A Study of Murine Vascular α_1-Adrenoceptors: A Functional Knockout Approach.

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Abstract

Introduction

Currently, traditional pharmacological techniques are being increasingly complimented by applying parallel transgenic approaches employing the use of functional knock-out, over-expressed and site-directed mutagenesis models of individual proteins in order to determine their functional role or distribution within cells and tissues.

Recent advances have provided individual functional knockout models of the three α_1-AR subtypes (α_{1A}-KO, α_{1B}-KO and α_{1D}-KO). All three have been implicated as having a role in the maintenance of blood pressure, as a result of studies of the phenotypes of the individual KO models. However, of the three α_1-ARs, only the α_{1D} and α_{1B}-AR have been reported to be involved in vasoconstriction of the mouse aorta. As such, the α_{1D}-AR is reported to be the major adrenergic vasoconstrictor with a minor role for α_{1B}-ARs.

The data presented in this thesis is the result of a detailed pharmacological study, aided by the use of transgenic mice of the α_1-ARs involved in the functional responses of the mouse aorta.

Chapter 1. Contractile responses of the mouse aorta: Effect of age and α_1-AR knockout

In Chapter 1 the functional alterations of contractile (KCl: single challenge; adrenergic and serotonergic responses done by cumulative concentration response curves [CCRCs]) and relaxant responses (acetylcholine-induced: single challenge) as a result of increasing age were tested by studying adult male mice at two age points: 4-month-old mice and 16-month-old mice.

As a whole, no significant alterations in function as a result of increased age were observed in the strains studied.

Since both α_{1B}-KO and α_{1D}-KO possibly had different genetic backgrounds it was essential to ensure that varied genetic background did not have a significant effect on functional responses. The control strains for each KO, α_{1B}-WT (wild-type) and α_{1D}-WT were compared and no differences were observed at either age point. Therefore for further experiments either strain of WT mice were used as control mice.

The α_{1B}-KO was not significantly different from its WT at either age point, but this was complicated by the apparent compensation by α_{1D}-ARs. The α_{1D}-KO had 33-fold and 44-fold reduced potency and 19% and 23% reduced maximum for phenylephrine (PE) induced contractile responses compared to its WT at 4m and 16m age point respectively.

5-Hydroxytryptamine (5-HT) and 125mM KCl contractile responses and acetylcholine (ACh) induced relaxations were unaltered by age.

Thus, this data confirmed that the α_{1D}-AR was the adrenergic vasoconstrictor with little or no role for α_{1B}-ARs and the phenotypic differences the α_{1D}-KO exhibited were unaffected by age.

Chapter 2. Subtyping the α_1-AR mediated contractile responses in mouse aorta.

The adrenergic responses of the WT and α_{1B}-KO have previously been subtyped pharmacologically and the α_{1D}-AR has been indicated as the adrenergic vasoconstrictor with the α_{1B}-AR playing a minor role in the WT. The PE response of the α_{1D}-KO was subtyped in this chapter.

The pK_B values for prazosin (non-selective α_1-AR antagonist) and 5-methylurapidil (5-MU: α_{1A}-AR selective) were 9.3 and 7.7 respectively. The pK_B for prazosin confirmed the response was α_1-AR mediated whilst the relatively low pK_B for 5-MU indicated that the α_{1B}-AR was responsible for contraction in the α_{1D}-KO aorta rather than α_{1A}-AR.

The use of the selective α_{1A}-AR agonist confirmed this. The order of potency of both PE and A61603 in the mouse strains was α_{1B}-KO > WT > α_{1D}-KO. In fact, it was not possible to establish a pEC_{50} value for A61603 in the α_{1D}-KO as A61603 has little or no

efficacy at α_{1B}-ARs. However, this data also demonstrated the significant potency of A61603 at α_{1D}-ARs contrary to previous reports.

To compliment the pharmacological study, the adrenergic response of the aortae of double-AR KO, the α_{1B}-α_{1D}-KO was tested. PE and A-61603 failed to cause contractile responses, whilst serotonergic responses remained unaltered. Therefore, there were no contractile functional α_1-ARs in the α_{1B}-α_{1D}-KO aortae. This confirmed that either α_{1B}-ARs or α_{1D}-ARs are the adrenergic vasoconstrictors in the mouse aorta

An attempt was then made, using a receptor protection protocol, to isolate the minor α_{1B}-AR response in WT mouse aorta. 10μM chloroethylclonidine (CEC: non-selective α_1-AR alkylating agent) completely ablated the adrenergic (PE) response and incubation with 10nM BMY 7378 (α_{1D}-AR competitive antagonist) prior to 10μM CEC treatment could not protect any of the response.

However, 1μM CEC reduced the maximal response by 50% but the entire response was protected by pre-incubation with 10nM BMY 7378 but not by 10nM 5-MU. These results indicated that there was little or no role for α_{1D}-AR in the WT mouse aorta and confirmed the lack of α_{1A}-AR role in functional responses. However in the α_{1D}-KO the response was due to α_{1B}-AR activation which could be due to α_{1B}-AR up-regulation in the absence of functional α_{1D}-ARs in the mouse aorta.

Chapter 3. Adrenergic and serotonergic synergy in the mouse aorta

The synergistic interaction between α_{1D}-AR mediated contractions and 5-HT_{2A} mediated contractions in the mouse aorta is shown in Chapter 3. 30nM PE and 5-HT synergistically amplified the serotonergic and adrenergic responses respectively in WT aortae. This was observed as sensitivity increases in the CCRC of PE and 5-HT curves.

However this was complicated by the finding that the 5-HT response is partially α_{1D}-AR mediated. Both 10nM prazosin and 10nM BMY 7378 decreased the sensitivity to 5-HT in WT aortae but no decrease in sensitivity was observed in α_{1D}-KO as a result of 10nM or 100nM prazosin treatment. Thus, the serotonergic response in the WT aorta is partially α_1-AR mediated and the two systems can synergistically interact.

However, the serotonergic responses of the WT and α_{1D}-KO were not significantly different. Use of ritanserin (5-HT_{2A} receptor insurmountable antagonist) and BRL 54443 (5-HT_{2A} agonist) confirmed that 5-HT_{2A} receptors appeared to be compensating for the lack of functional α_{1D}-ARs in the α_{1D}-KO aortae.

Chapter 4. The effect of L-NAME on contractile responses in the mouse aorta

100μM L-NAME (nitric oxide synthase blocker) treatment increased the maximal response to KCl in all three strains (WT, α_{1B}-KO and α_{1D}-KO). Serotonergic responses exhibited increased sensitivity but no increase in maxima as a result of L-NAME treatment.

The adrenergic responses of all three strains exhibited an increased maximum after L-NAME treatment. L-NAME treatment resulted in an increased PE sensitivity in the WT and α_{1B}-KO but not in the α_{1D}-KO. This indicated that the α_{1D}-AR is also involved in nitric oxide (NO) release, probably from endothelial cells, as well as being the major adrenergic vasoconstrictor.

The involvement of α_2-ARs in the PE response was also tested. Rauwolscine (α_2-AR antagonist) had no effect on the PE-induced response in WT aortae.

Conclusions

Thus, the α_{1D}-AR is the sole adrenergic vasoconstrictor in the WT aorta. Knocking out the functional α_{1D}-AR results in an α_{1B}-AR-mediated response that is probably the result of compensatory mechanisms leading to an upregulation of the α_{1B}-AR.

The α_{1D}-AR can synergistically interact with the serotonergic response as well as being involved in partially mediating the response to 5-HT. The α_{1D}-AR is also involved in NO release and hence vasodilatation as well as being a vasoconstrictor.

Thus the α_{1D}-AR has a complex but crucial role in modulating tone of the mouse aorta.

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I start in the name of Allah, The Most Gracious, The Most Merciful.

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Going into my senior Honours year, one of the best pieces of advice given to me by a friend was "choose your supervisor, before you choose your project".

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Declaration

The work presented within this thesis is entirely my own work.

This work has not been presented in part or alone for any other degree course.

Following is a list of publications which have arisen as a result of the research presented within this thesis:

1. **Ali, Z.**, McGrath, J.C. & Daly, C.J. (2002). Characterisation of the contractile α_1-adrenoceptor response in the mouse thoracic aorta. *Br. J. Pharmacol.*, **137**, 92P. (Abstract)

2. Daly, C.J., Deighan, C., McGee, A., Mennie, D., **Ali, Z.**, McBride, M. & McGrath, J.C. (2002). A knockout approach indicates a minor vasoconstrictor role for vascular α_{1B}-adrenoceptors in mouse. *Physiol. Genomics*, **9**, 85-91.

3. McGrath, J.C., Pediani, J.D., Macmillan, J., Mackenzie, J., Deighan, C., Woollhead, A., McGrory, S.P., McBride, M., **Ali, Z.**, Malekzadeh-Shafaroudi, M., Cotecchia, S., Arribas, S.M., Vila, E., Briones, A., Perez, D., Mullins, J., Tsujimoto, G. & Daly, C.J. (2002). Adventitial cells are identified as the major location of vascular alpha1B-adrenoceptors and may drive vascular remodelling. *Br. J. Pharmacol.*, **137**, 21P. (Abstract)

4. **Ali, Z.**, McGrath, J.C. Tsujimoto G. & Daly C.J. (2003) Inhibition of 5-hydroxytryptamine-mediated contraction by α_1-adrenoceptor antagonist in mouse thoracic aorta. *J. Physiol.*, **548P**, P83 (Abstract)

5. **Ali, Z.**, McGrath J.C & Daly C.J. (2004) Adrenergic and serotonergic synergism in the mouse thoracic aorta. *J. Physiol.*, **557P**, C34. (Abstract)

6. Miquel, M.R., **Ali, Z.**, D'Ocon, M.P., McGrath, J.C. & Daly, C.J. (2004). 3D image analysis of fluorescent drug binding. *Mol. Imaging*. (Accepted with minor revisions).

General Introduction

General Introduction

Adrenoceptors

Adrenoceptors mediate the responses of the endogenous catecholamines: the sympathetic neurotransmitter, norepinephrine (NE: noradrenaline) and the circulating hormone, epinephrine (adrenaline). In 1896, Oliver & Schafer observed that extracts of adrenal glands could effect a pressor response (Rang *et al.*, 1999) and from this initial observation, the study of adrenaline and its involvement in the cardiovascular system began.

Over a century later and the role of adrenoceptors has been uncovered in such organ systems of the body as the brain, spinal cord, lungs, liver, kidneys and many more, along with the heart and the vasculature. The effects of the adrenal extracts Oliver & Schafer observed, we now know, was due to the presence of epinephrine and its activity at receptors termed adrenoceptors. It is also now known that norepinephrine is the endogenous neurotransmitter which elicits its response via action at adrenoceptors.

Currently, nomenclature describes nine distinct subtypes of adrenoceptors (Alexander *et al.*, 2004), namely three α_1-AR subtypes (α_{1A}, α_{1B} & α_{1D}), three α_2-AR subtypes (α_{2A}, α_{2B} & α_{2C}) and three β-AR subtypes (β_1, β_2 & β_3). So how did we go from Oliver & Schafer's original observations to the current climate with 9 distinct subtypes mediating the responses of NE and epinephrine? Following is a brief history of the discovery and classification of ARs, in particular the α_1-AR subtypes as this is the focus of this thesis. However, preceding this is a note on nomenclature throughout the thesis.

Note on nomenclature

Throughout the thesis the individual AR subtypes will be indicated with uppercase letters such as α_{1A}, α_{1B} and α_{1D} or alternatively, with lower case letters i.e. α_{1a}, α_{1b} and α_{1d}. It is customary to use upper case letters for native receptor subtypes within tissues or cells. The lowercase letters are used for cloned receptors, i.e. those receptors that are not native to those tissues or cells, and have been artificially introduced. Therefore, throughout the thesis this nomenclature has been applied.

History of adrenoceptors subtypes: α-AR and β-AR subtypes

In 1913, Dale observed blood pressure responses to epinephrine after treatment with a crude ergot alkaloid substance (Rang *et al.*, 1999). The response went from a vasoconstrictor response to vasodilatation response once the tissue had been exposed to the ergot extracts. This was the first indication of the multiple receptor action of epinephrine.

In 1948, Ahlquist postulated the existence of two distinct populations of ARs, alpha (α) and beta (β). His conclusions were based on the effects of the endogenous agonists, epinephrine (Epi), NE and a synthetic derivative of epinephrine, isoprenaline (Iso).

Ahlquist (1948) reported administration of NE *in vivo*, in dogs, cats and rabbits, resulted in an elevation of mean arterial pressure (MAP), whilst isoprenaline administration caused a decrease in MAP. Epinephrine administration though, resulted in a biphasic response, an initial increase in MAP followed by a decrease below baseline before returning to normal.

This indicated to Ahlquist (1948) the existence of two distinct populations of receptors, the NE-sensitive receptors (α) and the isoprenaline-sensitive receptors (β), whilst epinephrine appeared to have efficacy at both. Due to the activity of epinephrine at both α and β adrenoceptors, he concluded that epinephrine was the endogenous sympathetic neurotransmitter, but further research later found the neurotransmitter was norepinephrine.

Ahlquist (1948) described two observed orders of agonist potency dependent on whether the agents resulted in pressor responses or depressor responses. The order of potency for vasoconstrictor responses was NE > Epi > Iso for activity at what Ahlquist (1948) called α-adrenotropic receptors (adrenoceptors). Conversely, the order of agonist potency for vasorelaxant responses at β-adrenoceptors was Iso > Epi > NE. However this division was only the first of more family divisions to come.

α-AR subtypes: further division into α_1- and α_2-AR subtypes

In 1957, Brown and Gillespie reported dibenamine treatment increased sympathetic outflow from nerve stimulation in cat spleens. In Starke's (1972) study of adrenergic and cholinergic transmission in the rabbit heart, he introduced the concept of α-ARs having a negative feedback action on the release of noradrenaline from sympathetic neurons. His conclusions were based on the observations that oxymetazoline and naphazoline could decrease NE overflow but phenylephrine caused an increase in the force of contraction of the heart indicating the α-ARs resulting in negative feedback of NE release were different from those α-ARs on the cardiac cells enhancing contractility.

Then Langer (1974) reviewed the evidence for the existence of pre- and post-junctional α-ARs, which he also argued were non-identical. His evidence for this was based on a previous report by Dubocovich & Langer (1974) where the authors reported phenoxybenzamine treatment resulted in an increase in sympathetic outflow at a 30-fold higher concentration than was required to block post-junctional α-receptors. Therefore he postulated the subdivision of α-ARs by their anatomical location and suggested dividing α-ARs into α_1 and α_2 for postjunctional and prejunctional respectively.

This division was an oversimplification, as Berthelsen & Pettinger (1977) reported α_2-ARs can be involved post-synaptically, reporting a role for α_2-AR activation in renin releases from kidneys and melanocyte dispersion in frog skin. They suggested the subtype division should be due to pharmacological properties and not anatomical location as previously suggested.

The α_1/α_2 division was pharmacologically confirmed by Timmermans & Van Zwieten (1980), when they established that α_1-ARs and α_2-ARs had different selective antagonists. In a study performed in pithed rats they reported that the prazosin blockade of pressor responses by various agonists had the following order:

$$\text{phenylephrine} > \text{clonidine} >> \text{B-HT 933}$$

In contrast yohimbine blockade had the following order:

$$\text{B-HT 933} > \text{clonidine} \geq \text{phenylephrine}$$

Timmermans & Van Zweiten (1980) had pharmacologically confirmed the two subtypes, the prazosin sensitive α_1-ARs and the yohimbine sensitive α_2-ARs.

Subdivision of α_1-ARs

In McGrath's (1982) review of the apparent heterogeneous distribution of α_1-ARs he suggested a subdivision of the α_1-ARs into two separate subtypes. His conclusion was based on the activity of phenylethanolamine and non-phenylethanolamine agonists in various tissues but in particular, the rabbit basilar artery and rat anococcygeus.

He reported phenylethanolamines resulted in biphasic responses in these tissues and suggested the subdivision of the phenylethanolamine-sensitive α_{1A}-ARs and the phenylethanolamine-insensitive α_{1B}-ARs. He proposed the first phase of the curve was due to α_{1A}-ARs and the second phase due to α_{1B}-ARs. The actions of non-phenylethanolamines further pointed to a difference in the pharmacology of the α_{1A} and α_{1B}-ARs. Non-phenylethanolamines had little efficacy at α_{1B}-ARs but were good agonists at α_{1A}-ARs.

McGrath (1982) was not alone in suggesting the subdivision of α_1-AR into two subtypes. Holck *et al.* (1983) had noted the differential blockade of verapamil, the calcium entry blocker, in rabbit pulmonary artery. They reported that verapamil and prazosin were better antagonists at blocking clonidine-induced responses than methoxamine-induced responses, postulating the existence of two receptors. However, their data also hinted that the dependence of extracellular calcium for the two postulated receptors may be different.

However, this subdivision was somewhat resisted mainly due to the lack of suitably selective antagonists. It wasn't until Morrow and Creese (1986) performed a binding study of rat brain that the division was confirmed. They reported [^{3}H]prazosin and [^{3}H]WB4101 dissociation curves were biphasic. [^{3}H]WB4101 had a 37-fold difference between K_{high} (α_{1A}-AR) and K_{low} (α_{1B}-AR) dissociation constants.

They reported the following antagonist potencies:

α_{1A}-AR: WB4101 ≥ prazosin > phentolamine

α_{1B}-AR: prazosin ≥ WB4101> phentolamine

Thus, WB4101 was confirmed as an α_{1A}-AR selective antagonist and could be used to establish which α_1-AR subtypes are involved in functional responses. Unlike WB4101, prazosin could not differentiate between α_{1A}-AR or α_{1B}-ARs.

Han *et al.* (1987) then further strengthened this hypothesis by reporting that the alkylating agent chloroethylclonidine (CEC) could differentiate between sites with high and low affinity for WB4101. Low affinity sites for WB4101 were preferentially alkylated by CEC, thus CEC alkylation was α_{1B}-AR selective.

Gross *et al.* (1988) then reported the urapidil analogue, 5-methylurapidil (5-MU) had affinity for α_{1A}-ARs over α_{1B}-ARs by performing binding studies in rat hippocampus, vas deferens, heart, liver and spleen. Thus α_{1A}-ARs could be defined by their sensitivity to 5-MU and WB4101, whilst α_{1B}-ARs could be defined by their sensitivity to CEC alkylation.

α_{1H} and α_{1L}-ARs subdivision

Around the time when McGrath (1982) suggested a subdivision of α_1-ARs into α_{1A} and α_{1B}-ARs, Medgett & Langer (1984) observed that α_1-ARs appeared to have two different populations based on their varying affinity of prazosin. In their study of the rat tail artery, Medgett & Langer (1984) noted an apparent '*kink in the Schild plot*' of prazosin blockade. They reviewed the literature and noted that in general two distinct affinities for prazosin tended to be reported, a population with prazosin affinity with a pK_B value >9.36 and a second population with pK_B values of 8.80.

Medgett and Langer's (1984) findings added weight for McGrath's (1982) call to subdivide the α_1-ARs. Flavahan and Vanhoutte (1986) reviewed the literature, providing additional evidence for the postulated heterogeneity of the α_1-AR responses but they suggested the two receptors had differential affinity for prazosin. Thus

Flavahan & Vanhoutte (1986) introduced the subtypes α_{1H} (high affinity for prazosin) and α_{1L} (low affinity for prazosin) into the nomenclature.

Contrast this with the binding study done by Morrow & Creese (1986) who reported prazosin could not distinguish between subtypes, but the response can be subtyped by WB4101 sensitivity. It is noteworthy that the three current α_1-AR subtypes, α_{1A}, α_{1B}, and α_{1D}-ARs all have high affinity for prazosin i.e. they are all α_{1H}-ARs.

Muramatsu *et al.* (1990) then added to the increasing complexity of α_1-AR subtypes. In their extensive study of various vessels from dogs, rabbits, guinea-pigs and rats they found the vessels broadly fell into three categories based on their variation of antagonist affinity.

Group II (dog carotid artery and rat thoracic aorta) had high affinity for prazosin (pA_2 = 9.5) which was more potent than both WB4101 and HV723. They associated this with α_{1H}-ARs, previously described by Flavahan & Vanhoutte (1986). The α_{1L}-AR was associated with group III (rabbit mesenteric artery, thoracic aorta, carotid artery and guinea-pig thoracic aorta). This group had pA_2 values for prazosin, HV723 and WB4101 ranging from 8-9 but no distinct order of potency was noted.

Group I (dog mesenteric artery, vein and saphenous vein) had affinity for HV723 and WB4101 over prazosin. The affinities in this group did not fit the above two groups as they had a low affinity for prazosin (pA_2 = approx 8.6) and these tissue were unusually insensitive to yohimbine (pA_2 values < approx 6.0) compared to the other two groups. Muramatsu *et al.* (1990) termed this group α_{1N}.

Although the evidence pointed to $\alpha_{1H/L/N}$ subtypes, no evidence from cloned studies had been provided to support this subdivision. It is now believed that the α_{1L}-AR may be a splice variant of the α_{1A}-AR of which four variants are believed to exist (Chang *et al.*, 1998). Therefore the $\alpha_{1H/L/N}$-AR subtypes have fallen into relative disuse.

Cloning studies of α_1-AR subtypes

α_{1b}-AR: The α_{1b}-AR was the first to be cloned. Cotecchia *et al.* (1988) cloned a 1545-base pair fragment of DNA that coded for the 515 amino-acid hamster α_{1b}-AR. As no other α_1-AR had been cloned at the time, Cotecchia *et al.* (1988) compared the protein sequence with α_2, β_1 and β_2-ARs. The most conserved regions were in the transmembrane regions of the receptors with 42-45% conservation with the other ARs.

They highlighted seven distinct hydrophobic regions of approximately 20 to 25 amino acids that they associated with the seven transmembrane regions GPCRs have (see section on Murine α_1-ARs). They also reported threonines and serines present in the third cytoplasmic loop as possible phosphorylation sites by protein kinase C.

Pharmacological studies revealed the cloned receptor had a high affinity for prazosin and low affinity for WB4101, consistent with the functional α_{1B}-AR.

α_{1a}-AR: The α_{1a}-AR was then cloned by Schwinn *et al.* (1990) but was provisionally termed the α_{1c}-AR as this protein could not be detected in tissues which had been shown to have α_{1A}-AR mediated responses such as, rat vas deferens and hippocampus (Gross *et al.*, 1988). The cloned bovine α_{1c}-AR (466 amino-acids: Schwinn *et al.*, 1990) was reported to have 72% sequence homology with the α_{1b}-AR (previously cloned by Cotecchia *et al.*, 1988: discussed earlier). Threonine and serine residues in the third intracellular loop indicated possible phosphorylation sites by protein kinases.

The pharmacology though was consistent with the α_{1A}-AR: high affinity for WB4101 and phentolamine (Morrow & Creese, 1986). Later Schwinn *et al.* (1991) reported the novel α_{1A}-AR antagonist, 5-MU (Gross *et al.*, 1988), could discriminate between α_{1b} and α_{1c}, having affinity for α_{1c} (Schwinn *et al.*, 1991).

α_{1d}-AR: The rat α_{1d}-AR was the last AR to be cloned (Perez *et al.*, 1991). The gene was a 1680 base pair fragment encoding 560 amino-acids. The α_{1d}-AR had 72% and 64% homology with hamster α_{1b}-ARs and bovine α_{1c}-ARs respectively.

However, pharmacological findings indicated the novel α_{1d}-AR had significantly lower affinity for 5-MU than native α_{1A}-ARs in tissue studies as well as being susceptible to CEC alkylation. They concluded the α_{1d}-AR was a new subtype distinct from the α_{1A}-AR that had been pharmacologically identified.

At the same time Lomansay *et al.* (1991) cloned a receptor from rat cortex which they termed the α_{1a}-AR but it was identical to the cloned α_{1d}-AR.

We had gone from having only two pharmacological subtypes to four cloned subtypes with apparently distinct pharmacology. The nomenclature was eventually cleared up by the International Union of Pharmacology (Byland *et al.*, 1994) when the α_{1a} and α_{1d}-ARs were accepted as a novel α_{1D}-AR, the α_{1c}-AR became the original α_{1A}-AR, whilst the cloned α_{1B}-AR remained as the pharmacologically distinct α_{1B}-AR.

Current subtype selective agonists

Selective agents for the individual α_1-AR subtypes are becoming increasingly available but the pharmacology remains complex. Following is some of the ligands that have been used in this project for α_1-AR subtyping studies.

Norepinephrine (NE) is the endogenous agonist for α-ARs. Ahlquist (1948) showed that NE has greater affinity for α-ARs over β-ARs. In 1995 Knepper *et al.* performed a binding study and reported that NE has partial affinity for α_{1d}-ARs over α_{1a} and α_{1b}-ARs (22-fold and 15-fold respectively). However NE has efficacy at α_2-ARs as well as partial efficacy at β-ARs. Using NE as the agonist, a host of blockers would have been required in order to isolate the α_1-AR mediated response i.e. propranolol to block β, rauwolscine for α_2-ARs, cocaine for uptake-1 and corticosterone for uptake-2. Therefore NE has not been used in the present study.

Phenylephrine (PE), an α_1-AR agonist with full efficacy, had slight affinity for α_{1d}-ARs over α_{1a} and α_{1b}-ARs (6-fold and 8-fold affinity respectively: Knepper *et al.*, 1995). Therefore, it is essentially a non-subtype selective α_1-AR selective full agonist and was therefore the agonist of choice for the study reported in this thesis, as the focus was the α_1-AR subtypes.

The R-enantiomer of A61603 is a currently accepted full agonist with selectivity for α_{1A}-ARs (Knepper *et al.*, 1995). R-A61603 had 163-fold and 58-fold affinity for cloned α_{1a}-ARs over α_{1b} and α_{1d}-ARs respectively. They also demonstrated this selectivity by performing binding studies using specific tissues from the rat (α_{1A}- vas deferens; α_{1B}- spleen; α_{1D}- aorta).

Furthermore, the racemate mix of A61603 was shown to have no efficacy at hamster α_{1b}-ARs as it could not raise IP_3 levels to a quantifiable level but increased IP_3 levels 132-fold 3.5-fold in cells transfected with bovine α_{1a}-ARs and rat α_{1d}-ARs respectively. However, R-A61603 was reported to have affinity for α_2-ARs but the authors made no indication of which α_2-AR subtype was involved or whether A61603 had efficacy at α_2-ARs. R-A61603 was used in Chapter 2 to compliment antagonist studies done using PE as the agonist, in order to determine functional roles for specific α_1-AR subtypes.

Current subtype selective antagonists

Phentolamine is a classical α-AR selective antagonist that has affinity for both α_1 and α_2-ARs. Morrow & Creese (1986) demonstrated that phentolamine could be used to discriminate between α_{1A} and α_{1B}-ARs but more selective antagonists are now available for subtyping. Unlike phentolamine which is a reversible antagonist, phenoxybenzamine, another α-selective antagonist is insurmountable. However, both phentolamine and phenoxybenzamine, remain useful tools in establishing α-AR selectivity of ligands, but have not been used in this study.

Prazosin is the most commonly used α_1-AR selective antagonist for determining the extent of α_1-AR involvement. Prazosin cannot discriminate between α_1-AR subtypes, but has potency at all three subtypes in the nanomolar range. It has been used in this project to ensure responses were α_1-AR mediated.

5-methylurapidil is an α_{1A}-AR selective antagonist with approximately 50-fold selectivity for cloned α_{1a}-ARs over α_{1b} and α_{1d}-ARs (see Table 2-A). Gross *et al.* (1988) was the first to show the selectivity of 5-MU at α_{1A}-ARs but this was pre-1994 when only two α_1-ARs had been pharmacologically profiled in native tissues. Schwinn *et al.* (1994) later confirmed that 5-MU had affinity for α_{1A}-AR over α_{1B} and α_{1D}-ARs.

No suitable α_{1B}-AR antagonist is currently available (Alexander *et al.*, 2004). Chloroethylclonidine (CEC), an irreversible alkylating agent which was postulated to be an α_{1B}-AR selective antagonist (Han *et al.*, 1987; Perez *et al.* 1994). However more recent data, particularly from cellular studies, has indicated that this apparent selectivity is due to heterogeneous distribution of α_1-AR subtypes at the cellular level.

Hirasawa *et al.* (1997) suggested that CEC preferentially alkylates the receptors presented on the cellular membrane irrespective of α_1-AR subtype. The authors postulated that the α_{1B}-ARs are present on the membrane whilst the α_{1A} or α_{1D}-ARs tend to be intracellular. However CEC can still be used as an irreversible α_1-AR alkylating agent.

Other α_{1B}-AR selective antagonists that have been postulated include cyclazosin (Giardina *et al.*, 1996) and L765,314 (Patane *et al.* 1998). However, both agents have been used by colleagues who have been unable to reproduce the selectivity reported by the initial authors.

Therefore there currently is no suitable α_{1B}-AR selective antagonist. However, after personal correspondence with Dr. Michael T. Piascik (University of Kentucky, Lexington, Kentucky, USA) and Prof. James E. Faber (University of North Carolina, Chapel Hill, North Carolina, USA), it was decided that the best approach to isolate the α_{1B}-AR mediated response would be a receptor protection protocol (See Chapter 2 for greater detail).

The hypothesis was, by pre-incubating with selective α_{1A} and α_{1D}-ARs selective antagonists, the α_{1B}-AR could be alkylated by CEC treatment. The antagonists could then be washed out and the response remaining should be a response minus the role of α_{1B}-ARs. This approach has been applied in Chapter 2.

For the study of α_{1D}-ARs the most commonly used antagonist is BMY 7378 (Saussy *et al.*, 1994). In a later study Goetz *et al.* (1995) reported selectivity of 126-fold and 110-fold for α_{1d}-ARs over α_{1a} and α_{1b}-ARs respectively. Therefore BMY has been used in this study for the isolation of the role of α_{1D}-ARs.

The mouse aorta

Murine models are being increasingly used in adrenoceptor pharmacology due to the availability of knockout models of each individual α_1-AR subtypes. However, such studies only highlight the current lack of background physiological studies done in mice. Recently, Russell & Watts (2000), performed a study of the vascular reactivity of the mouse thoracic aorta.

They used the C57 Black 6J strain of mice, as this strain is commonly used for the breeding of genetically manipulated models. Using helical strips of aorta they tested various vasoactive agonists and highlighted the high potency and efficacy of NE, PE and 5-HT. They also reported endothelium-dependent ACh-induced relaxation.

Other interesting notes included the low efficacy UK14,304 (α_2-AR selective agonist) and angiotensin II. There was a distinct lack of β-AR-mediated relaxation and no relaxation to histamine or adenosine which Russell & Watts (2000) noted was different to the effect of these agents in the rat aorta. Comparison of mouse and rat aortae revealed that the mouse aorta had little response to angiotensin II and endothelin, whilst in the rat aorta they both exhibited high efficacy and relatively high potency. Thus the mouse aorta exhibits distinct physiological responses indicating background work on the mouse was essential.

Interestingly, the 5-HT was equipotent to PE in the mouse aorta, whilst 5-HT has been shown to be less potent than PE in the rat aorta (Christ & Jean-Jacques, 1991). McKune & Watts (2001) then subtypes the serotonergic response of the mouse aorta. 5-HT_{2A} receptor activation was reported to be responsible for the 5-HT induced response.

The adrenoceptor response of the mouse aorta (albino ddY strain) has been subtyped pharmacologically (Yamamoto & Koike, 2001) using α_1-AR subtype selective antagonists. They reported pA_2 values of 9.7, 9.6, 7.5 and 8.4 for prazosin, WB4101, 5-

MU and BMY 7378 respectively and that the NE-response was sensitive to CEC alkylation. BMY 7378 was significantly more potent than 5-MU, indicating the α_{1D}-AR was the major adrenergic vasoconstrictor. This was later confirmed by studies done in transgenic mice but is discussed later.

Adrenoceptor responses in other murine vessels

Yamamoto & Koike (2001a) also performed a study of α_1-AR subtype of the abdominal aorta and mesenteric arteries. They suggested the adrenergic responses in the upper region of the abdominal aorta were α_{1D}-AR mediated whilst in the lower region were α_{1A}-AR mediated.

However they could not determine the α_1-AR subtype involved in vasoconstriction of the superior mesenteric artery as both BMY 7378 and 5-MU exhibited low affinity, whilst prazosin and WB4101 had high affinity. Thus, they concluded it was non-α_{1D} (tissue was not BMY sensitive), non-α_{1A} (tissue was not 5-MU sensitive), non-α_{1B} (tissue had high affinity for WB4101) and non-α_{1L} (tissue had high affinity for prazosin). Their suggestion was that it may have been a functional phenotype of the α_{1D}-AR, akin to the α_{1L}-AR being a splice variant of the α_{1A}-AR.

Daly *et al.* (2002) performed a study of four different vascular preparations (aorta, carotid artery, 1st-order mesenteric artery & tail artery) from mice. They also used transgenic models but the data provided on the effect of α_1-AR subtype selective antagonists in the WT alone indicated that the aorta and carotid artery adrenergic responses are primarily α_{1D}-AR mediated whilst the α_1-AR responses of the mesenteric and caudal arteries were due to mainly α_{1A}-AR activation.

Thus, it appears that in large conductance arteries in the mouse the adrenergic responses tend to be α_{1D}-AR mediated whilst the α_{1A}-AR seems to be responsible for the adrenergic responses of smaller resistance arteries such as the mesenteric arteries or the tail artery.

Murine α_1-ARs

The mouse (Mus musculus: Domestic mouse) genome consists of 40 chromosomes. Detailed information on individual proteins can be accessed on the SWISS-PROT© database by entering the appropriate protein code.

All ARs are metabotropic receptors belonging to a class of proteins known as G-protein-coupled receptors (GPCRs). GPCRs are single polypeptides chain proteins with seven α-helical regions that span across the cellular membrane. As such, GPCRs have an extracellular N-terminus, an intracellular C-terminus, with three intracellular and three extracellular loops. The 3rd intracellular loop is extended and is believed to be involved in the interaction of the GPCR with its appropriate G-protein (Rang *et al.* 1999).

All three α_1-AR subtypes are coupled to the $G_{q/11}$ subset of G-proteins and increase inositol trisphosphate (IP_3) production. As a result, they raise intracellular calcium and can recruit voltage-gated calcium channels.

Following is a brief description of the three murine α_1-AR subtypes. The amino-acid sequences are also shown with the seven transmembrane regions (potential) underlined and the extended third intracellular loops shown in bold.

α_{1A}-AR: The gene for the α_{1A}-AR is located on chromosome 14. It is now known that splice variants of the α_{1A}-AR now exist of which four distinct variants have been identified ($\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2}$, $\alpha_{1A\text{-}3}$ & $\alpha_{1A\text{-}4}$; Chang *et al.*, 1998). For this study the $\alpha_{1A\text{-}1}$ variant is considered as the sole α_{1A}-AR in the mouse although this may not be the case. The murine α_{1A}-AR is 466 amino-acids long (protein code: P97718; accession number: GI 20141255).

```
001 mvllsenase gsnethppaq vniskaillg vilggliifg vlgnilvils vachrhlhsv
061 thyyivnlav adllltstvl pfsaifellg ywafgrvfcn iwaavdvlcc tasimglcii
121 sidryigvsy plryptivtq rrgvrallcv walslvisig plfgwrgqap edeticqine
181 epgyvlfsal gsfyvpltii lvmycrvyvv akresrglks glktdksdse qvtlrihrkn
241 vpaegsgvss aknkthfsvr llkfsrekka aktlgivvgc fvlcwlpffl vmpigsffpn
301 fkppetvfki vfwlgylnsc inpiiypcss qefkkafqnv lriqclrrrq sskhalgytl
361 hppsqaveeq hrgmvripvq sqetfykisk tdgvcewkff ssmpqgsari tmpkdqsact
421 tarvrsksfi qvcccvgsst prpeenhqvp tikihtislg engeev
```

α_{1B}-AR: The gene for the α_{1B}-AR is located on chromosome 11. The murine α_{1B}-AR is 514 amino-acids long (protein code: P97717; accession number: GI 3023234).

```
001 mnpdldtghn tsapahwgel kdanftgpnq tssnstlpql dvtraisvgc lgafilfaiv
061 gnilvilsva cnrhlrtptn yfivnlaiad lilsftdlpf satlcvlgyw vlgrifcdiw
121 aavdvlccta silslcaisi dryigvrysl qyptlvtrrk allallsvwv istvisigpl
181 lgwkepapnd dkecgvteep fyalfsslgs fyiplavilv mycrvyivak rttknleagv
241 mkemsnskel tlrihsknfh edtlsstkak ghnprssiav klfkfsrekk aaktlgivvg
301 mfilcwlpff ialplgslfs tlkppdavfk vvfwlgyfns clnpiiypcs skefkrafmr
361 illgcqcrqgr rrrrrrrlga caytyrpwtr ggslersqsr kdsldasgsc msgsqrtlps
421 aspspgylgr gtqppvelca fpewkpgall slpeppgrrg rldsgplftf klqepespg
481 tegdasnggc dtttdlangq pqfksnmpla pghf
```

α_{1D}-AR: The gene for the α_{1D}-AR is located on chromosome 2. The murine α_{1D}-AR is 562 amino-acids long (protein code: P97714; accession number: GI 3121722).

```
001 mtfrdilsvt feqprassst ggsgagggag tvgpegpavg gvpgatggsa vvgtgsgedn
061 qsstaeagaa asgevngsaa vgglvvsaqg vgvgvflaaf iltavagnll vilsvacnrh
121 lqtvtnyfiv nlavadllls aavlpfsatm evlgfwpfgr tlcdvwaavd vlcctasils
181 lctisvdryv gvrhslkypa imterkaaai lallwavalv vsvgpllgwk epvppderfc
241 gitecvgyai fssvcsfylp mavivvmycr vyvvarsttr sleagikrep gkasevvlri
301 hcrgaatsak gnpgtqsskg htlrssisvr llkfsrekka aktlaivvgv fvlcwfpfff
361 vlplgslfpq lkpsegvfkv ifwlgyfnsc vnpliypcss refkraflrl lrcqcrrrrr
421 rlwpslrppl asldrrpalr lcpqpahrtp rgspsphctp rpglrrhagg agfglrpska
481 slrlrewrll gplqrpttql rakvsslshk frsggarrae tacalrseve avsinvpqdg
541 aeavicqaye pgdisnlret di
```

Generation of α_1-AR subtype knockout mice

The first α_1-AR subtype knockout (KO) mouse created was the α_{1B}-KO, reported by Cavalli *et al.* (1997). Using gene targeting vectors, Cavalli *et al.* (1997) removed exon 1 of the α_{1B}-AR gene. The disrupted gene was then electroporated into embryonic stem cells from the 129 mouse strain before microinjection into C57/Black/6J blastocytes. From this, homozygous progeny were obtained by further breeding with C57/Bl/6J mice. Using this approach, the mice produced had a mixed genetic background, part 129/Sv and part C57/Bl/6J. Therefore, Cavalli *et al.* (1997) obtained both wild-type (WT) and KO homozygous progeny to ensure a genetically matched control strain of mice with which to make a comparison.

The α_{1D}-KO and α_{1A}-KO mice were created in 2002 by Tanoue *et al.* (2002) and Rokosh & Simpson (2002) respectively. Tanoue *et al.* (2002) took an almost identical approach to Cavalli *et al.* (1997) creating the α_{1D}-KO by targeted gene disruption before using the 129/Sv stem cells microinjected into C57/Bl/6J blastocytes and bred the progeny with C57/Bl/6J mice. Using heterozygous progeny, several generations of breeding were done to ensure homozygous strains of WT and α_{1D}-KO mice were obtained. Thus both the α_{1B}-KO and α_{1D}-KO had mixed 129/Sv/C57/Bl/6J genetic backgrounds but it is unknown the extent of the genetic mixing and therefore the respective WT control strains were obtained in order to make appropriate comparisons.

Rokosh and Simpson (2002) created the α_{1A}-KO using a similar approach but additionally, they inserted the *Escherechia coli β-galactosidase* gene, LacZ. Having created the homozygous KO mice also using 129Sv stem cells but breeding mice on both C57/Bl/6J and FVB/N mice they went on to localise the expression of the *β-galactosidase* protein in the vasculature and demonstrate the heterogeneous expression of α_{1A}-ARs within blood vessels.

Disrupted gene expression in α_1-AR KO mice

In all three KO's created, only exon 1 was removed. For all three α_1-AR subtypes, exon 1 codes for the first five transmembrane spanning domains. The promoters for protein expression were unaffected and as such, some unanswered questions about the disrupted proteins expression remain.

Does the cell produce the disrupted AR? This question must be tackled at two levels, transcription and translation. Cavalli *et al.* (1997), Tanoue *et al.* (2002) and Rokosh & Simpson (2002) all performed RT-PCR of isolated RNA from mouse tissues, therefore the genes must have been transcribed for the RNA products to be present.

Rokosh & Simpson (2002) clearly demonstrated the disrupted gene was expressed through to protein level, i.e. translation had also occurred. No evidence for the translation of the disrupted α_{1B} and α_{1D}-AR RNA products into proteins is currently available. Therefore, it is currently unknown how far the production of these disrupted receptors proceeds, although if the findings of Rokosh & Simpson (2002) are consistent across the board then it may be fair to make this assumption.

What happens to the protein products of these mutant genes? Although Rokosh & Simpson (2002) demonstrated protein expression, they were unable to comment on the cellular location of the protein, as their protocol did not have the resolution to do so. Therefore, assuming the protein products for all three mutant genes are present, it is unknown whether or not the cells detect this mutant gene, and initiate the degradation of the protein or whether these gene products actually are transported to the cellular membrane, bearing in mind that the protein code for transmembrane regions 6 and 7 of the receptors were still present.

What can be confirmed though is that all three mutant gene products have lost the functional response associated with that native α_1-AR subtype. Therefore these KO are *functional knockouts* and not complete gene KOs as their name suggests.

Phenotypes resulting from α_1-AR subtype KO

α_{1A}-KO: Rokosh and Simpson (2002) reported α_{1A}-AR mice were moderately hypotensive (approx 10% reduction in systolic and mean arterial pressure; MAP), had significantly reduced pressor responses to both phenylephrine and A61603 and a decreased baroreceptor reflex. They also demonstrated a reduction in the number of [^{3}H]-prazosin binding sites in brains, hearts and kidneys isolated from α_{1A}-KO mice compared with WT mice.

Furthermore, by insertion of the β-galactosidase gene in place of exon 1 of the α_{1A}-AR in the α_{1A}-KO, Rokosh & Simpson (2002) were able to study the expression of the mutant gene throughout the arterial tree. They reported a distinct lack of β-galactosidase staining in the thoracic aorta and abdominal aorta with clear staining in celiac artery, mesenteric artery and both left and right renal artery branches. They concluded the α_{1A}-AR is involved in homeostatic control of blood pressure in mice whilst there was no significant α_{1A}-AR expression in the mouse aorta.

α_{1B}-KO: α_{1B}-KO mice exhibited a lessened number of α_1-ARs binding sites in the cerebral cortex, cerebellum, kidneys, liver & hearts compared to WT (Cavalli *et al.*, 1997). Although the α_{1B}-KO mice were normotensive, they

had significantly reduced pressor responses compared to WT mice. In aortic ring segments they reported a decrease in PE potency, therefore, they implicated the α_{1B}-AR as having a role in blood pressure maintenance and also suggested the α_{1B}-AR is the major adrenergic vasoconstrictor in the mouse aorta.

However, Daly *et al.* (2002) performed a comprehensive pharmacological study of four blood vessels from the mouse aorta, namely the aorta, carotid arteries, mesenteric arteries (1st order branch of superior mesenteric artery) and caudal (tail) arteries. They reported that the lack of functional α_{1B}-ARs did not adversely affect vascular contractility in any of the arteries studied, with Daly *et al.* (2002) suggesting that the α_{1B}-AR "*confuses the pharmacology*" of the WT rather than play a major role in vasoconstriction. However they did not completely rule out a role in vasoconstriction for the α_{1B}-AR, suggesting instead that it has a minor role in contractile responses of all four vascular preparations studied.

Vecchione *et al.* (2002) then attempted to make the α_{1B}-KO mice and their WT controls hypertensive by chronic administration of NE, PE and angiotensin II to selected mice over a number of days using osmotic pumps. PE infusion did not induce hypertension in either WT or α_{1B}-KO whilst angiotensin II induced hypertension in both. However, NE treatment only resulted in hypertension in WT mice whilst α_{1B}-KO mice remained normotensive.

Although PE treatment did not result in hypertension Vecchione and colleagues (2002) reported that in WT mice, PE-infusion resulted in inward eutrophic vascular remodelling, where vascular wall thickness remains unaltered whilst lumen diameter decreases. This can be observed as a significant increase in media: lumen ratio and Vecchione *et al.* (2002) reported a 45% increase in media: lumen ratio in WT mice, whilst the α_{1B}-KO did not exhibit any significant vascular remodelling. Thus, the α_{1B}-KO has been implicated in the induction of hypertension by raised catecholamine levels, and also has a role in vascular remodelling.

α_{1D}-KO: Compared to the WT, the α_{1D}-KO mouse had significantly less total α_1-AR protein binding (reduced B_{max} values) in whole brain and cerebral cortex, whilst no binding was detected in aorta (Tanoue *et al.*, 2002). However, binding studies indicated no loss of α_1-AR population in heart and kidney tissues. α_{1D}-KO mice were also moderately hypotensive and had reduced pressor responses to NE and PE. Aortae isolated from α_{1D}-KO mice were significantly less sensitive than WT to PE and NE and not susceptible to blockade with 100nM BMY 7378, whilst the pA_2 for BMY 7378 was 8.6. Thus, they concluded that the α_{1D}-AR is involved in blood pressure maintenance and the α_{1D}-AR is the major vasoconstrictor in the mouse aorta.

Tanoue *et al.* (2002a) then performed a study of salt-induced hypertension in subtotal nephrectomised WT and α_{1D}-KO mice. Of the 15 mice from each group used in the study, by day 35 following subtotal nephrectomy and 1% saline loading, only 8 of the WT mice had survived, whilst only 1 fatality was recorded in the α_{1D}-KO strain of mice. Furthermore, α_{1D}-KO mice were less susceptible to the onset of salt-induced hypertension, and Tanoue *et al.* (2002a) suggested this may be due, in part at least, to the reduced α_1-AR induced vascular reactivity.

Thus, each of the α_1-AR subtypes has been implicated in the pathophysiology of hypertension. The use of these transgenic mice has suggested that all three α_1-ARs play a critical role in hypertension but they may have distinct roles in its pathogenesis. The individual roles for each of the subtypes in vasoconstriction could help elucidate important functional differences.

Therefore the mouse aorta has been chosen as the vascular preparation in this study in an attempt to focus particularly on the role of the α_{1D}-AR in vasoconstriction.

Aims of Study

Aims of Study

Chapter 1

1. Establish if there were any significant age-related changes in vascular contractility or relaxant responses in WT aortae.

2. Ensure that there were no significant strain-related differences in the contractile or relaxant responses between the two WT strains.

3. Confirm that the α_{1D}-AR is the major adrenergic vasoconstrictor in the mouse aorta with little role for α_{1B}-ARs.

4. Ensure that no age-related changes in the phenotypes in the α_{1B}-KO or α_{1D}-KO (such as compensatory up-regulation) occurred.

Chapter 2

1. Establish that the PE-induced response in α_{1D}-KO aortae is α_1-AR mediated and subtype this response using

    a. Selective antagonists (prazosin, 5-MU)

    b. Selective agonists (A61603)

2. Test the adrenergic response of the double α_{1B}-α_{1D}-KO mouse aorta

3. Isolate the minor role of the α_{1B}-AR in mediating the contractile response of WT aortae, using a receptor protection protocol.

Chapter 3

1. Demonstrate synergy between the adrenergic and serotonergic vasoconstrictor systems in the mouse aorta.

2. Demonstrate that 5-HT responses in the mouse aorta are prazosin-sensitive and establish which α_1-AR subtype is responsible for this

3. Establish 5-HT receptor subtype responsible for the serotonergic responses of both WT and α_{1D}-KO aortae.

Chapter 4

1. Investigate the role of α_{1D}-ARs in NO-dependent vasodilatation by testing the effect of L-NAME on KCl, adrenergic and serotonergic responses on WT α_{1D}-KO and α_{1D}-KO.

2. Test the involvement of α_2-ARs in PE-induced vasoconstrictor responses.

General Methods

General Methods

Colony Maintenance

Breeding pairs of 129/Sv/C57Bl/6J control wild-type mice (WT) and mice lacking functional α_{1B}-ARs (α_{1B}-KO) were kindly supplied by Professor Susanna Cotecchia (University of Lausanne, Lausanne, Switzerland). Breeding pairs of 129/Sv/C57BL/6J control wild-type mice (WT) and mice lacking functional α_{1D}-ARs (α_{1D}-KO) mice were kindly supplied by Professor Gozoh Tsujimoto (National Children's Medical Research Center, Tokyo, Japan). The generation and background of α_{1B}-KO mice, α_{1D}-KO mice and their appropriate genetically-matched control WT counterparts have previously been described in detail (Cavalli et al., 1997; Tanoue et al., 2002 respectively). α_{1B}-α_{1D}-KO mice were created by cross breeding α_{1B}-KO and α_{1D}-KO mice (see below for more details). All mice were bred in the University of Glasgow, maintained on a 12:12-hour light/dark schedule at 22-25°C with 45-65% humidity and fed ad-libitum on a standard rodent diet and tap water.

