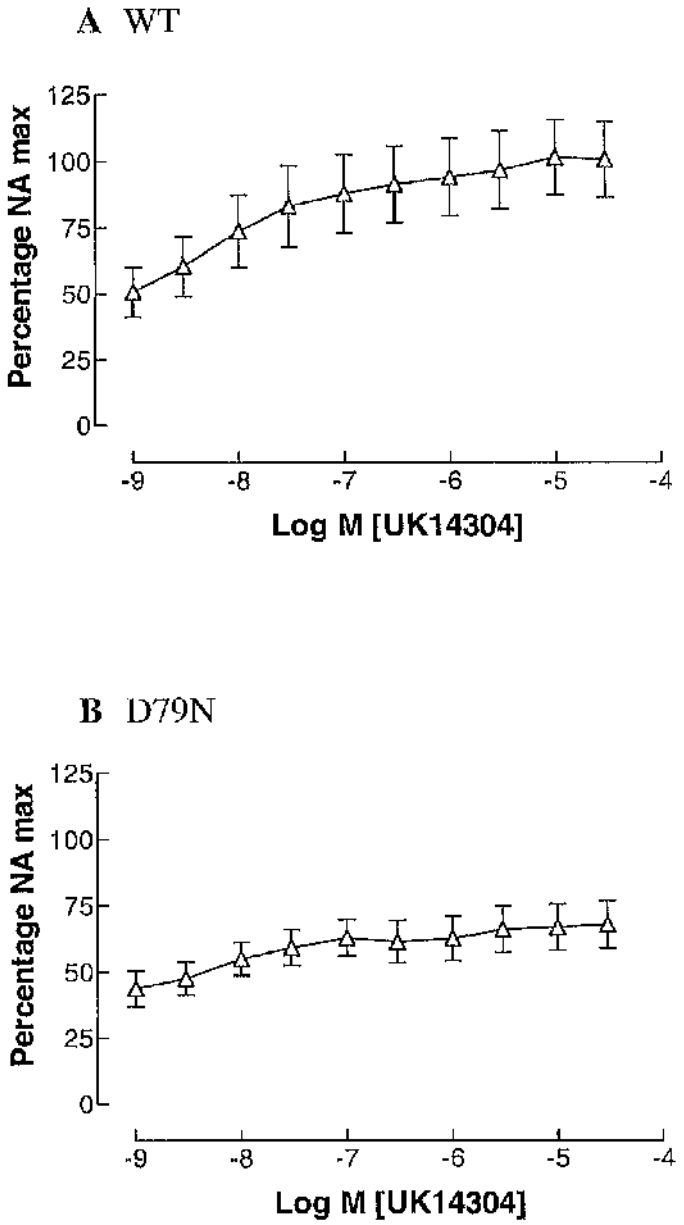



Figure 3.6: Combined contractile response to UK and U19 (to 50% of NA maximum), expressed as a percentage of the NA max ($1 \times 10^{-5}$M), in tail arteries from 4-month old WT and D79N mice. **A** The combined contractile response (△, n = 12) in WT arteries. **B** The combined contractile response (△, n = 13) in D79N arteries. Each point represents mean ± standard error.

expressed as a percentage of a noradrenaline maximum (1 x $10^{-5}$M). The combination of U46619-induced tone and UK14304 produced a considerable contraction, given the size of the vessels studied. Expressing the combined contraction as a percentage of the noradrenaline response, showed that in WT arteries UK14304-mediated contractions on top of U46619-induced tone reached 101 ± 14.0 % of the response to sighting concentrations of noradrenaline. However, in tail arteries from D79N mice the combined response to U46619-induced tone and UK14304 only reached 68 ± 8.8 % of the contraction to noradrenaline.

3.3.11 Effect of L-NAME on UK-induced contractions in the presence of high levels of elevated vascular tone

Having established that high levels of U46619-induced tone provide favourable conditions for studying $\alpha_2$-adrenoceptor-mediated responses in the murine tail artery, one additional protocol was tested. L-NAME was incubated with arterial rings before raising vascular tone to high levels. Response curves to UK14304 were then performed in tail arteries from four-month old WT and D79N mice.

In WT arteries (figure 3.7 **A**), contractions tended to be smaller in the presence of L-NAME and had a maximum response of 0.25 ± 0.05gms Force. However, when compared with responses gained in the absence of L-NAME no significant difference was found (p = 0.44). In tail arteries from D79N mice (figure 3.7 **B**), contractile responses were significantly potentiated in the presence of L-NAME. The maximum response gained in the presence of high U46619-induced tone was 0.21 ± 0.04gms Force, while with L-NAME the maximum reached was 0.41 ± 0.04gms Force (p = 0.025*). Comparing the responses gained in D79N with WT arteries showed that in the

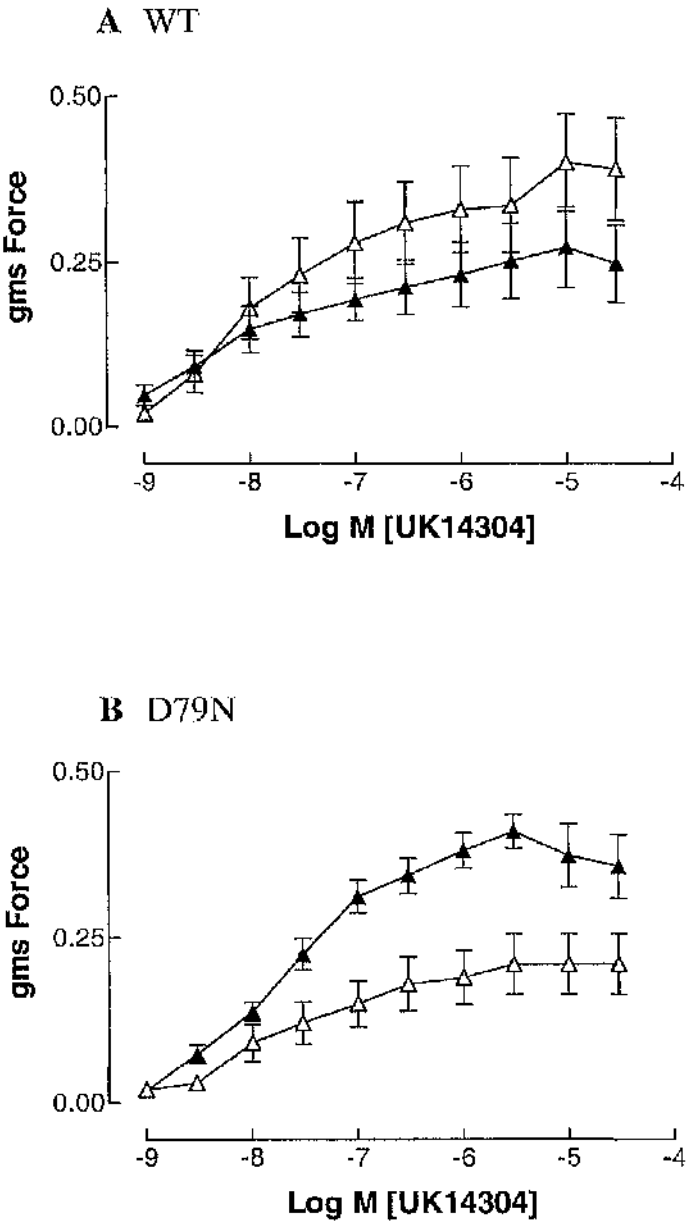



Figure 3.7: The effect of L-NAME (1 x $10^{-4}$M) on the UK response in tail arteries from 4-month old WT and D79N mice in the presence of high U19-induced tone. **A** The UK response in the presence of tone (△, n = 12), and tone with L-NAME (▲, n = 6) in the WT. **B** The UK response in the presence of tone (△, n = 13), and tone with L-NAME (▲, n = 8) in the D79N. Each point represents mean ± standard error.

presence of high levels of U46619-induced tone and L-NAME, contractile responses were significantly greater in the D79N ($p = 0.02^*$).

3.3.12 Effect of age on contractile responses in tail arteries from D79N mice

Contractile responses can change with increasing age. Therefore, when a number of mice aged twelve-months became available, I decided to investigate if UK14304-mediated contractions were altered in old D79N mice. Figure 3.8 shows the contractile responses at four and twelve-months in tail arteries from D79N mice. Figure 3.8 **A**, shows the contractile responses to UK14304 on top of U46619-induced tone at both age points. In arteries from four-month old mice, the maximum contraction was 0.21 ± 0.04gms Force, while at twelve-months the maximum response achieved was 0.17 ± 0.03gms Force. Analysis of the responses gained at the different age points confirmed that UK14304-mediated contractions are not significantly different at four and twelve-months ($p>0.05$). However, when the combined contractile response to U46619-induced tone and UK14304 was compared, the results were quite different.

The combined contractile responses in arteries from twelve-month old D79N mice (figure 3.8 **B**) was significantly greater than the response at four-months ($p = 0.01^*$). In arteries from twelve-month old mice, combined contractions reached a maximum of 0.79 ± 0.05gms Force, but at four-months the maximum was 0.45 ± 0.06gms Force. In arteries from four-month old D79N mice the U46619-induced contraction was 0.24 ± 0.04gms Force, at twelve-months contractions were significantly potentiated, and had a maximum response of 0.67 ± 0.06gms Force ($p<0.0001^{***}$).

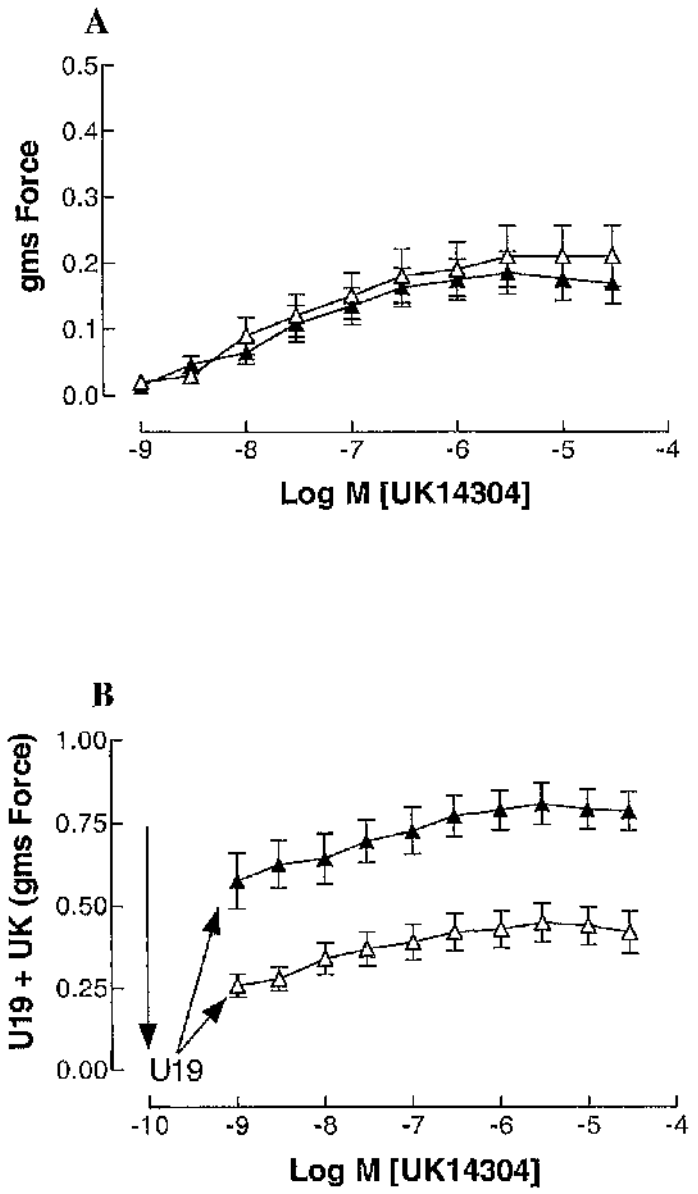



Figure 3.8: Responses in tail arteries from 4 and 12-month old D79N mice with high U19-tone. **A** UK curve on top of tone in arteries from 4 ( △, n = 10) and 12-month old (▲, n = 6) mice. **B** Combined response to UK and U19-tone in arteries from 4 ( △, n = 10) and 12-month old (▲, n = 6) mice. Arrows are indicative of the U19-induced increase in basal tone. Each point represents mean ± standard error.

Therefore, the combination of elevated tone, and UK14304-mediated contractions leads to a significantly enhanced contractile response in arteries from older mice. However, the potentiation of responses can be attributed to an enhancement of the U46619-induced contraction, and is not UK14304-mediated.

## 3.4 Discussion

### A small elevation in vascular tone uncovers UK14304-mediated contractions of the murine tail artery

In the rat tail artery elevation of vascular tone with either 5HT [Templeton et al, 1988] or arginine vasopression (AVP) provides an environment that is suitable for studying $\alpha_2$-adrenoceptor-mediated contractile responses [Templeton et al, 1989]. Given these findings, it seemed logical to determine if similar conditions are required for the study of $\alpha_2$-adrenoceptor-mediated responses of the murine tail artery.

In the mouse tail artery, UK14304 causes, little, if any contractile effect in arteries from WT and D79N mice. However, low levels of vascular tone, elevated with U46619 ($3 \times 10^{-9}$M) allow contractile responses to be studied; by providing conditions that are similar to those required for $\alpha_2$-adrenoceptor-mediated pressor response to be studied in the rat tail artery [Templeton et al, 1989].

The thromboxane mimetic, U46619 was used to elevate vascular tone because the contractions that occur are independent of $\alpha_1$ and $\alpha_2$-adrenoceptors. In the past, phenylephrine has been used to raise tone within a blood vessel. However, the complication of using phenylephrine is that $\alpha_2$-selective agonists, such as UK14304 can act as inverse agonists at $\alpha_1$-adrenoceptors in some rodent blood vessels (unpublished observation for our laboratory). Therefore, leading to an incorrect interpretation of experimental results. For example, activation of $\alpha_2$-adrenoceptors could be falsely proposed to mediate relaxant responses in arteries preconstricted with phenylephrine, when, in fact, the "$\alpha_2$-selective" agonist opposes $\alpha_1$-adrenoceptor-mediated contractions by acting as an inverse agonist. Under these experimental conditions, $\alpha_2$-adrenoceptor-

mediated relaxations and activation of $\alpha_1$-adrenoceptor with an inverse agonist both lead to a reduction in the force generated within a mounted arterial ring.

In addition to the effects in the rodent tail artery, elevated vascular tone has been shown to enhance $\alpha_2$-adrenoceptor-mediated responses in the isolated canine saphenous artery [Sulpizio & Hieble, 1987]. Furthermore, in the presence of an $\alpha_1$-selective antagonist, agonist-induced increases in blood pressure in the pithed rat, induced by agonists proposed to be selective for $\alpha_2$-adrenoceptor agonists are mediated by postjunctional $\alpha_2$-adrenoceptors, *in vivo* [Docherty & McGrath, 1980]. In the absence of a functional central nervous system, high circulating levels of angiotension II and catecholamines may act to maintain vascular tone. These conditions may provide a favourable environment for $\alpha_2$-adrenoceptor-mediated pressor effects.

*In vitro*, the elevation of vascular tone in the perfused rat tail is presumed to lead to an increase in intracellular calcium concentrations, which provides conditions suitable for $\alpha_2$-adrenoceptor-mediated contractions [Xiao et al, 1989]. Presumably, U46619 causes similar effects in the murine tail artery.

Elevated tone and inhibition of nitric oxide release provide no further enhancement of UK14304-mediated contractions

The contractile response to U46619 is not significantly greater in size in the presence of L-NAME. This data provides evidence that in the mouse tail artery, nitric oxide release is not constitutive. If nitric oxide release were constitutive in this artery, then it would act to oppose the contractions caused by U46619. Under these circumstances, L-

NAME would be expected to cause a significant potentiation in the size of U46619-induced contractions, which is not the case.

The combined use of low levels of synergism and inhibition of nitric oxide release, do not enhance UK14304-mediated contractile responses in WT and D79N tail arteries. Therefore, it is more advantageous to use only one of these techniques as opposed to using both, which add another variable to an already complex experimental set-up.

Inhibiting nitric oxide release enhances UK14304-mediated contractions of the murine tail artery

L-NAME can be used to prevent the release of nitric oxide *in vivo*, by adding the drug to an animal's drinking water. Arteries isolated from animals given L-NAME *in vivo* show enhanced contractions to $\alpha_2$-selective agonists, *in vitro* [Carter & Kanagy, 2002]. Constitutive nitric oxide release can occur in isolated *in vitro* preparations [Furchgott and Vanhoutte, 1989], and blockade of its release has been shown to enhance contractile responses *in vivo* [Conrad & Whittemore, 1992]. Therefore, I examined what effect L-NAME has on UK14304-mediated responses in the murine tail. L-NAME irreversibly binds to the enzyme nitric oxide synthase, which prevents the release of nitric oxide, which is a potent endothelial derived relaxing factor [Akaike et al, 1993]

L-NAME enhances UK14304-mediated contractions in tail arteries from WT and D79N mice. This could be caused by constitutive release of nitric oxide countering contractile responses that are mediated by $\alpha_2$-adrenoceptors. However this appears unlikely, because L-NAME does not potentiate contractile responses to other agonists in the murine tail artery.

If the release of nitric oxide in the murine tail artery is not constitutive, then it must be caused by exogenous UK14304. Stimulation of $\alpha_2$-adrenoceptors, located on the endothelium of blood vessels leads to nitric oxide release [Bockman et al, 1993]. In the mouse tail artery, L-NAME enhances contractile responses in both WT and D79N arteries. Therefore, this suggests that $\alpha_{2A/D}$-adrenoceptors do not mediate nitric oxide release, and that if UK14304 stimulates nitric oxide release by direct mechanisms, they may result from stimulation of $\alpha_{2B}$ and/or $\alpha_{2C}$-adrenoceptor subtypes. Alternatively, UK14304 may indirectly lead to the release of nitric oxide. In order to determine if this is the case further experiments are required.

The mechanism whereby L-NAME leads to a potentiation of $\alpha_2$-adrenoceptor-mediated contractions in the mouse tail artery, are likely to involve calcium. *In vivo*, blocking nitric oxide release leads to an enhancement of $\alpha_2$-adrenoceptor-mediated contraction *in vitro* by activating two calcium pathways. One is tyrosine kinase dependent, the other does not depend on the activation of tyrosine kinases [Carter & Kangy, 2002].

<u>High levels of vascular tone provide the most favourable conditions for the study of UK14304-mediated contractions</u>

Although low levels of U46619-induced tone permit UK14304-mediated contractions, the responses gained are still small, and highly variable. Therefore, I decided to increase the tone by using higher concentrations of the synergist, U46619. In an attempt to standardise the experimental conditions U46619-induced contractions were compared to a reference noradrenaline contraction.

In WT and D79N tail arteries the size of UK14304-mediated contractions (in the presence of high levels of U46619-induced tone) is significantly greater than any of the other protocols tested. In addition to enhancing the size of these responses, mounted arteries also become more sensitive to UK14304, evidenced by higher $pEC_{50}$ values. Given this, it appears that high levels of vascular tone are the most favourable conditions of all the protocols studied.

L-NAME potentiates UK14304-mediated contractions in the presence of high levels of vascular tone in the D79N but not in the WT

Having determined suitable conditions for the study of UK14304-induced contractions of the mouse tail artery, I then tested what effect L-NAME would have on the responses produced, under suitable experimental conditions.

In arteries from four-month old WT mice L-NAME has no effect on the size of the responses to UK14304. The variability within the contractions gained is high, and is most probably due to the development of rhythmic arterial contractions, which are a complication of studying contractile responses in tail and mesenteric arteries. However in the D79N, incubation with L-NAME, prior to U46619-induced tone and a UK14304 response curve causes a significant potentiation of the contractions gained.

The enhancement of responses by L-NAME may be the result of several different mechanisms, occurring alone, or in combination with each other. L-NAME may inhibit constitutive release of nitric oxide, which may occur in D79N mice, but not in the WT. This seems unlikely, as there is no evidence to support constitutive release, because L-NAME does not affect resting tone or U46619-induced contractions. Another

possibility is that in the absence of a fully functional $\alpha_{2A/D}$-adrenoceptor-pool, UK14304 may enhance nitric oxide release. The existence of normal, functional $\alpha_{2A/D}$-adrenoceptors in the WT mouse may explain the difference in the results gained. This suggests that $\alpha_2$-adrenoceptors may be involved in the regulation of nitric oxide release in murine blood vessels. A hypothesis that is supported by results gained for the D79N in chapter six of this thesis. Where $\alpha_2$-adrenoceptor-mediated vasodilatations in mesenteric resistance arteries are significantly attenuated by L-NAME in the D79N, but not the WT.

U46619-induced contractions are significantly enhanced with increasing age

Responses to endogenous and exogenous agonists can be affected by advancing age [Rodriguez-Martinez et al, 1999]. However, the mechanisms involved are often complex and have been attributed to a variety of physiological alterations.

UK14304 causes concentration-dependent contractions in tail arteries from four and twelve-month old D79N mice. Contractions gained are of comparable size and sensitivity at the different age points. This suggests that UK14304-mediated contractions of cutaneous murine blood vessels are not altered in older animals. However, the combined contractile response to U46619-induced tone and UK14304 is significantly greater at twelve-months when compared with the responses gained at four months. The enhanced contractions result from a significant potentiation in U46619-mediated responses.

U46619 is a thromboxane mimetic, and the responses gained are intended to mimic the effects of endogenous contracting factors. Altered contractile responses to endothelial

contracting factors are a common finding in blood vessels from elderly patients [Docherty, 1990]. Yet, it is often unclear if the elevation in vascular tone, that accompanies advancing age and leads to elevated blood pressure, results from increased release of vasoactive contracting factors, altered receptor numbers, or an alteration of the native receptor population within a blood vessel.

In tail arteries from old D79N mice, altered contractile responses may reflect a change in the sensitivity of endothelial receptor to U46619. A number of receptors are known to develop enhanced sensitivity to agonists with age, and under pathophysiological conditions. Therefore, this seems a probable hypothesis to explain the potentiation of U46619-induced contractions. Alternatively, U46619-mediated contractions may be enhanced by a reduction in the release of endothelial derived relaxing factors, which act to oppose contractile responses. In order to determine if this is the case, a further series of experiments are required. It would be interesting to determine what effect L-NAME and prostacyclin have in arteries from older mice. Unfortunately the completion of these studies was impossible during my experiments, as we had limited stocks of elderly mice, and the study of U46619-induced contractions is outwith the scope of this thesis.

Comparison of responses gained in WT and D79N tail arteries

UK14304-mediated contractions of the murine tail artery are consistently smaller in the D79N than WT, in almost all protocols tested. The one notable exception is when tone has been elevated with high concentrations of the synergist, U46619, and arteries have been preincubated with L-NAME prior to construction of a UK14304 response curve.

Excluding the last protocol, data strongly suggests that the point mutation in the $\alpha_{2A/D}$ adrenoceptor has a detrimental effect on contractile responses of the murine tail artery. Indicating that the $\alpha_{2A/D}$-adrenoceptor plays a role in $\alpha_2$-adrenoceptor-mediated contractions of peripheral blood vessels. The remaining contractile response in the tail artery of the D79N may result from stimulation of $\alpha_{2C}$-adrenoceptors, which are known to mediate vasoconstrictor responses in the rat tail [Craig et al, 1995]. Alternatively, they may result from non-selective stimulation of $\alpha_1$-adrenoceptors. However, the results contained in chapter four may suggest an alternative explanation for the altered responsiveness of the D79N tail artery (where the non-cumulative response in the WT and D79N is comparable). The results, to which I refer, will be discussed in detail in chapter four.

# Chapter four

## UK14304-induced contractions are complicated by receptor-desensitisation

## 4.1 Introduction

The development of persistent rhythmic contractility upon agonist administration is common in resistance arteries, and has been termed vasomotion [Peng et al, 2001]. Adrenoceptor-selective agonists and synergists such as U46619 can lead to the development of rhythmic contractions in murine blood vessels. Both of the resistance arteries studied in this thesis develop rhythmic contractions when stimulated with exogenous agonists. Although vasomotion is interesting in its own right, the development of rhythmic waves makes it extremely difficult to separate drug-induced contractions from spontaneous movements. In order to combat this, an adaptation of the original protocol was made. This was the addition of a low concentration of nifedipine ($1 \times 10^{-7}$M).

The UK14304-mediated response tends to 'fall off' at high agonist concentrations, which was indicative of agonist-induced receptor desensitisation and internalisation [Ferguson et al, 1997]. To ascertain whether desensitisation occurred in tail arteries challenged with UK14304, consecutive response curves were constructed in tail arteries from WT and D79N mice.

In addition to this, a series of non-cumulative response curves to UK14304 were performed. This was done to determine if the non-cumulative UK14304 response was less variable than that of the cumulative, and whether contractility was maintained at high agonist concentrations. The UK14304-mediated response to non-cumulative administration was compared with that of the cumulative, to determine if the size of contractile responses was affected by the way in which the agonist was administered.

Having established suitable conditions that uncovered UK-mediated contractile responses, the use of selective antagonists was employed. The $\alpha_2$-selective antagonist, rauwolscine was used to confirm that the contractile response mediated by UK14304 resulted from stimulation of vascular $\alpha_2$-adrenoceptors. Prazosin, an $\alpha_1$-selective antagonist ($3 \times 10^{-8}$M) was also used, to exclude the possibility that the UK14304 response arose from stimulation of $\alpha_1$-adrenoceptors.

## 4.2 Methods

### 4.2.1 Vessel dissection and mounting

Mice weighing between 28.9-33.6gms were euthanised by asphyxiation with $CO_2$, tails were removed and placed in fresh, cold Krebs solution until dissection. Tails were then pinned out on a petri dish, measured, and a 1.5cm section beginning 2.5cms from the base of the tail was marked. The thick, hairy, skin covering the tail was removed and the artery underneath exposed. The tail artery was then dissected using ultra fine forceps and dissecting scissors. Once removed, the section of artery was cleaned of excess connective tissue and fat and cut into 2mm rings. These rings were then mounted in 5ml stainless steel Mulvany/Halpern myograph baths (described in detail in chapter two) and allowed to equilibrate for 20-30 minutes before setting the resting tension. Arteries were then stretched to give a resting tension of 0.25gms Force, and a further 20-30 minute equilibration was allowed.

### 4.2.2 Wake-up protocol

Mounted arterial rings were stimulated with noradrenaline at a concentration of $1 \times 10^{-5}$M, and contractions were allowed to reach a plateau before being washed with fresh Krebs. Each bath was washed four times with warmed, gassed Krebs over a fifteen minute period. This procedure was then repeated. The last concentration of noradrenaline used was slightly lower than the first two, at $1 \times 10^{-6}$M. When the plateau of contraction was stable, acetylcholine at a concentration of $3 \times 10^{-6}$M was administered to each bath, to determine if the endothelium on the mounted vessel was intact.

4.2.3 Incubation with nifedipine

Nifedipine stock solution (1 x $10^{-2}$M) was prepared in 100 % alcohol, and diluted in distilled water before each experiment to give a solution at the desired concentration (1 x $10^{-4}$M, final bath concentration of 1 x $10^{-7}$M). Nifedipine was used in all of the experiments where UK14304-mediated responses were investigated. Mounted arterial segments were incubated with nifedipine for a minimum of twenty minutes before construction of a response curve.

4.2.4 Elevation of vascular tone

Prior to construction of an UK14304-mediated response curve, the tone within each mounted arterial ring was elevated with the thromboxane mimetic, U46619. I have already established that an increase in tone provides conditions that were suitable for the study of UK14304-induced contractions of the murine tail artery. In earlier experiments (shown in chapter three) U46619 was added in half log increments to give a contraction comparable to fifty percent of the noradrenaline response (1 x $10^{-5}$M). In most cases, a stable contraction, close to fifty percent of the noradrenaline maximum was achieved with U46619 at a concentration of 1 x $10^{-7}$M. Therefore, in order to reduce variability, this concentration was used for all of the experiments contained within this chapter.

4.2.5 Effect of rauwolscine and prazosin on UK14304-induced contractions

Rauwolscine and prazosin were prepared in accordance with the manufacturers guidelines (described in Chapter two). Both antagonists were used at the same concentration of 3 x $10^{-8}$M, and incubated for thirty minutes before construction of a response curve. For each antagonist the concentrations were chosen as being

significantly above the $pA_2$ concentration for the receptor being blocked, but below the $pA_2$ value for the other receptors, intended not to be blocked.

## 4.3 Results

### 4.3.1 Nifedipine prevents the development of unwanted rhythmic contractions in the tail artery

Figure 4.1 shows a trace of the effect of nifedipine on the development of rhythmic contractions in the murine tail artery from a four-month old WT mouse. Figure 4.1 **A** shows that in the absence of nifedipine constriction with the synergist U46619, followed by a series of washes, caused rhythmic contractions to develop. In figure 4.1 **B** nifedipine ($1 \times 10^{-7}$M) has been incubated for twenty minutes prior to contraction with U46619 ($1 \times 10^{-7}$M). In the presence of nifedipine basal tone is stable, and the development of rhythmic contractions was blocked.

Nifedipine ($1 \times 10^{-7}$M) was incubated with tail artery segment from four-month old WT (figure 4.2 **A**) and D79N (figure 4.2 **B**) mice before construction of a cumulative UK14304 response curve. Nifedipine prevented the development of vasomotion and tended to reduce the variability of the responses gained. In WT arteries the maximum UK14304 response was $0.27 \pm 0.03$gms Force at ($3 \times 10^{-6}$M) in the presence of nifedipine, whereas the maximum response at the same agonist concentration, without nifedipine was $0.33 \pm 0.07$gms Force ($p>0.05$). The size of the contractions gained in the D79N was unaffected by nifedipine, but the development of rhythmic contractions was halted. With UK14304 alone, the maximum UK14304 response at $3 \times 10^{-6}$M was $0.21 \pm 0.05$gms Force while in the presence of nifedipine the same agonist concentration produced a maximum of $0.19 \pm 0.02$gms Force ($p>0.05$). Thereafter, nifedipine was incubated with all mounted arteries used to study the UK14304-mediated response in the tail artery.

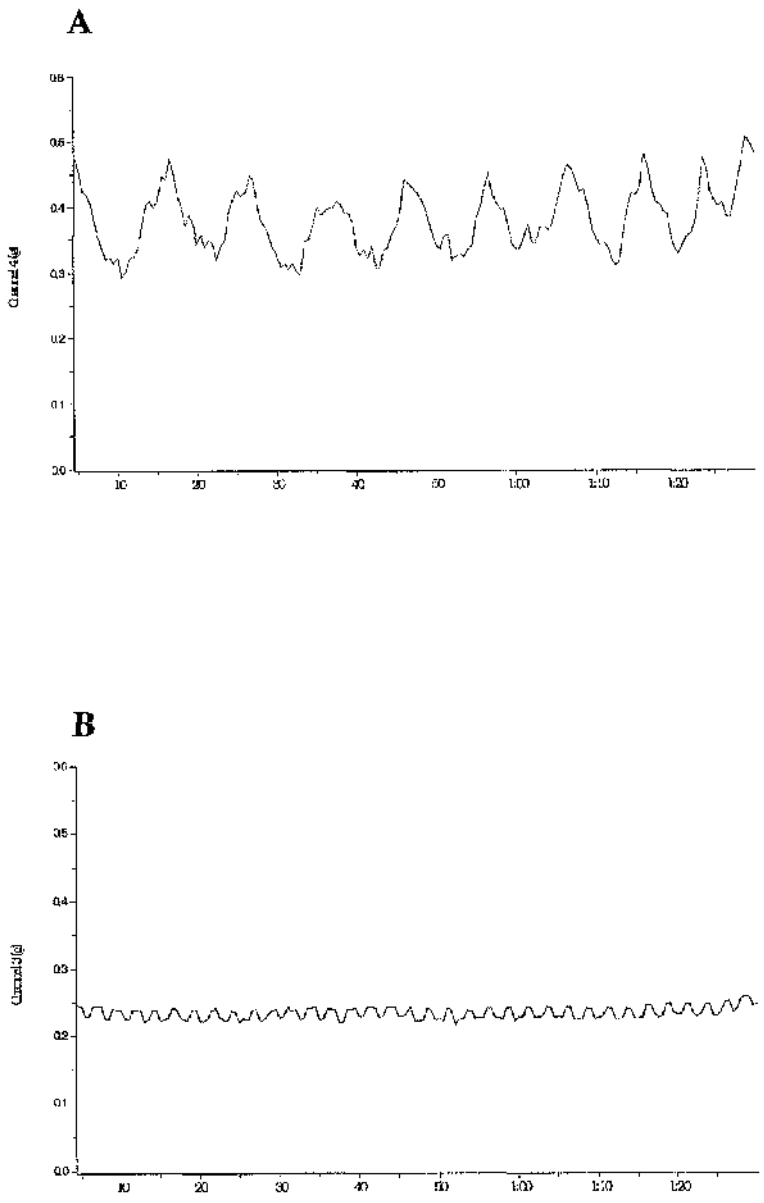



Figure 4.1: A representative trace of the effect of nifedipine on the development of rhythmic contractions in the tail artery of a 4-month old WT mouse. **A** Rhythmic contractions in a mounted tail artery, previously exposed to U19. **B** Abolition of rhythmic contractions in the presence of nifedipine ($1 \times 10^{-7}$M). The responses shown are in a single vessel.

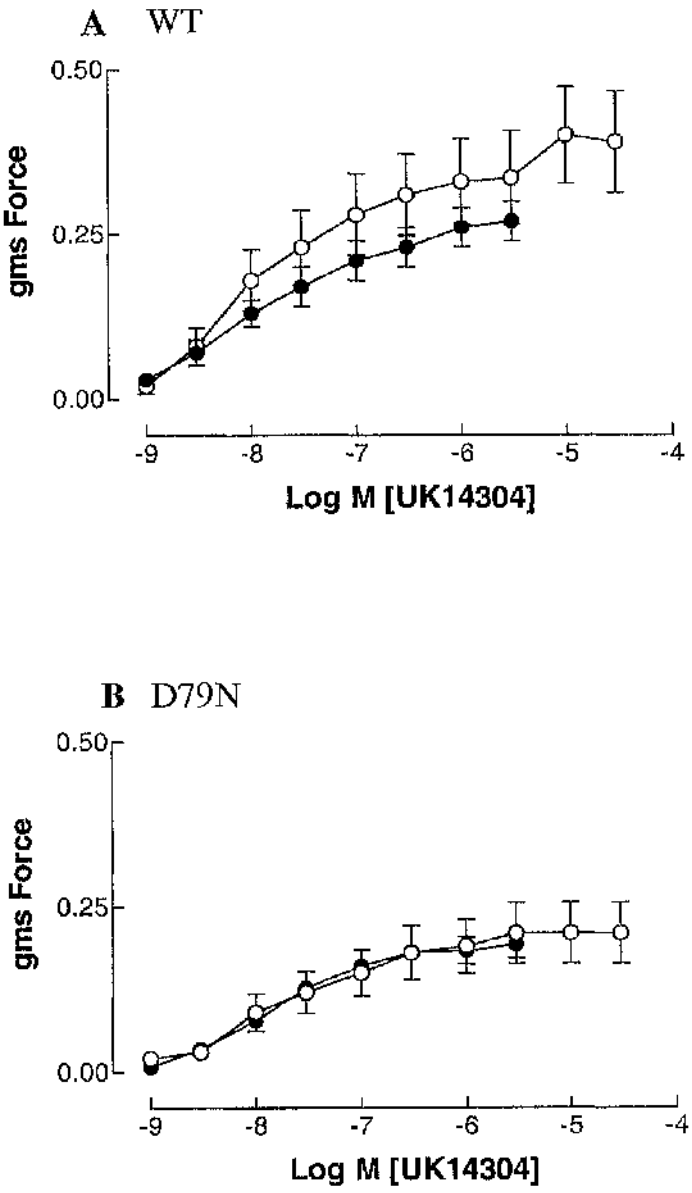



Figure 4.2: The UK response in tail arteries from 4-month old WT and D79N mice. **A** The cumulative UK response alone (○, n = 12), and with nifedipine (At 1 x $10^{-7}$M, ●, n = 12) in the WT. **B** The cumulative UK response alone (○, n = 12), and with nifedipine (●, n = 12) in the D79N. Each point represents mean ± standard error.

4.3.2 UK14304-induced responses are susceptible to profound desensitisation

Figure 4.3 shows the responses gained to consecutive, cumulative curves to UK14304 in tail arteries from four-month old WT (figure 4.3 **A**) and D79N mice (figure 4.3 **B**). In arteries from WT mice, UK14304 caused concentration-dependent contractions of tail arteries. However, a second curve, thirty minutes later to the $\alpha_2$-selective agonist could not be constructed. This showed that UK14304-mediated vasoconstrictor responses were profoundly desensitised. The maximum response gained in the first curve reached 0.27 ± 0.03gms Force, whereas in the second curve, the maximum was 0.03 ± 0.03gms Force, in tail arteries from WT mice. Responses obtained in tail arteries from D79N mice were similar. The maximum response gained in a first UK14304 curve was 0.19 ± 0.02gms Force, and is smaller than that of the WT ($p = 0.018^*$). Thirty minutes later a second curve produced significantly smaller contractions at all drug concentrations tested and had a maximum response of 0.10 ± 0.02gms Force. Again, this indicated that the receptors stimulated by UK14304 were desensitised.

4.3.3 Phenylephrine-induced responses are not affected by UK14304 desensitisation

In figure 4.4 the cumulative phenylephrine response, and phenylephrine-induced contractions after, a UK14304 curve, in tail arteries from four-month old WT mice are shown. In the vessels where phenylephrine was given alone one concentration response curve was constructed, while in those exposed to an initial UK14304 curve, two response curves were performed. Figure 4.4 **A** shows the absolute size of the responses gained to increasing concentrations of phenylephrine. Figure 4.4 **B** shows the phenylephrine response, expressed as a percentage of its own maximum. The first curve to phenylephrine, constructed before a cumulative UK14304 curve had a maximum of 1.08 ± 0.07gms Force, compared with the curve performed after

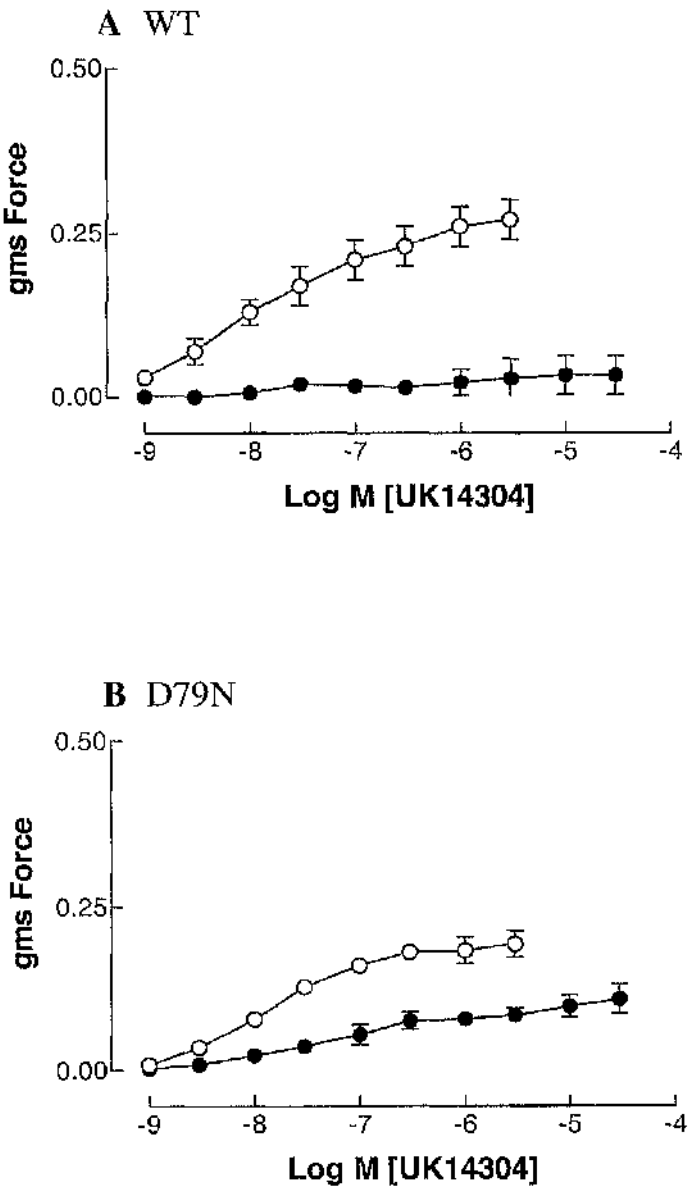



Figure 4.3: Cumulative UK response curves in tail arteries from 4-month old WT and D79N mice. **A** A first (○, n = 12) and second (●, n = 6) UK response curve in WT arteries. **B** A first (○, n = 12) and second (●, n = 6) UK response curve in D79N arteries. Each point represents mean ± standard error.

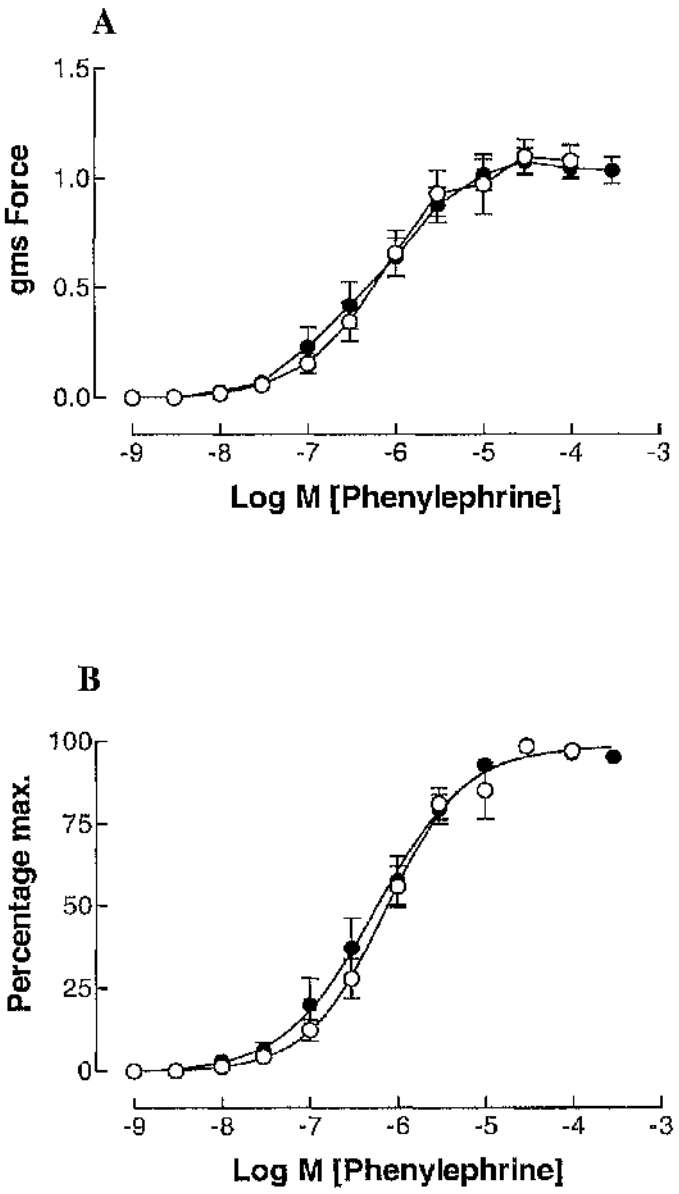



Figure 4.4: Cumulative phenylephrine response curves in tail arteries from 4-month old WT mice. **A** PE response curve before (○, n = 5), and after (●, n = 5) a cumulative UK curve. **B** PE response curves, expressed as a percentage of the maximum before (○, n = 5), and after (●, n = 5) a UK curve. Each point represents mean ± standard error.

UK14304, which had a maximum response of 1.04 ± 0.06gms Force. Thus, the size of contractions to phenylephrine were unaffected by previous exposure to UK14304. By expressing the data as a percentage of the maximum response, $pEC_{50}$ values could be calculated. In the curve carried out before a UK14304 curve, a $pEC_{50}$ value of 6.1 ± 0.05 was obtained, compared with a value of 6.2 ± 0.04 for the curve performed after. These $pEC_{50}$ values indicated that the sensitivity of the tail artery to phenylephrine is unchanged by UK14304 treatment ($p>0.05$).

Figure 4.5 shows the response to phenylephrine in a first curve, and the phenylephrine response curve constructed after an initial curve to UK14304, in tail arteries from four-month old D79N mice. Phenylephrine-induced contractions in the D79N tail arteries were generally smaller than those obtained in age matched WT arteries. Regardless of the smaller maximum when compared with the WT, the phenylephrine-mediated response was unaffected by prior exposure to UK14304. In phenylephrine curves, constructed before and after UK14304, the maximum contractile responses gained was of comparable size. The phenylephrine response curve, constructed before a cumulative UK14304 curve, had a maximum of 0.63 ± 0.05gms Force, compared with the maximum response after, which was 0.59 ± 0.09gms Force (figure 4.5 **A**). In figure 4.5 **B** the phenylephrine response curves have been expressed as a percentage of their own maximum. The $pEC_{50}$ value for the curve before a UK14034 response was 5.7 ± 0.02, compared with a $pEC_{50}$ value of 5.7 ± 0.02 calculated for the phenylephrine curve constructed after UK14304 treatment ($p>0.05$).

The phenylephrine response curves constructed after a first curve to UK14304 gave contractions that were of comparable size and sensitivity to those only exposed to

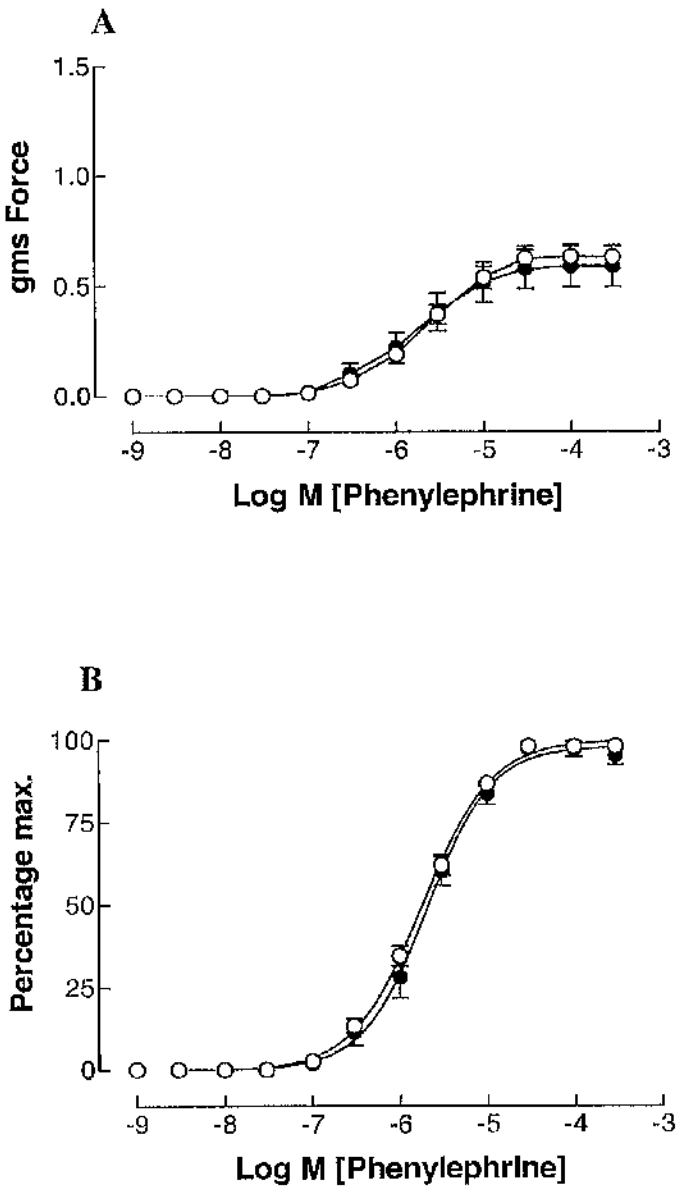



Figure 4.5: Cumulative phenylephrine response curves in tail arteries from 4-month old D79N mice. **A** PE response curve before (○, n = 5), and after (●, n = 5) a cumulative UK curve. **B** PE response curves, expressed as a percentage of control maximum before (○, n = 5), and after (●, n = 5) a UK curve. Each point represents mean ± standard error.

phenylephrine in the WT and D79N. This indicated that the desensitisation shown in figure 4.3 is specific to UK14304-induced responses, and was not caused by a generalised reduction in responsiveness to applied agonists.

The responses obtained in tail arteries from four-month old WT (figure 4.6 **A**) and D79N (figure 4.6 **B**) mice to the agonists phenylephrine and UK14304 are shown in figure 4.6. Comparison of the data showed that contractions to phenylephrine, in tail arteries from WT mice, were significantly greater than those obtained in tail arteries from age matched D79N mice ($p<0.0001$***). UK14304-induced contractions were smaller than phenylephrine-mediated vasoconstrictor responses in the WT and D79N. In both murine strains, a second response curve to UK14304 could not be obtained, because of profound, persistent, desensitisation of responses.

| | WT | D79N |
|---|---|---|
| | Maximum (gms Force) | Maximum (gms Force) |
| Cumulative (nifedipine -) | $0.33 \pm 0.07$ | $0.21 \pm 0.05$ |
| Cumulative (nifedipine +) | $0.27 \pm 0.03$ | $0.19 \pm 0.02$ |
| Second curve | $0.03 \pm 0.03$ | $0.10 \pm 0.02$ |
| Non-cumulative curve | $0.35 \pm 0.02$ | $0.36 \pm 0.02$ |

Table 4.1 The maximum UK response in tail arteries from the WT and D79N

4.3.4 The effect of rauwolscine on desensitised second curves to UK14304

Figure 4.7 shows the responses obtained to a second, cumulative UK14304 curve, constructed in the presence of rauwolscine ($3 \times 10^{-8}$M) in tail arteries from four-month

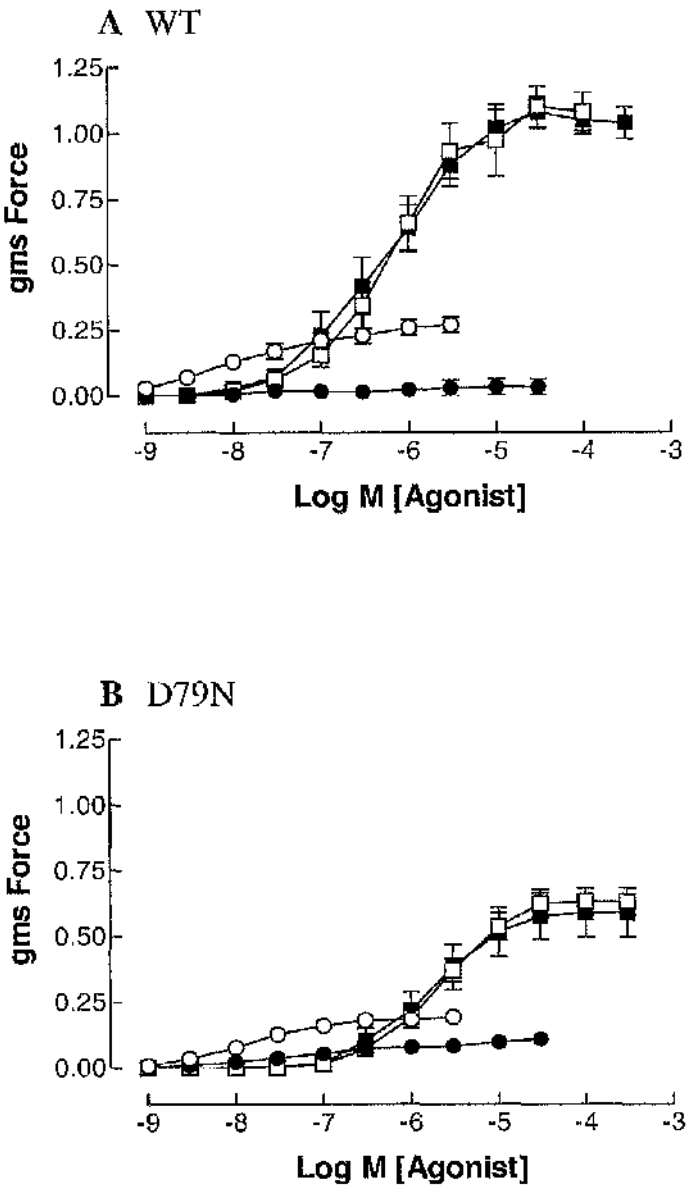



Figure 4.6: Agonist-induced responses in tail arteries from 4-month old WT and D79N mice. **A** A first (○) and second (●) cumulative UK curve, a first (□) and second (■) cumulative PE curve, respectively. **A** Agonist curves in WT arteries, n = 12, 6, 5, and 5. **B** Agonist curves in D79N arteries, n = 12, 6, 5, and 5. Each point represents mean ± standard error.

old WT (figure 4.7 **A**) and D79N (figure 4.7 **B**) mice. In WT arteries, a second cumulative response curve to UK14304 gave no measurable response. However, in the presence of rauwolscine, a second could be obtained. With rauwolscine, the UK14304-induced maximum was significantly greater at 0.47 ± 0.04gms Force than that of the first UK14304 curve maximum of 0.27 ± 0.03 ($p = 0.0004$***).

Responses in tail arteries from D79N also exhibit desensitisation in a second response curve, but responses in the first control curve were smaller than that of the WT. A second, cumulative curve to UK14304 was impossible. However, when tissues were incubated with rauwolscine ($3 \times 10^{-8}$M) for thirty minutes, a second UK14304 curve could be obtained. The maximum response gained for the second curve to UK14304, alone was 0.10 ± 0.02gms Force, compared with that obtained in a second curve in the presence of rauwolscine, which was 0.26 ± 0.03gms Force, and significantly greater than the first curve maximum ($p = 0.015$*).

Responses in a first curve to UK14304 alone, and a second curve constructed in the presence of rauwolscine, have been expressed as a percentage of the first curve maximum. The results gained for tail arteries from four-month old WT (figure 4.8 **A**) and D79N (figure 4.8 **B**) mice are shown in figure 4.8. In WT arteries, the maximum contraction obtained in the presence of rauwolscine reached 150.5 ± 13.5 % of the maximum gained in the control curve. Responses in tail arteries from D79N mice were also potentiated in maximum, and in the presence of rauwolscine reached 136.5 ± 10.0 % of the first UK14304 curve maximum. Although potentiated in maximum in the WT and D79N the presence of rauwolscine shifted the response curve (at lower agonist concentrations) to the right, indicative of a shift in sensitivity. In the WT, the first curve

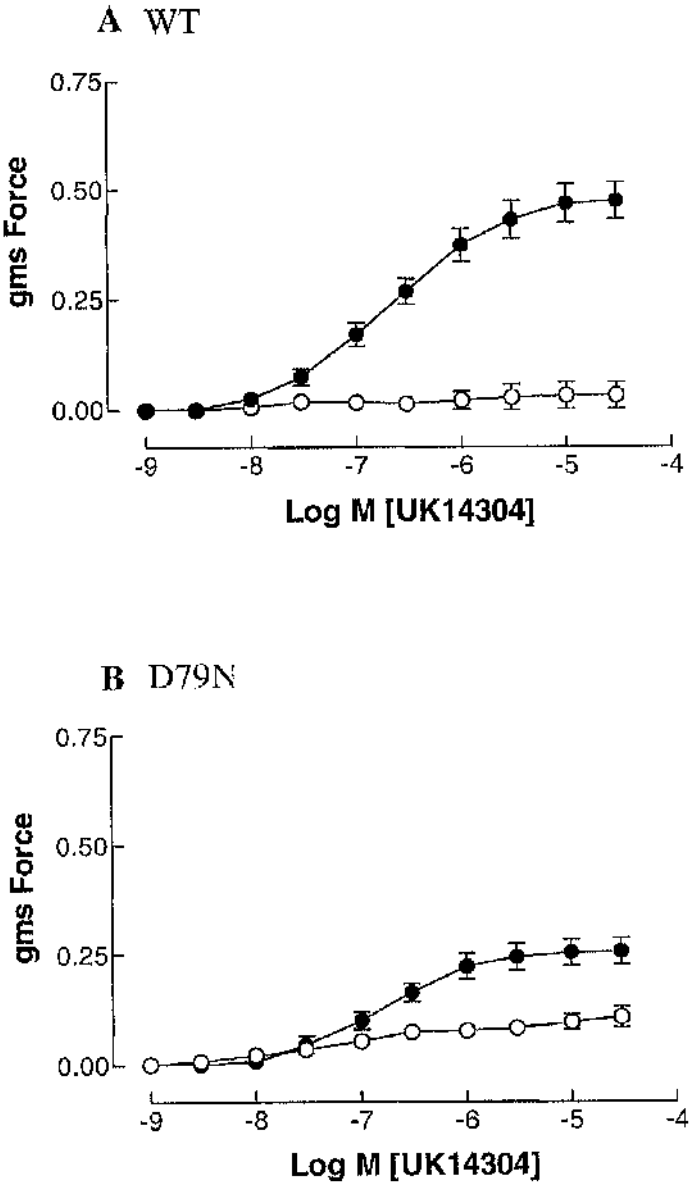



Figure 4.7: The effect of rauwolscine (3 x $10^{-8}$M) on a second cumulative UK response curves in tail arteries from 4-month old WT and D79N. **A** A second UK curve alone (○, n = 6 ), and with rauwolscine (●, n = 6) in WT arteries. **B** A second UK curve alone (○, n = 6 ), and with rauwolscine (●, n = 6). Each point represents mean ± standard error.