The α_{1B}-α_{1D}-KO was created by cross-breeding homozygous α_{1B}-KO with homozygous α_{1D}-KO. Since the genes for the α_{1B}-AR (Chromosome 11) and α_{1D}-AR (Chromosome 2) are located on separate chromosomes, the genetic makeup of future generations is predicted by Mendelian genetics.

Thus, the first generation (F_1) was heterozygous for WT alleles and both α_{1B}-KO and α_{1D}-KO mutant alleles and F_1 mice were genotyped, in order to confirm a heterozygous population. F_1 were then interbred and the second generation (F_2) was genotyped. F_2 mice that were homozygous for both mutant α_{1B}-AR and α_{1D}-AR alleles were selected and interbred, thus a population of α_{1B}-α_{1D}-KO mice were created. These mice should therefore only express α_{1A}-ARs. It was not deemed necessary to obtain a control strain of mice that were homozygous for the both the native α_{1B} and α_{1D}-ARs (Chapter 1).

The primers used for genotyping were those primers used by Cavalli *et al.* (1997) and Tanoue *et al.* (2002) who created the α_{1B}-KO and α_{1D}-KO mice respectively. This work was not done myself and is therefore not shown. However tissue was isolated from these mice and use for experiments presented herein.

Vessel isolation

4-month-old mice of weights 25-40g (4m, all chapters) or 16-month-old adult male mice of weights 35-50g (16m, Chapter 1 only) control (α_{1B}-WT or α_{1D}-WT), α_{1B}-KO, α_{1D}-KO or α_{1B}-α_{1D}-KO mice were euthanased by exposure to an increasing concentration of CO_2. Skin on the ventral surface of the thoracic cavity was carefully incised, the ribcage was removed and the heart and lungs were exposed. The aortic arch was located and cut in order to free the aorta from the heart. The thoracic aorta, from the aortic arch to the diaphragm was then excised directly into ice cold physiological saline solution (PSS).

The aorta was cleared of fat and connective tissue by dissection in an agar-filled Petri dish with the aid of a dissection stereomicroscope (Zeiss). The remains of the aortic arch and the first 2mm of descending aorta were removed and discarded. Four consecutive rings of 2mm in length were then cut and the remainder of the aorta discarded. No attempt was made to denude the vessels of endothelium.

Myograph mounting

The technique used for studying the vessels was small vessel wire myography, an extensively used and reliable *in vitro* technique used for pharmacological and physiological studies of small blood vessels. This method was originally described by Mulvany & Halpern (1977) and has been used for functional studies of blood vessels with passive lumen diameters as small as 100μm.

In brief, a 40μm diameter stainless-steel wire was carefully passed through the lumen and attached by screws to a mounting head, fixed to a force transducer. A second 40μm stainless-steel wire was then passed through the lumen and attached by screws to a mounting head which is fixed to an adjustable micrometer (Fig. I-1). The micrometer can be manually adjusted in order to apply circumferential tension to the vessel and the transducer measures the force (tension) generated by this stretching. Once a suitable tension has been set, any alterations in tone as a result of vascular activity can be measured as a change in isometric force.

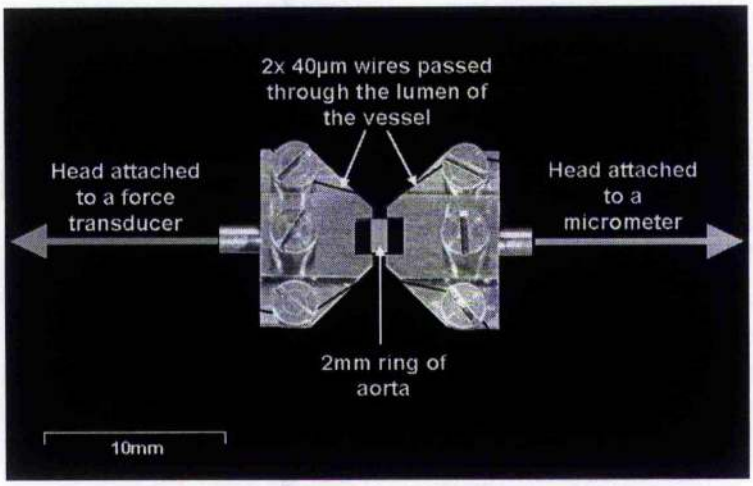


Fig I-1. The mounting heads of a myograph bath. The vessel sits between the two 'jaws' of the mounting heads and the screws are used to secure the 40µm wires.

Aortic rings were randomised and mounted on a 4-channel wire myograph (Danish Myo-Technologies) in PSS at 37°C bubbled with 95% O_2 /5% CO_2 gas mixture. Previously, colleagues had constructed passive-active length-tension curves in order to determine the optimal tension for contractile responses and tension equivalent to 1.0gram (9.81mN) was found to be suitable for the mouse aorta (data not shown). 20 minutes (min.) after vessels were mounted, 1.0grams of passive circumferential tension was applied by graduated increments of 0.33g, 3 min. apart. After a further 20 min. the tone was readjusted to 1.0g.

Myograph mounted vessels

Tissues were allowed 40 min. to equilibrate before any agonist challenges were made. A 'wake-up' protocol was then performed. Tissue viability was tested by replacing the PSS solution with a high KCl (125mM) PSS solution for 10 min. to allow contractions to reach plateau and then washed out with normal PSS. In the high KCl solution, NaCl was replaced by the molar equivalent of KCl (see Solution Used section).

After a further 20 min., adrenergic contractile responses were assessed by a single challenge to 10µM phenylephrine (PE) and 3µM acetylcholine (ACh) was added a few

minutes prior to washout in order to determine endothelial function. Serotonergic responses (where appropriate) were assessed by a single challenge to 1μM 5-HT. Cumulative concentration response curves (CCRCs) to the appropriate agonists were then constructed using half-log step increments (i.e. 1nM, 3nM, 10nM….. etc). Agonist concentrations were added at 3 min. intervals. Detail of the appropriate protocols used in each chapter are given in the following pages.

Protocol used: Chapter 1

A CCRC to PE was constructed (1nM-30μM for α_{1B}-WT, α_{1B}-KO and α_{1D}-WT; 10nM-300μM for α_{1D}-KO). Following a washout and equilibration period, a CCRC to 5-HT was constructed (1nM-3μM, all strains).

Protocol used: Chapter 2

A second challenge to PE was made (10μM for WT & α_{1B}-KO; 100μM for α_{1D}-KO and α_{1B}-α_{1D}-KO). In the experiments using antagonists in the α_{1D}-KO (see below), the PE CCRCs were normalised using this response as the maximum.

Initial experiments to establish the sustainability of the PE responses of α_{1D}-KO aortae were performed by constructing a CCRC to PE (100nM-300μM) and then after washout a second CCRC to PE was constructed (100nM-1mM)

The effects of 1nM, 10nM & 100nM prazosin and 1nM, 10nM , 100nM & 1μM 5-MU on the PE-induced contractile responses of the α_{1D}-KO aorta were tested in order to establish pK_B values for prazosin and 5-MU (see below). These experiments were done by incubating the agonist before constructing the first CCRC due to the loss of response on the second curve (see Chapter 2-Results, Figure 2-1, Table 2-1 and Chapter 2-Discussion).

A61603 responses in WT, α_{1B}-KO and α_{1D}-KO aortae were tested by constructing CCRCs (1nM-300μM) both in the absence and presence of 3nM or 30nM prazosin. For consistency across strains the effect of prazosin was tested on the initial CCRC. Where appropriate, a pK_B value for prazosin was estimated. The α_{1B}-WT, α_{1B}-KO and α_{1D}-

KO PE response curves from the previous chapter were used for comparison of the A61603 responses.

The α_{1B}-α_{1D}-KO was studied by constructing CCRCs to PE (100nM-3mM), A61603 (1nM-30μM) or 5-HT (1nM-3μM). Responses were compared with the α_{1B}-WT data from the previous chapter.

The isolation of α_{1B}-ARs and α_{1A}-ARs in the WT response was attempted by using a receptor protection protocol (all data from this protocol was pooled). After the initial agonist testing procedure, selected tissues were incubated with either 10nM BMY 7378 or 10nM 5-MU. 30min. later, 1μM or 10μM chloroethylclonidine (CEC) was added to baths, both with and without BMY 7378 or 5-MU present.

Following a further 30 min. incubation, CEC was washed out by repeated washes at 5 min. intervals over a 15 min. period. In baths where BMY 7378 or 5MU was already present, the CEC was washed using PSS with the BMY 7378 or 5-MU added at the appropriate concentration. Finally, all agonists were removed by repeated washing with normal PSS at 5 min. intervals over a 15 min. period. Following this CCRCs to PE were constructed (1nM-30μM). This protocol is shown in diagrammatic form in Figure I-2.

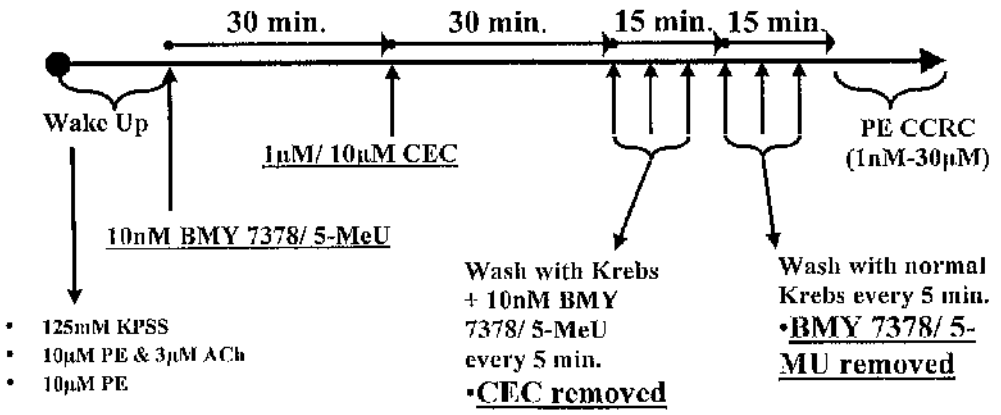


Figure I-2. Diagrammatic representation of the receptor protection protocol used.

Three distinct experiments were performed using the receptor protection protocol.

i. The effect of 10μM CEC and the protective effect of 10nM BMY 7378 on 10μM CEC treated tissues.
ii. The effect of 1μM CEC and the protective effect of 10nM BMY 7378 on 1μM CEC treated tissues.
iii. The effect of 1μM CEC and the protective effect of 10nM 5-MU on 1μM CEC treated tissues.

For each protocol four treatments were considered.

A. Control: no antagonist exposure.
B. BMY 7378/ 5-MU post-removal control: BMY 7378/ 5-MU incubated and washed away to ensure blockade with these antagonists was reversible.
C. CEC treated post-removal: CEC treatment alone with no other antagonists present.
D. CEC treated, BMY 7378/ 5-MU protected post-removal: CEC treatment in baths where BMY 7378 or 5-MU was present in order to protect the α_{1D}-AR and the α_{1A}-AR response respectively.

Protocol used: Chapter 3

Synergy studies were conducted in the WT by constructing CCRCs to PE (1nM-30μM) and then constructing a second PE CCRC in either control aortic rings or 5-HT treated rings (either 10nM or 30nM). Similarly 5-HT CCRCs (1nM-3μM) were constructed, then second CCRCs in the absence and presence of 10nM or 30nM PE were constructed. Synergist doses were added 10-minutes prior to the construction of the CCRC. Where the synergist treatment resulted in a contractile response, the magnitude of the CCRC was measured from this elevated baseline

The α_1-AR-mediated actions of 5-HT (1nM to 3μM) in the WT were examined by testing 5-HT sensitivity to 1nM, 10nM & 100nM prazosin and BMY 7378. 5-HT responses in the α_{1D}-KO were tested against prazosin (control, +1nM prazosin, +10nM prazosin & +100nM prazosin). For consistency, antagonist effects were tested on the first CCRC as initial experiments in the α_{1D}-KO showed a tendency for desensitisation, when a second CCRC to 5-HT was constructed (data not shown).

Serotonergic responses were subtyped by constructing CCRCs to 5-HT (1nM to 3μM) and BRL 54443, 5-HT_{2A} receptor agonist (1nM to 30μM). The effects of a selective concentration of ritanserin (10nM; 5-HT_{2A} receptor antagonist) on the 5-HT and BRL 54443 responses were investigated in both the WT and the α_{1D}-KO. The effects of 10nM ritanserin on 10μM PE and 100nM U46619 responses were also tested, in order to confirm selectivity of blockade.

Protocol used: Chapter 4

In this set of experiments 100μM N_{ω}-nitro-L-arginine methyl ester (L-NAME) was added to randomly selected baths for a further 40 min. before any agonist challenges were made (prior to the 'wake-up' protocol. Tissue viability was then tested as before (KCl, PE, ACh and 5-HT responses in the absence and presence of 100μM L-NAME).

CCRCs were then constructed for either PE (1nM-30μM for WT and α_{1B}-KO; 100nM-300μM for α_{1D}-KO) or 5-HT (1nM-3μM) in the absence and presence of 100μM L-NAME .

The involvement of α_2-ARs in the PE mediated response was investigated by testing the effects of 30nM rauwolscine on the PE response in both L-NAME treated and non-treated WT aortae (PE curves: 1nM-30μM).

Equilibration periods

A 30 min. equilibration period was allowed between single agonist challenges or successive CCRCs. With the exception of the receptor protection protocol (See previous *Chapter 2* section and Chapter 2- Introduction), antagonists were allowed 40 min. incubation before the CCRCs were constructed. Washouts using PSS were repeated every 5 min. until the initial baseline was re-established. Responses recorded were the isometric forces generated by the tissues and were expressed in grams.

Data recording, graphing and statistics

Responses were recorded either mechanically on a Linseis L6514-II 4-channel pen recorder or digitally via an ADI Powerlab 4/20 analogue-digital converter linked to a PC running the data capture software, Chart™. Calibration of the equipment, using a 1.0 gram force, was done regularly and little change in sensitivity of the equipment was observed.

Using Graphpad Prism, data from replicate experiments were grouped and the mean, standard deviation, standard error of the mean (S.E.M.) and 95% confidence intervals for responses at individual x-values were calculated. For normalised data, data was first normalised and then statistical operations were performed. Graphs were then constructed using the mean values and with the S.E.M values used to add in the error bars.

Graphpad Prism was also used in order to mathematically fit sigmoidal-curves to the mean data using non-linear regression. The equation for the fit is shown in Fig I-3.

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{Hillslope}(\log EC_{50} - X))}}$$

Fig I-3. The 'four parameter logistic equation' used by Graphpad Prism in order to perform a nonlinear regression, where X is the logarithm of the concentration of drug and Y is the response which starts at the bottom and rises to the top in a sigmoidal shape.

The non-linear regression returns EC_{50} values, Hill slopes and the errors of estimation for both. However only the Hill slopes were recorded, but not used for statistical analysis. The error values generated by this method were the error of the estimation of fit of the non-linear regression and not necessarily the geometric mean of the data spread. Therefore the Hill slopes were not statistically compared. However, it was deemed appropriate to calculate the geometric mean of the EC_{50} values of individual curves so that statistical analysis could be reliably performed.

pEC_{50} values were estimated by first normalising responses (y) of individual curves to their own maximum and expressing them as a percentage of maximum. pEC_{50}'s could then be calculated for individual experiments by returning the solution for the equation shown in figure I-4. The equation describes a straight line intercept equation using the x and y values both immediately below (x_1, y_1) and immediately above (x_2, y_2) the y = 50%.

$$pEC50 = -\left(\frac{x_1(y_2 - Y) + x_2(Y - y_1)}{(y_2 - y_1)}\right)$$

Fig I-4. The straight line equation used to calculate pEC_{50} values. In order to calculate the pEC_{50}, Y was substituted with '50' and the pEC_{50} was returned on solving the equation. Where pEC_{25} values are shown, Y was replaced in the equation with '25'.

Individual values for the maximal responses (E_{max}) and the pEC_{50} or pEC_{25} values were grouped and entered into Graphpad Prism. Statistical comparisons were done using either Student's t-test (unpaired) or a one-way-ANOVA with Bonferroni's multiple comparisons test. CCRCs were compared using a two-way-ANOVA with a Bonferroni post-test. A 'p' value less than 0.05 was taken to be statistically significant. Where no statistical significance was observed, it is clearly stated.

Graphs are shown throughout with the Y-axis units in grams, percentage of the maximum response (normalised to own curve maximum; % max) or percentage of the wake-up maximum (normalised to maximum response of single agonist challenge during wake-up protocol; % wake-up max) where appropriate. Tables throughout display the mean ± S.E.M except where Hill slopes are shown, in which case the mean is shown with the 95% confidence interval shown in parenthesis. The 'n' number (total number of animals) for each set of experiments is also given throughout.

Solutions used

PSS composition (in mM): 119 NaCl, 4.7 KCl, 1.2 $MgSO_4.H_2O$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, 2.5 $CaCl_2$, and 11.1 glucose.

125mM K^+ PSS composition (in mM): 125 KCl, 1.2 $MgSO_4.H_2O$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, 2.5 $CaCl_2$, and 11.1 glucose.

Drugs used

All drugs used were of analytical grade and purchased from SIGMA™ (unless otherwise stated). The following stock solutions were made from crystalline form using de-ionised H_20 (unless otherwise stated). All further dilutions were made in de-ionised H_20.

Agonists:

- 5-HT, 5-hydroxytryptamine (*3-[2-Aminoethyl]-5-hydroxyindole*): 10mM
- A61603 (*N-[5-(4,5-Dihydro-1H-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide*)- purchased from TOCRIS™: 100mM
- ACh (*Acetylcholine*): 10mM
- BRL 54443 (*3-(1-Methylpiperidin-4-yl)-1H-indol-5-ol*): 10mM
- PE, phenylephrine (*(R)-(-)-1-(3-Hydroxyphenyl)-2-methylaminoethanol*): 100mM
- U46619 (*9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2α}$*)- purchased from CALBIOCHEM™: liquid form made to 1mM in ethanol

Antagonists:

- 5-MU, 5-methylurapidil (*5-Methyl-6[[3-[4-(2-methoxyphenyl)-1-piperazinyl]-propyl]amino]-1,3-dimethyluracil*): 100μM
- BMY 7378 (*8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione*): 1mM
- CEC, chloroethylclonidine (*2-[2,6-Dichloro(N-β-chloroethyl-N-methyl)-4-aminomethyl]phenylimino-2-imidazolidine*) : 100mM
- L-NAME, (N_{ω}-*Nitro-L-arginine methyl ester*): 100mM
- Prazosin (*1-(4-Amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine*): 1mM
- Rauwolscine (*17α-Hydroxy-20α-yohimban-16β-carboxylic acid methyl ester*): 100μM
- Ritanserin (*6-[2-[4-(bis(4-Fluorophenyl)methylene]-1-piperidinyl]ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one*): 1mM in dimethylsulfoxide

Note: Due to the photosensitive nature of BRL 54443, care was taken during preparation and use to minimise exposure to light.

Chapter 1. Contractile responses of the mouse aorta: Effect of age and α_1-AR knockout.

Introduction- Chapter 1

Cardiovascular studies in mice

Murine models are increasingly being used in cardiovascular studies, primarily due to the numerous functional knockouts, over-expresse and transgenic mice that are now available. Recently, Russell & Watts (2000) published an extensive agonist profile of the "Vascular reactivity of the isolated thoracic aorta of the C57/BL/6J mouse." Perhaps surprisingly, such a study had previously not been performed and a useful reference of the specific activity of the mouse aorta to various vasoactive agents was provided. Both NE and phenylephrine (PE) were shown to be potent agonists, indicating that the mouse aorta had a significant α_1-AR mediated contractile response. In addition, it was observed that the aorta exhibited both a high degree of sensitivity and a substantial contractile response to 5-HT.

α_1-Adrenoceptors in the mouse aorta

Yamamoto & Koike (2001), pharmacologically characterised the adrenergic response of the mouse aorta, and suggested that the α_{1D}-AR is the major vasoconstrictor and the α_{1B}-AR has a minor vasoconstrictor role. The use of genetically altered mice has confirmed this, as Tanoue *et al.* (2000) demonstrated the α_{1D}-KO mouse aorta was approximately 50-fold and 40-fold less sensitive than the WT for NE and PE respectively. The ability of BMY 7378 (α_{1D}-AR selective antagonist) to competitively block the NE response in WT aorta was absent in the α_{1D}-KO aorta.

Cavalli *et al.* (1997) used the α_{1B}-KO mouse and observed a significantly reduced contraction in the mouse aorta, to about 75% of that observed in the WT, and suggested that the α_{1B}-AR plays an important role in contraction of the aorta. However, Daly *et al.* (2002) performed a detailed pharmacological study of four arteries from WT and α_{1B}-KO mice, namely the aorta, carotid artery, caudal (tail) artery and, 1st-order mesenteric artery. The authors concluded there that there was no significant role for the α_{1B}-AR in contractile responses as none of the four arteries studied had significantly reduced responses to PE. Some of the data provided in this chapter was included in the publication by Daly *et al.* (2002).

The use of transgenic mice has proved extremely useful in subtyping responses but detailed pharmacological analysis is still required. It is unknown if and what compensatory changes may take place where an important functional receptor is lost. Compensatory changes may also be affected by the age of the mice used in the study. Indeed, Deighan & McGrath (2002) previously demonstrated that expression of hepatic α_1-ARs was affected by age in α_{1B}-KO mice.

The effect of age on vasculature

The effect of senescence on vascular structure and function is of particular interest as advanced age is associated with an increased likelihood of the onset of various cardiovascular diseases. Many studies of age-related vascular changes in structure and function have been performed in numerous vessel preparations from several species (reviewed by Docherty, 1990; Marin, 1995; and Marin *et al.*, 1999).

In general ageing is associated with an increased vascular stiffness due to an increase in medial thickness (Virmani *et al.*, 1991) of the vessel and alterations in functional responses, both contractile and relaxant (see review by Docherty *et al.*, 1990). The results however are often variable, as different groups have report different findings. A more detailed discussion on the effects of ageing is provided in the Discussion section.

Aim of study

The aim was to establish if there were any age-related changes in vasoconstrictor and vasorelaxant responses both in WT and KO mice. Both WT and α_{1B}-KO and α_{1D}-KO mice were used in order to establish the differences as a result of receptor α_1-AR KO and ensure that these differences are maintained with age. In order to do this, both 4-month-old and 16-month-old mice were studied and cumulative concentration curves to PE constructed.

In order to ensure the loss of functional an α_1-AR did not have an affect on overall contractility, the effect of 5-HT and KCl were studied as examples of receptor-mediated and depolarising-induced contractions respectively. Furthermore the vasorelaxant response to ACh was also assessed.

Results- Chapter 1

KCl contractile responses

Contractile responses to 125mM KCl were: 4m α_{1B}-WT= 0.63 ± 0.03g (n = 18), 16m α_{1B}-WT = 0.79 ± 0.04g (n = 12), 4m α_{1B}-KO = 0.68 ± 0.06g (n = 13), 16m α_{1B}-KO = 0.90 ± 0.06g (n = 7), 4m α_{1D}-WT were 0.77 ± 0.06g (n = 5), 16m α_{1D}-WT 0.75 ± 0.04g (n = 12), 4m α_{1D}-KO = 0.66 ± 0.03g (n = 36) and 16m α_{1D}-KO = 0.67 ± 0.03g (n = 12). These results are shown in graphical form in Figure 1-1 and tabulated in Table 1-1.

KCl contractile responses: Effect of age

The 16m α_{1B}-WT KCl-induced response was 25% greater than the 4m α_{1B}-WT responses ($p<0.05$). Similarly, the 16m α_{1B}-KO KCl maximum response was 33% greater than the 4m α_{1B}-KO KCl response ($p<0.05$). However, 4m α_{1D}-WT mice had a similar magnitude of response as the 16m α_{1D}-WT, and the α_{1D}-KO was not significantly different at 4m or 16m age-points (one-way-ANOVA, Bonferroni post-test).

KCl contractile responses: Effect of adrenoceptor-knockout

The loss of the α_{1B}-AR (α_{1B}-KO) had no effect on the 125mM induced contractions at the 4m age-point or at the 16m age-point. Likewise the lack of functional α_{1D}-ARs (α_{1D}-KO) did not affect the response to KCl in 4m mice or in 16m mice. The α_{1B}-WT and α_{1D}-WT were not significantly different in their contractile responses to KCl at either age point. Generally, 125mM KCl-induced contractile responses were consistent and not affected by the loss of a functional α_1-AR or by the mouse strain (one-way-ANOVA, Bonferroni post-test).

Acetylcholine-mediated relaxant responses

The relaxant responses to ACh in the various groups were: 4m α_{1B}-WT = 51.3 ± 2.6% decrease in tone (n = 18), 16m α_{1B}-WT = 55.2 ± 4.4% (n = 12), 4m α_{1B}-KO = 61.2 ± 4.6% (n = 13), 16m α_{1B}-KO = 37.6 ± 6.1% (n = 7), 4m α_{1D}-WT = 63.1 ± 6.1% (n = 5), 16m α_{1D}-WT = 54.1 + 2.6% (n = 12), 4m α_{1D}-KO = 72.6 ± 4.6% (n = 36) and 16m α_{1D}-KO = 57.5 ± 5.4% (n = 12). These results are shown in graphical form in Figure 1-2 and tabulated in Table 1-2.

Acetylcholine-mediated relaxant responses: Effect of age

There was no significant difference in ACh-induced relaxations between the 4m α_{1B}-WT and 16m α_{1B}-WT. The ACh relaxant response responses of 16m α_{1B}-KO aortae were 38% less than 4m α_{1B}-KO relaxant responses ($p<0.01$). The 16m α_{1D}-WT had similar responses to their 4m α_{1D}-WT, whilst the 16m α_{1D}-KO had a 21% reduced relaxant response compared to the 4m α_{1D}-KO ($p<0.05$: one-way-ANOVA, Bonferroni post-test).

Acetylcholine-mediated relaxant responses: Effect of adrenoceptor-knockout

The α_{1B}-WT and α_{1B}-KO had similar relaxant response at 4m and at 16m. The α_{1D}-KO was not significantly different from its WT counterpart at 4m or at 16m age-points. The two WT strains exhibited similar relaxations to ACh at both age points. No significant differences in ACh relaxations as a result of age or strain differences were observed (one-way-ANOVA, Bonferroni post-test).

Adrenergic and serotonergic responses of the α_{1B}-WT

PE-induced contractile responses of the α_{1B}-WT had the following maxima: 4m = 0.74 ± 0.05g (n = 18) and 16m = 0.89 ± 0.04g (n = 12). Sensitivity (pEC_{50}) was: 4m = 6.33 + 0.07 and 16m = 6.34 ± 0.08, whilst the Hill slopes were: 4m = 0.60 (*95% Confidence Interval: 0.49 -0.74*) and 16m = 0.54 (*0.44-0.63*). The Hill slopes did not overlap with unity (1.0) at either age-point.

α_{1B}-WT serotonergic (5-HT) contractile E_{max} values were: 4m = 1.27 ± 0.15g (n = 15), 16m = 1.28 ± 0.07g (n = 11). The pEC_{50} values were: 4m = 6.91 ± 0.04 and 16m = 6.78 ± 0.05 and the Hill slopes were: 4m = 1.12 (*0.96-1.28*) and 16m = 1.02 (*0.87-1.74*). The Hill slopes overlapped with unity.

The response curves for PE and 5-HT of the α_{1B}-WT are shown in Figure 1-3 and the values shown above are tabulated in Table 1-3.

Adrenergic and serotonergic responses of the α_{1B}-KO

α_{1B}-KO adrenergic contractile responses were: E_{max}- 4m = 0.97 ± 0.19 (n = 16) and 16m = 1.03 ± 0.06 (n = 12); pEC_{50}- 4m = 6.73 ± 0.08 and 16m = 6.62 ± 0.09; Hill slopes- 4m = 0.61 (*0.49-0.72*) and 16m = 0.53 (*0.38-0.68*). The Hill slopes did not overlap with unity at either age-point.

α_{1B}-KO serotonergic contractile responses were: E_{max}- 4m = 1.36 ± 0.17 (n = 13) and 16m = 1.35 ± 0.09 (n = 11); pEC_{50}- 4m = 7.01 ± 0.12 and 16m = 6.89 ± 0.08; Hill slopes- 4m = 1.03 (*0.93-1.13*) and 16m = 0.98 (*0.67-1.28*). The Hill slopes overlapped with unity.

The response curves for PE and 5-HT of the α_{1B}-KO are shown in Figure 1-4 and the values shown above are tabulated in Table 1-4.

Adrenergic and serotonergic responses of the α_{1D}-WT

α_{1D}-WT adrenergic contractile responses were: E_{max}- 4m = 0.81 ± 0.08 (n = 5) and 16m = 0.66 ± 0.06 (n = 7); pEC_{50}- 4m = 6.49 ± 0.07 and 16m = 6.75 ± 0.06; Hill slopes- 4m = 0.77 (*0.63-0.91*) and 16m = 0.98 (*0.78-1.18*). The Hill slopes did not overlap with unity at 4m but overlapped with unity at 16m age-points.

α_{1D}-WT serotonergic contractile responses were: E_{max}- 4m = 1.27 ± 0.13 (n = 5) and 16m = 1.43 ± 0.09 (n = 6); pEC_{50}- 4m = 7.08 ± 0.09 and 16m = 7.02 ± 0.06; Hill slopes- 4m = 1.39 (*0.78-2.00*) and 16m = 1.20 (*1.14-1.26*). The Hill slopes for the 4m α_{1D}-WT overlapped with unity although the 16m α_{1D}-WT which did not overlap with unity.

The response curves for PE and 5-HT of the α_{1D}-WT are shown in Figure 1-5 and the values shown above are tabulated in Table 1-5.

Adrenergic and serotonergic responses of the α_{1D}-KO

α_{1D}-KO adrenergic contractile responses were: E_{max}- 4m = 0.66 ± 0.04 (n = 36) and 16m = 0.51 ± 0.04 (n = 12); pEC_{50}- 4m = 4.97 ± 0.04 and 16m = 5.11 ± 0.07; Hill slopes- 4m = 0.81 (*0.59-1.04*) and 16m = 0.95 (*0.69-1.21*). The Hill slopes overlapped with unity.

α_{1D}-KO serotonergic contractile responses were: E_{max}- 4m = 1.15 ± 0.11 (n = 16) and 16m = 1.24 ± 0.07 (n = 11); pEC_{50}- 4m = 7.18 ± 0.06 and 16m = 7.00 ± 0.08; Hill slopes- 4m = 1.56 (*1.21-1.91*) and 16m = 1.70 (*1.43-1.97*). The Hill slopes overlapped with unity.

The response curves for PE and 5-HT of the α_{1D}-KO are shown in Figure 1-6 and the values shown above are tabulated in Table 1-6.

Adrenergic and serotonergic responses: Effect of Age

No significant change in adrenergic responses between the 4m age point and 16m age points within each strain was observed. Similarly, serotonergic responses were not significantly different at the two age-points chosen to study (Student's t-test). The age comparisons can be seen in Figures 1-3, 1-4, 1-5 and 1-6 for the α_{1B}-WT, α_{1B}-KO, α_{1D}-WT and α_{1D}-KO respectively.

Serotonergic responses: Effect of adrenoceptor-knockout

The loss of the α_{1B}-AR did not alter serotonergic responses in the α_{1B}-KO as α_{1B}-WT and α_{1B}-KO were comparable in size and sensitivity at both 4m and 16m (one-way-ANOVA, Bonferroni post-test). Similarly, serotonergic responses were unaltered by a lack of functional α_{1D}-ARs as the α_{1D}-KO and its WT counterpart, the α_{1D}-WT, had similar contractile responses at both age points (one-way-ANOVA, Bonferroni post-test).

The serotonergic responses of the α_{1B}-WT, α_{1B}-KO, α_{1D}-WT and α_{1D}-KO at the 4m age point are shown in Figure 1-7 and the values for E_{max}, pEC_{50} and Hill slopes are tabulated in Table 1-7. 5-HT responses of the α_{1B}-WT, α_{1B}-KO, α_{1D}-WT and α_{1D}-KO at the 16m age point are shown in Figure 1-8 and the values for E_{max}, pEC_{50} and Hill slopes are tabulated in Table 1-8.

Adrenergic responses: Effect of adrenoceptor-knockout

The 4m α_{1B}-KO exhibited a 3-fold increase in sensitivity compared to the 4m α_{1B}-WT ($p<0.01$). However the 16m α_{1B}-KO was not different from the 16m α_{1B}-WT. Maximum responses were not significantly different in either strain (one-way-ANOVA, Bonferroni post-test).

The α_{1D}-KO was 33-fold less sensitive at 4m ($p<0.001$) and 44-fold less sensitive at 16m ($p<0.001$) to PE compared to the α_{1D}-WT. Maximum responses of α_{1D}-KO mice were significantly smaller than WT at both 4m (19% less: $p<0.05$) and 16m (23% less: $p<0.05$: one-way-ANOVA, Bonferroni post-test). The 4m α_{1D}-WT had hill a Hill slope significantly less than unity, whilst the 16m α_{1D}-WT, 4m α_{1D}-KO and 16m α_{1D}-KO all had hill slopes that were not different from unity.

The adrenergic responses of the α_{1B}-WT, α_{1B}-KO, α_{1D}-WT and α_{1D}-KO at the 4m age point are shown in Figure 1-9 and the values for Emax pEC_{50} and Hill slope are tabulated in Table 1-9. 5-HT responses of the α_{1B}-WT, α_{1B}-KO, α_{1D}-WT and α_{1D}-KO at the 16m age point are shown in Figure 1-10 and the values for E_{max}, pEC_{50} and Hill slope are tabulated in Table 1-10.

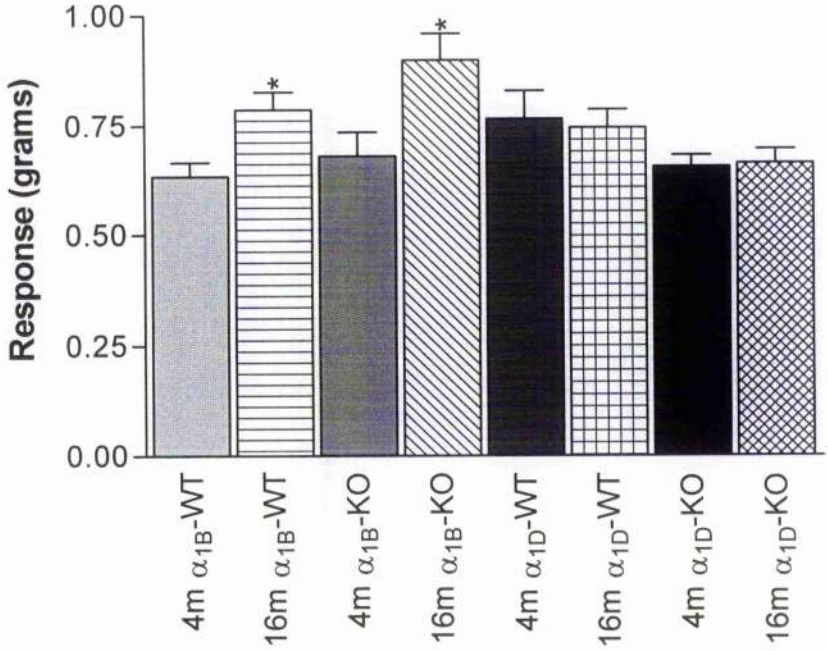


Figure 1-1. Maximum responses of 4-month-old & 16-month-old α_{1B}-WT, α_{1B}-KO, α_{1D}-WT & α_{1D}-KO aortae to a single 125mM KCl challenge.

Columns shown are the mean ± S.E.M of the generated responses expressed in grams (*$p<0.05$ against strain matched 4m aortae: one-way-ANOVA, Bonferroni post-test).

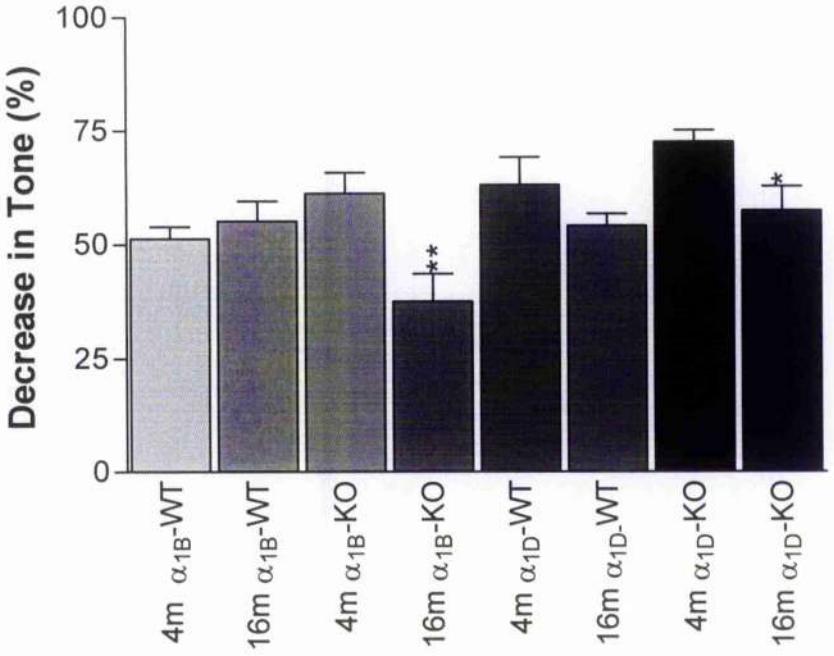


Figure 1-2. Relaxant responses to 3μM ACh (PE-induced precontraction) of 4-month-old & 16-month-old α_{1B}-WT, α_{1B}-KO, α_{1D}-WT & α_{1D}-KO aortae.

Columns shown are the mean ± S.E.M and expressed as a percentage decrease in tone (*$p<0.05$ age-matched WT: one-way-ANOVA, Bonferroni post-test).

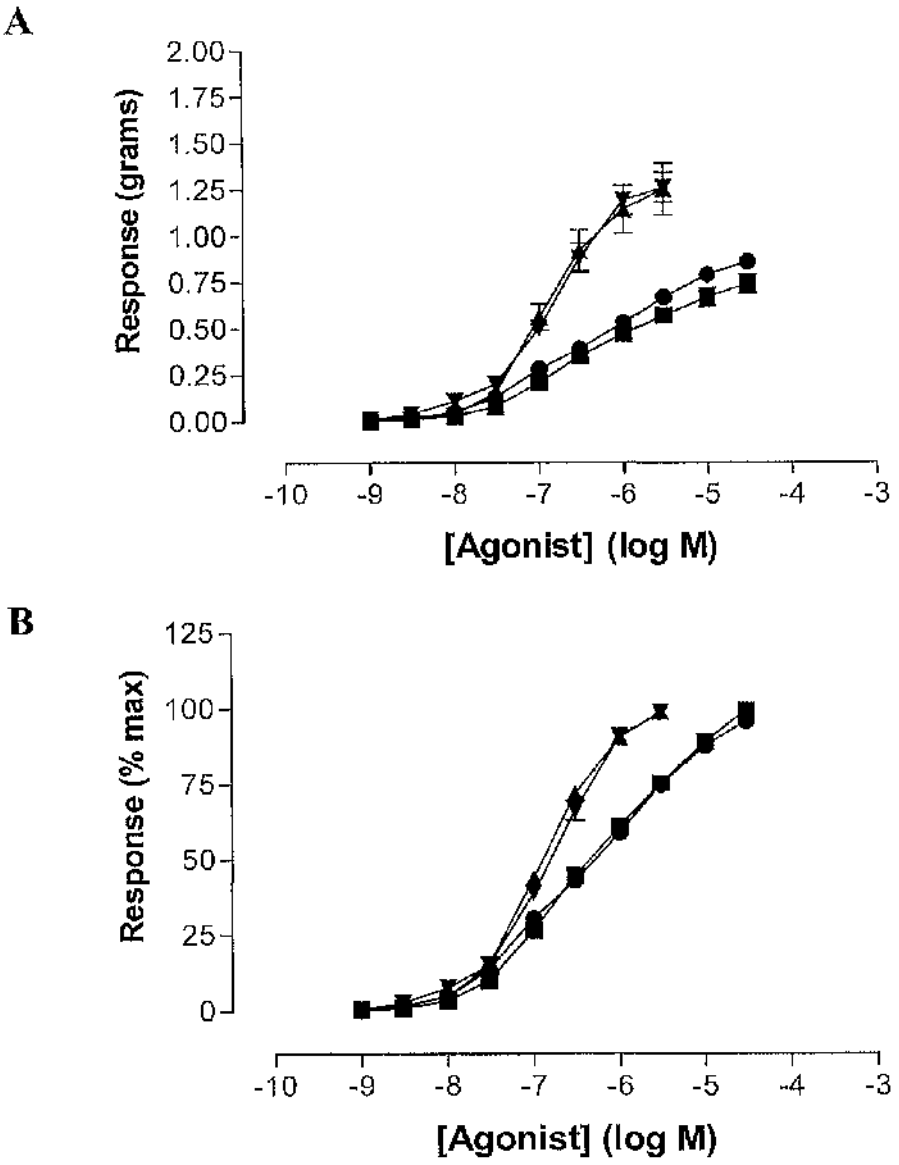


Figure 1-3. Contractile responses of 4-month-old & 16-month-old α_{1B}-WT aortae induced by PE (■; n =18 & ●; n =12 for 4m and 16m respectively) & 5-HT (▲; n =15 & ▼; n =11 for 4m and 16m respectively).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M (No statistical difference comparing 4m and 16m age points: two-way-ANOVA, Bonferroni post-test).

A

	E_{max} (grams)	
	PE	5-HT
4m α_{1B}-WT	0.74 ± 0.05, *18*	1.27 ± 0.15, *15*
16m α_{1B}-WT	0.89 ± 0.04, *12*	1.28 ± 0.07, *11*

B

	pEC_{50}	
	PE	5-HT
4m α_{1B}-WT	6.33 ± 0.07	6.91 ± 0.04
16m α_{1B}-WT	6.34 ± 0.08	6.78 ± 0.05

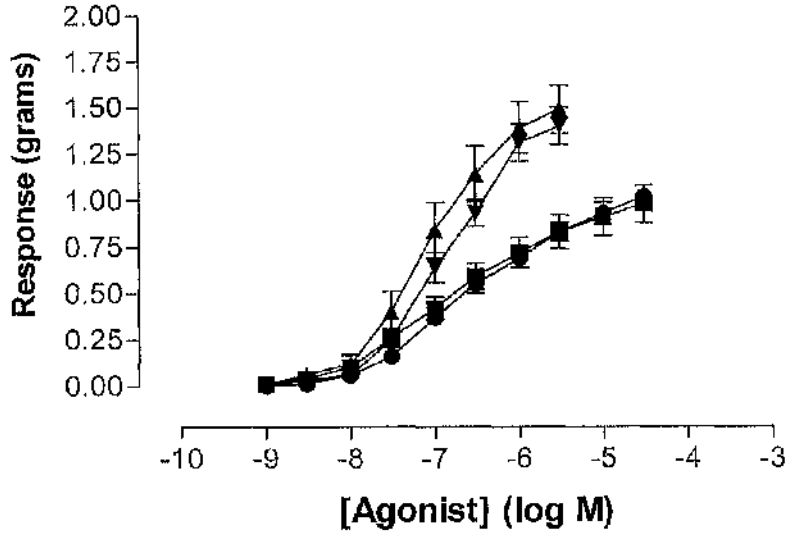
C

	Hill slope	
	PE	5-HT
4m α_{1B}-WT	0.60 (*0.49-0.74*)	1.12 (*0.96-1.28*)
16m α_{1B}-WT	0.54 (*0.44-0.63*)	1.02 (*0.87-1.74*)

Table 1-3. PE-induced & 5-HT-induced contractile responses of 4-month-old & 16-month-old α_{1B}-WT aortae.

The maximum responses generated, expressed in grams followed by the '*n*' number (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical difference comparing 4m and 16m age points: Student's t-test).

A



B

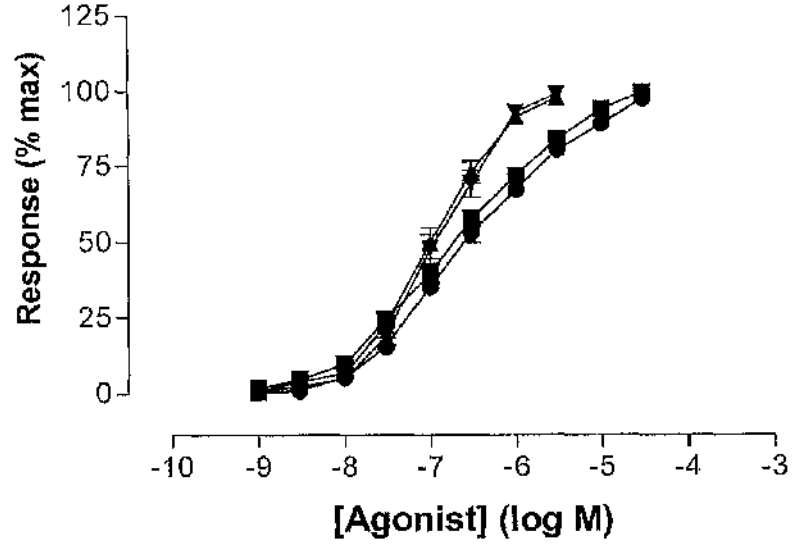


Figure 1-4. Contractile responses of 4-month-old & 16-month-old α_{1B}-KO aortae induced by PE (■; n =16 & ●; n =12 for 4m and 16m respectively) & 5-HT (▲; n =13 & ▼; n =11 for 4m and 16m respectively).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M (No statistical difference comparing 4m and 16m age points: two-way-ANOVA, Bonferroni post-test).

A

	E_{max} (grams)	
	PE	5-HT
4m α_{1B}-KO	0.97 ± 0.09, *16*	1.36 ± 0.17, *13*
16m α_{1B}-KO	1.03 ± 0.06, *12*	1.35 ± 0.09, *11*

B

	pEC_{50}	
	PE	5-HT
4m α_{1B}-KO	6.73 ± 0.08	7.01 ± 0.12
16m α_{1B}-KO	6.62 ± 0.09	6.89 ± 0.08

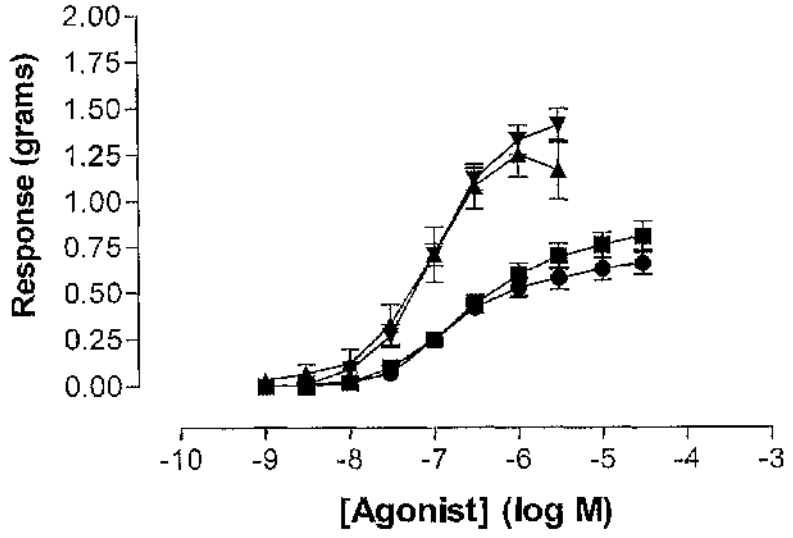
C

	Hill slope	
	PE	5-HT
4m α_{1B}-KO	0.61 (*0.49-0.72*)	1.03 (*0.93-1.13*)
16m α_{1B}-KO	0.53 (*0.38-0.68*)	0.98 (*0.67-1.28*)

Table 1-4. PE-induced & 5-HT-induced contractile responses of 4-month-old & 16-month-old α_{1B}-KO aortae.

The maximum responses generated, expressed in grams followed by the '*n*' number (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical difference comparing 4m and 16m age points: Student's t-test).

A



B

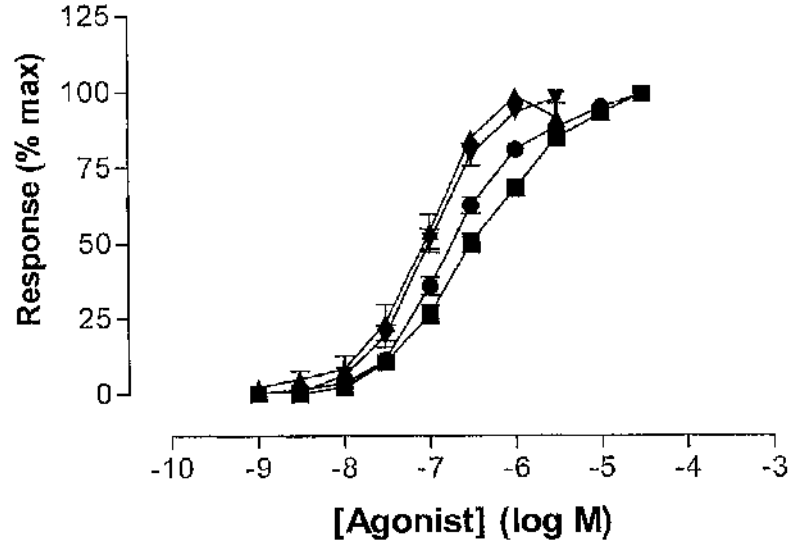


Figure 1-5. Contractile responses of 4-month-old & 16-month-old α_{1D}-WT aortae induced by PE (■; n =5 & ●; n =7 for 4m and 16m respectively) & 5-HT (▲; n =5 & ▼; n =6 for 4m and 16m respectively).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M (No statistical difference comparing 4m and 16m age points: two-way-ANOVA, Bonferroni post-test).