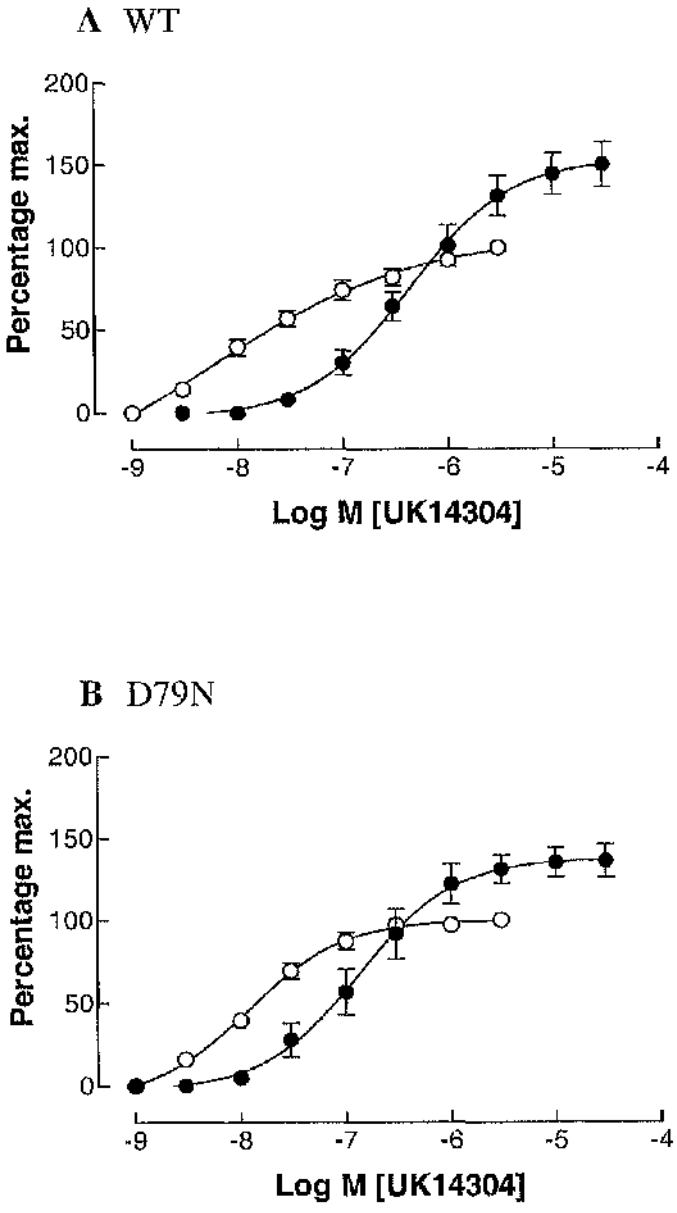



Figure 4.8: Responses to cumulative UK curves in tail arteries from 4-month old WT and D79N mice, expressed as percentage of the first curve maximum. **A** The UK response in a first (○, n =12 ) and second curve with rauwolscine (3 x $10^{-8}$M, ●, n = 6) in WT. **B** The UK response in a first (○, n = 12) and second curve with rauwolscine (●, n = 6) in D79N. Each point represents mean ± standard error.

has a $pEC_{50}$ of 8.2 ± 0.3, which was significantly higher than the value gained for rauwolscine, of 6.6 ± 0.02 (p = 0.0002***). In the D79N first curve, the $pEC_{50}$ was 8.1 ± 0.3, which was significantly higher than the $pEC_{50}$ value of 6.55 ± 0.09, gained in the presence of rauwolscine (p<0.0001***).

4.3.5 Cumulative versus non-cumulative drug administration

Contractile responses to UK14304 in the cumulative response tended to 'fall off' at high agonist concentrations. In constructing the non-cumulative curve, tail arteries were exposed to each UK14304 concentration for approximately three minutes; responses constructed in this way, were not susceptible to a reduction in response over time, unlike the cumulative response. Figure 4.9 shows the responses gained in cumulative and non-cumulative curves to UK14304, constructed in tail arteries from four-month old WT (figure 4.9 **A**) and D79N (figure 4.9 **B**) mice. In WT arteries responses tended to be slightly greater in size, when UK14304 is administered non-cumulatively. The maximum contraction obtained for the cumulative curves was 0.27 ± 0.03gms Force, compared with a maximum contraction of 0.35 ± 0.02gms Force in the curve constructed non-cumulatively. Contractions gained in arteries from four-month old D79N mice were significantly greater in size at all agonist concentrations tested, with the maximum contraction in a cumulative curve being 0.19 ± 0.02gms Force, while in the non-cumulative curve, the maximum response of 0.36 ± 0.02gms Force was significantly higher (p<0.0001***).

Constructing a UK14304 response curve non-cumulatively, lead to a significant enhancement of the contractile responses in tail arteries from D79N mice. Contractions of the WT tail artery had a slight reduction in variability, but were not significantly

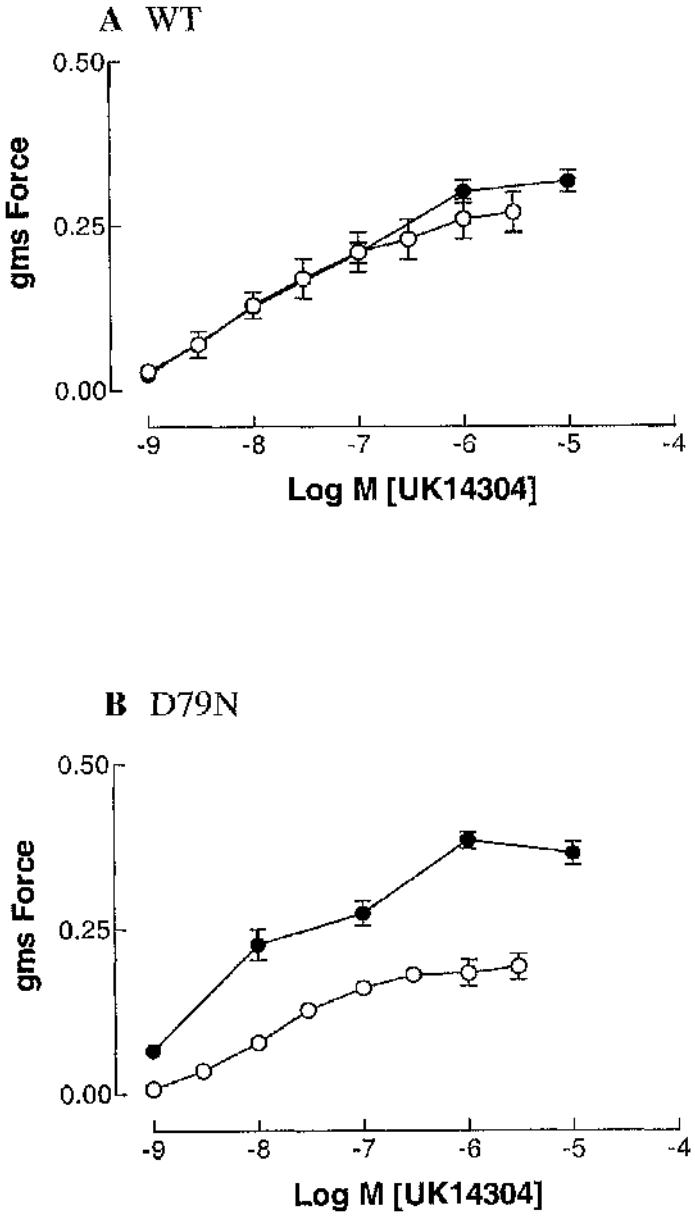



Figure 4.9: UK response curves in tail arteries from 4-month old WT and D79N mice (Both first curves). **A** A cumulative (○, n = 9) and non-cumulative (●, n = 11) UK response curve in WT arteries. **B** A cumulative (○, n = 7) and non-cumulative (●, n = 6) UK response curve in D79N arteries. Each point represents mean ± standard error.

potentiated in size, when curves were constructed non-cumulatively. Given this, and the fact that construction of a second curve is prevented by desensitisation, the decision was taken to study the effects of antagonists on first, non-cumulative curves, as opposed to the traditional method of using, consecutive, cumulative response curves.

Mounted segments of tail artery were incubated with rauwolscine, at a concentration of $3 \times 10^{-8}$M, before constructing a non-cumulative UK14304 response curve. The responses gained in tail arteries from four-month old WT (figure 4.10 **A**) and D79N (figure 4.10 **B**) mice are shown in Figure 4.10. In WT and D79N arteries rauwolscine caused a rightward shift in the concentration response curve to UK14304, without causing a reduction in the contractile maximum.

In figure 4.11 the responses to non-cumulative UK14304 alone, and with rauwolscine ($3 \times 10^{-8}$M), have been expressed as a percentage of their own maximum to determine $pEC_{50}$ values. In arteries from WT mice (figure 4.11 **A**) the $pEC_{50}$ value for the control curve was $7.5 \pm 0.3$, compared with a lower $pEC_{50}$ value of $6.5 \pm 0.07$ in the presence of rauwolscine, a difference that was highly significant ($p = 0.0005$***). This shift in the $pEC_{50}$ value indicates that rauwolscine is antagonising UK14304-induced contractions. As only a single concentration of rauwolscine was used, a $pA_2$ value was not determined, but a $pK_B$ value of $8.5 \pm 0.21$ was calculated by determining the shift in the response in unpaired vessels. Rauwolscine had a similar effect in D79N tail arteries (figure 4.11 **B**). A $pEC_{50}$ value of $8.1 \pm 0.3$ was calculated for the control curve and compared with a value of $6.5 \pm 0.09$ in the presence of rauwolscine ($p = 0.0002$***). The $pK_B$ value calculated for rauwolscine in the D79N tail artery was $8.7 \pm 0.30$.

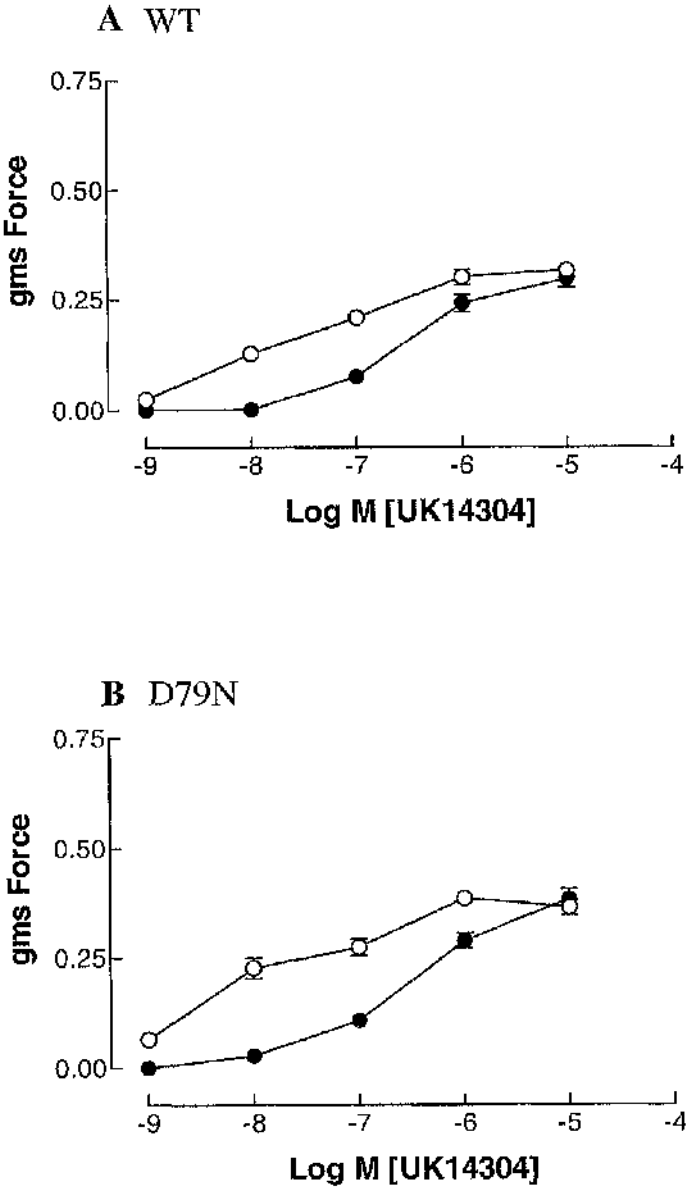



Figure 4.10: Non-cumulative UK response curves in unpaired tail arteries from 4-month old WT and D79N mice. **A** The UK response alone (○, n = 12) and with rauwolscine (3 x $10^{-8}$M, ●, n = 6) in WT arteries. **B** The UK response alone (○, n = 6) and with rauwolscine (●, n = 6) in D79N arteries. Each point represents mean ± standard error.

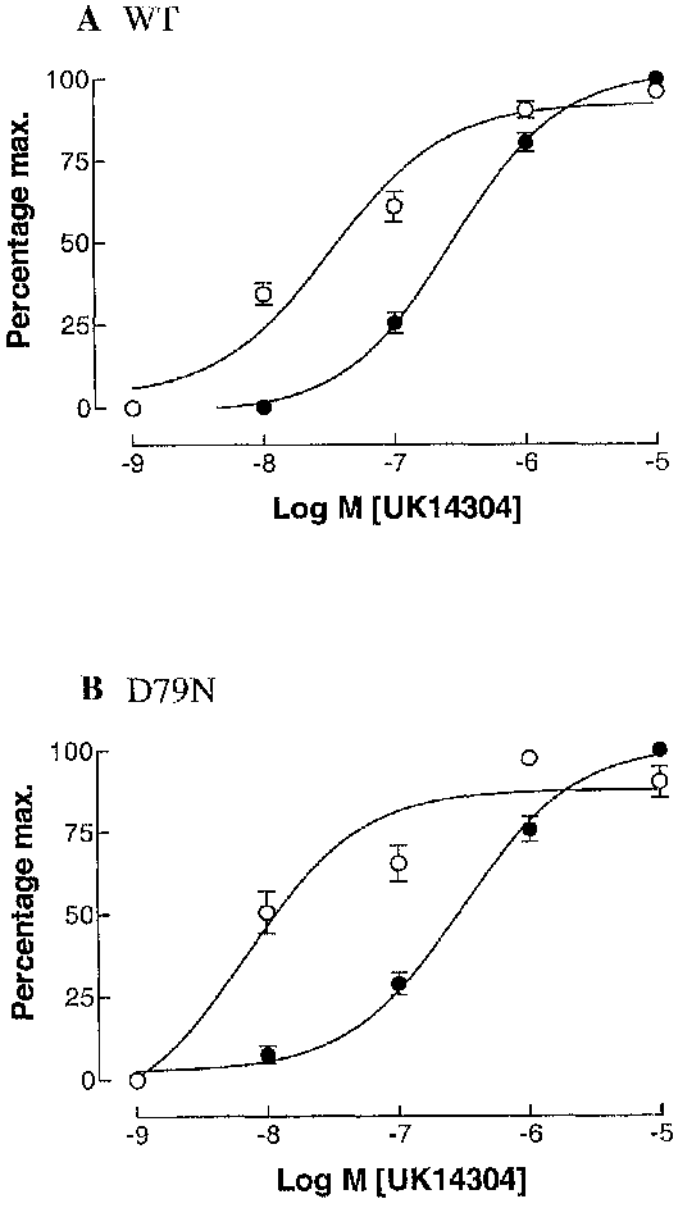



Figure 4.11: Non-cumulative UK response curves in unpaired tail arteries from 4-month old WT and D79N mice, expressed as a percentage of the maximum. **A** The UK response alone (○, n = 12) and with rauwolscine (●, n = 6) in WT arteries. **B** The UK response alone (○, n = 6) and with rauwolscine (●, n = 6) in D79N arteries. Each point represents mean ± standard error.

4.3.6 The effect of prazosin on non-cumulative UK14304 curves in murine tail arteries

Figure 4.12 shows responses gained to non-cumulative UK14304 curves, alone, and in the presence of prazosin (3 x $10^{-8}$M), in tail arteries from four-month old WT (figure 4.12 **A**) and D79N (figure 4.12 **B**) mice. The maximum contraction to UK14304, achieved in arteries from WT and D79N mice was unaffected by the presence of prazosin. In WT arteries, contractions reached a maximum response of 0.31 ± 0.01gms Force in controls, compared with a maximum of 0.28 ± 0.02gms Force, gained in the presence of prazosin. Comparison of the $pEC_{50}$ values gained for a control curve, with those incubated with prazosin showed no significant shift in the responses gained ($p = 0.10$). Prazosin, unlike rauwolscine, did not cause a rightward shift in the concentration response curve. This suggested that the vasoconstrictor responses induced by UK14304 are not due to the non-selective stimulation of $\alpha_1$-adrenoceptors, even at high agonist concentrations.

A similar picture emerged for the D79N mouse. The maximum contraction gained in the control curve was 0.37 ± 0.03gms Force, compared with the maximum response gained in the presence of prazosin of 0.38 ± 0.04gms Force. Prazosin did not cause a rightward shift in the concentration response curve to UK14304. The $pEC_{50}$ values gained were no different from that of a control curve ($p = 0.52$). Again, this suggested that, even at high agonists concentrations, UK14304 did not stimulate $\alpha_1$-adrenoceptors.

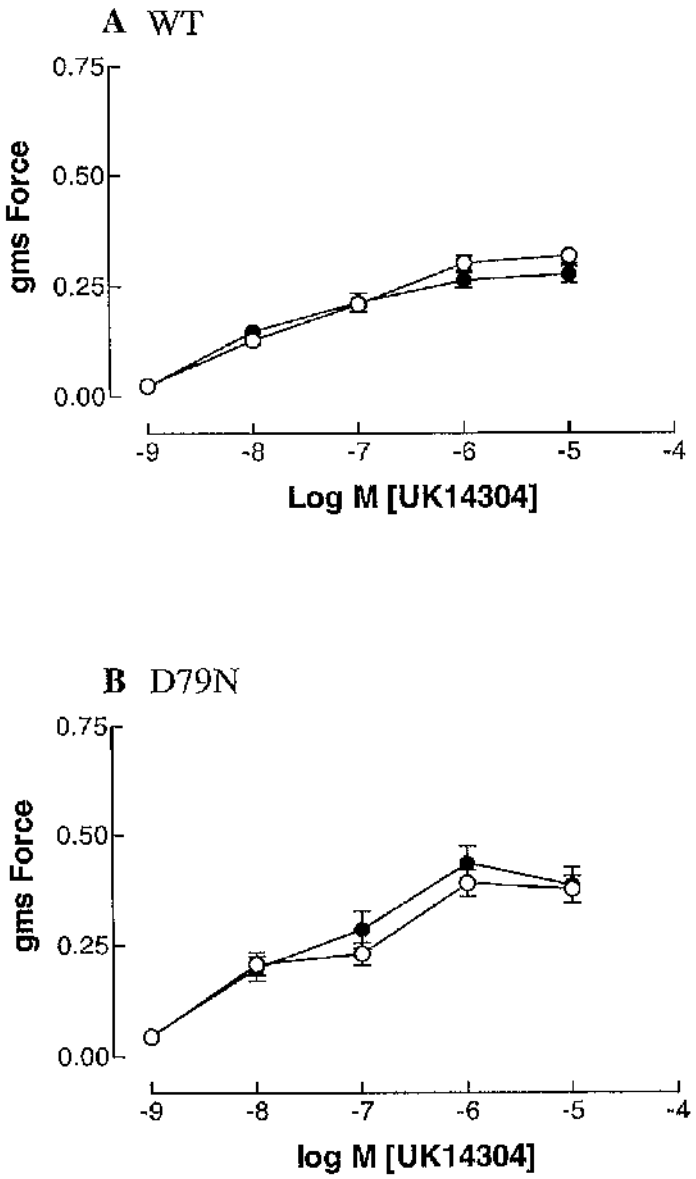



Figure 4.12: Non-cumulative UK response curves in unpaired tail arteries from 4-month WT and D79N mice. **A** The UK response alone (○, n = 12) and with prazosin ($3 \times 10^{-8}$M, ●, n = 6) in WT arteries. **B** The UK response alone (○, n = 4) and with prazosin (●, n = 4) in D79N arteries. Each point represents mean ± standard error.

## 4.4 Discussion

### Low concentrations of nifedipine abolish rhythmic contractility

Mounted segments of tail artery from WT and D79N mice develop rhythmic contractions in response to U46619 and the adrenergic agonists phenylephrine and UK14304. Incubation with the L type calcium channel antagonist, nifedipine ($1 \times 10^{-7}$M) prevents the development of rhythmic activity. In the presence of nifedipine, agonist-induced contractions still occur, but are slower to develop. Vascular contractions depend, in part, on calcium wave activity, which is regulated by the sarcoplasmic reticulum and mitochondria [Sward et al, 2002]. Exogenous agonists can stimulate calcium oscillations in vascular smooth muscle cells *in vitro*. The sarcloplasmic reticulum develops rhythmic oscillations in calcium release in response to $InsP_3$ [Ruehlmann et al, 2000]. The results gained with nifedipine, suggest that rhythmic contractions of the murine tail depend, in part, on extracellular calcium. In electrically excitable cells, stimulation of $\alpha_2$-adrenoceptors inhibits voltage-operated $Ca^{2+}$ channels, which is in part responsible for the inhibition of noradrenaline release [Holz et al, 1986]. In the rat tail artery and canine saphenous vein, $Ca^{2+}$ channel antagonists attenuate $\alpha_2$-adrenoceptor-mediated contractions, and when extracellular $Ca^{2+}$ is removed from the surrounding bathing solution, responses are abolished [Medgett & Rajanayagam, 1984]. This provides evidence that $\alpha_2$-adrenoceptor-mediated contractions rely upon the influx of extracellular calcium ions, a process that nifedipine blocks *in vitro* [Dunn et al, 1991]. By reducing the influx of calcium ions through L type channels in the mouse tail artery, nifedipine successfully blocks unwanted rhythmic movements, and in doing so provides experimental conditions that are suitable for further pharmacological analysis.

Agonist-induced receptor desensitisation

UK14304-induced contractions of WT and D79N tail artery are subject to profound, persistent, agonist-induced desensitisation. The desensitisation that occurs persists, even when UK14304 has been removed from the surrounding bathing solution, suggesting that the process of desensitisation begins during construction of the first UK14304 response curve. A series of washes with fresh Krebs and a rest period of thirty-minutes separate first and second curves. Yet a second, cumulative response to UK14304 is still not possible.

Agonist-dependent receptor desensitisation, like that occurring in the UK14304 response, is mediated by G protein coupled receptor kinases and arrestin molecules [Ferguson et al, 1997]. Once activated, receptor kinases and arrestins regulate the uncoupling and internalisation of agonist bound receptors. Active, agonist-bound receptors are internalised when they have been targeted for sequestration. Internalisation proceeds via dynamin-dependent clathrin coated vesicle endocytosis. A process that only proceeds when receptors tagged for internalisation have been phosphorylated, and are bound by arrestin molecules [Ferguson et al, 1997].

Pre and postjunctional $\alpha_2$-adrenoceptors are both susceptible to drug-induced desensitisation. Presynaptic $\alpha_2$-adrenoceptors located in the mouse atria are prone to receptor desensitisation [Bucheler et al, 2001]. $\alpha_{2C}$-adrenoceptor-mediated autoinhibition of noradrenaline release from nerve terminals is attenuated by prolonged expose to $\alpha_2$-selective agonists [Bucheler et al, 2002]. Desensitisation of postjunctional $\alpha_2$-adrenoceptors also occurs *in vitro*, evidenced by a progressive reduction in the noradrenaline-mediated response curve maximum in sequential concentration curves

[Rodriguez-Martinez et al, 1999]. A progressive reduction in the maximum vasoconstrictor response achieved is indicative of agonist-induced receptor desensitisation.

The $\alpha_1$-adrenoceptor-mediated response in the tail is free from agonist-induced desensitisation

The magnitude of the response gained and the sensitivity of the tissue to phenylephrine are unaffected by repetitive stimulation of $\alpha_1$-adrenoceptors, and by previous exposure to UK14304. Consecutive responses to the $\alpha_1$-selective agonist phenylephrine can be obtained in the WT and D79N. This suggests that the profound desensitisation that occurs with UK14304 is confined to $\alpha_2$-adrenoceptors, and does not result from a generalised reduction in tissue contractility.

The non-cumulative response to UK14304 overcomes reduced contractility in the D79N. All of the response curves shown in chapter three were constructed cumulatively. In each protocol tested, maximal contractions of the D79N are significantly smaller than those obtained in the WT. This initially led to the conclusion that the $\alpha_{2A/D}$-adrenoceptor is a major contributor to $\alpha_2$-adrenoceptor-mediated contractions of the murine tail artery. However, the results shown here refute this.

The non-cumulative response to UK14304 in the D79N is significantly greater than the response gained in a cumulative curve, giving a response that is of comparable magnitude to that of the WT. The size of contractile responses in WT arteries is unaffected by the method in which UK14304 is administered.

Incubation with rauwolscine permits a second cumulative curve with a potentiated maximum response

Consecutive, cumulative UK14304 response curves were constructed alone, and in the presence of the $\alpha_2$-selective antagonist, rauwolscine ($3 \times 10^{-8}$M). The control curve gained has already been shown (figure 4.1), and is subject to profound agonist-induced desensitisation. However, arteries incubated with rauwolscine have quite different responses.

In the presence of rauwolscine, the construction of a second cumulative response is possible. The responses gained are significantly potentiated in maximum, but are shifted rightward at lower agonist concentrations. So how does rauwolscine prevent, and essentially reverse UK14304-mediated desensitisation?

Stimulation of UK14304 sensitive receptors in the tail artery may lead to a response that is permanently 'switched on'. The binding of rauwolscine, which has affinity for the receptor without having efficacy, may 'switch off' the second messenger cascade activated by UK14304. Alternatively, the binding of the antagonist to active receptors may retain them at the plasma membrane, thus preventing internalisation and sequestration to intracellular binding sites. Adrenoceptor antagonists can alter the activation state of constitutively active G-protein coupled receptors [Zhu et al, 2000]. Alternatively, rauwolscine may 'switch on' a response that is inhibited by UK14304-mediated stimulation of $\alpha_2$-adrenoceptors. This would suggest that $\alpha_2$-adrenoceptors are constitutively turned off, a state which is overcome by U46619-induced tone.

The potentiation in size of the response to UK14304 is more pronounced in the WT, than the D79N, suggesting two possibilities. It may be that the $\alpha_{2A/D}$-adrenoceptor is a major contributor to vasoconstrictor responses of the murine tail artery. Alternatively, UK14304 may stimulate the release of a relaxing factor that acts to oppose the development of contractile responses. In the absence of a functional $\alpha_{2A/D}$-adrenoceptor, responses that oppose contractions may proceed unchecked. This suggests a regulatory role for the $\alpha_{2A/D}$ in mediating contractility at the vessel level.

Rauwolscine antagonises the UK14304 response in first curves constructed non-cumulatively

In an attempt to standardise the experimental conditions used to study $\alpha_2$-adrenoceptor-mediated responses in the tail artery, the decision was taken that first curves, constructed non-cumulatively are more suitable for pharmacological analysis. In WT and D79N arteries, rauwolscine shifts the UK14304-mediated response curve rightward, without affecting the maximum agonist-induced contraction. Under these experimental conditions, rauwolscine shifts the UK14304 response curve, therefore reducing the $pEC_{50}$ value gained. This demonstrates that UK14304-mediated vasoconstrictor responses result from stimulation of $\alpha_2$-adrenoceptors located on the vascular wall of the murine tail artery.

At high agonist concentrations, drugs marketed as subtype-selective, can stimulate other structurally related receptors. To determine if the contraction to UK14304 at high concentrations can be attributed to stimulation of vascular $\alpha_1$-adrenoceptors, the non-selective, potent $\alpha_1$-antagonist prazosin was used. Prazosin ($3 \times 10^{-8}$M) failed to shift the UK14304 response, and had no effect on the magnitude of contractions gained in the

WT and D79N. Although ineffective against the UK14304-mediated response, prazosin antagonises phenylephrine-induced contractions of the murine tail artery, causing a rightward shift in the concentration curve and a significant reduction in the maximum response at high antagonist concentrations (chapter seven). These results provide evidence that, at least in the murine tail artery, UK14304-mediated responses are not attributable to vascular $\alpha_1$-adrenoceptors.

# Chapter five

## The effect of cold temperatures on contractions of cutaneous blood vessels from WT and D79N mice

## 5.1 Introduction

Cutaneous blood vessels are primed to respond to subtle, physiological changes in the external environment. Most of the changes that occur are protective, and function to preserve vital physiological functions from cold-induced damage.

$\alpha_2$-adrenoceptors are expressed at high levels in cutaneous resistance arteries and veins. It is the high expression levels of $\alpha_2$-adrenoceptors at these sites that first lead to the hypothesis that these receptors may have a role in thermoregulation [Flavahan & Vanhoutte, 1986].

Cold temperatures stimulate a protective reflex that leads to an increase in sympathetic outflow and elevated levels of catecholamines, such as noradrenaline. In addition to the central effects induced by cold temperatures, local changes also occur [Vanhoutte, 1980]. Local alterations are thought to lead to an enhancement of vasoconstrictor responses at cold temperatures (at $28^0$C), by activating quiescent $\alpha_2$-adrenoceptors of the $\alpha_{2C}$ subtype [Chotani et al, 2000]. The model system for these studies was the mouse tail artery.

However, under normal physiological conditions the surface temperature of the rat tail artery will be significantly lower than core body temperature [Redfern et al, 1995], and will reflect the temperature of the external surroundings. This therefore raises the question of whether the $\alpha_{2C}$-adrenoceptor participates in vasoconstrictor responses "normal" physiological temperatures. This leads me to ask the question; "Should the study of functional responses in the rat and mouse tail artery be carried out at room temperature, as opposed to core body temperature of $37^0$C?"

The tail artery of the rat and mouse is believed to be involved in thermoregulation, so its sensitivity to changes in temperature may relate somehow to this function. The tail artery has been used here to study the effects of temperature on $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated responses. The D79N mouse presents the opportunity to study the effect of cold on $\alpha_2$-mediated responses in the absence of the $\alpha_{2A/D}$ and, therefore, potentially in a simpler situation regarding participation of different $\alpha_2$-subtypes

## 5.2 Methods

The middle section of the tail artery from WT and D79N weighting 27.4-32.9gms mice was dissected and mounted in 5ml, stainless steel Mulvany/Halpern myograph baths (as described in detail in Chapter two).

### 5.2.1 Effect of temperature on $\alpha_1$ and $\alpha_2$-adrenoceptor-mediated contractions of the murine tail artery

To determine the effect of cold temperatures on phenylephrine and UK14304-induced contractions, two experimental protocols were employed. The first series of these experiments was carried out at normal physiological temperatures of $37^0$C. In the second protocol, arterial rings were allowed to equilibrate in myograph baths at room temperature in a thermostatically controlled room, where the ambient temperature was set to $22^0$C (actual temperature ranged from 21.5-23.5$^0$C). Fresh Krebs and any drugs used were all stored at room temperature for the duration of the protocol.

### 5.2.2 Wake-up protocol

Mounted arterial rings were challenged with three concentrations of the non-selective adrenergic agonist, noradrenaline. Two concentrations of the drug were employed. The first ($1 \times 10^{-5}$M) was used to give a contraction near to the adrenergic maximum. When a stable contractile plateau was obtained, vessels were then washed four times, over a fifteen minute period, before the procedure was repeated with the same agonist concentration. A second lower concentration of noradrenaline ($1 \times 10^{-6}$M) was then added, the contraction was allowed to reach a plateau, and acetylcholine ($3 \times 10^{-6}$M) was used to check that the endothelium of mounted vascular rings was intact.

## 5.3 Results

### 5.3.1 Agonists-induced responses in the WT and D79N tail artery

Figure 5.1 shows the results gained for cumulative concentration curves to three agonists, in tail arteries from four-month old WT (figure 5.1 **A**) and D79N (figure 5.1 **B**) mice. Response curves to phenylephrine, noradrenaline and 5HT were constructed in mounted tail arteries to determine the potency order of these agonists in each murine strain. This was an attempt to determine any changes in the general sensitivity to agonists in the D79N mouse.

In arteries from WT (figure 5.1 **A**) and D79N (figure 5.1 **B**) mice, all three agonists produced concentration-dependent contractions. Within each strain, the sensitivity and maxima of curves to phenylephrine and noradrenaline were of comparable size with the phenylephrine maximum being 1.08 ± 0.14gms Force, compared with the noradrenaline maximum of 1.12 ± 0.13gms Force ($p>0.05$). In both strains the response to 5HT was susceptible to desensitisation at high agonist concentrations, which gave a bell-shaped response curve. For example, in the WT the peak contraction occurred at a concentration of $3 \times 10^{-6}$M and yielded a contraction of 0.84 ± 0.14gms Force. Each additional cumulative drug administration caused a further reduction in the contractile response, and at the highest 5HT concentration the contraction was reduced to 0.44 ± 0.18gms Force.

Agonist-induced responses in arteries from four-month old D79N mice tended to be smaller than those obtained in arteries from WT mice. The maximum response gained in a noradrenaline and phenylephrine response curve were significantly smaller than those gained in the WT (NA, $p = 0.04$*, PE, $p = 0.03$*). Both WT and D79N tissues

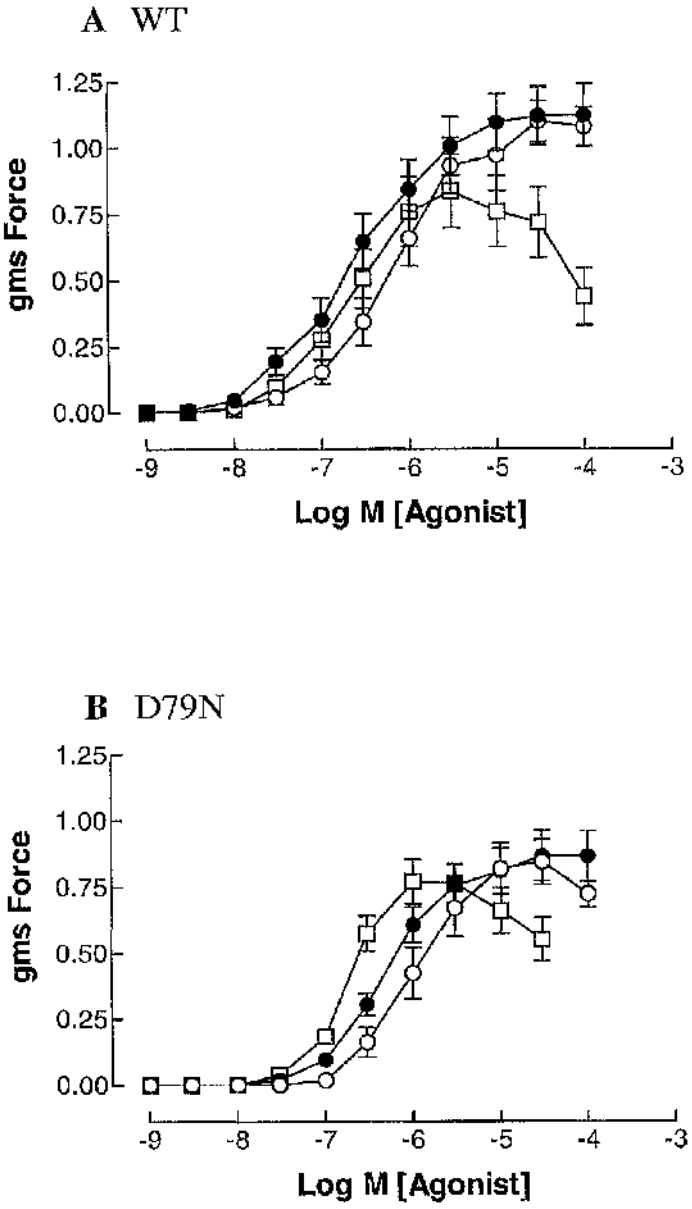



Figure 5.1: Agonist response curves in tail arteries from 4-month old WT and D79N mice. **A** PE (○, n = 12), NA (●, n = 7) and 5HT (□, n = 8) response curves in WT arteries. **B** PE (○, n = 6), NA (●, n = 6) and 5HT (□, n = 6) response curves in D79N arteries. Each point represents mean ± standard error.

were more sensitive to noradrenaline than to phenylephrine. The sizes of the contractile responses to each agonist are recorded in table 5.1.

| | WT | | D79N | |
|---|---|---|---|---|
| | Maximum | $pEC_{50}$ | Maximum | $pEC_{50}$ |
| Phenylephrine | 1.08 ± 0.14 n = 12 | 6.1 ± 0.05 | 0.84 ± 0.08* n = 6 | 5.9 ± 0.07 |
| Noradrenaline | 1.12 ± 0.13 n = 7 | 6.6 ± 0.04 | 0.87 ± 0.09* n = 6 | 6.3 ± 0.02 |
| 5HT | 0.84 ± 0.14 n = 8 | 6.8 ± 0.10 | 0.78 ± 0.08 n = 6 | 6.8 ± 0.09 |
| UK at $37^0$C(after PE) | 0.21 ± 0.03 n = 5 | 9.1 ± 0.5 | 0.22 ± 0.05 n = 6 | 9.0 ± 0.12 |
| UK at $22^0$C (after PE) | 0.54 ± 0.06* n = 7 | 8.5 ± 0.09 | 0.43 ± 0.04* n = 9 | 8.4 ± 0.15 |
| NA wake up at $37^0$C | 1.09 ± 0.03 n = 5 | - | 0.79 ± 0.09 n = 6 | - |
| NA wake up at $22^0$C | 1.27 ± 0.12*, n = 7 | - | 1.16 ± 0.08*, n = 9 | - |

Table 5.1 Response curves maxima and $pEC_{50}$ values in WT and D79N tail artery

Figure 5.2 shows the agonist response curves in tail arteries from four-month old WT (figure 5.2 **A**) and D79N (figure 5.2 **B**) mice to PE, NA and 5HT, expressed as a percentage of their own maximal response. In WT arteries, the agonist potency order was 5HT > NA > PE. The response curve to 5HT yielded a $pEC_{50}$ value of 6.8 ± 0.10, the NA curve was 6.6 ± 0.03, and the PE curve had the lowest $pEC_{50}$ value at 6.1 ± 0.05. In tail arteries from D79N mice, the agonist potency order was 5HT > NA > PE, and was the same as that gained for the WT. The $pEC_{50}$ values were as follows, 5HT 6.8 ± 0.09, NA 6.3 ± 0.02, and PE 6.0 ± 0.02. Although the potency order was the same as that of the WT, the $pEC_{50}$ value gained for the noradrenaline response curve was lower than the WT. A summary of maximal contractile responses gained and the $pEC_{50}$ values obtained for phenylephrine, noradrenaline, and 5HT in both strains are contained

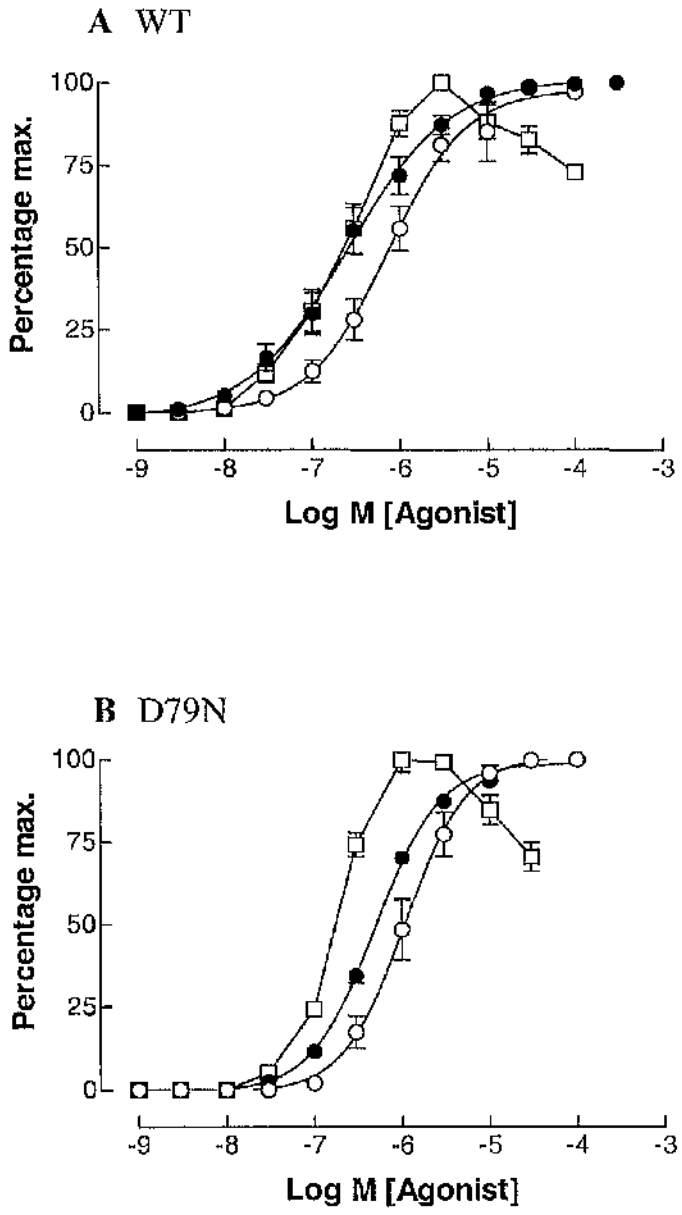



Figure 5.2: Agonist response curves in tail arteries from 4-month old WT and D79N mice, expressed as a percentage of their own maximum. **A** PE (○, n = 12), NA (●, n = 7) , and 5HT ( □, n = 8) response curves in WT arteries. **B** PE (○, n = 6), NA (●, n = 6) , and 5HT ( □, n = 8) response curves in D79N arteries. Each point represents mean ± standard error.

in table 5.1, in addition to the UK curve maxima at 37 and 22$^0$C and the noradrenaline wake-up response at both temperatures.

5.3.2 Effect of temperature on phenylephrine-induced contractions of WT and D79N arteries

Contractile responses to the $\alpha_1$-selective agonist phenylephrine were investigated at 37$^0$C and at 22$^0$C, in myograph mounted tail arterial segments from WT and D79N mice. Phenylephrine-induced contractions at room temperature are shown in figure 5.3. The contractile maxima at 22$^0$C, in arteries from both strains were of comparable size. In the WT the greatest response gained was 0.94 ± 0.08gms Force, and in the D79N, the maximal contractile effect of phenylephrine gave a response of 0.98 ± 0.10gms Force.

Figure 5.3 illustrates the effect that temperature had on phenylephrine responses in both murine strains. The maximum contraction in WT arteries (figure 5.3 **A**) was unaffected by temperature (22$^0$C maximum 0.94 ± 0.08gms Force, and the maximum at 37$^0$C was 1.1 ± 0.08gms Force), unlike the D79N (figure 5.3 **B**), where the maximum at 22$^0$C was significantly greater than the response at 37$^0$C. In the D79N, the maximum at 22$^0$C was 0.98 ± 0.10gms Force compared with a maximum of 0.63 ± 0.05gms Force at 37$^0$C. Thus when a comparison is made in the WT, a reduction in experimental temperature had no effect on the response gained. However, in arteries taken from D79N mice the maximum responses to phenylephrine was significantly larger at 22$^0$C compared with that at 37$^0$C ($p = 0.012^{**}$).

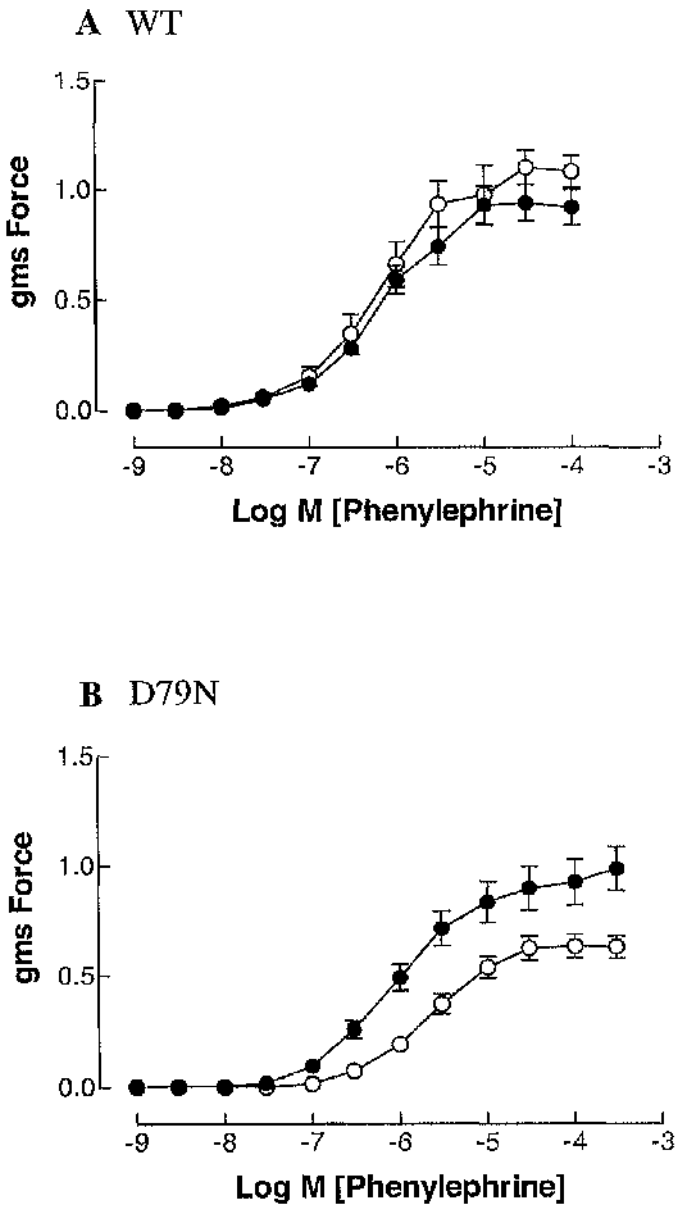



Figure 5.3: Phenylephrine response curves in tail arteries from 4-month old WT and D79N arteries at 37$^0$C and 22$^0$C. **A** PE response curves at 37$^0$C (○, n = 5), and 22$^0$C ( ●, n = 7) in WT arteries. **B** PE response curves at 37$^0$C (○, n = 5), and 22$^0$C ( ●, n = 9) in D79N arteries. Each point represents mean ± standard error.

5.3.3 The effect of temperature on UK14304-induced contraction in WT and D79N arteries

Agonist response curve to UK14304 and phenylephrine (constructed at $22^0$C) were compared in tail arteries from WT (figure 5.4 **A**) and D79N (figure 5.4 **B**) mice. The UK14304 response curve was non-sigmoid in shape, and the contractions obtained were small in comparison to those induced by phenylephrine. The maximum contraction obtained in WT arteries was 0.20 ± 0.03gms Force, while that achieved in D79N arteries was 0.22 ± 0.03gms Force. In contrast, the phenylephrine concentration curves gave a sigmoid curve, which tended to reach a plateau at the highest agonist concentrations tested.

Figure 5.5 shows the UK14304 response at room temperature in WT (figure 5.5 **A**) and D79N (figure 5.5 **B**) tail arteries, compared with the response gained at $37^0$C. In each strain responses follow the same pattern and were of comparable size. In the WT, the maximum contraction at $37^0$C was 0.23 ± 0.04gms Force compared with 0.20 ± 0.03gms Force at $22^0$C. In the D79N, the maximum at $37^0$C was 0.22 ± 0.05gms Force compared with 0.22 ± 0.03gms Force at $22^0$C.

In addition to having an effect on the UK and PE response curves, a reduction in temperature also caused a significant enhancement of the sighting noradrenaline response ($1 \times 10^{-5}$M), tested during the wake-up protocol. In both strains, a reduction in temperature to $22^0$C caused a significant increase in the response gained. In the WT the response at $37^0$C was 1.09 ± 0.02gms Force, compared with 1.27 ± 0.04gms Force at $22^0$C ($p = 0.014^*$). In D79N tail arteries the response gained at $37^0$C was 0.79 ±

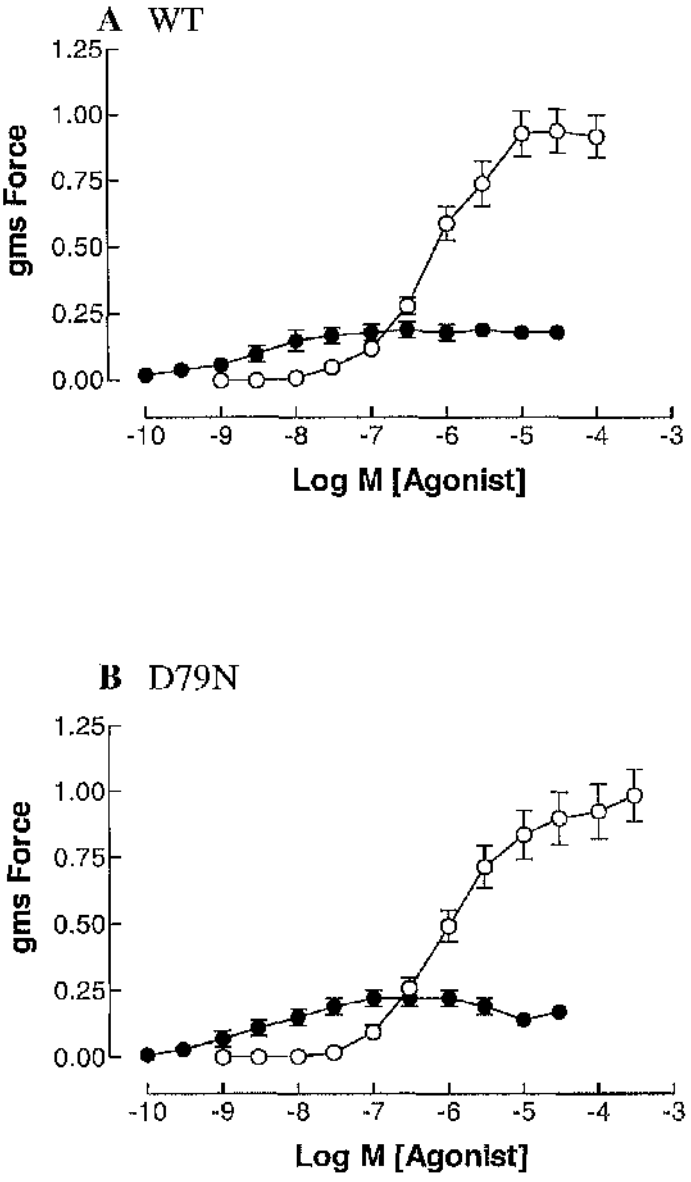



Figure 5.4: Agonist response curves in tail arteries from 4-month old WT and D79N mice at 22$^0$C. **A** PE (○, n = 7) and UK (●, n = 7) response curves in the WT. **B** PE (○, n = 9) and UK (●, n = 9) response curves in the D79N. Each point represents mean ± standard error.

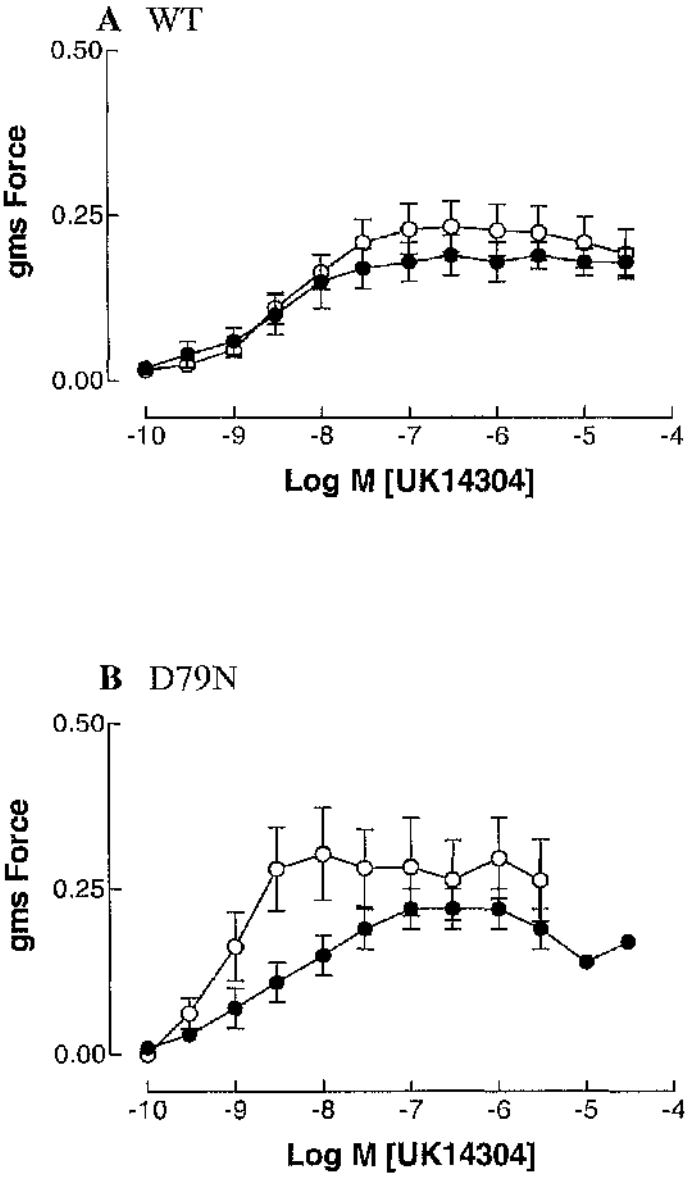



Figure 5.5: UK response curves in tail arteries from 4-month old WT and D79N mice. **A** UK responses curves at 37$^0$C (○, n = 5) and 22$^0$C ( ●, n = 7) in WT arteries. **B** UK responses curves at 37$^0$C (○, n = 6) and 22$^0$C ( ●, n = 9) in D79N arteries. Each point represents mean ± standard error.

0.09gms Force which was significantly potentiated to 1.16 ± 0.08gms Force at $22^0$C (p = 0.02*)

5.3.4 Responses following a curve to another adrenergic agonist

Due to the nature of the protocols that were performed cumulative concentrations curves to the agonists UK14304 and phenylephrine were often performed in succession. So these responses were then analysed to determine if construction of a curve to another adrenergic agonist affected the response gained.

Two consecutive curves to phenylephrine were constructed in arteries from WT and D79N mice maintained at $22^0$C, before and after a UK14304 response curve. Neither the $pEC_{50}$ nor the maximum showed a statistically significant difference. Figure 5.4 shows the responses gained in tail arteries from WT mice. The maximum response in the first curve was 0.94 ± 0.08gms Force and the maximum gained in the second cumulative concentration curve was 1.1 ± 0.10gms Force (figure 5.6 **A**). $pEC_{50}$ values were calculated for both curves. The first curve had a $pEC_{50}$ of 6.2 ± 0.04, while the second curve had a $pEC_{50}$ of 6.3 ± 0.01. This indicated that the sensitivity to phenylephrine was unchanged by a response curve to UK14304 (figure 5.6 **B**).

In the D79N, responses to phenylephrine, carried out at $22^0$C before and after a curve to UK14304 had similar maximal responses (figure 5.7 **A**) and sensitivity (figure 5.7 **B**) and are shown in figure 5.7. The maximum contraction gained to phenylephrine alone was 0.98 ± 0.1gms Force, while that obtained after a cumulative UK14304 curve was 0.89 ± 0.08gms Force. The first curve had a $pEC_{50}$ of 6.1 ± 0.02, in the second curve, after a UK14304 response curve, the $pEC_{50}$ was 5.9 ± 0.01.

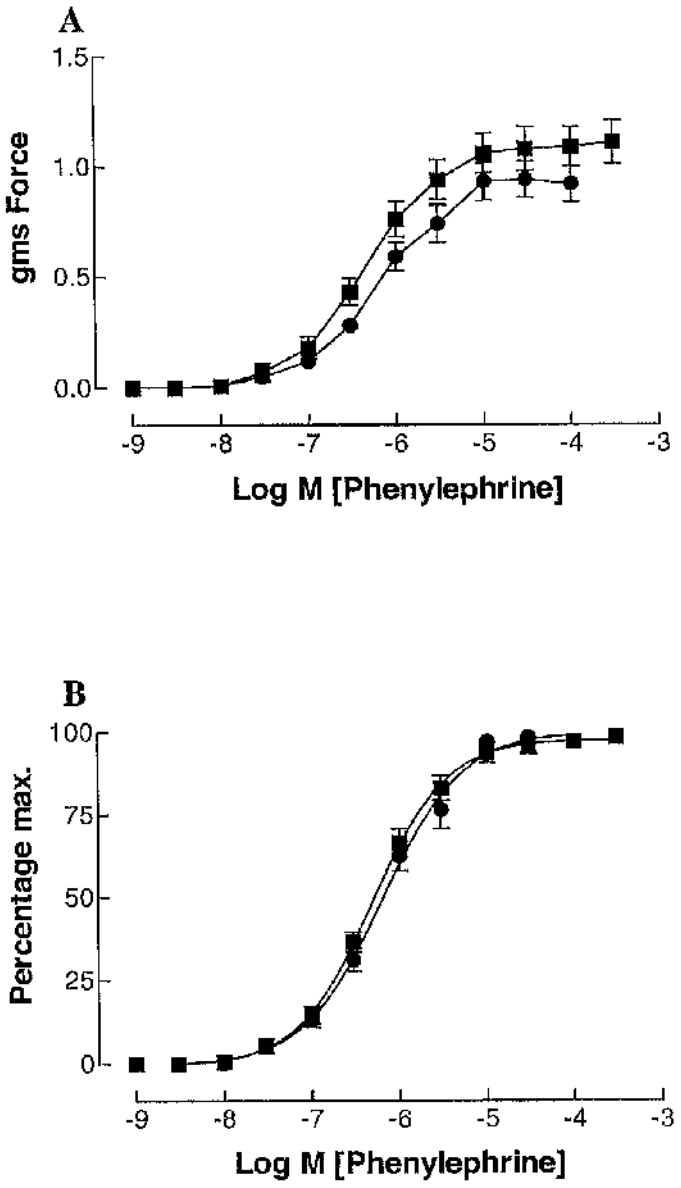



Figure 5.6: PE response curves in tail arteries from 4-month old WT mice, constructed before and after a cumulative UK curve at 22$^0$C. **A** PE curve before (●, n = 7) and after ( ■, n = 7) a cumulative UK curve. **B** PE response curves, expressed as a percentage of own maximum. Each point represents mean ± standard error.