A

	E_{max} (grams)	
	PE	5-HT
4m α_{1D}-WT	0.81 ± 0.08, *5*	1.27 ± 0.13, *5*
16m α_{1D}-WT	0.66 ± 0.06, *7*	1.43 ± 0.09, *6*

B

	pEC_{50}	
	PE	5-HT
4m α_{1D}-WT	6.49 ± 0.07	7.08 ± 0.09
16m α_{1D}-WT	6.75 ± 0.06	7.02 ± 0.06

C

	Hill slope	
	PE	5-HT
4m α_{1D}-WT	0.77 (*0.63-0.91*)	1.39 (*0.78-2.00*)
16m α_{1D}-WT	0.98 (*0.78-1.18*)	1.20 (*1.14-1.26*)

Table 1-5. PE-induced & 5-HT-induced contractile responses of 4-month-old & 16-month-old α_{1D}-WT aortae.

The maximum responses generated, expressed in grams followed by the '*n*' number (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical difference comparing 4m and 16m age points: Student's t-test).

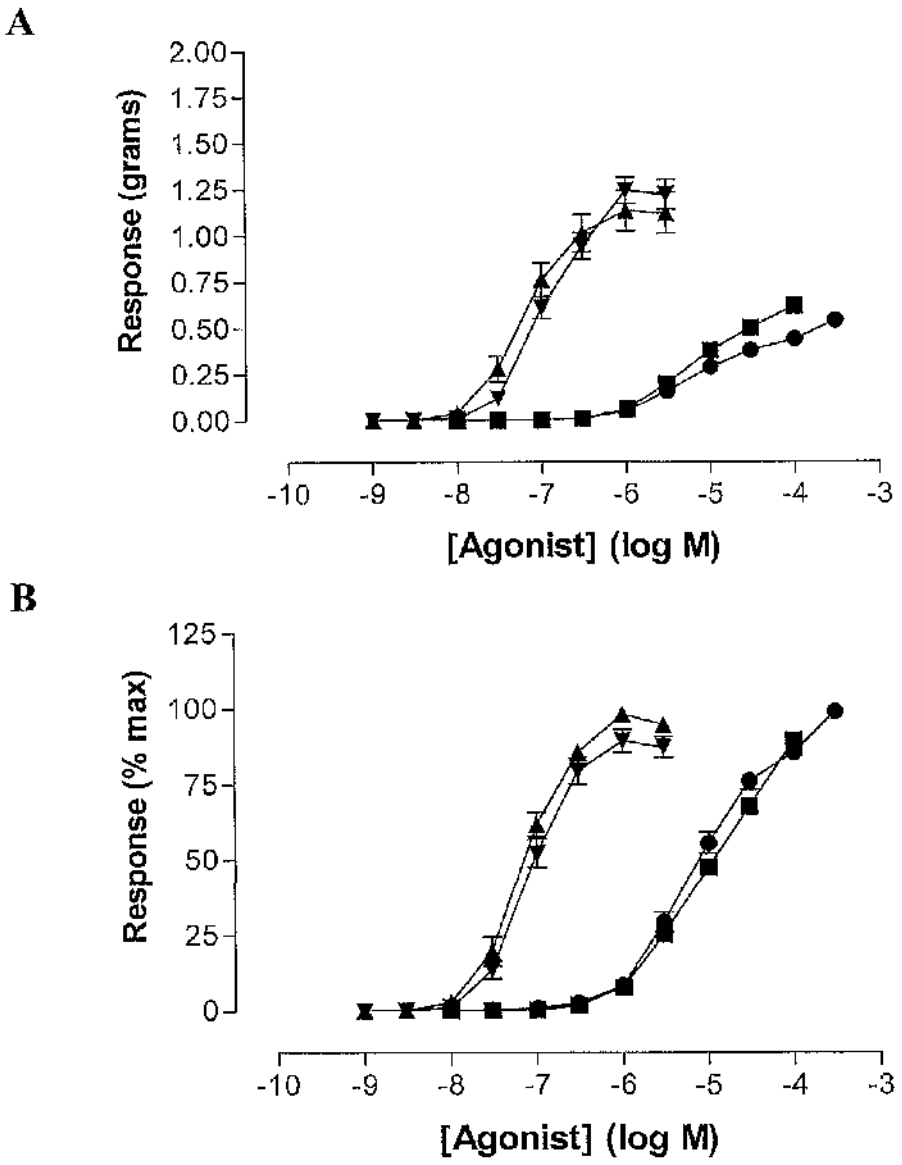


Figure 1-6. Contractile responses of 4-month-old & 16-month-old α_{1D}-KO aortae induced by PE (■; n =36 & ●; n =12 for 4m and 16m respectively) & 5-HT (▲; n =16 & ▼; n =11 for 4m and 16m respectively).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M (No statistical difference comparing 4m and 16m age points; two-way-ANOVA, Bonferroni post-test).

A

	E_{max} (grams)	
	PE	5-HT
4m α_{1D}-KO	0.66 ± 0.04, *36*	1.15 ± 0.11, *16*
16m α_{1D}-KO	0.51 ± 0.04, *12*	1.24 ± 0.07, *11*

B

	pEC_{50}	
	PE	5-HT
4m α_{1D}-KO	4.97 ± 0.04	7.18 ± 0.06
16m α_{1D}-KO	5.11 ± 0.07	7.00 ± 0.08

C

	Hill slopes	
	PE	5-HT
4m α_{1D}-KO	0.81 (*0.59-1.04*)	1.56 (*1.21-1.91*)
16m α_{1D}-KO	0.95 (*0.69-1.21*)	1.70 (*1.43-1.97*)

Table 1-6. PE-induced & 5-HT-induced contractile responses of 4-month-old & 16-month-old α_{1D}-KO aortae.

The maximum responses generated, expressed in grams followed by the '*n*' number (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical difference comparing 4m and 16m age points: Student's t-test).

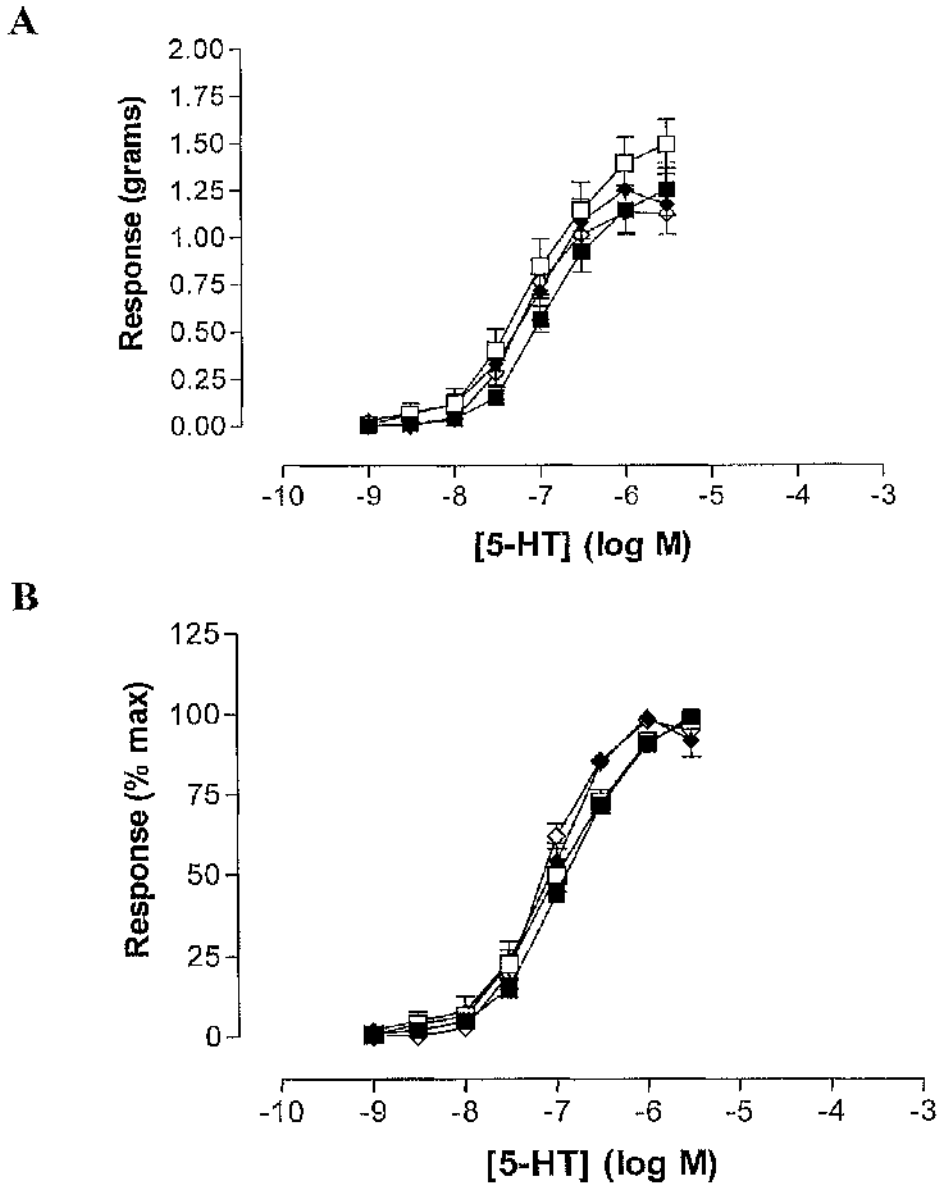


Figure 1-7. 5-HT-induced contractile responses of 4-month old α_{1B}-WT (■; n = 15), α_{1B}-KO (□; n = 13), α_{1D}-WT (◆; n = 5) & α_{1D}-KO (◇; n = 16) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M (No statistical difference comparing appropriate WT and KO: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
α_{1B}-WT	15	1.27 ± 0.15
α_{1B}-KO	13	1.36 ± 0.17
α_{1D}-WT	5	1.27 ± 0.13
α_{1D}-KO	16	1.15 ± 0.11

B

	pEC_{50}	Hill slope
α_{1B}-WT	6.91 ± 0.04	1.12 (*0.96-1.28*)
α_{1B}-KO	7.01 ± 0.12	1.03 (*0.93-1.13*)
α_{1D}-WT	7.08 ± 0.09	1.39 (*0.78-2.00*)
α_{1D}-KO	7.18 ± 0.06	1.56 (*1.21-1.91*)

Table 1-7. 5-HT-induced contractile responses of 4-month-old α_{1B}-WT, α_{1B}-KO, α_{1D}-WT & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed in grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical difference comparing 4m and 16m age points: Student's t-test: Student's t-test).

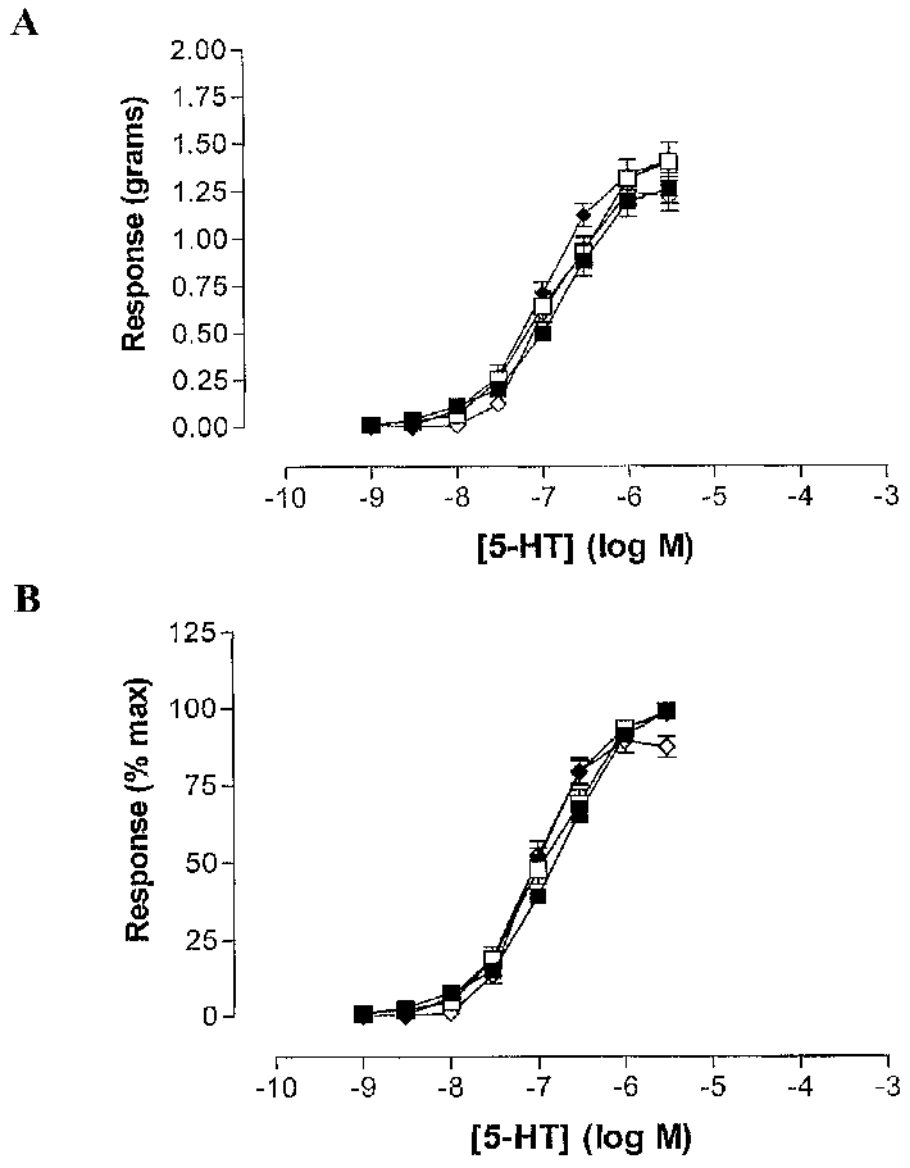


Figure 1-8. 5-HT-induced contractile responses of 16-month old α_{1B}-WT (■; n = 11), α_{1B}-KO (□; n = 11), α_{1D}-WT (◆; n = 6) & α_{1D}-KO (◇; n = 11) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean + S.E.M (No statistical difference appropriate WT and KO: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
α_{1B}-WT	11	1.28 ± 0.07
α_{1B}-KO	11	1.35 ± 0.09
α_{1D}-WT	6	1.43 ± 0.09
α_{1D}-KO	11	1.24 ± 0.07

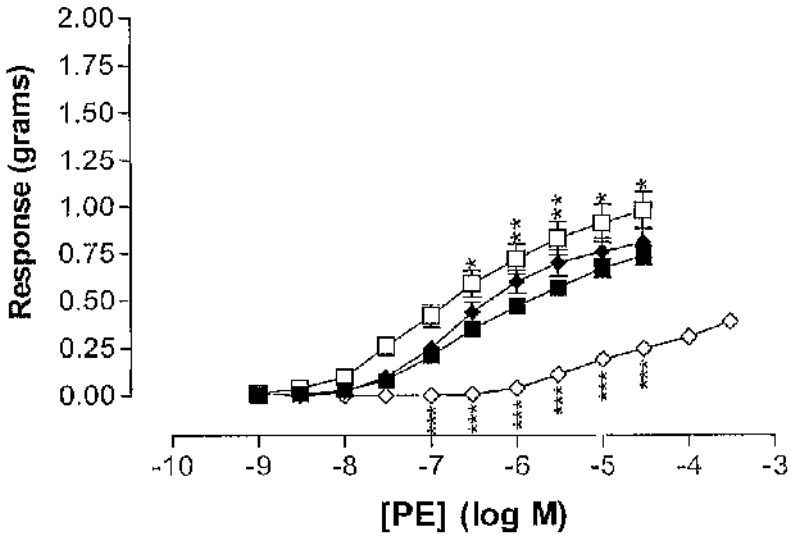
B

	pEC_{50}	Hill slope
α_{1B}-WT	6.78 ± 0.05	1.02 (*0.87-1.74*)
α_{1B}-KO	6.89 ± 0.08	0.98 (*0.67-1.28*)
α_{1D}-WT	7.02 ± 0.06	1.20 (*1.14-1.26*)
α_{1D}-KO	7.00 ± 0.08	1.70 (*1.43-1.97*)

Table 1-8. 5-HT-induced contractile responses of 16-month-old α_{1B}-WT, α_{1B}-KO, α_{1D}-WT & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed in grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical difference comparing 4m and 16m age points: Student's t-test: Student's t-test).

A



B

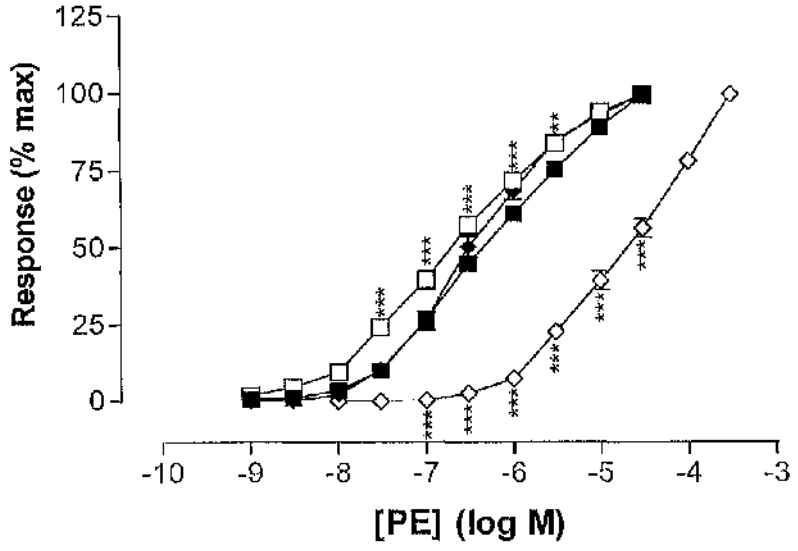


Figure 1-9. PE-induced contractile responses of 4-month old α_{1B}-WT (■; n = 18), α_{1B}-KO (□; n = 13), α_{1D}-WT (◆; n = 5) & α_{1D}-KO (◇; n = 36) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001 appropriate WT and KO: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
α_{1B}-WT	18	0.74 ± 0.05
α_{1B}-KO	16	0.97 ± 0.09
α_{1D}-WT	5	0.81 ± 0.08
α_{1D}-KO	36	0.66 ± 0.04*

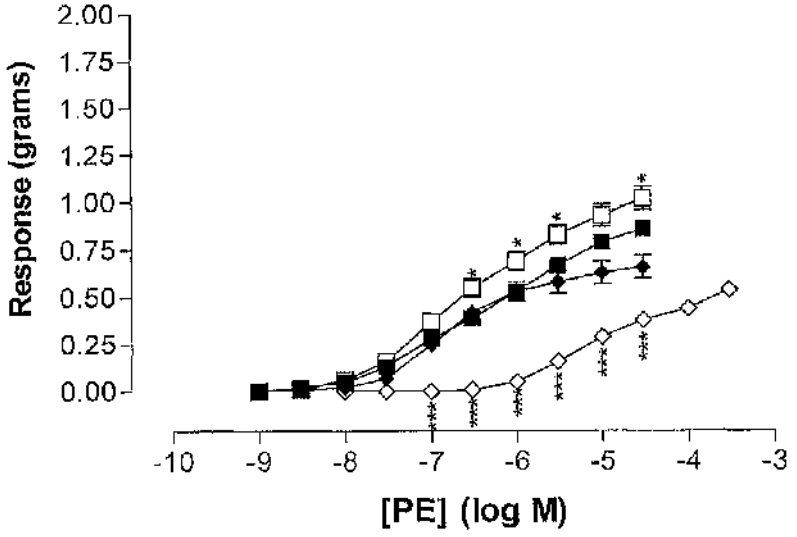
B

	pEC_{50}	Hill slope
α_{1B}-WT	6.33 ± 0.07	0.60 (*0.49-0.74*)
α_{1B}-KO	6.73 ± 0.08**	0.61 (*0.49-0.72*)
α_{1D}-WT	6.49 ± 0.07	0.77 (*0.63-0.91*)
α_{1D}-KO	4.97 ± 0.04***	0.81 (*0.59-1.04*)

Figure 1-9. PE-induced contractile responses of 4-month-old α_{1B}-WT, α_{1B}-KO, α_{1D}-WT & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed in grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*p<0.05, **p<0.01, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

A



B

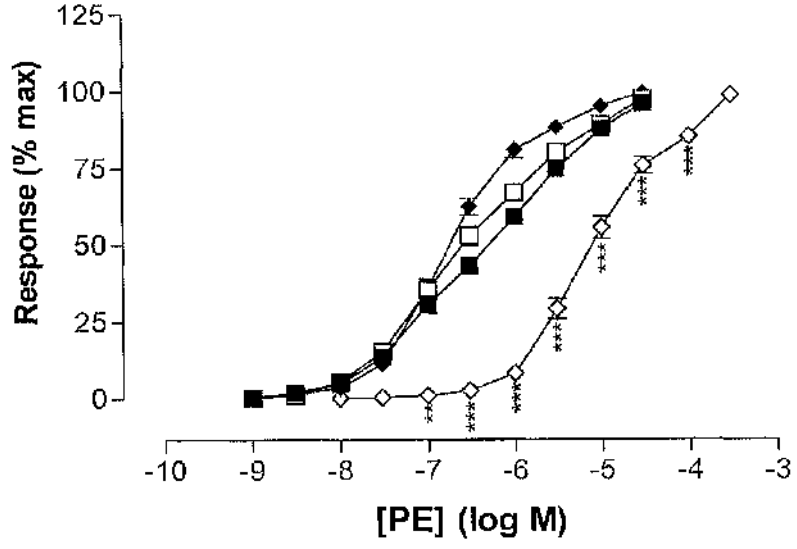


Figure 1-10. PE-induced contractile responses of 16-month old α_{1B}-WT (■; n = 12), α_{1B}-KO (□; n = 12), α_{1D}-WT (◆; n = 7) & α_{1D}-KO (◇; n = 12) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001 comparing appropriate WT and KO: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
α_{1B}-WT	12	0.89 ± 0.04
α_{1B}-KO	12	1.03 ± 0.06
α_{1D}-WT	7	0.66 ± 0.06
α_{1D}-KO	12	0.51 ± 0.04*

B

	pEC_{50}	Hill slope
α_{1B}-WT	6.34 ± 0.08	0.54 (*0.44-0.63*)
α_{1B}-KO	6.62 ± 0.09	0.53 (*0.38-0.68*)
α_{1D}-WT	6.75 ± 0.06	0.98 (*0.78-1.18*)
α_{1D}-KO	5.11 ± 0.07***	0.95 (*0.69-1.21*)

Table 1-10. PE-induced contractile responses of 16-month-old α_{1B}-WT, α_{1B}-KO, α_{1D}-WT & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed in grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*p<0.05, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

Discussion- Chapter 1

Effect of genetic background

The use of transgenic mice necessitates the availability of genetically-matched controls. The effect of genetic background on the cardiovascular system was discussed by Schlager (1966), who measured the systolic blood pressure in eight different inbred strains of mice. Cavalli *et al.* (1997) created the α_{1B}-KO by targeted gene disruption in embryonic stem cells from the SV/129 strain of mice. Stem cells were then microinjected into C57BL/6J blastocysts which were then transferred to pseudo-pregnant females. Tanoue *et al.* (2002) took an almost identical approach in creating the α_{1D}-KO. Thus both KOs had a mixed Sv/129 and C57Bl/6J genetic background. The WT controls were homozygous littermates that were not carrying the disrupted mutant gene.

For this chapter the α_{1B}-KO was compared with its WT, the α_{1B}-WT and the α_{1D}-KO was compared with its WT, the α_{1D}-WT. However the results for both WTs were not significantly different at either 4m or at 16m in their contractile KCl, PE and 5-HT responses or their ACh relaxant responses. Thus in the remaining chapters, the α_{1B}-WT and α_{1D}-WT are both used as WT controls but are not distinguished from one another.

Age-related changes in vascular structure

Structurally, old age appears to be associated with an increase in vascular stiffness (Roach & Burton, 1959) which is more pronounced in large-calibre elastic vessels, such as the aorta, than in muscular vessels, such as the brachial artery (Van der Heijden-Spek *et al.*, 2000) . The loss of distensibility of the vasculature can be clinically measured as an increase in pulse-wave velocity. In general a thickening of intimal-medial layers with increasing age is observed (Virmani *et al.*, 1991), which significantly contributes to the increase in stiffness. However, reductions in vascular compliance may not due to structural changes alone.

Age-related changes in vascular contractile responses

Age-related functional changes of contractile responses vary, dependent on the species and the artery studied. Furthermore, various groups studying the same vessel from the same species have reported dissimilar findings. For example, the effect of age on adrenergic responses in the rat aorta has been shown as a reduction in the potency of NE-induced contractility (Tuttle, 1966; Simpkins *et al.*, 1983). Conversely Hynes & Duckles (1987) have reported that there is no age related change in NE potency in the rat aorta. α_1-adrenoceptor responses in the rabbit aorta have been shown to be unaffected by age (Hayashi & Toda, 1978). Furthermore, 5-HT potency in the rat aorta has been reported to decrease (Docherty, 1988; Wanstall & O'Donnell, 1989) or increase with age (Emmick & Cohen, 1986). Hence, the effect of ageing on vasoconstrictor responses remains unclear in rat aorta but has not been studied in mice.

In the present study the KCl response of the α_{1B}-WT and α_{1B}-KO increased significantly with age, whereas the α_{1D}-WT and α_{1D}-KO did not. This was the first indication of a strain-dependent age related change in vascular function but no age-related change in the maximal response or potency of PE or 5-HT were observed. However, it is important to be aware of the difficulties in interpreting changes in absolute maximal contractile responses.

Structural vascular changes themselves could result in alterations in function, making comparisons of maximum responses unreliable. It may be necessary to normalise using a structural factor such as cross-sectional area, particularly in cases where there is a clear alteration in structure. In the present study, no differences in passive lumen diameter were observed both when no tension had been applied and when tension equivalent to 1.0grams (9.81mN) had been applied. Instead the absolute force generated by the vessel has been used as a comparison.

Thus the α_{1B}-WT and α_{1B}-KO both had an increased contractile response with age but comparison with the other control strain, the α_{1D}-WT, revealed no significant difference at either age point. Such a comparison would have revealed whether the 16m α_{1B}-WT and α_{1B}-KO KCl response was greater than the α_{1D}-WT or that the 4m α_{1B}-WT and

α_{1B}-KO KCl response was less than the α_{1D}-WT, so no sound conclusion could be made.

Importantly, the PE and 5-HT responses in all strains remained unaltered as a result of age, indicating that the receptor mediated contractile mechanism was independent of mouse age. Therefore in the mouse aorta, no significant changes with age, on receptor mediated contractile mechanisms are observed.

Age-related changes in vascular relaxant responses

The age-related alterations of relaxant responses have been extensively studied. In most studies performed, relaxant responses of conductance vessels appear to decrease with age. In particular, reduced relaxation responses mediated by β-AR agonists, such as isoprenaline, are well documented and have been shown in the rat aorta by Simpkins *et al.* (1983). Relaxations mediated by β-AR agonists, are primarily the result of receptor activation of β-AR on vascular smooth muscle cells (VSMCs) which causes them to relax.

The role of the endothelium in ACh-induced relaxations was originally described by Furchgott & Zawadzki (1980). It was discovered that the ACh indirectly leads to VSMC relaxation by initiating the release of nitric oxide (NO) from endothelial cells into surrounding tissue (Palmer *et al.*, 1987). Endothelial cells are now known to release a host of other modulating substances which are vasodilators (endothelium-derived hyperpolarising factor: EDHF) and vasoconstrictors (endothelin).

ACh responses, in general, are reduced with age which appears to be the result of a reduced ability of endothelial cells to release NO (Hongo *et al.*, 1988) as responses to nitrovasodilators are conserved (Tominaga *et al.*, 1994). Some discrepancy does exist in these findings as Hynes and Duckles (1987) found that aortic segments from rats showed an increased responsiveness to the cholinergic mimetic, methacholine.

The β-ARs response was not investigated as this was beyond the scope of this thesis. In hindsight, studies of the alterations of β-AR activity could have provided useful information about age related alterations. The present study does indicate that in control aortae (α_{1B}-WT, α_{1D}-WT), there were no significant age-related changes whilst the α_{1B}-

KO and α_{1D}-KO both had a significantly reduced ACh relaxant response, the significance of which is discussed later. Thus, although this indicated relaxant responses of the mouse aorta to ACh were not age-dependent this conclusion cannot be made from the present study. A more detailed study would be required, perhaps constructing relaxant CCRCs to ACh rather than the single challenge used in the current study. However, in the mouse aorta it appears that endothelial cells retain their ability to release NO and VSMCs retain their ability to relax to NO.

The age-related reduction of the ACh-induced relaxant response of α_{1B}-KO and α_{1D}-KO response initially indicated a clear difference in vasorelaxant responses which were dependent on the loss of a functional α_1-AR. However, once again analysis with the appropriate control strains, the α_{1B}-WT and α_{1D}-WT, revealed no significant difference at either age point. Therefore, there was no indication of whether the observed changes were due to a loss of normal relaxant response at 16m in these two KOs or due to an greater than normal relaxant response at 4m. Therefore, no further discussion is made on this topic.

α_1-ARs KOs: Effect on ACh relaxant responses

The relaxant responses to ACh, were not affected by the absence of functional α_{1B}-ARs or the α_{1D}-ARs. The loss of a vasoconstrictor receptor could upset the fine balance of tone within the vessel which could be compensated by a reduction in vasodilatator release or activity. The α_{1B}-KO was normotensive (Cavalli *et al.*, 1997) and the α_{1D}-KO mice were moderately hypotensive (Tanoue *et al.*, 2002). However, the level of hypotension may have been less than expected due to a homeostatic reduction in vasodilatation. This does not appear to be the case though, as we did not observe a significant alteration in the relaxation responses of these vessels. Hence, no effect of α_1-AR KO on ACh responses was observed.

α_1-ARs KOs: Effect on KCl and serotonergic responses

Neither the loss of the α_{1B}-AR or the α_{1D}-AR in the KO affected the KCl response. 125mM KCl initiates contraction by causing a depolarisation of all the cells in the arterial wall, including endothelial cells that could release NO, but the response is contractile since this depolarisation is mainly VSMCs activation. Therefore 125mM KCl provides a good indication of overall contractile ability. The loss of α_{1B}- or α_{1D}-ARs do not affect the overall contractility demonstrating that the contractile mechanisms of the VSMCs in the aortae of these KOs are intact and functional.

Serotonergic responses, induced by 5-HT, were also unaltered in the α_{1B}-KO and α_{1D}-KO aorta. The responses at 4m and 16m were consistent and not different from their WT counterparts at both these age points. 5-HT acts mainly through the 5-HT_{2A} receptor in the mouse aorta (McKune & Watts, 2001) and has been shown to be a potent and efficacious agonist in the mouse thoracic aorta (Russell & Watts, 2000) so can provide a useful gauge of receptor-mediated contractility of the mouse aorta.

The 5-HT_{2A} receptor is a membrane bound GPCR coupling to $G_{q/11}$ second messengers and raises IP_3 levels and intracellular Ca^{2+} (Martin, 1998). In this respect the 5-HT_{2A} receptor is similar to α_1-ARs which are all known to be $G_{q/11}$ (Byland *et al.*, 1998) coupled GPCRs (discussed in the General Introduction). Hence 5-HT_{2A} receptors and α_1-ARs utilise the initial trigger for their second messenger pathways, but the serotonergic response is unaltered in either KO. The contractile mechanism is intact and functional and the second messengers that the α_{1B}- or α_{1D}-ARs use remain functional so the individual KO had not affected the contractile machinery.

α_1-ARs KOs: Effect on adrenergic responses

Stassen *et al.* (1998) compiled a detailed binding study of α_{1A}-AR distribution in rats and the effect of chemical sympathectomy. They suggested that the α_{1A}-AR is the 'innervated' functional adrenoceptor, likely to be found on arteries with a great degree of sympathetic innervation, such as the mesenteric arteries. Accordingly, the other two receptors, the α_{1B}- and α_{1D}-AR, tend to provide a contractile function in large calibre, conductance arteries. The α_{1D}-AR has been shown to be the major adrenergic

vasoconstrictor in the rat aorta (Kenny *et al.*, 1995; Piascik *et al.*, 1995; Martinez *et al.*, 1999).

Both NE and PE have shown to be potent agonists in the mouse aorta (Russell & Watts 2000). The major adrenergic vasoconstrictor of the mouse aorta has been characterised pharmacologically by Yamamoto *et al.* (2002) and also by using transgenic mice. Pharmacological studies in both the α_{1B}-AR knockout mouse (Daly *et al.*, 2002) and α_{1D}-AR knockout mouse (α_{1D}-KO; Tanoue *et al.*, 2002) have been performed, with all groups agreeing that the adrenergic response was predominantly mediated by α_{1D}-ARs. Furthermore, Daly *et al.* (2002) suggested that the α_{1B}-AR may play a minor role in vasoconstriction.

The loss of functional α_{1B}-ARs did not significantly alter the response in the aorta suggesting there is little or no involvement for α_{1B}-ARs in contraction of this vessel. This was observed at both 4m and 16m age groups and no significant alterations with age occurred. Daly *et al.* (2002) did however suggest that the lack of α_{1B}-ARs simplifies the pharmacological analysis of the vasculature. In fact, Daly *et al.* (2002) reported the pA_2 values for prazosin increased from 9.8 in the WT to 10.6 in the α_{1B}-KO and the pA_2 for BMY 7378 (α_{1D}-AR selective antagonist) was increased from 8.8 (WT) to 9.3 (α_{1B}-KO).

This observed 6-fold increase and 3-fold increase in sensitivity for prazosin and BMY 7378 respectively may be due to an increase in the number of α_{1D}-ARs by a systemic compensatory up-regulation of α_{1D}-ARs as a result of the loss of α_{1B}-ARs. McBride *et al.* (2002) reported an uncovered α_{1D}-AR response in the mouse mesenteric artery of the α_{1B}-KO. The adrenergic contractile response of 1st order mesenteric arteries is mainly due to α_{1A}-ARs activation (Daly *et al.*, 2002) but a portion of the adrenergic response was found to be 5-MU resistant (α_{1A}-selective antagonist) and BMY 7378 sensitive in the α_{1B}-KO. This BMY 7378 sensitive response was absent in the WT, suggesting that there is some compensation by α_{1D}-ARs in α_{1B}-KO mice. Even then, there was no significant contractile role for α_{1B}-ARs in the mouse aorta.

The lack of functional α_{1D}-ARs, however, resulted in an observed 40-fold less sensitive PE response in the α_{1D}-KO (Tanoue *et al.*, 2002). The present findings were consistent

with this as we observed 33-fold and 44-fold reductions in potency at 4m and 16m respectively (Ali *et al.*, 2002). The removal of the major adrenergic subtype involved in vasoconstriction in the α_{1D}-KO, adversely affected the response to PE confirming that the adrenergic response is due to α_{1D}-AR activity. The reduced potency was observed at both 4m and 16m age points so no significant alteration due to age or any gradual upregulation of the remaining α_1-AR mediated was observed

Thus we have confirmed the α_{1D}-AR is the major adrenergic vasoconstrictor in the mouse aorta. These findings are consistent with the general consensus in the literature. We have also shown there is no effect of age on functional responses of aortae from the WTs and the KOs. Furthermore, the loss of either the α_{1B}-AR or the α_{1D}-AR has not affected the contractile machinery of the VSMCs or their second messenger pathway.

Hill slopes of agonist activation

Interestingly the slope of the PE responses in the α_{1B}-WT and α_{1D}-WT and the α_{1B}-KO were less than unity (1.0). The adrenergic response of the α_{1D}-KO to PE exhibits a slope which overlapped with unity. A shallow Hill slope can be indicative of more than one receptor subtype but can also provide some insight into either the intrinsic efficacy of the agonist used at the receptor or the kinetics of interaction of the receptor with second messengers, discussed by Kenakin (1989) in detail.

We have already discussed that the α_{1D}-AR is the major adrenergic vasoconstrictor in the mouse aorta and the α_{1B}-AR plays a minor role. The activation of two receptors could explain why the Hill slope was shallower than unity in both WTs. However, this explanation of multiple receptor activation giving arise to shallow Hill slopes, does not explain why the response in the α_{1B}-KO was still significantly less than unity. The WT response is due to the activation α_{1D}-ARs and α_{1B}-ARs, and the response in the α_{1B}-KO is only due to α_{1D}-ARs (Daly *et al.*, 2002) and no involvement of α_{1A}-ARs in the mouse aorta has yet been reported (Chapter 2). Thus, the response in the α_{1B}-KO is only α_{1D}-AR mediated and should then have had a Hill slope fitting unity, but this was not the case but it is possible to explain this.

The law of mass action describes the relationship between agonist concentration and occupancy. The relationship between occupancy and response, though, is dependent on the intrinsic efficacy of the agonist used and the kinetics of the receptor in its ability to initiate molecular changes, such as a channel opening or in the case of GPCRs, the receptor's ability to interact with a specific G-protein. Generally the occupancy of receptors by a full agonist and the response observed is a relationship that fits unity on a Hill slope of a log concentration response curve.

Thus the shallow slope of this response is an indication of either the intrinsic efficacy of PE at α_{1D}-ARs, or the transducer function of α_{1D}-ARs i.e. the coupling of α_{1D}-ARs to their second messengers via the $G_{q/11}$ proteins. However to clear this up it would be necessary to plot agonist concentration- occupancy curves and occupancy- response curves as well as the standard log-agonist concentration-response curves, but no data on the occupancy of the receptor pool of α_1-ARs in the mouse aorta was made.

Interestingly, although the α_{1D}-KO was less sensitive to PE, the Hill slopes were not different from unity, suggesting that the remaining α_1-AR (the α_{1B}-AR: see Chapter 2) had different kinetics than the α_{1D}-AR in its relationship of agonist concentration and response. Since PE is a non-selective full α_1-AR agonist it appears that perhaps the α_{1D}-AR has different kinetics, than the remaining AR(s) in the α_{1D}-KO, in its coupling to its second messenger pathway, via the $G_{q/11}$ protein.

Besides this, the serotonergic response is also due to the 5-HT_{2A} receptor increasing IP_3, PKC and tyrosine kinase activity via $G_{q/11}$ second messengers. However, the Hill slope value for this response was not significantly different from unity in most cases and indeed greater than unity in some cases. The serotonergic response seems to be more typical of a full agonist in its relationship between agonist concentration, occupancy and response.

Conclusions- Chapter 1

Conclusion

In the present study there was no clear cut general increase or decrease in contractile or vasorelaxant responses with age. However, most of the data indicates no change in vasoactive responses with age but a more detailed study of the vasoconstrictor and vasodilator systems may provide a clearer picture.

The adrenergic, serotonergic and KCl responses of the α_{1B}-KO were not significantly different from the WT, suggesting there is little or no role for the α_{1B}-AR in contractile responses. However, this is complicated by the apparent compensation by α_{1D}-ARs.

The α_{1D}-KO mouse exhibited a significantly reduced potency to PE at both 4m and 16m age-points. The overall contractility of the α_{1D}-KO to KCl and 5-HT was conserved suggesting the α_{1D}-KO VSMCs retain their contractile mechanism and the second messenger coupling associated with the response. Hence, the reduced adrenergic response of the α_{1D}-KO is due to the loss of the main contractile AR. Therefore, the α_{1D}-AR is the major vasoconstrictor in the mouse aorta.

Thus, the most pertinent question in the case of the α_{1D}-KO was 'what is the remaining adrenergic response in the α_{1D}-KO?' This is discussed in detail in chapter two, where various methods were used to establish which AR response remains in the α_{1D}-KO.

Chapter 2. Subtyping α_1-AR mediated contractile responses in mouse aorta.

Introduction- Chapter 2

The mouse aorta

Increasingly, cardiovascular studies are using murine models, no doubt due to the recent advances in molecular biology providing functional knockouts, over-expressed or transgenic models of single or multiple genes. However such studies only highlight the current lack of background knowledge of murine physiology. Recently, Russell and Watts (2000) provided a useful reference of the activity of various vasoactive agents in the mouse aorta. Observations indicated both NE and PE were potent and efficacious agonists.

Adrenoceptors in the mouse aorta

Yamamoto & Koike (2000) pharmacologically subtyped the adrenergic response in the mouse aorta using selective ligands. They reported the sensitivity (pA_2) of BMY 7378 (α_{1D}-AR selective antagonist: Saussy *et al.*, 1994) was approximately 9-fold greater than 5-methylurapidil (5-MU: α_{1A}-selective antagonist, Gross *et al.*, 1988; Schwinn *et al.*, 1995). Thus, they concluded that the α_{1D}-AR is the main adrenoceptor involved in NE or PE-induced vasoconstriction.

However the difficulty that can arise when using pharmacological approaches alone is demonstrated by the numerous studies done in the rat aorta. Using 5-MU and/or BMY 7378, various authors have come to different conclusions about which of the α_1-ARs subtypes are involved in vasoconstriction. The majority consensus implicated the α_{1D}-AR (Kenny *et al.*, 1995; Piascik *et al.*, 1995; Martinez *et al.*, 1999). However Testa *et al.* (1995) suggested the response was due to α_{1B}-ARs, whilst Aboud *et al.* (1993) discounted the roles of α_{1A}- and α_{1D}-ARs but did not go as far as implicating the α_{1B}-AR. Such pharmacological studies can be complimented by combining them with a transgenic approach, perhaps using functional KO models.

The use of transgenic murine models confirmed the α_{1D}-AR is the major vasoconstrictor in the mouse aorta. Tanoue *et al.* (2002) reported that the isolated aortae from α_{1D}-KO had significantly reduced responses to NE and PE compared to WT (approximately 50-

fold and 40-fold less sensitive for NE And PE respectively). Cavalli *et al.* (1997) initially showed a significant reduction in PE-induced contractility in the α_{1B}-KO aortae but a detailed pharmacological study by Daly *et al.* (2002), using α_{1B}-KO mice, confirmed that the lack of functional α_{1B}-ARs did not have an adverse effect on AR-mediated contractility, concluding the α_{1D}-AR is the major vasoconstrictor.

Role of α_{1B}-ARs in vasoconstriction

However, Yamamoto & Koike (2000), Daly *et al.* (2002) & Tanoue *et al.* (2002) did not completely rule out the α_{1B}-AR from having a role in vasoconstriction suggesting it may instead have a minor role. In the α_{1D}-KO aorta there remained a residual response to PE (Chapter 1) that was BMY 7378 resistant (Tanoue *et al.*, 2002). However, no attempt has yet been made to establish which α_1-AR subtypes are involved in the adrenergic response in α_{1D}-KO aortae.

Such a study is compelled further by recent findings of Deighan *et al.* (In Press), who reported an increased involvement of α_{1A}-ARs in the carotid arteries of α_{1D}-KO mice, whereas the α_{1D}-AR is the main adrenergic vasoconstrictor in WT mouse carotid arteries. Furthermore, McBride et al (2003) has shown that mesenteric resistance arteries from α_{1B}-KO mice had an α_{1D}-AR mediated component that was absent in the arteries taken from WT mice. Thus, an attempt to determine exactly which of the α_1-AR subtypes modulate the adrenergic response of α_{1D}-KO aortae was made.

α_1-AR subtype selective agents

5-MU (α_{1A}-AR selective: Gross *et al.*, 1988; Schwinn *et al.*, 1995), chloroethylclonidine (CEC- α_{1B}-AR selective: Perez *et al.*, 1994) and BMY 7378 (α_{1D}-AR selective: Saussy *et al.*, 1994) are currently the most commonly used subtype-selective antagonists used for α_1-AR studies. 5-MU and BMY 7378 are fairly reliable subtype selective competitive antagonists with 50-fold (α_{1A} over α_{1B} and α_{1D}) and 125-fold (α_{1D} over α_{1A} and α_{1B}) selectivity respectively (see Table 2-14 for pK_i for these antagonists at cloned receptors) and were used in establishing which of the α_1-ARs are involved in vasoconstriction in the α_{1D}-KO.

Alongside this antagonist study a selective agonist was used. A61603 is reported to be an α_{1A}-selective agonist. The more potent R-enantiomer has reported 163-fold and 58-fold affinity for cloned α_{1a}-ARs over α_{1b}-ARs and α_{1d}-ARs respectively (Knepper *et al.*, 1995). Therefore, the contractile responses induced by A61603 in WT, α_{1B}-KO, α_{1D}-KO and α_{1B}-α_{1D}-KO were compared. The effect of prazosin on A61603 was also tested to ensure responses were α_1-AR mediated.

To compliment the pharmacological approach, the α_{1B}-α_{1D}-AR-double-KO was also used. The α_{1B}-α_{1D}-KO was the result of cross-breeding of homozygous α_{1B}-KO and α_{1D}-KO mice (see Methods section for greater detail).

CEC selectivity and receptor protection

CEC is an irreversible alkylating agent (Leclerc *et al.*, 1980), therefore, establishing pK_B or pA_2 values, normally used for the analysis of the effects of competitive antagonists, is problematic. Although initially reported to have selectivity for α_{1B}-ARs (Perez *et al.*, 1994), recent research has indicated that the selectivity of CEC is less to do with subtype selectivity and more to do with receptor availability at the cell membrane. Hirasawa *et al.*, (1997) reported that CEC is a non-selective AR alkylating agent whose apparent selectivity is due to the different cellular distribution of α_1-AR subtypes. Thus, a more complex approach in isolating individual subtypes, in particular the α_{1B}-AR was required.

A more intricate approach to isolate α_{1B}-ARs is receptor protection. The aim with such a method is to protect specific receptor types using suitable concentrations of subtype-selective competitive antagonists (antagonists must be competitive to ensure blockade can be reversed by washing away the antagonist). Treatment with an irreversible antagonist, such as CEC, should then only affect non-protected receptors. Such a study has been nicely demonstrated by Ibarra *et al.* (2000) who reported that the effect of CEC, on NE induced responses in rat aorta, could be protected against, using BMY 7378, confirming the adrenergic response is α_{1D}-AR mediated

Aim of study

The aim was to pharmacologically establish which α_1-AR subtype is responsible for the residual PE-induced response of α_{1D}-KO mouse aortae. This was done by a combination of selective antagonist and agonist studies. To compliment this, functional responses of the α_{1B}-α_{1D}-KO aortae were also studied. Finally an attempt was made to isolate the α_{1B}-AR-mediated component of the PE response in the WT mouse aorta.

Results- Chapter 2

Consecutive PE response- curves in the α_{1D}-KO

E_{max} values for consecutive CCRCs to PE in α_{1D}-KO aortae were: 1st curve = 0.78 ± 0.14g (n = 6); 2nd curve = 0.87 ± 0.14g (n = 7). The maxima were not significantly different (Student's t-test).

The pEC_{50} values were: 1st curve = 5.17 ± 0.13; 2nd curve = 4.24 ± 0.15. The 2nd curve showed 9-fold less sensitivity than the control curve ($p<0.001$: Student's t-test).

The Hill slopes were: 1st curve = 0.93 (*0.62-1.23*); time control = 0.57 (*0.35-1.04*). The Hill slopes overlapped with unity.

The PE responses curves are shown in Figure 2-1 and the values shown above are tabulated in Table 2-1.

Effect of prazosin in α_{1D}-KO PE-induced responses

E_{max} values for PE responses (first curves) in the presence of 1nM, 10nM & 100nM prazosin were: control = 0.78 ± 0.14g (n = 6); +1nM prazosin = 0.53 ± 0.13g (n = 5); +10nM prazosin = 0.58 ± 0.16g (n = 6); +100nM prazosin = 0.77 ± 0.10g (n = 6). No significant differences in maximum responses were observed (one-way-ANOVA: Student's t-test).

Sensitivity of the PE responses expressed as pEC_{50} values were= 5.17 ± 0.13; +1nM prazosin = 4.14 ± 0.29; +10nM prazosin = 3.31 ± 0.29; +100nM prazosin = 3.11 ± 0.13. The 1nM prazosin-treated group was 11-fold less sensitive than the control ($p<0.05$). The 10nM and 100nM prazosin-treated groups were 72-fold and 115-fold less sensitive than control rings, respectively ($p<0.001$: one-way-ANOVA: Bonferroni post-test).

Hill slopes were: control = 0.93 (*0.62-1.23*). The Hill slopes for +1nM, +10nM & 100nM prazosin treated groups were not calculated (see Discussion). The control group Hill slope overlapped with unity.

A pK_B value for prazosin against PE in α_{1D}-KO aortae was estimated as **9.25 ± 0.26** (pK_B calculated using 1nM and 10nM prazosin data only: see Discussion).

The effect of prazosin on the PE response curves in α_{1D}-KO aortae is shown in Figure 2-2 and the values shown above are tabulated in Table 2-2.