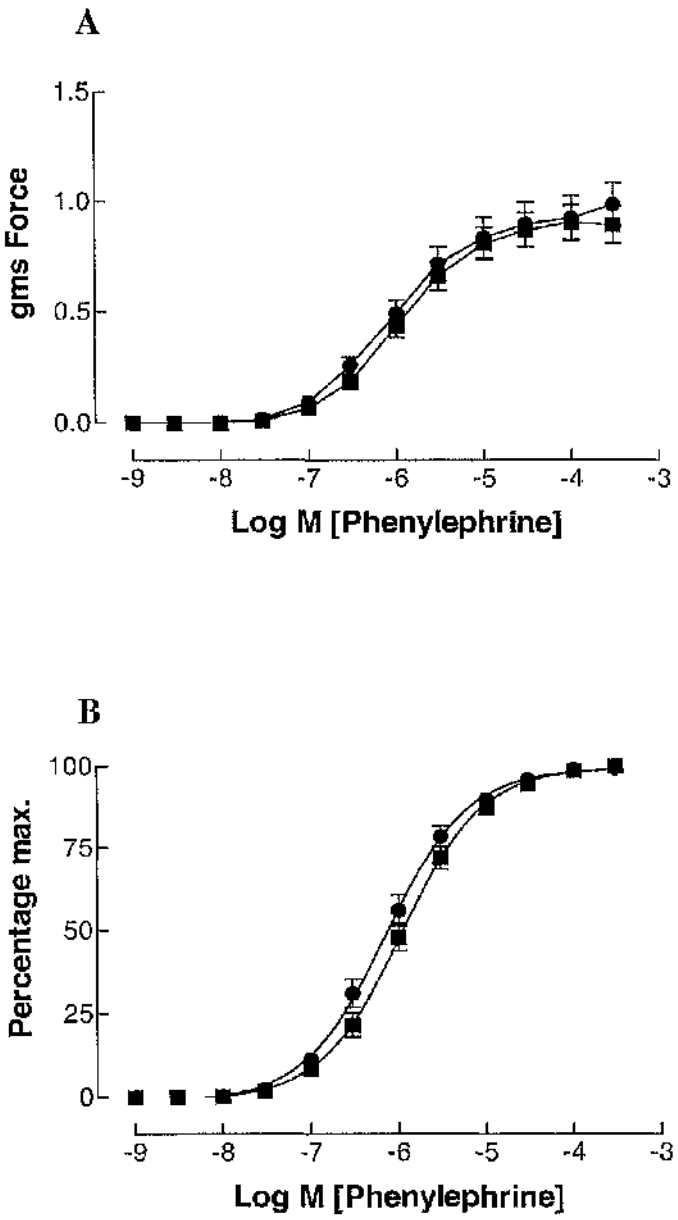



Figure 5.7: PE response curves in tail arteries from 4-month old D79N mice, constructed before and after a cumulative UK curve at 22$^0$C. **A** PE response before (●, n = 9) and after ( ■, n = 9) a cumulative UK curve. **B** PE response curves, expressed as a percentage of their own maximum. Each point represents mean ± standard error.

Figure 5.8 shows the responses to cumulative UK14304 curves constructed alone, and following a curve to the $\alpha_1$-selective agonist phenylephrine at $37^0$C in both murine strains. In WT arteries (figure 5.8 **A**) responses were of comparable size, with the maximum to UK14304 alone being 0.25 ± 0.04gms Force, and that gained after an initial curve to phenylephrine being 0.21 ± 0.03gms Force. Responses in D79N arteries (figure 5.8 **B**) were small and highly variable. The maximum responses gained to UK14304 alone, was 0.26 ± 0.06gms Force, compared to a maximum of 0.23 ± 0.05gms Force, when a cumulative UK14304 curve was constructed after an initial curve to phenylephrine.

The responses to cumulative UK14304 alone, and after a phenylephrine curve at $22^0$C are shown in figure 5.9. Unlike the response at $37^0$C, the UK14304-mediated response at $22^0$C was significantly potentiated after an initial phenylephrine curve. In WT arteries (figure 5.9 **A**), contractile responses were significantly greater when a cumulative concentration curve to phenylephrine had been constructed prior to UK14304. The maximum response obtained when the UK14304 curve was carried out after an initial phenylephrine curve was 0.54 ± 0.06gms, which was significantly greater than the maximum to UK14304 alone of 0.20 ± 0.03gms Force ($p = 0.0004^{***}$).

Responses in tail arteries from D79N mice (figure 5.9 **B**) also showed enhanced responses at $22^0$C, when an initial curve to phenylephrine had been constructed before the UK response. The maximum response gained in the curve after phenylephrine was 0.43 ± 0.04gms Force, compared with that obtained to UK14304 alone, which was 0.22 ± 0.03gms Force. Statistical analysis showed that these responses were significantly

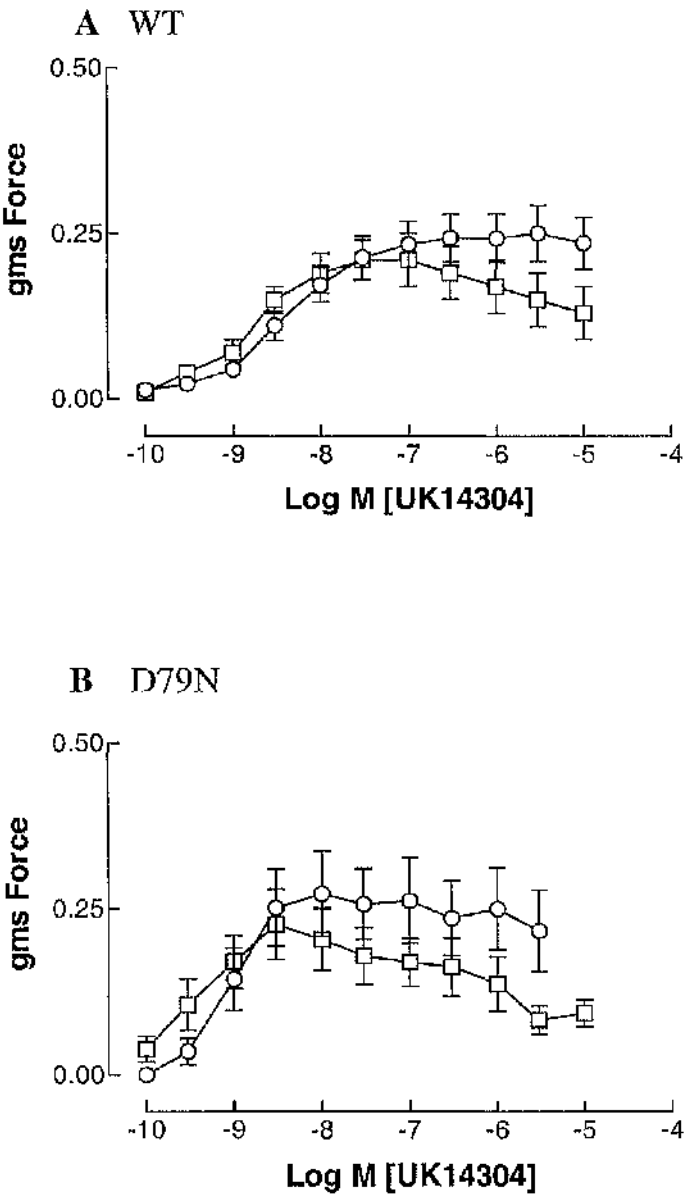



Figure 5.8: UK responses curves in tail arteries from 4-month old WT and D79N mice at 37$^{0}$C, constructed before and after a PE curve. **A** The UK response in WT arteries before (○, n = 5) and after ( □, n = 5) a PE curve. **B** The UK response in D79N arteries before (○, n = 6) and after ( □, n = 6) a PE curve. Each point represents mean ± standard error.

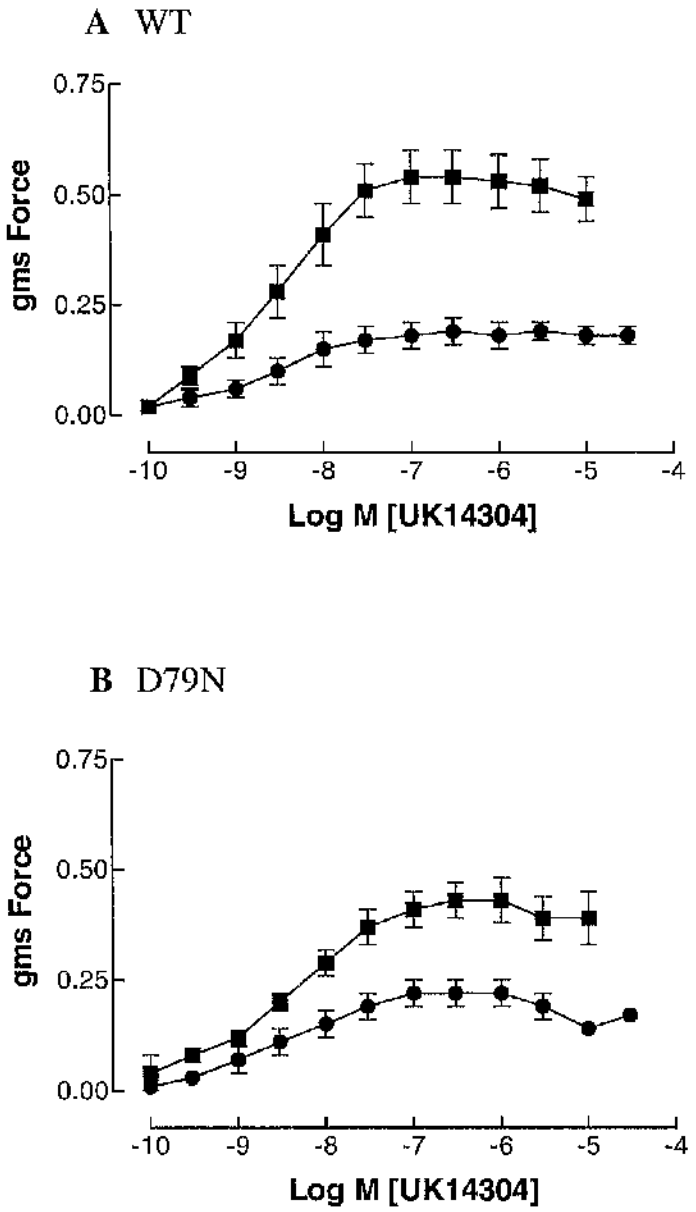



Figure 5.9: UK responses curves in tail artery from 4-month old WT and D79N mice at 22$^0$C, constructed before and after a PE curve. **A** The UK response in WT arteries before (●, n = 7) and after ( ■, n = 7) a PE curve. **B** The UK response in D79N arteries before (●, n = 9) and after (■, n = 9) a PE curve . Each point represents mean ± standard error.

different, generating a p value of 0.0035**. The proportional increase was larger in WT than the D79N. At $22^0$C the response in the WT reached 270 % of the curve constructed without exposing the tissue to phenylephrine. In the D79N, the percentage increase in the maximum response was 195 % of the UK14304 curve maximum, constructed at $22^0$C, without prior stimulation with phenylephrine.

5.3.5 Combined contractile responses to U46619 and UK14304 at $22^0$C

The combined contractile response to the synergist, U46619 and increasing concentrations of UK14304 in the WT (figure 5.10 **A**) and D79N (figure 5.10 **B**) are shown in figure 5.10. In WT arteries, the combined contraction alone reached a maximum of 0.64 ± 0.1gms Force, while after construction of a phenylephrine curve the maximum was significantly greater at 0.80 ± 0.05gms Force (p = 0.04*). In D79N arteries, the combined contractile response to U46619 and UK14304, without previous stimulation with phenylephrine, had a maximum response of 0.59 ± 0.08gms Force. After construction of a phenylephrine curve the combined contractile response in the D79N tail artery was 0.75 ± 0.05gms Force (p = 0.047*).

To determine how close contractile responses at $22^0$C were to an adrenergic maximum, the contractions in the WT (figure 5.11 **A**) and D79N (figure 5.11 **B**) to UK14304 alone, and to UK after a phenylephrine response curve, were expressed as a percentage of the noradrenaline maximum (1 x $10^{-5}$M). In the WT, maximum UK14304-induced contraction reached 15.4 ± 3.0 % of the noradrenaline maximum. After a phenylephrine curve the UK14304 curve maximum was increased to 44 ± 6.15 % of the noradrenaline response. In the D79N mouse, the UK14304-mediated contractile response at $22^0$C is 20.5 ± 3.3 % of the noradrenaline maximum (1 x $10^{-5}$M). However, after construction

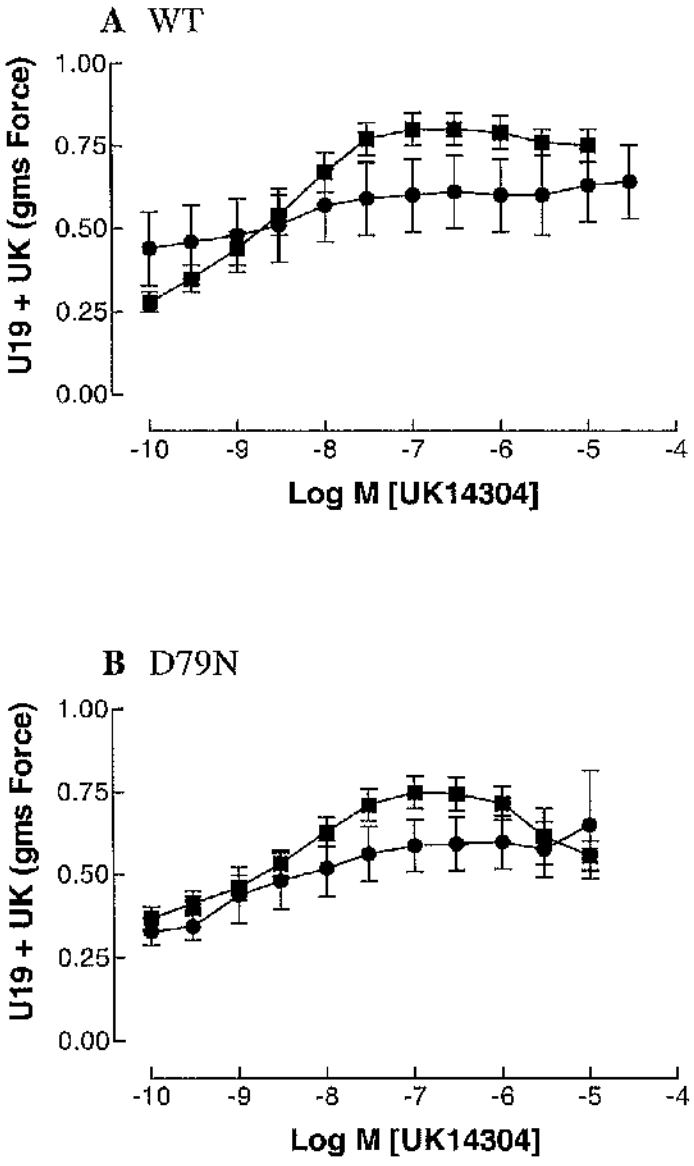



Figure 5.10: Combined contractile responses to U19 tone and UK in tail arteries from 4-month old WT and D79N mice at 22$^0$C. **A** The UK/U19 response in WT arteries before (●, n = 7), and after (■, n = 7) a PE curve. **B** The UK/U19 response in D79N arteries before (●, n = 9), and after (■, n = 9) a PE curve. Each point represents mean ± standard error.

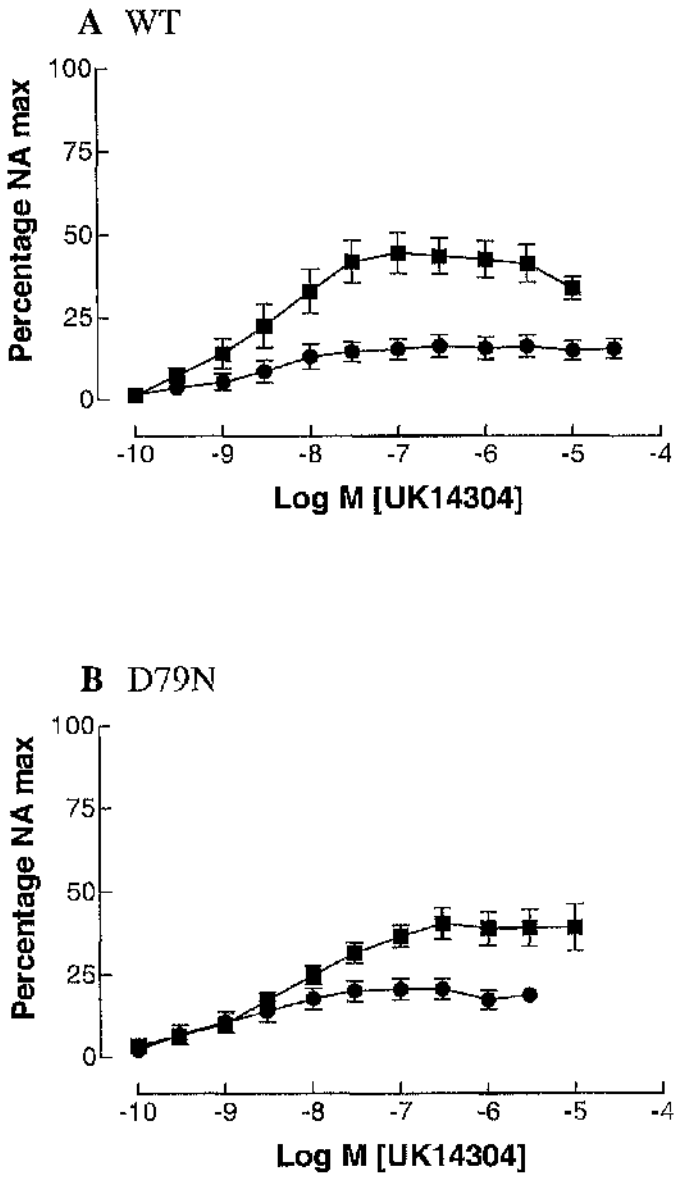



Figure 5.11: UK response curves in tail arteries from 4-month old WT and D79N mice at 22$^{0}$C, expressed as a percentage of the NA maximum (1 x $10^{-5}$M). **A** The UK response in WT arteries before (●, n = 7), and after ( ■, n = 7) a PE curve. **B** The UK response in D79N arteries before (●, n = 9), and after ( ■, n = 9) a PE curve. Each point represents mean ± standard error.

of a phenylephrine curve the UK14304-induced contraction increased to 40.3 ± 4.7 % of the noradrenaline response.

So how close is the combined U46619/UK14304 contraction to an adrenergic maximum? The combined contractile response to U46619 and UK14304 at $22^0$C for the WT (figure 5.12 **A**) and D79N (figure 5.12 **B**) have been expressed as a percentage of maximum response to a sighting concentration of noradrenaline (1 x $10^{-5}$M). In WT arteries, after a phenylephrine response curve, the combined contractile response reached 64.5 ± 6.1 % of the noradrenaline response. The combined contraction to U46619 and UK14304 without phenylephrine exposure was smaller, and reached 48.0 ± 6.4 % of the noradrenaline maximum. Similar results were obtained in tail arteries from the D79N mouse. Without an initial phenylephrine curve, the combined contraction reached 55.6 ± 9.5 % of the noradrenaline maximum, while after an initial phenylephrine curve the maximum was higher at 66.9 ± 6.3 %.

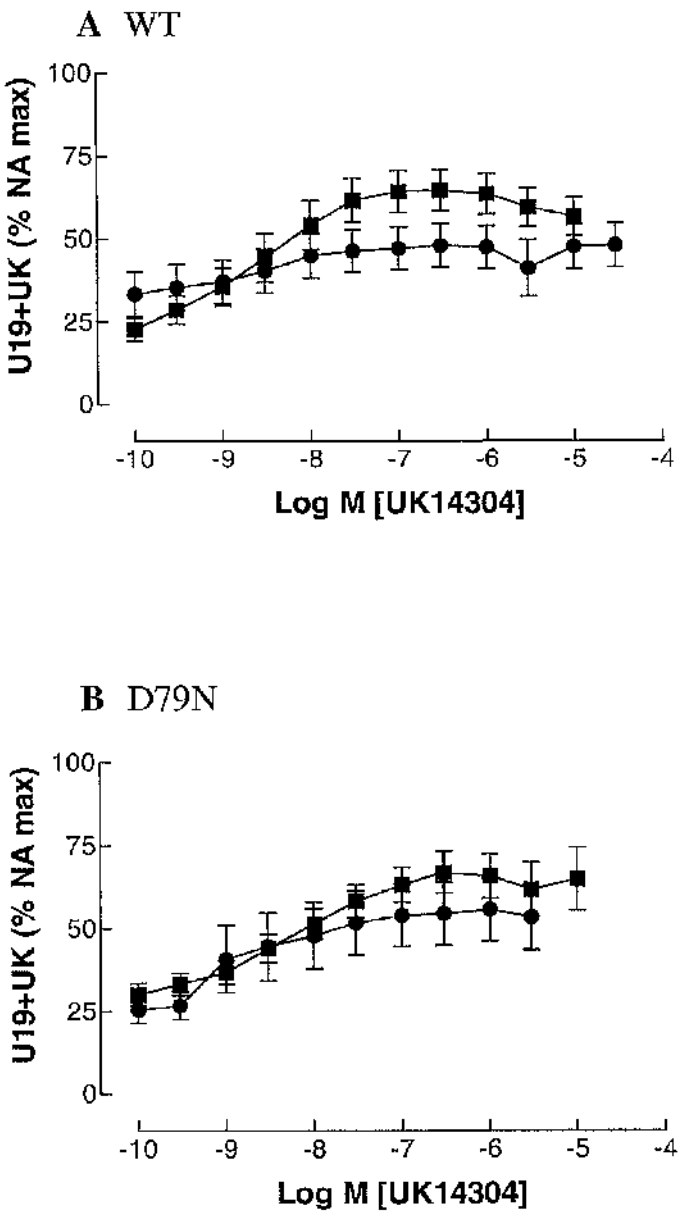



Figure 5.12: The combined UK/U19 response in tail arteries from 4-month old WT and D79N mice at 22$^0$C, expressed as a percentage of the NA maximum (1 x 10$^{-5}$M). **A** Responses in WT arteries before (●, n = 7), and after ( ■, n = 7) a PE curve. **B** Responses in D79N arteries before (●, n = 9), and after ( ■, n = 9) a PE curve. Each point represents mean ± standard error.

## 5.4 Discussion

### A temperature of 22$^0$C enhances UK and NA-mediated responses in the tail artery of the WT and D79N

In the presence of U46619-induced tone, a reduction in experimental temperature, to 22$^0$C, is insufficient to enhance UK14304-mediated vasoconstrictor responses in tail arteries from WT and D79N mice. These findings are in disagreement with those published by Chotani and co workers. What is the explanation for the discrepancy in our findings? The enhanced UK14304-mediated response described by Chotani and co workers was uncovered in distal segments of the murine tail artery, mounted in a Living Systems pressure myograph. My experiments investigated the responses to UK in the middle section of the tail artery, mounted in a Mulvany/Halpern wire myograph. It seems doubtful that the difference in results can be fully explained by the different experimental conditions.

Surprisingly, the size of UK14304-induced contractions is significantly potentiated at 22$^0$C, when an initial curve to phenylephrine has been constructed before the UK14304 response curve. The increase in size of vasoconstrictor responses occurs in the WT and D79N, and is of comparable size. This suggests that the $\alpha_{2A/D}$-adrenoceptor is not involved in the enhancement of responses at 22$^0$C, which in this instance supports the conclusions of Chotani and co workers [Chotani et al, 2000], that the response at cold temperatures (in their case 28$^0$C) is attributable to stimulation of the $\alpha_{2C}$-subtype.

Furthermore, the experiments performed by Chotani et al, also investigated the effect of cold temperatures (28$^0$C) on phenylephrine-induced responses. Like us, they found that phenylephrine-induced contractions in WT mice are unaffected by a decrease in

external temperature. Whether the response to UK14304 was studied after a phenylephrine stimulus is unclear from their publication. If phenylephrine-induced vasoconstrictions were investigated before the responses to UK14304, this would explain the discrepancy with the response shown here, and could explain the UK14304-mediated potentiation in contractility in pressure myograph mounted vessels.

But how does prior stimulation of $\alpha_1$-adrenoceptors lead to an enhancement of $\alpha_2$-adrenoceptor-mediated vasoconstrictor responses? Contractile responses, resulting from stimulation of $\alpha_1$-adrenoceptors are enhanced by $\alpha_2$-selective agonists in the rat tail artery. The potentiation in $\alpha_2$-adrenoceptor-mediated contractility results from an alteration in calcium levels [Xiao et al, 1989]. Whether reducing the experimental temperature to $22^0$C provides conditions whereby $\alpha_1$-stimulation can enhance calcium levels is unclear, but it does present a possible explanation for the results gained.

Cutaneous blood vessels are sensitive to subtle changes in temperature, and this is likely to be relevant to their role in thermoregulation. A reduction in external temperature to below $30^0$C can enhance vasoconstrictor responses in cutaneous blood vessels in rodents, and has been likened to the changes occurring in the vasculature of the human hand upon exposure to extremes of temperature [Thorington, 1966]. The elevation in contractility when cutaneous blood vessels are exposed to a reduction in temperature is thought to result from activation of vascular $\alpha_2$-adrenoceptors [Chotani et al, 2000]. Furthermore, $\alpha_2$-receptor antagonists have been shown to cause vasodilatation of the perfused rat tail, leading to an increase in surface temperature [Redfern et al, 1995]. Another factor affecting the noradrenaline response is the possible potentiation due to inhibition of the neuronal amine transporters at cold temperatures.

Enhanced contractions of the murine tail artery at cold temperatures have been proposed to result from the recruitment and activation of quiescent $\alpha_{2C}$-adrenoceptors, which at $37^0$C do not participate in vasoconstrictor responses [Chotani et al, 2000]. However, under normal physiological conditions, the rodent tail has an ambient temperature that is significantly lower than core body temperature, and generally reflects the external environment. This leads me to propose a hypothesis; under normal physiological conditions $\alpha_{2C}$-adrenoceptors will be involved in contraction of the murine tail artery. At $37^0$C, the $\alpha_{2C}$-adrenoceptor may play a lesser role in $\alpha_2$-adrenoceptor-mediated contractions [Chotani et al, 2000], but should the tail ever reach this temperature, arteries will most probably be vasodilated (and sympathetic nerve activity ceased) to allow dissipation of heat from the surface of the tail, negating the need for activation of this receptor-subtype.

At $22^0$C compared with $37^0$C, the size of the contractile response to sighting concentrations of noradrenaline ($1 \times 10^{-5}$M) is significantly potentiated in the WT and D79N. The enhanced response in the WT can be attributed solely to a potentiation of the $\alpha_2$-adrenoceptor-mediated contraction, because the phenylephrine response is unaffected by a reduction in experimental temperature to $22^0$C. However, in the D79N, responses to noradrenaline, phenylephrine and UK14304, are enhanced at $22^0$C. This suggests the involvement of $\alpha_1$- and $\alpha_2$-adrenoceptors in enhanced noradrenaline-mediated contractions. Furthermore, this data provides additional evidence that the $\alpha_{2A/D}$-adrenoceptor is not involved in the cold-induced potentiation in contractility, because the magnitude of the change in size of the noradrenaline response is comparable in the D79N and the WT.

The contractile response to UK14304 alone, and in the presence of U46619 has been expressed as a percentage of the noradrenaline maximum to give an indication of the size of the $\alpha_2$-adrenoceptor-mediated contraction in relation to the combined response to dual activation of $\alpha_1$ and $\alpha_2$-adrenoceptors. In the WT and D79N, the response to UK14304 and the combined contraction to UK and U46619 is significantly less than the noradrenaline response. This data suggests that the UK14304-mediated response at $22^0$C is small in comparison to the noradrenaline response, which results from non-selective stimulation of $\alpha_1$- and $\alpha_2$-adrenoceptors, suggesting that even at low temperatures the $\alpha_1$-adrenoceptor-mediated contraction is substantial in comparison to that mediated by $\alpha_2$-adrenoceptors.

Potency order for agonists in tail arteries from WT and D79N mice

Before investigating the effect of a reduction in temperature on the vasoconstrictor response to the $\alpha_2$-selective agonist, UK14304, the effect of cumulative addition of the agonists, phenylephrine, noradrenaline, and 5HT were studied in the murine tail artery. This was done to determine if responses to other agonists are affected by mutation of the $\alpha_{2A/D}$-adrenoceptors, because compensatory mechanisms cannot always be excluded as the reason for a unique phenotype. The potency order of the agonists tested is the same for the WT and D79N, and has been determined as 5HT > NA > PE. The responses to phenylephrine (smaller in size, but similar sensitivity) and 5HT were similar in size and sensitivity in arteries from both strains. This indicates no general change in sensitivity or contractility suggesting that $\alpha_1$-adrenoceptor and 5HT mechanisms are normal in the D79N mouse at $37^0$C.

However, the noradrenaline-mediated response in the D79N has a smaller maximum response and has reduced sensitivity, compared with the WT, at $37^0$C. This suggests that the presence of a fully functional $\alpha_{2A/D}$ receptor pool is necessary for normal, full sensitivity to noradrenaline. This provides evidence that, at this temperature, the $\alpha_{2A/D}$ receptor is involved in contractile responses. It would therefore be expected that stimulation of $\alpha_{2A/D}$-adrenoceptors is, in part, responsible for UK14304-mediated contractions of the murine tail artery. The results shown in chapter three support this hypothesis, where the response to UK14304 is significantly smaller in the D79N than in the WT in all of the protocols tested. However, the data from chapter four is contradictory. The results presented there, show that the response gained is critically dependent on the method in which the agonist, UK14304, is administered. When UK14304 curves are constructed non-cumulatively, the observed reduction in contractility in the D79N is abolished. This suggests that at $37^0$C the cumulative UK14304 response does involve the $\alpha_{2A/D}$ receptor, and that prolonged stimulation results in the development of desensitisation. At $22^0$C, responses to UK14304 in the WT and D79N are comparable, suggesting the involvement of a receptor other than the $\alpha_{2A/D}$-adrenoceptor in this response.

PE-induced response is potentiated at $22^0$C in the D79N but not the WT

Vasoconstrictor responses to UK14304 were studied by constructing cumulative response curves at a reduced experimental temperature, in keeping with the studies performed by Chotani and co workers. Reducing the experimental temperature to $22^0$C had no effect on the phenylephrine-induced contraction of the WT tail artery. However, in the D79N, in which the contraction to phenylephrine was smaller than in the WT at $37^0$C, reducing the temperature to $22^0$C, significantly enhanced contractile responses to

phenylephrine, giving a vasoconstriction that was of comparable size to that gained in the WT. This data suggests that at a reduced temperature of $22^0$C, the $\alpha_1$-adrenoceptor-mediated contraction in the D79N mouse is enhanced by a lack of functional $\alpha_{2A/D}$-adrenoceptors. How does this occur?

Enhancement of vasoconstrictor responses at cold temperatures is proposed to result from stimulation of quiescent $\alpha_{2C}$-adrenoceptors [Chotani et al, 2000]. According to that study, at $37^0$C stimulation of $\alpha_{2A/D}$-adrenoceptors contributes to vasoconstrictor responses in the murine tail artery, because antagonists proposed to be selective for the $\alpha_{2A/D}$-adrenoceptor-subtype, block vasoconstrictor responses. In the absence of a functional $\alpha_{2A/D}$-adrenoceptor pool, compensatory changes may permit and/or recruit $\alpha_1$-adrenoceptors to participate in the cold-induced response. At $22^0$C, the potentiation of phenylephrine-induced contractions in the D79N provides evidence in support of this hypothesis. Furthermore, given the location of the tail artery, it is reasonable to assume that at physiological temperatures, contraction of this artery results from dual activation of $\alpha_1$- and $\alpha_2$-adrenoceptors.

<u>$\alpha_1$-adrenoceptor-mediated responses are not cross-desensitised by previous exposure to UK14304</u>

Cumulative UK14304 response curves are susceptible to profound, agonist-induced receptor desensitisation. Given that the response to phenylephrine was investigated at $37^0$C and $22^0$C, this provided conditions where the effect of UK14304 on phenylephrine-induced contractions could be studied, to determine if the stimulation of UK14304 sensitive receptors results in cross desensitisation of the $\alpha_1$-adrenoceptor-mediated vasoconstrictor response.

In the WT and D79N tail, the response to phenylephrine at $22^0$C and at $37^0$C is unaffected by previous exposure to the $\alpha_2$-selective agonist, UK14304. This data confirms that, at cold temperatures and at $37^0$C (chapter four), $\alpha_1$-adrenoceptor-mediated responses are free from UK14304-induced receptor desensitisation. In essence, the desensitisation that affects UK14304-mediated responses is specific to the $\alpha_2$-adrenoceptor, and does not reflect a generalised reduction in responsiveness of the tail artery to exogenous agonists.

Summary and conclusions

In summary, the results shown here confirm that $\alpha_2$-adrenoceptor-mediated vasoconstrictor responses of WT and D79N tail arteries are enhanced by a reduction in the experimental temperature. However, the elevation in contractility develops only when vessels have previously been exposed to a phenylephrine response curve, a result that may reflect conditions *in vivo*. The elevation in the size of contractile responses occurs in the WT and D79N, providing strong evidence that the $\alpha_{2A/D}$-adrenoceptor does not participate in the cold-induced response. Considering the responses gained and the difficulties associated with the study of $\alpha_2$-adrenoceptor-mediated responses (shown in previous chapters) I have highlighted many questions about the experimental conditions, and temperature at which responses of the tail artery are studied. This leads me to pose the question, should all contractile, and nerve-induced responses in the rodent tail artery be performed at lower experimental temperatures, to better mimic conditions *in vivo*?

# Chapter six

## UK14304-induced relaxations of mesenteric resistance arteries from WT and D79N mice

## 6.1 Introduction

Resistance arteries play an important role in the maintenance of peripheral resistance, and as such contribute to the maintenance and control of blood pressure. Responses to exogenous agonists have been studied extensively in rat mesenteric resistance arteries, but to date, little is known about the pharmacology of murine blood vessels.

The initial aim of carrying out the experiments contained within this chapter was to study the effects of UK14304 on mesenteric blood vessels, and to determine if stimulation of $\alpha_2$-adrenoceptors causes contractile responses in another murine artery. However, a review of the available literature suggested that the response gained would be relaxant in nature.

In the rat, stimulation of endothelial $\alpha_2$-adrenoceptors on coronary, renal, and mesenteric arteries has a relaxant effect. In porcine coronary arteries, the $\alpha_{2A/D}$-adrenoceptor mediates agonist-induced reductions in vascular tone according to analysis employing various antagonists with varying selectivity for the $\alpha_2$-subtype. This is in spite of the $\alpha_{2C}$ subtype being expressed at much higher levels than the $\alpha_{2A/D}$-adrenoceptor in porcine coronary arteries [Bockman et al, 1993].

In the rat, EDHF appears to be the major contributor to endothelium-dependent relaxations of mesenteric resistance arteries but plays little, if any, role in relaxation responses of large calibre blood vessels [Hwa et al, 1994]. EDHF has been shown to cause relaxant effects by inhibiting the opening of voltage-gated calcium channels, leading to hyperpolarisation of vascular smooth muscle cells, which leads to a reduction in vascular contractility [Nagao et al, 1991]. To determine what role EDHF plays in

agonist-induced vasodilatations, the balance of positive ions in the surrounding bathing solution can be changed, this hyperpolarises the membrane potential in all cells and, relevantly, vascular smooth muscle cells which are thus prevented from responding to EDHF. Experimentally, when extracellular $K^+$ influx has been blocked, EDHF-induced relaxations were prevented [Chanhan et al, 2003].

When the response to UK14304 was established, the effect of L-NAME and rauwolscine were investigated, to determine the role of nitric oxide, and confirm that the response gained results from stimulation of $\alpha_2$-adrenoceptors. In addition, the D79N mouse was utilised to gain further insight into the subtype(s) mediating vascular responses in mouse mesenteric resistance arteries.

## 6.2 Methods

WT (C57BL/6c/129Sv) and D79N ($\alpha_{2A/D}$ mutant) mice aged four-months old were obtained from the University animal house for this study. Males of between 29.6-34.2g were killed by asphyxiation with carbon dioxide and the mesenteric arcade was removed, and placed in fresh Krebs.

### 6.2.1 Vessel isolation and mounting

The mesenteric arcade was pinned out on a petri dish containing cold Krebs and first order arteries located. Several arteries were dissected, cleaned of excess fat and tissue and stored at $4^0$C until use. These arteries were then set up in 5ml, stainless steel, Mulvany/Halpern wire myograph baths. After vessel mounting, a 30-35 minute rest period was allowed, during which time baths were gassed with a 95 % $O_2$ 5 % $CO_2$ mixture and heated to $37^0$C. Vessels were then washed with fresh Krebs, and a resting

tension of 0.17gms Force (optimum resting tension, unpublished observation) was placed on each mounted arterial segment. Vessels were then washed four times with fresh Krebs and allowed a further 20-30 minute rest period before commencing the wake-up protocol.

### 6.2.2 Wake-up protocol

Each vessel was challenged with noradrenaline ($1 \times 10^{-5}$M) and contractions were allowed to reach a plateau. Vessels were then washed with, fresh, gassed Krebs ($37^{0}$C) four times over a fifteen minute period. The entire process was then repeated once, with the same noradrenaline concentration, and a second time with a lower noradrenaline concentration of $1 \times 10^{-6}$M. When the contractile response to the lower noradrenaline response reached a plateau, acetylcholine at a concentration of $3 \times 10^{-6}$M was added to test the viability of the endothelium. After each drug addition, the process of washing with fresh Krebs four times over a fifteen minute period was performed.

### 6.2.3 Experimental protocols

#### Elevation of vascular tone

Cumulative concentration response curves to the $\alpha_2$-selective agonist UK14304 were constructed in mounted arterial rings. Alone, UK14304 produced no notable responses. Therefore the use of the synergist, U46619 was employed to elevate vascular tone. Vascular tone was elevated for two reasons. Firstly, to determine if contractile responses occur when vascular tone is enhanced. Secondly, to ascertain if U46619-induced tone will be reduced by increasing concentrations of UK14304 (that is, does stimulation of $\alpha_2$-adrenoceptors causes vasodilatation?).

U46619 ($1 \times 10^{-7}$M) was added to each bath containing a 2mm arterial ring, and the contractile response was allowed to reach a plateau. After this, UK14304 was added in half log units in a cumulative manner, alone, and in the presence of L-NAME ($1 \times 10^{-4}$M).

Cumulative UK14304 response curves

The experimental protocol was later changed because of the variability of responses to cumulative drug additions and the variability of U46619-induced tone over time. Furthermore, twenty experiments were set up to test the effects of cumulative UK14304 on U44619-induced tone in first order mesenteric arteries. Of the twenty experiments performed only twelve gave a measurable response (Shown in figure 6.1).

Thereafter, UK14304-induced responses were studied by constructing randomised, non-cumulative concentration curves. The wake-up protocol, tension applied, and U46619 concentration used, remained the same. Each non-cumulative concentration of UK14304 was given a minimum of twenty-five minutes apart (after the tone within the vessel had been elevated with U46619). All of the vessels in which a non-cumulative response curve was constructed were responsive to the applied drugs.

6.2.4 Effect of L-NAME and rauwolscine on UK14304-mediated responses

Given the problems associated with UK14304-mediated desensitisation (Chapter four) the effect of L-NAME and rauwolscine were assessed against first curves. L-NAME ($1 \times 10^{-4}$M) was made fresh daily, and stored on ice throughout the course of each experiment. To assess the effect of blocking nitric oxide synthase production, L-NAME

was incubated with mounted artery for a minimum of 20 minutes, prior to construction of an agonist curve.

The effect of rauwolscine on the UK14304-mediated response was assessed against randomised, non-cumulative first curves, in mesenteric resistance arteries from WT mice. Mounted arterial segments were incubated with rauwolscine ($3 \times 10^{-8}$M) for a minimum of thirty minutes prior to construction of a UK14304 response curve.

## 6.3 Results

### 6.3.1 The response to cumulative UK14304 in WT arteries

Figure 6.1 shows the cumulative response to UK14304 in first order mesenteric resistance arteries from four-month old WT mice, expressed as a percentage of U46619-induced tone. The cumulative administration of UK14304 caused concentration-dependent relaxations. The maximum relaxant response, at a UK14304 concentration of $3 \times 10^{-5}$M was a $48.1 \pm 6.4$ % reduction in U46619-induced tone.

### 6.3.2 Effect of L-NAME on the cumulative UK14304 response

Figure 6.2 illustrates the effect of L-NAME ($1 \times 10^{-4}$M) on UK14304-induced relaxations in unpaired arteries. At low agonist concentrations the relaxant effect of UK was significantly attenuated in the presence of L-NAME. In the control curve a UK14304 concentration of $3 \times 10^{-7}$M caused a $9.6 \pm 4.09$ % reduction in U46619-induced tone, but in the presence of L-NAME U46619-induced tone was unaffected ($p = 0.02$*). However, at high UK14304 concentrations the inhibitory effect of L-NAME became surmountable. The relaxations that occurred at high agonist concentrations were not significantly different from those in the control curve ($p>0.05$).

### 6.3.3 Non-cumulative UK14304 response curves in WT mice

With the exception of figures 6.1 and 6.2 all other curves shown were constructed in a randomised, non-cumulative manner. In Figures 6.3 **A** and 6.3 **B** representative traces of the effects of single concentrations of UK14304, investigated in first order mesenteric resistance arteries from a four-month old WT mice are shown. In figure 6.3 **A** basal tone was elevated with the synergist, U46619 ($1 \times 10^{-7}$M). UK14304 at a concentration of $1 \times 10^{-6}$M caused a significant reduction in U46619-induced tone. To

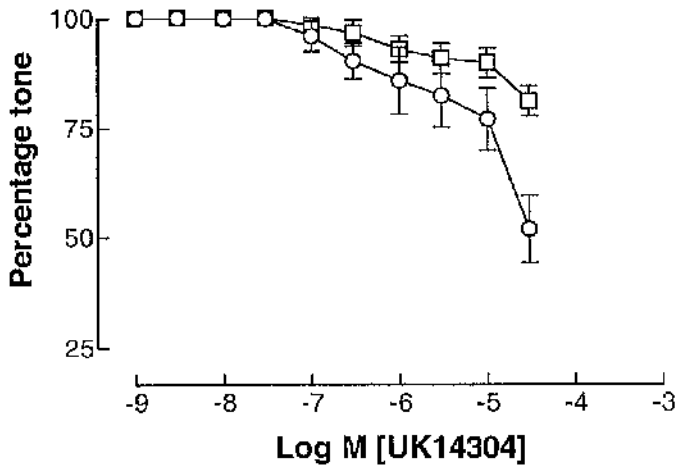



Figure 6.1: UK14304-mediated response in first order mesenteric arteries from 4-month old mice WT mice. The variation in U46619-induced tone over time (□, n = 7) and the effect of a cumulative UK curve (○, n = 12). Both data sets were expressed as a percentage of U46619-induced tone. Each point represents mean ± standard error.

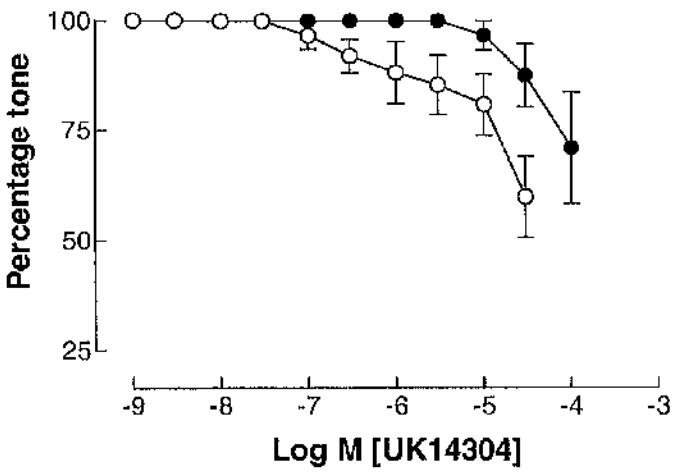



Figure 6.2: UK14304-mediated responses in first order mesenteric arteries from 4-month old WT mice. A cumulative UK response curve alone (○, n = 12), and in the presence of L-NAME (1 x $10^{-4}$M,●, n = 6). Both data sets were expressed as a percentage of U46619-induced tone.. Each point represents mean ± standard error.

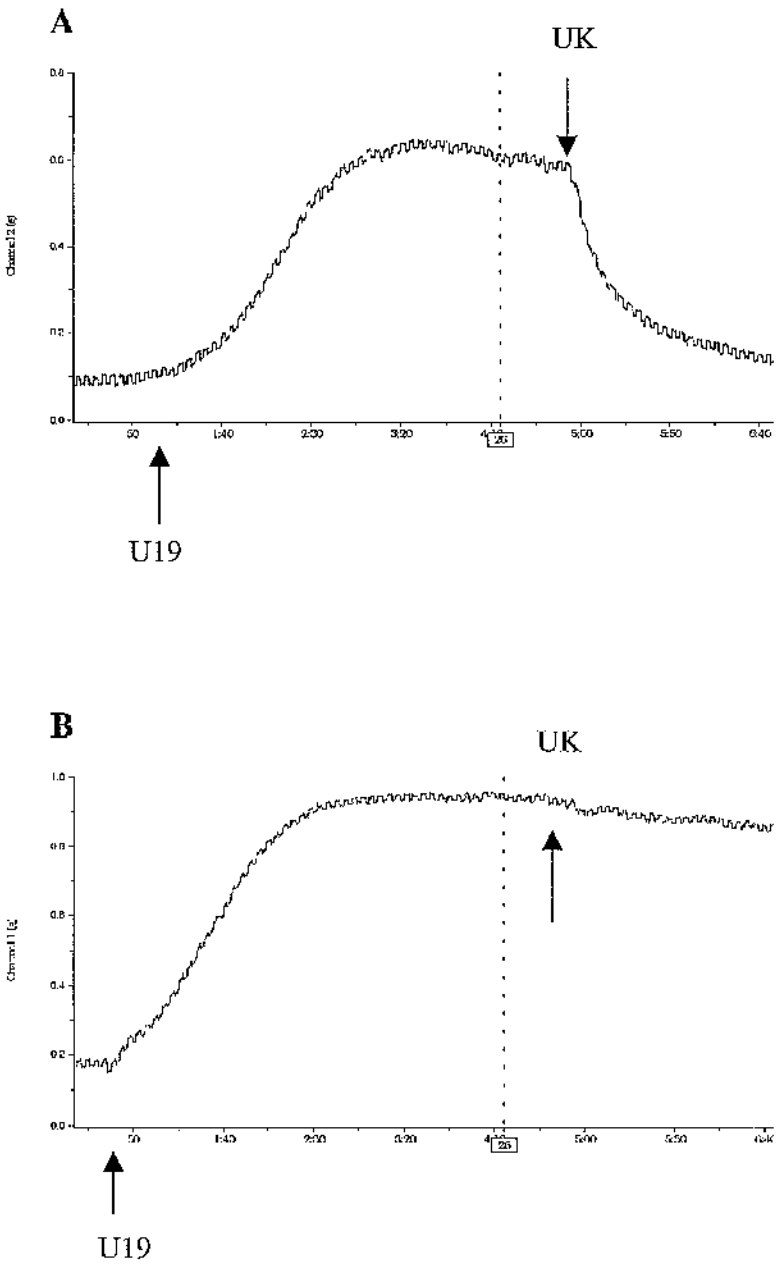



Figure 6.3: Representative trace of the relaxant response to a single concentration of UK in mesenteric resistance arteries from a 4-month old WT mouse. **A** The effect of 1 x $10^{-6}$M UK in the presence of U19-induced tone. **B** The non-cumulative response to UK at 1 x $10^{-6}$M, in a vessel previously exposed to a cumulative UK14304 response curve. The responses shown are in a single vessel.

determine whether UK14304-mediated responses were prone to agonist-induced desensitisation, like those of the murine tail artery, a non-cumulative response curve to UK14304 was constructed on top of U46619-induced tone in a mounted mesenteric artery. After a thirty minute rest period, the response to a single concentration (1 x $10^{-6}$M) of UK14304 was tested (figure 6.3 **B**). The relaxant response gained was slight in comparison to the response gained without previous exposure to UK14304 (figure 6.3 **A**). This indicated that in mesenteric arteries the UK14304-mediated response was prone to agonist-induced receptor desensitisation, much like the response in the mouse tail artery.

Figure 6.4 shows the non-cumulative response to UK14304 in first order mesenteric resistance arteries from four-month old WT mice. UK14304 causes concentration-dependent reductions in U46619-induced tone. At the highest agonist concentration tested, UK14304 (1 x $10^{-4}$M) caused a 59.0 ± 5.6 % reduction in U46619-induced tone.

Figure 6.5 shows the cumulative and non-cumulative UK14304 response curves, constructed in first order mesenteric resistance arteries from four-month old WT mice. The cumulative and non-cumulative response curves follow each other closely. However, in cumulative time control curves a reduction in U46619-induced tone was observed. The maximum reduction in tone in the cumulative curve was smaller at 40.1 ± 6.4 % (3 x $10^{-5}$M) of elevated tone but was achieved at a lower concentration of UK14304. Although the difference in the responses to cumulative and non-cumulative drug additions is not apparent from the graph, non-cumulative UK14304 always produced a measurable response, unlike curves constructed cumulatively.

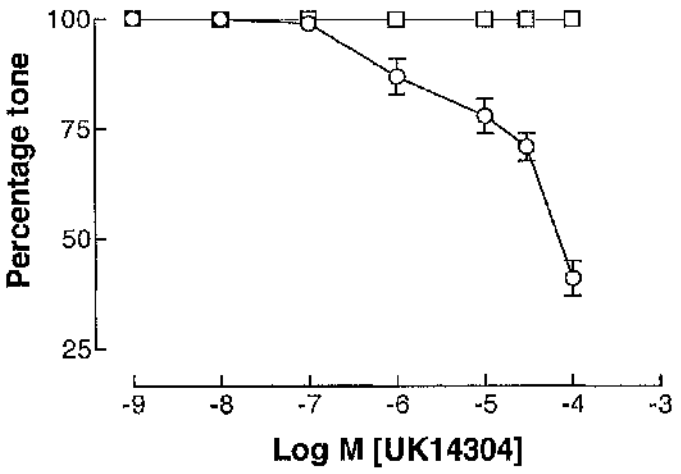



Figure 6.4: The non-cumulative UK14304-mediated response in first order mesenteric arteries from 4-month old WT mice. The variation in U46619-inducted tone over time (□, n = 12) and the non-cumulative response curve to UK (○, n = 12). Both data sets were expressed as a percentage of U46619-induced tone. Each point represents mean ± standard error.

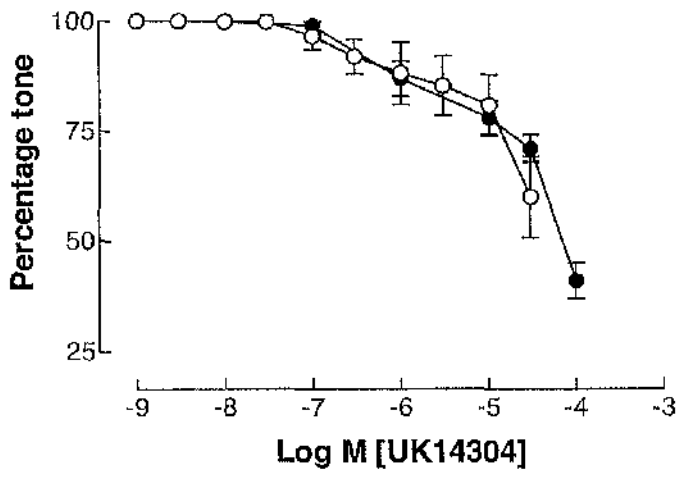



Figure 6.5: UK14304-mediated responses in first order mesenteric arteries from 4-month old WT mice. Cumulative (○, n = 12) and non-cumulative (●, n = 12) UK response curves, expressed as a percentage of U46619-induced tone. Each point represents mean ± standard error.

6.3.4 Effect of L-NAME on non-cumulative UK14304 in WT arteries

Figure 6.6 shows the effect of L-NAME on UK14304-mediated relaxant responses. L-NAME significantly attenuated agonist-induced relaxations at 1 x $10^{-6}$ ($p = 0.049$*) and 1 x $10^{-5}$M ($p = 0.008$**). At higher UK14304 concentrations the effects of L-NAME were surmountable.

6.3.5 Effect of L-NAME on U46619-induced elevations in vascular tone

To test whether L-NAME affects the contraction produced by U46619, responses were compared before and after incubation with L-NAME. In arteries from WT mice the size of U46619-induced contractions was unaffected by the presence of L-NAME ($p = 0.56$). Similarly, L-NAME had no effect on the contractile responses to U46619 in arteries from D79N mice ($p = 0.82$).

6.3.6 Effect of rauwolscine on the UK14304 response in WT arteries

Figure 6.7 shows the non-cumulative UK14304 response alone, and in the presence of rauwolscine (3 x $10^{-8}$M), in first order mesenteric resistance arteries from four-month old WT mice. Rauwolscine attenuated UK14304-mediated relaxations at low agonist concentrations. Incubating arterial rings with rauwolscine abolished agonist-induced relaxations at UK14304 concentrations of 1 x $10^{-6}$M, while in control vessels a 13 ± 4.0 % reduction in U46619-induced tone occurred ($p = 0.04$*). Rauwolscine also attenuated relaxations at a UK14304 concentration of 1 x $10^{-5}$M. In the control curve UK14304 reduced U46619-induced tone to 78 ± 3.9 % of basal levels, but in the presence of rauwolscine, tone was only reduced to 88.5 ± 2.6 % of the U46619-induced contraction ($p = 0.0003$***). At higher agonist concentrations, the antagonism produced by rauwolscine was surmountable, and at the highest UK14304 concentration,

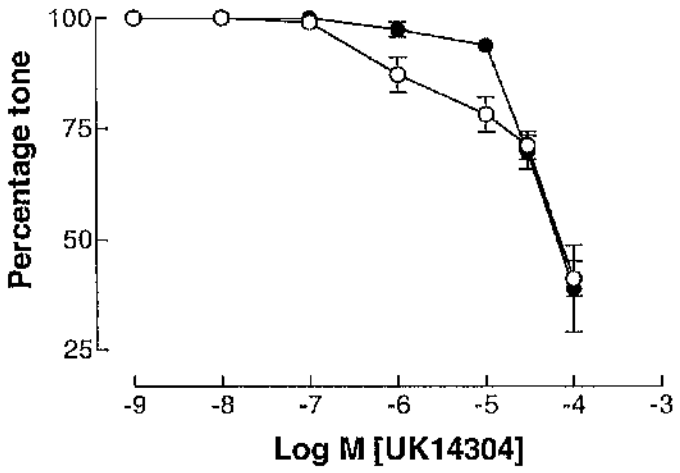



Figure 6.6: The non-cumulative UK14304-mediated responses in first order mesenteric arteries from 4-month old WT mice. A non-cumulative UK response curve alone (○, n = 12) and in the presence of L-NAME (1 x $10^{-4}$M,●, n = 6). Both data sets were expressed as a percentage of U46619-induced tone. Each point represents mean ± standard error.

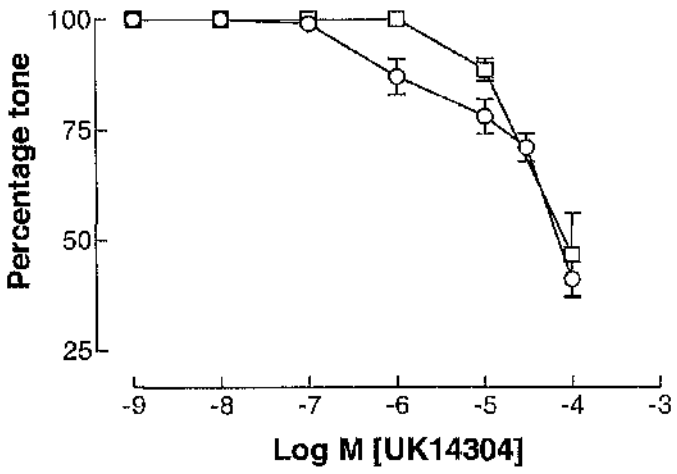



Figure 6.7:The non-cumulative UK14304-mediated responses in first order mesenteric arteries from 4-month old WT mice. A non-cumulative UK response curve alone (○, n = 12), and in the presence of rauwolscine (3 x $10^{-8}$M, □, n = 4). Both data sets were expressed as a percentage of U46619-induced tone. Each point represents mean ± standard error.

relaxant responses obtained in control vessels were no different from those gained in vessels incubated with rauwolscine ($p>0.05$).

6.3.7 The non-cumulative UK14304 response in D79N arteries

Figure 6.8 shows the non-cumulative response to UK14304 in first order mesenteric resistance arteries from four-month old D79N mice. UK14304 caused a concentration-related reduction in U46619-induced tone. At the highest agonist concentration tested ($1 \times 10^{-4}$M), UK14304 caused a $67.6 \pm 8.8$ % reduction in U46619-induced tone. A $pIC_{50}$ value was calculated in this instance, because the relaxation caused by UK14304 exceeded 50 %, and was determined to be $4.8 \pm 2.3$.

6.3.7 Effect of L-NAME on the non-cumulative UK14304 response in the D79N

Figure 6.8 also shows the effect of increasing concentrations of UK14304 alone, and in the presence of L-NAME ($1 \times 10^{-4}$M) on responses in first order mesenteric arteries from four-month old D79N mice. Incubation with L-NAME, prior to construction of a UK14304 curve, caused a significant reduction in the relaxant response obtained. Relaxations gained were significantly attenuated at $1 \times 10^{-6}$, $1 \times 10^{-5}$ and $1 \times 10^{-4}$M. At the maximum UK14304 concentration ($1 \times 10^{-4}$M), L-NAME significantly attenuated the relaxation responses. In the control curve, UK14304 at a concentration of $1 \times 10^{-4}$M caused a $67.6 \pm 8.8$ % reduction in tone, but in the presence of L-NAME the relaxation gained only reduced U46619-induced tone by $36 \pm 2.1$ % ($p = 0.0001$***).

6.3.8 Comparison of responses gained in WT and D79N arteries

Figure 6.9 shows the non-cumulative UK14304 response in WT and D79N arteries, to a UK14304 curve alone (figure 6.9 **A**), and in the presence of L-NAME (figure 6.9 **B**).

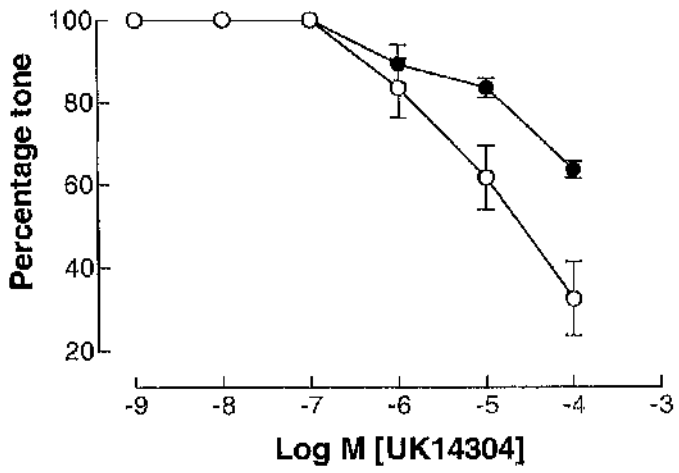



Figure 6.8: The non-cumulative UK14304-mediated responses in first order mesenteric resistance arteries from 4-month old D79N mice. A non-cumulative UK response curve alone (○, n = 6) and in the presence of L-NAME (1 x $10^{-4}$M, ●, n = 6). Both data sets were expressed as a percentage of U46619-induced tone. Each point represents mean ± standard error.

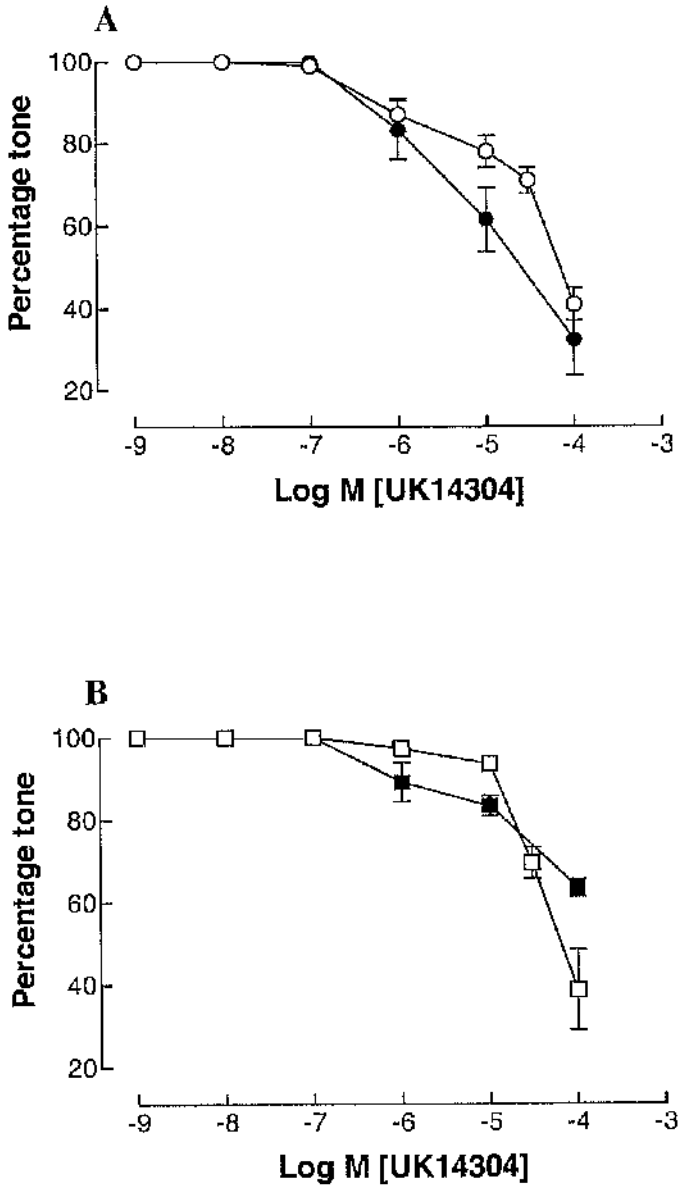



Figure 6.9: The non-cumulative UK14304-mediated responses in first order mesenteric resistance arteries from 4-month old WT and D79N mice. **A** A non-cumulative UK response curve in the WT (○, n = 12) and D79N (●, n = 6). **B** A non-cumulative UK response curve in the presence of L-NAME in the WT ( □, n = 6 ) and D79N ( ■, n = 6 ). Data expressed as a percentage of U19-induced tone. Each point represents mean ± standard error.

Comparison of the curves gained, showed that responses in the WT were shifted rightward in respect of the results gained in the D79N. At the highest UK14304 concentration tested (1 x $10^{-4}$M), there was no significant difference between relaxations in WT and D79N arteries (p = 0.328).

The most striking difference observed between UK14304-induced responses in mesenteric resistance arteries from WT and D79N mice occurred at higher UK14304 concentration. L-NAME had a more significant effect on UK14304-mediated relaxations in D79N arteries, than it did in the WT. At the highest agonist concentration tested, relaxant responses were significantly smaller in the D79N (the inhibition of relaxation by L-NAME was greater in the D79N) than the WT in the presence of L-NAME (p = 0.025*). Yet in control curves, the responses at the same UK14304 concentration were comparable.

6.3.9 Effect of elevated $K^+$ levels on UK14304-mediated relaxations

Figure 6.10 **A** shows the response to a single concentration of UK14304 in the presence of elevated $K^+$ levels (30mM). Following the elevation of extracellular $K^+$, tone was elevated with U46619 (1 x $10^{-7}$M). Figure 6.10 **B** shows the response in the same artery after removal of 30mM KPSS from the surrounding bathing solution. In comparison to the response shown in figure 6.10 **A**, the relaxation caused by UK14304 (1 x $10^{-6}$M) when $K^+$ levels were not elevated with KPSS are significantly greater.

In Figure 6.11 the results shown are similar to those in figure 6.10, but in this instance the concentration of KPSS used was lower (15mM). In the presence of elevated $K^+$ the relaxant response to a single concentration of UK14304 (1 x $10^{-6}$M) was greater than

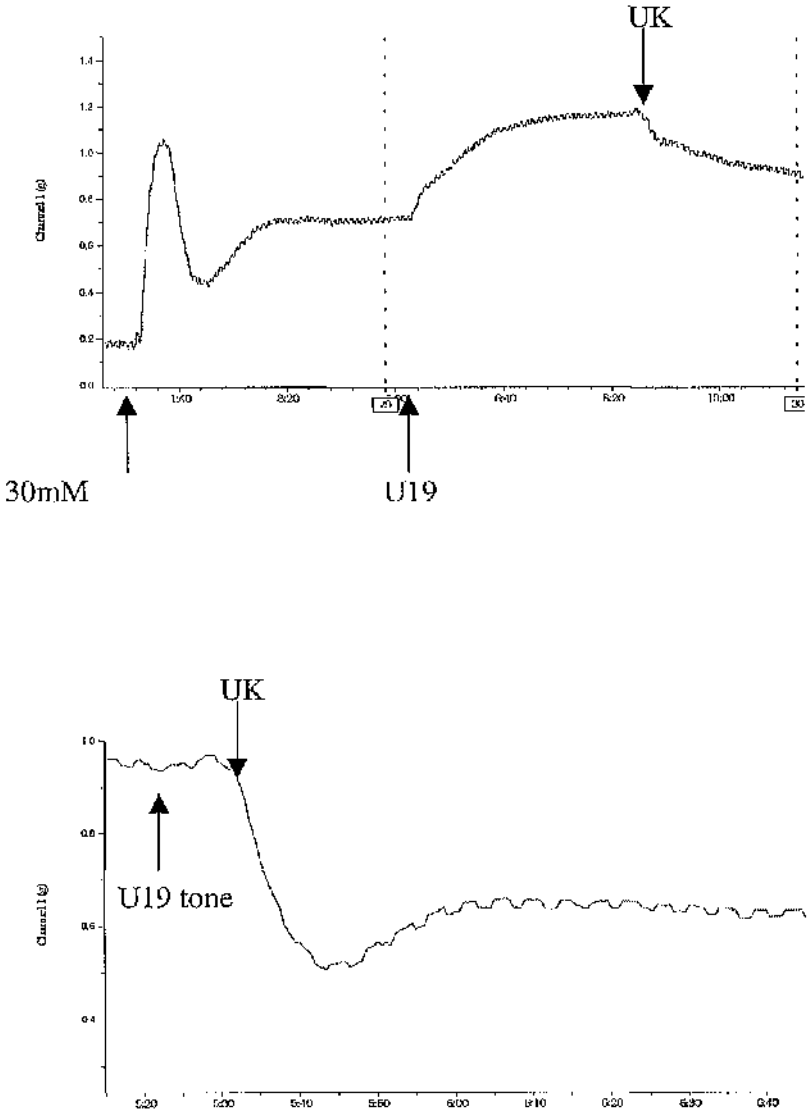



Figure 6.10: A trace of the effect of UK at 1 x $10^{-6}$M, alone, and in the presence of 30mM KPSS. **A** The relaxant response to UK in the presence of U19-induced tone and 30mM KPSS. **B** The relaxant response to the same UK concentration in the presence of U19-induced tone, after KPSS has been removed from the myograph bath. Responses shown are in a single vessel.

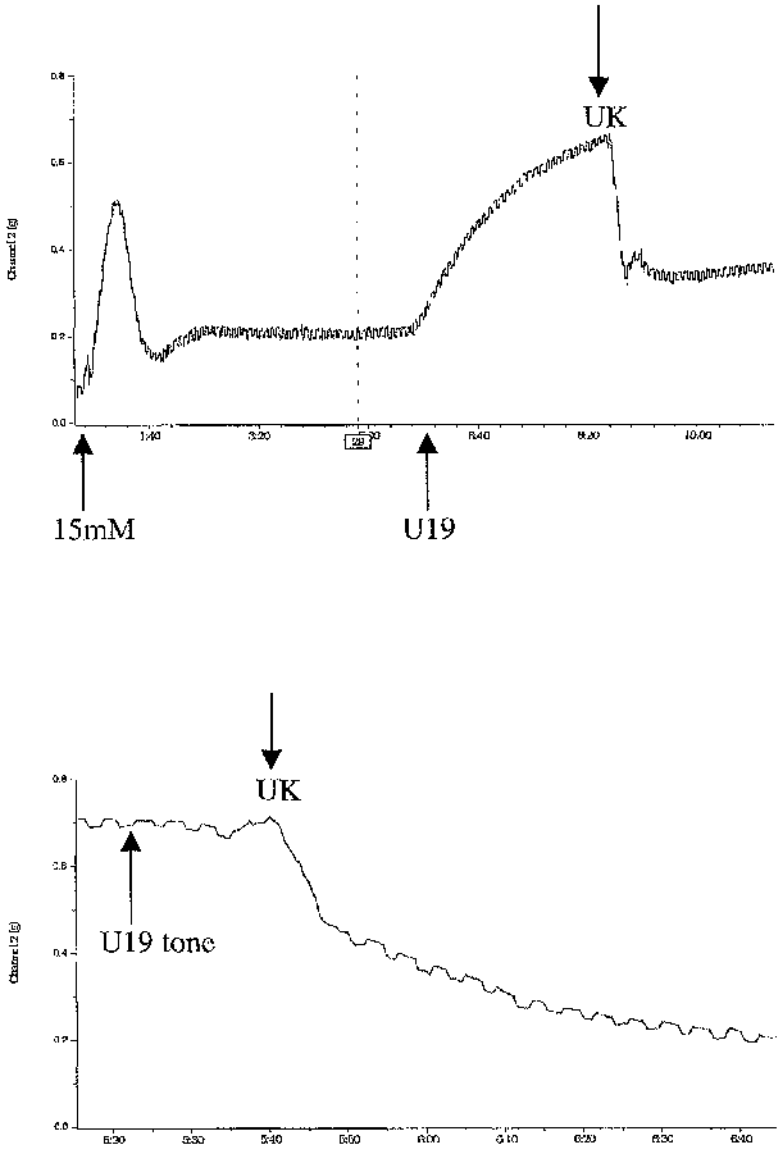



Figure 6.11: A trace of the effect of UK at 1 x $10^{-6}$M, alone, and in the presence of 15mM KPSS. **A** The relaxant response to UK in the presence of U19-induced tone and 15mM KPSS. **B** The relaxant response to the same UK concentration in the presence of U19-induced tone, after KPSS has been removed from the myograph bath. Responses shown are in a single vessel.

when $K^+$ levels were enhanced. Figure 6.11 **B** illustrates that as in figure 6.10 **B** in the absence of elevated $K^+$ the relaxant response to UK14304 was greater.

## 6.4 Discussion

In mouse mesenteric resistance arteries, UK14304 causes concentration-related relaxations of wire myograph-mounted first order mesenteric resistance arteries from the WT and D79N. Fortunately, the UK14304-mediated response in mesenteric resistance arteries is reproducible, and more easily studied than UK14304-induced contractions of the murine tail artery.

Stimulation of $\alpha_2$-adrenoceptors, located on endothelial cells of rat mesenteric resistance arteries, leads to vasodilatator responses of preconstricted isolated arteries. In porcine arteries, this has been attributed to activation of the $\alpha_{2A/D}$-adrenoceptor (based on the rank order of potency of a number of selective agonists), which leads to alterations in cAMP levels [Bockman et al, 1993].

### Cumulative UK14304 response in mesenteric resistance arteries

Initially, the effect of UK14304 on U46619-induced tone was investigated by constructing cumulative response curves. The responses gained were variable. In addition, a number of vessels were unresponsive to agonist stimulation, even when an acetylcholine-induced relaxation had been confirmed during the wake-up protocol. Given this, and in light of the desensitisation of UK14304-mediated contractions in the tail and mesenteric resistance arteries, all further experiments investigating the effect of UK14304 on mesenteric arteries, were studied by constructing randomised, non-cumulative response curves.

The non-cumulative UK14304-mediated response

UK14304 causes concentration-related vasodilatation of U46619-induced tone in first order mesenteric arteries from four-month old WT mice. The responses gained, tend to be greater in size than those observed in the cumulative response, and are less variable. This is shown by a measurable response being obtained in all arteries used, and a reduction in the standard error of the mean, which is indicative of a reduction in variability.

In D79N arteries, curves were only constructed non-cumulatively. UK14304 causes concentration-dependent reductions in U46619-induced tone, that are of comparable size to those gained in the WT, and at a UK14304 concentration of $1 \times 10^{-5}$M, are significantly greater. The relaxant response to UK14304 in D79N arteries, suggests that in murine mesenteric vessels, the $\alpha_{2A/D}$-adrenoceptor is not the major subtype mediating agonist-induced relaxations or, that another receptor has been up-regulated to compensate for its loss.

Endothelial cells contribute to vasodilatation by regulating the delicate balance between endothelium derived relaxing and contracting factors. Vasodilator responses occur when nitric oxide, prostacyclin, and endothelium derived hyperpolarizing factor (EDHF) are liberated or released. Nitric oxide appears to be the major contributor to relaxant responses in large calibre arteries, such as the aorta and carotid, while the relaxant effects of EDHF increases, the further you move down the vascular tree [Shimokawa et al, 1996].

Effect of L-NAME on UK-mediated vasodilatations

At low agonist concentrations, incubating mesenteric arteries with L-NAME (1 x $10^{-4}$M) prevents UK14304-induced relaxations in the WT and D79N. However, at high agonist concentrations, relaxations that are of comparable size to those gained in a control curve still occur. This suggests the involvement of additional relaxing factors, and uncovers a relaxant response that cannot be attributed solely to the release of nitric oxide.

At submaximal UK14304 (1 x $10^{-6}$M) concentrations, the relaxant response in the D79N is slightly, but significantly, greater than that of the WT. L-NAME abolishes the enhanced vasodilator response in the D79N, giving relaxations that are of comparable size to those produced in the WT. In the presence of L-NAME, UK14304-mediated relaxations at a concentration of 1 x $10^{-4}$M are significantly smaller in the WT than the D79N. This suggests that nitric oxide plays a more significant role in the regulation of vascular responses in the D79N than in the WT. Results gained in chapter three (figure 3. 11) show that blocking nitric oxide release significantly potentiates contractile responses in the D79N tail artery but has little, if any, effect in the WT. Although the difference in relaxant responses is not as obvious as the effect on contractility, this still provides further evidence in support of the hypothesis, that in the absence of a fully functional $\alpha_{2A/D}$-adrenoceptor pool, regulation of nitric oxide release is affected (and may be increased).

In rat mesenteric resistance arteries, blockade of nitric oxide alone does not inhibit endothelium-mediated relaxations, because prostacyclin and EDHF participate in the vasodilator response. Furthermore, acetylcholine-induced vasodilatations of rat

mesenteric resistance arteries are mediated, primarily by EDHF [Shimskawa et al, 1996]. However, when nitric oxide synthase is inhibited with L-NAME, and COX activity is blocked with indomethacin, NO scavengers can still significantly reduce acetylcholine-induced relaxations [Chauhan et al, 2003], refuting this hypothesis.

In the rat, EDHF is a major contributor to relaxant responses in mesenteric resistance arteries, but plays little, if any role in larger calibre blood vessels [Hwa et al, 1994]. In mouse mesenteric arteries, L-NAME is not maximally effective in the WT or the D79N, suggesting the involvement of other, as yet unidentified relaxing factors. In preliminary studies, raising extracellular potassium levels inhibits UK14304-mediated relaxations in a reversible manner. Reducing $K^+$ levels allows relaxations to resume. This provides evidence, which suggests that EDHF is involved in UK14304-mediated vasodilatations of mouse mesenteric resistance arteries. Experimentally, when extracellular $K^+$ influx is blocked, EDHF-induced relaxations are prevented [Chanhan et al, 2003].

In rat mesenteric and hepatic arteries, treatment with L-NAME and COX inhibition cause a significant reduction in acetylcholine-mediated vasodilatations. The remaining response has previously been attributed to EDHF, but in the presence of NO scavengers, a further inhibition of relaxations is observed [Chauhan et al, 2003]. Leading to the conclusion that nitric oxide, proposed to be stored in endothelial cells, can be released and mediate vasodilatations, by opening $K^+$ channels [Chauhan et al, 2003]. This hypothesis is supported by experiments by Simonsen and co workers. They have shown that in the presence of submaximal concentrations of L-NAME, that microsensors can still detect nitric oxide release [Simonsen et al, 1999].

The results presented here suggest that UK14304-mediated nitric oxide release contribute to relaxant responses of murine mesenteric resistance arteries. However, the surmountable effect of L-NAME, suggests the involvement of additional relaxing factors. Preliminary studies suggest the involvement of EDHF, but without COX inhibition and the use of NO scavengers, no clear conclusions as to the identity of additional relaxant mediators can be made.

Effect of rauwolscine on UK14304-mediated responses

Due to the high variability and lack of responsiveness when cumulative UK14304 curves are constructed, and the additional complication of agonist-induced receptor desensitisation, the effect of rauwolscine was investigated against first, non-cumulative response curves. Rauwolscine, at a concentration of $3 \times 10^{-8}$M, antagonises vasodilatations induced by low concentrations of UK14304. The antagonism produced by rauwolscine is surmountable, and at high UK14304 concentrations, relaxations, that are comparable to those gained in a control curve, proceed. The inhibitory effect of rauwolscine provides evidence that the UK14304-mediated response, results from selective stimulation of $\alpha_2$-adrenoceptors, which are probably endothelial in origin. The use of higher concentrations of rauwolscine was deemed unproductive since selectivity against $\alpha_2$-adrenoceptors could not be guaranteed. However, at high concentrations of UK14304, the lack of effect of rauwolscine suggests an effect that cannot be attributed to stimulation of $\alpha_2$-adrenoceptors.

Summary and conclusions

To summarise, unlike the rat, the $\alpha_{2A/D}$-adrenoceptor does not appear to be the major $\alpha_2$-adrenoceptor-subtype mediating vasodilatations of murine mesenteric resistance

arteries. This conclusion is made based on the results obtained in the D79N, where UK14304-mediated relaxations persist even in the absence of functional $\alpha_{2A/D}$-adrenoceptors. The inhibitory effect of rauwolscine confirms that UK14304-mediated effects are attributable to stimulation of $\alpha_2$-adrenoceptors, but at high agonist concentrations may involve another receptor. Taken together, the results in the D79N, and responses in the WT with rauwolscine, lead to the conclusion that the UK14304 response is mediated, in part, by the $\alpha_{2C}$ and/or $\alpha_{2B}$ subtype (s). We cannot exclude involvement of the $\alpha_{2A/D}$ in the control mouse, but, if it is significant there, it must have been compensated for, or replaced by another subtype when it was suppressed in the D79N.

Activation of $\alpha_2$-adrenoceptors in mesenteric resistance arteries stimulates nitric oxide release, which acts to oppose U46619-induced contractions. In addition, other relaxing factors, which are not susceptible to L-NAME, contribute to vasodilatations. Their identity has yet to be determined, but these responses are presumed to involve EDHF. However, the possibility that stored nitric oxide, and/or prostacyclin are involved, cannot, at this point, be excluded.

# Chapter seven

## The determination of $pA_2$ values for prazosin in the tail artery of young and old WT and $\alpha_{1B}$ KO mice

## 7.1 Introduction

Experiments in knockout mice provide evidence that the $\alpha_{1B}$-adrenoceptor is involved in the regulation of pressor responses in the mouse [McCafferty et al, 1999]. In the pithed mouse, phenylephrine-induced pressor effects can be antagonised by the $\alpha_1$-selective antagonist terazosin, but were resistant to antagonism with rauwolscine. B-HT 933, an $\alpha_2$-selective agonist, also caused an increase in peripheral blood pressure, which was antagonised by rauwolscine, but unaffected by low doses of terazosin. This data confirmed that as in the rat, pressor responses were mediated by a mixed population of $\alpha_1$ and $\alpha_2$-adrenoceptors in the mouse [McCafferty et al, 1999], and that *in vitro* analysis was required to clarify which arteries were major contributors to the control of blood pressure, and to delineate the role of each adrenoceptor subtype *in situ*.

$\alpha_{1B}$-adrenoceptors are expressed in a wide variety of arteries known to regulate blood pressure, yet the contractile responses mediated by this adrenoceptor subtype, are poorly defined. This is due in part to a lack of subtype selective ligands. In light of the current advances in molecular biology, mice have been generated with gene-targeted disruptions of each adrenoceptor subtype. This procedure has been used successfully to 'knockout' the $\alpha_{1B}$-adrenoceptor [Cavalli et al, 1997].

Studies that aimed to determine the changes in the expression levels of $\alpha_1$-adrenoceptors are often contradictory, and the changes that occurred in expression levels of adrenoceptors appear to depend on the species and vessel type studied. Data has been presented that proposes that age dependent alterations occur in the expression and function of the $\alpha_{1D}$ receptor subtype in the rat vasculature [Ibarra et al, 1997]. However, little if any information is available on what happens to responses mediated

by $\alpha_{1B}$-adrenoceptors, partly because of the lack of subtype selective compounds for this adrenoceptor.

It has been suggested that the increase in systemic blood pressure occurring with age, may be the result of an elevation in peripheral nerve activity. Evidence in support of this hypothesis came from studies that showed circulating levels of catecholamines increased in humans with advancing age [Buchholz & Duckles, 1990]. Age related increases in sympathetic nerve activity are known to occur in peripheral blood vessels [Buchholz et al, 1995]. Whether a change occurs in the expression or function of the adrenoceptors exposed to high levels of neurotransmitters is unclear.

There were two main aims in carrying out the experiments contained within this chapter. The first was to determine what, if any, role the $\alpha_{1B}$-adrenoceptor plays in phenylephrine-induced contractions of the murine tail artery. This aim was achieved by testing the effects of prazosin, in combination with studies where the subtype selective antagonists, 5MeU and BMY7378 were utilised [Daly et al, 2002]. Secondly to determine, if $\alpha_1$-adrenoceptor responses were altered with increasing age and, if so, what affect does a lack of functional $\alpha_{1B}$-adrenoceptors have on these responses?

Previous work has documented a change in catecholamine-induced contractions of the rat tail artery. With increasing age, contractile responses became significantly potentiated in maximum. In addition to an alteration in the size of the responses achieved, the sensitivity to exogenous agonists was also affected. The increase in tissue sensitivity has been attributed to an increase in oxidative stress with advancing age [Janero et al, 1990].

## 7.2 Methods

Four and sixteen-month old WT (C57BL/6c/129Sv) and $\alpha_{1B}$ KO ($\alpha_{1B}$ -/- C57BL/6c/129Sv, background) mice were killed by asphyxiation with $CO_2$. Immediately after, tails were removed and immersed in fresh, cold Krebs until dissection. Tails arteries were dissected, and mounted in Mulvany/Halpern wire myograph baths, containing 5mls of fresh Krebs, gassed with a 95 % $O_2$ 5 % $CO_2$ mixture. Following an equilibration period a resting tension of 0.25gms Force was applied to each mounted arterial ring (Detailed description contained in chapter two).

### 7.2.1 Wake-up protocol

Each mounted, arterial ring was challenged with phenylephrine at a concentration of 1 x $10^{-5}$M. Once the contractile response reached a plateau, arteries were washed with fresh Krebs four times over a fifteen minute period. This procedure was repeated twice, once with the same concentration of phenylephrine, and a second time with phenylephrine at a concentration of 1 x $10^{-6}$M. When the third, and final, phenylephrine response reached a plateau, acetylcholine (3 x $10^{-6}$M) was added to test the viability of the endothelium. After a series of washes over a fifteen minute period, arteries were allowed a 30-35 minute rest period before construction of the first phenylephrine curve.

### 7.2.2 Effect of nifedipine on phenylephrine-induced contractions

Tail arteries from WT and $\alpha_{1B}$ KO mice were incubated with nifedipine at a concentration of 1 x $10^{-7}$M (final bath concentration), for a minimum of twenty minutes prior to construction of a phenylephrine response curve. After construction of a control curve, to determine the effect nifedipine had on contractile responses, all subsequent experiments were carried out in the presence of nifedipine: this was done for two

reasons. Firstly, experiments were carried out in the presence of nifedipine to complement an earlier study carried out in the laboratory [Daly et al, 2002]. Secondly, as in the earlier publication to prevent the development of rhythmic contractions that occur when exogenous agonists are applied in the tail artery.

### 7.2.3 Effect of prazosin on phenylephrine-induced contractions

The effect of increasing concentrations of prazosin has been determined against the phenylephrine-induced response in tail arteries from WT and $\alpha_{1B}$ KO mice, at two age points. To permit the calculation of a $pA_2$ value, prazosin was used at three concentrations, ranging from $1 \times 10^{-9}$ to $1 \times 10^{-7}$M. Each mounted vessel was subjected to an initial phenylephrine curve, which was followed by a phenylephrine curve in the presence of nifedipine ($1 \times 10^{-7}$M). Where one vessel was then used as a time control, receiving a curve in the absence of antagonists. The remaining three vessels were incubated with a single concentration of prazosin for a minimum of thirty minutes before construction of a further response curve. Therefore, each mounted vessel was subjected to two consecutive response curves.

## 7.3 Results

### 7.3.1 Effect of nifedipine in arteries from 4-month old WT and $\alpha_{1B}$KO mice

Figure 7.1 shows the cumulative phenylephrine response alone, and in the presence of nifedipine; curves were constructed in tail arteries from four-month old WT (figure 7.1 **A**) and $\alpha_{1B}$ KO (figure 7.1 **B**) mice. In WT arteries, contractions to phenylephrine reached a maximum of 0.64 ± 0.06gms Force, while in the presence of nifedipine the maximum contraction was significantly reduced to 0.51 ± 0.05gms Force ($p = 0.006$*).

Nifedipine has a more pronounced effect on the phenylephrine-induced response in tail arteries from the $\alpha_{1B}$ KO. The maximum contraction in the phenylephrine control curve was 0.73 ± 0.09gms Force, while in the presence of nifedipine it was reduced significantly to 0.39 ± 0.05gms Force ($p<0.001$***).

Figure 7.2 shows the responses gained in WT and $\alpha_{1B}$ KO tail arteries in a control curve (figure 7.2 **A**) and in the presence of nifedipine (figure 7.2 **B**), on the same graph for ease of comparison. In the control curve the maximum response in the $\alpha_{1B}$ KO was significantly greater than that of the WT. The WT maxima was 0.64 ± 0.06gms Force, compared to the maximum response gained in the $\alpha_{1B}$ KO, that was 0.73 ± 0.09gms Force ($p = 0.02$*). This order is reversed after nifedipine; WT 0.51 ± 0.05gms Force, $\alpha_{1B}$ KO significantly smaller at 0.39 ± 0.05gms Force ($p = 0.0098$**).

Nifedipine caused a greater reduction in the maximum phenylephrine-induced response in the KO than in the WT. The mean change in maximum in the WT was 0.13 ± 0.1gms Force, while in the $\alpha_{1B}$ KO the mean reduction in maximum was 0.42 ± 0.04gms Force.

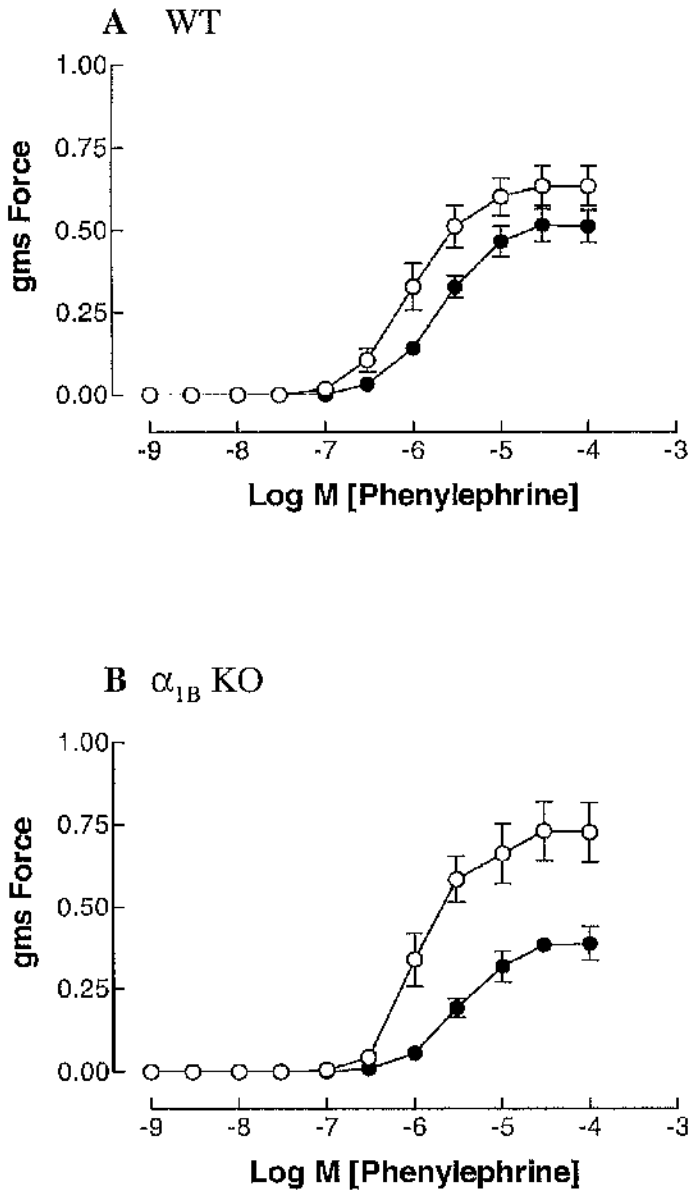



Figure 7.1: Responses in tail arteries from 4-month old WT and $\alpha_{1B}$ KO mice. **A** A PE response curve alone (○, n = 9) and with nifedipine (1 x $10^{-7}$M, ●, n = 6) in the WT. **B** A PE response curve alone (○, n = 9) and with nifedipine (●, n = 6) in the $\alpha_{1B}$ KO. Each point represents mean ± standard error.

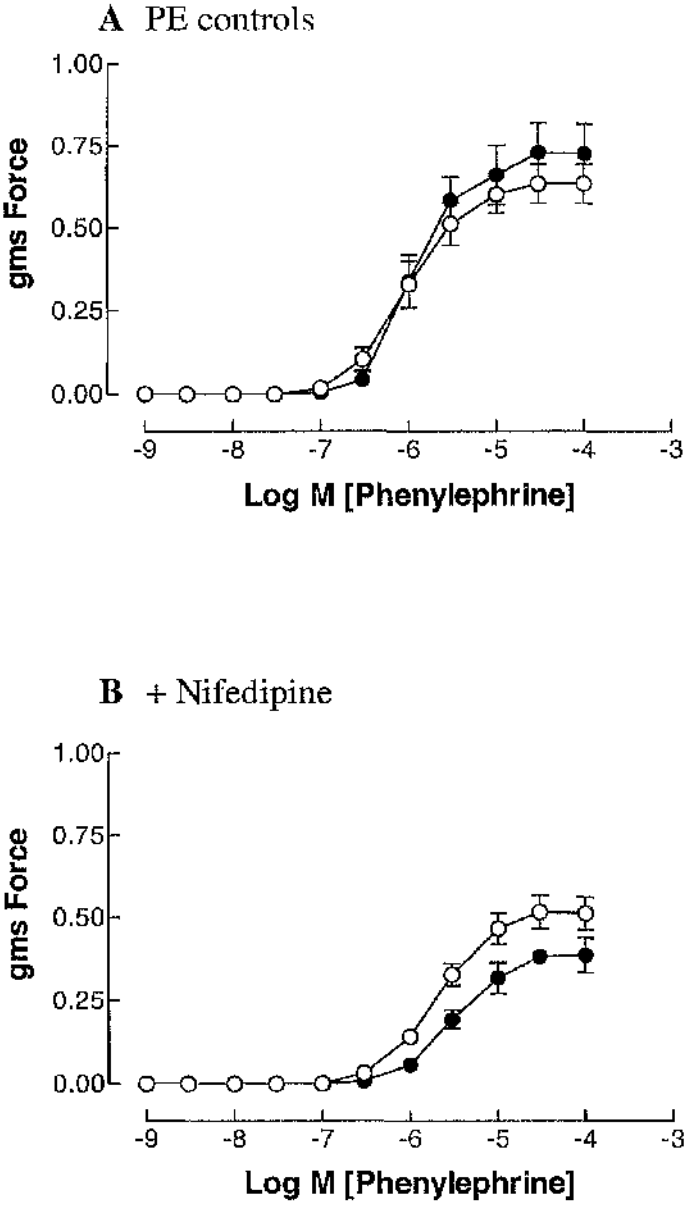



Figure 7.2: Responses in tail arteries from 4-month old WT and $\alpha_{1B}$KO mice. **A** A PE control curve in the WT (○, n = 9) and $\alpha_{1B}$KO (●, n = 9). **B** A PE response curve in the presence of nifedipine ( 1 x $10^{-7}$M) in the WT (○, n = 6) and $\alpha_{1B}$KO (●, n = 6). Each point represents mean ± standard error.

Statistical analysis of the reduction in the maximum between strains revealed that in the $\alpha_{1B}$ KO, nifedipine caused a significantly greater reduction in maximal contractions ($p<0.0001$***).

Comparison of responses gained in the presence of nifedipine revealed two things. Firstly, that nifedipine caused a significant reduction in phenylephrine-induced contractions in the WT and $\alpha_{1B}$ KO tail artery. Secondly, that the reduction in the maximum response in the $\alpha_{1B}$ KO was significantly greater than in the WT. In the control curve, the maximum agonist-induced contraction was greater in the $\alpha_{1B}$ KO than in the WT. However, in the presence of nifedipine, contractile responses in the WT tail artery exceeded those of the $\alpha_{1B}$ KO. This situation is unique amongst the vessels that our laboratory has compared in the WT and $\alpha_{1B}$ KO. In aorta, carotid and first order mesenteric resistance arteries responses have been shown to be consistently larger in the $\alpha_{1B}$ KO, but the study of responses in these arteries was not complicated by the development of rhythmic contractions, unlike the mouse tail artery.

Figure 7.3 shows the phenylephrine response in control curves, and in the presence of nifedipine in tail arteries from WT (figure 7.3 **A**) and $\alpha_{1B}$ KO (figure 7.3 **B**) mice, expressed as a percentage of their own maximum. In WT arteries, the control curve had a $pEC_{50}$ of $6.0 \pm 0.01$, which was significantly reduced in the presence of nifedipine to $5.7 \pm 0.01$ ($p<0.0001$***). Nifedipine had a similar effect on the $pEC_{50}$ value gained in the $\alpha_{1B}$ KO. The $\alpha_{1B}$ KO control curve yielded a $pEC_{50}$ value of $6.0 \pm 0.02$, while in the presence of nifedipine the $pEC_{50}$ value was significantly reduced to $5.5 \pm 0.02$ $\alpha_{1B}$ KO ($p<0.0001$***).

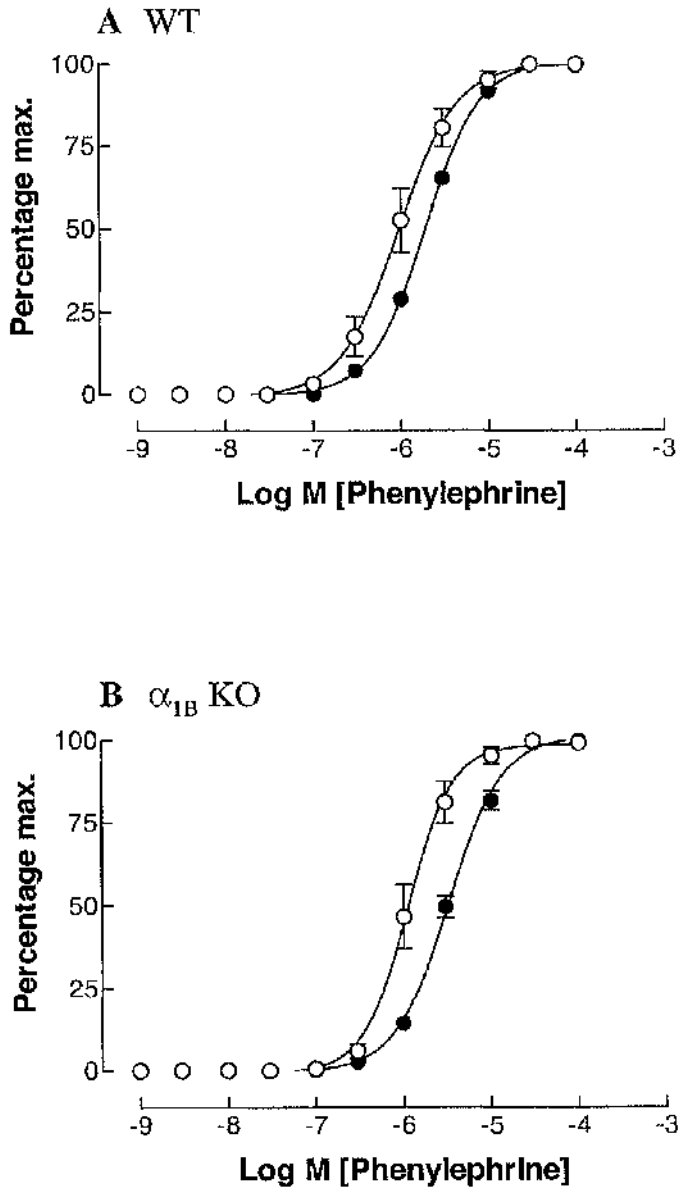



Figure 7.3: Responses in tail arteries from 4-month old WT and $\alpha_{1B}$ KO mice, expressed as percentage maximum. **A** A PE response curve alone (○, n = 9) and with nifedipine (1 x $10^{-7}$M, ●, n = 6) in the WT. **B** A PE response curve alone (○, n = 9) and with nifedipine (●, n = 6) in the $\alpha_{1B}$ KO. Each point represents mean ± standard error.

7.3.2 Effect of prazosin in 4-month old WT and $\alpha_{1B}$ KO tail arteries

All of the response curves constructed to investigate the effect of prazosin on the phenylephrine-induced response were performed in the presence of nifedipine (1 x $10^{-7}$M). Prazosin caused a rightward shift in the concentration response curve to phenylephrine in tail arteries from WT mice, and a significant reduction in the maximum response at a prazosin concentration of 1 x $10^{-7}$M. The WT control curve maximum was 0.51 ± 0.05gms Force, in the prazosin curve (1 x $10^{-9}$M) was 0.38 ± 0.04gms Force, at 1 x $10^{-8}$M was 0.45 ± 0.05gms Force (no significant change at this concentration), and at 1 x $10^{-7}$M, prazosin caused a significant reduction to 0.29 ± 0.03gms Force ($p<0.01$**) although a true maximum was not established even at phenylephrine concentrations of 3 x $10^{-4}$M. Additionally, the maximum response at 1 x $10^{-7}$M was also significantly smaller than the response gained in the presence of prazosin at a concentration of 1 x $10^{-8}$M ($p<0.05$*).

In tail arteries from the $\alpha_{1B}$ KO, increasing concentrations of prazosin caused a rightward shift in the phenylephrine response curve, and at higher antagonist concentrations (1 x $10^{-7}$M) a significant reduction in the maximum response, though again this was not a true maximum. The $\alpha_{1B}$ control curve maximum was 0.39 ± 0.05gms Force, with 1 x $10^{-9}$M prazosin was 0.27 ± 0.04gms Force, at 1 x $10^{-8}$M was 0.36 ± 0.04gms Force (no significant change to this concentration), and at 1 x $10^{-7}$M prazosin the response was significantly reduced to 0.12 ± 0.05gms Force ($p<0.05$*). In addition to a reduction in contractility when compared with the control curve, the maximum response in the presence of prazosin at 1 x $10^{-7}$M was significant smaller than the response gained with prazosin at 1 x $10^{-8}$M ($p<0.05$*). As figure 7.4 (effect of 1 x

$10^{-9}$ and 1 x $10^{-8}$M prazosin) and 7.5 (effect of 1 x $10^{-7}$M prazosin) show the pattern of effects of prazosin was similar in the two strains.

7.3.3 $pA_2$ values for prazosin in tail arteries from 4-month old mice

Figure 7.6 shows the Schild regression plot for prazosin in tail arteries from four-month old WT (figure 7.6 **A**) and $\alpha_{1B}$ KO (figure 7.6 **B**) mice. To determine the $pA_2$ (detailed description contained in chapter two) value the log (dose ratio – 1) was calculated and plotted for each result gained from a sample size of six, in each strain. Where the line intercepts the x-axis determines the $pA_2$ value. Arteries from WT mice had a $pA_2$ value of 8.8, and the regression line had a slope of 0.95 ± 0.13, indicative of competitive antagonism. The $pA_2$ value calculated for prazosin in tail arteries from $\alpha_{1B}$ KO mice appeared to be higher than that obtained for the WT at 9.2, with a slope of 0.99 ± 0.14, again the slope of the line was not significantly different from unity, again indicative of competitive antagonism.

| | WT | $\alpha_{1B}$ KO |
|---|---|---|
| | Maximum (gms Force) | Maximum (gms Force) |
| Control | 0.64 ± 0.06, n = 9 | 0.73 ± 0.09, n = 9 |
| Nifedipine 1 x $10^{-7}$M | 0.51 ± 0.05, n = 6 | 0.39 ± 0.05, n = 6 |
| Prazosin 1 x $10^{-9}$M | 0.38 ± 0.04, n = 6 | 0.27 ± 0.04, n = 6 |
| Prazosin 1 x $10^{-8}$M | 0.45 ± 0.05, n = 6 | 0.36 ± 0.04, n = 6 |
| Prazosin 1 x $10^{-7}$M | 0.29 ± 0.03*, n = 6 | 0.12 ± 0.05*, n = 6 |

Table 7.1 Highest response attained in tail arteries from four-month old WT and $\alpha_{1B}$ KO

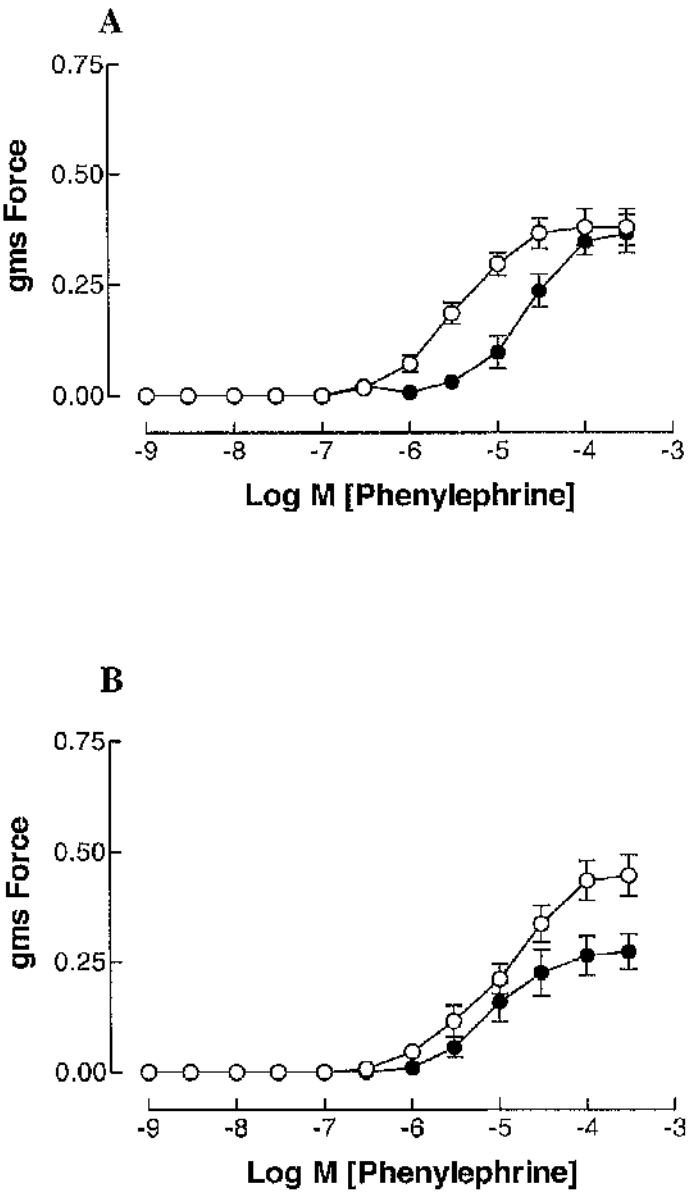



Figure 7.4: Responses in tail arteries from 4-month old WT and $\alpha_{1B}$KO mice (with nifedipine). **A** A PE response curve in the WT (○, n = 6) and $\alpha_{1B}$KO (●, n = 6) with prazosin at 1 x $10^{-9}$M. **B** A PE response curve in the WT (○, n = 6) and $\alpha_{1B}$KO (●, n = 6) with prazosin at 1 x $10^{-8}$M. Each point represents mean ± standard error.

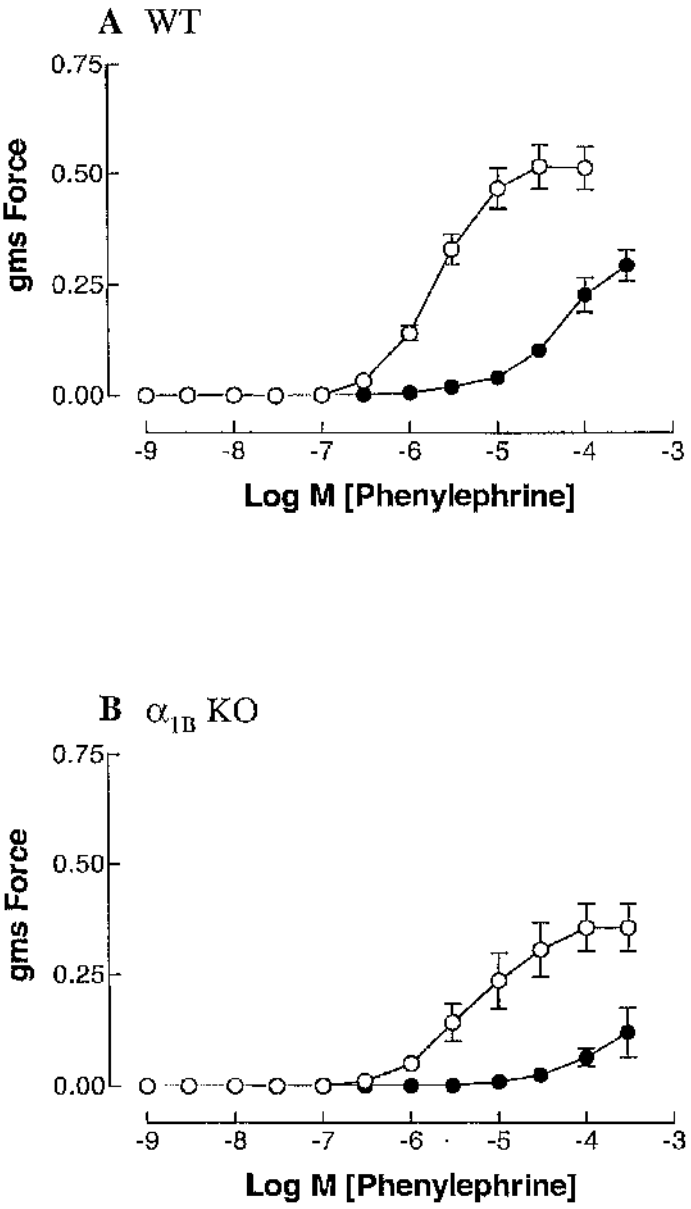



Figure 7.5: Responses in tail arteries from 4-month old WT and $\alpha_{1B}$ KO mice (with nifedipine). **A** PE control curve (○, n = 6), and with prazosin (1 x $10^{-7}$M, ●, n = 6)) in the WT. **B** PE control curve (○, n = 6), and with prazosin (1 x $10^{-7}$M, ●, n = 6)) in the $\alpha_{1B}$ KO. Each point represents mean ± standard error.

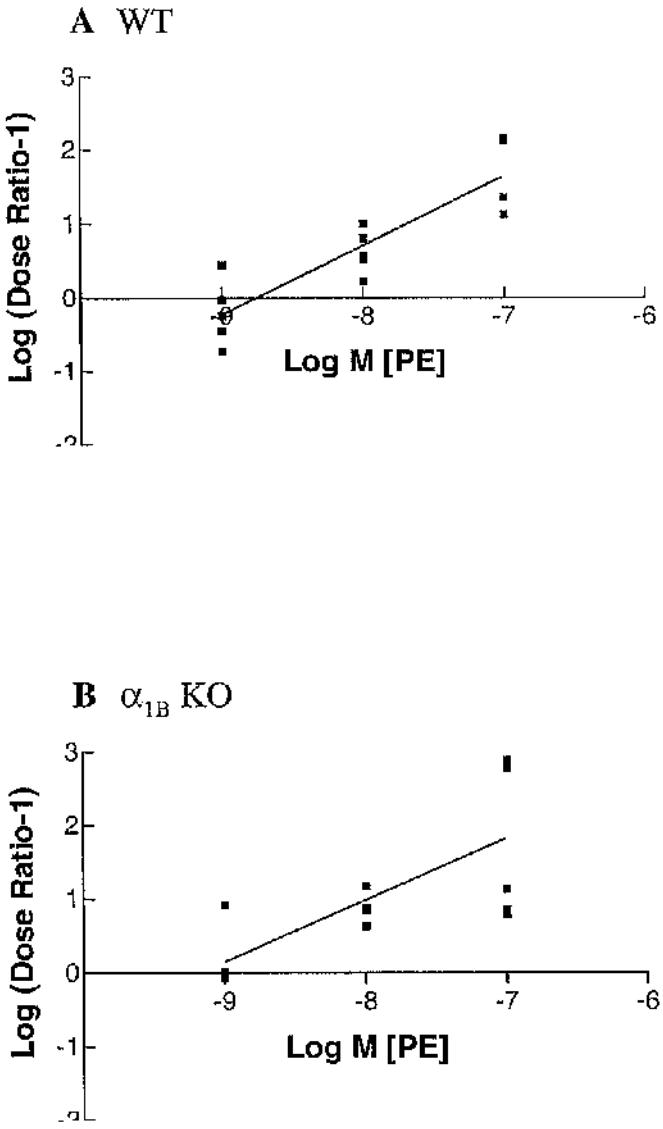



Figure 7.6: Schild regression plots for prazosin in tail arteries from 4-month old WT and $\alpha_{1B}$KO mice. **A** In WT arteries (■, n = 6), yielding a $pA_2$ value of 8.8 with a slope of 0.95. **B** In $\alpha_{1B}$KO arteries (■, n = 6), yielding a $pA_2$ value of 9.2 with a slope of 0.99. Each point shows a single results for a given antagonist concentration.

7.3.4 Effect of nifedipine in tail arteries from 16month old WT and $\alpha_{1B}$ KO mice

Figure 7.7 illustrates the effect of nifedipine ($1 \times 10^{-7}$M) on phenylephrine-induced contractions of tail arteries from four (figure 7.7 **A**) and sixteen-month old WT (figure 7.7 **B**) and four (figure 7.7 **C**) and sixteen-month old $\alpha_{1B}$ KO (figure 7.7 **D**) mice. At sixteen-months in the WT, the maximum response gained was $0.81 \pm 0.07$gms Force. In the presence of nifedipine, the maximum was reduced to $0.67 \pm 0.04$gms Force ($p = 0.075$), which is not significantly different from the control curve. However in the $\alpha_{1B}$ KO, the nifedipine-induced reduction in the maximum response was significant, with the control maximum being reduced from $0.86 \pm 0.07$gms Force, to $0.60 \pm 0.08$gms Force ($p = 0.038$*). Figure 7.7 **A** and figure 7.7 **C** are replicates of the graphs shown in figure 7.1, but were presented here for easy of comparison with the responses gained at sixteen-months.

Figure 7.8 compares the responses gained in a phenylephrine control curve at four (figure 7.8 **A**) and sixteen-months (figure 7.8 **B**), and the effect of nifedipine at four (figure 7.8 **C**) and sixteen-months (figure 7.8 **D**) on the phenylephrine-induced responses in the WT and $\alpha_{1B}$ KO. At sixteen-months in the WT control curve, phenylephrine produced a maximum response of $0.81 \pm 0.07$gms Force in WT arteries, compared with $0.86 \pm 0.07$gms Force for the $\alpha_{1B}$ KO, which were not significantly different ($p>0.05$). In the presence of nifedipine, contractions were generally smaller, but of comparable size in both strains. WT arteries gave a maximum contraction of $0.67 \pm 0.04$gms Force, compared with $0.60 \pm 0.08$gms Force in the sixteen-month old $\alpha_{1B}$ KO mice ($p>0.05$). Again, figures 7.8 **A**, and figure 7.8 **C** have already been shown in figure 7.2, but appear again for comparison with the responses at sixteen-months.