Effect of 5-MU in α_{1D}-KO PE-induced responses

E_{max} values for PE responses (first curves) in the presence of 1nM, 10nM, 100nM & 1μM 5-MU were: control = 0.94 ± 0.09g (n = 10); +1nM 5-MU = 0.90 ± 0.10g (n = 6); +10nM 5-MU = 1.03 ± 0.15g (n = 7); +100nM 5-MU = 0.88 ± 0.10g (n = 11); 1μM 5-MU = 0.78 ± 0.20g (n = 7). No significant differences in maximum responses were observed (one-way-ANOVA: Student's t-test).

Sensitivity of the PE responses expressed as pEC_{50} values were: control = 5.34 ± 0.12; +1nM 5-MU = 5.51 ± 0.21; +10nM 5-MU = 4.77 ± 0.10; +100nM 5-MU = 4.48 ± 0.16; +1μM 5-MU = 3.81 ± 0.14. The 1nM 5-MU-treated group was not significantly different from the control. The rings treated with 10nM, 100nM & 1μM 5-MU were 4-fold ($p<0.05$), 11-fold ($p<0.001$) and 50-fold ($p<0.001$) less sensitive than the control PE responses respectively (one-way-ANOVA, Bonferroni post-test).

Hill slopes were: control = 0.73 (*0.45-1.03*); +1nM 5-MU = 0.54 (*0.10-0.99*); +10nM 5-MU = 1.06 (*0.80-1.31*); +100nM 5-MU = 0.80 (*0.58-1.21*); +1μM 5-MU = 1.38 (*1.03-1.74*). The Hill slopes overlapped with unity.

A pK_B value for 5-MU against PE in α_{1D}-KO aortae was estimated as **7.65 ± 0.13** (pK_B calculated using 10nM, 100nM, 1μM 5-MU data only: see Discussion).

The effect of 5-MU on the PE response curves in α_{1D}-KO aortae is shown in Figure 2-3 and the values shown above are tabulated in Table 2-3.

PE-induced responses in WT, α_{1B}-and α_{1D}-KO aortae

The maximum responses to PE in WT, α_{1B}-KO and α_{1D}-KO aortae were: 0.74 ± 0.05 (n = 18), 0.97 ± 0.09 (n = 16), 0.66 ± 0.04 (n = 36) respectively. The α_{1B}-KO maximum was 34% greater than the WT ($p<0.05$). The α_{1D}-KO E_{max} was 11% less than the WT ($p<0.05$: one-way-ANOVA, Bonferroni post-test).

pEC_{50} values of the PE responses: WT = 6.33 ± 0.07; α_{1B}-KO = 6.73 ± 0.08; α_{1D}-KO = 4.97 ± 0.04. The α_{1B}-KO and α_{1D}-KO were 3-fold more sensitive ($p<0.01$) and 23-fold less sensitive ($p<0.001$) than the WT respectively (one-way-ANOVA, Bonferroni post-test).

Hill slopes were: WT = 0.60 (*0.49-0.74*); α_{1B}-KO = 0.61 (*0.49-0.72*); α_{1D}-KO 5-MU = 0.81 (*0.59-1.04*). The Hill slope for the WT and α_{1B}-KO aortae did not overlap with unity, whilst the Hill slope of the α_{1D}-KO overlapped with unity.

The PE curves for WT, α_{1B}-KO and α_{1D}-KO are shown in Figure 2-4 and the above values are tabulated in Table 2-4. The above data is taken from Figure 1-9 and Table 1-9 of Chapter 1.

A61603-induced responses in WT, α_{1B}-and α_{1D}-KO aortae

The maximum responses to A61603 were: WT = 0.95 ± 0.08 (n = 6); α_{1B}-KO = 1.26 ± 0.07 (n = 8); α_{1D}-KO = 0.14 ± 0.04 (n = 8). The α_{1B}-KO maximum was 33% greater than the WT ($p<0.05$), but the α_{1D}-KO maximum for A61603 was 85% less than the WT ($p<0.001$: one-way-ANOVA, Bonferroni post-test).

pEC_{50} values of the PE responses: WT = 6.17 ± 0.10; α_{1B}-KO = 6.79 ± 0.10. It was not possible to calculate a pEC_{50} value for the α_{1D}-KO A61603 response. The A61603 response in the α_{1B}-KO was 4-fold more sensitive than the WT ($p<0.01$: one-way-ANOVA, Bonferroni post-test).

Hill slopes were: WT = 0.38 (*0.28-0.48*); α_{1B}-KO = 0.41 (*0.23-0.61*). It was not possible to estimate a Hill slope value for the α_{1D}-KO A61603 response. The Hill slope for the WT and α_{1B}-KO aortae did not overlap with unity.

The A61603 curves for WT, α_{1B}-KO and α_{1D}-KO are shown in Figure 2-5 and the above values are tabulated in Table 2-5.

Effect of prazosin on A61603-induced contractions of WT aortae

The maximum responses to A61603, in the absence and presence of prazosin (first curves) and the control PE responses in WT aortae were: A61603 control = 0.95 ± 0.08 (n = 6); +3nM prazosin = 0.92 ± 0.18 (n = 6); +30nM prazosin = 0.77 ± 0.11 (n = 6); PE = 0.74 ± 0.05 (n = 18). There was no significant difference in maxima (one-way-ANOVA, Bonferroni post-test).

pEC_{50} values of the A61603 and PE responses were: A61603 control = 6.17 ± 0.10; +3nM prazosin = 5.94 ± 0.13; +30nM prazosin = 5.35 ± 0.25; PE = 6.33 ± 0.07. The +3nM prazosin-treated group and PE response were similar to the control A61603 responses. The +30nM prazosin-treated group had a 7-fold less sensitive response to A61603 ($p<0.01$: one-way-ANOVA, Bonferroni post-test).

Hill slopes were: A61603 curve = 0.38 (*0.28-0.48*); +3nM prazosin = 0.35 (*0.22-0.49*); +30nM prazosin = 0.38 (*0.29-0.48*); PE = 0.60 (*0.49-0.74*). The Hill slopes for all groups did not overlap with unity.

A pK_B value for prazosin against A61603 in WT aortae was estimated as **8.45 ± 0.07** (pK_B calculated using 30nM prazosin data only: see Discussion).

The A61603 curve, the effect of prazosin on the A61603 responses and the PE responses in WT aortae are shown in Figure 2-6 and the above values are tabulated in Table 2-6.

Effect of prazosin on A61603-induced contractions of α_{1B}-KO aortae

The maximum responses to A61603, in the absence and presence of prazosin (first curves) and the control PE responses in α_{1B}-KO aortae were: A61603 control = 1.26 ± 0.07 (n = 8); +3nM prazosin = 1.13 ± 0.10 (n = 8); +30nM prazosin = 1.13 ± 0.08 (n = 8); PE = 0.97 ± 0.09 (n = 16). There was no significant difference in maxima (one-way-ANOVA, Bonferroni post-test).

pEC_{50} values of the A61603 and PE responses were: A61603 control = 6.79 ± 0.10; +3nM prazosin = 6.36± 0.21; +30nM prazosin = 5.79 ± 0.09; PE = 6.73 ± 0.08. The +3nM prazosin-treated group and PE response were similar to the control A61603 responses. The +30nM prazosin-treated group was 10-fold less sensitive ($p<0.001$: one-way-ANOVA, Bonferroni post-test).

Hill slopes were: A61603 curve = 0.41 (*0.23-0.61*); +3nM prazosin = 0.40 (*0.22-0.59*); +30nM prazosin = 0.50 (*0.29-0.70*); PE = 0.61 (*0.49-0.72*). The Hill slopes for all groups did not overlap with unity.

A pK_B value for prazosin against A61603 in α_{1B}-KO aortae was estimated as **8.46 ± 0.12** (pK_B calculated using 30nM prazosin data only: see Discussion).

The A61603 curve, the effect of prazosin on the A61603 responses and the PE responses in α_{1B}-KO aortae are shown in Figure 2-7 and the above values are tabulated in Table 2-7.

Effect of prazosin on A61603-induced contractions of α_{1D}-KO aortae

The maximum responses to A61603, in the absence and presence of prazosin (first curves) and the control PE responses in α_{1D}-KO aortae were: A61603 control = 0.14 ± 0.02 (n = 8); +3nM prazosin = 0.14 ± 0.04 (n = 8); +30nM prazosin = 0.10 ± 0.03 (n = 8); PE = 0.66 ± 0.04 (n = 36). The PE response was 371% greater than the A61603 ($p<0.001$: one-way-ANOVA, Bonferroni post-test).

It was not possible to estimate pEC_{50} values for the A61603 responses. The pEC_{50} of the PE response was 4.97 ± 0.04.

It was not possible to estimate Hill slope values for the A61603 responses. The Hill slope for PE was 0.93 (*0.59-1.04*). The Hill slope overlapped with unity.

It was not possible to estimate a pK_B for prazosin against A61603 in α_{1D}-KO aortae (see Discussion).

The A61603 curve, the effect of prazosin on the A61603 responses and the PE responses in α_{1D}-KO aortae are shown in Figure 2-8 and the above values are tabulated in Table 2-8.

Adrenergic response in the α_{1B}-α_{1D}-KO

The maximum PE and A61603 responses in WT and α_{1B}-α_{1D}-KO were: WT PE = 0.74 ± 0.05 (n = 18); α_{1B}-α_{1D}-KO PE = 0.01 ± 0.01 (n = 6); WT A61603 = 0.95 ± 0.08 (n = 6); α_{1B}-α_{1D}-KO A61603 = 0.04 ± 0.01 (n = 6). The PE and A61603 responses of the α_{1B}-α_{1D}-KO aortae were 99% and 96% respectively less than the WT ($p<0.001$ for both: Student's t-test).

It was not possible to estimate pEC_{50} values or Hill slopes for PE and A61603-induced response in α_{1B}-α_{1D}-KO aortae.

The comparison of the PE and A61603 responses of the α_{1B}-α_{1D}-KO with the WT responses is shown in Figure 2-9 and relevant values are tabulated in Table 2-9.

Serotonergic response in the α_{1B}-α_{1D}-KO

The maxima for the serotonergic responses in WT and α_{1B}-KO were 1.27 ± 0.14 (n = 17) and 1.31 ± 0.15 (n = 6) respectively. No significant difference was observed (Student's t-test).

pEC_{50} values were: WT = 6.91 ± 0.14; α_{1B}-α_{1D}-KO = 6.83 ± 0.15. The sensitivity was not significantly different (Student's t-test).

The Hill slopes were: WT = 1.17 (*0.95-1.28*); α_{1B}-α_{1D}-KO = 1.43 (*1.02-1.84*). The Hill slopes overlapped with unity.

The comparison of the WT and α_{1B}-α_{1D}-KO serotonergic response is shown in Figure 2.10 and the above values are tabulated in Table 2.10.

Effect of 10μM CEC and the protective effect of 10nM BMY 7378

The E_{max} values were: control = 0.89 ± 0.08 (n = 23); 10nM BMY 7378 post-removal control = 0.83 ± 0.08 (n = 18); +10μM CEC post-removal = 0.07 ± 0.04 (n = 7); +10μM CEC, 10nM BMY 7378 protected post-removal = 0.13 ± 0.05 (n = 7). The controls were not significantly different. The 10μM CEC-treated group and the 10μM CEC, 10nM BMY 7378-treated group were 92% and 85% respectively less than the control ($p<0.001$ for both). The 10μM CEC-treated group and the 10μM CEC-treated, 10nM BMY 7378 protected groups were not significantly different from one another (one-way-ANOVA, Bonferroni post-test).

The pEC_{50} values were: control = 6.73 ± 0.07; 10nM BMY 7378 control post-removal = 6.62 ± 0.07. It was not possible to estimate pEC_{50} values for the 10μM CEC-treated groups. The two controls were not significantly different.

The Hill slopes were: control = 0.84 (*0.62-1.07*); 10nM BMY 7378 control post-removal = 0.83 (*0.64-1.02*). Hill slopes for the 10μM CEC-treated group and the 10μM CEC-treated, 10nM BMY 7378 protected groups were not estimated. The Hill slopes overlapped with unity.

The protection study conducted using 10μM CEC and 10nM BMY 7378 is shown in Figure 2-11 and the above values are tabulated in Table 2-11.

Effect of 1μM CEC and the protective effect of 10nM BMY 7378

The E_{max} values were: control = 0.89 ± 0.08 (n = 23); 10nM BMY 7378 control post-removal = 0.83 ± 0.08 (n = 18); +1μM CEC post removal = 0.49 ± 0.07 (n = 18); +1μM CEC, 10nM BMY 7378 protected post removal = 0.76 ± 0.10 (n = 11). The controls were not significantly different. The 1μM CEC-treated group was 45% less sensitive than the control ($p<0.001$). The 1μM CEC-treated, 10nM BMY 7378 protected group was not significantly different from the control but significantly larger than the 1μM CEC-treated group ($p<0.05$: one-way-ANOVA, Bonferroni post-test).

The pEC_{50} values were: control = 6.73 ± 0.07; 10nM BMY 7378 control post-removal = 6.62 ± 0.07; +1μM CEC post-removal = 6.60 ± 0.07; +1μM CEC, 10nM BMY 7378 protected post-removal = 6.73 ± 0.09. The pEC_{50} values of all groups were not significantly different (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: control = 0.84 (*0.62-1.07*); 10nM BMY 7378 control = 0.83 (*0.64-1.02*); +1μM CEC post-removal = 1.11 (*0.87-1.36*); +1μM CEC, 10nM BMY 7378 protected post-removal = 0.88 (*0.64-1.12*). The Hill slopes overlapped with unity.

The protection study conducted using 1μM CEC and 10nM BMY 7378 is shown in Figure 2-12 and the above values are tabulated in Table 2-12.

Effect of 1μM CEC and the protective effect of 10nM 5-MU

The E_{max} values were: control = 0.89 ± 0.08 (n = 23); 10nM 5-MU control post-removal = 1.03 ± 0.10 (n = 5); +1μM CEC post-removal = 0.49 ± 0.07 (n = 18); +1μM CEC, 10nM 5-MU protected post-removal = 0.47 ± 0.14 (n = 6). The controls were not significantly different. The 1μM CEC-treated group and the 1μM CEC, 10nM 5-MU - protected group were 45% and 47% respectively less than the control ($p<0.001$ for both). The two 1μM CEC-treated groups were not significantly different (one-way-ANOVA, Bonferroni post-test).

The pEC_{50} values were: control = 6.73 ± 0.07; 10nM 5-MU control post-removal = 6.95 ± 0.06; +1μM CEC = 6.60 ± 0.07; +1μM CEC post-removal, 10nM 5-MU protected post-removal = 6.90 ± 0.06. The pEC_{50} values of all groups were not significantly different (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: control – 0.84 (*0.62-1.07*); 10nM 5-MU control post-removal = 0.95 (*0.52-1.38*); +1μM CEC post-removal = 1.11 (*0.87-1.36*); +1μM CEC, 10nM 5-MU protected post-removal = 1.84 (*1.04-2.62*). The Hill slopes overlapped with unity.

The protection study conducted using 1μM CEC and 10nM 5-MU is shown in Figure 2-13 and the above values are tabulated in Table 2-13.

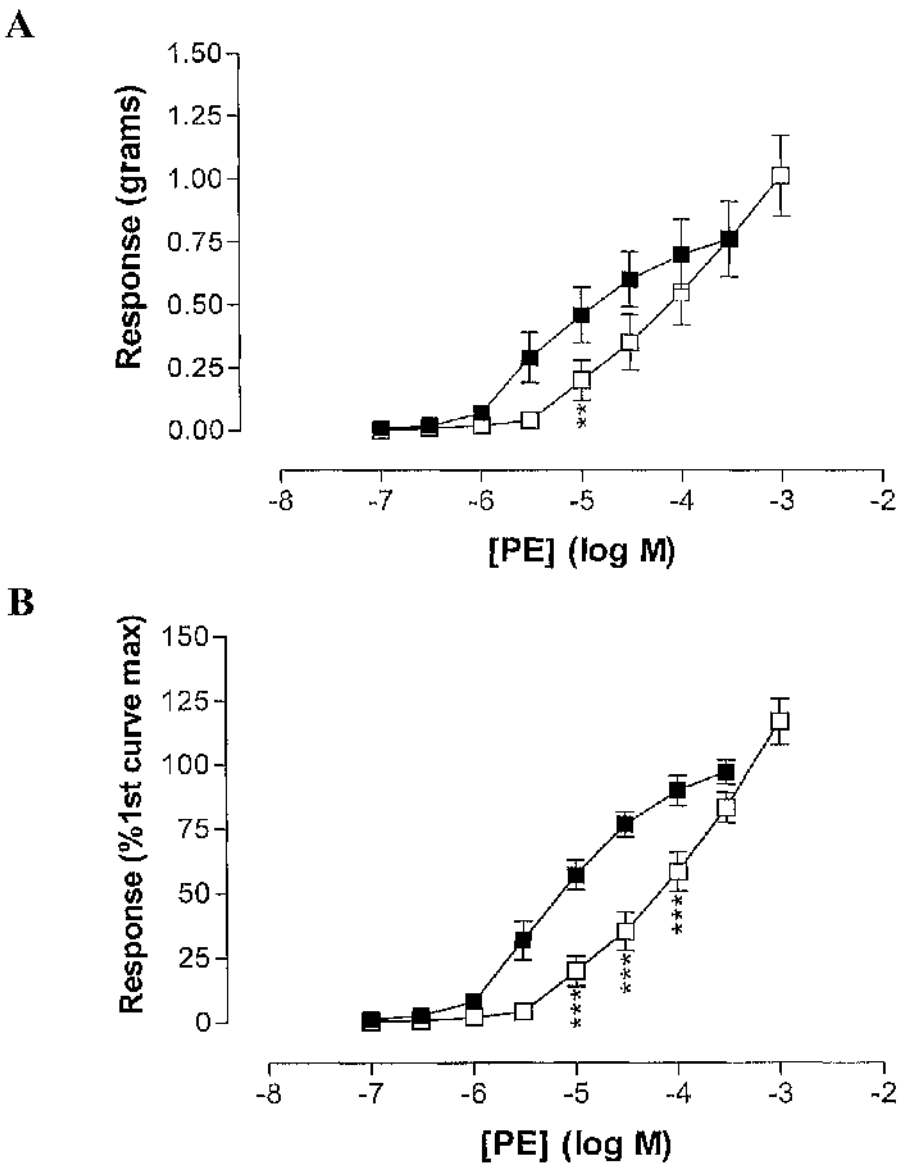


Figure 2-1. Comparison of consecutive PE curves constructed in α_{1D}-KO mouse aorta (■ 1st curve, $n = 6$; □ 2nd curve, $n = 7$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of the maximum response of the initial curve (**B**). Data points are the mean ± S.E.M. (**p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
1st curve	6	0.78 ± 0.14
2nd curve	7	0.87 ± 0.14

B

	pEC_{50}	Hill slope
1st curve	5.17 ± 0.13	0.93 (*0.62-1.23*)
2nd curve	4.24 ± 0.15***	0.57 (*0.35-1.04*)

Table 2-1. Comparison of consecutive PE curves constructed in α_{1D}-KO mouse aorta.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (***$p<0.001$: Student's t-test).

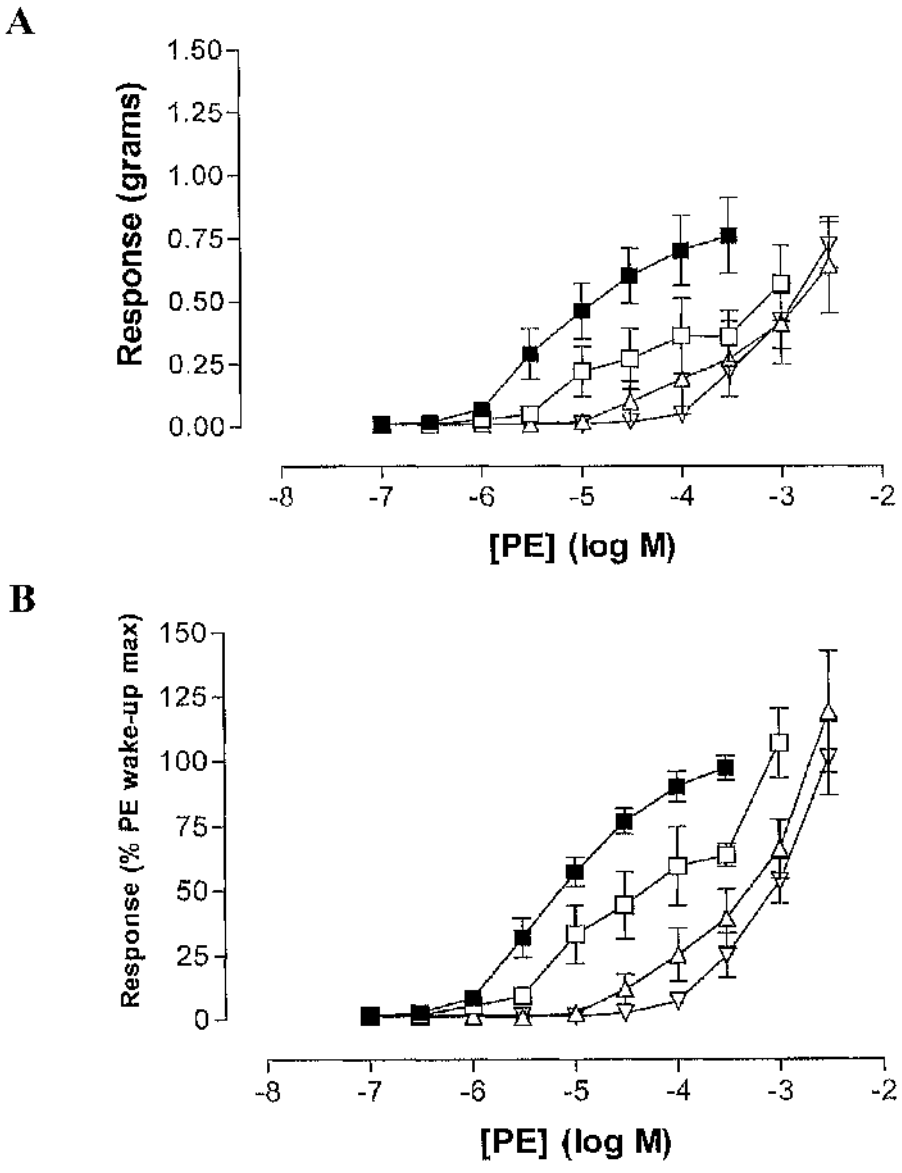


Figure 2-2. The effect of 1nM, 10nM & 100nM prazosin on PE-induced contractile responses (first curves) of α_{1D}-KO aortae (■ control, $n = 6$; ⊓ 1nM prazosin, $n = 5$; △ 10nM prazosin, $n = 6$; ▽ 100nM prazosin $n = 6$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of the maximum PE wake-up response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	6	0.78 ± 0.14
+1nM prazosin	5	0.53 ± 0.13
+10nM prazosin	6	0.58 ± 0.16
+100nM prazosin	6	0.77 ± 0.10

B

	pEC_{50}	Hill slope
Control	5.17 ± 0.13	0.93 (*0.62-1.23*)
+1nM prazosin	4.14 ± 0.29*	*n.c.*
+10nM prazosin	3.31 ± 0.29***	*n.c.*
+100nM prazosin	3.11 ± 0.13***	*n.c.*

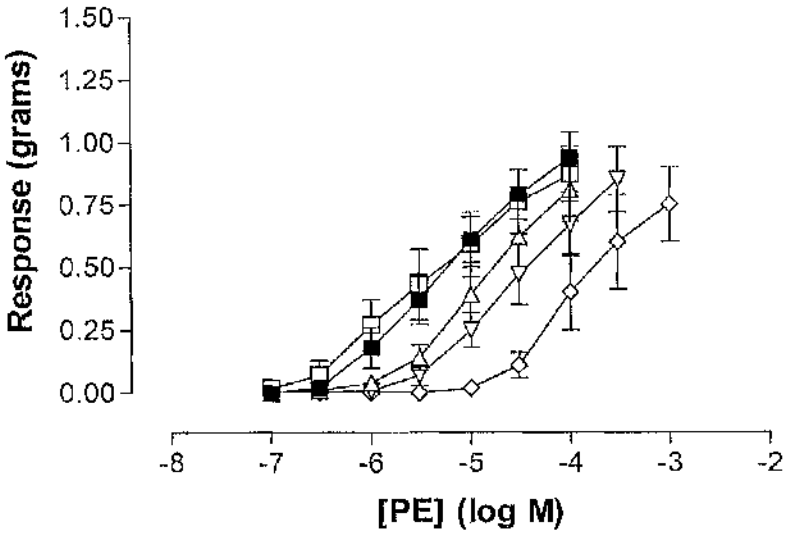
Table 2-2. The effect of 1nM, 10nM & 100nM prazosin on PE-induced contractile responses of α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*n.c.*: not calculated, *p<0.05, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

Estimated pK_B for prazosin = **9.25 ± 0.26**.

(pK_B calculated using 1nM and 10nM prazosin data only: see Discussion)

A



B

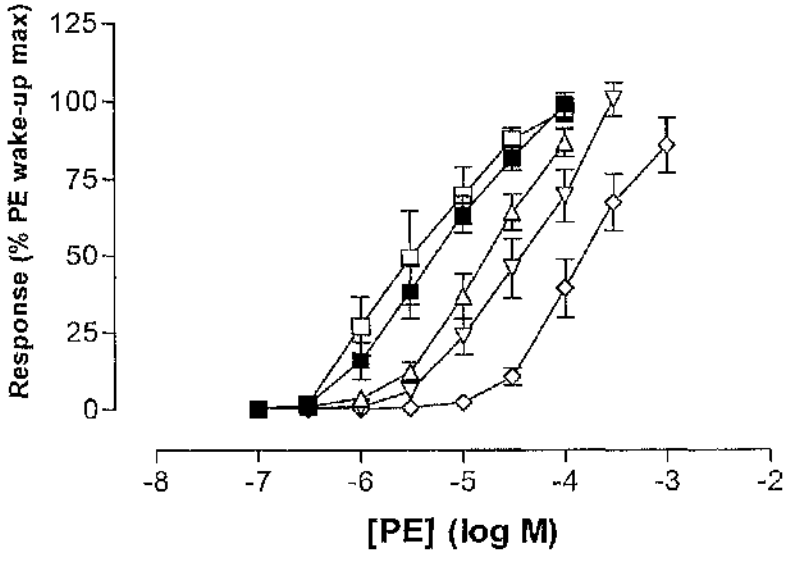


Figure 2-3. The effect of 1nM, 10nM, 100nM & 1μM 5-MU on PE-induced contractile responses (first curves) of α_{1D}-KO aortae (■ control, $n = 10$; □ 1nM 5-MU, $n = 6$; △ 10nM 5-MU, $n = 7$; ▽ 100nM 5-MU $n = 11$; ◇ 1μM 5-MU, $n = 7$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of the maximum PE wake-up response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	10	0.94 ± 0.09
+1nM 5-MU	6	0.90 ± 0.10
+10nM 5-MU	7	1.03 ± 0.15
+100nM 5-MU	11	0.88 ± 0.10
+1μM 5-MU	7	0.78 ± 0.20

B

	pEC_{50}	Hill slope
Control	5.34 ± 0.12	0.73 (*0.45-1.03*)
+1nM 5-MU	5.51 ± 0.21	0.54 (*0.10-0.99*)
+10nM 5-MU	4.77 ± 0.10*	1.06 (*0.80-1.31*)
+100nM 5-MU	4.48 ± 0.16***	0.80 (*0.58-1.21*)
+1μM 5-MU	3.81 ± 0.14***	1.38 (*1.03-1.74*)

Table 2-3. The effect of 1nM, 10nM, 100nM & 1μM 5-MU on PE-induced contractile responses of α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*p<0.05, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

Estimated pK_B for 5-MU = **7.65 ± 0.13**.

(pK_B calculated using 10nM, 100nM, 1μM 5-MU data only: see Discussion)

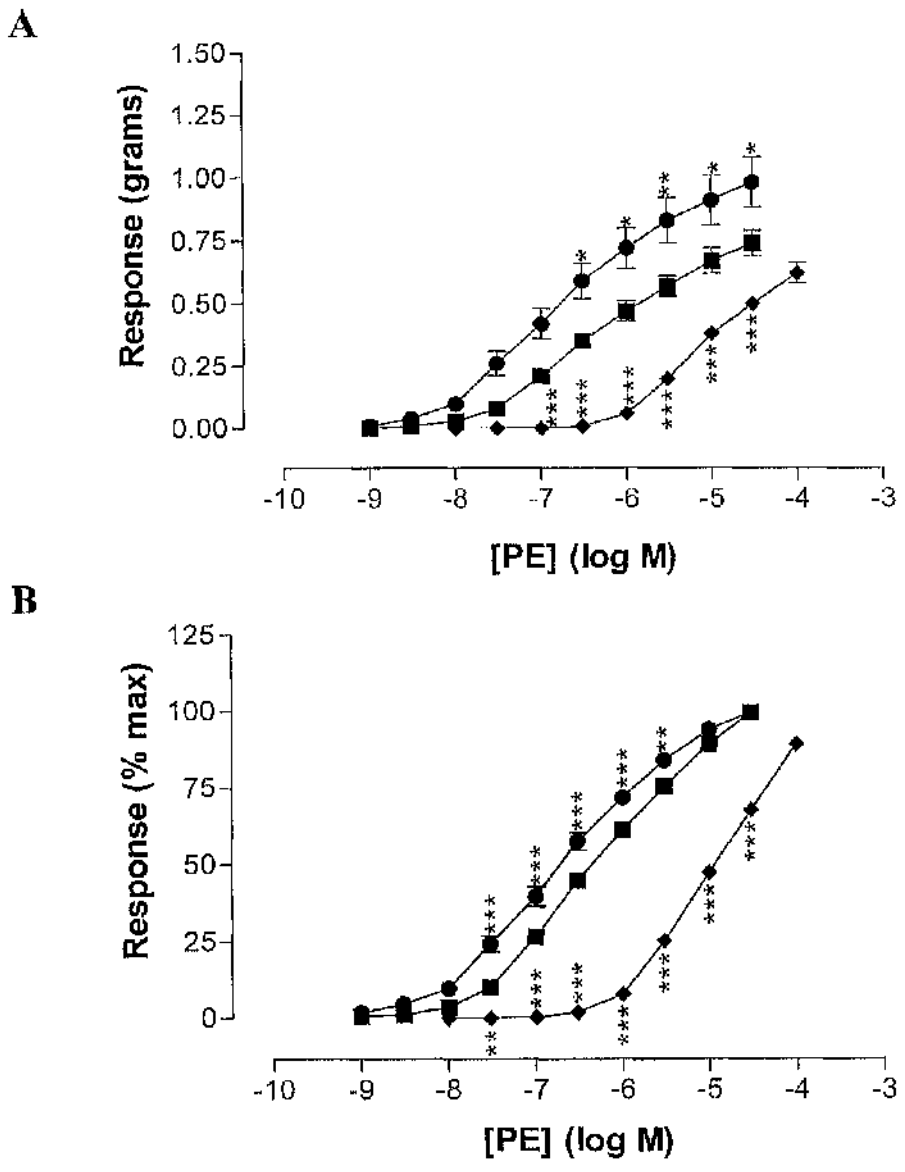


Figure 2-4. PE-induced contractile responses of WT (■; n = 18), α_{1B}-KO (●; n = 16) & α_{1D}-KO (◆; n = 36) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
WT	18	0.74 ± 0.05
α_{1B}-KO	16	0.97 ± 0.09*
α_{1D}-KO	36	0.66 ± 0.04*

B

	pEC_{50}	Hill slope
WT	6.33 ± 0.07	0.60 (*0.49-0.74*)
α_{1B}-KO	6.73 ± 0.08**	0.61 (*0.49-0.72*)
α_{1D}-KO	4.97 ± 0.04***	0.81 (*0.59-1.04*)

Table 2-4. PE-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$, **$p<0.01$, ***$p<0.001$: one-way-ANOVA, Bonferroni post-test).

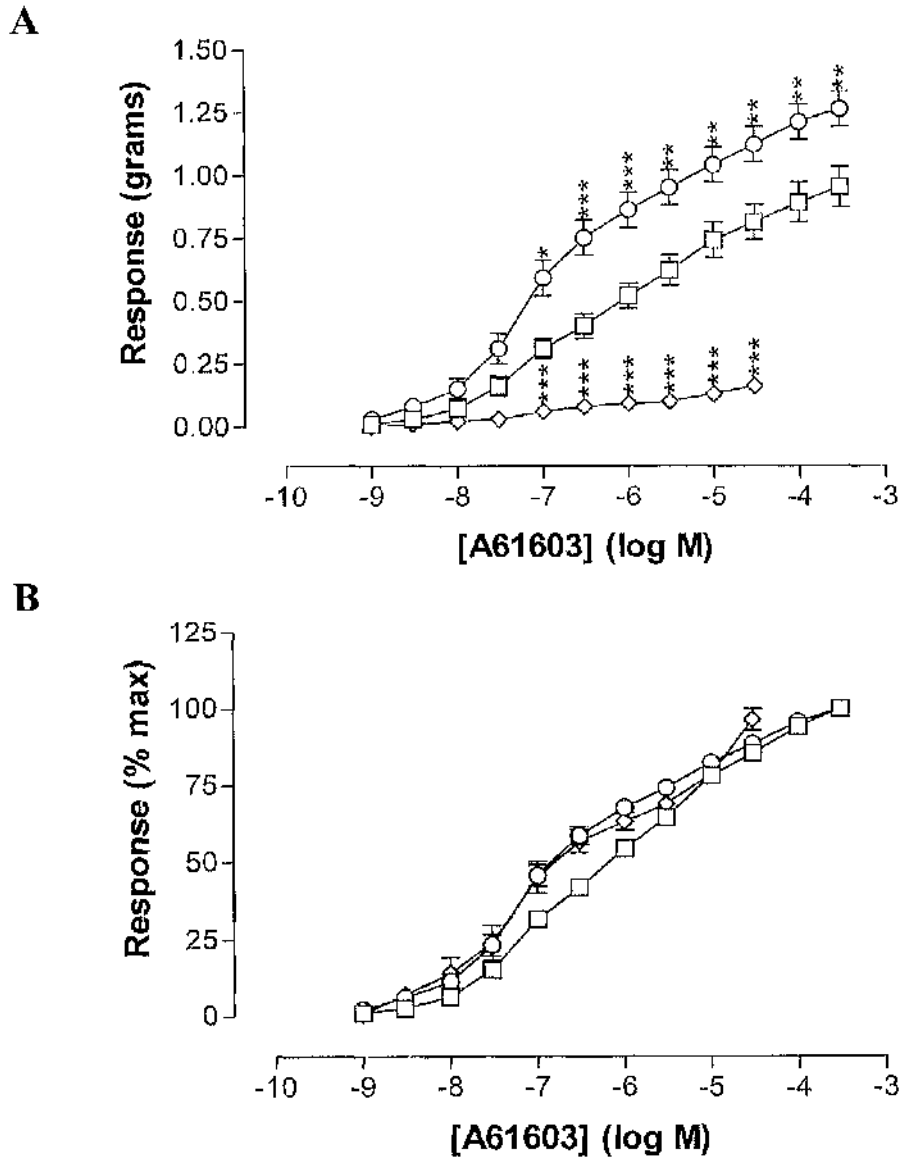


Figure 2-5. A61603-induced contractile responses of WT (□; n = 6), α_{1B}-KO (○; n = 8) & α_{1D}-KO (◇; n = 8) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown in **B** for clarity, *p<0.05, **p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
WT	6	0.95 ± 0.08
α_{1B}-KO	8	1.26 ± 0.07*
α_{1D}-KO	8	0.14 ± 0.08***

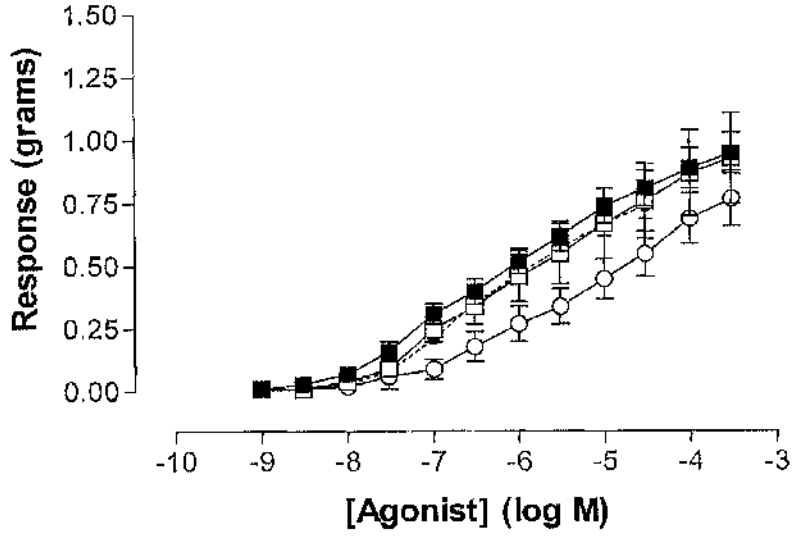
B

	pEC_{50}	Hill slope
WT	6.17 ± 0.10	0.38 (*0.28-0.48*)
α_{1B}-KO	6.79 ± 0.10**	0.41 (*0.23-0.61*)
α_{1D}-KO	*n.c.*	*n.c.*

Table 2-5. A61603-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*n.c.*: not calculated, *p<0.05, **p<0.01, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

A



B

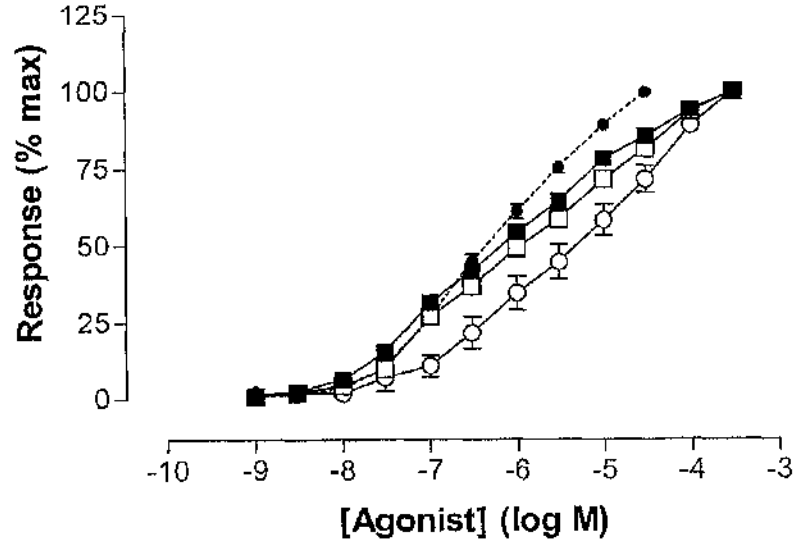


Figure 2-6. The effect of 3nM & 30nM prazosin on A61603-induced contractile responses of WT aortae (solid lines: ■ control, $n = 6$; □ +3nM prazosin, $n = 6$; ○ +30nM prazosin, $n = 6$). The PE response is also shown for comparison (dashed line: ●; $n = 18$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
A61603	6	0.95 ± 0.08
+3nM prazosin	6	0.92 ± 0.18
+30nM prazosin	6	0.77 ± 0.11
PE	18	0.74 ± 0.05

B

	pEC_{50}	Hill slope
A61603	6.17 ± 0.10	0.38 (*0.28-0.48*)
+3nM prazosin	5.94 ± 0.13	0.35 (*0.22-0.49*)
+30nM prazosin	5.35 ± 0.25**	0.38 (*0.29-0.48*)
PE	6.33 ± 0.07	0.60 (*0.49-0.74*)

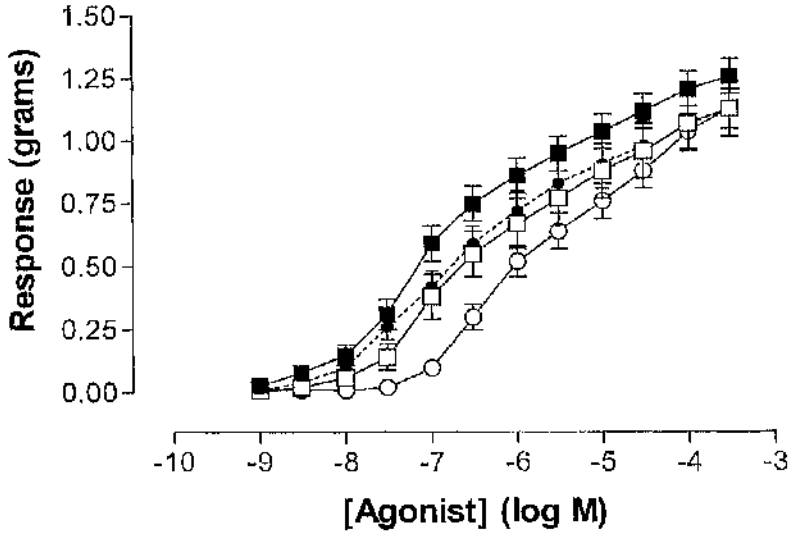
Table 2-6. The effect of 3nM & 30nM prazosin on A61603-induced contractile responses of WT aortae. The PE response is also shown for comparison.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (**p<0.01: one-way-ANOVA, Bonferroni post-test).

Estimated pK_B for prazosin = **8.45 ± 0.07**.

(pK_B calculated using 30nM prazosin data only: see Discussion)

A



B

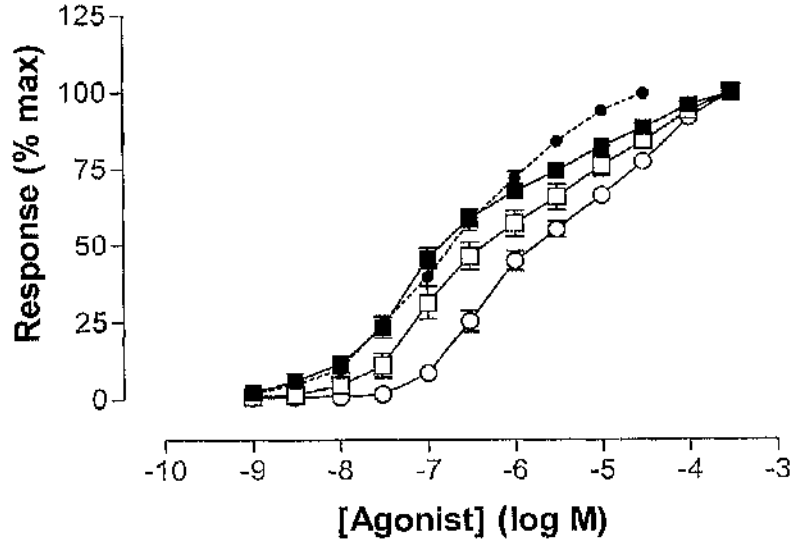


Figure 2-7. The effect of 3nM & 30nM prazosin on A61603-induced contractile responses of α_{1B}-KO aortae (solid lines: ■ control, $n = 8$; □ +3nM prazosin, $n = 8$; ○ +30nM prazosin, $n = 8$). The PE response is also shown for comparison (dashed line: ●; $n = 16$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
A61603	8	1.26 ± 0.07
+3nM prazosin	8	1.13 ± 0.10
+30nM prazosin	8	1.13 ± 0.08
PE	16	0.97 ± 0.09

B

	pEC_{50}	Hill slope
A61603	6.79 ± 0.10	0.41 (*0.23-0.61*)
+3nM prazosin	6.36 ± 0.21	0.40 (*0.22-0.59*)
+30nM prazosin	5.79 ± 0.09***	0.50 (*0.29-0.70*)
PE	6.73 ± 0.08	0.61 (*0.49-0.72*)

Table 2-7. The effect of 3nM & 30nM prazosin on A61603-induced contractile responses of α_{1B}-KO aortae. The PE response is also shown for comparison.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (***p<0.001: one-way-ANOVA, Bonferroni post-test).

Estimated pK_B for prazosin = **8.46± 0.12**.

(pK_B calculated using 30nM prazosin data only: see Discussion)

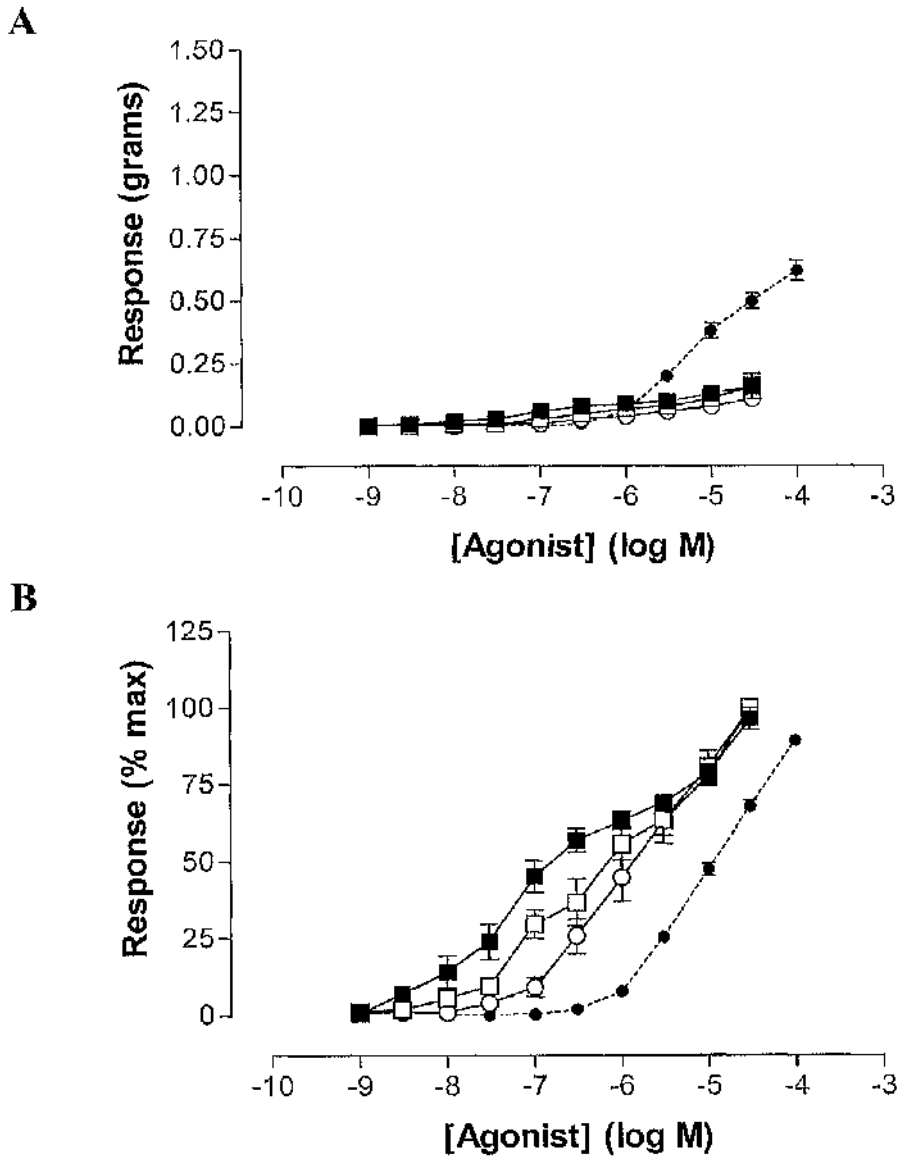


Figure 2-8. The effect of 3nM & 30nM prazosin on A61603-induced contractile responses of α_{1D}-KO aortae (solid lines: ■ control, n = 8; □ +3nM prazosin, n = 8; ○ +30nM prazosin, n = 8). The PE response is also shown for comparison (dashed line: ●; n = 36).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
A61603	8	0.14 ± 0.02
+3nM prazosin	8	0.14 ± 0.04
+30nM prazosin	8	0.10 ± 0.03
PE	36	0.66 ± 0.04***

B

	pEC_{50}	Hill slope
A61603	*n.c.*	*n.c.*
+3nM prazosin	*n.c.*	*n.c.*
+30nM prazosin	*n.c.*	*n.c.*
PE	4.97 ± 0.04	0.93 (*0.59-1.04*)

Table 2-8. The effect of 3nM & 30nM prazosin on A61603-induced contractile responses of α_{1D}-KO aortae. The PE response is also shown for comparison.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*n.c.*: not calculated, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

No pK_B estimated for prazosin: see Discussion.