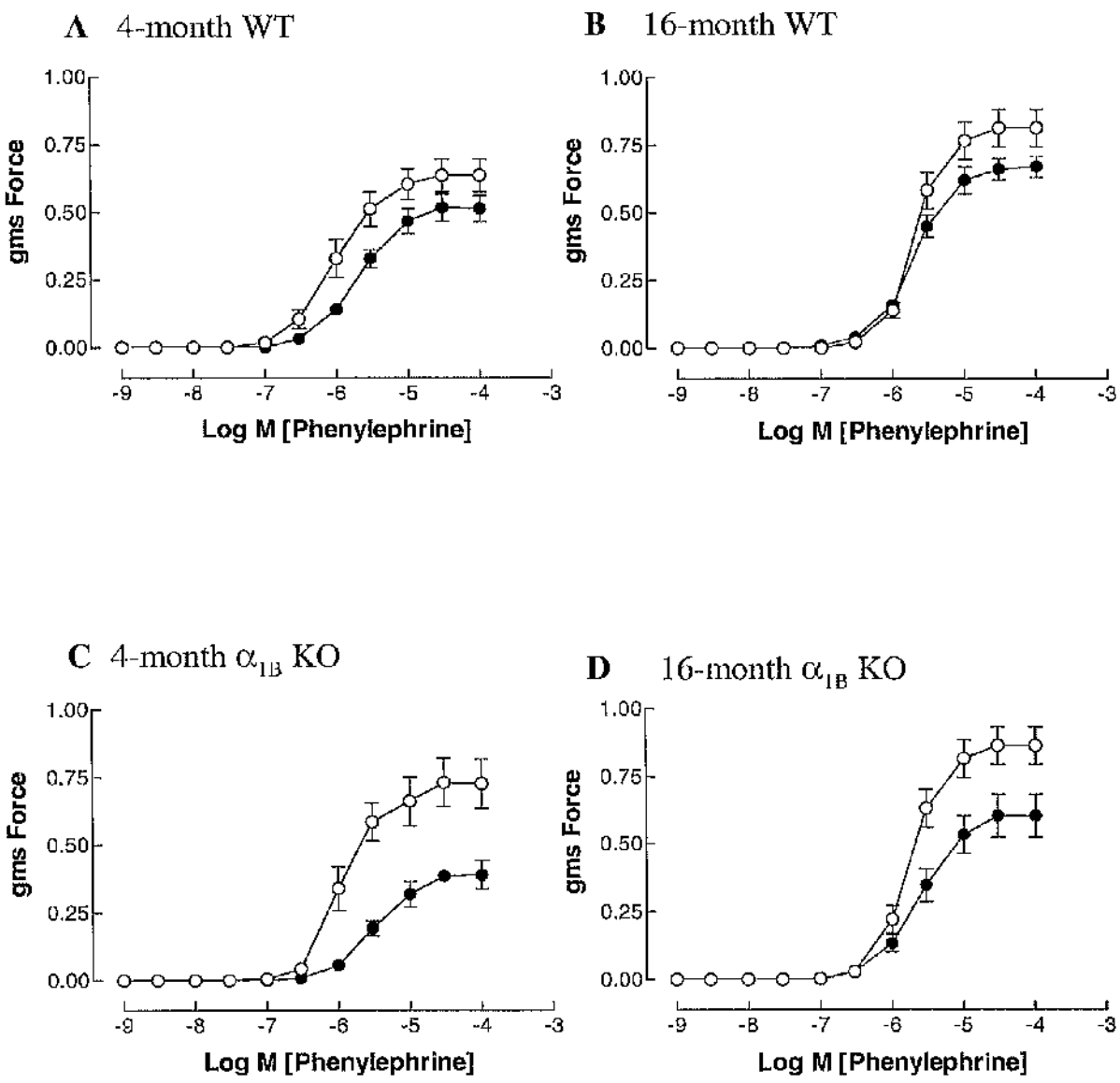



Figure 7.7: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$KO mice. Figures **A** and **C** are a copy of figure 7.1 (4-month data). **B** A PE response curve alone (○, n = 10) and with nifedipine (1 x $10^{-7}$M, ●, n = 9) in the WT at 16-months. **D** A PE response curve alone (○, n = 10) and with nifedipine (●, n = 9) in the $\alpha_{1B}$KO at 16-months. Each point represents mean ± standard error.

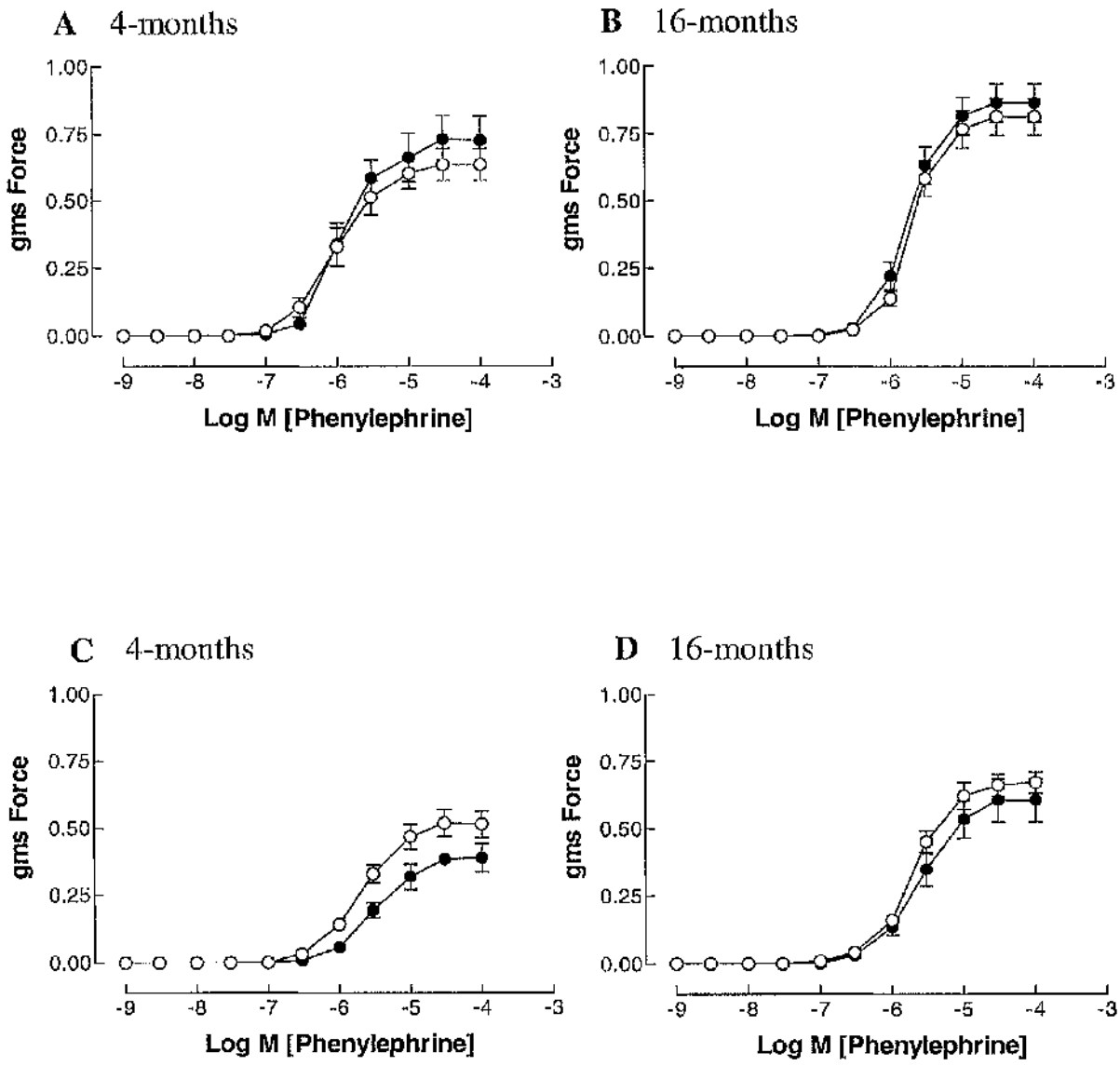



Figure 7.8: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$KO mice. Figures **A** and **C** are a copy of figure 7.2 (4-month data). **B** A PE control curve in the WT (○, n = 10) and $\alpha_{1B}$KO (●, n = 10) at 16-months. **D** A PE response curve with nifedipine in the WT (○, n = 9) and $\alpha_{1B}$KO (●, n = 9) at 16-months. Each point represents mean ± standard error.

The phenylephrine control curve in tail arteries from sixteen-month old mice, and the response gained in the presence of nifedipine ($1 \times 10^{-7}$M), were expressed as a percentage of the maximum for the WT at four (figure 7.9 **A**) and sixteen-months (figure 7.9 **B**) and $\alpha_{1B}$ KO at four (figure 7.9 **B**) and sixteen-months (figure 7.9 **D**), and have been shown in figure 7.9. Figure 7.9 **A**, and figure 7.9 **C** are duplicates of the four-month data but have been shown again for ease of comparison with the responses gained at sixteen-months. At sixteen-months, nifedipine had no effect on tissue sensitivity in the WT. The $pEC_{50}$ value in the WT control curve was $5.7 \pm 0.01$, which was no different to the $pEC_{50}$ gained in the presence of nifedipine of $5.7 \pm 0.005$ ($p>0.05$). In arteries from $\alpha_{1B}$ KO mice the control curve $pEC_{50}$ value was $5.7 \pm 0.004$, while in the presence of nifedipine the $pEC_{50}$ value was slightly lower, at $5.5 \pm 0.01$ ($p>0.05$).

7.3.5 Effect of prazosin in tail arteries from 16month old WT and $\alpha_{1B}$ KO mice

In the WT at sixteen-months, prazosin caused a rightward shift in the concentration response curve at all antagonist concentrations. Furthermore, prazosin also caused a progressive reduction in the maximum phenylephrine-mediated response in WT arteries, although this was significant only at the highest prazosin concentration tested and where no true maximum was attained. The control curve maximum was $0.67 \pm 0.04$gms Force, compared with $0.54 \pm 0.07$gms Force for $1 \times 10^{-9}$M ($p = 0.21$), $0.45 \pm 0.06$gms Force for $1 \times 10^{-8}$M ($p = 0.73$), and $0.23 \pm 0.06$ with prazosin at $1 \times 10^{-7}$M ($p = 0.02$*).

In tail arteries from sixteen-month old $\alpha_{1B}$ KO mice, prazosin caused a rightward shift in the phenylephrine response curve, but did not reduce the maximum response, unlike the WT. The maximum contraction in the control curve was $0.60 \pm 0.08$gms Force,

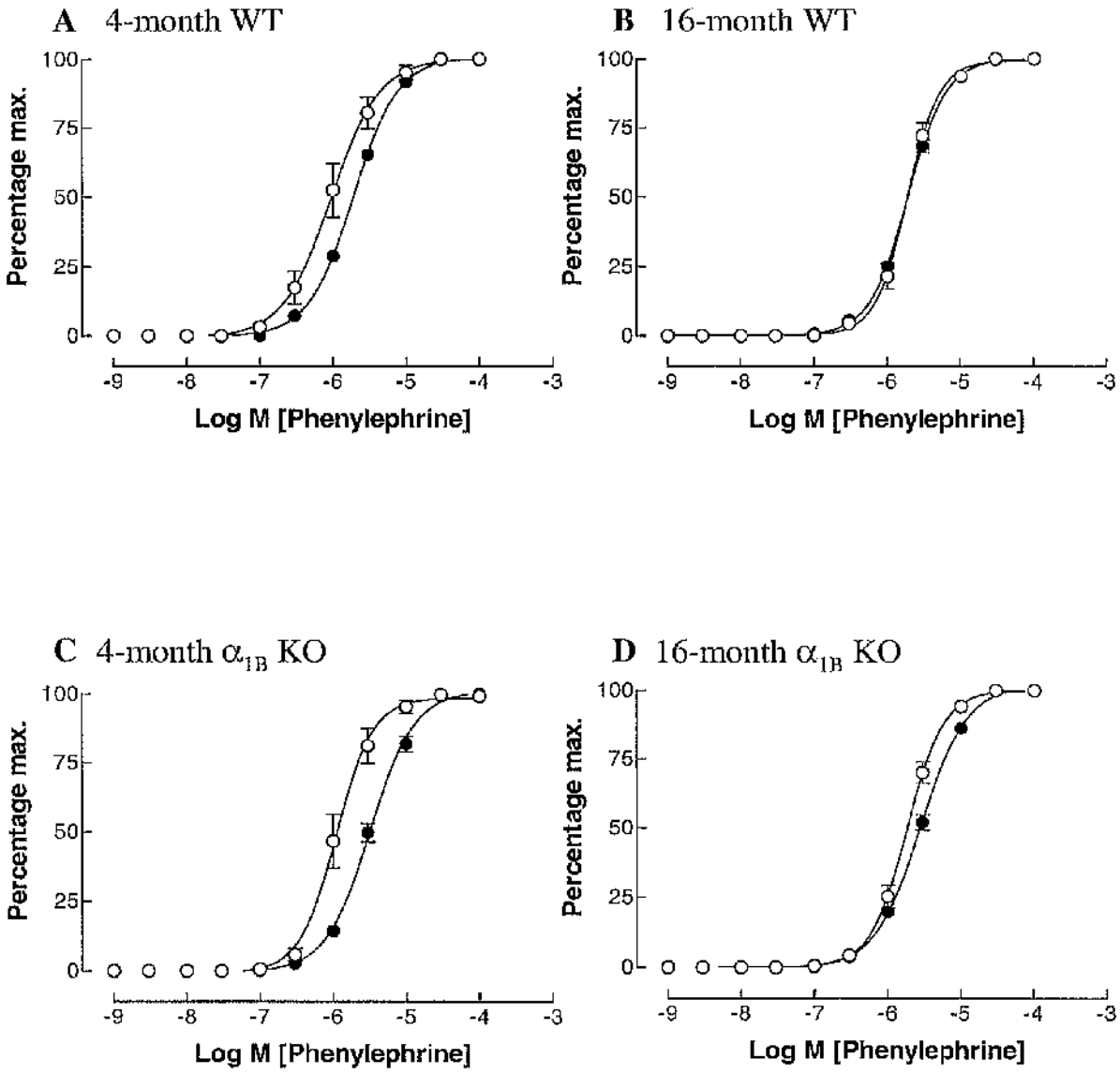



Figure 7.9: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$ KO mice, expressed as percentage maximum. **A** and **C** are a copy of figure 7.3 (4-month data). **B** A PE response curve alone (○, n = 10) and with nifedipine (1 x $10^{-7}$M, ●, n = 9) in the WT. **D** A PE response curve alone (○, n = 10) and with nifedipine (●, n = 9) in the $\alpha_{1B}$ KO both at 16-months. Each point represents mean ± standard error.

compared with 0.49 ± 0.1gms Force for 1 x $10^{-9}$M, 0.58 ± 0.09gms Force for 1 x $10^{-8}$M, and 0.52 ± 0.1gms Force for 1 x $10^{-7}$M prazosin.

Figure 7.10 shows the comparison of responses in the WT and $\alpha_{1B}$ KO, and at both age points with prazosin at a concentration of 1 x $10^{-9}$ and 1 x $10^{-8}$M. Figure 7.10 **A** compares the response in the WT and $\alpha_{1B}$ KO with prazosin at 1 x $10^{-9}$M at four-months, while figure 7.10 **C** compares the responses between WT and $\alpha_{1B}$ KO with prazosin at 1 x $10^{-8}$M at four-months. Figure 7.10 **B** shows the response with prazosin at 1 x $10^{-9}$M in the WT and $\alpha_{1B}$ KO at sixteen-months, while figure 7.10 **D** compares the responses in the WT and $\alpha_{1B}$ KO with prazosin at 1 x $10^{-8}$M, at sixteen-months.

At four-months, the lowest prazosin concentration tested (1 x $10^{-9}$M) shifted the response in the $\alpha_{1B}$ KO further to the right than in the WT. At a concentration of 1 x $10^{-8}$M prazosin, the maximum response in the WT was 0.45 ± 0.05gms Force, which was significantly greater than the maximum in the $\alpha_{1B}$ KO of 0.27 ± 0.04gms Force (p = 0.037*).

Figure 7.10 also shows the comparison between responses gained in WT and $\alpha_{1B}$ KO at 16-months with two concentrations of prazosin, those gained at 1 x $10^{-9}$M (figure 7.10 **B**), and those at a concentration of 1 x $10^{-8}$M (figure 7.10 **D**). The responses gained at both antagonist concentrations were comparable in size and sensitivity in the WT and $\alpha_{1B}$ KO.

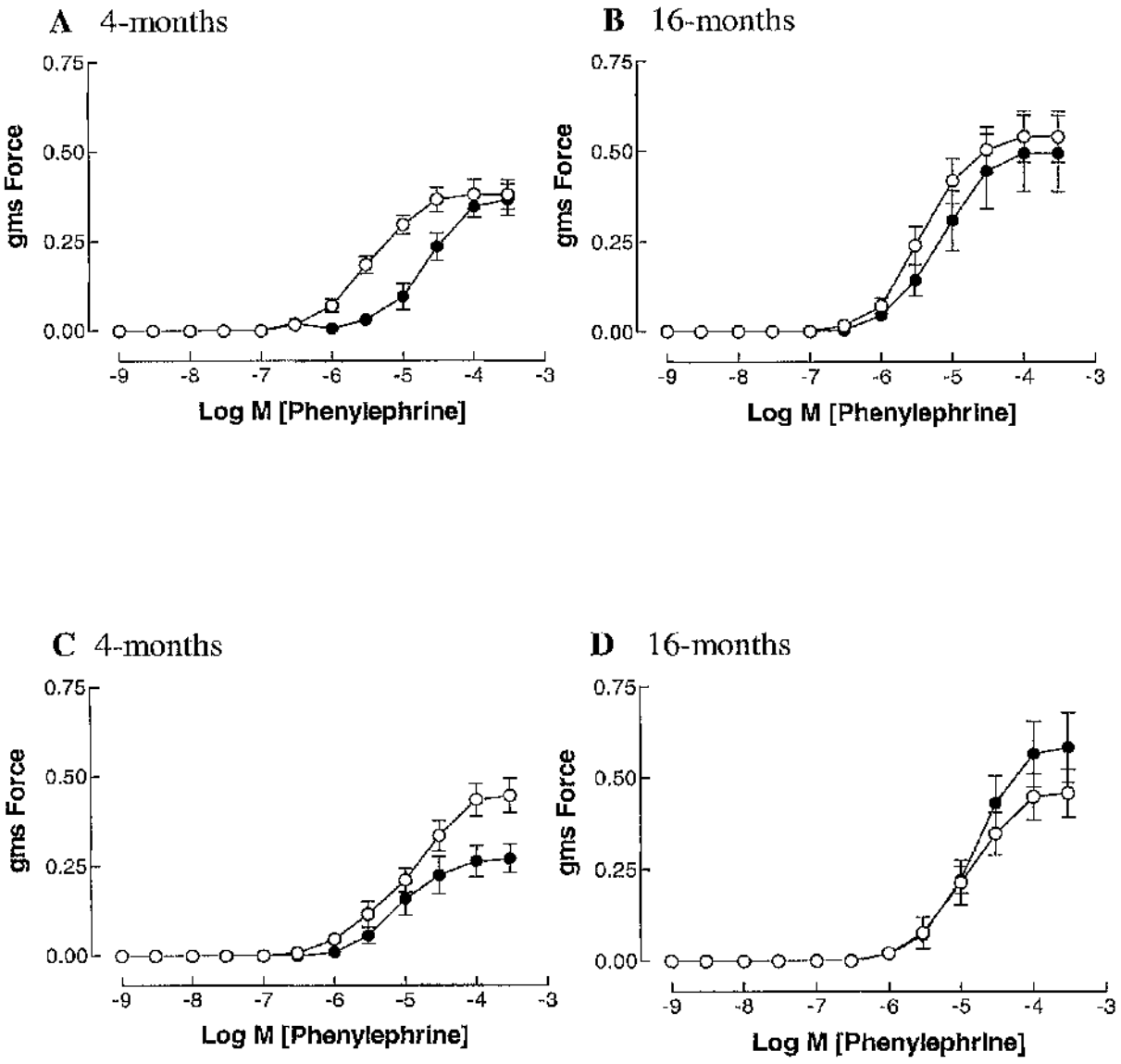



Figure 7.10: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$KO mice. Figures **A** and **C** show responses at 4-months in WT and $\alpha_{1B}$ KO arteries. **B** A PE response curve in the WT (○, n = 6) and $\alpha_{1B}$KO (●, n = 6) with prazosin at 1 x $10^{-9}$M, at 16-months. **D** A PE response curve in the WT(○, n = 6) and $\alpha_{1B}$KO (●, n = 6) with prazosin at 1 x $10^{-8}$M, at 16-months. Each point represents mean ± standard error.

Figure 7.11 shows the phenylephrine-mediated response in tail arteries from four-month old WT (figure 7.11 **A**) and $\alpha_{1B}$ KO (figure 7.11 **C**) mice in the presence of prazosin at a concentration of 1 x $10^{-7}$M. At this concentration, prazosin caused a significant reduction in the maximum response in both strains. In the WT, the control curve maximum was reduced from 0.51 ± 0.06gms Force, to 0.29 ± 0.03gms Force in the presence of prazosin ($p<0.02$*). Responses in the $\alpha_{1B}$ KO were reduced from 0.39 ± 0.05gms Force in a control curve, to 0.12 ± 0.05gms Force in the presence of prazosin ($p<0.012$*).

Figure 7.11 also shows the phenylephrine-induced control response, and the response gained in the presence of prazosin at a concentration of 1 x $10^{-7}$M in tail arteries from sixteen-month old WT (figure 7.11 **B**) and $\alpha_{1B}$ KO (figure 7.11 **D**) mice. The prazosin-induced reduction in the control curve maximum was obvious in WT arteries. In contrast, contractile responses, although shifted rightward retain their maximum in arteries from the $\alpha_{1B}$ KO. Statistical analysis of the contractile responses in the presence of prazosin confirmed that contractile responses in the WT were significantly smaller than those of the $\alpha_{1B}$ KO ($p = 0.02$*).

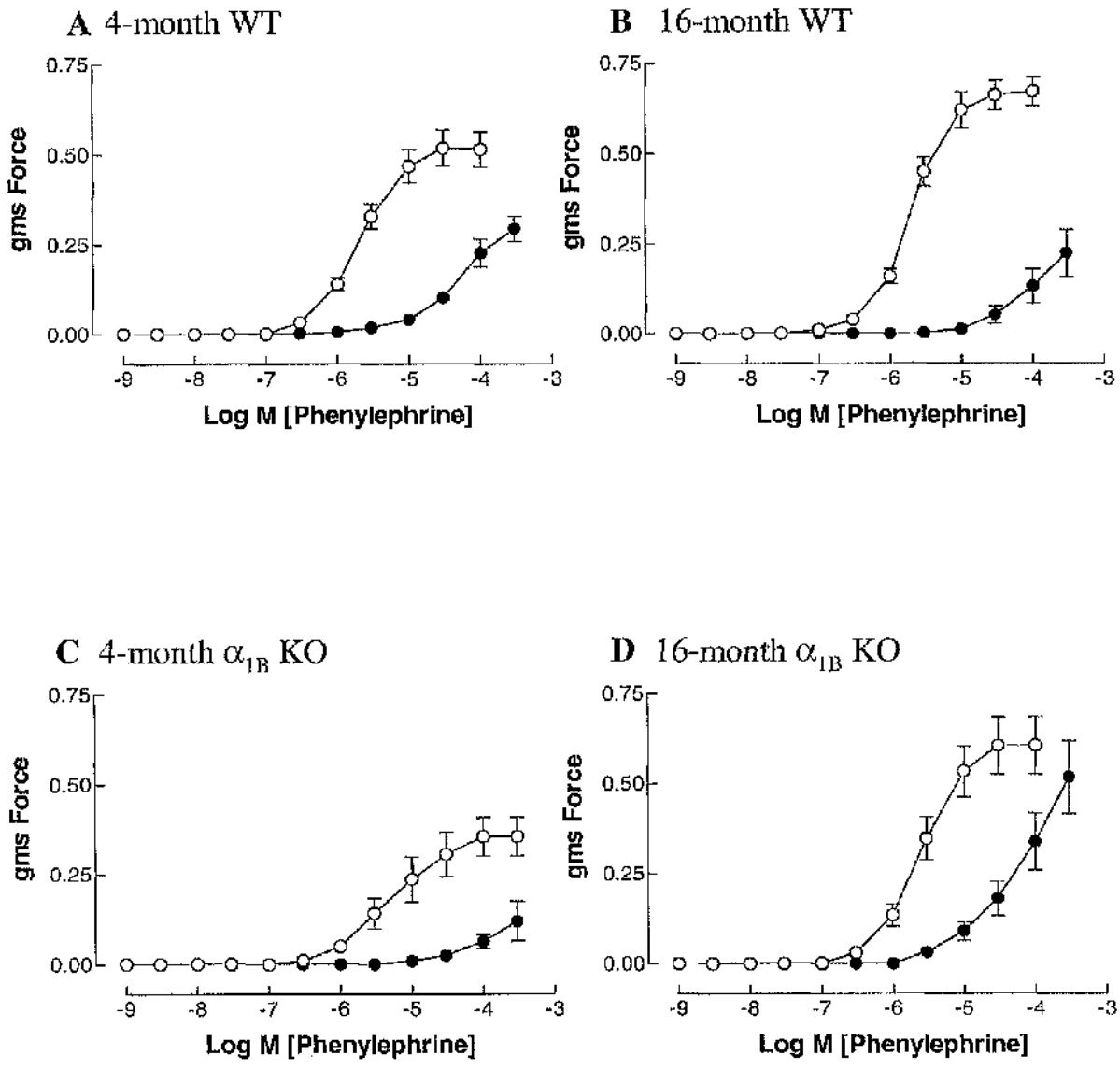



Figure 7.11: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$ KO mice. Figures **A** and **C** show responses at 4-months in WT and $\alpha_{1B}$ KO. **B** PE response in the WT control (○, n = 9) and with prazosin at 1 x $10^{-7}$M (●, n = 6) at 16-months. **D** PE response in the $\alpha_{1B}$ KO control (○, n = 9) and with prazosin at 1 x $10^{-7}$M (●, n = 6) at 16-months. Each point represents mean ± standard error.

| | WT | $\alpha_{1B}$ KO |
|---|---|---|
| | Maximum (gms Force) | Maximum (gms Force) |
| Control curve | 0.81 ± 0.07, n = 10 | 0.86 ± 0.07, n = 10 |
| Nifedipine 1 x $10^{-7}$M | 0.67 ± 0.04, n = 9 | 0.60 ± 0.08, n = 9 |
| Prazosin 1 x $10^{-9}$M | 0.54 ± 0.07, n = 6 | 0.49 ± 0.10, n = 6 |
| Prazosin 1 x $10^{-8}$M | 0.45 ± 0.06, n = 6 | 0.58 ± 0.09, n = 6 |
| Prazosin 1 x $10^{-7}$M | 0.23 ± 0.06*, n = 6 | 0.52 ± 0.10*, n = 6 |

Table 7.2 Highest response attained in tail arteries at sixteen-months in WT and $\alpha_{1B}$ KO.

7.3.6 $pA_2$ values for prazosin in tail arteries from 16month old WT and $\alpha_{1B}$ KO mice

Figure 7.12 shows Schild regression plots for prazosin in tail arteries from four (figure 7.12 **A**) and sixteen-month old (figure 7.12 **B**) WT mice and four (figure 7.12 **C**) and sixteen-month old (figure 7.12 **D**) $\alpha_{1B}$ KO mice. In WT arteries, the intercept of the regression line with the x-axis gave a $pA_2$ value of 8.8, with a slope of 0.84 ± 0.13 at 16-months. While in the $\alpha_{1B}$ KO the $pA_2$ value was slightly higher than the WT at 9.0, with a slope of 0.72 ± 0.12 at 16-months. In both strains the regression lines had a slope that was significantly different from unity, indicative of non-competitive antagonism.

| | tail artery (4-month old) | | tail artery (16-month old) | |
|---|---|---|---|---|
| | $pA_2$ | Slope | $pA_2$ | Slope |
| WT | 8.8 | 0.95 | 8.8 | 0.84 |
| $\alpha_{1B}$ KO | 9.2 | 0.99 | 9.0 | 0.72 |

Table 7.3 $pA_2$ values for prazosin at both age points in tail arteries from WT and $\alpha_{1B}$ KO mice.

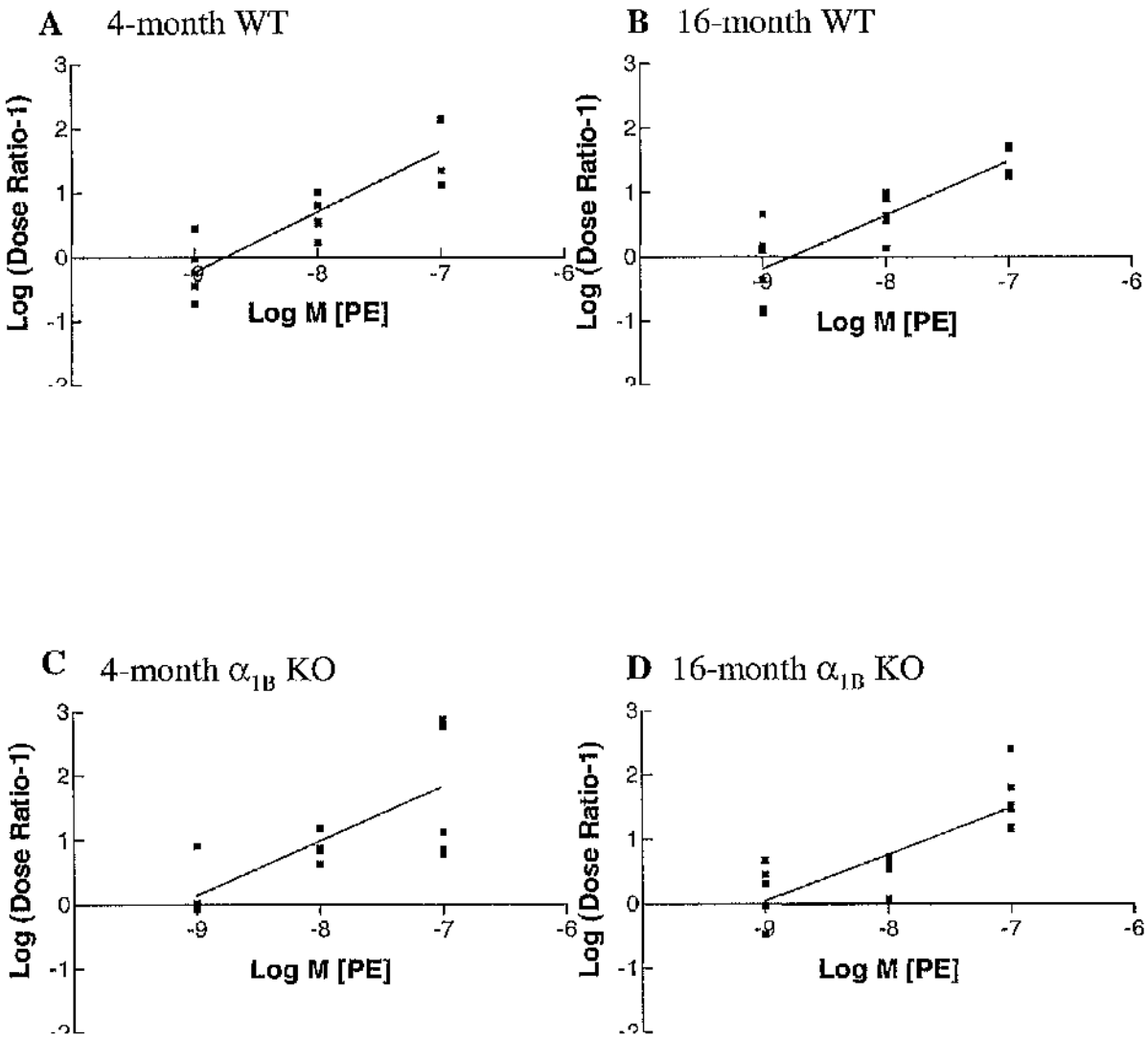



Figure 7.12: Schild regression plots for prazosin in tail arteries from 16-month old WT and $\alpha_{1B}$KO mice. Figures **A** and **C** are a copy of figure of the 4-months in the WT and $\alpha_{1B}$ KO. **B** Schild plot for WT arteries (■, n = 6) at 16-months, prazosin has a $pA_2$ of 8.8 with a slope of 0.84. **D** Schild plot for $\alpha_{1B}$KO arteries (■, n = 6) at 16-months, prazosin yields a $pA_2$ of 9.0 with a slope of 0.72. Each point shows a single results for a given antagonist concentration.

7.3.7 Comparison of results gained in arteries from 4 and 16month old mice

Figure 7.13 compares the phenylephrine control curves from four and sixteen-month old WT (figure 7.13 **A**) and $\alpha_{1B}$ KO mice (figure 7.13 **B**). In arteries from WT mice, contractile responses reached a greater maximum at sixteen-months than at four. At four-months the maximum response gained in the control curve was 0.64 ± 0.06gms Force, compared with 0.81 ± 0.07gms Force obtained in arteries from sixteen-month old WT mice (p = 0.02*).

In the $\alpha_{1B}$ KO, contractile responses tended to be greater at sixteen-months, but were not significantly different from those gained at four-months. At sixteen-months the maximum response was 0.86 ± 0.07gms Force, compared with a maximum of 0.73 ± 0.09gms Force in tail arteries from four-month old $\alpha_{1B}$ KO mice (p = 0.50).

The phenylephrine control curves for the WT (figure 7.14 **A**) and $\alpha_{1B}$ KO (figure 7.14 **B**) at each age point were expressed as a percentage of their own maximum and are shown in Figure 7.14. In WT arteries, the $pEC_{50}$ value at 4-months was 6.0 ± 0.01, compared with a value of 5.7 ± 0.01 for curves constructed in arteries from sixteen-month old mice (p = 0.0007***). The significant shift in $pEC_{50}$ values, suggested that arteries are slightly less sensitive to phenylephrine with increasing age. A similar result was obtained in the $\alpha_{1B}$ KO, with a $pEC_{50}$ of 6.0 ± 0.01 at four-months, compared with a $pEC_{50}$ of 5.7 ± 0.004 in the response curve from sixteen-month old mice (p<0.0001***).

At both age points, and in both strains, nifedipine reduced the contractile maximum, but had only a slight effect on tissue sensitivity. Figure 7.15 shows phenylephrine response curves in the presence of nifedipine ($1 \times 10^{-7}$M) constructed in arteries from four-month

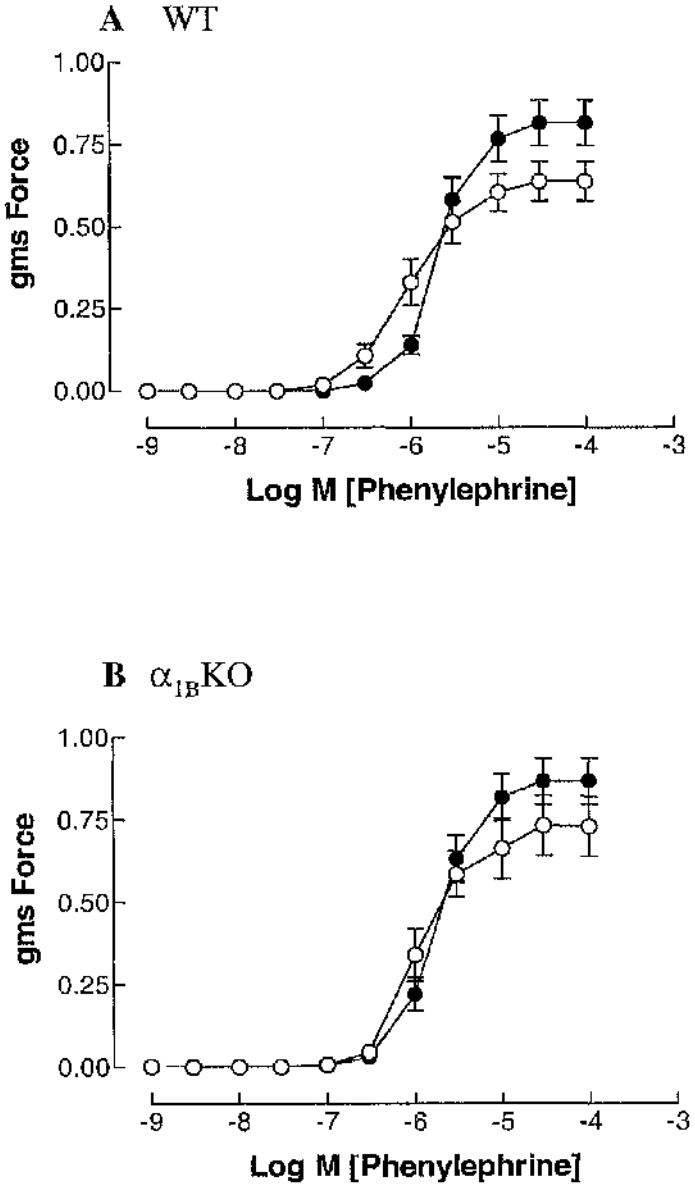



Figure 7.13: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$ KO mice. **A** A PE control curve at 4 (○, n = 9) and 16-months (●, n = 10) in the WT. **B** A PE control curve at 4 (○, n = 9) and 16-months (●, n = 10) in the $\alpha_{1B}$KO. Each point represents mean ± standard error.

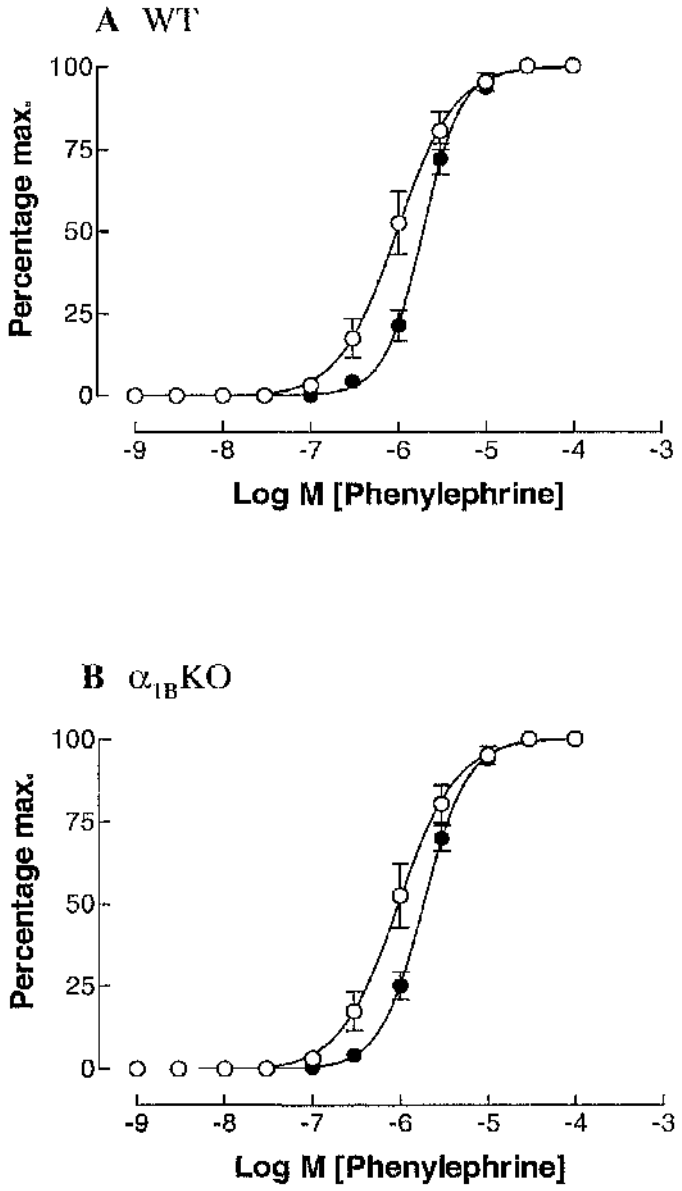



Figure 7.14: The phenylephrine response in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$KO mice, expressed as a percentage of the maximum response. **A** In WT controls at 4 (○, n = 9) and 16months (●, n = 10). **B** In $\alpha_{1B}$ KO controls at 4 (○, n = 9) and 16months (●, n = 10). Each point represents mean ± standard error.

old and sixteen-month old WT (figure 7.15 **A**) and $\alpha_{1B}$ KO (figure 7.15 **B**) mice. In WT arteries, the contractile maximum at four-months was 0.51 ± 0.05gms Force, which was significantly lower than the maximum response at sixteen-months of 0.67 ± 0.04gms Force ($p = 0.009^{**}$). In $\alpha_{1B}$ KO mice, the maximum phenylephrine-induced response at four-months was 0.39 ± 0.05gms Force, which was significantly smaller than the response at sixteen-months, which was 0.60 ± 0.08gms Force ($p = 0.014^{*}$).

Figure 7.16 shows a comparison of the phenylephrine-induced contractions in tail arteries from four (figure 7.16 **A**) and sixteen-month (figure 7.16 **B**) old WT and $\alpha_{1B}$ KO mice in the presence of the highest concentration of prazosin tested ($1 \times 10^{-7}$M). At four-months the phenylephrine-induced response was small in tail arteries from the WT and $\alpha_{1B}$ KO, but in the $\alpha_{1B}$ KO the maximum response gained was significantly smaller than that of the WT ($p = 0.02^{*}$). At sixteen-months the converse was true. The phenylephrine-induced contraction in the WT was significantly smaller than that of the $\alpha_{1B}$ KO ($p = 0.017^{*}$)

In the WT, the size of contractile responses tended to increase at sixteen-months, but comparison with responses gained at four-months has shown no significant difference. However, comparison of the response curves gained in the $\alpha_{1B}$ KO at both age points revealed that in the presence of all three concentrations of prazosin, the contractile responses were significantly greater at sixteen-months (at $1 \times 10^{-9}$M, $p = 0.048^{*}$, $1 \times 10^{-8}$M, $p = 0.04^{*}$, and $1 \times 10^{-7}$M, $p = 0.004^{**}$)

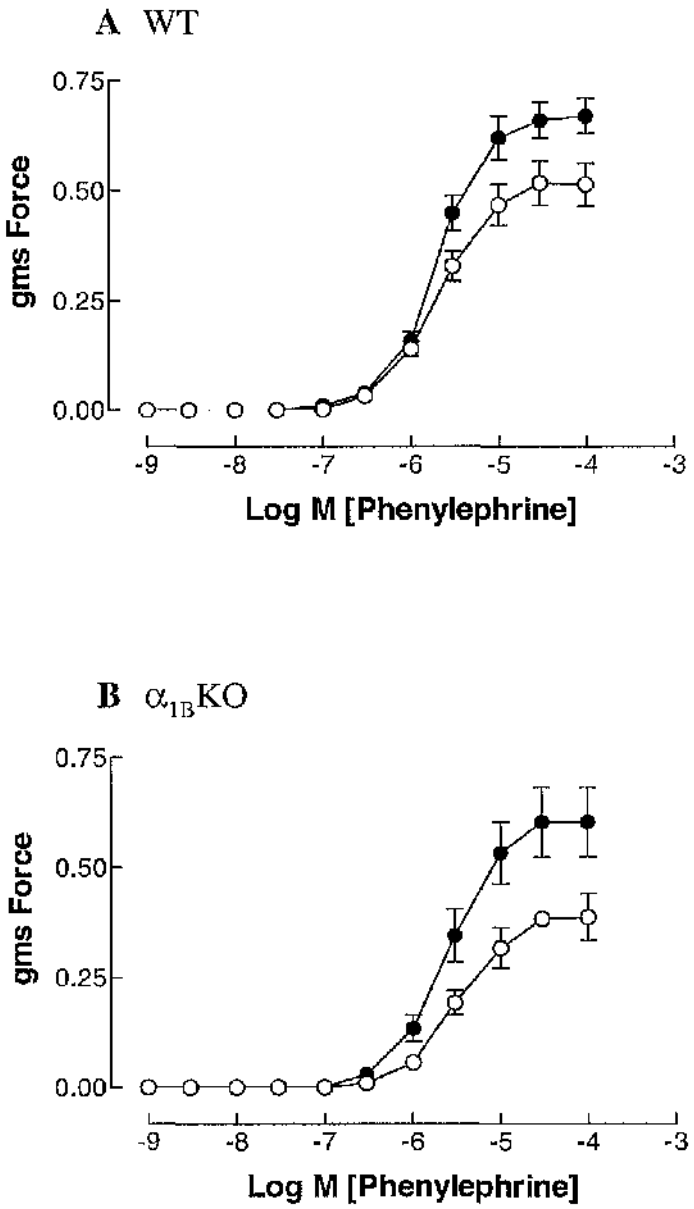



Figure 7.15: The phenylephrine response in the presence of nifedipine in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$KO. **A** A PE response curve at 4 (○, n = 6) and 16-months (●, n = 9) in the WT. **B** A PE response curve at 4 (○, n = 6) and 16-months (●, n = 9) in the $\alpha_{1B}$KO. Each point represents mean ± standard error.

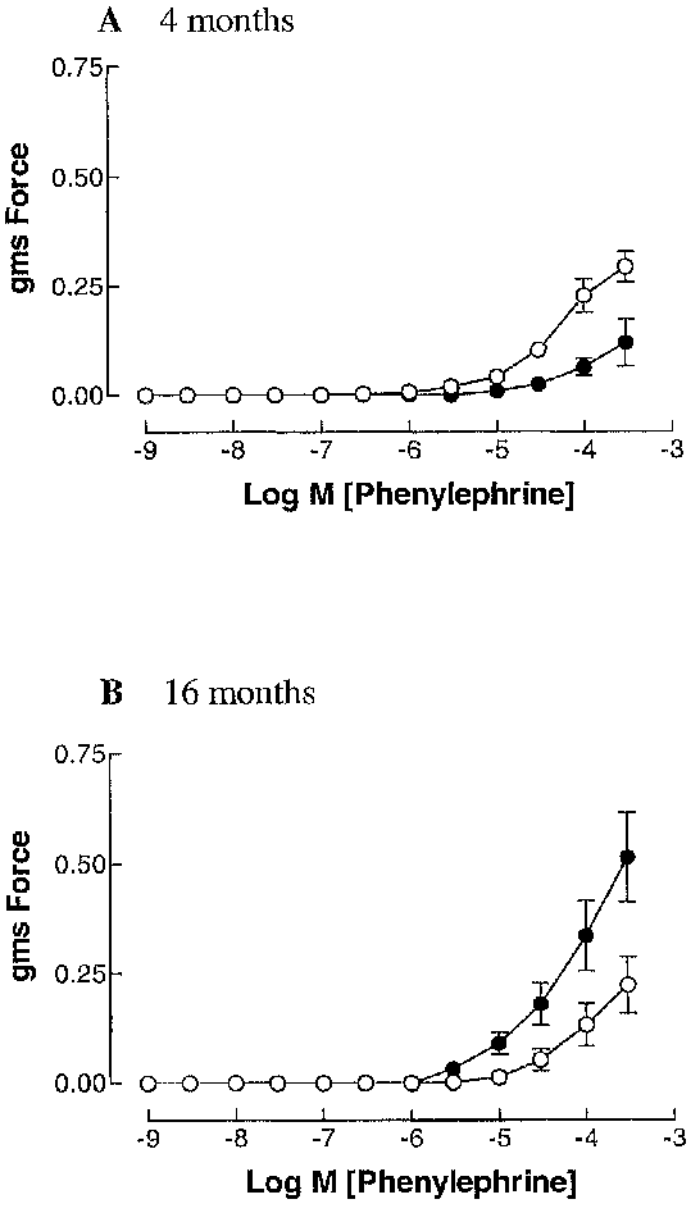



Figure 7.16: Responses in tail arteries from 4 and 16-month old WT and $\alpha_{1B}$KO mice (with nifedipine). **A** PE response at 4-months in the WT (○, n = 6) and $\alpha_{1B}$KO (●, n = 6) with prazosin at 1 x $10^{-7}$M **B** PE response at 16-months in the WT (○, n = 6) and $\alpha_{1B}$KO (●, n = 6), with prazosin at 1 x $10^{-7}$M. Each point represents mean ± standard error.

7.4 Discussion

Work presented in this thesis provides evidence that the murine tail artery contracts in response to activation of a mixed population of $\alpha_1$ and $\alpha_2$-adrenoceptors. However, to date the role of each adrenoceptor subtype in mediating contractile responses is poorly defined, in particular, the role of the $\alpha_{1B}$-adrenoceptor. It has previously been proposed that the $\alpha_{1B}$-adrenoceptor plays only a minor role in the control of peripheral blood pressure [Piascik et al, 1993]. The work shown here in combination with studies using subtype selective antagonists from our laboratory has been used to argue in support of this hypothesis [Daly et al, 2002]. Given that prazosin has been used widely as a pharmacological tool to assign receptor functions, I have assessed the effects of this drug in the mouse tail artery.

Prazosin is an $\alpha_1$-selective antagonist, that causes potent inhibition of phenylephrine-induced contractions in a number of murine blood vessels, including the tail artery, mesenteric, carotid and aorta [Daly et al, 2002]. The data shown here confirms that prazosin antagonises phenylephrine-induced contractions of the murine tail artery and, at the highest drug concentrations used, causes a significant reduction in the contractile maximum in arteries from WT and KO mice, at four and sixteen-months.

The data presented in this chapter forms part of a recent publication by Daly et al, where the effect of prazosin, and a number of selective antagonists were used to determine the major $\alpha_1$-adrenoceptor subtype causing contraction of the mouse tail artery, aorta, carotid, and first order mesenteric resistance arteries. The effects of prazosin alone, provide little information on the adrenoceptor subtypes involved in mediating contractions of the murine tail artery, but combined with the complimentary information

provided by the other antagonists helps to determine the major $\alpha_1$-adrenoceptor subtype causing contraction. Thus providing clarification on the role of the $\alpha_{1B}$-adrenoceptor in the murine tail artery.

The responses shown in chapter three and four, where $\alpha_2$-adrenoceptor-mediated contractions of the tail artery were investigated, are complicated by the development of rhythmic contractions of the murine tail artery. In a similar way, stimulation of $\alpha_1$-adrenoceptors also leads of the development of unwanted rhythmic contractility in the mouse tail artery. Low concentration of nifedipine prevents rhythmic contractions from developing, and causes a reduction in the absolute size of the contractile response.

Nifedipine is a dihydropyridine calcium channel blocker, known to cause a significant reduction in noradrenaline-induced contractions of rabbit veins [Dunn et al, 1991]. The effect of nifedipine on contractions of the murine tail artery is similar, and predictable, because the influx of extracellular calcium is retarded. Although contractions are reduced in size, they are still substantial in both strains, providing evidence that $\alpha_1$-adrenoceptor-mediated contraction of the tail artery depends on both extra and intracellular calcium. Extracellular calcium enters the cell via nifedipine sensitive channels, while intracellular calcium probably originates from the sarcoplasmic reticulum. Nifedipine significantly reduces the magnitude of the phenylephrine-induced response in the $\alpha_{1B}$ KO at both age points, and in the WT at sixteen-months, but sensitivity remains similar.

In complementary studies carried out in our laboratory on mesenteric resistance arteries, 5MeU has been shown to competitively antagonise the phenylephrine-induced response

in WT and $\alpha_{1B}$ KO arteries. In the KO, the $pA_2$ value for 5MeU (8.5) is higher than that of the WT (8.3). This is a very small difference, but there was also a change in the slope of the Schild plot from the WT (1.4) to KO (1.1) [Daly et al, 2002]. Taken together, this is consistent with a minor contractile role for the $\alpha_{1B}$-adrenoceptor-subtype. Furthermore, increasing concentrations of BMY7378, ranging from $1 \times 10^{-9}$ to $1 \times 10^{-7}$M, have no effect on phenylephrine-induced contractions of the murine tail artery [Daly et al, 2002]. This provides evidence that the $\alpha_{1D}$-adrenoceptor does not contribute to contractile responses in the murine tail artery, unlike first order mesenteric resistance arteries where a small, but significant contractile function has been uncovered (Chapter eight).

In the $\alpha_{1B}$ KO, nifedipine causes a reduction in contractility that is of greater magnitude than the reduction observed in the WT. This represents an unexplained phenotype. It may reflect a greater dependency of the remaining adrenoceptor subtypes on extracellular calcium, or could be attributable to compensatory mechanisms that result in this phenotype because of the germline disruption of the $\alpha_{1B}$-adrenoceptor.

Effect of prazosin on PE-induced contractions in the WT and $\alpha_{1B}$ KO tail artery

Agonist-induced contractions of the rat tail artery appear to result from the activation of more than one $\alpha_1$-adrenoceptor subtype [Lachnit et al, 1997]. The combined use of CEC and RS17053, respectively considered to be $\alpha_{1B}$ and $\alpha_{1A}$-selective antagonists, has been taken to demonstrate the existence of functional $\alpha_{1B}$ and $\alpha_{1A}$-adrenoceptors in the rat tail. In the presence of RS17053 alone, phenylephrine curves are biphasic. However preincubation with CEC gives a monophasic response curve. The data gained from functional studies in the rat indicates that the major $\alpha_1$-subtype leading to contraction of

the rat tail artery is the $\alpha_{1A}$-subtype, with a small, but significant contractile function being mediated by the $\alpha_{1B}$-adrenoceptor [Lachnit et al, 1997]. The data presented here, combined with the effect of 5MeU on the phenylephrine response [Daly et al, 2002] provides evidence that indicates that responses in the mouse tail artery are similar to those of the rat.

The study of responses in the mouse tail artery have shown that prazosin shifts the phenylephrine-induced response curve to the right in the WT and $\alpha_{1B}$ KO at four and sixteen-months. At high antagonist concentrations, prazosin causes a significant reduction in phenylephrine-induced contractions of the WT tail artery at four and sixteen-months, and at four-months in the $\alpha_{1B}$ KO, despite the agonist concentration being increased to $3 \times 10^{-4}$M. High concentration of $\alpha_1$-antagonists can cause an insurmountable reduction in agonist-induced contractions, a phenomenon that also occurs in mesenteric resistance arteries in the presence of the $\alpha_{1A}$-selective antagonist 5MeU (Chapter eight).

The effect of increasing age

In young rats, ligands that selectively inhibit $\alpha_{1A}$-adrenoceptor-mediated pressor responses in the pithed rat are more effective antagonists than those that block $\alpha_{1D}$ and or $\alpha_{1B}$-adrenoceptor-mediated responses [Ibarra et al, 1997]. However, in old rats the $\alpha_{1D}$ and $\alpha_{1B}$ selective antagonists BMY7378 and CEC, antagonise agonist-induced increases in diastolic blood pressure, while blockade of $\alpha_{1A}$-adrenoceptors is ineffective [Ibarra et al, 1997]. This suggests that $\alpha_{1D}$ and or $\alpha_{1B}$-adrenoceptors play a more significant role in contraction of the rat vasculature with increasing age. Studies where

the expression levels of $\alpha_1$-adrenoceptors have been studied often provide contradictory results. In the rat expression of the $\alpha_{1B}$-adrenoceptor is proposed to decrease with age, while expression of the $\alpha_{1D}$-adrenoceptor is unchanged [Gurdal et al, 1995 a, b]. Yet, in the rat aorta, the expression of $\alpha_{1A}$-adrenoceptors increases with age, while the expression of the $\alpha_{1B}$ decreases [Ibarra et al, 1997]. Although informative, data presenting a change in expression levels of a given receptor does not clarify whether the responses of the existing receptor population change with increasing age, so investigating the functional response is imperative, as is determining the outcome of responses *in vivo*.

Increases in blood pressure occur with age. This has been proposed to result from an alteration in sympathetic nerve activity. For example, in the rat tail artery, stimulation evoked release of noradrenaline increases significantly with advancing age [Buchholz et al, 1998]. An alternative hypothesis to an altered sympathetic nerve response, leading to changes in blood pressure regulation with age, is that increased blood pressure may result from altered sensitivity of adrenoceptors to endogenous catecholamines. If receptors become supersensitive to endogenous agonists, then contractions mediated by vascular smooth muscle cells will ultimately be increased, leading to a decrease in vessel lumen and an elevation in resting pressure.

Data from the WT suggests that the sensitivity of the tail artery to antagonism with prazosin is unaffected by increasing age. In the $\alpha_{1B}$ KO the decrease in potency of prazosin suggests that the remaining receptor population may be slightly more resistant to the effects of prazosin. Whether this reflects a true phenotypic change in the function of the $\alpha_{1A}$ and $\alpha_{1D}$-adrenoceptors with increasing age, or is the result of an unexplained

phenotype that arises from deletion of germline $\alpha_{1B}$-adrenoceptors, requires clarification.

Catecholamine-induced contractions of rodent arteries have been shown to increase with advancing age [Buchholz et al, 1998]. In the tail artery, contractile responses to phenylephrine are not significantly affected by age. The obvious assumption may then be to hypothesise that the potentiation of catecholamine-induced responses may be attributable to enhanced responsiveness of vascular $\alpha_2$-adrenoceptors. However, the data presented in chapter three of this thesis refutes this hypothesis. In the D79N tail artery from young and old mice, UK14304-mediated contractions are of comparable size and sensitivity. This data suggests, that at least in the D79N mouse, $\alpha_2$-adrenoceptor-mediated contractions do not increase with age.

The one significant change that does occur at sixteen-months, is a potentiation of phenylephrine-induced contractile responses in the presence of prazosin in the $\alpha_{1B}$ KO. At sixteen-months the control curve in WT arteries is significantly greater in maximum than at four-months, but is not enhanced in the presence of nifedipine and/or prazosin. The results in arteries from sixteen-month $\alpha_{1B}$ KO mice present an unexplained phenotype, as a similar alteration in contractility does not occur in the WT. Whether this represents enhanced contractility of the major $\alpha_1$-adrenoceptor-subtype, the $\alpha_{1A}$ receptor, or reflects the involvement of the $\alpha_{1D}$-adrenoceptor in contractile responses in arteries from older animals, cannot be clarified here without the use of subtype selective antagonists.

Although potentiated in size, tail arteries from the $\alpha_{1B}$ KO are slightly less sensitive to phenylephrine at sixteen-months, evidenced by a reduction in the $pEC_{50}$ value. A similar decrease in sensitivity to phenylephrine occurs in the WT at sixteen-months. This data suggest that although agonist-induced contractions can be potentiated with age, higher concentrations of agonist are required to produce a measurable response. This may represent a protective mechanism whereby the concentration of agonist required to produce a response is elevated when responses become potentiated in size with increasing age.

The expression levels of $\alpha_1$-adrenoceptor subtypes have been shown to change with increasing age, and expression of the $\alpha_{1B}$-adrenoceptor has been proposed to increase [Ibarra et al, 1997]. The data presented here indicates that $\alpha_1$-adrenoceptor-mediated contractions have a tendency to increase in size with age, a phenomenon that is accompanied by a slight reduction in sensitivity. Whether the increase in size of responses is attributable to the $\alpha_{1B}$-adrenoceptor remains to be determined.

Summary and conclusions

In summary, phenylephrine causes concentration-dependent contractions of the murine tail artery from four and sixteen-month old mice, contractions that are potently inhibited by prazosin, a non-selective $\alpha_1$-adrenoceptor antagonist. In the WT, although responses in a control curve tend to be greater in maximum, they are relatively unaffected by advancing age. However, in the $\alpha_{1B}$ KO an unexplained potentiation of contractile responses in the presence of all three concentrations of prazosin occurs. Prazosin provides slightly lower $pA_2$ values for arteries from KO mice than for the WT. Alone, this provides little information on the subtypes involved in responses, but combined

with the data published by Daly et al with 5MeU, data points toward the $\alpha_{1A}$-adrenoceptor as the major contractile $\alpha_1$-adrenoceptor-subtype. It appears that, like the rat tail artery [Lachnit et al, 1997], the $\alpha_{1B}$-adrenoceptor plays only a minor role in contraction of the mouse tail artery.