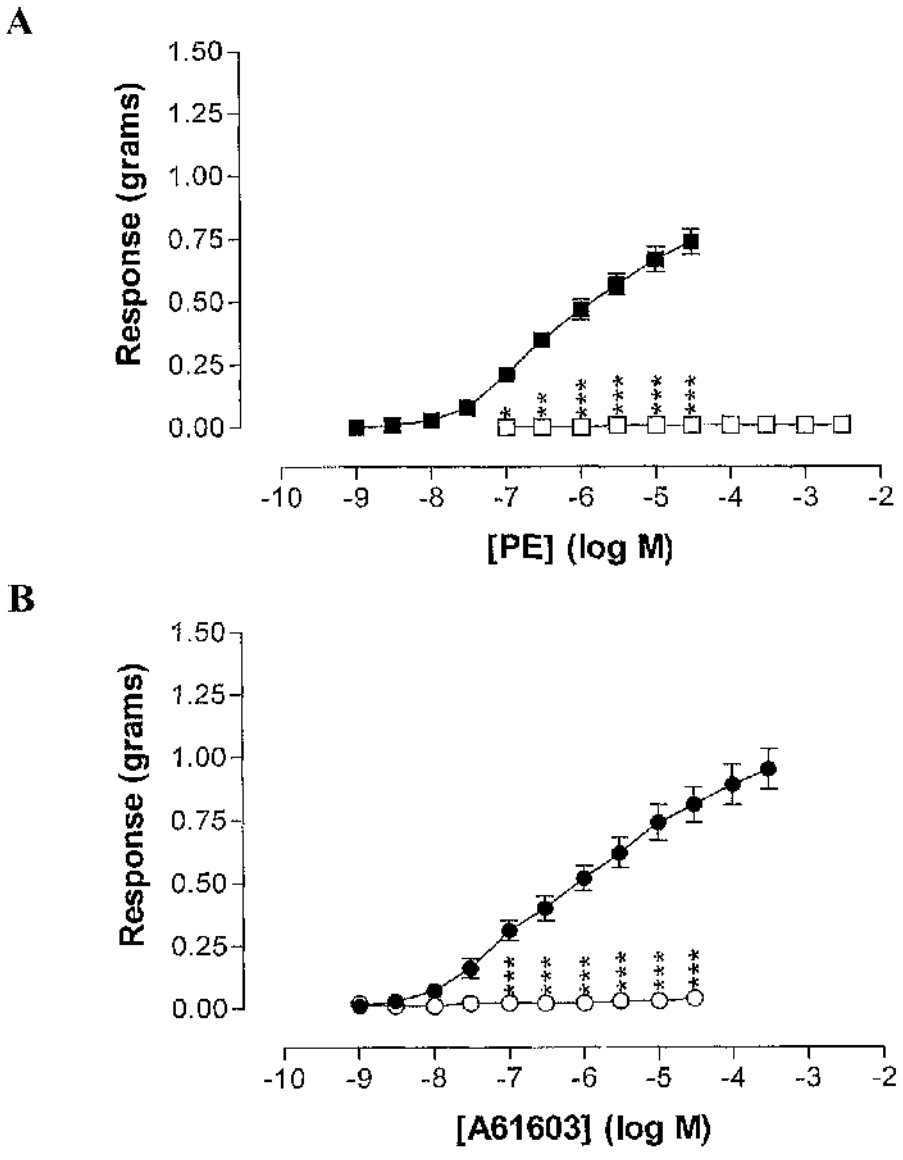


Figure 2-9. PE-induced (**A**) and A61603-induced (**B**) contractile responses of WT and α_{1B}-α_{1D}-KO aortae (■ WT- PE, n = 18; □ α_{1B}-α_{1D}-KO, n = 6; ● WT-PE, n = 6; ○ α_{1B}-α_{1D}-KO- A61603, n = 6).

Responses are shown in grams. Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

		n	E_{max} (grams)
PE	WT	18	0.74 ± 0.05
	α_{1B}-α_{1D}-KO	6	0.01 ± 0.01***
A61603	WT	6	0.95 ± 0.08
	α_{1B}-α_{1D}-KO	6	0.04 ± 0.01***

B

		pEC_{50}	Hill slope
PE	WT	6.33 ± 0.07	0.60 (*0.49-0.74*)
	α_{1B}-α_{1D}-KO	*n.c.*	*n.c.*
A61603	WT	6.17 ± 0.10	0.38 (*0.28-0.48*)
	α_{1B}-α_{1D}-KO	*n.c.*	*n.c.*

Table 2-9. Comparison of consecutive PE curves constructed in α_{1D}-KO mouse aorta.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*n.c.*: not calculated, ***p<0.001; Student's t-test).

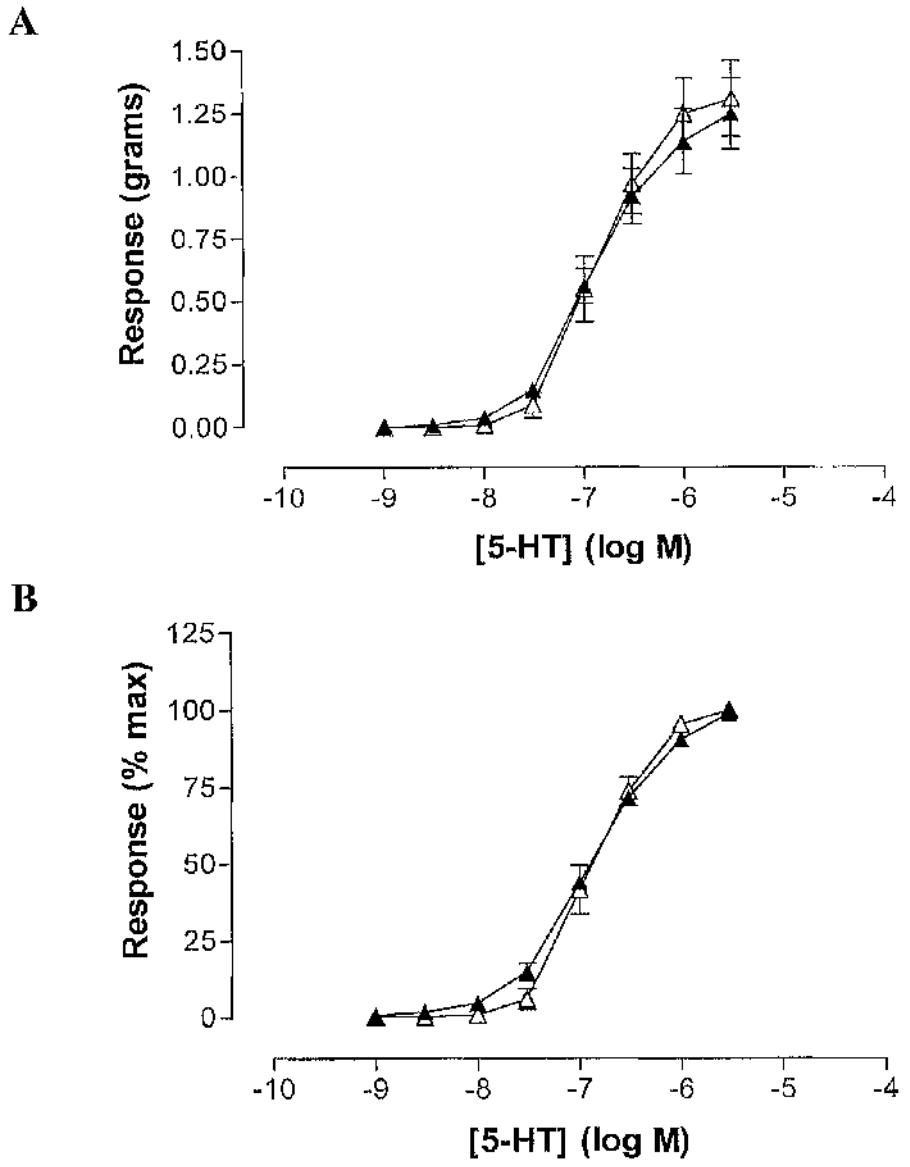


Figure 2-10. 5-HT-induced contractile responses of WT (▲; n = 17) & α_{1B}- α_{1D}-KO (△; n = 6) aortae.

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (no statistical significance: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
WT	17	1.27 ± 0.14
α_{1B}-α_{1D}-KO	6	1.31 ± 0.15

B

	pEC_{50}	Hill slope
WT	6.91 ± 0.14	1.17 (*0.95-1.28*)
α_{1B}-α_{1D}-KO	6.83 ± 0.15	1.43 (*1.02-1.84*)

Table 2-10. . 5-HT-induced contractile responses of WT & α_{1B}-α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (no statistical significance: one-way-ANOVA, Bonferroni post-test).

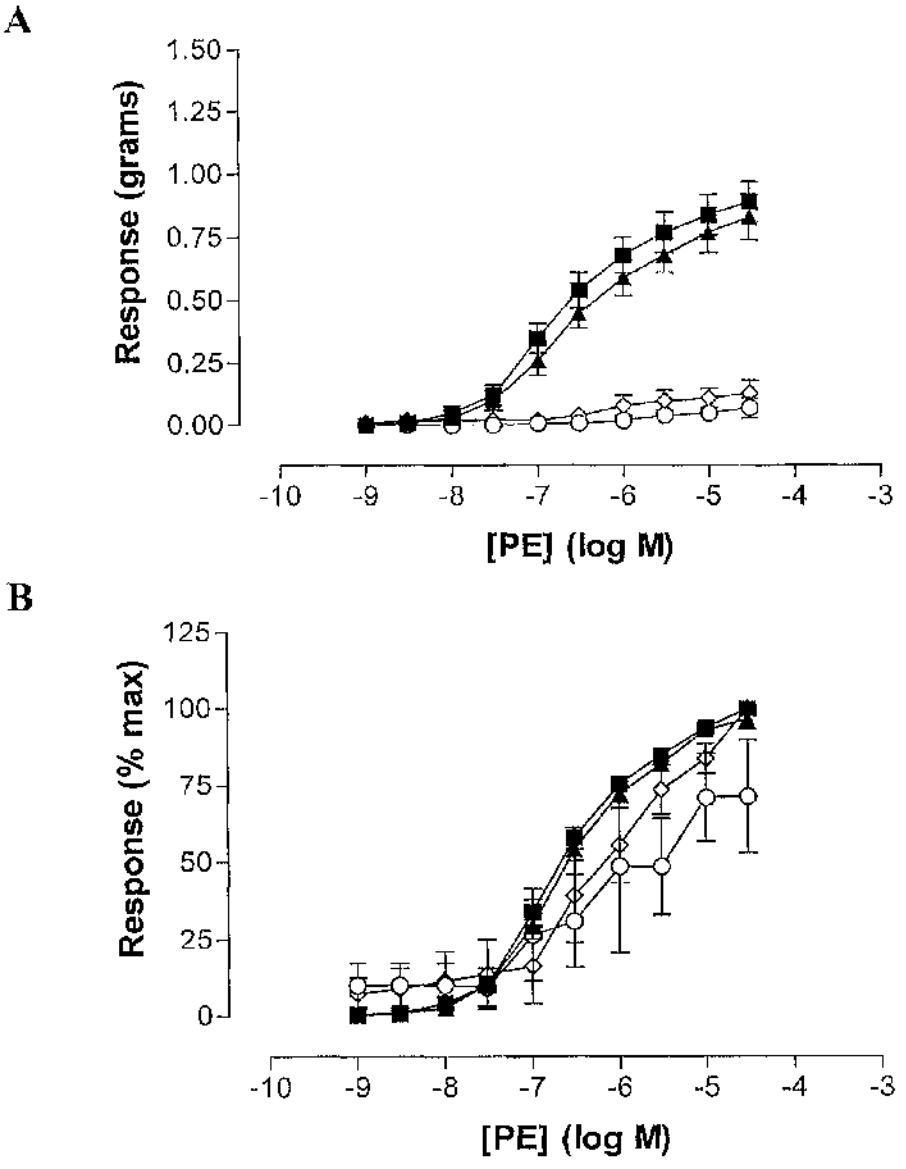


Figure 2-11. Receptor protection study: The effect of 10μM CEC treatment and the protective effect of 10nM BMY 7378 on PE-induced responses of WT aortae (■ control, $n = 23$; ▲ 10nM BMY 7378 control post-removal, $n = 18$; ○ 10μM CEC treated post-removal, $n = 7$; ◇ 10μM CEC treated, +10nM BMY 7378 protected post-removal, $n = 7$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	23	0.89 ± 0.08
+10nM BMY control *p.r.*	18	0.83 ± 0.08
+10μM CEC *p.r.*	7	0.07 ± 0.04***
+10μM CEC, +10nM BMY *p.r.*	7	0.13 ± 0.05***

B

	pEC_{50}	Hill slope
Control	6.73 ± 0.07	0.84 (*0.62-1.07*)
+10nM BMY control *p.r.*	6.62 ± 0.07	0.83 (*0.64-1.02*)
+10μM CEC *p.r.*	*n.c.*	*n.c.*
+10μM CEC, +10nM BMY *p.r.*	*n.c.*	*n.c.*

Table 2-11. Receptor protection study: The effect of 10μM CEC treatment, and the protective effect of 10nM BMY 7378 on PE-induced responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*p.r.*: post-removal; *n.c.*: not calculated, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

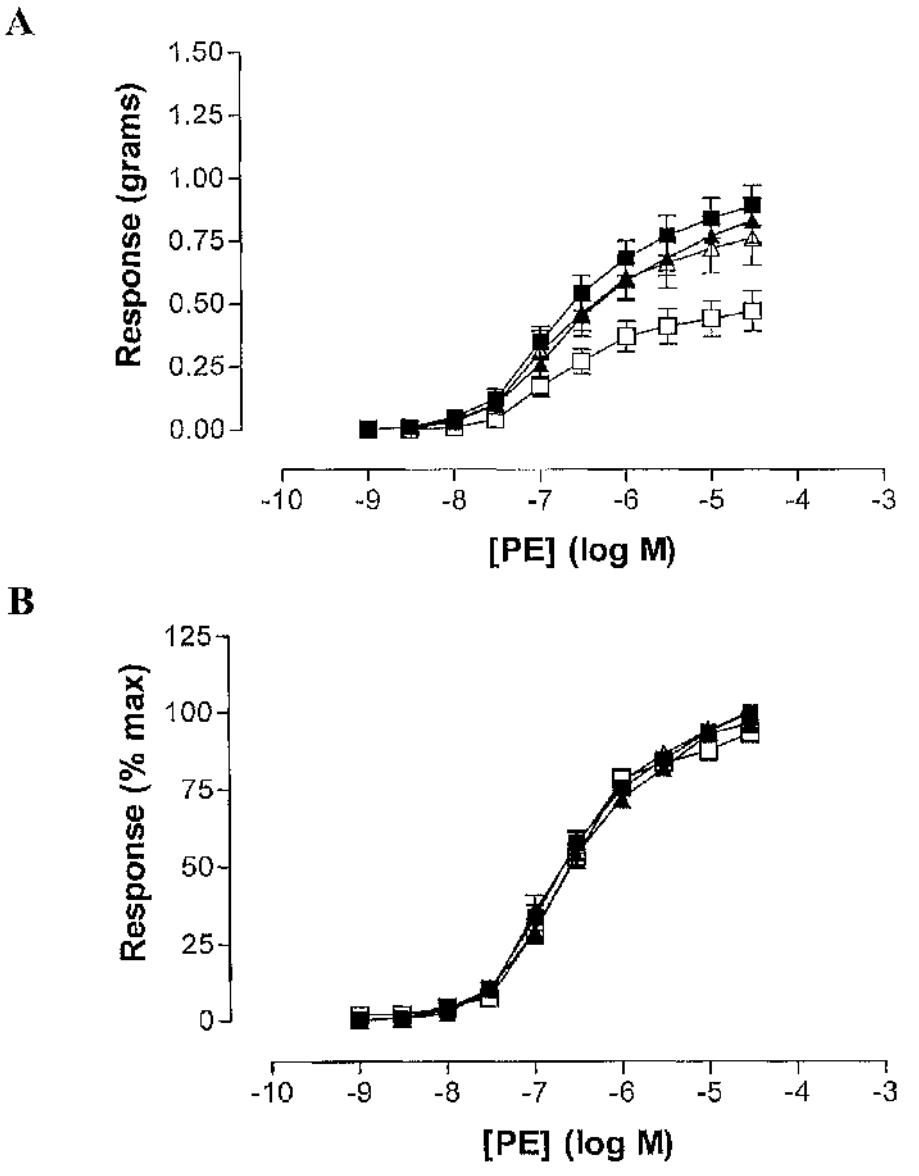


Figure 2-12. Receptor protection study: The effect of 1μM CEC treatment, and the protective effect of 10nM BMY 7378 on PE-induced responses of WT aortae (■ control, $n = 23$; ▲ 10nM BMY 7378 control post-removal, $n = 18$; □ 1μM CEC treated post-removal, $n = 18$; △ 1μM CEC treated, +10nM BMY 7378 protected post-removal, $n = 11$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	23	0.89 ± 0.08
+10nM BMY control *p.r.*	18	0.83 ± 0.08
+1μM CEC *p.r.*	18	0.49 ± 0.07***
+1μM CEC, +10nM BMY *p.r.*	11	0.76 ± 0.10§

B

	pEC_{50}	Hill slope
Control	6.73 ± 0.07	0.84 (*0.62-1.07*)
+10nM BMY control *p.r.*	6.62 ± 0.07	0.83 (*0.64-1.02*)
+1μM CEC *p.r.*	6.60 ± 0.07	1.11 (*0.87-1.36*)
+1μM CEC, +10nM BMY *p.r.*	6.73 ± 0.09	0.88 (*0.64-1.12*)

Table 2-12. Receptor protection study: The effect of 1μM CEC treatment, and the protective effect of 10nM BMY 7378 on PE-induced responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*p.r.*: post-removal; ***$p<0.001$ against control; §$p<0.05$ against 1μM CEC treated group: one-way-ANOVA, Bonferroni post-test).

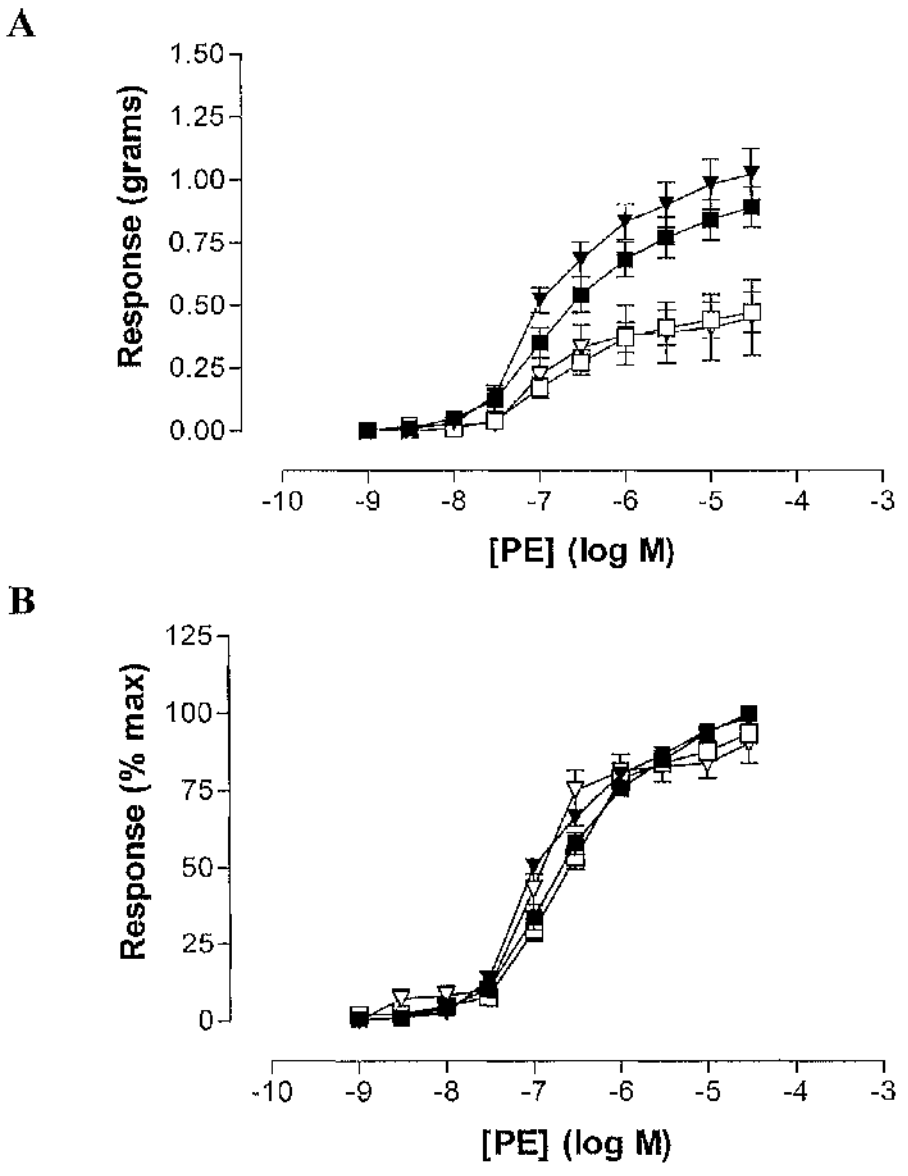


Figure 2-13. Receptor protection study: The effect of 1μM CEC treatment, and the protective effect of 10nM 5-MU on PE-induced responses of WT aortae (■ control, $n = 23$; ▲ 10nM 5-MU control, $n = 5$; □ 1μM CEC treated post-removal, $n = 18$; ▽ 1μM CEC treated post-removal, +10nM 5-MU protected post-removal, $n = 6$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	23	0.89 ± 0.08
+10nM 5-MU control *p.r.*	5	1.03 ± 0.10
+1μM CEC *p.r.*	18	0.49 ± 0.07***
+1μM CEC, +10nM 5-MU *p.r.*	6	0.47 ± 0.14***

B

	pEC_{50}	Hill slope
Control	6.73 ± 0.07	0.84 (*0.62-1.07*)
+10nM 5-MU control *p.r.*	6.95 ± 0.06	0.95 (*0.52-1.38*)
+1μM CEC *p.r.*	6.60 ± 0.07	1.11 (*0.87-1.36*)
+1μM CEC, +10nM 5-MU *p.r.*	6.90 ± 0.06	1.84 (1.04-*2.62*)

Table 2-13. Receptor protection study: The effect of 1μM CEC treatment, and the protective effect of 10nM 5-MU on PE-induced responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis. (*p.r.*: post-removal; *n.c.*: not calculated, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

Discussion- Chapter 2

α_1-AR in mouse aorta

The α_1-AR mediated response in the mouse aorta has previously been subtyped pharmacologically by Yamamoto & Koike (2001). The rank order of potency of 5-MU is $\alpha_{1a} > \alpha_{1b} = \alpha_{1d}$ (see following Table 2-14). Conversely the rank order of potency of BMY 7378 is $\alpha_{1d} > \alpha_{1a} = \alpha_{1b}$. Yamamoto & Koike (2001) reported an order of antagonist potency of BMY > 5-MU in mouse aorta with respective pA_2 values of 8.4 and 7.5, implicating the α_{1D}-ARs as the major adrenergic vasoconstrictor. Daly *et al.* (2002) and Tanoue *et al.* (2002) also subtyped the response using functional KO models. Their studies confirmed that the α_{1D}-AR is the major adrenergic vasoconstrictor in the mouse aorta, and our data in the previous chapter supports this.

However, all these authors suggested that the α_{1B}-AR has a minor vasoconstrictor component in the mouse aorta. Cavalli *et al.* (1997) had initially shown that PE was significantly less sensitive in α_{1B}-KO aortae. Later, Daly *et al.* (2002) reported that the α_{1B}-KO was not significantly different from the WT in contractile responses to α_1-AR agonists. Although the α_{1B}-AR has been implicated as having a minor role in vasoconstriction in mouse aorta by these authors, it had not yet been directly shown.

In contrast, Daly *et al.* (2002) reported a relatively high potency for 5-MU, an α_{1A}-AR subtype selective antagonist (Gross *et al.*, 1988; Schwinn *et al.*, 1995). 5-MU has an approximately 50-fold selectivity for cloned α_{1a}-ARs (pK_i = 8.8, see following Table 2-14) over cloned α_{1b}-ARs and α_{1d}-ARs (pK_i = 7.1 for both). Daly *et al.* (2002) reported 5-MU pA_2 values of 8.3 and 8.1 for WT and α_{1B}-KO aortae respectively. These values suggested a possible role for α_{1A}-ARs in function, yet Daly *et al.* (2000) concluded that there was no α_{1A}-AR mediated response.

Furthermore, recently, Deighan *et al.* (In Press) reported an α_{1A}-AR mediated response in α_{1D}-KO mouse carotid arteries, whilst the adrenergic response in WT mouse carotid arteries is primarily due to α_{1D}-AR activation. Thus, the suggestion that the α_{1A}-AR may be involved in the α_1-AR mediated response in mouse aortae is further

strengthened. Therefore, it was essential that the role of α_{1A}- and α_{1B}-ARs in mouse aorta be investigated. As such, the α_{1D}-KO mouse provided an excellent starting point.

Time Control

Before any detailed pharmacological analysis of the adrenergic PE-induced response of the α_{1D}-KO could be made, it was necessary to establish if the PE responses within a single aortic ring were repeatable. Thus, in order to make a fair comparison, the second curve was expressed as the percentage of the maximum of the first curve. Figure 2-1 shows that the second PE curve was significantly less sensitive.

The aim was to establish the receptor(s) involved in the residual adrenergic response in α_{1D}-KO aortae, not the residual response after desensitisation in α_{1D}-KO aortae. Although the desensitisation of the response was intriguing and merited further investigation, it was not followed up as it was out with the aims for this chapter. However, it was now clear that the PE-induced response in α_{1D}-KO aorta was prone to desensitisation.

Therefore, due to this desensitisation, the construction of consecutive PE curves in the aortic rings from α_{1D}-KO mice was something to be avoided. As a result, the antagonist studies done in the α_{1D}-KO were done by testing the effect of the antagonists on the first curve. The curves were normalised using the response of the single dose of PE during the wake-up as the maximal response. The sensitivity shifts of the antagonists could then be calculated, allowing the determination of the pK_B values.

Effect of prazosin

It was essential to establish whether PE-induced responses in α_{1D}-KO aorta were α_1-AR mediated or not before doing any detailed antagonist studies, therefore, the effect of prazosin was tested. The pK_B estimate for prazosin was 9.25. This value was calculated using the 1nM and 10nM data only. Although 100nM prazosin caused a shift, it had not significantly shifted the PE curve further than the shift produced by 10nM prazosin (Figure 2-2). Including this data would have artificially lowered the pK_B value calculated for prazosin.

The responses in the presence of 10nM and 100nM prazosin are at concentrations of PE at and above 100μM, bringing into question the selectivity of PE at α_1-ARs at these concentration. Laher *et al.* (1986) reported that at very high concentrations of AR agonists (100μM and higher), the responses are non-selective and do not appear to be susceptible to blockade by classical α_1-AR antagonists.

Laher *et al.* (1986) suggested that at high concentrations of α_1-AR agonists the response was due to the presence of 'extrareceptors'. This explains why 100nM prazosin did not have a significantly greater effect than 10nM prazosin as the PE may have lost selectivity for α_1-ARs.

Furthermore, the PE curves in the α_{1D}-KO particularly in the presence of prazosin did not achieve a true maximum. The presence of extrareceptors (Laher *et al.*, 1986) again explains this as at such high concentration of PE selectivity had been lost. This also further reinforces the rationale behind normalising the curves using the PE response during the wake-up as the maximum response, as this would clearly identify a shift in agonist sensitivity independent of curve maximum.

The control PE response in α_{1D}-KO was already significantly less sensitive than WT aortae and was approaching such concentrations where PE response may be due to activation of non-α_1-ARs. 10nM prazosin pushed the curve further to the right, to the point that 100nM prazosin could not block the response any further. Therefore the 100nM data was not included when estimating a pK_B for prazosin.

However, Table 2-14 shows a comparison of the affinities of various agonists and antagonists in aortic rings from WT, α_{1B}-KO, α_{1D}-KO and α_{1B}-α_{1D}-KO mice. The pA_2 value for prazosin in WT and α_{1B}-KO mice is 9.7 and 10.6 respectively (Daly *et al.*, 2002). The pK_i value for prazosin at cloned at cloned α_{1a}, α_{1b} and α_{1d} receptors is 9.0 (Mackenzie *et al.*, 2001).

Thus, although the pK_B for prazosin (9.25) in the α_{1D}-KO is about 3-fold and 30-fold less than in the WT and α_{1B}-KO respectively (Daly *et al.*, 2002), the pK_B value is still consistent with the action of prazosin at α_1-ARs. As there were no functional α_{1D}-ARs in the α_{1D}-KO, that prazosin could block, the adrenergic response must have been due

to either α_{1A}- or α_{1B}-ARs activation. The next step was an attempt to subtype the α_1-AR mediated response of the α_{1D}-KO.

A

Strain	PE	A-61603	praz.*	5-MU	BMY 7378	CEC sensitive	Concl.
albino ddY[1]	6.7[1]	*ND*[1]	9.7[1](*NE*)	7.5[1](*NE*)	8.4[1](*NE*)	yes[1]	α_{1D}, α_{1B}
WT	6.3	6.2	9.8[2]	8.3[2]	8.8[2]	yes[2]	α_{1D}, α_{1B}
α_{1B}-KO	6.7	6.8	10.6[2]	8.1[2]	9.2[2]	yes[2]	α_{1D}
α_{1D}-KO	5.0	*nr*	9.3(pK_B)	7.7(pK_B)	<7.0[3](*NE*)	yes[§]	α_{1B}
α_{1B}-α_{1D}-KO	*nr*	*nr*	*nd*	*nd*	*nd*	*nd*	No α_1-ARs

B

α_1-subtype	species		praz*	5-MU	BMY 7378	Reference
α_{1a}	Human		9.0	9.2	7.1	MacKenzie *et al.* (2001)
	Bovine			8.6	6.1	Saussy *et al.* (1994)
	Bovine			8.5		Goetz *et al.* (1993)
	Bovine				6.1	Goetz *et al.* (1995)
		MEAN	**9.0**	**8.8**	**6.4**	
α_{1b}	Human		9.0	7.2	6.8	MacKenzie *et al.* (2001)
	Hamster			7.0	6.2	Saussy *et al.* (1994)
	Hamster			7.0		Goetz *et al.* (1993)
	Hamster				6.2	Goetz *et al.* (1995)
		MEAN	**9.0**	**7.1**	**6.4**	
α_{1d}	Human		9.0	7.9	9.3	MacKenzie *et al.* (2001)
	Rat			7.3	8.1	Saussy *et al.* (1994)
	Rat			6.1		Goetz *et al.* (1993)
	Rat				8.2	Goetz *et al.* (1995)
		MEAN	**9.0**	**7.1**	**8.5**	

Table 2-14. The affinities of several α_1-selective agents in transgenic mice (**A**) and their appropriate affinities in cloned receptors (**B**). The conclusions are shown in the right-hand column of (**A**).

A. For PE and A61603, the pEC_{50} values are shown. For the antagonists the pA_2 value is shown unless otherwise indicated (agonist used was PE, unless otherwise stated). Greyed out boxes indicate values taken from reference sources ([1]Yamamoto & Koike, 2001; [2]Daly *et al.*, 2002; [3]Tanoue *et al.*, 2002 demonstrated no shift at 100nM BMY 7378; [§]observed in initial experiments- not shown).

B. pK_i values from several authors are shown. The mean pK_i values for the antagonists at each cloned receptor have been calculated. These values are used for discussion.

(Abbreviations: prazosin*; NE – agonist used was norepinephrine; *nr*- no response; *nd*- not determined)

Effect of 5-MU

The estimated pK_B for 5-MU was 7.65. Previously Daly *et al.* (2002) reported pA_2 values of 8.3 and 8.1 in WT and α_{1B}-KO aortae. They concluded that the mouse aorta adrenergic response is α_{1D}-AR mediated as BMY 7378 (α_{1D}-selective antagonist: Saussy *et al.*, 1994) was 3-fold and 13-fold more sensitive than 5-MU in WT and α_{1B}-KO aortae respectively.

In their study of the α_{1D}-KO, Tanoue *et al.* (2002) reported that in α_{1D}-KO aortae, BMY 7378 demonstrated no significant antagonism even when used at 100nM, suggesting the pK_B be greater than 7.0 and at most, equal to 7.0. Therefore the antagonist potency in the α_{1D}-KO was 5-MU > BMY 7378, indicating that α_{1A}-ARs may be involved.

Yet, even with a pA_2 value of 8.3 for 5-MU in WT mice, Daly *et al.* (2002) discounted the role of α_{1A}-ARs in vasoconstriction in mouse aorta, instead suggesting the response was mainly due to α_{1D}-AR activation with a minor role for α_{1B}-ARs. Therefore the response subtyped in the present study is likely to be α_{1B}-AR mediated.

This hypothesis is further strengthened by the affinity for 5-MU at cloned receptors. 5-MU has pK_i values of 8.8 and 7.1 at cloned bovine α_{1a}-ARs and cloned hamster α_{1b}-ARs respectively (see Table 2-14). Thus the pK_B estimate of 7.65 in α_{1D}-KO aortae is more consistent with the binding of 5-MU at α_{1b}-ARs, further reinforcing the presented hypothesis that the α_{1B}-AR is the adrenergic vasoconstrictor in α_{1D}-KO aortae.

It must be noted that the pK_B for 5-MU was calculated with the 10nM, 100nM and 1μM 5-MU data. No significant effect of 1nM 5-MU was observed therefore the data was not included as it was not always possible to calculate a pK_B.

However, the estimation of a pK_B for an antagonist is dependent on competitive antagonism which assumes a Schild slope of unity (Arunlakshana & Schild, 1959). This describes a direct relationship between antagonist concentration and the blockade produced, i.e. a tenfold increase in antagonist concentration should result in a tenfold increase in blockade, which would be visualised by a tenfold shift of the log-drug concentration-response curve.

The 5-MU data does not fit this model though. At 10nM BMY 7378 a 4-fold decrease in PE sensitivity was observed, whilst a 7-fold decrease at 100nM 5-MU and a 34-fold decrease in PE sensitivity at 1μM 5-MU were observed. From the estimated pK_B of 7.65, the shifts should have been the following: 10nM 5-MU = no shift, 100nM 5-MU = 9-fold decreased PE sensitivity and 1μM 5-MU = 90-fold decreased sensitivity. These estimations are based on the assumptions that the calculated pK_B was accurate, and assuming the Schild slope for 5-MU fits unity and therefore the $pK_B = pA_2$, which results in a two-fold shift of the PE response.

Therefore, a shadow is cast over the confidence that can be placed in the pK_B for 5-MU in α_{1D}-KO aortae. As such, another approach taken was the use of the α_{1A}-AR selective agonist, A61603 in an effort to confirm the hypothesis that the response in α_{1D}-KO aortae was α_{1B}-AR mediated.

PE & A61603 responses in WT, α_{1B}-KO and α_{1D}-KO

A61603 is reported to be an α_{1A}-AR agonist. Knepper *et al.* (1995) reported that the A61603 has a chiral carbon, and as such, has two enantiomers. The R-enantiomer was found to be the most active with an affinity of 163-fold and 58-fold selectivity for cloned α_{1a}-ARs over α_{1b}- and α_{1d}-ARs respectively (the discussion about A61603 following in this section is about the R-enantiomer unless otherwise stated). Thus, the affinity for A61603 for cloned receptors is $\alpha_{1a} > \alpha_{1d} \geq \alpha_{1b}$.

On the other hand, PE was reported to have an affinity for α_{1d}-ARs, over α_{1b}- and α_{1a}-ARs (6-fold and 8-fold respectively: Knepper *et al.*, 1995) but this selectivity is not as profound as the selectivity of A61603 and, therefore, is less useful for analysis.

As was discussed in the previous chapter, α_{1B}-KO aortae were more sensitive to PE than WT aortae whilst, α_{1D}-KO aortae were less sensitive (shown in Figure 2-4). This trend is continued with A61603, where the α_{1B}-KO was more sensitive than the WT. However, the α_{1D}-KO did not exhibit a notable contractile response to A61603. This result confirmed that there was no significant α_{1A}-AR mediated component in the α_{1D}-KO, but the relatively high sensitivity of A61603 in WT and α_{1B}-KO aortae was somewhat surprising.

PE and A61603 exhibited similar sensitivity in WT and α_{1B}-KO aortae. In contrast, using the racemate mix of A61603 and comparing it to PE in rat aorta (vasoconstriction due to α_{1D}-ARs), Knepper *et al.* (1995) reported the EC_{50} for PE was 33-fold less than A61603 (i.e. PE was significantly more sensitive). Therefore it was deemed necessary to establish if A61603 was acting only at α_1-ARs.

The effect of prazosin on A61603 responses

A61603 is an imidazoline derivative. Classically, imidazoline derivatives have been associated with α_2-AR activation or blockade, but recent research findings indicate that imidazolines can elicit responses independent of α-ARs, but through a new category of receptors, imidazoline or I receptors (Reviewed by Molderings & Gother, 1999). Furthermore, Minyan *et al.* (2000) recently reported that contractile responses to oxymetazoline in porcine rectal arteries, were I_3 receptor mediated, and not α_2-AR mediated as initially hypothesised, whilst Willems *et al.* (2001) reported A61603 could elicit vasoconstriction in porcine carotid arteries through a non-α_1-AR mechanism. Therefore, it was essential to verify that the A61603-induced contractile responses were α_1-AR mediated, which was done by testing the effect of prazosin.

The pK_B estimates for prazosin in WT and α_{1B}-KO aortae were 8.45 and 8.46 respectively. Therefore the A61603 response was α_1-AR mediated, but no attempt to pharmacologically subtype this response was made. However, the indications are that the A61603 response was due to α_1-AR activation.

A61603 and PE had similar pEC_{50} values in WT aortae, where the response is believed to be α_{1D}-AR mediated, with a minor role for α_{1B}-ARs in vasoconstriction. In α_{1B}-KO aortae A61603 and PE also had similar sensitivity and efficacy. Daly *et al.* (2002) reported that the response in α_{1B}-KO aortae is only α_{1D}-AR mediated, therefore A61603 must be activating α_{1D}-ARs as no role for α_{1A}-ARs has been reported. Furthermore, A61603 did not elicit a significant response in α_{1D}-KO aortae, confirming that the contractile response is α_{1D}-AR mediated, and that there were no functional α_{1A}-ARs.

Knepper *et al.* (1995) reported that there was no detectable increase in IP_3 levels when cells transfected with cloned α_{1b}-ARswere stimulated with the racemate mix of A61603,

whereas the EC_{50} for A61603 (racemate mix) for α_{1a}-ARs was 14.5nM. Therefore, although A61603 had affinity for α_{1b}-ARs it did not have efficacy. In the present study, no activity of A61603 was observed in the α_{1D}-KO. Had there been functional α_{1A}-ARs in α_{1D}-KO aortae A61603 should have had functional response but it did not. Therefore, the lack of reactivity of α_{1D}-KO aortae to A61603, indicates no role for α_{1A}-ARs, leaving only the α_{1B}-AR subtype.

Response of the α_{1B}-α_{1D}-KO

The serotonergic responses in the double-KO, the α_{1B}-α_{1D}-AR KO, were not significantly different from WT serotonergic responses, confirming the contractile machinery was intact and functional. However, there was a distinct lack of α_1-AR mediated responses. Neither PE nor A61603 was able to elicit a significant response that could be pharmacologically studied.

This evidence further supports the present hypothesis, that the adrenergic response in mouse aorta is α_{1D}-AR mediated, with a minor role for α_{1B}-ARs, as the lack of functional α_{1B}-ARs does not have a significantly adverse effect. In the absence of functional α_{1D}-ARs the response is α_{1B}-AR mediated. The lack of both α_{1B}- and α_{1D}-ARs results in a complete loss of adrenergic response.

However a few unresolved issues remained. The relatively high estimated pA_2 values for 5-MU in WT aortae reported by Daly *et al.* (2002), merited further investigation, whilst an attempt to evaluate the minor role of α_{1B}-ARs in WT aortae was also made.

Receptor protection

No suitable α_{1B}-AR antagonist is available currently, so a more intricate approach was taken. Chloroethylclonidine (CEC) was initially reported to be an α_{1B}-AR selective alkylating agent (Perez *et al.*, 1987). A more recent report by Hirasawa *et al.* (1997) argued that α_1-ARs present on the cell membrane are preferentially alkylated, irrespective of α_1-AR subtype. They suggested the apparent selectivity is due to the heterogeneous distribution of α_1-ARs subtypes at the cellular level.

However, the irreversible nature of CEC was useful in the receptor protection protocol used. By pre-incubating the tissue with a subtype-selective surmountable antagonist, before CEC treatment and then removing CEC and then the competitive antagonist, the assumption is that the only response remaining was subtype protected by the competitive antagonist used. Such a receptor protection technique has successfully been used previously by Ibarra *et al.* (2000) to demonstrate that α_{1D}-ARs are the major adrenergic vasoconstrictors in mouse aorta.

In the present study, it was necessary to ensure that the competitive antagonists used could be washed away without adverse effects on contractility. Figures 2-11 and 2-12 show that 10nM BMY 7378 could be successfully washed away, so that the response was not significantly affected. Similarly, when 10nM 5-MU was used it could also be washed away (Figure 2-13). Thus, the antagonism of these two ligands is reversible by removal of the antagonist from the baths.

The next aim was to establish a suitable concentration of CEC required for the study. 10μM CEC almost completely ablated the PE response. However, 1μM CEC treatment halved the maximal PE response. CEC treatment was able to decrease the efficacy of PE in mouse aorta in a concentration-dependent manner.

Normalising the responses revealed there was no significant difference in sensitivity when treated with CEC, consistent with the effects of a classical insurmountable antagonist. CEC alkylation decreased the total number of receptors available without affecting the affinity of PE. The pEC_{50} is a measure of the affinity of the agonist, not of the efficacy, and was unchanged.

10nM BMY 7378 was unable to protect any of the response, when the tissue was treated with 10μM CEC indicating the concentration of CEC was too high. This result also further highlighted the non-selective nature of CEC. In contrast, 1μM CEC reduced the response by about 50%. Pre-incubation with 10nM BMY 7378 was able to protect the response, such that the entire response was maintained, and no effect of CEC treatment was observed. Therefore the effect of CEC observed at both 1μM and 10μM was due to α_{1D}-AR alkylation. The concentration of BMY 7378 was selectively chosen so as not to have an effect at either α_{1A}- or α_{1B}-ARs. Since 10nM BMY 7378 was able to protect the entire response from 1μM CEC treatment, no significant role for α_{1B}-ARs was detected.

10nM 5-MU was unable to protect any of the response from 1μM CEC treatment, confirming the original hypothesis that there were no α_{1A}-ARs in the mouse aorta. However, this result still does not explain the relatively high affinity of 5-MU in WT mouse aorta reported by Daly *et al.* (2002).

This new evidence, from the receptor protection study, compels a revision of the previous hypothesis that α_{1B}-ARs have a minor role in vasoconstriction. The indications are that neither the α_{1A}- nor α_{1B}-ARs are involved in contraction of WT mouse aorta. Thus the α_{1D}-AR is the sole adrenergic vasoconstrictor in WT mouse aorta. However, in the absence of α_{1D}-ARs, the α_{1B}-AR is the adrenergic vasoconstrictor.

Conclusion- Chapter 2

Conclusion

A61603 is a potent and efficacious agonist at α_{1D}-ARs, bringing into question its usefulness as an α_{1A}-AR selective agonist. It has no significant α_{1B}-AR activity.

No α_{1A}-AR mediated contractile response has been detected in any of the studies performed.

From this data presented in this study, it is clear that there is no significant role for α_{1A}- or α_{1B}-ARs in WT mouse aorta. A lack of functional α_{1B}-ARs did not affect the response. A lack of α_{1D}-ARs in α_{1D}-KO aortae, uncovered an α_{1B}-AR mediated response which has not been detected in the WT.

The lack of both α_{1B}- and α_{1D}-ARs in the α_{1B}-α_{1D}-KO resulted in no functional adrenergic responses, whilst the serotonergic responses remained unaltered confirming that the contractile machinery was functional.

Thus, the adrenergic response in WT mouse aorta is only α_{1D}-AR mediated, with no significant contractile role for α_{1A}- or α_{1B}-ARs.

Chapter 3. Adrenergic and serotonergic synergy in the mouse aorta.

Introduction- Chapter 3

Serotonergic and adrenergic synergism

The vasoactive nature of serotonin and its synergistic interactions have been studied extensively (reviewed by Yildiz *et al.*, 1998). The effect of co-activation of the serotonergic and adrenergic vasoconstrictor systems was originally discussed by de la Lande *et al.* (1966) who demonstrated a functional interaction between 5-hydroxytryptamine (5-HT) and norepinephrine (NE). In particular, α_1-adrenergic and serotonergic contractile synergy has been described in several vascular preparations, such as the rabbit aorta (Stupecky *et al.*, 1986), rabbit femoral artery, (Chen *et al.*, 2000), rat aorta (Christ and Jean-Jacques, 1990) and the rat caudal artery (Van Nueten *et al.*, 1981).

5-HT and α_1-adrenoceptors

It has previously been reported by Purdy *et al.* (1987) that 5-HT-induced responses in the rabbit aorta are due to the partial involvement of α_1-adrenoceptors (α_1-ARs). Recently, Shaw *et al.* (2000) established concentration-dependent prazosin blockade of 5-HT responses in rat pulmonary arteries, estimating a pK_B of 10.2, consistent with the sensitivity of prazosin at α_1-ARs.

However, such studies, perhaps due to a lack of suitably selective ligands, have not ascertained which of the α_1-AR subtypes are involved in the adrenoceptor mediated response of 5-HT. Commonly used α_1-AR ligands such as the α_{1A}-AR antagonist, 5-methylurapidil (5-MU: Gross *et al.*, 1988, Schwinn *et al.*, 1995) and the α_{1D}-AR antagonist, BMY 7378 (Saussy *et al.*, 1994) are known to be 5-HT_{1A} partial agonists (Gross *et al.*, 1987 and Yocca *et al.*, 1987 for 5-MU and BMY 7378 respectively). Therefore results obtained using these ligands could be ambiguous. On a similar note, kitanserin, a widely used 5-HT_2 receptor antagonist has been shown to have a high affinity for α_1-ARs (Van Neuten *et al.*, 1981).

The mouse aorta

Murine models are increasingly being used in cardiovascular studies, primarily due to the numerous functional knockouts, overexpressing and transgenic mice that are now available. Recently, Russell & Watts (2000) demonstrated an extensive agonist profile of the "Vascular reactivity of the isolated thoracic aorta of the C57/BL/6J mouse." It was observed that the aorta exhibited both a high degree of sensitivity and a substantial contractile response to 5-HT. McKune & Watts (2001) subtyped this response, concluding the serotonergic response of the mouse aorta was primarily the result of 5-HT_{2A} receptor activation.

In addition, both NE and phenylephrine (PE) were shown to be potent agonists, indicating that the mouse aorta had a significant α_1-AR mediated contractile response. The major adrenergic vasoconstrictor of the mouse aorta has been characterised pharmacologically by Yamamoto *et al.* (2002) and also by using transgenic mice. Pharmacological studies in both the α_{1B}-AR knockout mouse (Daly *et al.*, 2002) and α_{1D}-AR knockout mouse (α_{1D}-KO; Tanoue *et al.*, 2002) have been performed, with all groups agreeing that the adrenergic response was predominantly mediated by α_{1D}-ARs.

Hence, studying synergy and the AR-mediated response of 5-HT in the mouse aorta using both traditional pharmacological techniques and transgenic mice may provide useful insights into the roles and interactions of the serotonergic and α_1-adrenergic vasoconstrictor systems.

Aim of study

Initially, an attempt was made to uncover any functional interactions between the serotonergic and adrenergic vasoconstrictors in the mouse aorta. The activation of ARs by 5-HT in the mouse aorta was then studied in detail by subtyping the α_1-AR component of the 5-HT-mediated response, employing a combination of selective pharmacological agents and transgenic α_{1D}-KO mice. Finally, the 5-HT receptor subtypes involved in the 5-HT responses of both the WT and α_{1D}-KO mice were established.

Results- Chapter 3

Effect of 5-HT on PE-induced contractions of WT aortae

10nM 5-HT caused a response of 0.04 ± 0.03g (n = 6) whilst 30nM 5-HT resulted in a response of 0.14 ± 0.02g (n = 7).

E_{max} values for PE-induced contractile responses in the presence and absence of 5-HT were: 1st curve = 0.85 ± 0.04g (n = 13); time control = 0.97 ± 0.06g (n = 12); +10nM 5-HT = 1.01 ± 0.12g (n = 6); +30nM 5-HT = 1.01 ± 0.07g (n = 7). None of the groups were significantly different from the time control in maximum responses (one-way-ANOVA, Bonferroni post-test).

The sensitivities of the PE responses were:
pEC_{25} values- 1st curve = 6.98 ± 0.06; time control = 7.18 ± 0.12; +10nM 5-HT = 7.07 ± 0.13; +30nM 5-HT = 7.98 ± 0.14.
pEC_{50} values- 1st curve = 6.26 ± 0.06; time control = 6.47 ± 0.09; +10nM 5-HT = 6.44 ± 0.15; +30nM 5-HT = 7.29 ± 0.14.
The 1st curve, and +10nM 5-HT treated groups were not significantly different from the time control in sensitivity. The +30nM 5-HT treated group was 6-fold ($p<0.001$) and 7-fold ($p<0.001$) more sensitive than the time control at the EC_{25} and EC_{50} respectively (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: 1st curve = 0.56 (*0.39-0.73*); time control = 0.56 (*0.43-0.70*); +10nM 5-HT = 0.58 (*0.41-0.75*); +30nM 5-HT = 0.57 (*0.49-0.66*). The Hill slopes did not overlap with unity.

The PE responses curves are shown in Figure 3-1 and the values shown above are tabulated in Table 3-1.

Effect of PE on 5-HT-induced contractions of WT aortae

10nM PE caused a response of 0.05 ± 0.03g (n = 6) whilst 30nM PE resulted in a response of 0.19 ± 0.05g (n = 7).

E_{max} values for 5-HT-induced contractile responses in the presence and absence of PE were: 1st curve = 1.41 ± 0.07g (n = 13); time control = 1.33 ± 0.10g (n = 12); +10nM PE = 1.53 ± 0.18g (n = 6); +30nM PE = 1.12 ± 0.09g (n = 7). None of the groups were significantly different from the time control in maximum responses (one-way-ANOVA, Bonferroni post-test).

The sensitivities of the 5-HT responses were:
pEC_{25} values- 1st curve = 7.26 ± 0.05; time control = 7.20 ± 0.12; +10nM PE = 7.23 ± 0.09; +30nM PE = 7.87 ± 0.21.
pEC_{50} values- 1st curve = 6.91 ± 0.05; time control = 6.84 ± 0.02; +10nM PE = 6.86 ± 0.06; +30nM PE = 7.34 ± 0.20.
The 1st curve, and +10nM PE treated groups were not significantly different from the time control in sensitivity. The +30nM PE treated group was 5-fold ($p<0.01$) and 3-fold ($p<0.05$) more sensitive than the time control at the EC_{25} and EC_{50} respectively (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: 1st curve = 1.22 (*1.05-0.38*); time control = 1.23 (*1.00-1.45*); +10nM PE = 1.19 (*1.07-1.32*); +30nM PE = 0.68 (*0.38-0.99*). The Hill slopes overlapped with unity but the Hill slope of the +30nM PE treated group was shallower than the other groups (not statistically tested).

The 5-HT responses curves are shown in Figure 3-2 and the values shown above are tabulated in Table 3-2.

Effect of prazosin on 5-HT-induced contractions of WT aortae

E_{max} values for 5-HT responses in the absence and presence of various concentrations of prazosin in WT aortae were: control = 1.27 ± 0.13g (n = 5); +1nM prazosin = 1.00 ± 0.30g (n = 3); +10nM prazosin = 1.07 ± 0.17g (n = 5); +100nM prazosin = 0.91 ± 0.14g (n = 5). None of the groups were significantly different from the control in maximum responses (one-way-ANOVA, Bonferroni post-test).

The sensitivities were:
pEC_{25} values- control = 7.53 ± 0.13; +1nM prazosin = 7.03 ± 0.18; +10nM prazosin = 6.95 ± 0.08; +100nM prazosin = 6.89 ± 0.02.
pEC_{50} values- control = 7.08 ± 0.09; +1nM prazosin = 6.76 ± 0.15; +10nM prazosin = 6.70 ± 0.07; +10nM prazosin = 6.69 ± 0.02.
The 1nM prazosin treated group was 3-fold less sensitive ($p<0.05$) than the control at the EC_{25} but not significantly different at EC_{50}. Both the 10nM prazosin and 100nM prazosin treated groups were 4-fold ($p<0.01$) and 2-fold ($p<0.05$) less sensitive than the WT at the EC_{25} and EC_{50} respectively (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: control = 1.39 (*0.78-2.00*); +1nM prazosin = 1.78 (*1.23-2.32*); +10nM prazosin = 2.25 (*1.78-2.72*); +100nM prazosin = 3.00 (*1.03-4.97*). The control curve overlapped unity. The 1nM, 10nM and 100nM prazosin treated groups did not overlap with unity.

The effects of prazosin on the 5-HT response curve in WT aortae are shown in Figure 3-3 and the values shown above are tabulated in Table 3-3.

Effect of BMY 7378 on 5-HT-induced contractions of WT aortae

E_{max} values for 5-HT responses in the absence and presence of various concentrations of BMY 7378 in WT aortae were: control – 1.45 ± 0.19g (n = 6); +1nM BMY 7378 = 1.35 + 0.14g (n = 5); +10nM BMY 7378 = 1.19 ± 0.12g (n = 6); +100nM BMY 7378 = 1.15 ± 0.13g (n = 6). None of the groups were significantly different from the control in maximum responses (one-way-ANOVA, Bonferroni post-test).

The sensitivities were:
pEC_{25} values- control = 7.73 ± 0.11; +1nM BMY 7378 = 7.44 ± 0.05; +10nM BMY 7378 = 7.21 ± 0.11; +100nM BMY 7378 = 7.05 ± 0.11.
pEC_{50} values- control = 7.34 ± 0.11; +1nM BMY 7378 = 7.02 ± 0.04; +10nM BMY 7378 = 6.90 ± 0.09; +100nM BMY 7378 = 6.77 ± 0.08.
The 1nM BMY 7378 treated group was 2-fold less sensitive ($p<0.05$) at the EC_{25} but not significantly different at the EC_{50}.The 10nM BMY 7378 treated group was 3-fold less sensitive at both the EC_{25} ($p<0.01$) and EC_{50} ($p<0.05$). The 100nM BMY 7378 treated group was 5-fold ($p<0.001$) and 4-fold ($p<0.01$) less sensitive at the EC_{25} and EC_{50} respectively (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: control = 1.03 (*0.66-1.38*); +1nM BMY 7378 = 1.40 (*1.12-1.67*); +10nM BMY 7378 = 1.59 (*1.35-1.84*); + BMY 7378 prazosin = 1.80 (*1.57-2.03*). The control curve overlapped unity. The 1nM, 10nM and 100nM BMY 7378 treated groups did not overlap with unity.

The effects of prazosin on the BMY 7378 response curve in WT aortae are shown in Figure 3-4 and the values shown above are tabulated in Table 3-4.

Effect of prazosin on 5-HT-induced contractions of α_{1D}-KO aortae

E_{max} values for 5-HT responses in the absence and presence of various concentrations of prazosin in WT aortae were: control = 0.99 ± 0.14g (n = 7); +1nM prazosin = 0.90 ± 0.13g (n = 6); +10nM prazosin = 1.21 ± 0.10g (n = 7); +100nM prazosin = 1.10 ± 0.10g (n = 7). None of the groups were significantly different from the control in maximum responses (one-way-ANOVA, Bonferroni post-test).

The sensitivities were:
pEC_{25} values- control = 7.33 ± 0.08; +1nM prazosin = 7.17 ± 0.07; +10nM prazosin = 7.38 ± 0.09; +100nM prazosin = 7.28 ± 0.04.
pEC_{50} values- control = 7.05 ± 0.07; +1nM prazosin = 6.91 ± 0.06; +10nM prazosin = 7.08 ± 0.07; +100nM prazosin = 7.00 ± 0.05.
No significant difference in sensitivity between the control and the prazosin treated groups was observed (one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: control = 1.56 (*1.18-1.95*), +1nM prazosin = 1.91 (*1.46-2.35*), +10nM prazosin = 1.53 (*1.33-1.73*), +100nM prazosin = 1.80 (*1.15-2.45*). The Hill slopes did not overlap with unity.

The effects of prazosin on the 5-HT response curve in α_{1D}-KO aortae are shown in Figure 3-5 and the values shown above are tabulated in Table 3-5.

Comparing the effects of prazosin & BMY 7378 in WT & α_{1D}-KO aortae

Figure 3-6 and Table 3-6 compares the effects of 10nM prazosin and 10nM BMY 7378 in the WT with the effect of 10nM and 100nM prazosin in the α_{1D}-KO. In the WT 10nM prazosin resulted in a serotonergic response which was 4-fold ($p<0.01$) and 2-fold ($p<0.05$) less sensitive than the control at the EC_{25} and EC_{50} respectively. Furthermore, 10nM BMY 7378 in the WT resulted in a 5-HT response which was 3-fold less sensitive at both the EC_{25} ($p<0.01$) and EC_{50} ($p<0.05$), but 10nM and 100nM prazosin had no effect on the serotonergic response of α_{1D}-KO aortae (one-way-ANOVA, Bonferroni post-test).

The effect of α_{1D}-AR activation on 5-HT contractile responses

Figure 3-7 and Table 3-7 shows a comparison between the effects of 30nM PE and 10nM prazosin on the serotonergic response of the WT. The presence of 30nM PE caused the 5-HT response to be 5-fold ($p<0.01$) and 3-fold ($p<0.05$) more sensitive than the control at both the pEC_{25} and pEC_{50} values respectively and the Hill slope was shallower. 10nM prazosin resulted in the serotonergic response to be 4-fold ($p<0.01$) and 2-fold ($p<0.05$) less sensitive than the WT at the EC_{25} and EC_{50} respectively (one-way-ANOVA, Bonferroni post-test). Furthermore, in the presence of prazosin, the Hill slope steepened.

Serotonergic and adrenergic responses of WT and α_{1D}-KO aortae

No significant difference between the control 5-HT responses of the WT and α_{1D}-KO were observed (one-way-ANOVA, Bonferroni post-test). The comparison is shown in Figure 3-8 and values are tabulated in Table 3-8.

Effect of ritanserin on 5-HT & BRL 54443-induced contractions of WT aortae

The E_{max} values for 5-HT or BRL 54443 responses in the absence and presence of 10nM ritanserin in WT aortae were: 5-HT, control – 1.49 ± 0.13 (n = 7); 5-HT +10nM ritanserin = 0.43 ± 0.11 (n = 6); BRL 54443, control = 1.04 ± 0.24 (n = 7); BRL 54443 +10nM ritanserin = 0.01 ± 0.01 (n = 7). The presence of 10nM ritanserin significantly reduced the maximal responses for 5-HT ($p<0.001$) and BRL 54443 ($p<0.001$). 5-HT and BRL 54443 maxima were not significantly different from one another (one-way-ANOVA, Bonferroni post-test).

The sensitivities (pEC_{50} values) of the responses were: 5-HT control = 7.23 ± 0.13; BRL 54443 control – 6.47 ± 0.16. It was not possible to estimate the EC_{50} values for the responses in the presence of 10nM ritanserin. BRL 54443 responses were 6-fold less sensitive than 5-HT responses ($p<0.05$: one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: 5-HT control = 1.37 (*1.00-1.74*); BRL 54443 control = 1.03 (*0.71-1.35*). It was not possible to estimate the Hill slopes for the responses in the presence of 10nM ritanserin. The Hill slopes overlapped unity.

The effects of 10nM ritanserin on serotonergic responses of the WT are shown in Figure 3-9 and the above values are tabulated in Table 3-9.

Effect of ritanserin on 5-HT & BRL 54443-induced contractions of α_{1D}-KO aortae

The E_{max} values for 5-HT or BRL 54443 responses in the absence and presence of 10nM ritanserin in α_{1D}-KO aortae were: 5-HT, control = 1.67 ± 0.23 (n = 7); 5-HT +10nM ritanserin = 0.16 ± 0.09 (n = 6); BRL 54443, control = 1.18 ± 0.24 (n = 7); BRL 54443 +10nM ritanserin = 0.02 ± 0.01 (n = 7). The presence of 10nM ritanserin significantly reduced the maximal responses for 5-HT ($p<0.001$) and BRL 54443 ($p<0.001$). 5-HT and BRL 54443 maxima were not significantly different from one another (one-way-ANOVA, Bonferroni post-test).

The sensitivities (pEC_{50} values) of the responses were: 5-HT control = 7.48 ± 0.23; BRL 54443 control = 6.49 ± 0.13. It was not possible to estimate the EC_{50} values for the responses in the presence of 10nM ritanserin. BRL 54443 responses were 10-fold less sensitive than 5-HT responses ($p<0.05$: one-way-ANOVA, Bonferroni post-test).

The Hill slopes were: 5-HT control = 0.96 (*0.47-1.10*); BRL 54443 control = 1.02 (*0.59-1.45*). It was not possible to estimate the Hill slopes for the responses in the presence of 10nM ritanserin. The Hill slopes overlapped unity.

The effects of 10nM ritanserin on serotonergic responses of the α_{1D}-KO are shown in Figure 3-10 and the above values are tabulated in Table 3-10.

Comparison of the effects of ritanserin on 5-HT-induced contractions of WT & α_{1D}-KO

The control 5-HT responses of WT and α_{1D}-KO aortae were not significantly different in maxima or sensitivity (one-way-ANOVA, Bonferroni post-test). In the presence of ritanserin, the WT (0.43 ± 0.11g) had a significantly greater ($p<0.05$) maximal response than the α_{1D}-KO (0.16 ± 0.09). The comparison of 5-HT responses in WT and α_{1D}-KO aortae is shown in Figure 3-11 and Table 3-11.

Comparison of the effects of ritanserin on BRL 54443-induced contractions of WT & α_{1D}-KO

Comparison of the control BRL 54443-induced contractile responses in WT and α_{1D}-KO revealed no significant difference. The effect of ritanserin was almost identical in both strains (one-way-ANOVA, Bonferroni post-test). The comparison of BRL 54443 responses in WT and α_{1D}-KO aortae is shown in Figure 3-12 and Table 3-12.

Effect of ritanserin on PE-induced and U46619-induced contractile responses of WT

Responses to a single challenge of 10μM PE in WT aortae in the absence and presence of 10nM ritanserin were: control = 0.85 + 0.07g (n = 6), +10nM ritanserin = 0.69 ± 0.09g (n = 6). Responses induced by a single U46619 (100nM) challenge were: control = 1.74 ± 0.10g (n = 5), +10nM ritanserin = 1.81 ± 0.10g (n = 5). Responses of WT aortae to single challenges of PE and U46619 aortae were not affected by the presence of 10nM ritanserin (one-way-ANOVA, Bonferroni post-test). The effect of 10nM ritanserin on PE and U46619 responses in WT aortae are shown in Figure 3-13 and Table 3-13.

Effect of ritanserin on PE-induced and U46619-induced contractile responses of α_{1D}-KO aortae

Responses to a single challenge of 10μM PE in α_{1D}-KO aortae in the absence and presence of 10nM ritanserin were: control = 0.57 ± 0.08g (n = 7), +10nM ritanserin = 0.40 ± 0.07g (n = 7). Responses induced by a single U46619 (100nM) challenge were: control = 1.73 ± 0.07g (n = 7), +10nM ritanserin = 1.60 ± 0.06g (n = 7). Responses of α_{1D}-KO aortae to single challenges of PE and U46619 aortae were not affected by the presence of 10nM ritanserin (one-way-ANOVA, Bonferroni post-test). The effect of 10nM ritanserin on PE and U46619 responses in α_{1D}-KO aortae are shown in Figure 3-14 and Table 3-14.

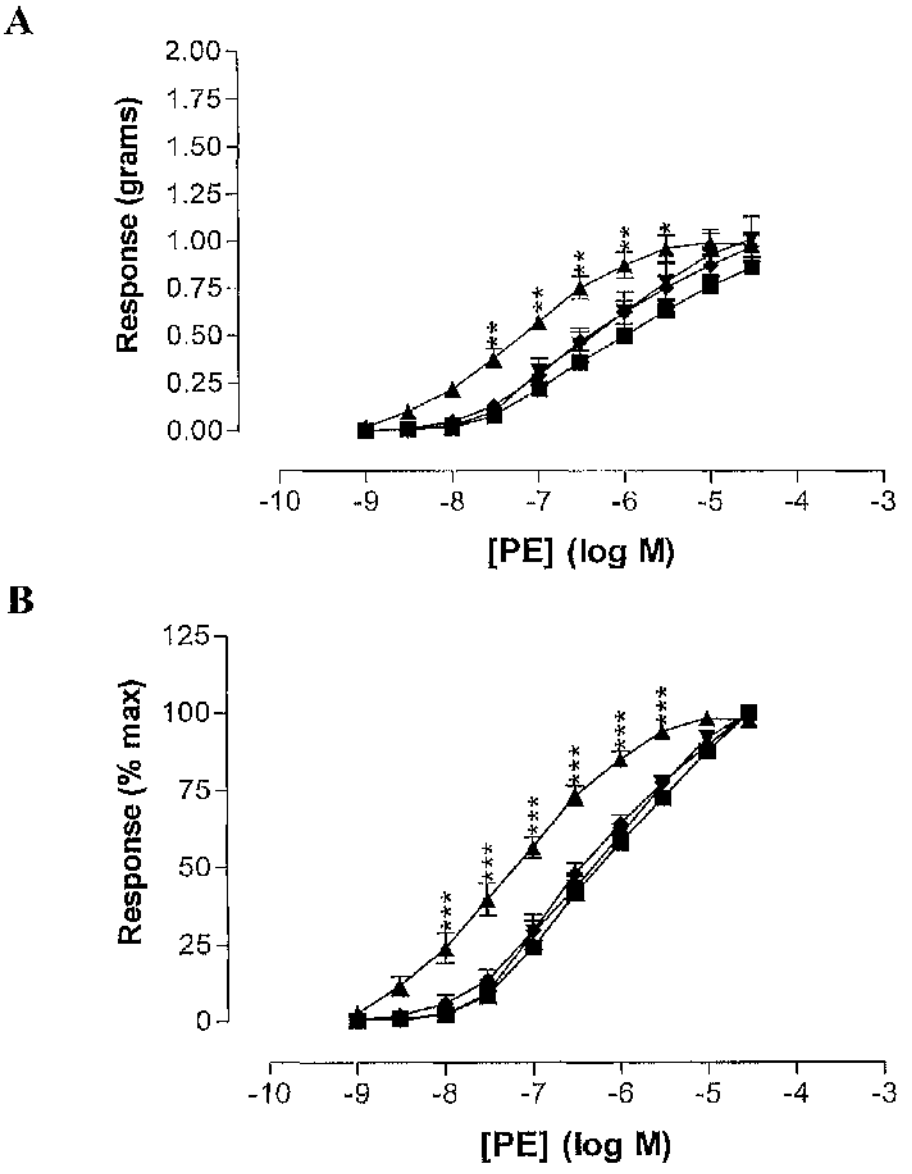


Figure 3-1. The effect of 10nM and 30nM 5-HT on PE-induced contractile responses of WT aortae (■ 1st curve, n = 13; ● time control, n = 12; ▼ 10nM 5-HT, n = 6; ▲ 30nM 5-HT, n = 7).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001 against time control: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
1st Curve	13	0.85 ± 0.04
Time control	12	0.97 ± 0.06
10^{-8}M 5-HT	6	1.01 ± 0.12
3x10^{-8}M 5-HT	7	1.01 ± 0.07

B

	pEC_{25}	pEC_{50}
1st Curve	6.98 ± 0.06	6.26 ± 0.06
Time control	7.18 ± 0.12	6.47 ± 0.09
10^{-8}M 5-HT	7.07 ± 0.13	6.44 ± 0.15
3x10^{-8}M 5-HT	7.98 ± 0.14***	7.29 ± 0.14***

C

	Hill slope
1st Curve	0.56 (*0.39-0.73*)
Time control	0.56 (*0.43-0.70*)
10^{-8}M 5-HT	0.58 (*0.41-0.75*)
3x10^{-8}M 5-HT	0.57 (*0.49-0.66*)

Table 3-1. The effect of 10nM and 30nM 5-HT on PE-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (***$p<0.001$ against time control: one-way-ANOVA, Bonferroni post-test)

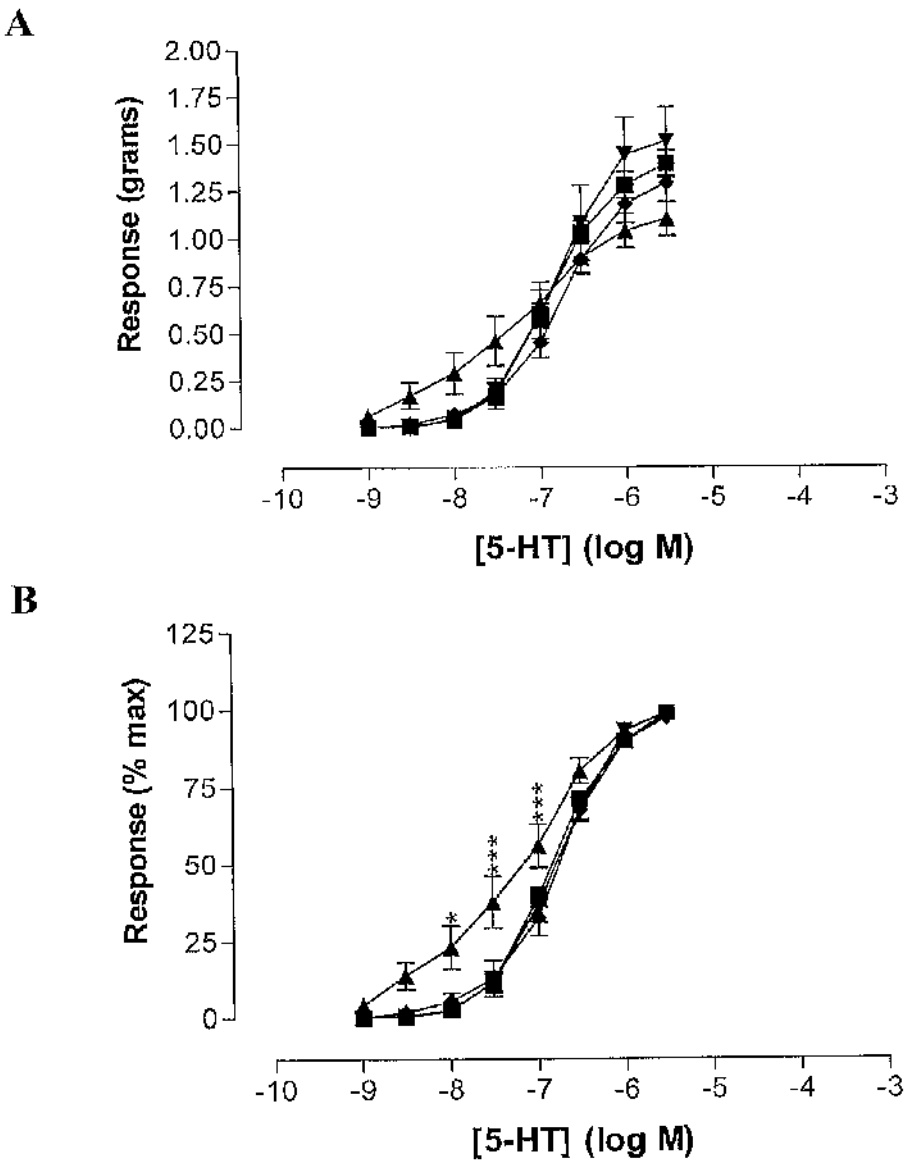


Figure 3-2. The effect of 10nM and 30nM PE on 5-HT-induced contractile responses of WT aortae (■ 1st curve, n = 13; ● time control, n = 12; ▼ 10nM PE, n = 6; ▲ 30nM PE, n = 7).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*$p<0.05$, ***$p<0.001$ against time control: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
1st Curve	13	1.41 ± 0.07
Time control	12	1.33 ± 0.10
10^{-8}M PE	6	1.53 ± 0.18
$3x10^{-8}$M PE	7	1.12 ± 0.09

B

	pEC_{25}	pEC_{50}
1st Curve	7.26 ± 0.05	6.91 ± 0.05
Time control	7.20 ± 0.12	6.84 ± 0.02
10^{-8}M PE	7.23 ± 0.09	6.86 ± 0.06
$3x10^{-8}$M PE	7.87 ± 0.21**	7.34 ± 0.20*

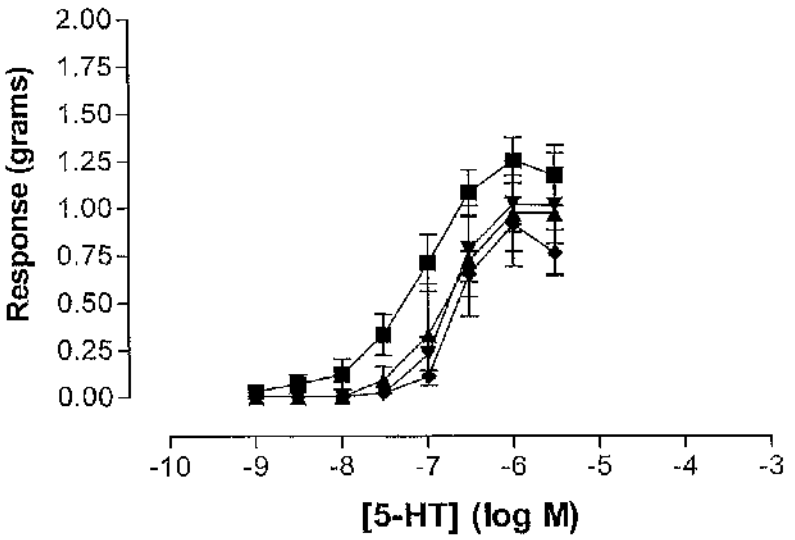
C

	Hill slope
1st Curve	1.22 (*1.05-1.38*)
Time control	1.23 (*1.00-1.45*)
10^{-8}M PE	1.19 (*1.07-1.32*)
$3x10^{-8}$M PE	0.68 (*0.38-0.99*)

Table 3-2. The effect of 10nM and 30nM PE on 5-HT-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$, **$p<0.01$ against time control: one-way-ANOVA, Bonferroni post-test).

A



B

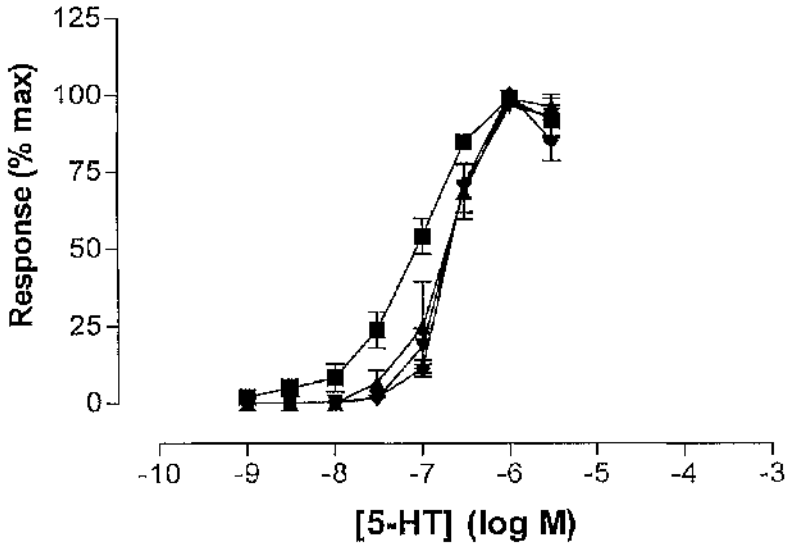


Figure 3-3. The effect of 1nM, 10nM and 100nM prazosin on 5-HT-induced contractile responses of WT aortae (■ control, n = 5; ▲ 1nM prazosin, n = 3; ▼ 10nM prazosin, n = 5; ◆ 100nM prazosin, n = 5).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (Statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	5	1.27 ± 0.13
10^{-9}M prazosin	3	1.00 ± 0.30
10^{-8}M prazosin	5	1.07 ± 0.17
10^{-7}M prazosin	5	0.91 ± 0.14

B

	pEC_{25}	pEC_{50}
Control	7.53 ± 0.13	7.08 ± 0.09
10^{-9}M prazosin	7.03 ± 0.18*	6.76 ± 0.15
10^{-8}M prazosin	6.95 ± 0.08**	6.70 ± 0.07*
10^{-7}M prazosin	6.89 ± 0.02**	6.69 ± 0.02*

C

	Hill slope
Control	1.39 (*0.78-2.00*)
10^{-9}M prazosin	1.78 (*1.23-2.32*)
10^{-8}M prazosin	2.25 (*1.78-2.72*)
10^{-7}M prazosin	3.00 (*1.03-4.97*)

Table 3-3. The effect of 1nM, 10nM & 100nM prazosin on 5-HT-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$, **$p<0.01$: one-way-ANOVA, Bonferroni post-test).

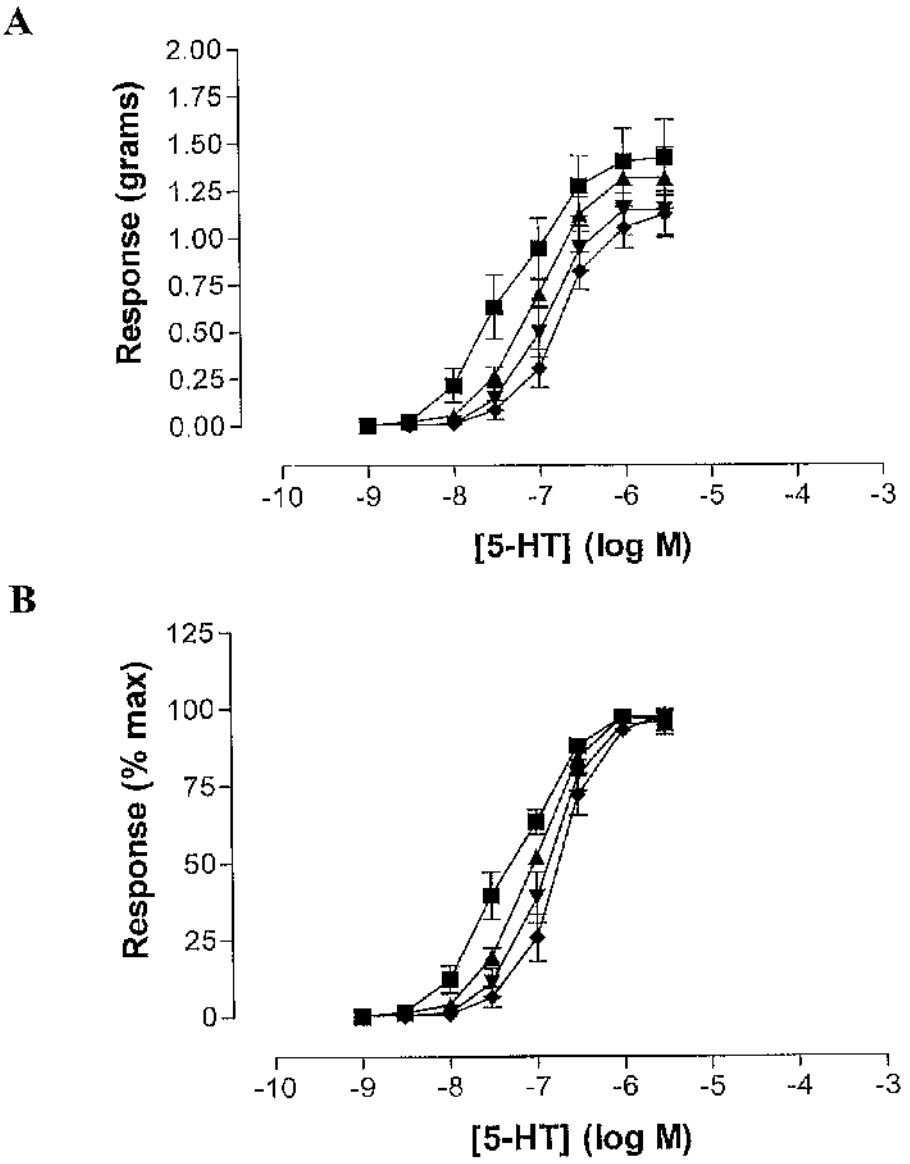


Figure 3-4. The effect of 1nM, 10nM and 100nM BMY 7378 on 5-HT-induced contractile responses of WT aortae (■ control, $n = 6$; ▲ 1nM BMY 7378, $n = 5$; ▼ 10nM BMY 7378, $n = 6$; ◆ 100nM BMY 7378, $n = 6$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (Statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	'n'	E_{max} (grams)
Control	6	1.45 ± 0.19
10^{-9}M BMY 7378	5	1.35 ± 0.14
10^{-8}M BMY 7378	6	1.19 ± 0.12
10^{-7}M BMY 7378	6	1.15 ± 0.13

B

	pEC_{25}	pEC_{50}
Control	7.73 ± 0.11	7.34 ± 0.11
10^{-9}M BMY 7378	7.44 ± 0.05*	7.02 ± 0.04
10^{-8}M BMY 7378	7.21 ± 0.11**	6.90 ± 0.09*
10^{-7}M BMY 7378	7.05 ± 0.11***	6.77 ± 0.08**

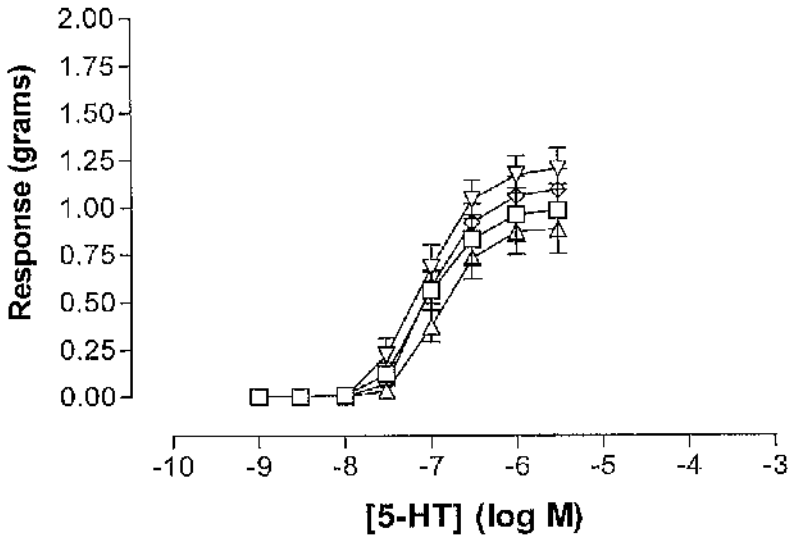
C

	Hill slope
Control	1.03 (*0.66-1.38*)
10^{-9}M BMY 7378	1.40 (*1.12-1.67*)
10^{-8}M BMY 7378	1.59 (*1.35-1.84*)
10^{-7}M BMY 7378	1.80 (*1.57-2.03*)

Table 3-4. The effect of 1nM, 10nM & 100nM BMY 7378 on 5-HT-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean + S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*p<0.05, **p<0.01, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

A



B

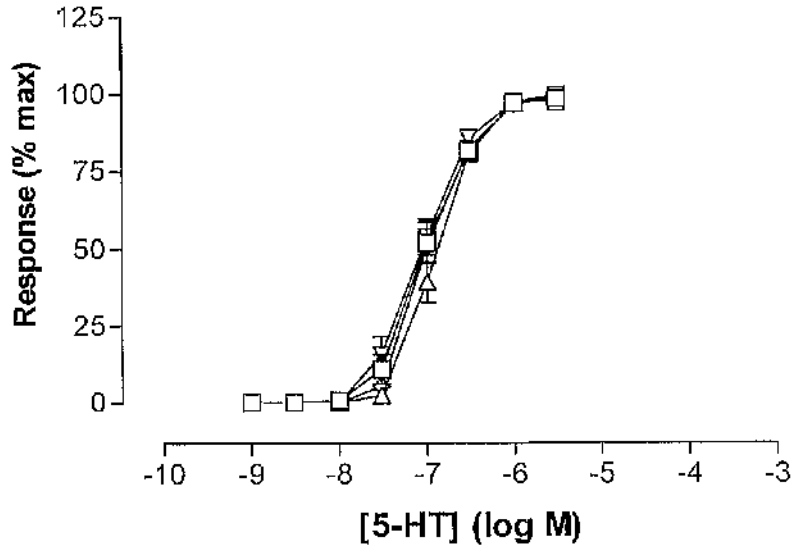


Figure 3-5. The effect of 1nM, 10nM and 100nM prazosin on 5-HT-induced contractile responses in α_{1D}-KO aortae (□ control, n = 5; △ 1nM prazosin, n = 3; ▽ 10nM prazosin, n = 5; ◇ 100nM prazosin, n = 5).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (No statistical significance: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	7	0.99 ± 0.14
10^{-9}M prazosin	6	0.90 ± 0.13
10^{-8}M prazosin	7	1.21 ± 0.10
10^{-7}M prazosin	7	1.10 ± 0.10

B

	pEC_{25}	pEC_{50}
Control	7.33 ± 0.08	7.05 ± 0.07
10^{-9}M prazosin	7.17 ± 0.07	6.91 ± 0.06
10^{-8}M prazosin	7.38 ± 0.09	7.08 ± 0.07
10^{-7}M prazosin	7.28 ± 0.04	7.00 ± 0.05

C

	Hill slope
Control	1.56 (*1.18-1.95*)
10^{-9}M prazosin	1.91 (*1.46-2.35*)
10^{-8}M prazosin	1.53 (*1.33-1.73*)
10^{-7}M prazosin	1.80 (*1.15-2.45*)

Table 3-5. The effect of 1nM, 10nM & 100nM prazosin on 5-HT-induced contractile responses of α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical significance: one-way-ANOVA, Bonferroni post-test).

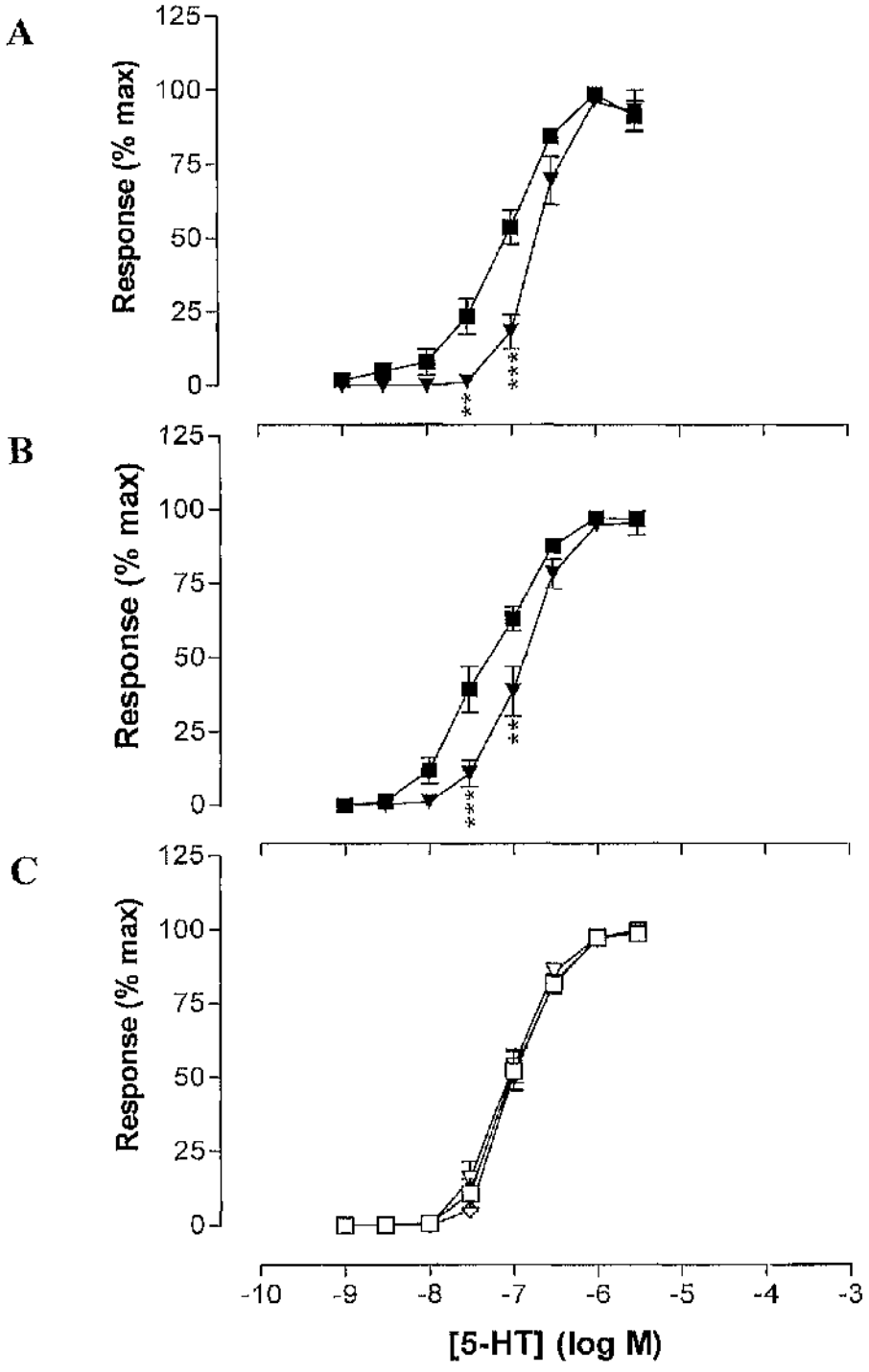


Figure 3-6. Comparison of the effects of 10nM prazosin (**A**: ■ control; ▼ 10nM prazosin) & 10nM BMY 7378 (**B**: ■ control; ▼ 10nM BMY 7378) in WT aortae and 10nM & 100nM prazosin in α_{1D}-KO aortae (**C**: □ control; ▽ 10nM prazosin; ◇ 100nM prazosin) on 5-HT-induced contractile responses.

Responses are shown normalised, expressed as percentage of maximum. Data points are the mean ± S.E.M. (**p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

WT	pEC_{25}	pEC_{50}	Hill slope
Control	7.53 ± 0.13	7.08 ± 0.09	1.39 (*0.78-2.00*)
10^{-8}M prazosin	6.95 ± 0.08**	6.70 ± 0.07*	2.25 (*1.78-2.72*)

B

WT	pEC_{25}	pEC_{50}	Hill slope
Control	7.73 ± 0.11	7.34 ± 0.11	1.03 (*0.66-1.38*)
10^{-8}M BMY 7378	7.21 ± 0.11**	6.90 ± 0.09*	1.59 (*1.35-1.84*)

C

α_{1D}-KO	pEC_{25}	pEC_{50}	Hill slope
Control	7.33 ± 0.08	7.05 ± 0.07	1.56 (*1.18-1.95*)
10^{-8}M prazosin	7.38 ± 0.09	7.08 ± 0.07	1.53 (*1.33-1.73*)
10^{-7}M prazosin	7.28 ± 0.04	7.00 ± 0.05	1.80 (*1.15-2.45*)

Table 3-6. Comparison of the effects of 10nM prazosin (**A**) & 10nM BMY 7378 (**B**) in WT aortae and 10nM & 100nM prazosin in α_{1D}-KO aortae (**C**) on 5-HT–induced contractile responses.

The pEC_{25} & pEC_{50} values are shown as the mean ± S.E.M., whilst the Hill slopes are shown as the mean with the 95% confidence intervals shown in parenthesis. (*p<0.05, **p<0.01: one-way-ANOVA, Bonferroni post-test).

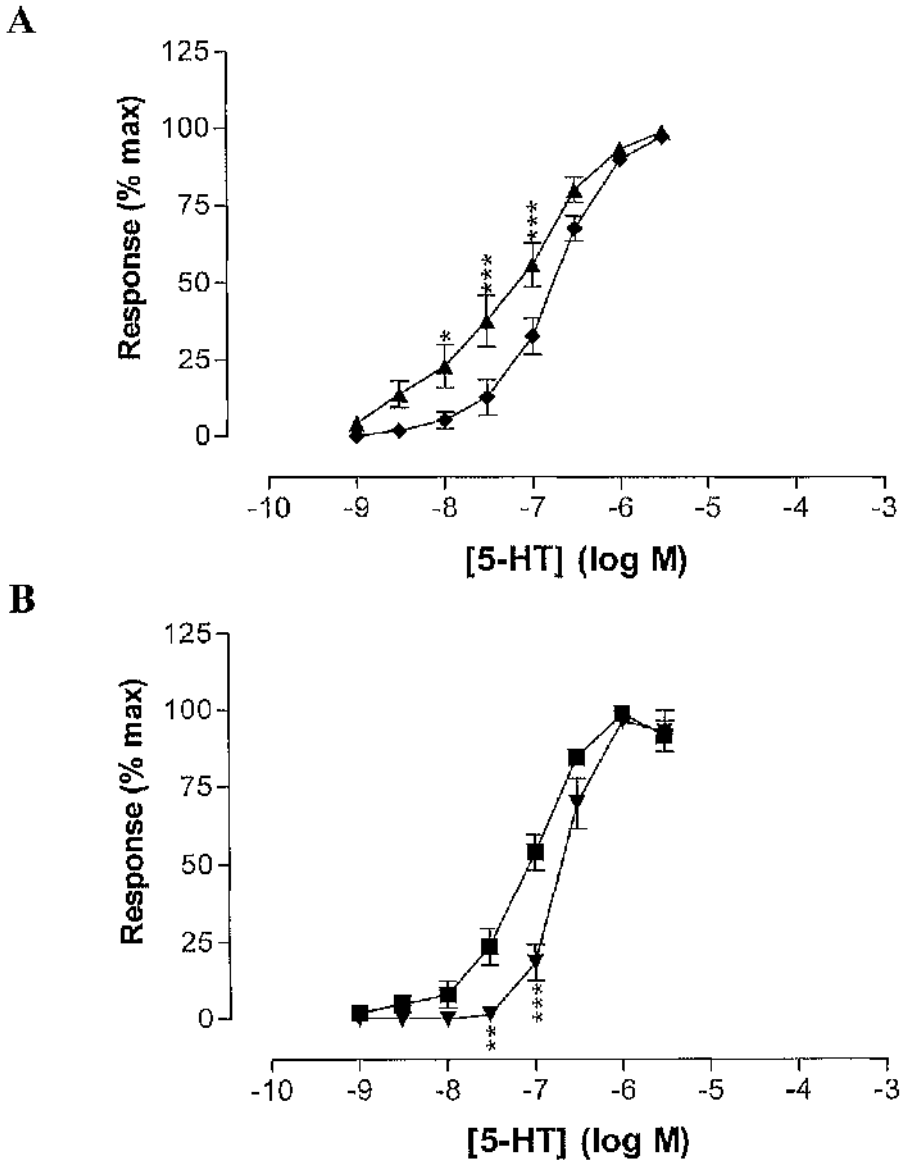


Figure 3-7. Comparison of the effect of α_{1D}-AR activation with 30nM PE (**A**: ◆ control; ▲ 30nM PE treated) and α_{1D}-AR blockade with 10nM prazosin (**B**: ■ control; ▼ 10nM prazosin treated) on the 5-HT-induced contractile responses of WT aortae.

Responses are shown normalised, expressed as percentage of maximum response. Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control 1	12	1.33 ± 0.10
+ 3x10^{-8}M PE	7	1.12 ± 0.09
Control 2	5	1.27 ± 0.13
+ 10^{-8}M prz	5	1.07 ± 0.17

B

	pEC_{25}	pEC_{50}
Control 1	7.20 ± 0.12	6.84 ± 0.02
+ 3x10^{-8}M PE	7.87 ± 0.21**	7.34 ± 0.20*
Control 2	7.53 ± 0.13	7.08 ± 0.09
+ 10^{-8}M prz	6.95 ± 0.08**	6.70 ± 0.07*

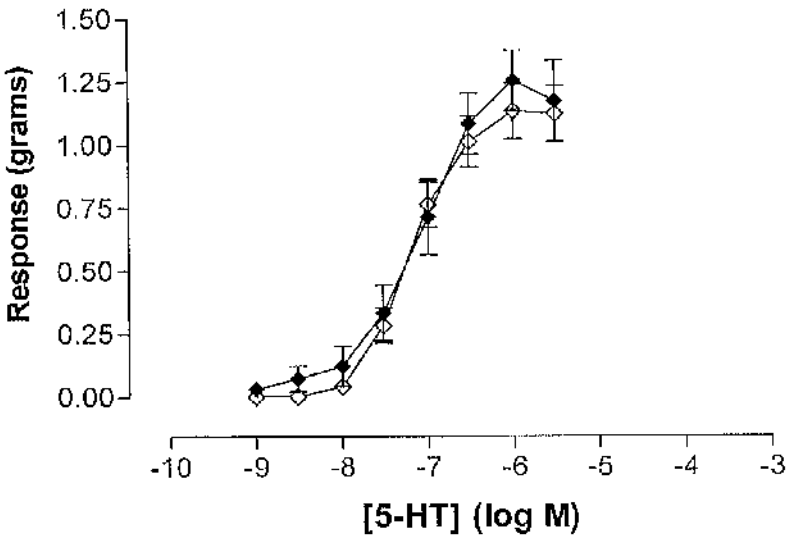
C

	Hill slope
Control 1	1.23 (*1.00-1.45*)
+ 3x10^{-8}M PE	0.68 (*0.38-0.99*)
Control 2	1.78 (*1.23-2.32*)
+ 10^{-8}M prz	2.25 (*1.78-2.72*)

Table 3-7. Comparison of the effect of α_{1D}-AR activation with 30nM PE and α_{1D}-AR blockade with 10nM prazosin on the 5-HT-induced contractile response of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean ± S.E.M. Hill slopes (**C**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$, **$p<0.01$: one-way-ANOVA, Bonferroni post-test).

A



B

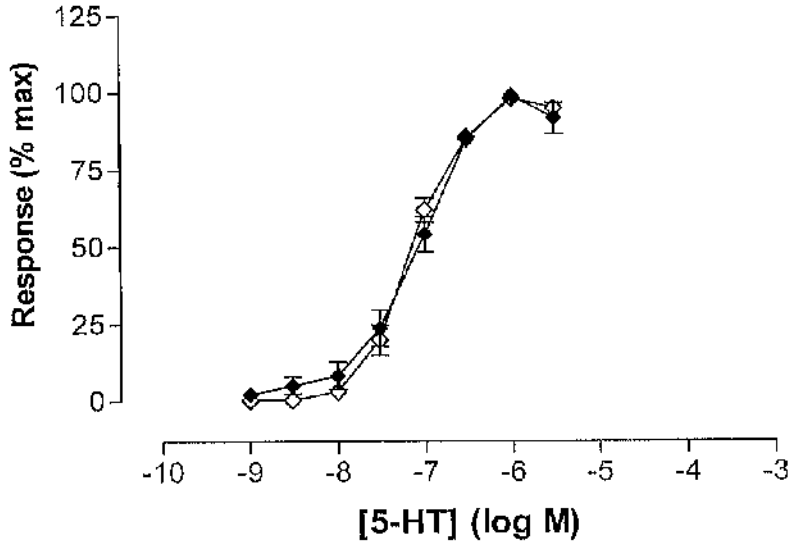


Figure 3-8. 5-HT-induced contractile responses of WT (◆; $n = 5$) & α_{1D}-KO (◇; $n = 16$) aortae.