# Chapter eight

## The $\alpha_{1D}$-adrenoceptor mediates a small but significant contractile response in mouse mesenteric resistance arteries

## 8.1 Introduction

Preliminary studies have suggested that phenylephrine-induced contractions of mesenteric resistance arteries from WT and $\alpha_{1B}$ knockout mice are, in part, resistant to antagonism with the $\alpha_{1A}$-selective antagonist 5MeU. The following experiments were carried out to determine whether a 5MeU resistant contractile component does exists, and if so, what adrenoceptor subtype was responsible for the 'minor' contractile response?

To answer these questions, 5MeU was administered at a concentration known to antagonise $\alpha_{1A}$-adrenoceptor-mediated contractile responses. These experiments were carried out in WT, $\alpha_{1B}$ and $\alpha_{1D}$ KO mice. In addition to the use of transgenic mice, the $\alpha_{1D}$-selective antagonist BMY7378 was used to determine if the $\alpha_{1D}$-adrenoceptor mediates contractile responses in mesenteric resistance arteries of the mouse.

Identification of the major adrenoceptor subtype leading to vascular contraction is often complicated by the presence of multiple receptor subtypes. $\alpha_1$-adrenoceptors are coexpressed in a variety of different blood vessels. Regardless of this, it appears to be a single subtype that is the major receptor leading to contraction of a given artery or tissue [Hrometz et al, 1999]. Mesenteric resistance arteries are no exception to this. The major $\alpha_1$-adrenoceptor subtype causing contraction of first order mesenteric resistance arteries from the mouse is the $\alpha_{1A}$-adrenoceptor (based on selectivity of subtype selective ligands) [Daly et al 2002]. So what functions do the remaining adrenoceptors perform? The work contained within this chapter goes some way to uncovering a secondary contractile response that was not attributable to the $\alpha_{1A}$-adrenoceptor.

## 8.2 Methods

WT controls (C57BL/6c/129Sv), $\alpha_{1B}$ and $\alpha_{1D}$ knockout mice (C57BL/6c/129Sv background) aged four and fourteen-sixteen months were used for these studies. All the mice used were euthanised by asphyxiation with $CO_2$. Immediately after death, the mesentery of each mouse was removed and placed in fresh, cold Krebs. The mesenteric arcade was then pinned out on a petri dish containing Krebs solution. The main mesenteric arcade was located and several first order arteries were identified, dissected, cleared of any excess fat and connective tissue and rings of approximately 2mm in length were mounted in 5ml stainless steel Mulvany/Halpern myograph baths (detailed description in Chapter two). Following a 30-35 minute resting period, vessels were stretched to give a resting tension of 0.17gms Force (determined as a suitable resting tension, unpublished observation).

### 8.2.1 Wake-up protocol

Each arterial segment was stimulated with phenylephrine at an initial concentration of 1 x $10^{-5}$M. Once the contraction reached a plateau, each bath was washed four times over a fifteen minute period, before repeating the entire procedure. Then arteries were administered a lower phenylephrine concentration, of 1 x $10^{-6}$M, following which, acetylcholine (3 x $10^{-6}$M) was used to test the viability of the endothelium.

### 8.2.3 Effect of 5-methylurapadil and BMY7378 on arterial contractions

5MeU and BMY7378 were made up according to the manufacturers' guidelines (described in Chapter two). Both of the drugs were used at a concentration of 1 x $10^{-7}$M known to be subtype selective [Daly et al, 2002]. Each drug was administered a minimum of thirty minutes before construction of a phenylephrine concentration

response curve. When two antagonists were added to a single artery, the drugs were administered five-ten minutes apart, and the thirty minute equilibration period began after the last antagonist has been added to the bath.

## 8.3 Results

### 8.3.3 Time controls in mesenteric arteries from four-month old mice

In figure 8.1 the time controls for 5MeU, and 5MeU with BMY7378 curves in four-month old WT mice have been shown. Unlike vessels treated with antagonists, second, and third, consecutive curves showed no reduction in maximum (figure 8.1 **A**). The maximum in the first curve was 0.32 ± 0.04gms Force, compared with that of the second 0.33 ± 0.06gms Force, and third of 0.30 ± 0.06gms Force. When this data was expressed as a percentage of the control curve, the responses gained were relatively consistent, but showed a slight reduction in sensitivity in the third curve (figure 8.1 **B**). The $pEC_{50}$ values for the first, second, and third curves were 5.6 ± 0.04, 5.5 ± 0.04, and 5.1 ± 0.04 respectively.

In figure 8.2, the time controls for the drug treatments tested in mesenteric arteries from $\alpha_{1B}$ (figure 8.2 **A**) and $\alpha_{1D}$ KO (figure 8.2 **B**) mice have been shown. In arteries from $\alpha_{1B}$ and $\alpha_{1D}$ KO mice the maximum response to phenylephrine was unchanged in consecutive response curves. The maximum responses gained in a first, second, and third curve, constructed in arteries from $\alpha_{1B}$ KO mice were as follows, 0.65 ± 0.05, 0.63 ± 0.06, 0.64 ± 0.05gms Force. In $\alpha_{1D}$ KO the first curve maximum was 0.53 ± 0.035gms Force, compared with those of the second and third curves, which were 0.55 ± 0.02, 0.58 ± 0.05gms Force respectively.

### 8.3.4 Effect of BMY7378 on phenylephrine-induced contractions in 4-month old WT mice

Cumulative response curves to phenylephrine alone, and with the $\alpha_{1D}$-selective antagonist BMY7378, were constructed in first order mesenteric resistance arteries from

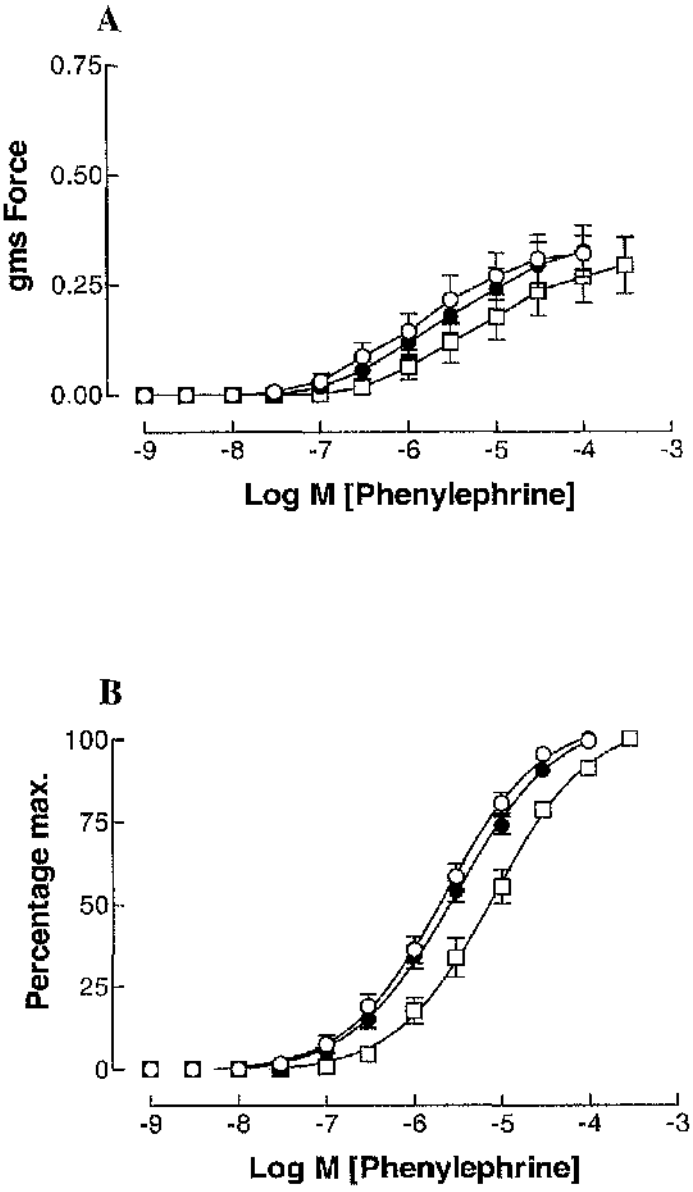
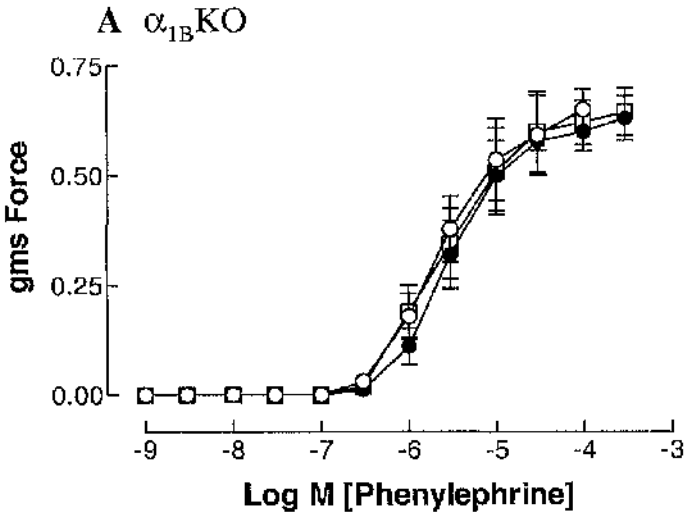



Figure 8.1: Consecutive time controls in mesenteric arteries from 4-month old WT mice. **A** PE-induced responses in a first (○, n = 11), second (●, n = 11), and third (□, n = 12) cumulative curve. **B** PE curves, expressed as percentage of the maximum in a first, second, and third response curve. Each point represents mean ± standard error.



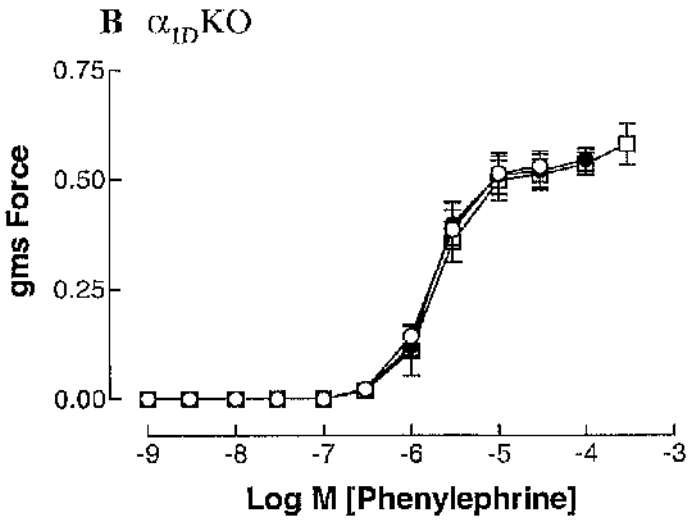




Figure 8.2: Consecutive time controls in mesenteric arteries from 4-month old $\alpha_{1B}$ and $\alpha_{1D}$ KO mice. **A** A first (○, n = 12), second (●, n = 6), and third (□, n = 6) PE curve in $\alpha_{1B}$ KO arteries. **B** A first (○, n = 10), second (●, n = 7), and third (□, n = 7) PE curve in $\alpha_{1D}$ KO arteries. Each point represents mean ± standard error.

four-month old WT mice. The effect of BMY7378 was studied at three concentrations, ranging from 1 x $10^{-8}$M to 1 x $10^{-6}$M.

In figure 8.3, the effect of BMY7378 (1 x $10^{-8}$M) on phenylephrine-induced contractions is shown. In the control curve the maximum response was 0.41 ± 0.03gms Force, compared with a maximum of 0.41 ± 0.09gms Force in the presence of BMY7378 (figure 8.3 **A**). In figure 8.3 **B**, responses to phenylephrine, have been expressed as a percentage of the control maximum for calculation of $pEC_{50}$ values. In the control curve a $pEC_{50}$ value of 5.5 ± 0.02 is gained, while in the presence of the 1 x $10^{-8}$M BMY7378, the $pEC_{50}$ was slightly lower at 5.06 ± 0.03.

Figure 8.4, shows phenylephrine response curves alone, and with BMY7378 (1 x $10^{-7}$M) in mesenteric arteries from four-month old WT mice. BMY7378 caused a slight reduction in the maximum response. The control curve maximum was 0.41 ± 0.03gms Force, compared with 0.30 ± 0.03gms Force, in the presence of BMY7378 (figure 8.4 **A**). Statistical analysis of the maximum contractions to phenylephrine alone, and with BMY7378 at 1 x $10^{-7}$M has shown that although contractions tend to be lower, they failed to reach statistical significance ($p = 0.07$). A $pEC_{50}$ value of 5.0 ± 0.03 was determined in the presence of 1 x $10^{-7}$M BMY7378, which was significantly lower than the control value of 5.5 ± 0.02 ($p<0.0001$***, figure 8.4 **B**)

Figure 8.5 shows the effect of the highest concentration of BMY7378 (1 x $10^{-6}$M) on the size (figure 8.5 **A**) and sensitivity (figure 8.5 **B**) of phenylephrine-induced contractions of mesenteric resistance arteries. At the highest agonist concentration tested, the size of the maximum response was no different from that gained in the

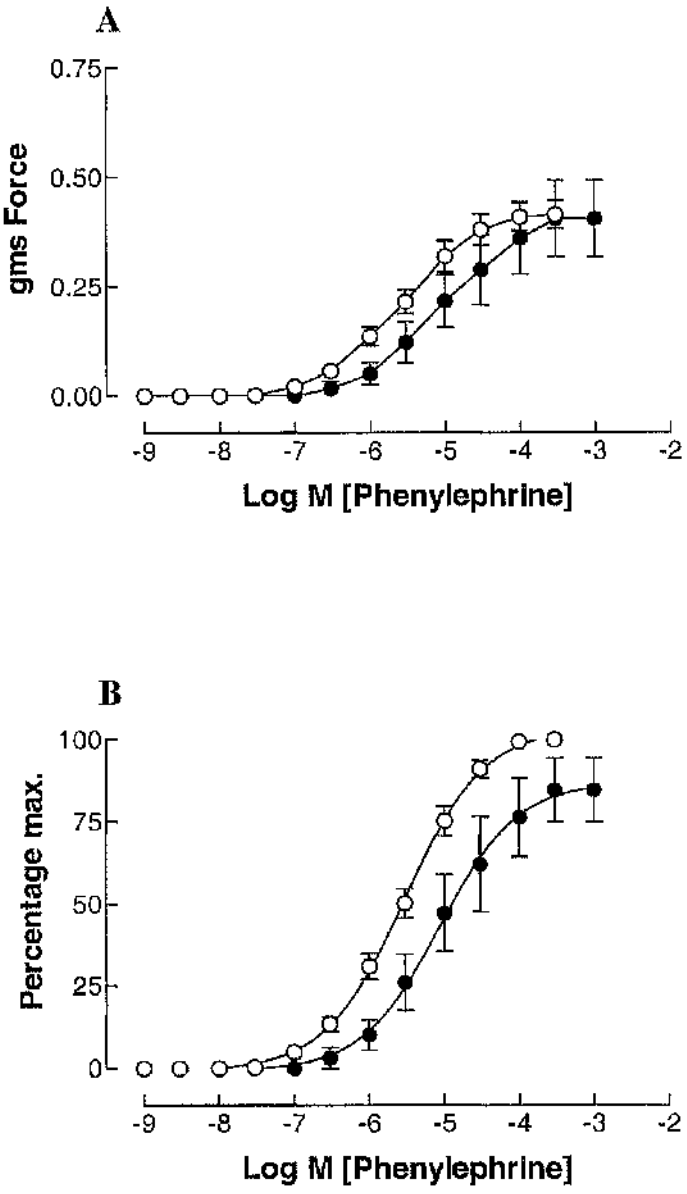



Figure 8.3: Responses in mesenteric arteries from 4-month old WT mice. **A** PE response curves alone (○, n = 12), and with BMY7378 at 1 x $10^{-8}$M (●, n = 3). **B** PE response curves, expressed as a percentage of the control curve maximum. Each point represents mean ± standard error.

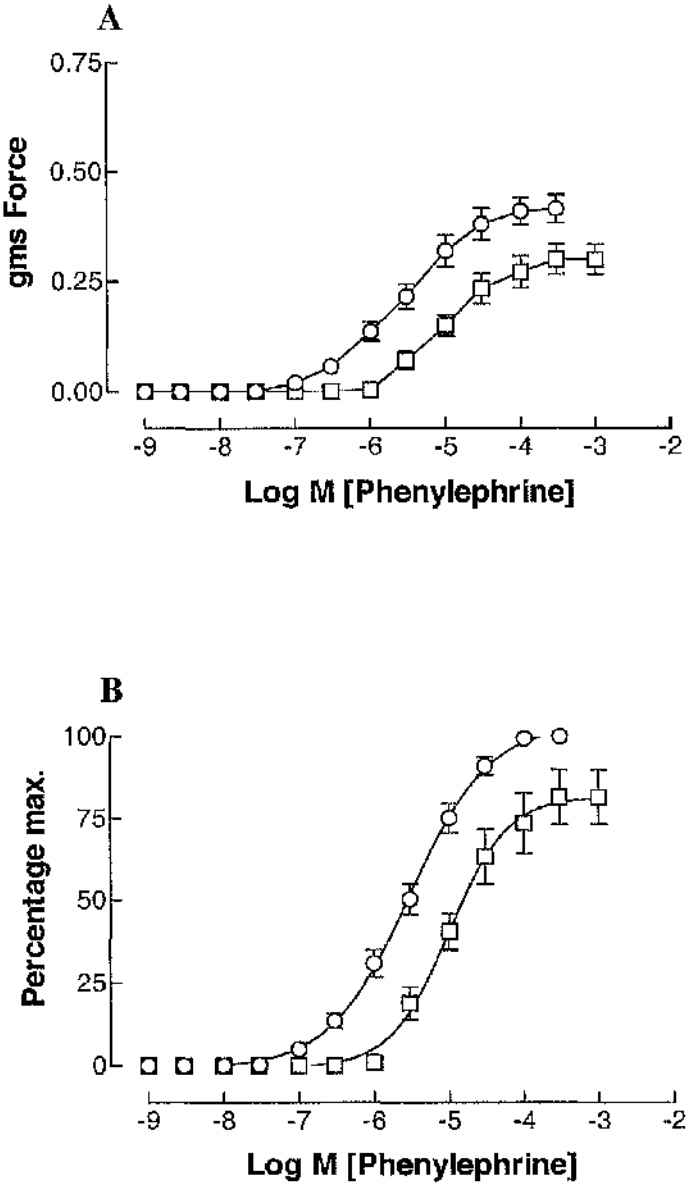



Figure 8.4: Responses in mesenteric arteries from 4-month old WT mice. **A** A PE curve alone (○, n = 12), and with BMY7378 at 1 x $10^{-7}$M ( □, n = 4). **B** PE response curves, expressed as a percentage of control maximum. Each point represents mean ± standard error.

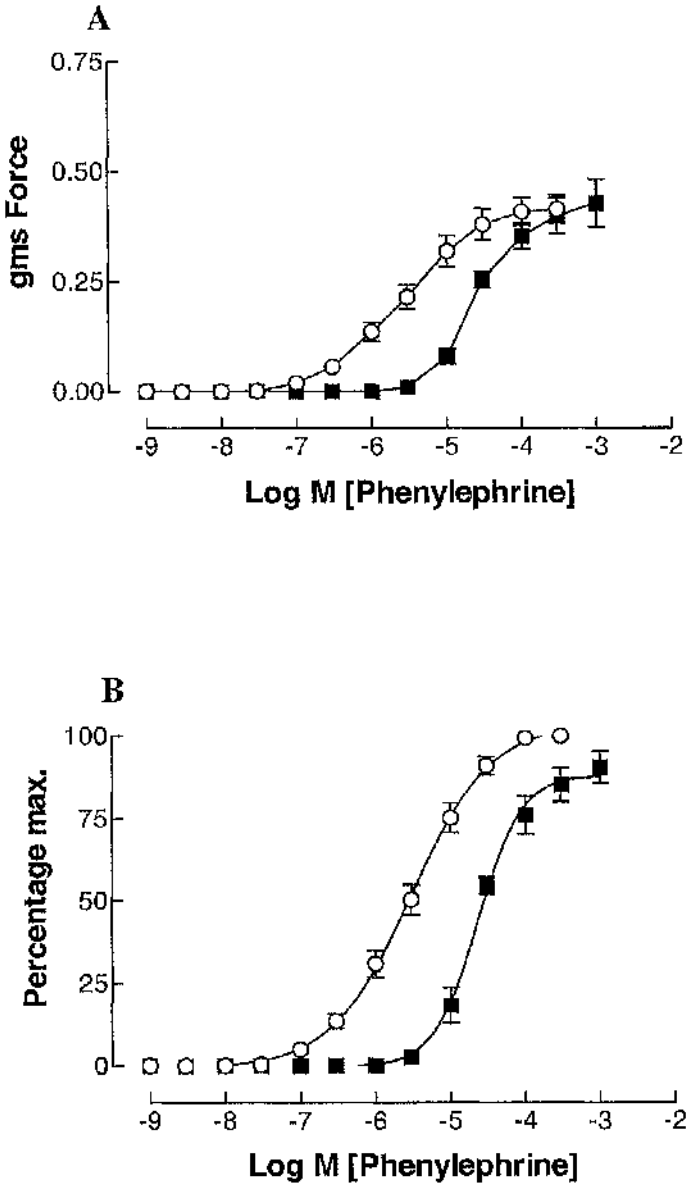



Figure 8.5: Responses in mesenteric arteries from 4-month old WT mice. **A** A PE curve alone (○, n = 12) and with BMY7378 at 1 x $10^{-6}$M (■, n = 4). **B** PE response curves, expressed as a percentage of control maximum. Each point represents mean ± standard error.

control curve (1 x $10^{-6}$M BMY7378 maximum 0.43 ± 0.01gms Force, control maximum 0.41 ± 0.03gms Force ($p>0.05$)). The $pEC_{50}$ value was shifted from 5.5 ± 0.02 in the control curve, to 4.6 ± 0.02 in the presence of BMY7378.

At the lowest antagonist concentration tested (1 x $10^{-8}$M) a shift in the response curve could not be calculated, because of the small sample size, and the variability within the results. Due to this a $pA_2$ value was not calculated, because a minimum of three antagonist concentrations are necessary to construct a Schild regression plot. Therefore, a $pK_B$ value was calculated for the two remaining antagonist concentrations. At a concentration of 1 x $10^{-7}$M a $pK_B$ value of 7.0 ± 0.37 was determined, which was not dissimilar from the value gained at 1 x $10^{-6}$M, found to be 6.9 ± 0.08.

8.3.1 Effect of 5MeU in mesenteric resistance arteries

Figure 8.6 shows the phenylephrine response in first order mesenteric resistance arteries from four-month old WT (figure 8.6 **A**) and $\alpha_{1B}$ KO mice (figure 8.6 **B**) in the presence of increasing concentrations of 5MeU. In both strains, 5MeU caused a rightward shift in the concentration response curve. In the WT, at the highest antagonist concentration (1 x $10^{-7}$M) a 5MeU resistant component of the contraction was uncovered. In the $\alpha_{1B}$ KO this appears at a lower antagonist concentration of 1 x $10^{-8}$M. The results shown provided evidence that a receptor other than the $\alpha_{1A}$-adrenoceptor-subtype was responsible for the 5MeU resistant component of the phenylephrine-induced contraction.

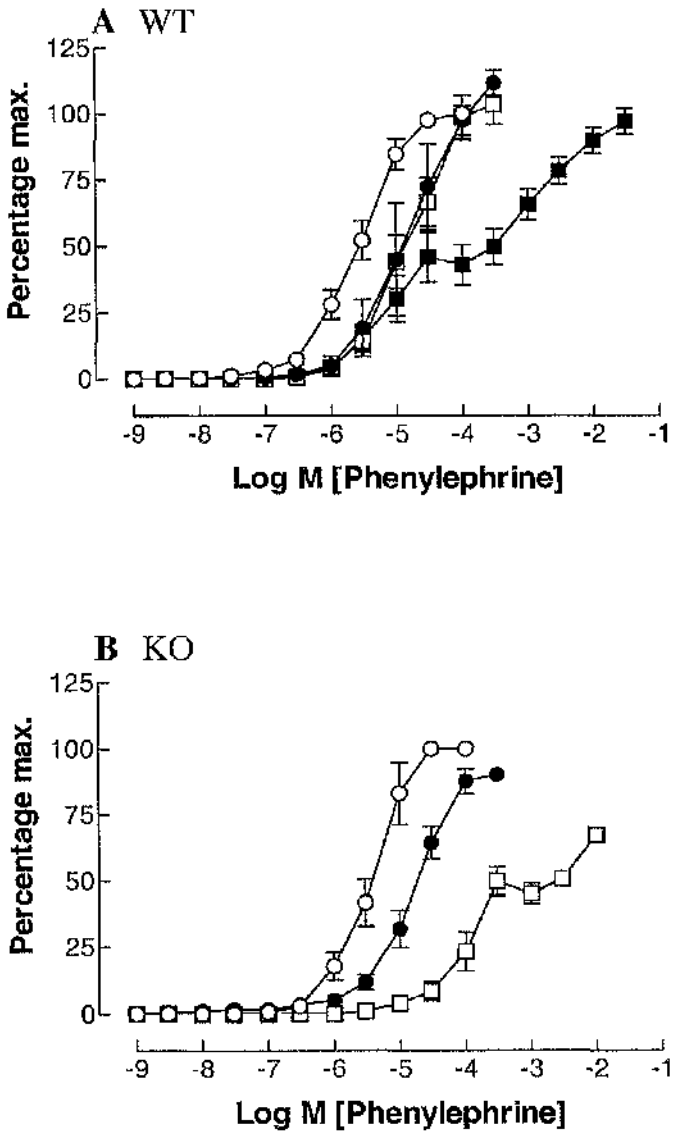



Figure 8.6: Phenylephrine response in first order mesenteric resistance arteries from 4-month old WT and $\alpha_{1B}$ KO mice in the presence of 5MeU. **A** PE response in the WT alone (○, n= 13) and with 5MeU at 1 x $10^{-9}$ (●, n =4) 1 x $10^{-8}$ (□, n = 5), and 1 x $10^{-7}$M (■, n = 6). **B** In the $\alpha_{1B}$ KO, alone (○, n = 3), and with 5MeU at 1 x $10^{-8}$ ( □, n = 3), and 1 x $10^{-7}$M (■, n = 2) 5MeU. Each point represents mean ± standard error.

8.3.2 Effect of 5MeU and BMY7378 in mesenteric arteries from 4-month old mice

Figure 8.7 shows responses to consecutive, cumulative phenylephrine curves constructed in first order mesenteric resistance arteries from four-month old WT mice, alone, and in the presence of 5MeU ($1 \times 10^{-7}$M), and 5MeU with BMY7378 (Both at $1 \times 10^{-7}$M). Data has been shown in two ways. Firstly, the absolute size of contractile responses has been shown in figure 8.7 **A**. Data was then expressed as a percentage of the control maximum, and is depicted in figure 8.7 **B**. In the control curve, the maximum response was 0.44 ± 0.06gms Force. In the presence of 5MeU the maximum contraction obtained was significantly smaller at 0.23 ± 0.04gms Force ($p = 0.001$ **), even in the presence of a higher agonist concentration. In addition to antagonism produced by 5MeU, BMY7378 shifted responses further to the right, and the largest response obtained was 0.22 ± 0.03gms Force in size ($p>0.05$, not significantly different from the effect of 5MeU).

When expressed as a percentage of the first control curve, the reduction in phenylephrine-induced responses was shown more easily (figure 8.7 **B**). In the presence of 5MeU alone, the largest response gained at the highest agonist concentration was 51.9 ± 4.8 % of the control maximum. In the presence of 5MeU and BMY7378, the maximum was 53.6 ± 6.1 % of the control curve maxima.

Contractile responses to consecutive, cumulative phenylephrine curves constructed in first order mesenteric resistance arteries from four-month old $\alpha_{1B}$ KO mice, alone, in the presence of an $\alpha_{1A}$, and $\alpha_{1D}$-selective antagonists have been shown in figure 8.8. Figure 8.8 **A**, shows the size of contractile responses in the three response curves. In the control curve, responses reached a maximum of 0.65 ± 0.05gms Force. In the presence

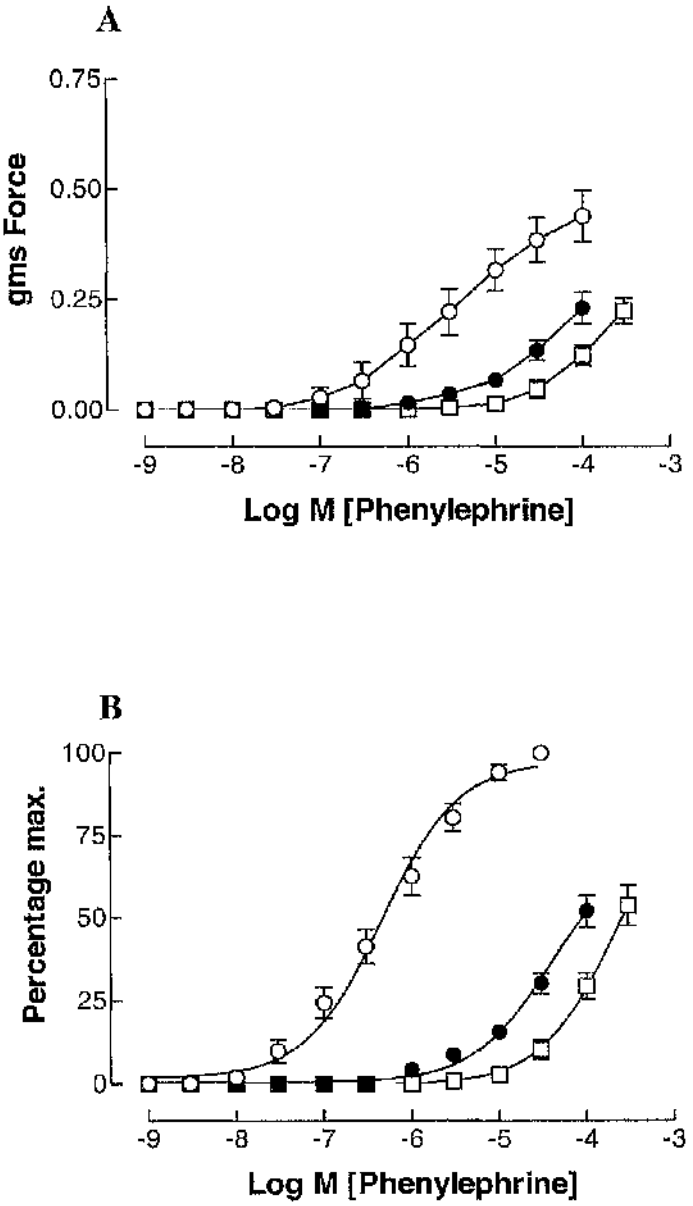



Figure 8.7: Responses in mesenteric arteries from 4-month old WT mice. **A** PE curve alone (○, n = 10), with 5MeU (1 x $10^{-7}$M, ●, n = 10), and 5MeU and BMY7378 (Both 1 x $10^{-7}$M, □, n = 10). **B** PE response curves, expressed as a percentage of control curve maximum. Each point represents mean ± standard error.

of 5MeU a maximum was obtained that was significantly reduced to 0.30 ± 0.07gms Force ($p = 0.0005$ ***). When arteries were incubated with 5MeU and BMY7378 before construction of a response curve the maximum (0.42 ± 0.11gms Force) was smaller than in controls but was no different than that obtained with 5MeU alone.

Responses to phenylephrine in $\alpha_{1B}$ KO arteries, have been expressed as a percentage of the control curve maximum, and are shown in figure 8.8 **B**. 5MeU caused a rightward shift in the concentration response curve, and a significant reduction in maximum. In the presence of 5MeU contractile responses reached 55.1 ± 10.2 % of those gained in the control curve. With 5MeU and BMY7378, the maximum contraction achieved was 76.6 ± 14.7 % of the control curve maximum. So BMY7378 caused no further reduction in the maximum response obtained.

Phenylephrine-induced response curves, constructed in mesenteric resistance arteries from four-month old $\alpha_{1D}$ KO mice, alone, and in the presence of the antagonists 5MeU and 5MeU with BMY7378, have been shown in figure 8.9. Figure 8.9 **A** shows the size of contractile responses. In a first control curve, the maximum contraction obtained was 0.53 ± 0.03gms Force. In the presence of 5MeU, contractile responses were shifted rightward, and had a maximum of 0.45 ± 0.04gms Force, which was not significantly different from that obtained in the control curve ($p = 0.14$). BMY7378, an $\alpha_{1D}$-selective antagonist, had no further effect on contractile responses, on either size, with a maximum of 0.46 ± 0.04gms Force, or sensitivity. In figure 8.9 **B** phenylephrine-induced contractions alone, with 5MeU, and 5MeU with BMY7378 in $\alpha_{1D}$ KO arteries, were expressed as a percentage of the control curve. The control $pEC_{50}$ was 5.3 ± 0.02,

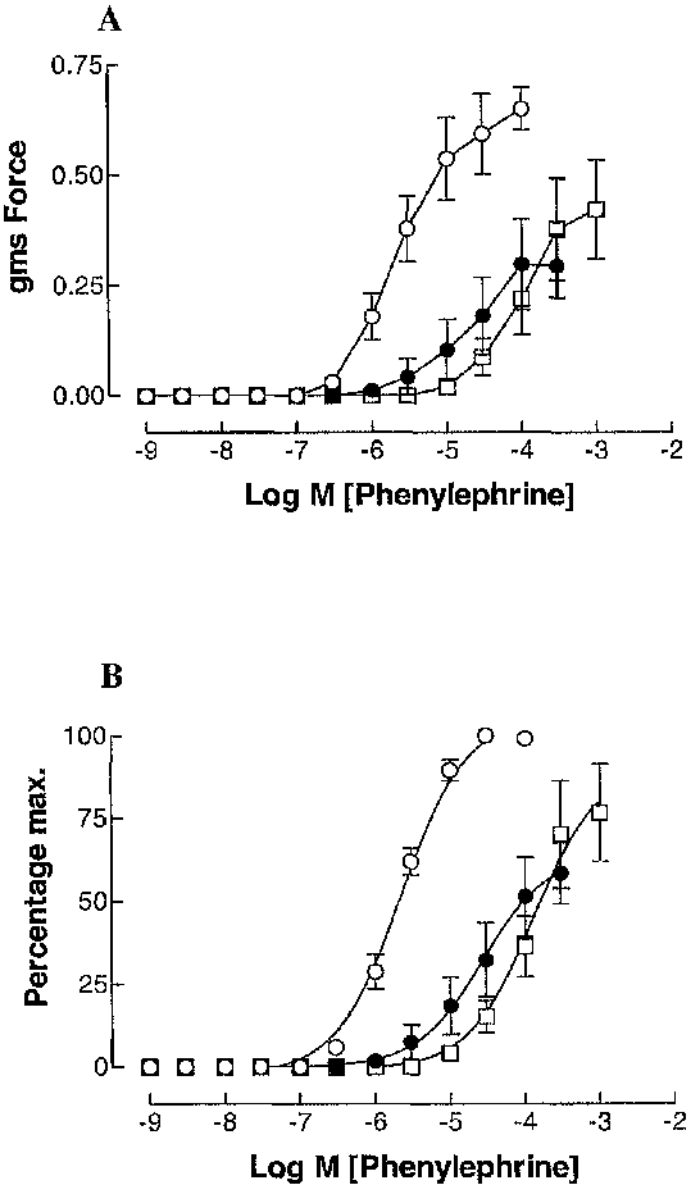



Figure 8.8: Responses in mesenteric arteries from 4-month old $\alpha_{1B}$ KO mice. **A** PE curve alone (○, n = 7), with 5MeU (1 x $10^{-7}$M, ●, n = 7), and 5MeU and BMY7378 (Both at 1 x $10^{-7}$M, □, n = 7). **B** PE response curves, expressed as a percentage of control curve maximum. Each point represents mean ± standard error.

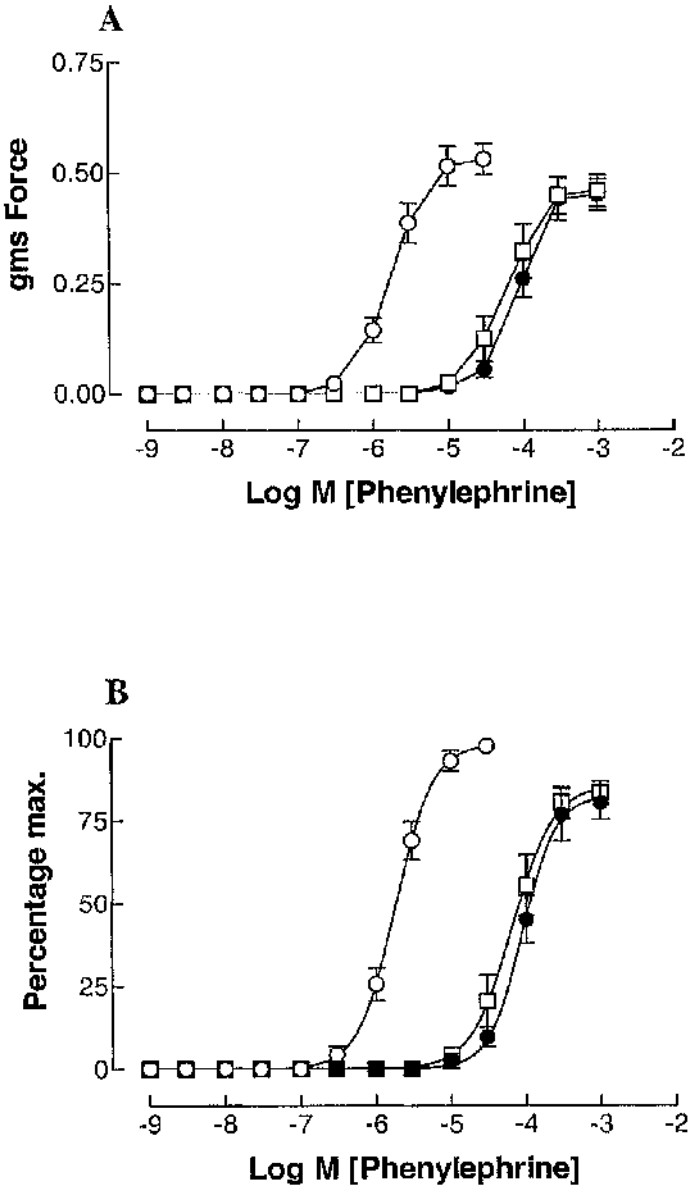



Figure 8.9: Responses in mesenteric arteries from 4-month old $\alpha_{1D}$KO mice. **A** PE-induced responses alone (○, n = 10), with 5MeU (1 x $10^{-7}$M, ●, n = 10), and 5MeU and BMY7378 (Both at 1 x $10^{-7}$M, □, n = 10). **B** PE response curves, expressed as a percentage of control curve maximum. Each point represents mean ± standard error.

in the presence of 5MeU. The maximum response was reduced to 69.05 ± 4.3 % of the control maximum, and when 5MeU and BMY7378 was present, responses were 68.2 ± 7.6 % of the maximum response gained for the control curve.

8.3.7 Time controls for 14-16month old mice

Figure 8.10, shows the time controls for 5MeU, and 5MeU with BMY7378 in mesenteric arteries from fourteen-sixteen-month old WT mice. Responses to a first, second, and third curve were of comparable size, with maximum responses of 0.31± 0.02, 0.34 ± 0.02, and 0.32 ± 0.05gms Force, respectively (figure 8.10 **A**). Phenylephrine-induced contractions in consecutive time controls were expressed as a percentage of the maximum response, and have been depicted in figure 8.10 **B**. The sensitivity of the second response curve was slightly lower than that of the first, and third, were the first curve had a $pEC_{50}$ value of 5.6 ± 0.06, the second a $pEC_{50}$ of 5.2 ± 0.06, and the third curve had a $pEC_{50}$ value of 5.6 ± 0.05.

Figure 8.11, shows the responses gained in time controls for 5MeU alone, and 5MeU with BMY7378, constructed in mesenteric arteries from 14-16-month old $\alpha_{1B}$ (figure 8.11 **A**) and $\alpha_{1D}$ KO (figure 8.11 **B**) mice. In arteries from both strains neither the maximum gained, or the sensitivity to phenylephrine were changed in a second or third consecutive response curve. In the $\alpha_{1B}$ KO the first, second and third curve maximums were 0.45 ± 0.07, 0.47 ± 0.1, and 0.43 ± 0.1gms Force, respectively. The time control curves were then expressed as a percentage of their maximum to permit calculation of $pEC_{50}$ values. The $pEC_{50}$ values for $\alpha_{1B}$ KO time controls were 5.7 ± 0.01 for a first, 5.5 ± 0.02 for a second, and 5.5 ± 0.04 for the third response curve (not shown graphically).

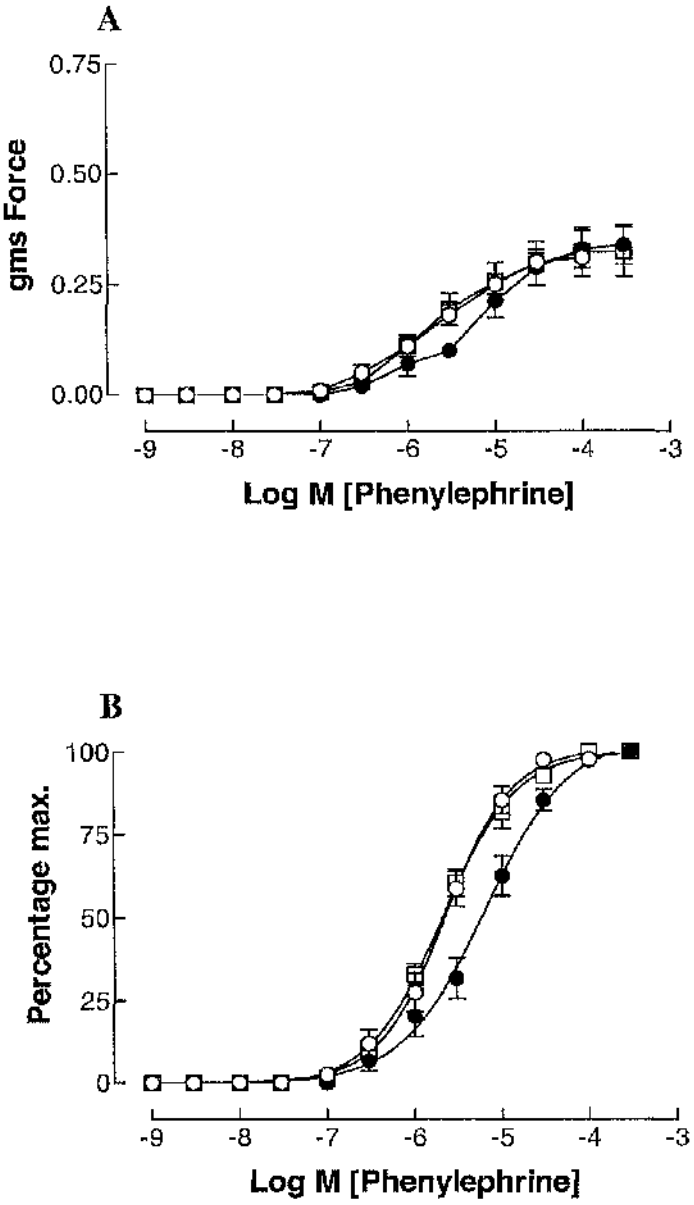
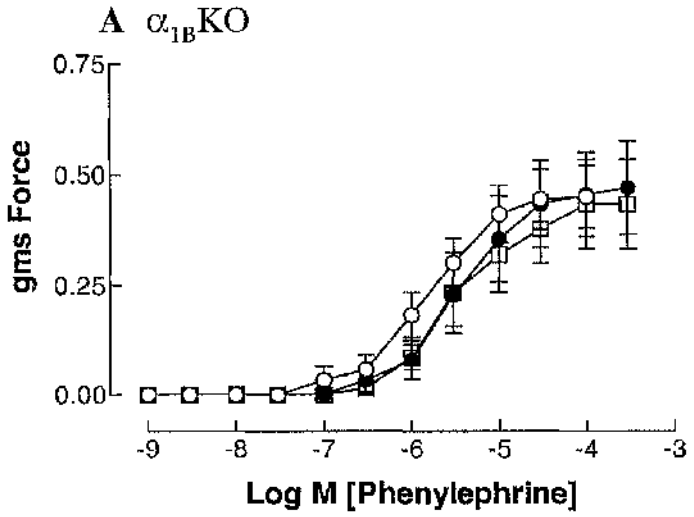



Figure 8.10: Consecutive time controls in mesenteric arteries from 14-16-month old WT mice. **A** First (○, n = 11), second (●, n = 10), and third ( □, n = 10) PE response curves. **B** First, second, and third PE response curves, expressed as a percentage of the maximum response. Each point represents mean ± standard error.



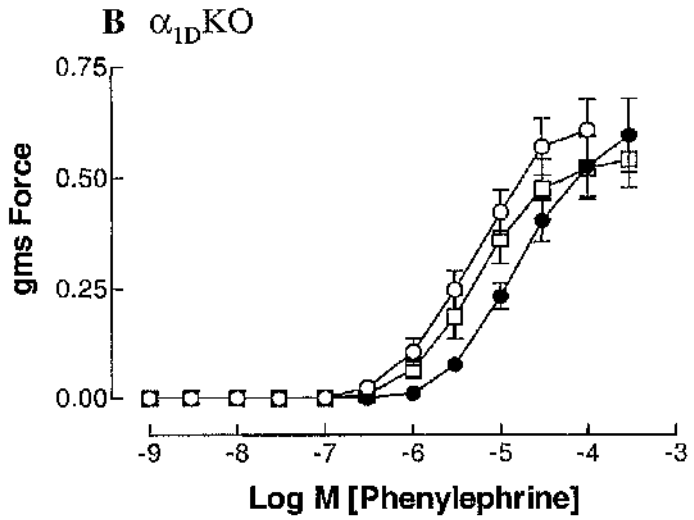




Figure 8.11: Consecutive time controls in mesenteric arteries from 14-16-month old $\alpha_{1B}$ and $\alpha_{1D}$ KO mice. **A** Responses to a first (○, n = 12), second (●, = 6) and third (□, = 6) PE time control in $\alpha_{1B}$ KO arteries. **B** Responses to a first (○, n = 10), second (●, = 8) and third (□, = 8) PE time control in $\alpha_{1D}$ KO arteries. Each point represents mean ± standard error.

Similarly, the maximum contractions gained in the time controls for mesenteric arteries from $\alpha_{1D}$ KO mice were unchanged. In the first, second and third consecutive response curves the maximum contractions gained were as follows, 0.61 ± 0.07, 0.60 ± 0.08, and 0.54 ± 0.06. The responses gained were then expressed as a percentage of the control curve maxima for calculation of $pEC_{50}$ values. The $pEC_{50}$ values for the first, second and third curves were as follows 5.2 ± 0.02, 4.5 ± 0.03 and 4.7 ± 0.02 (not shown graphically).

8.3.5 Contractions in arteries from 14-16-month old transgenic mice

Cumulative, consecutive response curves to phenylephrine alone, in the presence of 5MeU, and with 5MeU and BMY7378 (both at concentrations of 1 x $10^{-7}$M) were constructed in first order mesenteric resistance arteries from fourteen-sixteen-month old WT (figure 8.12 **A**) and $\alpha_{1D}$ KO (figure 8.12 **B**) mice, the responses gained are shown in figure 8.12. The contractions obtained in WT arteries were generally smaller than those of the transgenics, with the control curve reaching a maximum of 0.36 ± 0.04gms Force. 5MeU caused a rightward shift of the response curve, and a reduction in the maximum response in the WT to 0.23 ± 0.04gms Force. The reduction in the maximum response caused by 5MeU was significant (p = 0.036*). The addition of BMY7378 produced a slight rightward shift without reducing the contractile maximum, which was 0.23 ± 0.05gms Force.

Phenylephrine-induced contractions of mesenteric arteries from fourteen-sixteen-month old $\alpha_{1D}$ KO mice were greater in size than that those obtained in age matched WT arteries. In the $\alpha_{1D}$ KO the maximum phenylephrine response in a control curve was

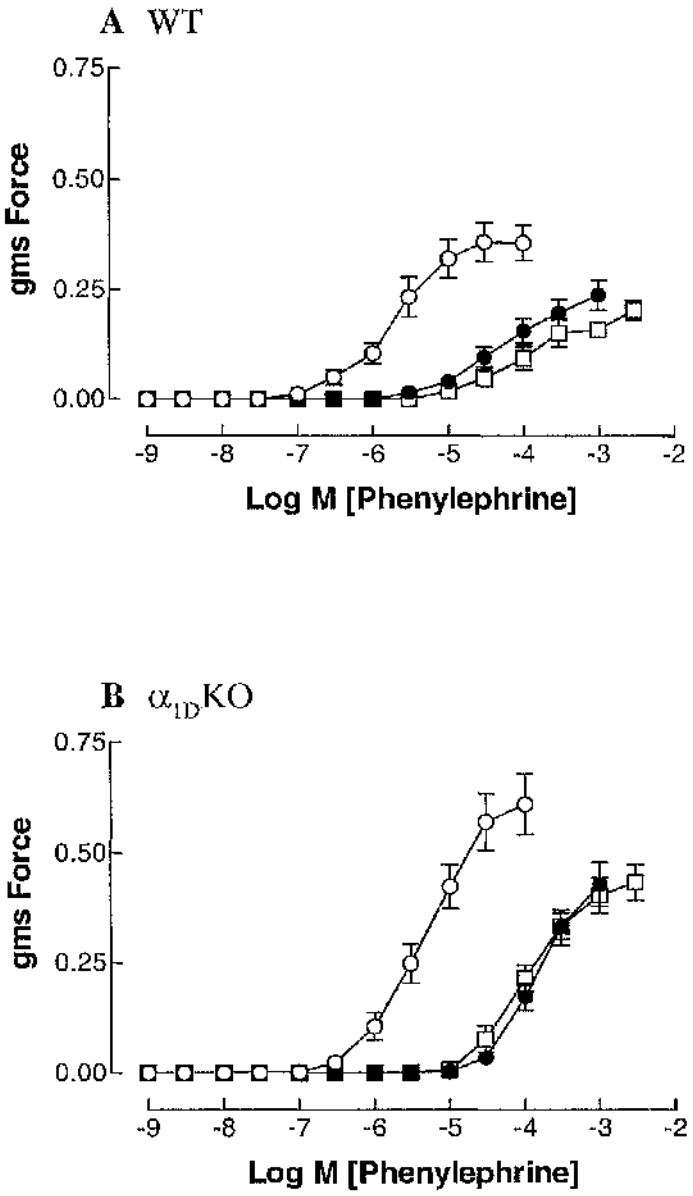



Figure 8.12: Responses in mesenteric arteries from 14-16-month old WT and $\alpha_{1D}$ KO mice. PE curve alone (○), with 5MeU (1 x $10^{-7}$M, ●), and 5MeU/BMY7378 (Both 1 x $10^{-7}$M, □), respectively. **A** In WT arteries, (○, n = 11), (●, n = 11), (□, n = 11). **B** In $\alpha_{1D}$KO arteries, (○, n = 10), (●, n = 10), (□, n = 10). Each point represents mean ± standard error.

0.61 ± 0.07gms Force, which was significantly greater than the WT maximum of 0.36 ± 0.04gms Force ($p<0.01$**). In arteries from $\alpha_{1D}$ KO mice, 5MeU caused a rightward shift of the concentration response curve, and a small, but significant reduction in maximum response from 0.61 ± 0.07gms Force to 0.43 ± 0.05gms Force ($p = 0.025$*). BMY7378 had no effect on the size or sensitivity of the remaining contractile response, with a maximum contraction of 0.43 ± 0.04gms Force being achieved in the third, and final response curve.

8.3.6 5MeU resistant contraction in mesenteric arteries from $\alpha_{1B}$KO mice

Figure 8.13 shows the responses to consecutive, cumulative phenylephrine response curves alone, with 5MeU, and in the presence of 5MeU and BMY7378 in mesenteric resistance arteries from $\alpha_{1B}$ KO mice. In the control curve, the maximum response gained was 0.45 ± 0.02gms Force. The presence of the $\alpha_{1A}$-selective antagonist 5MeU caused a rightward shift in the concentration curve, but at lower agonist concentrations, a component of the contraction that was resistant to 5MeU antagonism, was obvious. The maximum response obtained in the presence of 5MeU was 0.43 ± 0.12gms Force. The addition of BMY7378 ($1 \times 10^{-7}$M) removed the resistant phase of the phenylephrine contraction, shifting the curve further to the right (at low agonist concentrations).

When compared to a control curve, the presence of 5MeU with BMY7378 tended to reduce the size of contractile responses from 0.45 ± 0.02gms Force in the control curve to 0.32 ± 0.08gms Force, with both antagonists. However, statistical analysis of the responses gained in the control curve, compared with that of the curve constructed in the presence of 5MeU and BMY7378 showed that responses were not significantly smaller in size ($p = 0.76$).

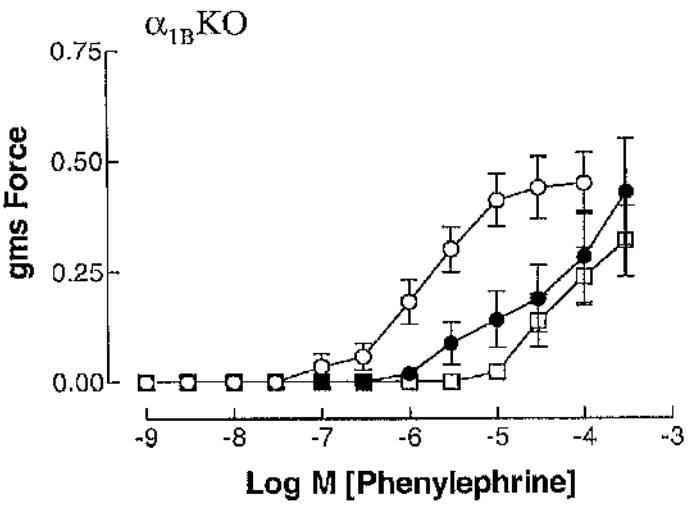



Figure 8.13: Responses in mesenteric arteries from 14-16-month old $\alpha_{1B}$KO mice. Responses to PE alone (○, n = 12), with 5MeU (1 x $10^{-7}$M, ●, n = 12) and with 5MeU and BMY7378 (Both 1 x $10^{-7}$M, □, n = 12) in $\alpha_{1B}$ KO arteries. Each point represents mean ± standard error.

The following three figures (8.14 to 8.16, inclusive) compare the responses gained in $\alpha_{1B}$ KO arteries to the other strains studied. Figure 8.14 shows phenylephrine-induced response curves, constructed in the presence of 5MeU in mesenteric arteries from $\alpha_{1B}$ and $\alpha_{1D}$ KO mice (figure 8.14 **A**). The curve produced in the $\alpha_{1D}$ KO was clearly shifted further to the right, than that of the $\alpha_{1B}$ KO. The addition of BMY7378 on top of 5MeU (figure 8.14 **B**) shifted responses in $\alpha_{1B}$ KO arteries, leaving a response that was comparable in shape and size, to that gained in the $\alpha_{1D}$ KO.

Figure 8.15 shows the contractile effect of cumulative phenylephrine response curves in mesenteric resistance arteries from all three murine strains, expressed as a percentage of their control curve maximum. In figure 8.15 **A**, the responses to phenylephrine in the presence of 5MeU are shown for WT, $\alpha_{1B}$ and $\alpha_{1D}$ KO arteries. Mesenteric arteries from $\alpha_{1D}$ KO mice are the least sensitive to phenylephrine, while responses in WT arteries, although smaller in size, were more sensitive to phenylephrine. Although responses obtained in arteries from $\alpha_{1B}$ KO mice lie somewhere between the two strains, a clear 'bump' in the curve was observed between 10-25% of the contractile maximum. When BMY7378 was present the 5MeU resistant phase of contraction was removed (figure 8.15 **B**). In the presence of 5MeU alone a $pEC_{25}$ value of $6.6 \pm 0.3$ had been calculated for the $\alpha_{1B}$ KO, in the presence of 5MeU and BMY7378 the $pEC_{25}$ was shifted to $4.8 \pm 0.1$ ($p<0.0001$***).