Responses are shown in both grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (No statistical significance: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
WT	5	1.27 ± 0.13
α_{1D}-KO	16	1.15 ± 0.11

B

	pEC_{50}	Hill slope
WT	7.08 ± 0.09	1.39 (*0.78-2.00*)
α_{1D}-KO	7.18 ± 0.06	1.56 (*1.21-1.91*)

Table 3-8. 5-HT-induced contractile responses of WT & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as both pEC_{25} and pEC_{50} values (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical significance: one-way-ANOVA, Bonferroni post-test).

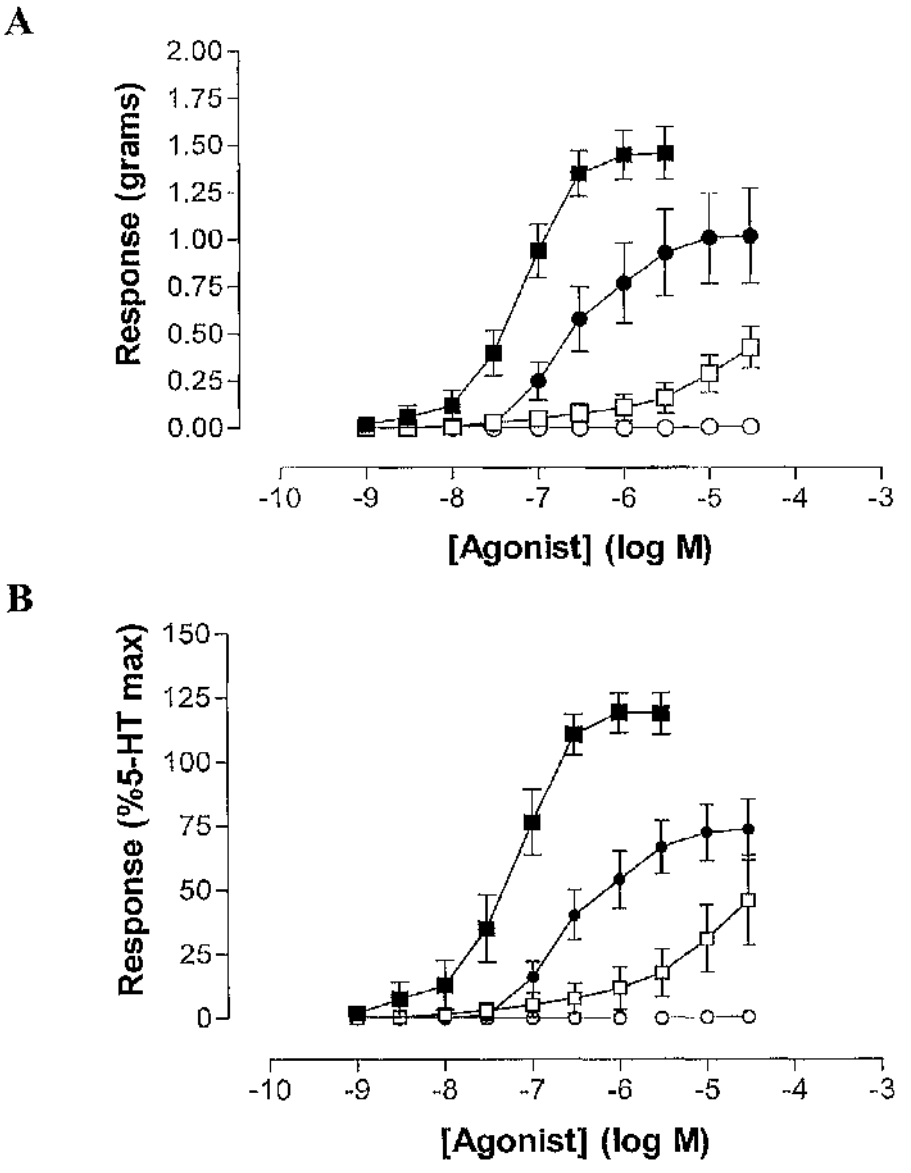


Figure 3-9. The effect of 10nM ritanserin on 5-HT-induced (■ control, n = 7; □ 10nM ritanserin treated, n = 6) & BRL 54443-induced contractile responses (● control, n = 7; ○ 10nM ritanserin treated, n = 7) of WT aortae.

Responses are shown both in grams (**A**) and normalised as a percentage of the maximum response of the single 1μM 5-HT challenge (**B**). Data points are the mean ± S.E.M. (Statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
5-HT	7	1.49 ± 0.13
5-HT + 10^{-8}M Rtn	6	0.43 ± 0.11***
BRL 54443	7	1.04 ± 0.24
BRL + 10^{-8}M Rtn	7	0.01 ± 0.01†††

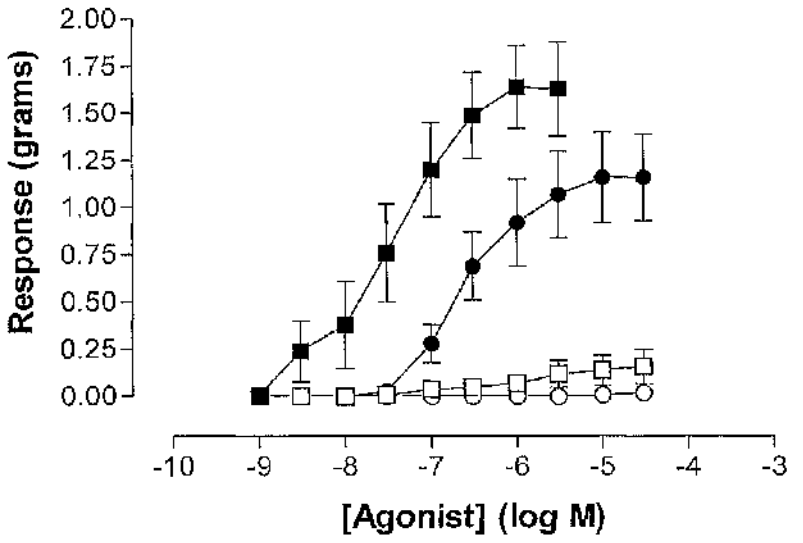
B

	pEC_{50}	Hill slope
5-HT	7.23 ± 0.13	1.37 (*1.00-1.74*)
5-HT + 10^{-8}M Rtn	n.c.	n.c.
BRL 54443	6.47 ± 0.16*	1.03 (*0.71-1.35*)
BRL + 10^{-8}M Rtn	n.c.	n.c.

Table 3-9. The effect of 10nM ritanserin on 5-HT-induced & BRL 54443-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis; n.c.: not calculable (*$p<0.05$, ***$p<0.001$ against 5-HT control; $^{\dagger\dagger\dagger}p<0.05$ against BRL 54443 control: one-way-ANOVA, Bonferroni post-test).

A



B

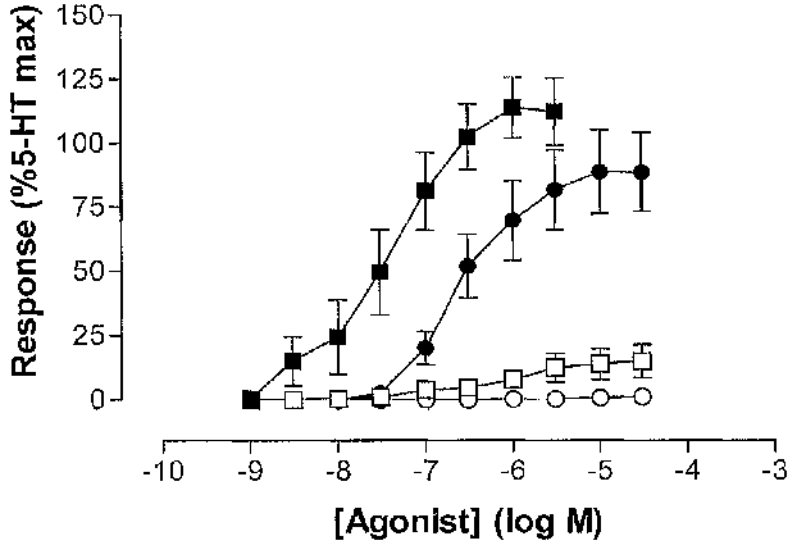


Figure 3-10. The effect of 10nM ritanserin on 5-HT-induced (■ control, n = 7; □ 10nM ritanserin treated, n = 6) & BRL 54443-induced contractile responses (● control, n = 7; ○ 10nM ritanserin treated, n = 7) of α_{1D}-KO aortae.

Responses are shown both in grams (**A**) and normalised as a percentage of the maximum response of the single 1μM 5-HT challenge (**B**). Data points are the mean ± S.E.M. (Statistical significance not shown for clarity: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
5-HT	7	1.67 ± 0.23
5-HT + 10^{-8}M Rtn	6	0.16 ± 0.09***
BRL 54443	7	1.18 ± 0.24
BRL + 10^{-8}M Rtn	7	0.02 ± 0.01†††

B

	pEC_{50}	Hill slope
5-HT	7.48 ± 0.23	0.96 (*0.47-1.10*)
5-HT + 10^{-8}M Rtn	n.c.	n.c.
BRL 54443	6.49 ± 0.13*	1.02 (*0.59-1.45*)
BRL + 10^{-8}M Rtn	n.c.	n.c.

Table 3-10. The effect of 10nM ritanserin on 5-HT-induced & BRL 54443-induced contractile responses of α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean + S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis; n.c.: not calculable (*$p<0.05$, ***$p<0.001$ against 5-HT control; $^{\dagger\dagger\dagger}p<0.05$ against BRL 54443 control: one-way-ANOVA, Bonferroni post-test).

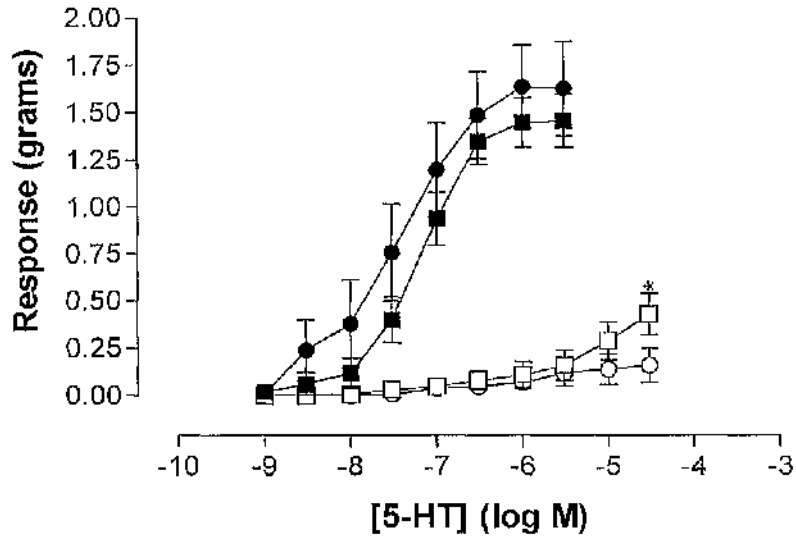


Figure 3-11. Comparison of the effect of 10nM ritanserin on 5-HT-induced contractile responses of WT (■ control, n = 7; □ 10nM ritanserin treated, n = 6) and α_{1D}-KO (● control, n = 6; ○ 10nM ritanserin treated, n = 7) aortae.

Responses are shown in grams. Data points are the mean ± S.E.M. (*p<0.05 WT vs. α_{1D}-KO: two-way-ANOVA, Bonferroni post-test).

		E_{max} (grams)	pEC_{50}	Hill slope
WT	5-HT	1.49 ± 0.13	7.23 ± 0.13	1.37 (*1.00-1.74*)
	5-HT + 10^{-8}M Rtn	0.43 ± 0.11	n.c.	n.c.
α_{1D}-KO	5-HT	1.67 ± 0.23	7.48 ± 0.23	0.96 (*0.47-1.10*)
	5-HT + 10^{-8}M Rtn	0.16 ± 0.09*	n.c.	n.c.

Table. 3-11 Comparison of the effect of 10nM ritanserin on 5-HT-induced contractile responses in WT and α_{1D}-KO aortae.

The, maximum response generated, expressed as grams and agonist sensitivity, expressed as a pEC_{50} value are shown as the mean ± S.E.M whilst the Hill slopes are shown as the mean with the 95% confidence intervals shown in parenthesis; n.c.: not calculable (*p<0.05, WT vs. α_{1D}-KO one-way-ANOVA, Bonferroni post-test).

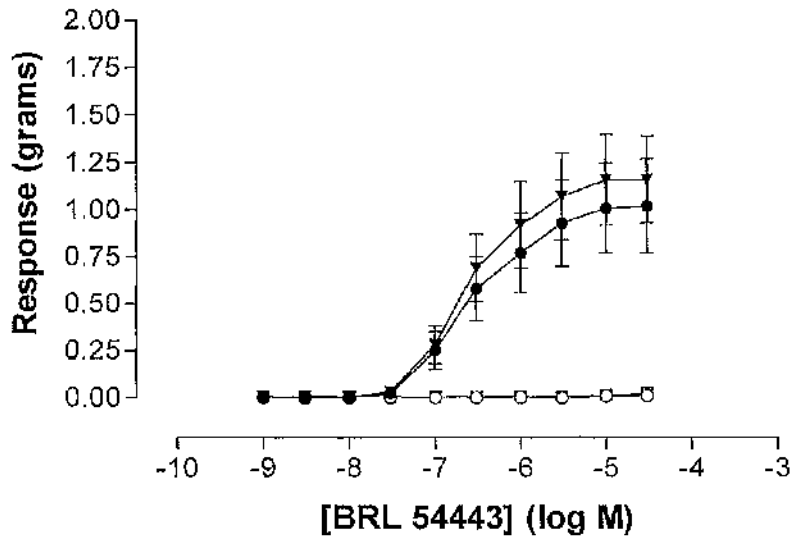


Figure 3-12. Comparison of the effect of 10nM ritanserin on BRL 54443-induced contractile responses of WT (■ control, n = 7; □ 10nM ritanserin treated, n = 6) and α_{1D}-KO (● control, n = 6; ○ 10nM ritanserin treated, n = 7) aortae.

Responses are shown in grams. Data points are the mean ± S.E.M. (no significant difference, WT vs. α_{1D}-KO: two-way-ANOVA, Bonferroni post-test).

		E_{max} (grams)	pEC_{50}	Hill slope
WT	BRL 54443	1.04 ± 0.24	7.23 ± 0.13	1.03 (*0.71-1.35*)
	BRL + 10^{-8}M Rtn	0.01 ± 0.01	n.c.	n.c.
α_{1D}-KO	BRL 54443	1.18 ± 0.24	7.48 ± 0.23	1.02 (*0.59-1.45*)
	BRL + 10^{-8}M Rtn	0.02 ± 0.01	n.c.	n.c.

Table. 3-12 Comparison of the effect of 10nM ritanserin on BRL 54443-induced contractile responses in WT and α_{1D}-KO aortae.

The, maximum response generated, expressed as grams and agonist sensitivity, expressed as a pEC_{50} value are shown as the mean ± S.E.M whilst the Hill slopes are shown as the mean with the 95% confidence intervals shown in parenthesis; n.c.: not calculable (no significant difference, WT vs. α_{1D}-KO one-way-ANOVA, Bonferroni post-test).

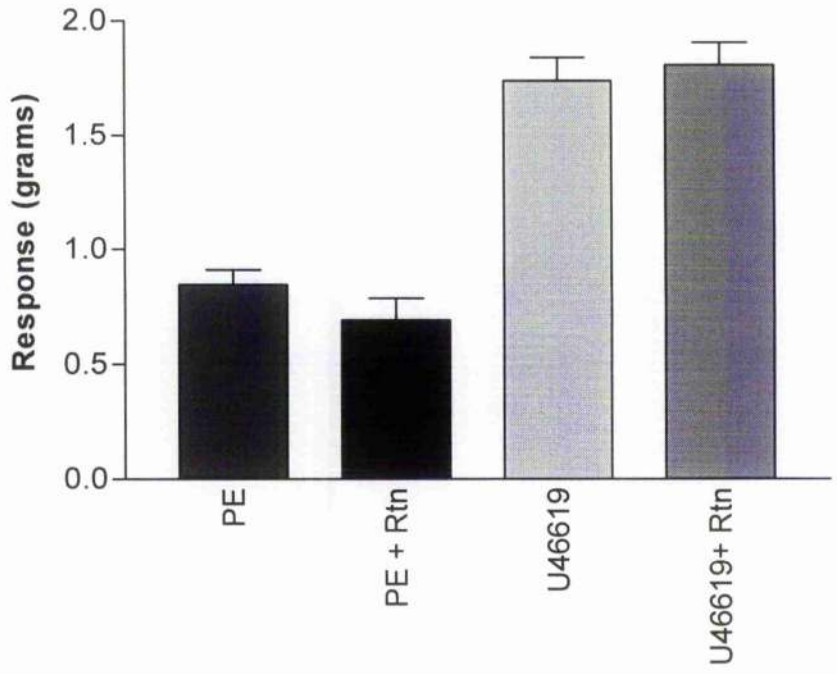


Figure 3-13. The effect of 10nM ritanserin on PE-induced (10μM) & U46619-induced (100nM) contractile responses of WT aortae.

Columns shown the mean ± S.E.M of the generated responses expressed in grams. No statistical significance: one-way-ANOVA, Bonferroni post-test.

	n	E_{max} (grams)
PE	6	0.85 ± 0.07
PE + 10^{-8}M Rtn	6	0.69 ± 0.09
U46119	5	1.74 ± 0.10
U46619 + 10^{-8}M Rtn	5	1.81 ± 0.10

Table 3-13. The effect of 10nM ritanserin on PE-induced (10μM) & U46619-induced (100nM) contractile responses of WT aortae.

The *n* number and the maximum response generated expressed in grams are shown. Values are the mean ± S.E.M.

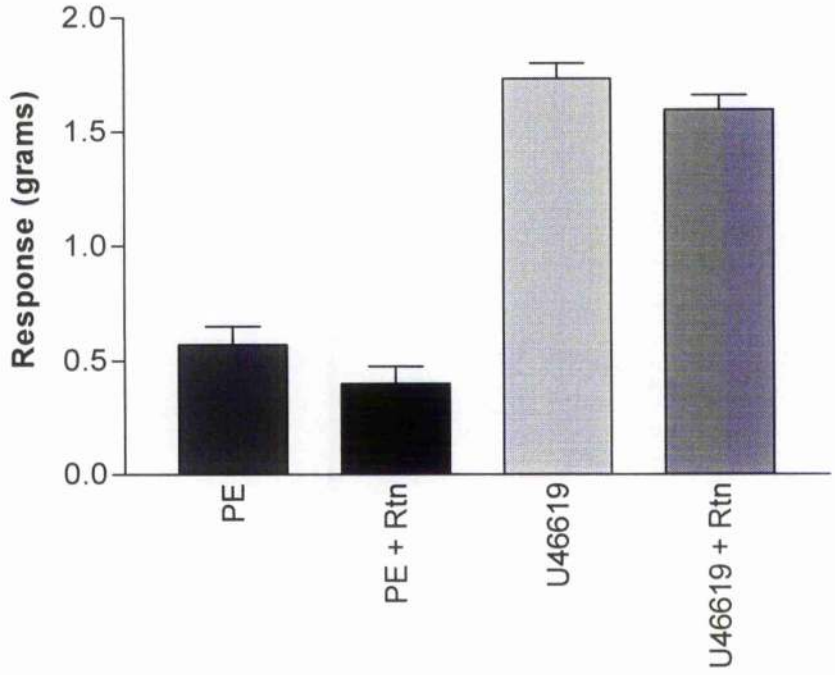


Figure 3-14. The effect of 10nM ritanserin on PE-induced (10μM) & U46619-induced (100nM) contractile responses of α_{1D}-KO aortae.

Columns shown the mean ± S.E.M of the generated responses expressed in grams. No statistical significance: one-way-ANOVA, Bonferroni post-test.

	n	E_{max} (grams)
PE	7	0.57 ± 0.08
PE + 10^{-8}M Rtn	7	0.40 ± 0.07
U46619	7	1.73 ± 0.07
U46119 + 10^{-8}M Rtn	7	1.60 ± 0.06

Table 3-14. The effect of 10nM ritanserin on PE-induced (10μM) & U46619-induced (100nM) contractile responses of α_{1D}-KO aortae.

The *n* number and the maximum response generated expressed in grams are shown. Values are the mean ± S.E.M.

Discussion- Chapter 3

Synergy in the mouse aorta

Synergy can be defined as the application of two or more agonist concentrations (or doses) in combination resulting in a response which is much larger than the sum of the responses of the individual agonists alone. Synergy between the adrenergic and serotonergic vasoconstrictor systems in the mouse aorta has now been demonstrated. The responses to PE and 5-HT were significantly more sensitive in the presence of 30nM 5-HT and PE respectively.

Types of synergy studies

Synergy can be studied by either combining single doses or concentrations of agonists or by constructing agonist concentration-response curves. Stupecky *et al.* (1986) performed both types of study in the rabbit aorta. They used single equi-effective concentrations of agonists that resulted in a response of 0.1grams and combined these individual doses and demonstrated synergism, since the resulting responses varied from 0.5g to 2.7g.

They also constructed CCRCs in the presence and absence of synergists. The synergist displaced the concentration-response curve of the agonist to the left indicating an increased sensitivity. Therefore, concentration response curves to PE and 5-HT in the mouse aorta were constructed in order to observe synergy. Crucially, the amplification of the response was only observed when the synergist concentration used was able to cause a contraction.

Threshold stimulus

Ariens *et al.* (1960) performed an early study of synergy and discussed a hidden precontraction, suggesting the contractile elements had to reach a particular contractile "inertia" before a measurable contraction occurred. Stupecky *et al.* (1986) associated this inertia with a biochemical event, arguing an agonist has to elevate second messengers to reach threshold concentrations for contraction. They termed this "threshold stimulus".

The synergist concentrations used in Stupecky's study were all suprathreshold, as they raised tone within the vessel. In the mouse aorta, 10nM concentrations of PE or 5-HT resulted in a contractile response on occasion but generally they did not, hence, no synergy was observed. 30nM PE or 5-HT was able to cause a contraction, i.e. the tissue passed the threshold stimulus required for contraction. Thus in the mouse aorta synergy is only observed when the synergist concentrations used caused a contractile response but this does not always need to be the case. However addition of an agonist on top of this tone the synergist had produced resulted in an amplification of the contractile response of that agonist. The dose response curves were measured using the elevated tone as the baseline and a larger response was observed, i.e. for any given concentration of agonist, the response was significantly greater than normal.

Curve shape in relation to synergy

The effect a synergist has on the shape of the concentration-response curve of an agonist can provide a useful insight into the synergist-agonist interaction. Drasckozy & Trendelenburg (1968) studied synergy by comparing agonist curves in the presence and absence of a synergist and described two types of synergy. Stupecky *et al.* (1986) also discussed the resulting changes in the agonist curves. A parallel shift of the curve to the left was considered to be "potentiation", which is likely to be found where the two agonists used initiate their responses by acting through separate receptors.

The type of synergy where a synergist caused the curve to shift to the left at threshold but converged at higher agonist concentrations was thought to be the result of an additive effect (Drasckozy & Trendelenburg, 1968). Stupecky *et al.* (1986) termed this "threshold synergism" and suggested that this type of synergy is likely to occur when the two agonists used act through the same receptor system, essentially a two-agonist-one-receptor model. The example the authors used was the effect of methoxamine on the concentration-curve curve to NE.

By applying these terms to the shape changes in the CCRCs of PE and 5-HT in the mouse aorta, it is apparent that the effect of 30nM 5-HT on the PE response was potentiation and the effect of 30nM PE on the 5-HT response was threshold synergy. The significance of these differing conclusions is discussed later, but first a possible explanation of the synergistic interaction is discussed.

Mutual effect amplification

Leff (1987) mathematically modelled synergy, using a system where the two agonists effect a response by coupling through the same second-messenger pathway and discussed the resulting "mutual effect amplification". Leff demonstrated this experimentally using 5-HT and histamine in the rabbit aorta. A 2-fold increase in sensitivity was observed when 0.3μM 5-HT and 1μM histamine were used as synergist concentrations for histamine and 5-HT responses respectively.

Christ *et al.* (1990) evaluated Leff's model with relation to adrenergic and serotonergic interaction in the rabbit aorta. Using a 'Fixed Molar Equivalent Ratio (FMR)' protocol, where PE was substituted for the molar equivalent of 5-HT in various ratios, Christ *et al.* (1990) showed that adrenergic and serotonergic co-activation resulted in a 2 to 3-fold increase in PE sensitivity.

Later, Christ & Jean-Jacques (1991) demonstrated the Leff model accurately predicted the synergy between α_1-AR and 5-HT receptor co-activation in the rat aorta. The rat aorta was 10-fold more sensitive for PE than 5-HT, so 5-HT was partially substituted with PE using the novel FMR protocol developed by Christ *et al.* (1990). A 2-3 fold increase in 5-HT sensitivity was observed.

The α_{1D}-AR and the 5-HT_{2A} receptors in the mouse aorta both couple through the $G_{q/11}$ protein in order to elevate $[Ca^{2+}]_i$ by increasing PI hydrolysis and IP_3 turnover (Byland *et al.*, 1988 & Martin, 1998 for α_{1D}-ARs and 5-HT_{2A} receptors respectively), so they fit Leff's two-receptor-one-transducer model. The sensitivity increases were of a magnitude to be expected by mutual effect amplification, ranging from 3-fold to 7-fold. However, no attempt was made to establish the mathematical fit of Leff's model of mutual effect amplification. But the synergy we observed is most likely due to mutual effect amplification.

The mathematical model of Leff (1987) lies somewhere between the two types of synergy discussed by Stupecky *et al.* (1986) i.e. potentiation and threshold synergy. Leff's two-receptor-one-transducer model is most closely related to threshold synergy, which describes a two-agonist-one-receptor system. However, the Leff model is limited by the assumption that the two receptors couple only to a single transducer. In the

mouse aorta, McKune & Watts (2001) showed that L-type Ca^{2-} channels and tyrosine kinases are also involved in the response mediated by 5-HT_{2A} receptors as well as $G_{q/11}$ protein activation to elevate $[Ca^{2+}]_i$ by increasing PI hydrolysis and IP_3 turnover. Hence, the vasoconstrictor system in the mouse aorta did not completely fulfil the criteria of Leff's model. Therefore, although the synergy is due to mutual effect amplification, the difference in the shape changes of the PE and 5-HT curves suggest there is a fundamental difference in the interaction at the receptor level, and Stupecky's models of potentiation and threshold synergy may explain this and is discussed later.

Synergy and the involvement of 'silent receptors'

In contrast to the Leff model where small (less than 10-fold) increases in sensitivity are observed, occasionally large 100-fold or 1000-fold, sensitivity increases in the agonist curve are apparent when a synergist is added. In such cases the synergy is believed to be the result of the activation of "silent receptors". This is a very powerful type of amplification resulting in 100-fold plus increases in sensitivity to an agonist. Generally this type of augmentation of the response is seen when the two agonists couple through receptors that preferentially couple with different G-proteins i.e. $G_{q/11}$ with $G_{i/0}$ (Selbie & Hill, 1998).

Movahedi *et al.* (1995), Chen *et al.* (2000) and Yildiz & Tuncer (1995) studied the serotonergic responses of the rabbit ear artery, femoral artery and iliac artery respectively, and uncovered "silent" 5-$HT_{1\text{-like}}$ receptor responses to 5-HT which were only unmasked when a contractile threshold concentration of PE or NE was applied to the tissue. Such interactions have been associated with the pathophysiology of conditions such as pulmonary hypertension (Maclean & Morecroft, 2001). However, little evidence for synergists uncovering "silent" α_1-ARs or 5-HT_{2A} receptors exists, and no such large shifts were observed.

Heterodimerisation of GPCRs

Although the synergy observed was probably due to mutual effect amplification via the activation of a mutual second messenger system, current cellular studies are revealing the heterodimerisation properties of G-protein-coupled-receptors (GPCRs; reviewed by Rios *et al.*, 2001). Recently, research has found that all three α_1-AR subtypes have the

ability for direct receptor-receptor interactions. The α_{1B}-AR can form both homodimers and heterodimers with both the α_{1A}-AR and the α_{1D}-AR, but no α_{1A}/α_{1D}-AR heterodimers have been observed (Stanasila *et al.*, 2003; Uberti *et al.*, 2003; Hague *et al.*, 2004). Thus the α_{1D}-AR has the ability to interact with other GPCRs.

Some of the family of 5-HT receptors have also been shown to form heterodimers. Xie *et al.* (1999) found that 5-HT_{1B} and 5-HT_{1D} receptors form homodimers when expressed on their own, in cultured cell lines, but when co-expressed, they can form heterodimers. No evidence of 5-HT_{2A} receptors having the ability to form heterodimers is currently available but such an interaction cannot be ruled out. It is known that 5-HT_{2A} receptors have the ability to cross-talk with 5-HT_{1A} receptors although this is attributed to post-receptor events (Zhang *et al.*, 2004).

Thus, although there is no evidence yet available of whether there is direct interaction between α_{1D}-ARs and 5-HT_{2A} receptors, it may be possible that these two receptors can heterodimerise. Such a study was beyond the scope of this thesis but it is not possible to completely rule out this type of direct receptor-receptor interaction, although the synergy we observed is most likely due to the downstream amplification of the response.

Potentiation

Thus, by applying the terms used by Stupecky *et al.* (1986) to our data, the effect of 30nM 5-HT on the PE curve is 'potentiation' as the PE curve shifted to the left in a parallel fashion. This is consistent with the effect of NE and 5-HT in combination in the rabbit aorta (Stupecky *et al.*, 1986). In the mouse aorta, PE induces its response through α_{1D}-ARs but when 5-HT is used as a synergist, a new receptor is introduced into the response, namely the 5-HT_{2A} receptor, so we see potentiation. As previously discussed, it is likely that the potentiation observed is due to "mutual effect amplification".

Threshold synergy

On the other hand, incubation with 30nM PE before constructing a CCRC to 5-HT does not result in a parallel shift but a curve that is enhanced at lower agonist concentrations resulting in threshold synergism. This curve shifts to the left at threshold concentrations and converges with the control curve at higher 5-HT concentrations, effectively making the slope of the curve shallower (Fig 3-2).

This is contrary to the findings of Stupecky *et al.* (1986) in the rabbit aorta. They demonstrated that the 5-HT curve was shifted in a parallel fashion when the tissue had NE or methoxamine as a synergist present. But threshold synergy is observed where the two synergists used are either full or partial agonists of the same receptor, for example methoxamine and NE acting at α_1-ARs. There have been many reports of 5-HT having activity at α_1-ARs. 5-HT acting at α_{1D}-ARs would help explain the threshold synergy observed when PE is used as a synergist with 5-HT.

5-HT and α_1-ARs

It has previously been demonstrated that 5-HT responses can be mediated via α_1-ARs. The 5-HT response of the rabbit ear artery is believed to be due to the significant involvement of α_1-ARs (Purdy *et al.*, 1981). Furthermore, the 5-HT response in the rabbit aorta is partly mediated by α_1-ARs (Purdy *et al.*, 1987). Recently Shaw *et al.* (2000) demonstrated concentration-dependent prazosin blockade of 5-HT responses in rat pulmonary arteries and concluded that 5-HT is acting through α_1-ARs. However, they were unable to elaborate specifically which α_1-AR subtypes are involved.

The complexity of subtyping an α_1-AR mediated response to 5-HT is due to the nature of the selective ligands available. BMY 7378 is the most commonly used α_{1D}-AR selective antagonist (Saussy *et al.*, 1994) but is also known to be a 5-HT_{1A} partial agonist (Yocca et al., 1987). Similarly the α_{1A}-selective antagonist 5-Methylurapidil, (Gross *et al.*, 1988; Schwinn *et al.*, 1995), was originally reported to be a partial 5-HT_{1A}-receptor agonist (Gross *et al.*, 1987), thus making the detailed study of adrenoceptor mediated 5-HT responses problematic. Furthermore the adrenergic

response may be masked by responses driven by 5-HT_{2A} receptor activation, making it difficult to uncover the adrenergic component of the 5-HT response.

However, the serotonergic response of the WT mouse aorta was antagonised by prazosin. The effect of BMY 7378 on the 5-HT response was very similar to the effect of prazosin. The blockade of α_{1D}-ARs using both antagonists resulted in a rightward shift at threshold, which converged with the control curve. Attempts to establish pA_2 values for prazosin and BMY 7378 against 5-HT were unsuccessful as shallow Schild slopes were observed (not shown). This is most likely due to the majority of the response being 5-HT_{2A} receptor-mediated, making it difficult to accurately isolate the smaller α_1-AR component.

Although these observations confirm an α_1-AR mediated serotonergic response, they are inconclusive as to which subtype is involved. As well as being a commonly used α_{1D}-selective antagonist BMY 7378 is known to be a partial agonist of the 5-HT_{1A}. Although no evidence for the role of 5-HT_{1A}-receptors is yet available the use of BMY is overshadowed by its activity at other receptors than the α_{1D}-AR, but the use of the α_{1D}-KO helped resolve this issue. Circumstantially though, BMY 7378 had a similar effect as prazosin, indicating that α_{1D}-ARs have a role in the serotonergic response in the mouse aorta.

The use of the α_{1D}-KO mouse confirmed this conclusion as the serotonergic response of the α_{1D}-KO mouse aorta was not antagonised by prazosin, verifying the α_{1D}-AR is involved in the serotonergic response. The adrenergic response α_{1D}-KO mouse aorta is α_{1B}-AR mediated. The lack of blockade of the serotonergic response by prazosin in the α_{1D}-KO excludes the α_{1B}-AR from the serotonergic response.

Role of constitutively active α_{1D}-ARs in the 5-HT response

The effect of prazosin or BMY 7378 in the WT appears to be most apparent in the 30nM to 100nM region of the 5-HT curve, suggesting 5-HT has a high degree of sensitivity for α_1-AR. Recently Gisbert et al. (2000) discussed the evidence for a constitutively active population of α_{1D}-ARs in rat aorta. In their study, the α_{1D}-AR's were activated using NE in Ca^{2+}-free PSS and the NE was then washed out using Ca^{2+}-

free PSS. On exposure to Ca^{2+}, an increase in tone was observed suggesting a constitutively active population of receptors. A population of constitutively active α_{1D}-ARs in the mouse aorta, may be why the α_1-AR activity of 5-HT was so sensitive, as only a small amount of agonist was required to 'switch on' the α_{1D}-ARs. The constitutive activity of α_{1D}-AR along with the synergistic interaction with 5-HT_{2A} receptors, may explain why the response at α_{1D}-ARs is apparently at such low concentrations of 5-HT.

The concept of constitutively active α_{1D}-ARs provides another possible explanation to the sensitivity of 5-HT responses to prazosin or BMY 7378. It is possible that the 5-HT has no effect at α_{1D}-ARs but that the sensitivity is simply due to the inverse agonist effect of prazosin or BMY 7378 at α_{1D}-ARs. Thus, in the WT the less sensitive 5-HT curve in the presence of prazosin or BMY 7378, may have been due to the 'switching off' of constitutively active α_{1D}-ARs which were synergistically amplifying the 5-HT_{2A} receptor response. The absence of such a shift in α_{1D}-KO aortae would also be consistent with this hypothesis as well as the presented hypothesis of the activity of 5-HT at α_{1D}-ARs.

However, the 5-HT curve was constructed after a 40-minute incubation with antagonist, once the original baseline had been re-established. Thus, the time difference between the initial PE challenge and the construction of the 5-HT curve was approximately 2 hours, allowing time for the constitutively active population of receptors to become "silent". Additionally, the baseline was established in normal Ca^{2+} PSS so any significant activity of α_{1D}-ARs would show up as a contraction. It is possible that there may have been a residual sub-threshold activity of α_{1D}-ARs, but this activity of α_{1D}-ARs should not have a synergistic interaction with the 5-HT_{2A} receptors. As previously discussed, a supra-threshold concentration of synergist, and hence, an elevation in tone is required in order to amplify the agonist response.

Therefore, 5-HT has activity at α_1-ARs and the serotonergic response in the WT mouse aorta is partly mediated by the α_{1D}-AR.

Hence, the threshold synergy observed when PE is used as a synergist for 5-HT can now be explained due to the activation of the α_{1D}-AR, a receptor involved in both responses. Stupecky et al., (1986) suggested that two agonists of the same receptor would result in threshold synergy consistent with the findings in the mouse aorta as a common receptor is involved in both responses, the α_{1D}-AR. Figure 3-7 compares the effect of α_{1D}-AR activation on the serotonergic response, showing the effect of an increased level of activation with 30nM PE present and the effect of a decreased level of activation with 10nM prazosin.

The application of PE primarily involves the activation of α_{1D}-ARs, a receptor shown to be involved in the response to 5-HT. 5-HT primarily acts through 5-HT_{2A} receptors but the presence of PE affected the curve by increasing the level of activation of a receptor, the α_{1D}-AR, which was already involved in the 5-HT response. The resulting curve was leftward shifted at threshold, converged at 300nM 5-HT and the slope was shallower.

In contrast, the blockade of α_{1D}-ARs results in a rightward shift of the curve, converging with the control curve at 300nM 5-HT and a steeper slope. Hence the synergistic effect of PE on the 5-HT response is similar to the synergistic effect of using another agonist of the same receptor (Stupecky *et al.*, 1986). A diagrammatic representation of the interaction is provided in Figure 3-15.

Thus, the relatively sensitive nature of the mouse aorta to 5-HT can be explained due its effects at both 5-HT_{2A} receptors and α_{1D}-ARs. The normal 5-HT response is itself synergistically amplified at threshold as α_{1D}-ARs are co-activated along with 5-HT_{2A} receptors for the 5-HT-induced response. Since both receptors are coupled through the same second messenger pathway they are likely to be interacting in a synergistic fashion due to "mutual effect amplification" as described by Leff (1987) and Christ & Jean-Jacques (1991).

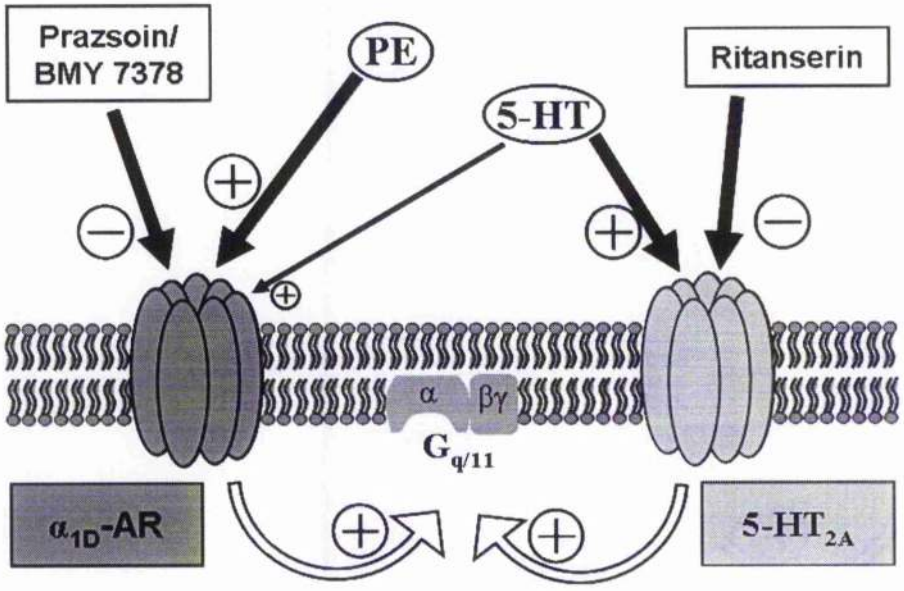


Figure 3-15. A diagrammatic representation of the activity of PE, 5-HT, prazosin, BMY 7378, and ritanserin on the activity of the α_{1D}-AR and the 5-HT_{2A} receptor. Both the α_{1D}-AR and 5-HT_{2A} receptor preferentially couple to the $G_{q/11}$ protein and is shown.

Serotonergic responses of the α_{1D}-KO

Interestingly the overall serotonergic response of the α_{1D}-KO was not significantly different from the WT although the adrenergic (PE) response of the α_{1D}-KO mouse was significantly less sensitive than the WT, as previously shown by Tanoue *et al.* (2002). The serotonergic responses in the α_{1D}-KO should have been analogous to the serotonergic response of WT aortae in the presence of prazosin or BMY 7378. But the α_{1D}-KO appears to have 'reset' its serotonergic response back to the WT serotonergic response.

There may have been some compensatory mechanism in the α_{1D}-KO to adjust for the loss of the α_{1D}-AR mediated vasoconstrictor response. The lack of one vasoconstrictor may have been offset by the alteration in function of another vasoconstrictor. 5-HT is very potent in the mouse aorta (Russell & Watts, 2000) and the serotonergic response in the mouse aorta is mainly 5-HT_{2A} receptor-mediated (McKune & Watts, 2001) so it is possible that the 5-HT_{2A} receptor is involved in the compensatory mechanism.

The commonly used 5-HT_{2A} receptor antagonist, ketanserin, has been shown to have an affinity for α_1-ARs (Cohen *et al.*, 1988), thus was unsuitable. The activity of 5-HT at both 5-HT_{2A} receptors and α_{1D}-AR would have been blocked by ketanserin making it incompatible with the aim of isolating the contribution of the 5-HT_{2A} receptors to the 5-HT induced response. Cohen *et al.* (1988) provided a pK_B value for ketanserin (7.9) at α_1-ARs. They also demonstrated that an insurmountable 5-HT_2 receptor antagonist, ritanserin had a pK_B value of 6.0 at α_1-ARs. Therefore ritanserin was chosen as the selective antagonist for isolating the 5-HT response.

Ritanserin completely ablated the 5-HT response of the α_{1D}-KO. Ritanserin did not affect the adrenergic responses of PE or the responses of the thromboxane mimetic, U46619 confirming its selectivity in blocking only the 5-HT_{2A}-mediated response. A residual serotonergic response remained in the WT and is likely to be the adrenergic α_{1D}-AR-mediated response of 5-HT. Thus, the serotonergic response of the α_{1D}-KO was ritanserin-sensitive and prazosin-resistant, suggesting the 5-HT_{2A} receptor compensates for the missing α_{1D}-AR in the α_{1D}-KO.

BRL 54443-induced contractions

Ritanserin completely ablated the 5-HT-induced response in both WT and α_{1D}-KO aortae. Therefore, it was necessary to use another ligand to study the function of the 5-HT_{2A} receptor. BRL 54443 is a full agonist at the 5-HT_{1E} and 5-HT_{1F} receptors (Brown *et al.*, 1998) but McKune and Watts (2001) demonstrated that BRL 54443 also has affinity for 5-HT_{2A} receptors, acting as a partial agonist in the mouse aorta.

BRL 54443 was used in an attempt to establish and quantify any differences in the 5-HT_{2A} mediated serotonergic responses of the WT and α_{1D}-KO aortae. However, BRL 54443 was equipotent in both the WT and the α_{1D}-KO and the responses were completely abolished by ritanserin in both strains. This confirmed that BRL 54443 has affinity for 5-HT_{2A} receptors but suggested that there was no significant difference in the 5-HT_{2A}-mediated response between WT and α_{1D}-KO aortae.

Although the maximum responses to BRL 54443 were not significantly different from 5-HT, there was a tendency for the maximum responses induced by BRL 54443 to be

smaller than the maximum 5-HT responses in both WT and α_{1D}-KO (consistent with McKune and Watts, 2001). The lack of significant difference is probably due to the large variability observed in these experiments. The inability of BRL 54443 to highlight differences in the 5-HT_{2A} receptor mediated contractions between WT and α_{1D}-KO was probably due to the fact that BRL 54443 is a partial agonist.

A characteristic of a partial agonist is that even with receptor occupancy of 1.0 or 100%, the partial agonist may still not be able to induce a maximal response (Rang *et al.*, 1999). Since we observed only a minor role for the α_{1D}-AR in the serotonergic response in WT mouse aorta, there may have been compensatory alterations 5-HT_{2A} receptor population in α_{1D}-KO aortae that weretoo subtle to distinguish using BRL 54443 as an agonist.

Conclusions- Chapter 3

Conclusion

A synergistic interaction between adrenergic and serotonergic vasoconstrictor systems has now been demonstrated in the mouse aorta. The α_{1D}-ARs and the 5-HT_{2A} receptors both couple through the same G-protein, in order to initiate a response, thus the synergy is due to mutual effect amplification. However, it is not possible to completely rule out a direct receptor-receptor interaction.

The sensitivity of the PE response was increased 6- to 7-fold in the presence of 30nM 5-HT. The PE curve was shifted to the left in a parallel fashion exhibiting 'potentiation'. The introduction of 5-HT synergist concentrations involves the recruitment of 5-HT_{2A} receptors, which are 'new' receptors in the PE response.

Conversely the sensitivity of the 5-HT response was increased 3- to 5-fold when a synergist concentration of 30nM PE was applied. However, the curve was shifted to the left primarily at threshold, converging at higher concentrations, demonstrating 'threshold synergy'. The threshold synergism of the 5-HT response was due to the increased involvement of α_{1D}-ARs.

The 5-HT response in the WT mouse aorta is partly mediated by α_{1D}-ARs. The relatively sensitive nature of the 5-HT response in the mouse aorta was, therefore, the result of the co-activation of both 5-HT_{2A} receptors and α_{1D}-ARs, which synergistically interact to amplify the functional response of 5-HT. Thus the threshold synergy observed was due to increased participation of α_{1D}-ARs, which are already involved in the 5-HT-induced response.

The serotonergic response of the α_{1D}-KO was not significantly different from the WT serotonergic response. However, the 5-HT response of the WT was sensitive to both 5-HT and prazosin, whilst the α_{1D}-KO response was prazosin-resistant and ritanserin-sensitive suggesting that in the α_{1D}-KO mouse aorta 5-HT_{2A} receptors compensate for the lack of functional α_{1D}-ARs.

Chapter 4. The effect of L-NAME on contractile responses in the mouse aorta

Introduction- Chapter 4

Role of endothelium in the vasculature

Historically, the endothelium had been primarily considered to be a physical barrier controlling the movement of cells, proteins and molecules between blood fluid and interstitial fluid within and beyond the vascular wall. Research over the last 25 years has now indicated the functional importance of the endothelium in modulating vascular tone. Endothelial cells (ECs) can release a myriad of modulators, both contractile, such as endothelin and some prostaglandins, and relaxatory, such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarising factor (EDHF). Furthermore the endothelium is an important site for the enzymatic conversion of angiotensin I to angiotensin II as well as affecting the activity of other vasoactive peptides. The endothelium has also been linked to the pathology of such conditions as hypertension (reviewed by Marin, 1995).

Nitric oxide

One of the most profound discoveries was that endothelial cells have the ability to release the simple molecule nitric oxide (NO) which acts as a dilator of vascular smooth muscle cells. Originally, NO was termed endothelium-derived relaxing factor (EDRF) by Furchgott & Zawadski (1980) who reported that the functional responses of ACh in the rabbit aorta were either contractile or relaxatory dependent on endothelial preservation during dissection and setup.

Initially EDRF was suspected to be a peptide, but was later identified as NO (Palmer *et al.*, 1987). Palmer *et al.* (1988) then discovered the metabolic pathway for NO synthesis. NO is synthesised by an enzyme-controlled five-electron oxidation of the terminal guanidino nitrogen atom of L-arginine. The recognition of NO as EDRF lead to an explosion in the study of NO, and its associated metabolic pathways and actions, as potential therapeutic targets. As such, blockers of the class of nitric oxide synthase (NOS) enzymes such as N_ω-nitro-L-arginine methyl ester (L-NAME: Rees *et al.*, 1990) are now available for pharmacological and physiological studies.

Endothelial α-adrenoceptors

Vascular α_1-adrenoceptors (ARs) are generally considered to be vasoconstrictor receptors, being primarily distributed amongst the medial layer of blood vessels. Conversely, in large conductance arteries such as the aorta, α_2-ARs have been associated with endothelium-dependent vasodilatation (Cocks & Angus, 1983). Vanhoutte & Miller (1989) then related α_2-AR activation with the release of nitric oxide from ECs.

However, authors are beginning to report the distribution of α_1-ARs amongst the adventitial layer (Faber *et al.*, 2001) and in endothelial cells (Filippi *et al.*, 2001). Indeed, Filippi *et al.* (2001) demonstrated, in perfused rat mesenteric vascular bed, that the activation of α_{1D}-ARs on ECs results in NO release, and hence, vasodilatation. Therefore, the α_1-AR-mediated contractile responses to agonists such as PE were somewhat attenuated by the simultaneous activation of α_{1D}-ARs on ECs which released NO. Similarly Zschauer *et al.* (1997) showed NE-induced vasoconstriction in rabbit bronchial artery was modulated by NO release from ECs which were stimulated by α_1- and α_2-AR activation.