Figure 8.16 shows the comparison between the size (figure 8.16 **A**) and sensitivity (figure 8.16 **B**) of control curves to phenylephrine in mesenteric resistance arteries from

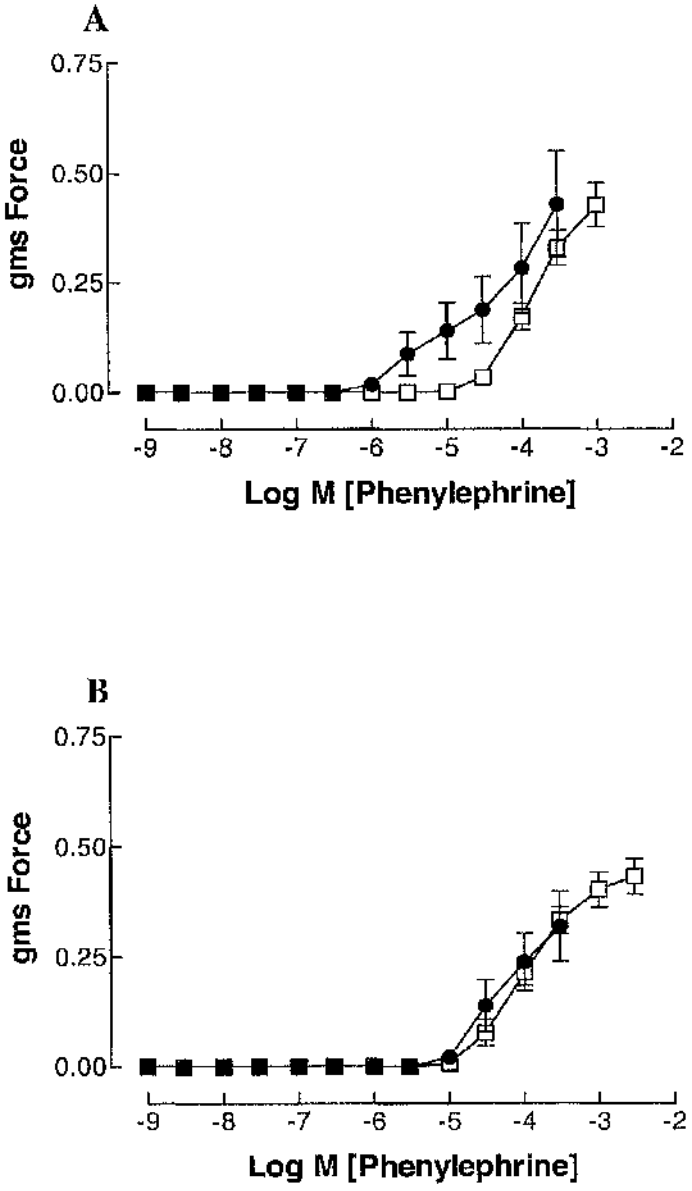



Figure 8.14: Responses in mesenteric arteries from 14-16-month old mice, in $\alpha_{1B}$ (●) and $\alpha_{1D}$ KO (□), respectively. **A** PE curves with 5McU at 1 x $10^{-7}$M, (●, n = 12), ( □, n = 10). **B** PE curves with 5MeU and BMY7378 both at 1 x $10^{-7}$M, (●, n = 12), ( □, n = 10). Each point represents mean ± standard error.

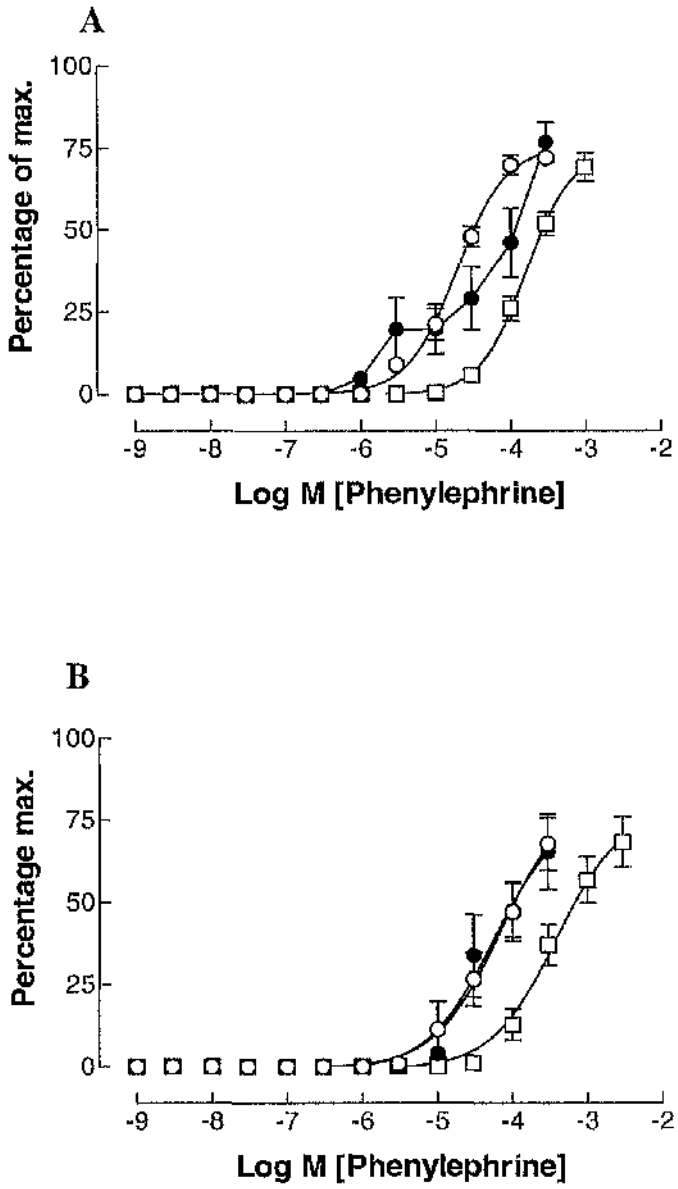



Figure 8.15: Responses in mesenteric arteries from 14-16-month old mice, expressed as a percentage of control maximum for WT (○), $\alpha_{1B}$ (●) and $\alpha_{1D}$ KO (□), respectively. **A** With 5MeU at 1 x $10^{-7}$M, (○, n = 11), (●, n = 12), ( □, n = 10). **B** With 5MeU and BMY7378, both at 1 x $10^{-7}$M, (○, n = 11), (●, n = 12), ( □, n = 10) arteries. Each point represents mean ± standard error.

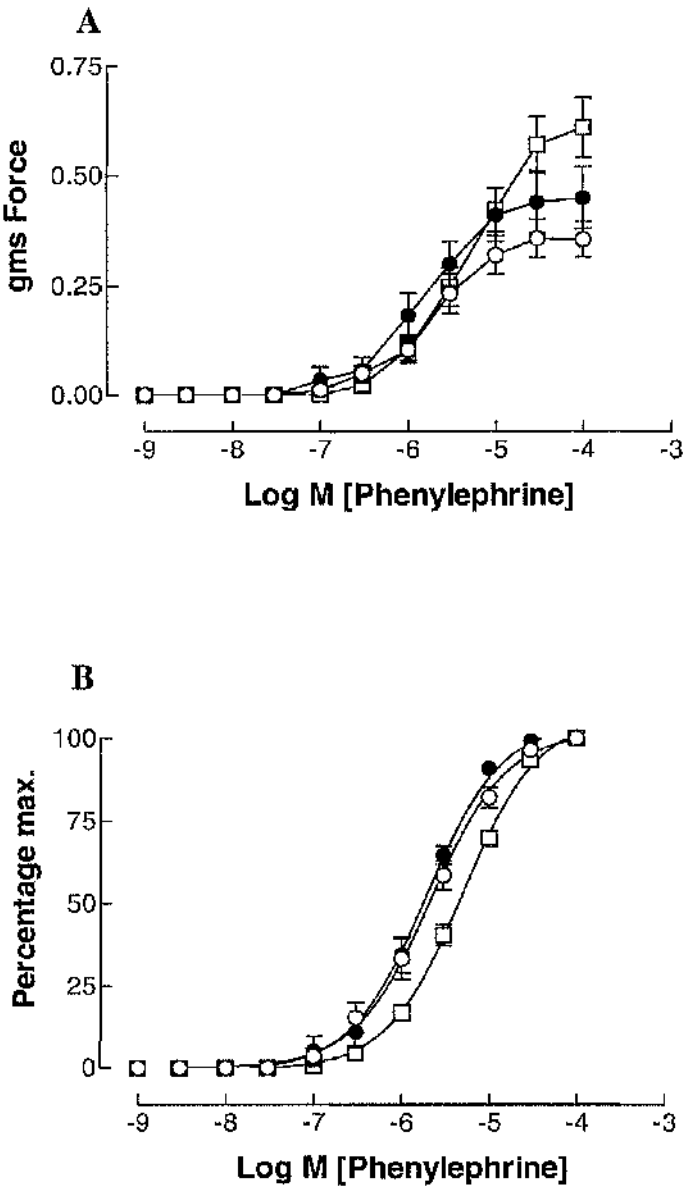



Figure 8.16: The PE response in mesenteric arteries from 14-16-month old WT mice. **A** First curves to PE in WT (○, n = 11), $\alpha_{1B}$KO (●, n = 12) and $\alpha_{1D}$KO ( □, n = 10) arteries. **B** First curves to PE in the WT, $\alpha_{1B}$ and $\alpha_{1D}$ KO arteries, expressed as a percentage of their own maximum. Each point represents mean ± standard error.

fourteen-sixteen-month old WT, $\alpha_{1B}$ and $\alpha_{1D}$ KO mice. Arteries from $\alpha_{1D}$ KO mice gave the greatest contractile responses, and had a maximum of 0.61 ± 0.07gms Force. The smallest contractile response was obtained in arteries from WT mice, with a maximum response of 0.36 ± 0.04gms Force. Contractions in $\alpha_{1B}$ KO arteries were between those gained in WT and $\alpha_{1D}$ KO mesenteric arteries; having a maximum of 0.45 ± 0.02gms Force. Statistical analysis confirmed that contractile responses in arteries from $\alpha_{1D}$ KO mice were significantly greater in maximum than those obtained in age matched WT ($p< 0.01^{**}$), and $\alpha_{1B}$ KO arteries ($p<0.01^{**}$). While the maximum contractions produced in WT and $\alpha_{1B}$ KO arteries was not significantly different ($p>0.05$)

In figure 8.16 **B**, phenylephrine-induced contractions in control curves from WT, $\alpha_{1B}$, and $\alpha_{1D}$ KO mice, have been expressed as a percentage of their own maximum response. Arteries from WT and $\alpha_{1B}$ KO mice had comparable sensitivity, with a $pEC_{50}$ value of 5.7 ± 0.02 for WT, and a $pEC_{50}$ of 5.7 ± 0.02 for the $\alpha_{1B}$ KO. While $\alpha_{1D}$ arteries were least sensitive to phenylephrine, yielding a $pEC_{50}$ value of 5.3± 0.02.

Figure 8.17 shows the absolute size of contractions to phenylephrine in the presence of 5MeU alone (figure 8.17 **A**), and 5MeU with BMY7378 (figure 8.17 **B**) in the three murine strains studied. Contractions gained in the presence of 5MeU were of comparable size in arteries from knockout mice, with an $\alpha_{1B}$ maximum of 0.43 ± 0.12gms Force, and an $\alpha_{1D}$ maximum of 0.43 ± 0.05gms Force arteries. However contractile responses in WT arteries were significantly smaller than both the $\alpha_{1B}$ ($p<0.01$ **) and $\alpha_{1D}$ KO ($p<0.01$ **), having a maximum of 0.23 ± 0.04gms Force.

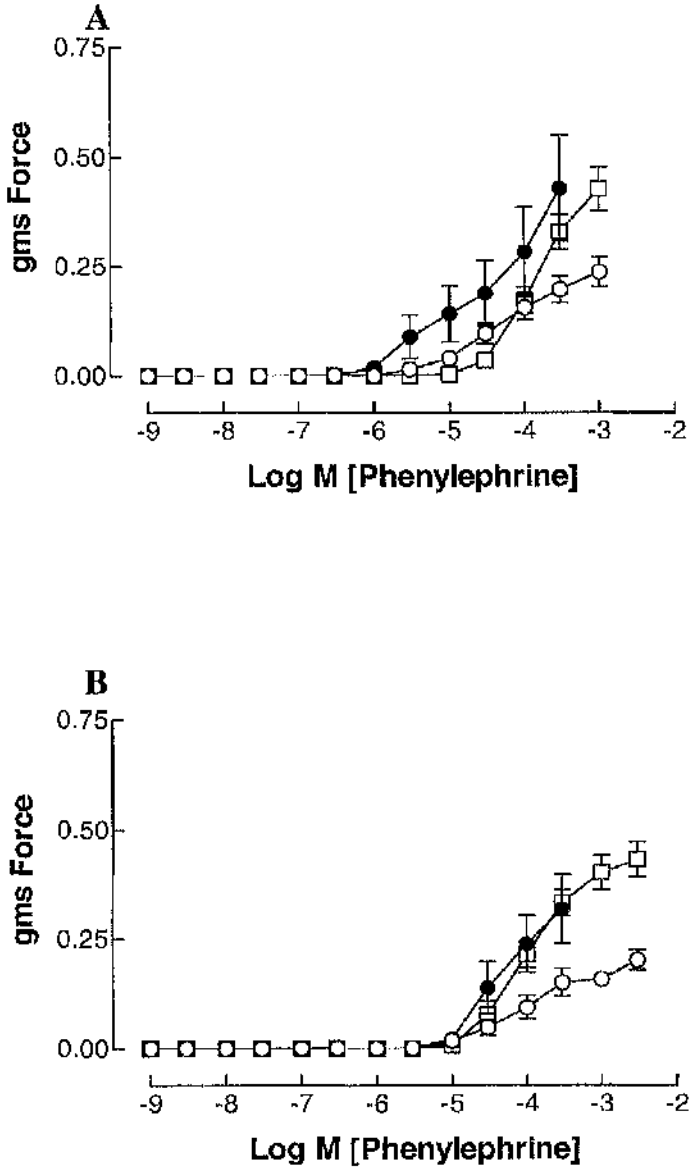



Figure 8.17: The PE response in mesenteric arteries from 14-16-month old mice. **A** PE curves with 5MeU, in WT (○, n = 11), $\alpha_{1B}$ (●, n = 12), and $\alpha_{1D}$ KO ( □, n = 10) arteries. **B** PE curves with 5MeU and BMY7378, in WT (○, n = 11), $\alpha_{1B}$ (●, n = 12), and $\alpha_{1D}$ KO ( □, n = 10) arteries. Each point represents mean ± standard error.

Comparison of the maximum responses gained in $\alpha_{1B}$ and $\alpha_{1D}$ KO arteries confirmed that there was no significant difference in the maximum response between these two strains ($p>0.05$).

Figure 8.17 **B** shows the effect of BMY7378 (in addition to 5MeU) on phenylephrine-induced contractions in mesenteric arteries from WT, $\alpha_{1B}$, and $\alpha_{1D}$ KO mice at 14-16-months. Again responses in $\alpha_{1B}$ and $\alpha_{1D}$ KO arteries were of comparable size and sensitivity. The contractile responses in arteries from the $\alpha_{1B}$ KO were significantly greater in size, than those obtained in WT mesenteric arteries ($p = 0.027$*). The contractile responses gained in $\alpha_{1D}$ KO arteries were also significantly greater than those of the WT ($p<0.01$**).

8.3.8 Comparison of responses gained in arteries from 4 and 14-16-month old mice

Figure 8.18, shows the responses gained in four-month (figure 8.18 **A**) and fourteen-sixteen-month (figure 8.18 **B**) old mesenteric arteries from WT mice. Contractile responses were not significantly different at the age points studied. The maximum response in arteries from four-month old WT mice was $0.44 \pm 0.06$gms Force, compared with that at fourteen-sixteen-months, which was $0.36 \pm 0.04$gms Force ($p = 0.20$). However, the first phenylephrine-induced response curve constructed in mesenteric arteries from four-month old WT mice, failed to reach a contractile plateau. 5MeU at a concentration of $1 \times 10^{-7}$M caused a rightward shift in the concentration response curves at both age points, and a reduction in maximum. The addition of BMY7378 in the third response curve caused a slightly greater shift, again, at both age points.

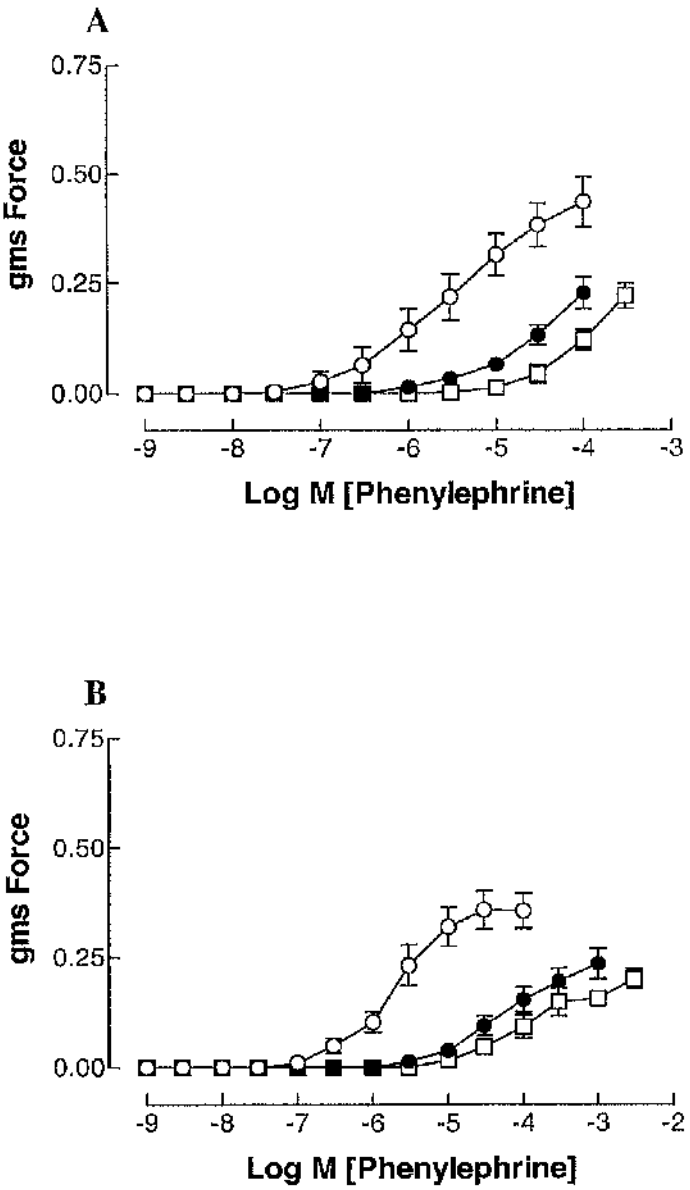



Figure 8.18: The PE response in mesenteric arteries from 4 and 14-16-month old WT mice. **A** PE curve alone (○, n = 10), with 5MeU (1 x $10^{-7}$M, ●, n = 10), and 5MeU/BMY7378 (Both 1 x $10^{-7}$M, □, n = 10) at 4-months. **B** PE curve alone (○, n = 11), with 5MeU (1 x $10^{-7}$M, ●, n = 11), and 5MeU/BMY7378 (Both 1 x $10^{-7}$M, □, n = 11) at 14-16-month. Each point represents mean ± standard error.

Consecutive, cumulative phenylephrine response curves constructed in mesenteric arteries from four (figure 8.19 **A**) and fourteen-sixteen-month (figure 8.19 **B**) old $\alpha_{1B}$ KO mice, are shown in figure 8.19. Responses obtained in arteries from four-month old mice were significantly greater in size than those obtained in older mice. With the maximum response gained in the control curve from four-month old mice being 0.65 ± 0.05gms Force, compared with 0.45 ± 0.02gms Force in mesenteric arteries from fourteen-sixteen-month old mice ($p = 0.02^*$). The major difference between the responses gained in four and 14-16months old $\alpha_{1B}$ KO arteries, was that at four-months the 5MeU resistant component of contraction was not as obvious. However, at 14-16months, it could clearly be seen.

Figure 8.20 shows the results gained for $\alpha_{1D}$ KO mice at four (figure 8.20 **A**) and fourteen-sixteen-months (figure 8.20 **B**). In the control curves, the size of responses in arteries from fourteen-sixteen-month old mice appeared to be greater than the maximum achieved at four-months. The maximum contractile effect of phenylephrine in fourteen-sixteen-month arteries was 0.61 ± 0.07gms Force, compared with 0.53 ± 0.03gms Force at four-months. However, statistical analysis showed that responses at four-months were not significantly smaller than those at fourteen-sixteen-months ($p = 0.27$). 5MeU caused a significant rightward shift in the response curves to phenylephrine in arteries from $\alpha_{1D}$ KO mice, at both age points. BMY7378 had little, if any, effect on phenylephrine-induced contractions, at either age point.

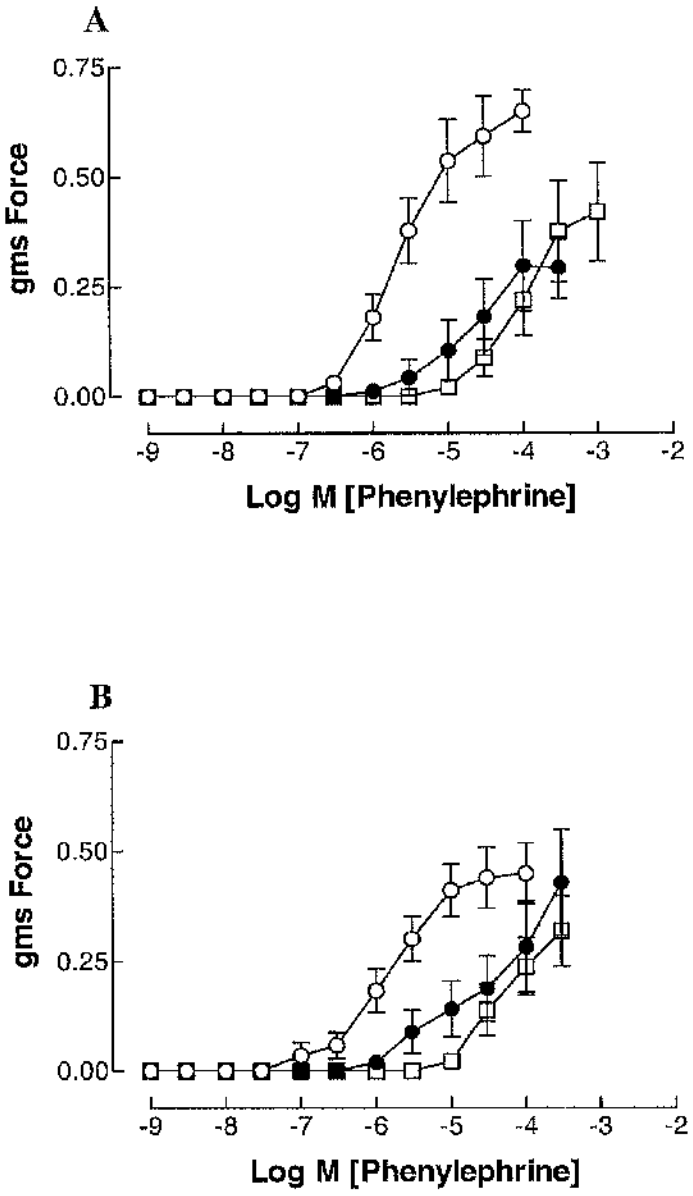



Figure 8.19: The PE response in mesenteric arteries from 4 and 14-16-month old $\alpha_{1B}$ KO mice. **A** PE curve alone (○, n = 7), with 5MeU (1 x $10^{-7}$M, ●, n = 7), and 5MeU/BMY7378 (Both 1 x $10^{-7}$M, □, n = 7) at 4-months. **B** PE curve alone (○, n = 12), with 5MeU (1 x $10^{-7}$M, ●, n = 12), and 5MeU/BMY7378 (Both 1 x $10^{-7}$M, □, n = 12) at 14-16-months. Each point represents mean ± standard error.

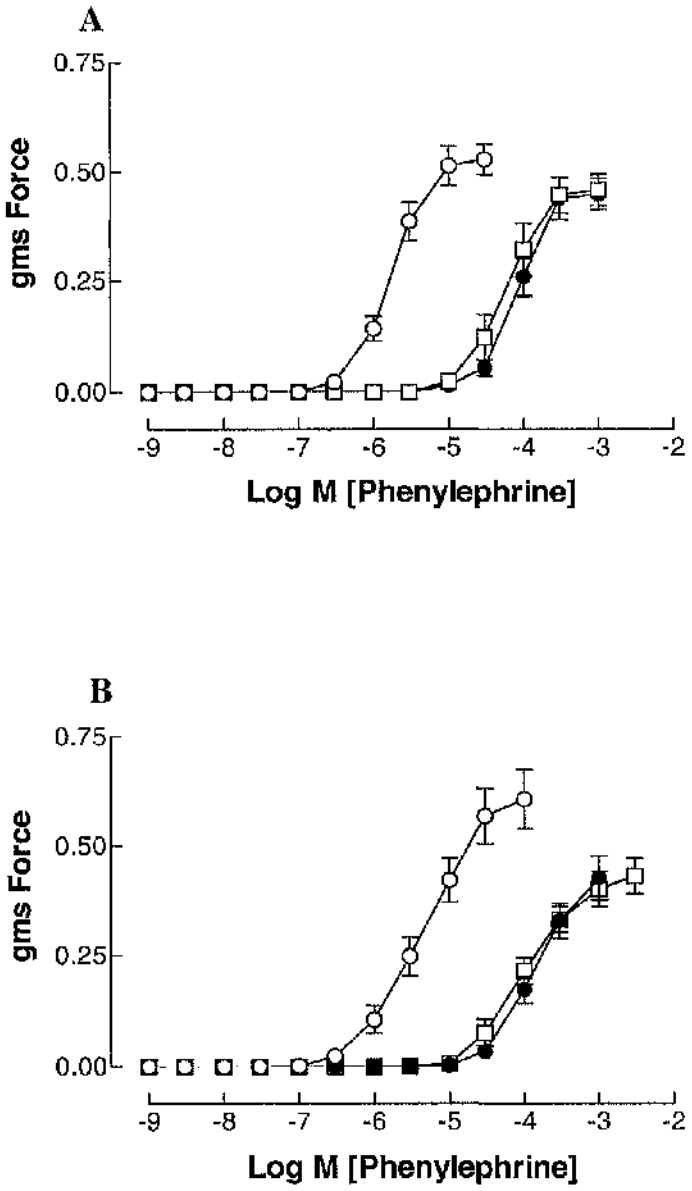



Figure 8.20: The PE response in mesenteric arteries from 4 and 14-16-month old $\alpha_{1D}$KO mice. **A** PE curve alone (○, n = 5), with 5MeU (1 x $10^{-7}$M, ●, n = 5) and 5MeU/BMY7378 (Both at 1 x $10^{-7}$M, □, n = 5) at 4-months. **B** PE curve alone (○, n = 10), with 5MeU (1 x $10^{-7}$M, ●, n = 5) and 5MeU/BMY7378 (Both at 1 x $10^{-7}$M, □, n = 10) at 14-16-months. Each point represents mean ± standard error.

## 8.4 Discussion

### The major $\alpha_1$-adrenoceptor leading to contraction

The $\alpha_{1A}$-adrenoceptor has previously been shown to be the major $\alpha_1$-adrenoceptor subtype leading to contraction of first order mesenteric resistance arteries in WT and $\alpha_{1B}$ KO mice [Daly et al, 2002]. The data presented here provides further evidence in support of this hypothesis. 5MeU ($1 \times 10^{-7}$M) causes potent, insurmountable antagonism of phenylephrine-induced contractions in mesenteric resistance arteries from WT, $\alpha_{1B}$ and $\alpha_{1D}$ KO mice, at four and fourteen-sixteen months.

Having established the major $\alpha_1$-adrenoceptor responsible for mediating contractile responses, the main aim was to determine, what, if any contractile function the remaining $\alpha_1$-adrenoceptor subtypes perform. In order to negate the use of multiple $\alpha_1$-adrenoceptor antagonists, 5MeU and BMY7378 (at concentrations known to be subtype selective) were used in combination with three murine strains.

### Time controls

Time control experiments were carried out in mesenteric arteries from WT, $\alpha_{1B}$, and $\alpha_{1D}$ KO mice aged four and fourteen-sixteen months of age. At both age points, and in all strains studied, phenylephrine-induced contractions showed no reduction in the maximal response gained, and little if any change in tissue sensitivity. The data gained from the time control experiments confirms that the actions of 5MeU and BMY7378 are drug-induced, and are not due to a generalised reduction in responsiveness.

Effect of increasing concentrations of BMY7378 in WT mesenteric resistance arteries

BMY7378 causes a small, but significant rightward shift in phenylephrine-induced concentration response curves. At a concentration of 1 x $10^{-8}$M, the small sample size, and variability of the data prevented calculation of a dose ratio. Therefore, this prevented the calculation of a $pA_2$ value for BMY7378 in this blood vessel. However, at higher antagonist concentrations dose ratio values could be determined, permitting calculation of a $pK_B$. At 1 x $10^{-7}$M a $pK_B$ of 7.0 ± 3.7 was determined, and at 1 x $10^{-6}$M the value was 6.9 ± 0.08. Therefore, at a concentration of approximately 1 x $10^{-7}$M, BMY7378 occupies fifty percent of the available receptor binding sites. Although this antagonist concentration is relatively high, when compared with the values gained for drugs such as 5MeU and prazosin, it does provide evidence that the $\alpha_{1D}$-receptor is involved, in part, in contractile responses of mesenteric resistance arteries from WT mice.

Size of responses and tissue sensitivity at both age points

A high degree of variability exists between the size of responses gained between strains at both age points, and across the species. These might be simply explained by differences between batches and biological variability; or, may reflect altered responsiveness of the remaining population of $\alpha_1$-adrenoceptors in the absence of one of the other subtypes.

Arteries from four-month old mice $\alpha_{1B}$ KO mice give the greatest maximal contractions, while WT arteries produce the lowest response. At fourteen-sixteen-months, the $\alpha_{1D}$ KO gives the greatest contraction but, again contractile responses are smallest in arteries from WT mice. What becomes clear is that contractile responses in arteries from WT

mice consistently produce responses that have a smaller maximum response than either knockout species.

The tissue sensitivity of the first cumulative curve to phenylephrine has been compared in mesenteric arteries from all three strains, and at both age points. WT arteries have the greatest sensitivity to phenylephrine at four and fourteen-sixteen months, which may be explained by the existence of all three $\alpha_1$-adrenoceptor subtypes. Mesenteric arteries from $\alpha_{1D}$ KO mice, at both age points, show the lowest sensitivity to phenylephrine in their control curves. This provides further evidence that the $\alpha_{1D}$-adrenoceptor contributes to contractile responses, mediated by $\alpha_1$-selective ligands in mesenteric resistance arteries.

The $\alpha_{1D}$-adrenoceptor plays a minor contractile role in mesenteric resistance arteries

5MeU causes potent antagonism of phenylephrine-induced contractions in first order mesenteric resistance arteries from four and fourteen-sixteen-month old WT, $\alpha_{1B}$, and $\alpha_{1D}$ KO mice. 5MeU is a subtype selective antagonist at $\alpha_{1A}$-adrenoceptors [Hieble et al, 1995], and is proposed to inhibit contractions mediated by the $\alpha_{1A}$-receptor subtype in a competitive manner. However in the data shown here, 5MeU causes a significant reduction in the maximal response, indicative of non-competitive antagonism.

At four-months, the addition of the $\alpha_{1D}$-selective antagonist BMY7378 causes a further rightward shift in arteries from the WT and $\alpha_{1B}$ KO, without causing a reduction in the size of responses obtained. BMY7378 has no effect on phenylephrine-induced contractions of mesenteric arteries from four-month old $\alpha_{1D}$ KO mice, which lack functional $\alpha_{1D}$ receptors. This suggests that in the WT and $\alpha_{1B}$ KO the shift produced

by BMY7378 indicates the presence of the $\alpha_{1D}$-adrenoceptor, and therefore that it plays a small, but significant role in mediating contractile responses of mesenteric resistance arteries in WT and $\alpha_{1B}$ KO mice.

At fourteen-sixteen months the role of the $\alpha_{1D}$-adrenoceptor in mediating contractile responses becomes even clearer. In arteries from WT mice, BMY7378 produces a biphasic curve, by inhibiting phenylephrine-induced contractions at high agonist concentrations. As at four-months and fourteen-sixteen months BMY7378 has no effect on phenylephrine-induced contraction of mesenteric resistance arteries from $\alpha_{1D}$ KO mice. The compelling evidence in support of a $\alpha_{1D}$ contractile component comes from data gained in the $\alpha_{1B}$ KO. 5MeU causes a rightward shift of the phenylephrine response curve without reducing maximal responses. At lower agonist concentrations a 5MeU resistant contractile response is evident. This gives rise to a biphasic response curve, which is indicative of receptor heterogeneity. BMY7378 completely abolishes the secondary contractile response, and shifts the response curve further to the right. Given that BMY7378 is a selective $\alpha_{1D}$-adrenoceptor antagonist [Saussy et al, 1994], this provides strong evidence that the $\alpha_{1D}$ receptor contributes to contractile responses in first order mesenteric resistance arteries from the mouse.

The $\alpha_{1D}$-adrenoceptor is the major contractile receptor that leads to contractions of large calibre murine blood vessels, such as the aorta and carotid [Piascik et al, 1997]. In studies carried out in the rat, a number of smaller downstream arteries have been shown to contract following stimulation of $\alpha_{1D}$-adrenoceptors; these include femoral, iliac, and superior mesenteric arteries [Hrometz et al, 1999]. The work shown here presents

another contractile response that can be attributed to stimulation of vascular $\alpha_{1D}$-adrenoceptors.

In summary, the $pK_B$ values calculated and the inhibitory effect of BMY7378 on 5MeU resistant contractions provide evidence that the $\alpha_{1D}$-adrenoceptor mediates contractile responses in mesenteric resistance arteries, and plays a secondary role to the dominant $\alpha_{1A}$ receptor subtype. Data gained from WT mice is often complex because of the presence of all three $\alpha_1$-adrenoceptor subtypes, because of this, a combination of transgenic mice and subtype selective antagonists simplify responses. In the presence of $\alpha_{1A}$ antagonism, and when functional $\alpha_{1B}$-adrenoceptors are absent, a $\alpha_{1D}$ contractile component is unmasked.

**General discussion**

The main aim of this study was to determine the effects of stimulation of $\alpha_2$ and $\alpha_1$-adrenoceptors on two murine blood vessels, the tail artery and first order mesenteric resistance arteries. These aims have been achieved by using a combination of transgenic mice and classical pharmacological techniques. The recent advances in molecular biology have provided us with mice harbouring gene directed deletions or mutations of given receptor subtypes. Because of this, the mouse has now become the main focus of research in many laboratories. Transgenic technology combined with the use of subtype selective ligands has provided data, not only in this thesis but also in a wealth of recent publications, which clarifies the function of postjunctional $\alpha_1$ and $\alpha_2$-adrenoceptors.

Responses mediated by postjunctional $\alpha_2$-adrenoceptors are difficult to study because the experimental conditions provided *in vitro* do not mimic conditions *in vivo*. To overcome this, I used published literature on the rat tail artery [Templeton et al, 1989], to develop a number of complex protocols intended to permit the study of responses mediated by postjunctional $\alpha_2$-adrenoceptors in the mouse tail artery.

Elevation of vascular tone with the synergist U46619, to levels comparable to fifty percent of the noradrenaline response, provides conditions where a clear $\alpha_2$-adrenoceptor-mediated contractile response can be observed. This shows that, as in the rat tail artery, elevated vascular tone is advantageous for the study of $\alpha_2$-adrenoceptor-mediated responses. Having established that the $\alpha_2$-selective agonist UK14304 causes a contraction of the murine tail artery, the use of subtype-selective antagonists was applied to confirm that the contractile response to UK14304 is the result of stimulation

of receptors belonging to the $\alpha_2$-subfamily, and cannot be attributed to non-selective stimulation of $\alpha_1$-adrenoceptors at high agonist concentrations. A further complication of studying $\alpha_2$-adrenoceptor-mediated responses was also uncovered during this work. Once activated, $\alpha_2$-adrenoceptor-mediated responses are susceptible to profound agonist-induced desensitisation, which can, in part, be delayed by limiting the length of time receptors are exposed to the agonist UK14304.

To overcome the lack of subtype selective antagonists for the three $\alpha_2$-adrenoceptor subtypes, the D79N mouse has been used to clarify the role of the $\alpha_{2A/D}$ receptor subtype in responses of the tail and first order mesenteric resistance arteries. The D79N mouse carries a point mutation of the $\alpha_{2A/D}$-adrenoceptor, which prevents activation of $K^+$ channels, and has therefore been proposed to act as a functional 'knockout' [MacMillan et al, 1996].

In a protocol designed to give the most suitable conditions for the investigation of the UK14304-mediated response in the tail artery, the contractile effect of UK14304 was significantly smaller in the D79N than in the WT. This leads to the conclusion that the $\alpha_{2A/D}$-adrenoceptor plays a role in $\alpha_2$-adrenoceptor-mediated contractions of the murine tail artery. However, given that consecutive response curves were impossible due to profound desensitisation of postjunctional $\alpha_2$-adrenoceptors, response curves thereafter, were constructed non-cumulatively. Under these conditions, the contractile response in the D79N is of comparable size to that of the WT. This data indicates that it is not necessarily a lack of functional $\alpha_{2A/D}$-adrenoceptors that leads to a reduction in the UK14304-mediated response, but some consequence of the method in which the agonist is administered in the mutant mouse. These findings present an unusual phenotype in

the mutant mouse, where the remaining $\alpha_2$-adrenoceptor subtypes (and possibly some functional $\alpha_{2A/D}$-adrenoceptors) are more sensitive to the desensitising effect of stimulation with UK14304.

Physiologically, the tail is an important thermoregulatory organ in rodent species [Rand et al, 1965]. In the perfused rat tail, there is an increase in surface temperature in response to $\alpha_2$-adrenoceptor antagonists, providing evidence that $\alpha_2$-adrenoceptors are involved in contraction of the rat tail artery [Redfern et al, 1995]. Furthermore, contractile responses in distal segments of the murine tail artery appear to be enhanced by a reduction in temperature (from 37 to $28^0$C), which has been attributed to activation of quiescent $\alpha_{2C}$-adrenoceptors [Chotani et al, 2000]. The data presented in this thesis in chapter five contradicts the findings presented by Chotani and co workers. I have found that a reduction in temperature, alone, is insufficient to alter UK14304-mediated contractions of wire myograph mounted vessels. However, prior exposure to the $\alpha_1$-selective agonist, phenylephrine, does lead to a significant potentiation of UK14304-mediated contractions at $22^0$C.

Tail arteries from D79N mice respond in the same way to those of the WT. This suggests that the elevation in contractility, induced by cold temperatures, is probably not due to the $\alpha_{2A/D}$-adrenoceptor, a hypothesis that is supported by the Chotani et al publication. Therefore the data presented here, yet again, presents a response that is mediated by $\alpha_2$-adrenoceptors, which is only detectable under specific experimental conditions. Enhanced contractility of cutaneous blood vessels in cold temperatures would reduce the blood flow and hence maintain a low surface temperature of the tail, or in the case of humans, the surface temperature of the skin of the hands. Therefore,

these protective mechanisms reduce the amount of heat lost from the tail and digits respectively. The findings that I have shown indicate that enhanced $\alpha_2$-adrenoceptor mediated contractions do not act in isolation, and are only significantly potentiated when stimulation of closely associated vascular $\alpha_1$-adrenoceptors precedes the UK14304-mediated response. Given the location of the mouse tail artery, it is reasonable to assume that the physiological temperature of the tail is lower than core body temperature. In light of this, I propose that the $\alpha_{2C}$-adrenoceptor, known to be involved in enhanced contractility at cold temperature [Chotani et al, 2000], will not be quiescent under "normal" conditions, but actively involved in the development of contractions of the mouse tail artery. This also leads me to ask the question "Should all experiments on the tail artery be carried out at lower experimental temperature?"

In first order mesenteric resistance arteries, stimulation of $\alpha_2$-adrenoceptors, most probably located on endothelial cells, leads to relaxant responses, which are due, in part, to stimulation of the $\alpha_{2A/D}$-adrenoceptor-subtype. The relaxant effect of activating $\alpha_2$-adrenoceptors leads to a response that is quite distinct from the contractile effect in the murine tail artery. The physiological significance of the differential effects of stimulating $\alpha_2$-adrenoceptors in the tail and mesenteric arteries are complex. It would appear that the ability of these receptors to mediate quite distinct responses is critically dependent of the location and the physiological function of the artery studied.

The physiological function of the tail artery is such that under conditions of stress, which includes extremes of cold, additional contractile ability is required for the maintenance of a homeostatic balance. In the mouse, there is strong evidence that this function is fulfilled by activation of quiescent $\alpha_2$-adrenoceptors [Chotani et al, 2000].

Mesenteric arteries supply the intestine with a rich blood supply and nutrients, and given the size and location of these arteries it is reasonable to assume that they may be involved in the maintenance of peripheral blood pressure. In this instance the role of the $\alpha_2$-adrenoceptor is quite distinct from that of the tail artery. The differential responses in the tail and mesenteric arteries highlight the intricate balance between contractile and relaxant responses in peripheral arteries. The relaxant responses to UK14304 are antagonised by rauwolscine and L-NAME, but are not abolished be either. Preliminary studies in which the $[K^+]$ is elevated, suggest the involvement of EDHF in mediating relaxant responses in first order mesenteric resistance arteries, in addition to nitric oxide.

In the tail artery stimulation of $\alpha_1$-adrenoceptors causes contractile responses that are more sensitive and significantly greater in maximum than responses mediated by $\alpha_2$-adrenoceptors. The $pA_2$ values determined for prazosin and the subtype selective antagonists 5MeU and BMY7378, proposed to be selective for the $\alpha_{1A}$ and $\alpha_{1D}$-adrenoceptors respectively, provide little evidence that the $\alpha_{1B}$-adrenoceptor is a major contributor of phenylephrine-induced contractions of the tail artery from WT and $\alpha_{1B}$ KO mice.

Adrenoceptor-mediated contractile responses can be enhanced with increasing age [Docherty, 1988]. However, most of the studies providing evidence in support of this hypothesis have been carried out in the rat, while the effects of age on contractile responses are poorly defined in the mouse. Contractions that result from stimulation of $\alpha_1$-adrenoceptors, with the selective ligand phenylephrine, are similar in tail arteries from young and old WT mice. However in the $\alpha_{1B}$ KO, contractile responses are

significantly greater in size in the presence of all three concentrations of prazosin tested against phenylephrine-induced contractions at sixteen-months when compared with the response in young animals. This represents a phenotype that is unexplained and which cannot be attributed solely to increasing age, since a similar potentiation in the size of responses does not occur in the WT. Whether this response is a manifestation of a compensatory mechanism that results from the germline deletion of the $\alpha_{1B}$-adrenoceptor, or reflects an increase in the responsiveness of one of the remaining $\alpha_1$-adrenoceptor subtypes is interesting, but unresolved, and merits further investigation!

The murine tail artery and first order mesenteric resistance arteries contract when vascular $\alpha_{1A}$-adrenoceptors are stimulated with selective agonists [Daly et al, 2002]. In mesenteric resistance arteries from young and old mice the $\alpha_{1A}$-selective antagonist 5MeU, causes a significant shift in the phenylephrine-induced response curve, and like prazosin in the tail artery, tends to reduce the maximum response. Agonist-induced responses and the effects of antagonist drugs are simplified in arteries from older animals. In the presence of 5MeU a component of contraction that cannot be attributed to the $\alpha_{1A}$-adrenoceptor is obvious in the $\alpha_{1B}$ KO mouse. Yet this resistant phase of contraction is notably absent in the $\alpha_{1D}$ KO. The addition of BMY7378, an $\alpha_{1D}$-selective antagonist, removes the 5MeU resistant contraction in the $\alpha_{1B}$ KO. This finding confirms that the $\alpha_{1D}$-adrenoceptor mediates a small, but significant contractile response in first order mesenteric resistance arteries of the $\alpha_{1B}$ knockout mouse.

In this instance, the use of transgenic mice combined with subtype selective ligands has provided strong evidence that the $\alpha_{1D}$-adrenoceptor contributes to contractile responses in murine mesenteric resistance arteries. If the transgenic mice, or the subtype selective

ligands had been used in isolation, a clear conclusion as to the function of the $\alpha_{1D}$-adrenoceptor in this blood vessel would have been impossible.

I have no doubt the future of cardiovascular research has been, and will continue to advance with the aid of transgenic technology. Future work will undoubtedly focus heavily on the use of transgenic animals, and given the success of germline manipulation in the mouse, may be applied to other species. The most significant advantage of molecular technology is that it will help provide clarification on responses mediated not only by adrenoceptors, but also by a plethora of other G-protein coupled receptors and enzymes. Yet caution is still required, as it has been highlighted here that transgenic mice, alone, cannot provide all of the answers. However, when experimental conditions and the method in which drugs are applied are questioned, our understanding of complex physiological responses can be greatly increased.

In summary, the work presented in this thesis has achieved several things. In the tail artery, suitable conditions for the investigation of contractile responses mediated by $\alpha_2$-adrenoceptors have been devised, allowing some analysis of their responses and a partial subtyping of the receptors involved. At $37^0$C, and when UK14304 curves are constructed cumulatively the $\alpha_{2A/D}$-receptor subtype appears to mediate part of the contractile response in the mouse tail artery. However, at $22^0$C, and when curves are constructed non-cumulatively, the role of the $\alpha_{2A/D}$-adrenoceptor in contractility declines. Furthermore the effect of increasing age on responses mediated by $\alpha_1$- and $\alpha_2$-adrenoceptors has been investigated in the tail artery. The data presented has shown that contractility, mediated by the $\alpha_1$ and $\alpha_2$-adrenoceptor subfamilies is not significantly enhanced by advancing age up to sixteen-months.

Stimulation of $\alpha_2$-adrenoceptors in murine mesenteric resistance arteries produces a vasodilator response that is quite distinct from the contractile effect in the tail. Relaxations of mesenteric resistance arteries are receptor specific, and appear to depend on both the release of nitric oxide, and the endothelial derived relaxing factor, EDHF. Furthermore, a novel role for the $\alpha_{1D}$-adrenoceptor, which is contractile in nature, has been described in mesenteric resistance arteries. The identification of several of the novel receptor-mediated responses, determined here, would have been considered impossible just ten years ago.

**References**

Aalkjaer C, Heagerty A. M, Mulvany M. J (1987). In vitro characteristic of vessels from patients with essential-hypertension. *Journal of Clinical Investigation* **3**: 317-322.

Allgaier C and Meder W (1996). Alpha (2)-adrenoceptor-mediated modulation of noradrenaline (NA) release and voltage-sensitive $Ca^{2+}$-channels (VSCC) in cultured sympathetic neurones. *Nauyny-Schmiedebergs Archives of Pharmacology* **354**: 147-147 Supplement 1.

Ahlquist R. P (1948). A study of adrenotropic receptors. *American Journal of Physiology* **153**: 586-600.

Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H (1993). Antagonistic action of imidazolicoxyl N-oxides against endothelium-derived relaxing factor. NO through a radical reaction. *Biochemistry* **32**: 827-832.

Altman J. D, Trendelenburg A. U, MacMillan J., Bernstein D, Limbird L, Starke K, Kobilka B.K, Hein L (1999). Abnormal regulation of the sympathetic nervous system in $\alpha_{2A/D}$-adrenergic receptor knockout mice. *Molecular Pharmacology* **56**: 154-161.

Anderson L. C, Sinkey C. A, Lawton W. J, Mark A. L (1989). Elevated sympathetic-nerve activity in borderline hypertensive humans-evidence from direct intranueral recordings. *Hypertension* **14**: 177-183

Argyle S. A, McGrath J. C (2000). An $\alpha_{1A}/\alpha_{1L}$-Adrenoceptor mediates contaction of canine subcutaneous resistance arteries. *The Journal of Pharmacology and Experimental Therapeutics* **295**: 627-633.

Arner M, Hogestatt E. D (1986). Contractile effect of noradrenaline and 5-hydroxytryptamine in human veins: a pharmacological receptor characterization. *Acta Physiologica Scandinavia* **128 (2)**: 209-217.

Arribas S. M, Hillier C, Gonzalez C, McGrory S, Dominiczak A. F, McGrath J. C (1997). Cellular aspects of vascular remodelling in hypertension revealed by confocal microscopy. *Hypertension* **30**: 1455-1464.

Arunlakshana O and Schild H. O (1959). Some quantitative used of drug antagonists. *British Journal of Pharmacology* **14**: 48-58.

Balzo U. D, Rosen M. R, Malfatto G, Kaplan L. M, Steinberg S. F (1990). *Circulation Research* **67**: 1535-1551.

Banbury Conference on Genetic Background in Mice. Mutant mice and neuroscience: recommendations concerning genetic background (1997). *Neuron*. **19**: 755-759.

Bartlett I. S, Marshall J. M. (2002). Analysis of the effect of graded levels of hypoxia on noradrenaline-evoked contraction in the rat iliac artery *in vitro*. *Experimental Physiology* **87.2**: 171-184.

Baumgart D, Haude M, Gorge G, Liu F, Ge J, CrosseEggebreht C, Erbel R, Heusch G (1999). Augmented α-adrenergic constriction of atherosclerotic human coronary arteries. *Circulation* **99**: 2090-2097.

Bevan R. D, Dodge J, Nichols P, Penar P. L, Walters C. L, Wellman T, Bevan J. A (1998a). Weakness of sympathetic neural control of human pial compared with superficial temporal arteries reflects low innervation density and poor sympathetic responsiveness. *Stroke* **29**: 212-221.

Bezard E, Brefel C, Tison F, Peyro-Saint Paul H, Ladure P, Rascol O, Gross C (1999). Effect of the $\alpha_2$ adrenoceptor antagonist, idazocan, on motor disabilities in MPTP-treated monkey. *Progressive Neuro-Psychopharmacology & Biological Psychiatry* **23**: 1237-1246.

Bezprozvanny I, Tsien R. W (1995). Voltage-dependent blockade of diverse types of voltage-gated $Ca^{2+}$ channels expressed in Xenopus oocytes by the $Ca^{2+}$ channel antagonist mibefradil. *Molecular Pharmacology* **48**: 540-549.

Bishai J. M, Penninga L, Nijland R, Meulenaar R, Gheorghe C. P, Zhao Y, Buchholz J. N, Zhang L, Longo L. D (2002). Pre- and postjunctional alpha (2)-adrenergic receptors in fetal and adult ovine cerebral arteries. *American Journal of Physiology-Regulatory Integrative and Comparative physiology* **282**: R1654-1662.

Bjorklund M, Sirvio J, Riekkinen M, Sallinen J, Scheinin M, Riekkinen P (2000). Overexpression of $\alpha_{2C}$-adrenoceptors impairs water maze navigation. *Neuroscience* **95**: 481-487.

Blue D. R, Bonhaus D. W, Ford A. P. D. W, Pfister J. R, Sharif N. A, Shieh L. A, Vimont R. L, Williams T. J, Clarke D. E (1995). Functional evidence equating the pharmacologically-defined $\alpha_{1A}$- and cloned $\alpha_{1C}$-adrenoceptor: studies in the isolated perfused kidney of the rat. *British Journal of Pharmacology* **115**: 283-294.

Bockman C. S, Jeffries W. B, Abel P. W (1993). Binding and functional characterization of $\alpha_2$-adrenergic receptor subtypes in pig vascular endothelium. *Journal of Pharmacology and Experimental Therapeutics* **267**: 1126-1133.

Bockman C. S, GonzalezCabrera I, Abel P. W (1996). $\alpha_2$-adrenoceptor subtype causing nitric oxide-mediated vascular relaxations in rats. *Journal of Pharmacology and Experimental Therapeutics* **278**: 1235-1243.

Boer C, Scheffer G. J, de Lange J. J, Westerhof N, Sipkema P (1999). $\alpha_1$-adrenoceptor stimulation induces nitric oxide release in rat pulmonary arteries. *Journal of Vascular Research* **36**: 79-81.

Bolton T. B, Lang R. J, Takewaki T (1984). Mechanism of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *Journal of Physiology* **351**: 549-572.

Bruban V, Estato V, Schann S, Ehrhardt J-D, Monassiert L, Renard P, Scalbert E, Feldman J, Bousquet P (2002). Evidence for synergy between $\alpha_2$-adrenergic and noadrenergic mechanisms in central blood pressure regulation. *Circulation* **105**: 1116-1121.

Bucheler M. M, Hadamek K, Hein L (2002). Two $\alpha_2$-adrenergic receptor subtypes, $\alpha_{2A/D}$ and $\alpha_{2C}$, inhibit transmitter release in the brain of gene-targeted mice. *Neuroscience* **109**: 819-826.

Bunemann M, Buchcler M. M, Philipp M, Lohse M. J, Hein L (2001). Activation and deactivation kinetics of $\alpha_{2A/D}$ and $\alpha_{2C}$-adrenergic receptor-activated G protein-activated inwardly rectifying $K^+$ channel currents. *Journal of Biological Chemistry* **276**: 47512-47517.

Bunemann M, Philipp M, Bucheler M, Lohse M. J, Hein L (2001). Kinetic analysis of $\alpha_{2A}$- and $\alpha_{2C}$-adrenergic receptor mediated signalling. *Naunyn-Schmiedebergs Archives of Pharmacology* **363**: 376 Supplement S.

Burt A. R, Sautel M, Wilson M. A, Rees S, Wise A, Milligan G (1998). Agonist occupation of an $\alpha_2$-adrenoceptor-G (i1)$\alpha$ fusion protein results in activation of both receptor-linked and endogenous G (i) proteins-Comparison of their contributions to GTPase activity and signal transduction and analysis of receptor-G protein activation stoichiometry. *Journal of Biological Chemistry* **273**: 10367-10375.

Busija D. W, Heistad D. D (1984). Factors involved in the physiological regulation of cerebral circulation. *Reviews of Physiological Biochemistry & Pharmacology* **101**: 161-211.

Busse R, Fleming I (1998). Regulation of NO synthesis in endothelial cells. *Kidney & Blood Pressure Research* **21**: 264-266.

Bylund D. B, Blaxall H. S, Iversen L. J, Caron M. G, Lefkowitz R. J, Lomasney J. W (1992). Pharmacological characteristics of $\alpha_2$-adrenergic receptors-Comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Molecular Pharmacology* **42**: 1-5.

Bylund D. B, Eikenberg D. C, Hieble J. P, Langer S. Z, Lefkowitz R. J, Minneman K. P, Mollinof P. B, Ruffolo R. R, Trendelenburg U (1994). International Union of Pharmacology: Nomenclature of Adrenoceptors. *Pharmacological Reviews* **46(2)**: 121-136.

Carter R. W, Kanagy N. L (2002). Tyrosine kinases regulate intracellular calcium during alpha (2)-adrenergic contraction in rat aorta. *American Journal of Physiology – Heart & Circulatory Physiology* **283(4)**: H1673-H1680.

Cavalli A. Lattion A-L, Hummler E, Nenniger M, Pedrazzini T, Aubert J-F, Michel M. C, Yang M, LemboG, Vecchione C, Mostardini M, Schmidt A, Beermann F, Cotecchia S (1997). Decreased blood pressure response in mice deficient of the alpha 1b-adrenergic receptor. *Proceedings of the National Academy of Science USA* **94**: 11589-11594.

Chalothorn D, McCune D. F, Edelmann S. E, Garcia-Cazarin M. L, Tsujimoto G, Piascik M (2002). Differences in the cellular localization and agonist-mediated internalization properties of the $\alpha_1$-adrenoceptor subtypes. *Molecular Pharmacology* **61**: 1008-1016.

Chalothorn D, McCune D, Edlemann S. E, Tobita K, Keller B. K, Lasley R. D, Tanoue A, Tsujimoto G, Perez D. M, Post G. R, Piascik M. T (2003). Differential cardiovascular regulatory activities of the $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor subtypes. *FASEB Journal* **17**: A213-A213 (Part 1 Suppl. S).

Chang H. Y, Klein R. M, Kunos G (1982). Selective desensitisation of cardiac – adrenoceptors by prolonged in vivo infusion of catecholamines in rats. *The Journal of Pharmacology and Experimental Therapeutics* **221**: 784-789.

Chang D. J, Chang T. K, Yamanishi S. S, Salazar F. H. R, Kosaka A. H, Khare R, Bhakta S, Jasper J. R, Shieh I-S, Lesnick J. D, Ford A. P. D. W, Daniels D. V, Eglen R. M, Clarke D. E, Bach C, Chan H. W (1998). Molecular cloning, genomic characterisation and expression of novel human $\alpha_{1A}$-adrenoceptor isoforms. *FEBS* **422**: 279.

Chauhan S, Rahman A, Nilsson H, Clapp L, MacAllister R, Ahluwalia A (2003). NO contributes to EDHF-like responses in rat small arteries: a role for NO stores. *Cardiovascular Research* **57**: 207-216.

Chen L, Xin X, Eckhart A. D, Yang N, Faber J. E (1995). Regulation of vascular smooth muscle growth by $\alpha_1$-adrenoceptor subtypes *in vitro* and in situ. *Journal of Biological Chemistry* **270**: 30980-30988.

Chotani M. A, Flavahan S, Mitra S, Daunt D, Flavahan N. A (2000). Silent $\alpha_{2C}$-adrenergic receptors enable cold-induced vasoconstriction in cutaneous arteries. American *Journal of Physiol Heart Circ Physiol* **278**: H1075-H1083.

Cocks T. M and Angus J. A (1983). Endothelium-dependent relaxation of coronary-arteries by noradrenaline and serotonin. *Nature* **305**: 627-630.

Conrad K. P and Whittemore S. L (1992). $N^G$ – monmethyl-$_L$-arginine and nitro-arginine potentiate pressor responsiveness of vasoconstrictors in conscious rats. *American Journal of Physiologoy* **262**: R1137-R1144.

Cooper A, Heagerty A. M (1997). Blood pressure parameters as determinants of small artery structure in human essential hypertension. *Clinical Science* **92**: 551-557.

Cottone S, Vadala A, Vella M. C, Nardi E, Mule G, Contorno A, Riccobene R, Cerasola G (1998). Changes in plasma endothelin and growth factor levels, and of left ventricular mass, after chronic AT (1) receptor blockade in human hypertension. *American Journal of Hypertension* **11**: 548-553.

Couet J, Sargiacomo M, Lisanti M. P (1997). Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. *Caveolin binding negatively regulates tyrosin and serine/threonin kinase activities. Journal of Biological Chemistry* **272**: 30429-30438.

Cowlen M. S, and Toews M. L (1988). Evidence for $\alpha_1$-adrenergic receptor internalisation in DDT1MF-2 cells following exposure to agonists plus protein kinase C activators. *Molecular Pharmacology* **34**: 340-346.

Crabbe J. C, Wahlsten D, Dudek B. C (1999). Genetics of mouse behaviour: Interactions with laboratory environment. *Science (Washington D. C)* **284**: 1670-1672.

Craig D, Iacolina M, Forray C (1995). $\alpha_{2C}$-Adrenoceptors mediated norepinephrine-induced contraction of rat caudal artery. *FASEB Journal* **9**: A106.

Daly C. J (1993). MSc Thesis. University of Glasgow.

Daly C.J, Deighan C, McGee A, Mennie D, Ali Z, **McBride M**, McGrath J.C. A knockout approach indicates a minor vasoconstrictor role for vascular $\alpha_{1B}$-adrenoceptors in mouse. Physiological Genomics **9**: 85-91, 2002.

Dao T. T, Kailasam M. T, Parmer R. J, Le H. V, Le Verge R, Kennedy B. P, Ziegler M. G, Insel P. A, Wright F. A, O'Connor D. T (1998). Expression of altered $\alpha_2$-adrenergic phenotypic traits in normotensive humans at genetic risk of hereditary (essential) hypertension. *Journal of Hypertension* **16**: 779-792.

Daunt D. A, Hurt C, Hein L, Kllio J, Feng F, Kobilka B. K (1997). Subtype-specific intracellular trafficking of $\alpha_2$-adrenergic receptors. *Molecular Pharmacology* **51**: 711-720.

Deighan C, Slattery D. A, MacKenzie J. F, Cotechia S, McGrath J. C (1999). The characterisation of $\alpha_1$-adrenoceptors in murine liver using radioligand binding and transgenic mice. *British Journal of Pharmacology* **128**: 91P (Suppl. S).

Delmas P, Abogadie F. C, Milligan G, Buckley N. J, Brown D. A (1999). βγ-dimmers derived from $G_o$ and $G_i$ proteins contribute to different components of adrenergic inhibition of $Ca^{2+}$ channels in rat sympathetic neurons. *Journal of Physiology* **518**: 23-36.

Deng J. T, Chemtob S, Varma D. R (1996). Characterization of $\alpha_{1D}$-adrenoceptor subtypes in rat myocardium, aorta and other tissues. *British Journal of Pharmacology* **119**: 269-276.

Diviani D, Lattion A-L, Larbi N, Kunapuli P, Pronin A, Benovic J. L, Cotecchia S (1996). Effect of different G protein-coupled receptor kinases on phosphorylation and desensitisation of the $\alpha_{1B}$-adrenergic receptor. *Journal of Biological Chemistry* **271**: 5049-5058.

Diviani D, Lattion A-L, Cotecchia S (1997). Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitisation of the $\alpha_{1B}$-adrenergic receptor. The Journal of Biological Chemistry **272**: 28712-28719.

Docherty J. R and McGrath J. C (1980). A comparison of the effects of pancuronium bromide and its monoquaternary analogue, ORG NC 45, on autonomic and somatic neurotransmission in the rat. *British Journal of Pharmacology* **71**: 225-233.

Docherty J. R (1988). Pertussis toxin and pre-junctional $\alpha_2$-adrenoceptors in rat-heart and vas-deferens. *Journal of Autonomic Pharmacology* **8**: 197-201.

Docherty J. R (1990) Cardiovascular responses in ageing: A Review. *Pharmacological Reviews* **42**: 103-125.

Docherty J. R (1998). Subtypes of functional $\alpha_1$ and $\alpha_2$-adrenoceptors. *European Journal of Pharmacology* **361**: 1-15.

Duka I, Gavras I, Johns C, Handy D. E, Gavras H (2000). Role of the postsynaptic $\alpha_2$-adrenergic receptor subtypes in catecholamine-induced vasoconstriction. *General Pharmacology-The Vascular System* **34**: 101-106.

Dunn W. R, Daly C. J, McGrath J. C, Wilson V. G (1991). The effects of nifedipine on $\alpha_2$-adrenoceptor-mediated contractions in several isolated blood-vessels from the rabbit. *British Journal of Pharmacology* **103**: 1493-1499.

Eason M. G, Liggett S. B (1992). Subtype-selective desensitization of $\alpha_2$-adrenergic receptors. Different mechanisms control short and long term agonist-promoted desensitization of $\alpha_2$C10, $\alpha_2$C4, and $\alpha_2$C2. *Journal of Biological Chemistry* **267**: 25473-25479.

Elawary A. M, Pettinger W. A, Wolff D. W (1992). Subtype-selective $\alpha_1$-adrenoceptor alkylation in the rat-kidney and its effect on the vascular pressor response

Eri L. M, Tveter K. J, (1995). $\alpha$-Blockade in the treatment of symptomatic benign prostatic hyperplasia. *Journal of Urology* **154**: 923-934.

Exton J. H (1996). Regulation of phosphinositide phospholipases by hormones, neurotransmitters, and other agonists liked to G proteins. *Annual Review of Pharmacology & Toxicology* **36**: 481-509.

Fairbanks C. A, Wilcox G. L (1999). Moxonidine, a selective $\alpha_2$-adrenergic and imidazoline receptor agonist, produces spinal antinociception in mice. *The Journal of Pharmacology and Experimental Therapeutics* **290**: 403-412.

Faure C, Pimoule C, Vallancien G, Langer S. Z, Graham D (1994). Identification of $\alpha_1$-adrenoceptor subtypes in vas deferens, kidney, and aorta of rat. *European Journal of Pharmacology* **224**: 125-136.

Fauz L. Feres T, Borges A. C. R, Paiva T. B (2000). Alpha-2 adrenoceptors are present in rat aorta smooth muscle cells and their action is mediated by ATP-sensitive $K^+$ channels. *British Journal of Pharmacology* **131**: 788-794.

Ferguson S, Zhang J, Barak L. S, Caron M. G (1997). Pleiotrophic role for GRKs and arrestins in receptor regulation. *News in Physiological Science* **12**: 145-152.

Ferrier C, Cox H, Esler M (1993). Elevated total-body noradrenaline spillover in normotensive members of hypertensive families. *Clinical Science* **84**: 225-230.

Ferrier C, Jennings G. L, Eisenhofer G, Lambert G, Cox H. S, Kalff V, Kelly M, Esler M. D (1993). Evidence for increased noradrenaline release from subcortical brain-regions in essential-hypertension. *Journal of Hypertension* **11**: 1217-1227.

Filippi S, Parenti A, Donnini S, Granger H. J, Fazzini A, Ledda F (2001). $\alpha_{1D}$-Adrenoceptors cause endothelium-dependent vasodilation in the rat mesenteric vascular bed. *The Journal of Pharmacology and Experimental Therapeutics* **296**: 869-875.

Flavahan N. A, and Vanhoutte P (1986). $\alpha_1$-Adrenoceptor subclassification in vascular smooth muscle. *TIPS* **7(9)**: 347-349.

Flavanhan N. A and Vanhoutte (1986). Effect of cooling on $\alpha_1$ and $\alpha_2$-adrenergic responses in canine saphenous and femoral veins. *Journal of Pharmacology and Experimental Therapeutics* **238**: 139-147.

Foglar R, Shibata K, Hone K, Hirasawa A, Tsujimoto G (1995). Use of recombinant alpha 1-adrenoceptors to characterise subtype selectivity of drugs for the treatment of prostatic hypertrophy. *European Journal of Pharmacology* **288**: 201-207.

Ford A. P. D. W, Arrendondo N. F, Blue D. R, Bonhaus D. W, Jasper J, Kava M. S, Lesnick J, Pfister J. R, Shieh I. A, Williams T. J, McNeal J. E, Stamey T. A, Clarke D. E (1996a). RS-17053, a selective $\alpha_{1A}$-adrenoceptor antagonist, displays low affinity for

functional $\alpha_1$-adrenoceptors in prostate of man: implications for adrenoceptor classification. *Molecular Pharmacology* **49**: 209-215.

Ford A. P. D. W, Chang D. J, Clarke D. E, Daniels D. V, Eglen R. M, Gever J. R, Japer J. R, Kava M. S, Lachnit W. G, Lesnick J. D, Melov T. D, Stepan G. T, Williams T. J (1998). *Alpha*-1A versus *alpha*-1L-adrenoceptors: a pharmacological comparison. *Pharmacology and Toxicology* **83**: 12-15.

Forray C, Bard J. A, Wetzel J. M, Chiu G, Shapiro E, Tang R, Lepor H, Hartig P. R, Weinshank R. L, Branchek T. A, Gluchowski C (1994). The $\alpha_1$-adrenoceptor that mediates smooth muscle contraction in human prostate has the pharmacological properties of the cloned human $\alpha_{1C}$-adrenoceptor subtype. *Molecular Pharmacology* **45**: 703-708.

Freedman R. R, Sabharal S. C, Desai N, Wenig P, Mayes M (1989). Increased $\alpha$-adrenergic responsiveness in idopathic Raynaud's disease. *Arthrtis Rheum.* **32**: 61-65.

Freedman R. R, Baer R. P, Mayes M. D (1995). Blockade of vasospaestic attacks by $\alpha_2$-adrenergic but not $\alpha_1$-adrenergic antagonists in idiopathic Raynaud's disease. Circulation **92**: 1448-1451.

Friedman J. L, Adler D. N, Davis K. L (1999). The role of norepinephrine in the pathophysiology of cognitive disorders: potential applications to the treatment of cognitive dysfunction in schizophrenia and Alzheimers's disease. *Biological Psychology* **46**: 1243-1252.

Fujita T, Toya Y, Iwatsubo K, Onda T, Kimura K, Umemura S, Ishikawa Y (2001). Accumulation of molecules involved in $\alpha_1$-adrenergic signal within caveolae: caveolin expression and the development of cardiac hypertrophy. *Cardiovascular Research* **51**: 709-716.

Furchgott R. F and Vanhoutte P. M (1989). Endothelium-derived relaxing and contracting factors. *FASEB* **3**: 2007-2018.

Furukawa K, Rosario D. J, Smith D. J, Chapple C. R, Uchiyawa K, Chess-Williams R (1995a). $\alpha_1$-Adrenoceptor-mediatd contractile responses of the human vas deferens. *British Journal of Pharmacology* **116**: 1605-1610.

Furukawa K, Chess-Williams R, Noble A. J, Rosario D. J, Chapple C. R, Uchiyama T (1995b). Non-surmountable antagonist effects of tamsulosin on the $\alpha_{1A}$-adrenoceptor-mediated responses of the rat and human vas deferens. *British Journal of Pharmacology* **115**: 127P

Garcia-Sainz J. A, Vazquez-Prado J, Vilalobos-Molina R (1999). $\alpha_1$-Adrenoceptors: Sybtypes, signalling, and role in health and disease. *Archives of Medical Research* **30**: 449-458.

Gavin K. T, Colgan M-P, Moore D, Shanik D, Docherty J. R (1997). $\alpha_{2C}$-Adrenoceptors mediate contractile responses to noradrenaline in human saphenous vein. *Naunyn-Schmiedeberg's Archive of Pharmacology* **355**: 406-411.

Gavras I, Manolis A. J, Gavras H (2001). The $\alpha_2$-adrenergic receptors in hypertension and heart failure: experimental and clinical studies. *Journal of Hypertension* **19**: 2115-2124.

Gisbert R, Noguera M. A, Ivorra M. D, D'Ocon P. (2000). Functional evidence of a constituitively active population of $\alpha_{1D}$-adrenoceptors in the rat aorta. *Journal of Pharmacology & Experimental Therapeutics* **295**: 810-817.

Gilman A. G (1987). G-proteins – transducers of receptor-generated signals. *Annual Review of Biochemistry* **56**: 615-649.

Grady E. F, Bohm S. K, Bunnett N. W (1997). Turning off the signal: mechanisms that attenuate signalling by GPCRs. *American Journal of Physiology* **273**(3 PART 1): G586-G601.

Grassic G, Seravalle G, Stella M. L, Turrie C, Zanchetti A, Mancia G (2000). Sympathocxcitatory responses to the acute blood pressure fall induced by central or peripheral antihypertensive drugs. *American Journal of Hypertension* **13**: 29-34.

Graham R. M, Perez D. M, Hwa J (1996). $\alpha_1$-Adrenergic receptor subtypes-molecular structure, function and signalling. *Circulation Research* **78**: 737-749.

Griffith T. M, Edwards (1997). $Ca^{2+}$ sequestration as a determinant of chaos and mixed-mode dynamics in agonist-induced vasomotion. *American Journal of Physiology* **272**: H1696-H1709.

Guarino R. D. Perez D. M, Piascik M. T (1996). Recent advances in the molecular pharmacology of the $\alpha_1$-adrenergic receptors. *Cell Signalling* **8(5)**: 323-333.

Guimaraes S, Moura D (2001). Vascular Adrenoceptor: An Update. *Pharmacological Reviews* **53**: 319-356.

Guo T-Z, Davies F. M, Kingery W. S, Patterson A. J, Limbird L. E, Maze M (1999). Nitrous oxide produces antinociceptive response via $\alpha_{2B}$ and/or $\alpha_{2C}$ adrenoceptor subtypes in mice. *Anesthesiology* **90 (2)**: 470-476.

Gupta R. C, Neumann J, Watanabe A. M, Sabbah H. N (1998). Muscarinic-cholinoceptor mediated attenuation of phosphlamban phosphorylation induced by inhibition of phosphodiesterase in ventricular cardiomyocytes: Evidence against a cAMP-dependent effect. *Molecular and Cellular Biochemistry* **187**: 155-161.

Guthrie R. M, Siegel R. L. (1999). A multicenter, community-based study of doxazosin in the treatment of concomitant hypertension and symptomatic benign prostatic hyperplasia: The hypertension and BHP intervention trial *Clinical Therapeutics* **21**: 1732-1748.

Gurdal H, Cai G, Johnson M. D (1995a). $\alpha_1$-Adrenoceptor responsiveness in the aging aorta. *European Journal of pharmacology* **272**: R5-R6.

Gurdal H, Tilakaratne N, Brown R. D, Fonseca M, Friedman E, Johnson M. D (1995b). The expression of $\alpha_1$-adrenoceptor subtypes changes with age in the rat aorta. *Journal of Pharmacology and Experimental Therapeutics* **275**: 1656-1662.

Guyenet P. G (1991). Central noradrenergic neurons: the autonomic connection. *Progressive Brain Research* **88**: 365-380.

Gyires K, Mullner K, Ronai A. Z (2000b). Functional evidence that gastroprotection can be induced by activation of central $\alpha_{2B}$-adrenoceptor subtypes in the rat. *European Journal of Pharmacology* **396**: 131-135.

Gyires K, Ronai A. Z, Mullner K, Furst S (2000c). Intracerebroventricular injection of clonidine releases B-endorphin to induce mucosal protection in the rat. *Neuropharmacology* **39**: 961-968.

Han C, Abel P. W, Minneman K. P (1987). $\alpha_1$-Adrenoceptor subtypes linked to different mechanisms of increasing intracellular $Ca_2^+$ in smooth muscle. *Nature* **329**: 333-335.

Harrison J. K, Dangelo D. D, Zeng D. W, Lynch K. R (1991). Pharmacological characterization of rat $\alpha_2$-adrenergic receptors. *Molecular Pharmacology* **40**: 407-412.

Haynes J. M, Hill S. J. (1996). $\alpha_1$-adrenceptor mediated responses of the cauda epididymis of guinea-pig. *British Journal of Pharmacology* **119**: 1203-1210.

Haynes J. M, Hill S. J. (1997). β-Adrenoceptor-mediated inhibition of $\alpha_1$-adrenoceptor-mediated and field stimulation-induced contractile responses in the prostate of the guinea pig. *British Journal of Pharmacology* **122**: 1067-1074.

Haynes J. M, Selbie L. A, Hill S. J (1999). Gi-protein α-subunit mRNA antisense oligonucleotide inhibition of Gi-coupled receptor contractile activity in the epididymis of the guinea-pig. *British Journal of Pharmacology* **127**: 85-90.

Heijnen C. J, van der Voort C. R, van de Pol M, Kavelaars A (2002). Cytokines regulate $\alpha_1$-adrenergic receptor mRNA expression in human monocytic cells and endothelial cells. *Journal of Neuroimmunology* **125**: 66-72.

Hein L., Altman J. D, Kobilka B. K (1999). Two functionally distinct $\alpha_2$-adrenergic receptors regulate sympathetic neurotransmission. *Nature* **402**: 181-184.

Hein L, Spindler M, Altman J. D, Kobilka B. K (1998). $\alpha_2$-adrenoceptor subtypes: distribution and function. *Pharmacology and Toxicology Reviews* **83**: 26-28.

Hein P, Goepel M, Cotecchia S, Michel M. C (2000). Comparison of [$^3$H] tamsulosin and [$^3$H] prazosin binding to wild-type and constitutively active $\alpha_{1B}$-adrenoceptors. *Life Sciences* **67**: 503-508.

Hicks P. E, Barras M, Herman G, Mauduit P, Armstrong J. M, Rossignol B (1991). $\alpha$-adrenoceptor subtypes in dog saphenous-vein that mediate contraction an inositol phosphate production. *British Journal of Pharmacology* **102**: 151-161.

Hieble J. P, Bylund D. B, Clarke D. E, Eikenberg D. C, Langer S. Z, Lefkowitz R. J, Minneman K. P (1995). International Union of Pharmacology X. Recommendation for nomenclature of $\alpha_1$-adrenoceptors: Consensus update. *Pharmacological Reviews* **47(2)**: 267-270.

Hieble J. P, Ruffolo R. R (1996). Subclassification and nomenclature of alpha 1- and alpha 2-adrenoceptors. *Progress in Drug Research* **47**: 81-130.

Hieble J. P (2000). Adrenoceptor subclassification: an approach to improved cardiovascular therapeutics. *Pharmaceutica Acta Helvetiae* **74**: 163-171

Ho S. L, Honner V, Docherty J. R (1998). Investigation of the subtypes of $\alpha_2$-adrenoceptor mediating prejunctional inhibition in rat atrium and cerebral cortex. *Naunyn-Schmeidelberg's Archive of Pharmacology* **357**: 634-639.

Holz G. G, Rane S. G, Dunlap K (1986). GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature* **319**: 670-372.

Hrometz S. L, Edelman S. E, McCune D. F, Olges J. R, Hadley R. W, Perez D. M, Piascik M. T (1999). Expression of multiple $\alpha_1$-adrenoceptors on vascular smooth muscle; correlation with the regulation of contractions. *Journal of Pharmacology and Experimental Therapeatics* **290**: 452-456

Hu Z. W, Shi X, Y, Hoffman B. B (1996). Insulin and insulin-like growth factor 1 differentially induce $\alpha_1$-adrenergic receptor subtype expression in rat vascular smooth muscle cell. *Journal of Clinical Investigation* **98**: 1826-1834.

Hunt R. D, Arnsten A. F. T, Asbell M. D (1995). An open trial of guanfacine in the treatment of attention-deficit hyperactivity disorder. *Journal of American Academy of Child and Adolescent Psychiatry* **34**: 50-54.

Hunter J. C, Fontana D. J, Hedley L. R, Jasper J. R, Lewis R, Link R. E, Secchi R, Sutton J, Eglen R. M (1997). Assessment of the role of the $\alpha_2$-adrenoceptor subtypes in the antinociceptive, sedative and hypothermic action of dexmedetomidine in transgenic mice. *British Journal of Pharmacology* **122**: 1339-1344.

Ibarra M, Terron J. A, Lopez-Guerrero J. J, Villalobos-Molina R (1997). Evidence for an age-dependent functional expression of $\alpha_{1D}$-adrenoceptor in the rat vasculature. *European Journal of Pharmacology* **322**: 221-224.

Ishikawa Y, Homey C. J (1997). The adenylyl cyclases as integrators of transmembrane signal transduction. *Circulation Research* **80**: 297-304.

Izzo J. L, Horwitz D, Keiser H. R (1981). Physiologic mechanisms opposing the haemodynamic-effects of prazosin. *Clinical Pharmacology & Therapeutics* **29**: 7-11.

Janero D. R, Burghadrdt C (1990). Production and release of platelet-activating factor by the injured heart-muscle cell (cardiomyocyte). *Research Communications in Chemical Pathology and Pharmacology* **67**: 201-218.

Janumpalli S, Butler L. S, MacMillan L. B, Limbird L. E, McNamara J. O (1998). A point mutation (D79N) of the $\alpha_{2A/D}$ adrenergic receptor abolished the antiepileptogenic action of endogenous norepinephrine. *Journal of Neuroscience* **18**: 2004-2008.

Javoy-Agid F, Ruberg M, Pique L, Bertagna X, Taquet H, Studler J. M, Cesselin F, Epelbaum J, Agid Y (1984). Biochemistry of the hypothalamus in Parkinson's disease. *Neurology* **34**: 672-675.

Jeyaraj S. C, Chotani M. A, Mitra S, Gregg H. E, Flavahan N. A, Morrison K. J (2001). Cooling evokes redistribution of $\alpha_{2C}$-adrenoceptors from golgi to plasma membrane in transfected human embryonic kidney 293 cells. *Molecular Pharmacology* **60**: 1195-1200.

Johansson M, Elam M, Rundqvist B, Eisnehofer G, Herlitz H, Jensen G, Frisberg P (2000). Differentiated response of the sympathetic nervous system to angiotensin-converting enzyme inhibition in hypertension. *Hypertension* **36**: 543-548.

Kable J. W, Murrin L. C, Bylund D. B (2000). In vivo gene modification elucidates subtype-selective functions of $\alpha_2$-adrenergic receptors. *Journal of Pharmacology and Experimental Therapeutics* **293**: 1-7.

Kenny B. A, Chalmers D. H, Philpott P. C, Naylor A. M (1995). Characterization of an $\alpha_{1D}$-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. *British Journal of Pharmacology* **115**: 981-986.

Kintsurashvili E, Gavras I, Johns C, Gavras H (2001). Effects of antisense oligodeoynucleotide targeting of the $\alpha_2$-adrenergic receptor messenger RNA in the central nervous system. *Hypertension* **38**:1075-1080.

Knepper S, Buckner S. A, Brune M. A, Deernardis J. F, Meyer M. D, Hancock A. A (1995). A-61603, a potent $\alpha_1$-adrenergic receptor agonist, selective for the $\alpha_{1A}$ receptor subtype. *Journal of Pharmacology and Experimental Therapeutics* **274**: 97-103.

Knowlton K. U, Baracchini E, Chien K. R, Harris A. N, Henderson S. A, Evans S. M, Glembotski C. C (1991). Co-regulation of the atrial natriuretic factor and cardiac myosin light chain-2 genes during α-adrenergic stimulation of neonatal rat ventricular cells. *Journal of Biological Chemistry* **266**: 7759-7768.

Knowlton K. U, Michel M. C, Itani M, Shubeita H. E, Ishihara K, Brown J. H, Chien K. R (1993). The $\alpha_{1A}$-adrenergic receptor subtype mediates biochemical, molecular and morphologic features of cultured myocardial cell hypertrophy. *Journal of Biological Chemistry* **268**: 15374-15380.

Kunos G and Ishac E. J. N (1987). Mechanism of inverse regulation of $\alpha_1$-adrenergic and β-adrenergic receptors. *Biochemical Pharmacology* **36**: 1185-1191.

Lakhlani P. P, MacMillan L. B, Guo T. Z, McCool B. A, Lovinger D. M, Maze M, Limbird L. E (1997). Substitution of a mutant $\alpha_{2A/D}$-adrenergic receptor via "hit and run" gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo. *Proc Natl Acad Sci USA* **94**: 9950-9955.

Lachnit W. G, Clarke D. E, Ford A. P. D. W (1995). Pharmacological studies with A-61603 and RS 107053 expose a putative $\alpha_{1A}$-adrenoceptor in caudal artery of rat. *British Journal of Pharmacology* **116**: 300P.

Lachnit W. G, Ford A. P. D. W, Clarke D. E (1996). SDZ NVI085, an $\alpha_{1A}$-adrenoceptor agonist with $5HT_2$ receptor antagonist properties. *European Journal of Pharmacology* **297**: 83-86.

Lachnit W. G, Tran A. M, Clarke D. E, Ford A. P. D. W (1997). Pharmacological characterization of an $\alpha_{1A}$-adrenoceptor mediating contractile responses to

noradrenaline in isolated caudal artery of rat. *British Journal of Pharmacology* **120**: 819-826.

Lawton W. J, Fitz A. E, Anderson E. A, Sinkey C. A, Coleman R. A (1990). Effect of dietary potassium on blood pressure, renal-function, muscle sympathetic-nerve activity, and forearm vascular-resistance and flow in normotensive, and borderline hypertensive humans. *Circulation* **81**: 173-184.

Lei D, Zhang Y, Han C (2002). Changes in mRNA expression induced by sustained noradrenaline stimulation are different for $\alpha_1$-adrenoceptor subtypes in HEK293 cells. *Clinical and Experimental Pharmacology and Physiology* **29**: 1084-1090.

Link R, Daunt D. A, Barsh G. S, Chruscinski A, Kobilka B. K (1992). Cloning of two mouse genes encoding $\alpha_2$-adrenergic receptor subtypes and identification of a single amino acid in the mouse $\alpha_2$-C10 homolog responsible for an interspecies variation in antagonist binding. *Molecular Pharmacology* **42**: 16-27.

Link R. E, Stevens M. S, Kulatunga M, Scheinin M, Barsh G. S, Kobilka B. K (1995). Targeted inactivation of the gene encoding the mouse $\alpha_2$-adrenoceptor homolog. *Molecular Pharmacology* **48**: 48-55.

Link R, Desai K, Hein L, Stevens M. E, Chruscinski A, Bernstein D, Barsh G. S, Kobilka B. K (1996). Cardiovascular regulation in mice lacking $\alpha_2$-adrenergic receptor subtypes b and c. *Science* **273**: 803-805.

Little R. A, Kirkman E (1997). Cardiovascular control after injury. In: Cooper G. J, Dudley H. A. F, Gann D. S, Little R. A, Maynard R. L, eds *Scientific Foundation of Trauma*. Oxford, UK: Butterworth, Heineman: 551-563.

Lomasney J. W, Lorenz W, Allen L. F, King K, Regan J. W, Yang-Feng T. L, Caron M. G, Lefkowitz R. J (1990). Expansion of the $\alpha_2$-adrenergic receptor family: cloning and

expression of a human $\alpha_2$-adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proceedings of the National Academy of Science* **87**: 5094-5098.

Luckhoff A, Pohl W, Mulsch A, Busse R (1988). Differential role of extra-and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *British Journal of Pharmacology* **95**: 189-196.

Lundberg M. S, Crow M. T (1999). Age-related changes in the signalling and function of vascular smooth muscle cells. *Experimental Gerontology* **34**: 549-557.

MacKenzie J. F, Daly C. J, Pediani J. D, McGrath J. C (2000). Quantitative imaging in live human cells reveals intracellular $\alpha_1$-adrenoceptor ligand-binding sites. *The Journal of Pharmacology and Experimental Therapeutics* **294**: 434-443.

MacDonald E and Scheinin M (1995). Distribution and pharmacology of $\alpha_2$-adrenoceptors in the central nervous-system. *Journal of Physiology and Pharmacology* **46**: 241-258.

MacMillan L. B, Hein L, Smith M. S, Piascik M. T, Limbird L. E (1996). Central hypotensive effects of the $\alpha_{2A/D}$-adrenergic receptor subtype. *Science* **273**: 801-803.

Makaritsis K. P, Handy D. E, Johns C, Kobilka B, Gavras I, Gavras H (1999). Role of the $\alpha_{2B}$-adrenergic receptor in the development of salt-induced hypertension. *Hypertension* **33**: 14-17.

Mark S. D, Wang W, Fraumeni J. F, Li J. Y, Taylor P. R, Wang G. Q, Guo W, Dawsey S. M, Li B, Blot W. J (1996). Lowered risks of hypertension and cerebrovascular disease after vitamin mineral supplementation- The Linxian nutrition intervention trial. *American Journal of Epidemiology* **143**: 658-664.

Marriot J. F, Marshall J. M. (1989). Comparisons between the effects of hypoxia upon noradrenaline-induced contractions of arteries from the rat and rabbit. *British Journal of Pharmacology* **96**: 821P.

Marriott J. F (1987). An investigation of the actions of diltiazem on rat aorta exposed to acute hypoxia followed by re-oxygenation. *British Journal of Pharmacology* **92**: 451-456.

Marriott J. F (1989). The effects of verapamil upon noradrenaline-induced contraction of the rat isolate aorta following acute and prolonged alterations in $PO_2$. *British Journal of Pharmacology* **98**: 1101-1108.

Marshall I, Burt R, P, Chapple C. R (1995). Noradrenaline contractions of human prostate mediated by $\alpha_{1A}$-($\alpha_{1C}$) subtype. *British Journal of Pharmacology* **115**: 781-786.

Medgett I. C & Langer S. Z (1984). Heterogeneity of smooth muscle alpha adrenoceptors in rat tail artery *in vitro*. *Journal of Pharmacology and Experimental Therapeutics* **229**: 823-830.

Megdett I. C, Hicks P. E, Langer S. Z (1984). Smooth muscle $\alpha_2$-adrenoceptors mediate vasoconstrictor responses to exogenous norepinephrine and to sympathetic stimulation to a greater extent in spontaneously hypertensive than in Wistar-Kyoto rat tail arteries. *Journal of Pharmacology and Experimental Therapeutics* **231**: 159-165.

Medgett I C and Rajanayagam M. A. S (1984). Effect of reduced calcium ion concentration and of diltiazem on vasoconstrictor responses to noradrenaline and sympathetic nerve stimulation in rat isolated tail artery. *British Journal of Pharmacology* **83**: 889-898.

Melchiorre C, Angeli P, Bolognesi M. L, Chiarini A, Giardina D, Gulini U, Leonardi A, Marucci G, Minarini A, Pigini M, Quaglia W, Rosini M, Tumiatti V (2000). $\alpha_1$-Adrenceptor antagonists bearing a quinazoline or a benzodioxane moiety. *Pharmaceutica Acta Helvetiae* **74**: 181-190.

McCafferty G. P, Naselsky D. P, Hieble P. (1999). Characterization of postjunctional α-adrenoceptors in the pithed mouse. *General Pharmacology* **33**: 99-105.

McCune D. F, Edelmann S. E, Olges J. R, Post G. R, Waldrop B. A, Waugh D. J, Perez D. M, Piascik M. T. (2000). Regulation of the cellular localization and signalling properties of the $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor by agonist and inverse agonists. *Molecular Pharmacology* **57**: 659-666.

McDougall J. J (2001). Abrogation of α-adrenergic vasoactivity in chronically inflamed rat knee joints. *American Journal of Physiology Regulatory Integrative Comparative Physiology* **281**: R821-R827.

McGrath J. C (1982). Evidence for more than one type of post-junctional α-adrenoceptor. *Biochemical Pharmacology* **31**: 1277-1282

McPherson R. W, Koehler R. C, Traystman R. J (1994). Hypoxia, $\alpha_2$-adrenergic, and nitric oxide-dependent interactions on canine cerebral blood flow. *American Journal of Physiology – Heart Circulatory Physiology* **266**: H476-H482.

McWhinney C, Wenham D, Kanwai S, Kalman V, Hansen C, Robinshaw J. D (2000). Constitutively active mutant of the $\alpha_{1a}$- and $\alpha_{1b}$-adrenergic receptor subtypes reveal coupling to different signalling pathways and physiological responses in rat cardiac myocytes. *The Journal of Biological Chemistry* **275**: 2087-2097.

Michel M. C, Kerker J, Branchek T. A, Branchek T. A, Forray C (1993). Selective irreversible binding of chloroethyclonidine at alpha1-and alpha-2-adrenoceptor subtypes. *Molecular Pharmacology* **44**: 1165-1170.

Michelotti G. A, Bauman M. J, Smith M. P, Schwinn D. A [2003]. Cloning and characterization of the rat $\alpha_{1a}$-Adrenergic receptor gene promoter: demonstration of cell specificity and regulation by hypoxia. *The Journal of Biological Chemistry* **278 (10)**: 8693-8705.

Milano C. A, Dobler P. C, Rockman H. A, Bond R. A, Venable M. E, Allen L. F, Lefkowitz R. J (1994). Myocardial expression of a constitutively active alpha $\alpha_{1B}$-

adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proceedings of the National Academy of Science USA* **91**: 10109-10113.

Millan M. J, Dekeyne A, Newman-Tancredi A, Cussac D, Audinot V, Milligan G, Duqueyroix D, Girardon S, Mullot J, Boutin J. A, Nicolas J. P, Renouard-try A, Lacoste J. M, Cordi A (2000). S18616, a highly potent, spiroimidazoline agonist at $\alpha_2$-adrenoceptors: Receptor profile, antinociceptive and hypothermic actions in comparison with dexmedetomidine and clonidine. *Journal of Pharmacology and Experimental Therapeutics* **295**: 1192-1205.

Milligan G, Bond R. A, Lee M (1995). Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? *Trends in Pharmacological Science* **16**: 10-13.

Minneman . P (1988). $\alpha_1$-Adrenergic receptor subtypes, inositol phosphates and sources of cell $Ca^{2+}$. *Pharmacological Reviews* **40(2)**: 87-119.

Miriel V. A, Mauban J. R, Blaustein M. P, Wier W. G (1999). Local and cellular $Ca^{2+}$ transients in smooth muscle of pressurized rat resistance arteries during myogenic and agonist stimulation. *Journal of Physiology* **518**: 815-824.

Morrow A. L and Creese I (1986). Characterisation of $\alpha_1$-adrenergic receptor subtypes in rat brain: a reevaluation of [$^3$H] WB4101 and [$^3$H] Prazosin. *Molecular Pharmacology* **29**: 321-330.

Mullner K, Ronai A. Z, Fulop K, Furst S, Gyires K (2002). Involvement of central $K_{ATP}$ channels in the gastric antisecretory action of $\alpha_2$-adrenoceptor agonists and $\beta$-endorphin in rats. *European Journal of Pharmacology* **435**: 225-229.

Mulvany M. J (1992). The development and regression of vascular hypertrophy. *Journal of Cardiovascular Pharmacology* **19**: S22-S27.

Nase G. P and Boegehold M. A (1998). Postjunctional $\alpha_2$-adrenoceptors are not present in proximal arterioles of rat intestine. *American Journal of Physiology-Heart and Circulatory Physiology* ***274***: H202-H208.

North R. A, Williams J. T, Surprenant A, Christie M. J (1987). μ- and δ-receptors belong to a family of receptors that are coupled to potassium channels. *Proceedings of the National Academy of Science* **84**: 5487-5491.

Nunes J. P, Guimaraes S (1993). Chloroethylclonidine irreversibly activates postjunctional alpha$_2$-adrenoceptors in the dog saphenous vein. *Naunyn-Schmiedebergs Archive of Pharmacology* **348**: 264-268.

Oparil S, Chen Y. F, Meng Q. C, Yang R. H, Jin H, Wyss J. M (1988). The neural basis of salt sensitivity in the rat: altered hypothalamic function. *American Journal of Medical Science* **295**: 360-369.

O'Rourke M, Kearns S, Docherty J. R (1995). Investigation of the actions of chloroethylclonidine in rat aorta. *British Journal of Pharmacology* **115**: 1399-1406.

Parkinson N. A and Hughes A. D (1995). The mechanism of action of $\alpha_2$-adrenoceptors in human isolated subcutaneous resistance arteries. *British Journal of Pharmacology* **115**: 1463-1468.

Parkinson N. A, Thom S. M, Hughes A. D, Sever P. S, Mulvany M. J, Neilsen H (1992). Neurally evoked responses of human isolated resistance arteries are mediated by both $\alpha_1$- and $\alpha_2$-adrenoceptors. *British Journal of Pharmacology* **106**: 568-573.

Pediani J. D, MacKenzie J. F, Heeley R. P, Daly C. J, McGrath J. C (2000). Single-cell recombinant pharmacology: Bovine $\alpha_{1a}$-adrenoceptors in rat-1 fibroblasts release intracellular $Ca^{2+}$, display subtype-characteristic antagonism and antagonism, and exhibit an antagonist-reversible inverse concentration-response phase. *The Journal of Pharmacology and Experimental Therapeutics* **293**: 887-895.

Peng H, Matchkov V, Ivarsen A, Aalkjaer C, Nilsson H. (2001). Hypothesis for the initiation of vasomotion. *Circulation Research* **88**: 810-815.

Peng H, Mathckov V, Ivarsen A, Aalkjaer C, Nilsson H (2001). Hypothesis for the initiation of vasomotion. *Circulation Research* **88**: 810-815.

Perez D. M, DeYoung M. B, Graham R. M (1993). Coupling of expressed $\alpha_{1B}$- and $\alpha_{1D}$-adrenergic receptors to multiple signalling pathways is both G protein and cell type specific. *Molecular Pharmacology* **44**: 784-795.

Pettinger W. A (1987). Renal alpha$_2$-adrenergic receptors and hypertension. *Hypertension* **9**: 3-6.

Piascik M. T, Guarino R. D, Smith M. S, Soltis E. E, Perez D. M (1995). The specific contribution of the novel $\alpha_{1D}$-adrenoceptor to the contraction of vascular smooth muscle. *Journal of Pharmacology and experimental Therapeutics* **275**: 1583-1589.

Piascik M. T, Hrometz S. L, Edelmann S. E, Guarino R. D, Hadley R. W, Brown R. D (1997). Immunocytochemical localization of the $\alpha_{1b}$-adrenergic receptor and the contribution of this and other subtypes on vascular smooth muscle contraction: analysis with selective ligands and antisense oligonucleotides. *Journal of Pharmacology and Experimental Therapeutics* **283**: 854-868.

Piascik M. T, Perez D. M (2001). $\alpha_1$-Adrenergic Receptors: New insights and directions. *The Journal of Pharmacology and Experimental Therapeutics* **298**: 403-410.

Picciotto M. R, Wickman K. (1998). Using knockout and transgenic mice to study neurophysiology and behaviour. *Physiological Reviews* **78**: 1131-1163.

Planitz V (1986). Intraindividual comparison of moxonidine and prazosin in hypertensive patients. *European Journal of Clinical Pharmacology* **29**: 645-650.

Pozzoli C, Todorov S, Schunack W, Timmerman H, Coruzzi G, Poli E (2002). Role of histamine $H_3$ receptors in control of mouse intestinal motility *in vivo* and *in vitro*: Comparison with $\alpha_2$-Adrenoceptors. *Digestive Diseases and Sciences* **47 (5)**: 1065-1072.

Preston A, Haynes J. M (2003). $\alpha_1$-Adrenoceptor effects mediated by protein kinase C $\alpha$ in human cultured prostatic stromal cells. *British Journal of Pharmacology* **138**: 218-224.

Price D. T, Lefkowitz R. J, Caron M. G, Berkowitz D, Schwinn D. A (1994b). Localisation of mRNA for three distinct $\alpha_1$-adrenergic receptor subtypes in human tissues: implications for human adrenergic physiology. *Molecular Pharmacology* **45**: 171-175.

Queiroz D. B. C, Mendes F. R, Porto C. S, Avellar M. C. W. (2002). $\alpha_1$-Adrenoceptor subtypes in rat epididymis and the effect of sexual maturation. *Biology of Reproduction* **66**: 508-515.

Rand R. P, Burton A. C, Ing T (1965). The tail of the rat, in temperature regulation and acclimatization. *Canadian Journal of Physiology and Pharmacology* **43**: 257-267.

Rang H. P, Dale M. M, Ritter J. M (1995) Pharmacology (3rd Edition). Churchill Livingstone.

Ratnasooriya W. D. Wadsworth R. M. (1990). Impairment of fertility of male rats with prazosin. *Contraception* **41**: 441-447.

Redfern W. S, MacLean M R, Clague R. U, McGrath J. C (1995). The role of $\alpha_2$-adrenoceptors in the vasculature of the rat tail. *British Journal of Pharmacology* **114**: 1724-1730.

Robert L (1999). Ageing of the vascular-wall and atherosclerosis. *Experimental Gerontology* **34**: 491-501.

Rodriguez-Martinez M. A, Garcia-Cohen E. C, Briones A, Baena A. B, Marin E, Salaices M, Marin J (1999). Changes in plasma oxidative state with age and their influence on contractions elicited by noradrenaline in the rat tail artery. *Life Sciences* **56**: 915-924.

Rokosh D. G and Simpson D. (2002). Knockout of the $\alpha_{1A/C}$-adrenergic receptor subytpe: The $\alpha_{1A/C}$ is expressed in resistance arteries and is required to maintain arterial blood pressure. *PNAS* **99 (14)**: 9474-9479.

Rokosh D. G, Stewart A. F. R, Chang K. C, Bailey B. A, Karliner J. S, Camacho S. A, Long C. S, Simpson P. C (1996). $\alpha_1$-adrenergic receptor subtype mRNAs are differentially regulated by $\alpha_1$-adrenergic and other hypertrophic stimuli in cardiac myocytes in culture and in vivo-Repression of $\alpha_{1B}$ and $\alpha_{1D}$ but induction of $\alpha_{1C}$. *Journal of Biological Chemistry* **271**: 5839-5843.

Rosin D. L, Zeng D, Stornetta R. L, Norton F. R, Riley T, Okusa M. D, Guyenet P. G, Lynch K. R (1993). Immunohistochemical localization of alpha $\alpha_{2A/D}$-adrenergic receptors in catecholaminergic and other brainstem neurons in the rat. *Neuroscience* **56**: 139-155.

Rossier O, Abuin L, Fanelli F, Leonardi A, Cotecchia S. (1999). Inverse agonism and neutral antagonism at $\alpha_{1a}$- and $\alpha_{1b}$-adrenoceptors subtypes. *Molecular Pharmacology* **56**: 858-866.

Rudner X. L, Berkowitz D. E, Booth J. V, Funk B. L, Cozart K. L, D'Amico E. B, El-Moalem D, Page S. O, Richardson C. D, Winters B, Marucci L, Schwinn D. A (1999). Subtype specific regulation of human vascular $\alpha_1$-adrenergic receptor by vessel bed and age. *Circulation* **100**: 2336-2343.

Ruchlmann D. O, Lee C. H, Proburko D, van Breeman C (2000). Asynchronous $Ca^{2+}$ waves in intact venous smooth muscle. *Circulation Research* **86**: e72-e79.

Ruffolo R. R, Nichols A. J, Stadel J. M, Hieble J. P (1993). Pharmacologic and therapeutic applications of $\alpha_2$-adrencoeptor subtypes. *Annual Review of Pharmacology & Toxicology* **32**: 243-279.

Sallinen J, Kink R. E, Haapalinna A, Viitamaa T, Kulatunga M, Sjoholm B, MacDonald E, Pelto-Huikko M, Leino T, Barsh G. S, Kobilka B. K, Scheinn M (1997). Genetic alteration of the $\alpha_{2C}$-adrenoceptor expression in mice: Influence on locomotor, hypothermic, and neurochemical effect of dexmedetomidine, a subtype-non-selective $\alpha_2$-adrenoceptor agonist. *Molecular Pharmacology* **51**: 36-46.

Sallinen J, Haapalinna A, Viitamaa T, Kobilka B. K, Scheinin M (1998). Adrenergic $\alpha_{2C}$-receptors modulate the acoustic startle reflex, prepulse inhibition, and aggression in mice. *Journal of Neuroscience* **18**: 3035-3042.

Sallinen J, Haapalinna A, MacDonald E, Viitamaa T, Lahdesmaki J, Rybnikova E, Pelto-Huikko M, Kobilka B. K, Scheinin M (1999). Genetic alteration of the alpha-2 adrenoceptor subtype C in mice affects the development of behavioural despair and stress-induced increases in plasma corticosterone levels. *Molecular Pharmacology* **4**: 443-452.

Sanchez A, Vidal M. J, Martinezsierra R, Saiz J (1986). Ontogeny of renal $\alpha_1$ and $\alpha_2$ adrenoceptors in the spontaneously hypertensive rat. *Journal of Pharmacology and Experimental Therapeutics* **237**: 972-979.

Sanderson K. J, Van Rij A. M, Wade C. R, Sutherland W. H (1995). Lipid-peroxidation of circulating low-density lipoproteins with age, smoking and in peripheral vascular-disease. *Atherosclerosis* **118**: 45-51.

Saussy D. L, Goetz A. S, King H. K, Truc T. A (1994). BMY7378 is a selective antagonist of $\alpha_{1D}$-adrenoceptors. Further evidence that vascular $\alpha_1$-adrenoceptors are of the $\alpha_{1D}$-subtype. *Canadian Journal of Physiology and Pharmacology* **72** (Supplement 1) 323.

Sawamura S, Kingery W. S, Davies M. F, Agashe G. S, Clark J. D, Kobilka B. K, Hashimoto T, Maze M (2000). Antinociceptive action of nitrous oxide is mediated by stimulation of noradrenergic neurons in the brainstem and activation of $\alpha_{2B}$ adrenoceptors. *Journal of Neuroscience* **20**: 9242-9251.

Scheibner J, Trendelenburg A. U, Hein L, Starke K, Blandizzi C (2002). $\alpha_2$-adrenoceptors in the enteric nervous system: a study in $\alpha_{2A/D}$-adrenoceptor deficient mice. *British Journal of Pharmacology* **135**: 697-704.

Schelb V, Gobel I, Khairallah L, Zhou H, Cox S. L, Trendelenburg A. U, Hein L, Starke K (2001). Postnatal development of presynaptic receptors that modulate noradrenaline release in mice. *Naunyn Schmiedebergs Archive of Pharmacology* **364**: 359-371.

Shimokawa Shimokawa,H., Yasutake,H., Fujii,K (1996) The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *Journal of Cardiovascular Pharmacology* **28**: 703-711.

Shyrock J. C, Ozeck M. J, Belardinelli L (1998). Inverse agonists and neutral antagonists of recombinant human $A_1$ adenosine receptors stably expressed in Chinese hamster ovary cells. *Molecular Pharmacology* **53**: 886-893.

Smith J. D and Breslow J. L (1997). The emergence of mouse models of atherosclerosis and their relevance to clinical research. *Journal of Internal Medicine* **242**: 99-109.

Smith K. M, MacMillan J. B, McGrath J. C (1997). Investigation of $\alpha_1$-adrenoceptor subtypes mediating vasoconstriction in rabbit cutaneous resistance arteries. *British Journal of Pharmacology* **122**: 825-832.

Starke K, Endo T, Taube H. D (1975). Pre- and postsynaptic components in effect of drugs with $\alpha$-adrenoceptor affinity. *Nature* **254**: 440-441.

Starke K (2001). Presynaptic autoreceptors in the third decade: focus on $\alpha_2$-adrenoceptors. *Journal of Neurochemistry* **78**: 673-675.

Starke K, Gothert M, Kilbinger H (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiological Reviews* **69**: 864-989.

Starke K, Trendelenburg A. U, Limberger M (1995). Presynaptic $\alpha_2$-adrenoceptors: subtype determination. *Pharmacological Communication* **6**: 99-108.

Sugawara T, Hirasawa A, Hashimoto K, Tsujimoto G. (2002). Differences in the subcellular localization of $\alpha_1$-adrenoceptor subtypes can affect the subtype selectivity of drugs in a study the fluorescent ligand BOPIDY FL-prazosin. *Life Sciences* **70**: 2113-2124.

Sullivan A. F, Dashwood M.R, Dickenson A. H (1987), $\alpha_{2C}$-Adrenoceptor modulation of nociception in rat spinal cord: location, effects and interaction with morphine. *European Journal of Pharmacology* **138**: 169-177.

Sulpizio A, Hieble J. P (1987). Demonstration of $\alpha_2$-adrenoceptor-mediated contraction in the isolated canine saphenous artery treated with BAY K 8644. *European Journal of Pharmacology* **135**: 107-110.

Summers R. J, and McMartin L. R (1993). Adrenoceptors and their second messenger systems. *Journal of Neurochemistry* **60** (1): 10-23.

Suprenant A, Horstmas D. A, Akbaraali H, Limbird L. E (1992). A point mutation of the $\alpha_2$-adrenoceptor that blocks coupling to potassium but not calcium currents. *Science* **257**: 977-980.

Sward K, Dreja K, Lindqvist A, Persson E, Hellstrand P. (2002). Influence of mitochondrial inhibition on global and local $[Ca^{2+}]i$ in rat tail artery. *Circulation Research* **90**: 792-799.

Tanilla H. K, Mustonen K, Sallinen J, Scheinin M, Riekkinen P (1999). Role of the $\alpha_{2C}$-adrenoceptor subtype in spatial working memory as revealed by mice with targeted disruption of the $\alpha_{2C}$-adrenoceptor gene. *European Journal of Neuroscience* **11**: 599-603.

Tavares A, Handy D. E, Bogdanova N. N, Rosene D. L, Gavras H (1996). Localization of $\alpha_{2A/D}$ and $\alpha_{2B}$-adrenergic receptor subtypes in brain. *Hypertension* **27**: 449-455.

Tazi-Saad K, Chariot J, Tacca M. D, Roze C (1992). Effects of $\alpha_2$-adrenoceptor agonists on gastric pepsin and acid secretion in the rat. *British Journal of Pharmacology* **106**: 790-796.

Thompson S, Clarke A. R, Pow A. M, Hooper M. L, Melton D. W. (1989). Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell* **56**: 313-321.

Thorington R. W (1966). The biology of rodent tails: A study of form and function. Report of project **832**, *Document number AAL-TR-65-8 from Arctic Aeromedical Laboratory*, Fort Wainwright, Alaska.

Trendelenburg A. U, Sutej I, Wahy C. A, Molderings G. J, Rump L. C, Starke K (1997). A re-investigation of questionable subclassifications of presynaptic $\alpha_2$-autoreceptors: rat vena cava, rat atria, human kidney and guniea-pig urethra. *Naunyn-Schmiedeberg's Archive of Pharmacology* **356**: 721-737.

Trendelenburg A. U, Starke K, Limberger N (1994). Presynaptic $\alpha_{2A/D}$-adrenoceptors inhibit the release of endogenous dopamine in rabbit caudate nucleus slices. *Naunyn-Schmiedebergs Archive of Pharmacolgy* **350**: 473-481.

Tsai B. S, Lefkowitz J (1978). Agonist-specific effects of monovalent and divalent cations on adenylate cyclase-coupled alpha-adrenergic receptors in rabbit platelets. *Molecular Pharmacology* **14**: 540-548.

Tsai H, Buchholz J, Duckles S. P (1993). Postjunctional $\alpha_2$-adrenoceptors in blood vessels: effect of age. *European Journal of Pharmcology* **237**: 311-316.

Ungvari Z, Csiszar A, Edwards J. G, Kaminski P. M, Wolin M. S, Kaley G, Koller A (2003). Increased superoxide production in coronary arteries in hyperhomocysteinemia. *Vascular Biology* **23**: 418.

Usui J, Fujiwara M, Tsukahara T, Taniguchi T, Kurahashi L (1985). Differences in contractile responses to electrical stimulation and α-adrenergic binding sites in isolated cerebral arteries in humans, cows, dogs and monkeys. *Journal of Cardiovascular Pharmacology* **7(Supplement 3):** S47-S52.

Valet P, Grujic D, Wade J, Ito M, Zingaretti M. C, Soloveva V, Ross S. R, Graves R. A, Cinti S, Lafontan M, Lowell B. B (2000). Expression of human $\alpha_{2C}$-adrenergic receptors in adipose tissue of $\beta_3$-adrenergic receptor-deficient mice promotes diet-induced obesity. *Journal of Biological Chemistry* **275**: 34797-34802.

Vanhoutte P. M, Cooke J. P, Lindblad L. E, Shepherd J. T, Flavahan N. A (1985). Modulation of postjunctional α-adrenergic responsiveness by local changes in temperature. *Clinical Science* **68** (Suppl. 10): S121-S123.

Vanhoutte P. M and Flavahan N. A (1986). Effects of temperature on α-adrenoceptors in limb veins – role of receptor reserve. *Federation Proceedings* **45**: 2347-2354.

Van der Graff P. H, Shankley N. P, Black J. W (1996b). Analysis of the effect of $\alpha_1$-adrenoceptro antagonists on noradrenaline-mediated contraction of rat small mesenteric artery. *British Journal of Pharmacology* **118**: 531-536.

Van der Lee R, Pfaffendorf M, De Mey J. G. R, van Zwieten P. A (2000). Inhibitory effect of mibefradil on contractions induced by sympathetic neurotransmitter release in the rat tail artery. *Naunyn-Schmiedeberg's Archive of Pharmacology* **361**: 74-79.

Van Zwieten P. A, Peters S. L. M (1999). Central $I_2$-imidazoline receptors as targets of centrally acting antihypertensive drugs -- Clinical pharmacology of moxonidine and rilmenidine. *Annals of the New York Academy of Science* **881**: 420-429.

Vandeputte C, Docherty J. R (2001). Modulation of contraction by $\alpha_{2A/D}$-adrenoceptor in mouse aorta: evidence employing knockout technology. *British Journal of Pharmacology* **00**: 0.

Vandeputte C & Docherty J. R (2001). Modulation of contraction by $\alpha_{2A/D}$-adrenoceptors in mouse aorta: evidence employing knockout technology. *British Journal of Pharmacology* **00**: 000-000.

Vanhoutte P. M and Luscher T. F (1986). Serotonin and the blood-vessel wall. *Journal of Hypertension* **4**: S29-S35.

Villalobos-Molina R, Ibarra M (1996). $\alpha_1$-Adrenoceptors mediating contraction in arteries of normotensive and spontaneously hypertensive rats are the $\alpha_{1D}$ or $\alpha_{1A}$ subtypes. *European Journal of Pharmacology* **298**: 257-263.

Villalobos-Molina R, Lopez-Guerrero J. J, Ibarra M (1997). $\alpha_{1D}$- and $\alpha_{1A}$-Adrenoceptors mediate contraction in rat renal artery. *European Journal of Pharmacology* **322**: 225-227.

Volgin D. V, Mackiewicz M, Kubin L (2001). $\alpha_{1B}$ receptors are the main postsynaptic mediators of adrenergic excitation in brainstem motoneurons, a single-cell RT-PCR study. *Journal of Chemical Neuroanatomy* **22**: 157-166.

Wada T, Hasegawa Y, Ono H (1997). Characterization of $\alpha_1$-adrenoceptor subtypes in facilitation of rat spinal motoneuron activity. *European Journal of Pharmacology* **340**: 45-52.

Walden P. D, Gerardi C, Lepor H (1999). Localization and expression of the alpha$_{1A}$, alpha $_{1B}$ and alpha$_{1D}$-adrenoceptors in hyperplastic and non-hyperplastic human prostate. *Journal of Urology* **161**: 635-640.

Wood C. L, Arnett C. D, Clarke W. R, Tsai B. S, Lefkowitz R. J (1979). Subclassification of α-adrenergic receptors by direct binding studies. *Biochemical Pharmacology* **28**: 1277-1282.

Wu D, Katz A, Lee C. H, Simon M. I (1992). Activation of phospholipase C by $\alpha_1$-adrenergic receptors is mediated by the alpha subunits of Gq family. *Journal of Biological Chemistry* **267**: 25798-25802.

Xia R. P, Tomhaye E. D, Wang D. J, Boluyl M. O, Jinadl S, Gupta R. S, Dietrich H, Wick G (1996). Regression of arteriselerotic lesions induced by immunization with heat shock protein 65-containing material in normocholesterolemic, but not hypercholesterolemic, rabbits. *Atherosclerosis* **123**: 145-155.

Xiao X. H, Rand M. J (1989) $\alpha_2$-Adrenoceptor agonists enhance vasoconstrictor responses to $\alpha_1$-adrenergic agonists in the rat tail artery by increasing the influx of $Ca^{2+}$. *British Journal of Pharmacology* **98**: 1032-1038.

Xin X, Nengyu Y, Eckhart A. D, Faber J. E (1997). $\alpha_{1D}$-adrenergic receptors and mitogen-activated protein kinase mediate increased proteins synthesis by arterial smooth muscle. *Molecular Pharmacology* **51**: 764-775.

Yu Y & Koss M. C (2002). $\alpha_{1A}$-Adrenoceptors mediate sympathetically evoked pupillary dilation in rats. *Journal of Pharmacology and Experimental Therapeutics* **300**: 521-525.

Zandberg P, De Jong W, De Wied D (1979). Effect of catecholamine-receptor stimulating agents on blood pressure after local application in the nucleus tractus solitarii of the medulla oblongata. *European Journal of Pharmacology* **55**: 43-56.

Zhang L, Takanobu T, Tanaka T, Shinozuka K, Kunitomo M, Nishiyama M, Kamata K, Muramatsu I. (2002). Alpha-1 adrenoceptor up-regulation induced by prazosin but not KMD3213 or reserpine in rats. *British Journal of Pharmacology* **135**: 1757-1764.

Zheng W. P, Lei L. P, Lalchandani S, Sun G. P, Feller D. R, Miller D. D (2000). Yohimbine dimmers exhibiting binding selectivities for human $\alpha_{2a}$- versus $\alpha_{2b}$-adrenergic receptor. *Bioorganic & Medicinal Chemistry Letters* **10**: 627-630.

Zhu J, Taniguchi T, Takauji R, Suzuki R, Tanaka T, Muramatsu I (2000). Inverse agonism and neutral antagonism at a constitutively active alpha-1a adrenoceptor. *British Journal of Pharmacology* **131**: 546-552.

Zuscik M, Sands S, Ross S. A, Waugh D. J. J, Gaivin R. J, Morilak D, Perez D. M (2000). Overexpression of the $\alpha_{1B}$-adrenergic receptor causes apoptotic neurodegeneration: Multiple system atrophy. *Nature Medicine* **6**: 1388-1394.

Zuscik M, Chaolthorn D, Hellard D, Deighan C, McGee A, Daly C. J, Waugh D. J. J, Ross S. A, Gaivin R. J, Morehead A. J, Thomas J. D, Plow E. F, McGrath J. C, Piascik M. T, Perez D. M (2001). Hypertension, autonomic failure, and cardiac hypetrophy in transgenic mice overexpressing the $\alpha_{1B}$-adrenergic receptor. *The Journal of Biological Chemistry* **276**: 13738-13743.