The mouse aorta

As yet, no studies on the effect of NO release on contractility in the mouse aorta have been performed. The mouse aorta has a significant α_1-adrenoceptor mediated contractile response (Russell & Watts, 2000) which is primarily α_{1D}-AR mediated (Yamamoto & Koike, 2001). The role of the individual α_1-AR subtypes has been studied using transgenic mice (Daly *et al.*, 2002; Tanoue *et al.*, 2002). 5-HT also exhibits a high degree of sensitivity and efficacy in the mouse aorta (Russell & Watts, 2000), thus providing a useful gauge of contractile responses.

Aim of Study

The aims were to determine the effects of NO release on PE, 5-HT and KCl-induced responses in the mouse aorta by blocking NO production using L-NAME. The involvement of α_{1B}- and α_{1D}-ARs in NO release was investigated using WT, α_{1B}-KO and α_{1D}-KO aortae. The PE-mediated involvement of α_2-ARs in contraction or relaxation was tested in both control and L-NAME treated aortic rings.

Results- Chapter 4

Effect of L-NAME on ACh-induced relaxations

The ACh-induced relaxations in the absence and presence of 100μM L-NAME were: WT control = 52.8 ± 2.6% decrease in tone (n = 25); WT + L-NAME = 14.5 ± 3.1% (n = 21); α_{1B}-KO control = 61.7 ± 3.8% (n = 10); α_{1B}-KO + L-NAME = 1.4 ± 3.9% (n = 9); α_{1D}-KO control = 70.3 ± 7.6% (n = 5); α_{1D}-KO + L-NAME = 10.3 ± 14.1% (n = 7).

The ACh-induced relaxations were significantly reduced by L-NAME in WT ($p<0.001$), α_{1B}-KO ($p<0.001$) and α_{1D}-KO aortae ($p<0.01$). These results are shown in graphical form in Figure 4-1.

The ACh response of WT and α_{1B}-KO were not significantly different, whilst the α_{1D}-KO ACh response was significantly less sensitive than WT ($p<0.01$: one-way-ANOVA, Bonferroni post-test).

Effect of L-NAME on KCl-induced contractile responses

The KCl-induced contractile responses in the absence and presence of 100μM L-NAME were: WT control = 0.72 ± 0.02 (n = 25); WT + L-NAME = 0.95 ± 0.04 (n = 21); α_{1B}-KO control = 0.69 ± 0.04 (n = 16); α_{1B}-KO + L-NAME = 1.25 ± 0.08 (n = 9); α_{1D}-KO control = 0.74 ± 0.05 (n = 8); α_{1D}-KO + L-NAME = 0.95 ± 0.05 (n = 6). The KCl-induced contractile responses were significantly greater in the L-NAME treated groups than in the controls (WT- $p<0.001$; α_{1B}-KO- $p<0.001$; α_{1D}-KO- $p<0.01$: Student's t-test). These results are shown in graphical form in Figure 4-2.

Effect of L-NAME on 5-HT-induced contractions of WT aortae

E_{max} values for 5-HT-induced contractile responses of WT aortae in the absence and presence of 100μM L-NAME were: control = 1.62 ± 0.09 (n = 11); + L-NAME = 1.66 ± 0.09 (n = 7). The maximum responses were not significantly different (Student's t-test).

pEC_{50} values were: control = 7.00 ± 0.07; + L-NAME = 7.58 ± 0.21. The L-NAME treated group was 4-fold more sensitive ($p<0.01$; Student's t-test) than the control group.

Hill slopes were: control = 1.15 (0.96-1.34); + L-NAME = 0.82 (0.69-0.95). The Hill slopes overlapped with unity.

The effect of L-NAME on the 5-HT curve in the WT aorta is shown in Figure 4-3 and the values shown are tabulated in Table 4-3.

Effect of L-NAME on 5-HT-induced contractions of α_{1B}-KO aortae

E_{max} values for 5-HT-induced contractile responses of α_{1B}-KO aortae in the absence and presence of 100μM L-NAME were: control = 1.51 ± 0.13 (n = 12); + L-NAME = 1.80 ± 0.17 (n = 9). The maximum response to 5-HT in the α_{1B}-KO were not significantly altered by L-NAME (Student's t-test).

pEC_{50} values were: control = 7.04 ± 0.10; + L-NAME = 7.48 ± 0.13. L-NAME treatment resulted in a 3-fold more sensitive response ($p<0.05$; Student's t-test).

Hill slopes were: control = 0.98 (0.79-1.16); + L-NAME = 0.80 (0.65-1.04). The Hill slopes overlapped with unity.

The 5-HT curves of the α_{1B}-KO aorta and the effect of L-NAME are shown in Figure 4-4 and the values shown are tabulated in Table 4-4

Effect of L-NAME on 5-HT-induced contractions of α_{1D}-KO aortae

E_{max} values for 5-HT-induced contractile responses of α_{1D}-KO aortae in the absence and presence of 100μM L-NAME were: control = 1.35 + 0.09 (n = 6); + L-NAME = 1.40 ± 0.08 (n = 7). There was no significant difference in the maximal 5-HT-induced response of α_{1D}-KO aortae as a result of L-NAME treatment (Student's t-test).

pEC_{50} values were: control = 7.39 ± 0.07; + L-NAME = 7.62 ± 0.05. The serotonergic response of the L-NAME treated group was 2-fold more sensitive than the control ($p<0.05$: Student's t-test).

Hill slopes were: control = 1.04 (0.35-1.73); + L-NAME = 1.02 (0.60-1.44). The Hill slopes overlapped with unity.

The serotonergic responses in the absence and presence of L-NAME in the α_{1D}-KO are shown in Figure 4-5 and the values shown are tabulated in Table 4-5.

Comparison of 5-HT-induced contractions of WT, α_{1B}-KO &α_{1D}-KO aortae

The comparison of the serotonergic responses of the WT, α_{1B}-KO & α_{1D}-KO aortae are shown in Figure 4-6 and the respective values are tabulated in Table 4-6. The E_{max} and pEC_{50} values of the 5-HT-induced responses of the α_{1B}-KO and α_{1D}-KO were not significantly different from the WT (one-way-ANOVA).

Comparison of 5-HT-induced contractions of WT, α_{1B}-KO &α_{1D}-KO aortae in the presence of L-NAME

The comparison of the serotonergic responses in the presence of L-NAME in WT, α_{1B}-KO & α_{1D}-KO aortae are shown in Figure 4-7 and the respective values are tabulated in Table 4-7. L-NAME treated WT, α_{1B}-KO & α_{1D}-KO aortae exhibited similar maxima and sensitivity (pEC_{50}) (one-way-ANOVA).

Effect of L-NAME on PE-induced contractions of WT aortae

E_{max} values for PE-induced contractile responses of WT aortae in the absence and presence of 100μM L-NAME were: control = 1.01 ± 0.05 (n = 26); + L-NAME = 1.37 ± 0.07 (n = 21). The maximum PE response was 36% significantly greater in the L-NAME treated group compared to the control group ($p<0.001$: Student's t-test).

pEC_{50} values were: control = 6.66 ± 0.07; + L-NAME = 7.11 ± 0.11. The PE response was 4-fold sensitive in the presence of L-NAME ($p<0.001$: Student's t-test).

Hill slopes were: control = 0.59 (0.44-0.74); + L-NAME = 0.55 (0.42-0.67). The Hill slopes did not overlap with unity.

The effect of L-NAME on the PE curve in the WT aorta is shown in Figure 4-8 and the values shown are tabulated in Table 4-8.

Effect of L-NAME on PE-induced contractions of α_{1B}-KO aortae

E_{max} values for PE-induced contractile responses of α_{1B}-KO aortae in the absence and presence of 100μM L-NAME were: control = 0.97 ± 0.09 (n = 16); + L-NAME = 1.73 ± 0.11 (n = 9). L-NAME treatment resulted in a 78% increase in sensitivity of the PE response of the α_{1B}-KO ($p<0.001$: Student's t-test).

pEC_{50} values were: control = 6.73 ± 0.08; + L-NAME = 7.21 ± 0.11. A 3-fold increase in sensitivity was observed in the presence of L-NAME ($p<0.01$: Student's t-test).

Hill slopes were: control = 0.55 (0.45-0.66); + L-NAME = 0.67 (0.51-1.82). The Hill slopes did not overlap with unity.

The effect of L-NAME on the PE curve in the α_{1B}-KO aorta is shown in Figure 4-9 and the values shown are tabulated in Table 4-9.

Effect of L-NAME on PE-induced contractions of α_{1D}-KO aortae

E_{max} values for PE-induced contractile responses of α_{1D}-KO aortae in the absence and presence of 100μM L-NAME were: control = 0.64 + 0.12 (n = 6); + L-NAME = 0.98 ± 0.09 (n = 7). L-NAME treatment resulted in a 53% increase in the maximum PE-induced contractile responses of the α_{1D}-KO aortae ($p<0.05$: Student's t-test)

pEC_{50} values were: control = 5.52 ± 0.13; + L-NAME = 5.29 ± 0.13. There was no significant alteration in sensitivity as a result of L-NAME treatment (Student's t-test)

Hill slopes were: control = 0.85 (*0.57-1.13*); + L-NAME = 0.86 (*0.57-1.17*). The Hill slopes overlapped with unity.

The effect of L-NAME on the PE curve in the α_{1D}-KO aorta is shown in Figure 4-10 and the values shown are tabulated in Table 4-10.

Comparison of the PE-induced contractions of WT, α_{1B}-KO &α_{1D}-KO aortae

A comparison of the PE-induced responses of the WT, α_{1B}-KO & α_{1D}-KO aortae are shown in Figure 4-11 and the respective values are tabulated in Table 4-11. The WT and α_{1B}-KO exhibited similar maxima and sensitivity (pEC_{50}) (one-way-ANOVA, Bonferroni post-test). The maximum of the α_{1D}-KO PE response was decreased 37% ($p<0.05$: one-way-ANOVA, Bonferroni post-test) and the pEC_{50} was 14-fold less sensitive than the WT ($p<0.001$).

Comparison of the PE-induced contractions of WT, α_{1B}-KO &α_{1D}-KO aortae in the presence of L-NAME

A comparison of the WT, α_{1B}-KO and α_{1D}-KO PE-induced responses in the presence of L-NAME is shown in Figure 4-12 and the relevant values are tabulated in Table 4-12. In the presence of L-NAME, α_{1B}-KO aortae exhibited a significantly larger (26%, $p<0.05$: one-way-ANOVA, Bonferroni post-test) but no significant alteration in sensitivity (pEC_{50}). The α_{1D}-KO aorta maxima to PE was decreased 28% and the sensitivity was 66-fold less sensitive than the PE responses of the WT.

Effect of rauwolscine in the absence and presence of L-NAME on adrenergic responses of WT aortae

E_{max} values for PE-induced contractile responses of α_{1D}-KO aortae in the absence and presence of 30nM rauwolscine and/or were 100μM L-NAME: normal control = 1.01 ± 0.05 (n = 26); normal + rauwol. = 1.06 ± 0.09 (n = 8); + L-NAME control = 1.37 ± 0.07 (n = 21); + L-NAME + rauwol. = 1.45 ± 0.09 (n = 7). The rauwolscine treated groups did not exhibit significantly different maxima from their appropriate control (Student's t-test).

pEC_{50} values were: normal control = 6.67 ± 0.07; + rauwol. = 6.71 ± 0.08; + L-NAME = 7.11 ± 0.11; + L-NAME & rauwol. = 6.98 ± 0.19. Rauwolscine treatment did not affect the sensitivity of the non-L-NAME treated and L-NAME treated groups (Student's t-test).

Hill slopes were: normal control = 0.59 (*0.44-0.74*); + rauwol. = 0.60 (*0.43-0.77*); + L-NAME = 0.54 (*0.41-0.67*); + L-NAME & rauwol. = 0.58 (*0.47-0.69*). The Hill slopes did not overlap with unity.

The above data is shown in graphical form in Figure 4-13 and the relevant values are tabulated in Table 4-13.

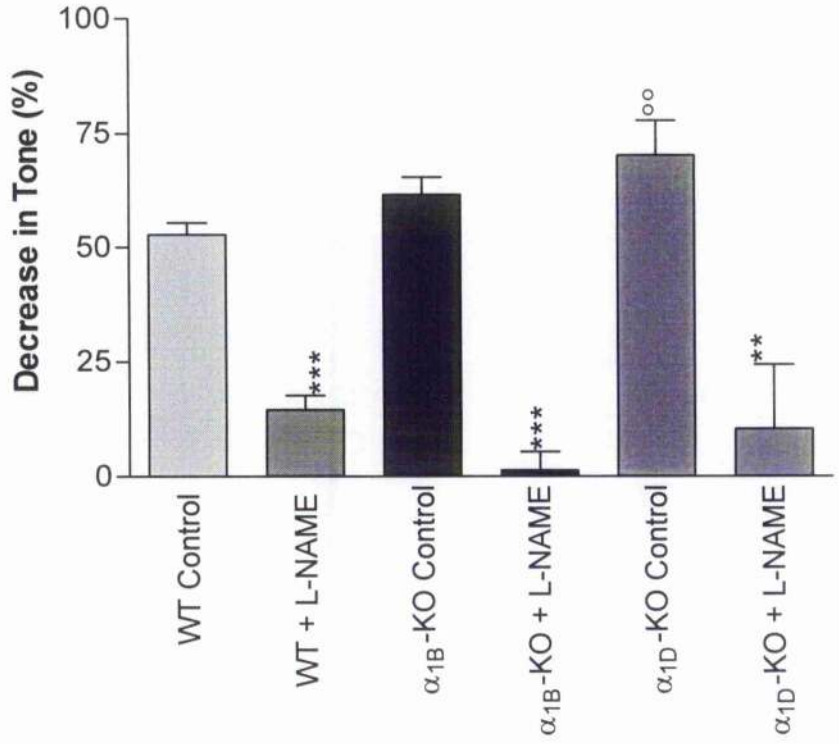


Figure 4-1. The effect of 100μM L-NAME on 30μM ACh-induced relaxant responses of WT, α_{1B}-KO and α_{1D}-KO aortae.

Columns shown are the mean ± S.E.M of the generated responses expressed in grams force (**p<0.01, ***p<0.001, against strain-matched control; °°p<0.01 against WT control: one-way-AVOVA, Bonferroni post-test).

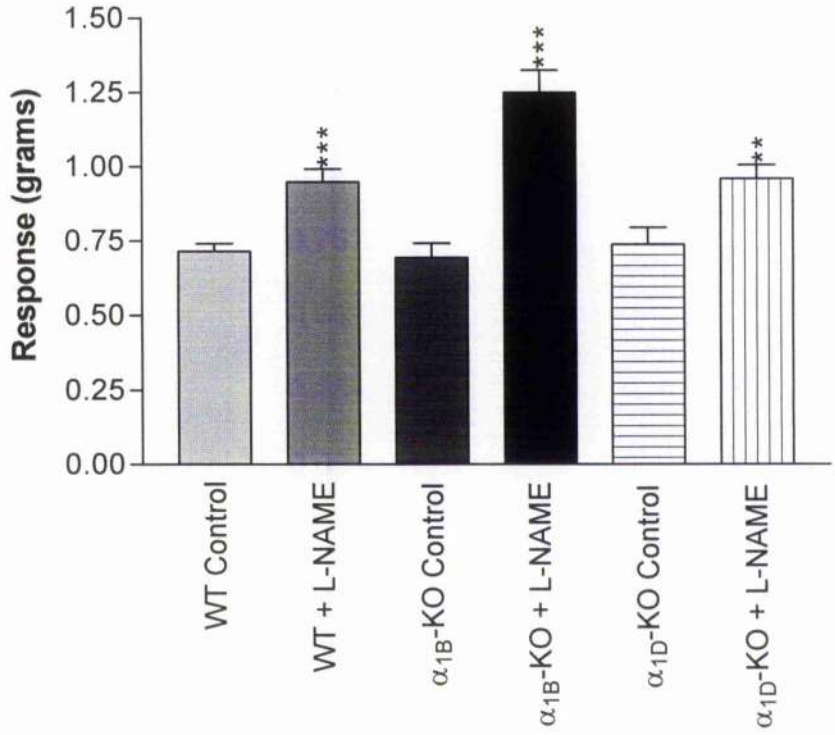


Figure 4-2. The effect of 100μM L-NAME on 125mM KCl-induced contractile responses of WT, α_{1B}-KO and α_{1D}-KO aortae.

Columns shown are the mean ± S.E.M of the generated responses expressed in grams force. (**p<0.01, ***p<0.001 against strain-matched control: one-way-AVOVA, Bonferroni post-test)

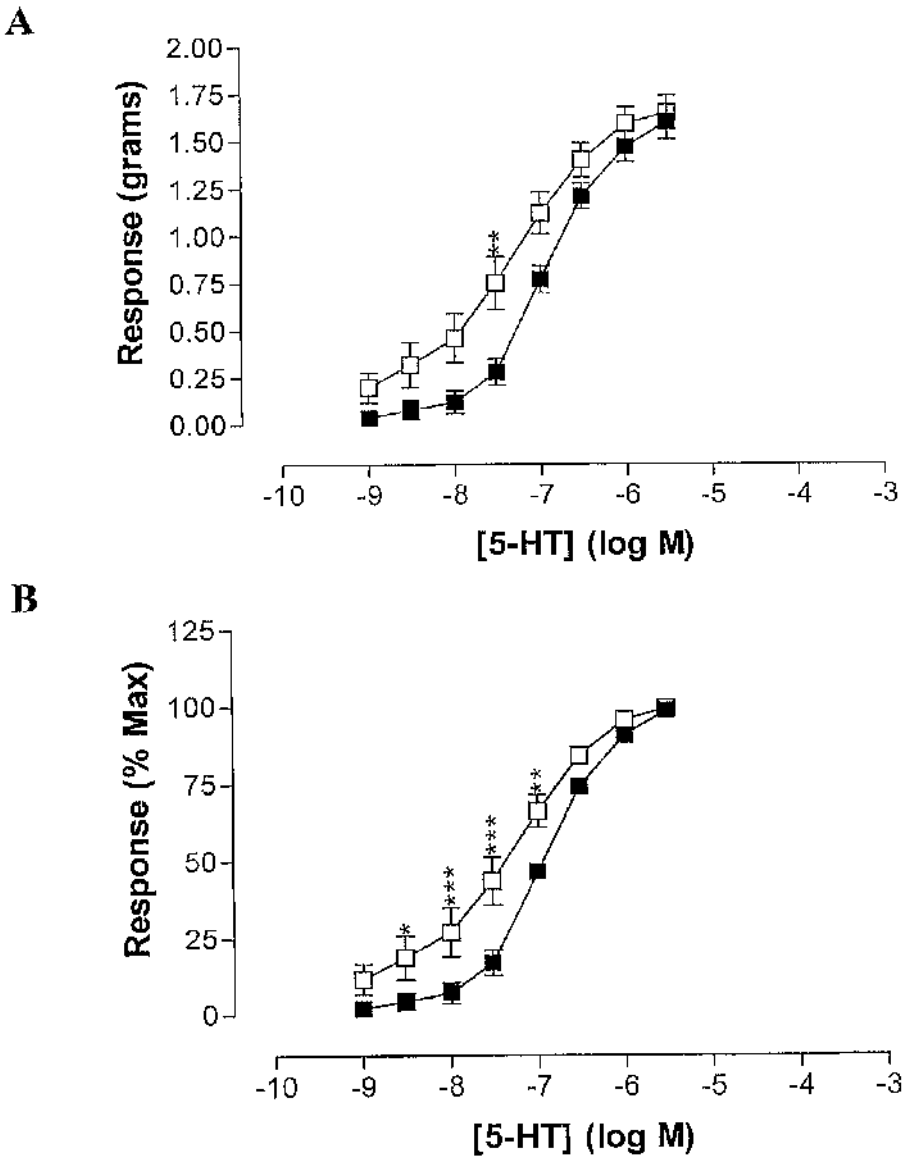


Figure 4-3. The effect of 100μM L-NAME on 5-HT-induced contractile responses of WT aortae (■ control, $n = 11$; □ 100μM L-NAME, $n = 7$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	11	1.62 ± 0.09
+ L-NAME	7	1.66 ± 0.09

B

	pEC_{50}	Hill slope
Control	7.00 ± 0.07	1.15 (*0.96-1.34*)
+ L-NAME	7.58 ± 0.21**	0.82 (*0.69-0.95*)

Table 4-3. The effect of 100μM L-NAME on 5-HT-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (**p<0.01: Student's t-test).

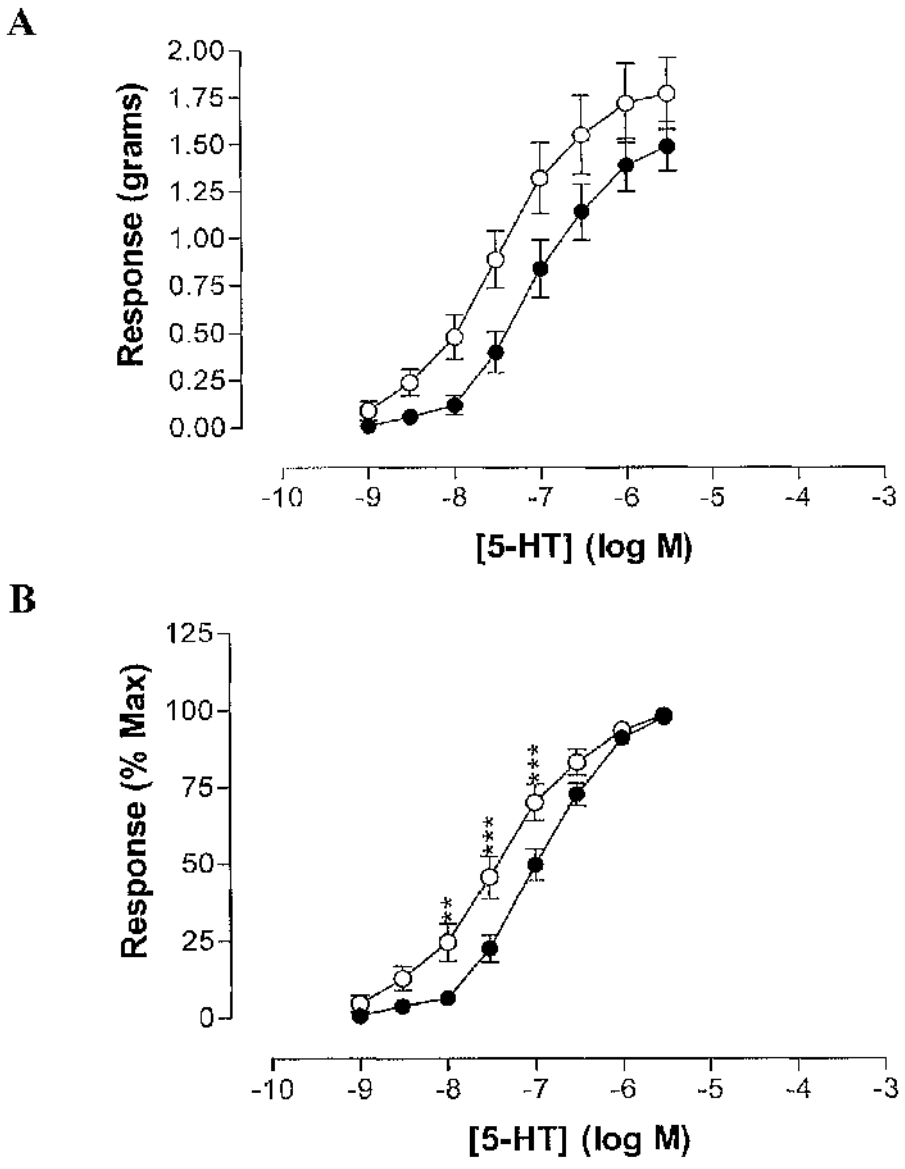


Figure 4-4. The effect of 100μM L-NAME on 5-HT-induced contractile responses of α_{1B}-KO aortae (● control, n = 12; ○ 100μM L-NAME, n = 9).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (**p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	12	1.51 ± 0.13
+ L-NAME	9	1.80 ± 0.17

B

	pEC_{50}	Hill slope
Control	7.04 ± 0.10	0.98 (*0.79-1.16*)
+ L-NAME	7.48 ± 0.13*	0.80 (*0.65-1.04*)

Table 4-4. The effect of 100μM L-NAME on 5-HT-induced contractile responses of α_{1B}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$: Student's t-test).

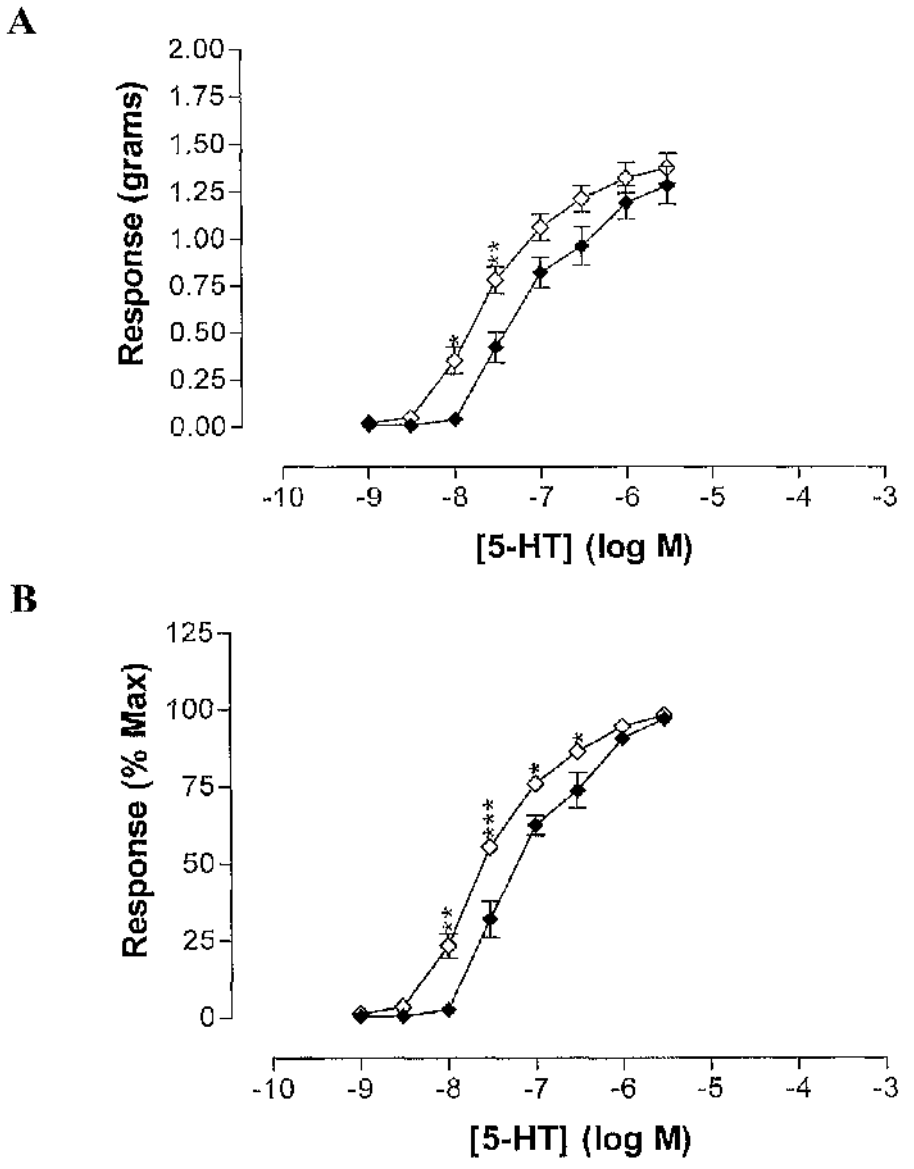


Figure 4-5. The effect of 100μM L-NAME on 5-HT-induced contractile responses of α_{1D}-KO aortae (◆ control, n = 12; ◇ 100μM L-NAME, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	6	1.35 ± 0.09
+ L-NAME	7	1.40 ± 0.08

B

	pEC_{50}	Hill slope
Control	7.39 ± 0.07	1.04 (*0.35-1.73*)
+ L-NAME	7.62 ± 0.05*	1.02 (*0.60-1.44*)

Table 4-5. The effect of 100μM L-NAME on 5-HT-induced contractile responses of α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$: Student's t-test).

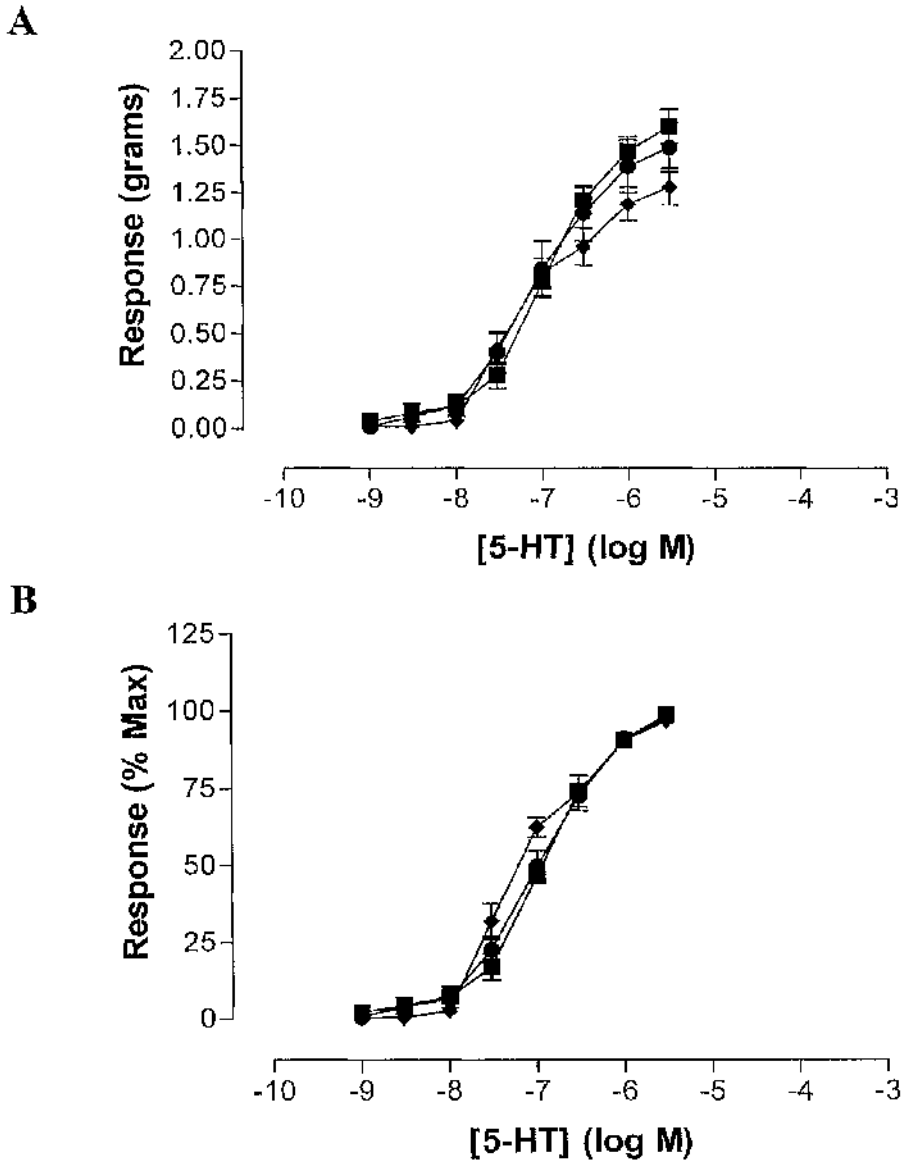


Figure 4-6. Comparison of the 5-HT-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae (■ WT, n = 12; ● α_{1B}-KO, n = 6; ◆ α_{1D}-KO, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (No statistical significance against WT: two-way-ANOVA, Bonferroni).

A

	n	E_{max} (grams)
WT	11	1.62 ± 0.08
α_{1B}-KO	12	1.51 ± 0.13
α_{1D}-KO	6	1.36 ± 0.09

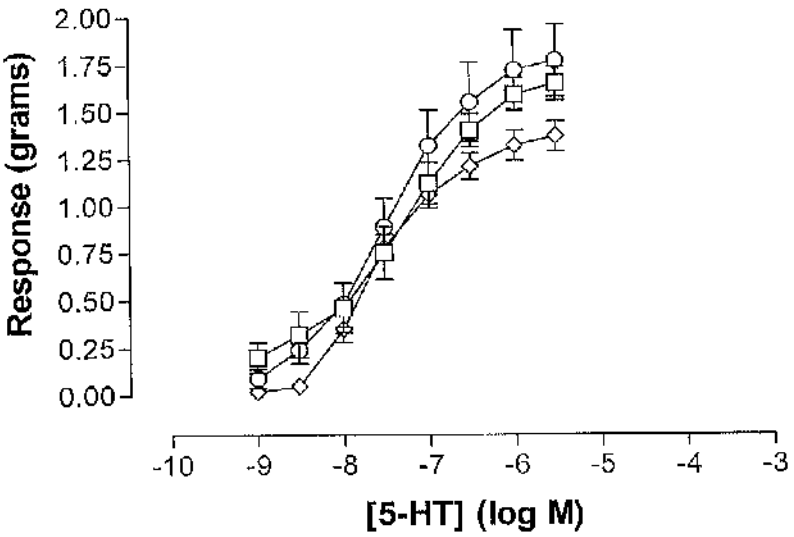
B

	pEC_{50}	Hill slope
WT	7.00 ± 0.07	1.15 (*0.96-1.34*)
α_{1B}-KO	7.04 ± 0.10	0.97 (*0.79-1.16*)
α_{1D}-KO	7.39 ± 0.07	1.04 (*0.35-1.72*)

Table 4-6. Comparison of the 5-HT-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical significance against WT: one-way-ANOVA, Bonferroni post-test).

A



B

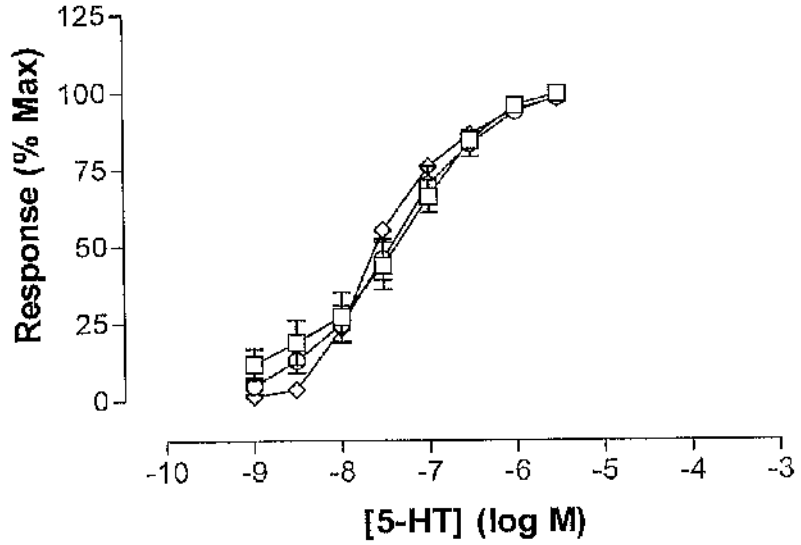


Figure 4-7. Comparison of the 5-HT-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae in the presence of 100μM L-NAME (┌┐ WT, $n = 12$; ○ α_{1B}-KO, $n = 6$; ◇ α_{1D}-KO, $n = 6$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (No statistical significance against WT: two-way-ANOVA, Bonferroni).

A

	n	E_{max} (grams)
WT	7	1.66 ± 0.09
α_{1B}-KO	9	1.80 ± 0.17
α_{1D}-KO	7	1.40 ± 0.08

B

	pEC_{50}	Hill slope
WT	7.58 ± 0.21	0.82 (*0.69-0.95*)
α_{1B}-KO	7.48 ± 0.13	0.80 (*0.65-1.04*)
α_{1D}-KO	7.62 ± 0.05	1.02 (*0.60-1.44*)

Table 4-7. Comparison of the 5-HT-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae in the presence of 100μM L-NAME.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical significance against WT: one-way-ANOVA, Bonferroni post-test).

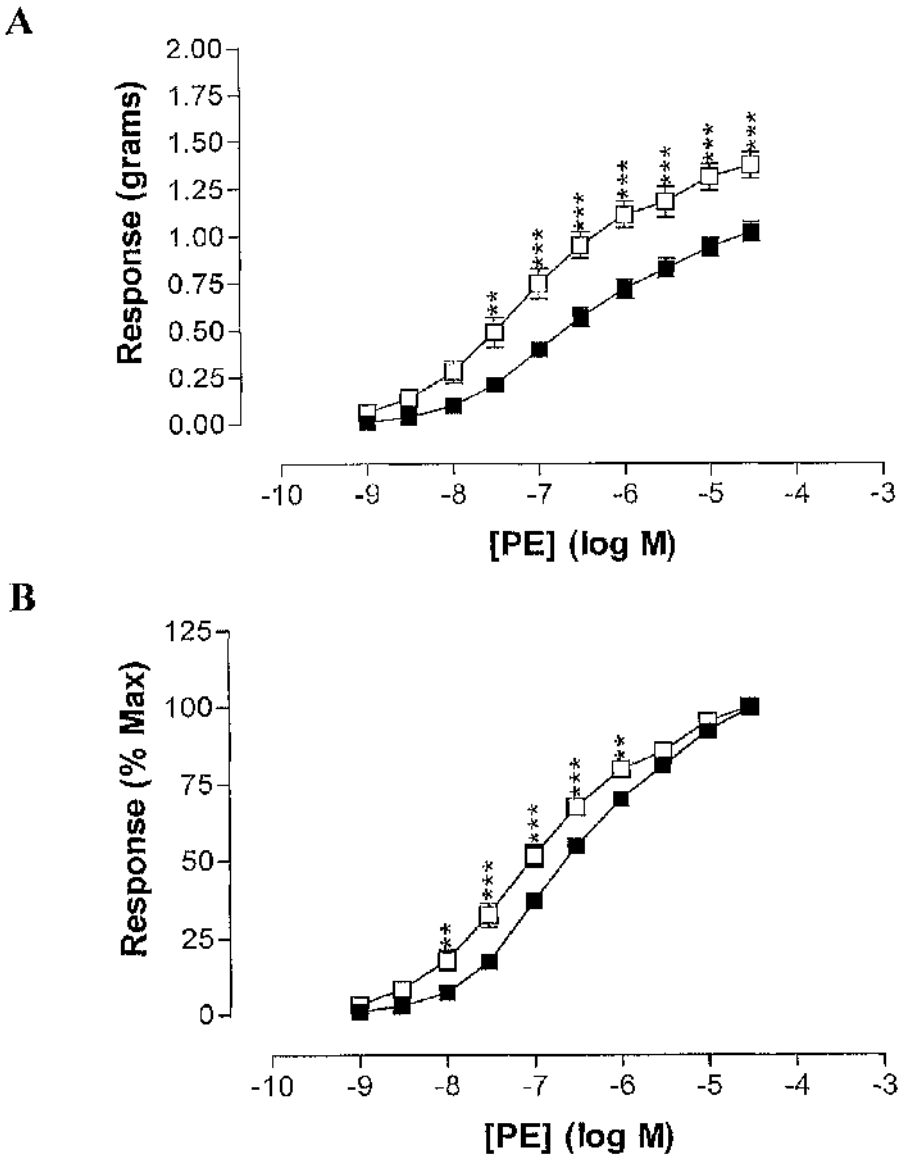


Figure 4-8. The effect of 100μM L-NAME on PE-induced contractile responses of WT aortae (■ control, n = 12; □ 100μM L-NAME, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (**p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	26	1.01 ± 0.05
+ L-NAME	21	1.37 ± 0.07***

B

	pEC_{50}	Hill slope
Control	6.66 ± 0.07	0.59 (*0.44-0.74*)
+ L-NAME	7.11 ± 0.11***	0.55 (*0.42-0.67*)

Table 4-8. The effect of 100μM L-NAME on PE-induced contractile responses of WT aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (***$p<0.001$: Student's t-test).

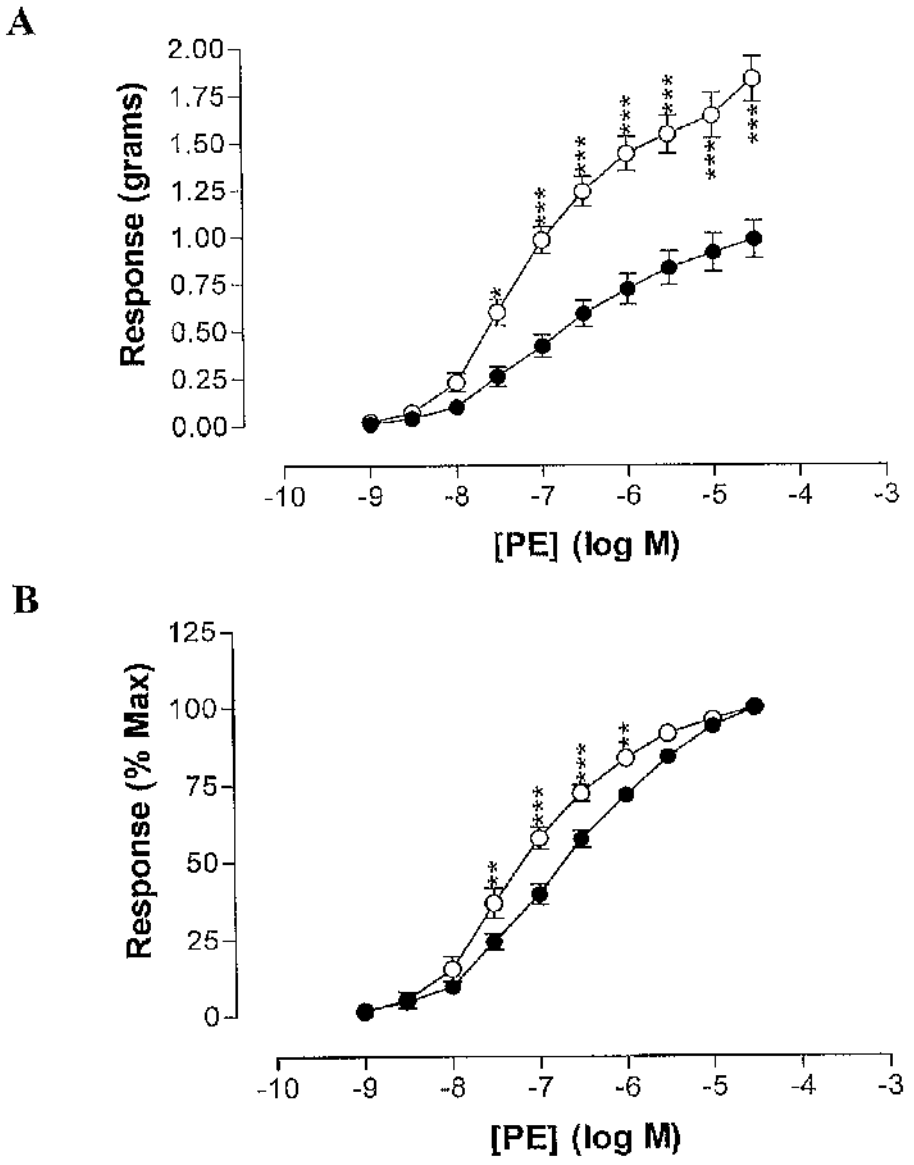


Figure 4-9. The effect of 100μM L-NAME on PE-induced contractile responses of α_{1B}-KO aortae (● control, n = 12; ○ 100μM L-NAME, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	16	0.97 ± 0.09
+ L-NAME	9	1.73 ± 0.11***

B

	pEC_{50}	Hill slope
Control	6.73 ± 0.08	0.55 (*0.45-0.66*)
+ L-NAME	7.21 ± 0.11**	0.67 (*0.51-1.82*)

Table 4-9. The effect of 100μM L-NAME on PE-induced contractile responses of α_{1B}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (**p<0.01, ***p<0.001: Student's t-test).

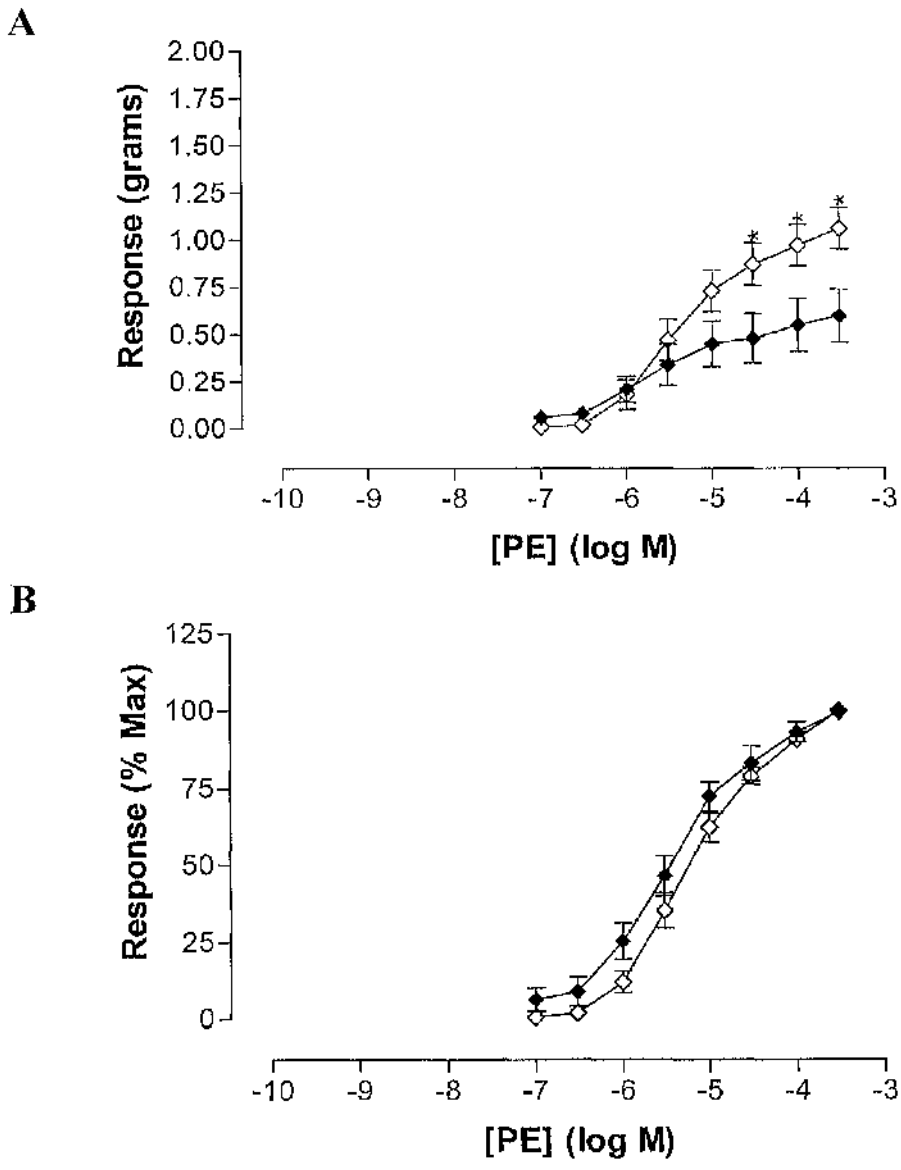


Figure 4-10. The effect of 100μM L-NAME on PE-induced contractile responses of α_{1D}-KO aortae (◆ control, n = 12; ◇ 100μM L-NAME, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*$p<0.05$: two-way-ANOVA, Bonferroni post-test).

A

	n	E_{max} (grams)
Control	6	0.64 ± 0.12
+ L-NAME	7	0.98 ± 0.09*

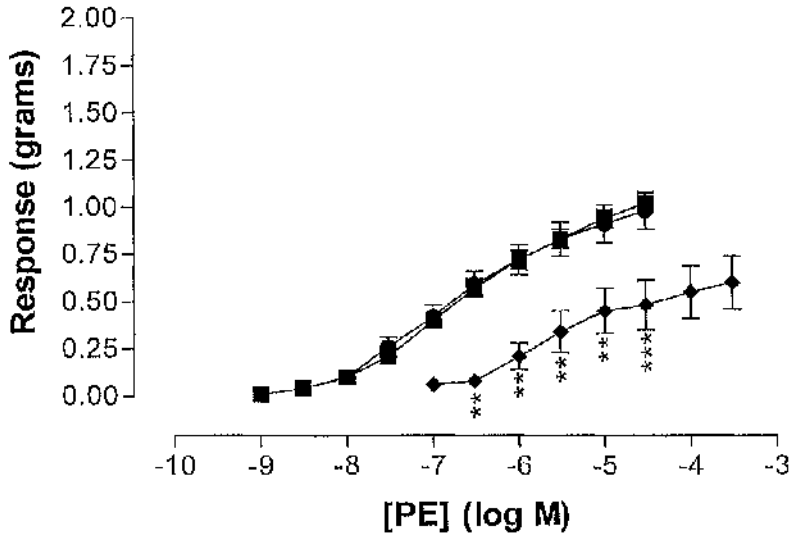
B

	pEC_{50}	Hill slope
Control	5.52 ± 0.13	0.85 (*0.57-1.13*)
+ L-NAME	5.29 ± 0.13	0.86 (*0.57-1.17*)

Table 4-10. The effect of 100μM L-NAME on PE-induced contractile responses of α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$: Student's t-test).

A



B

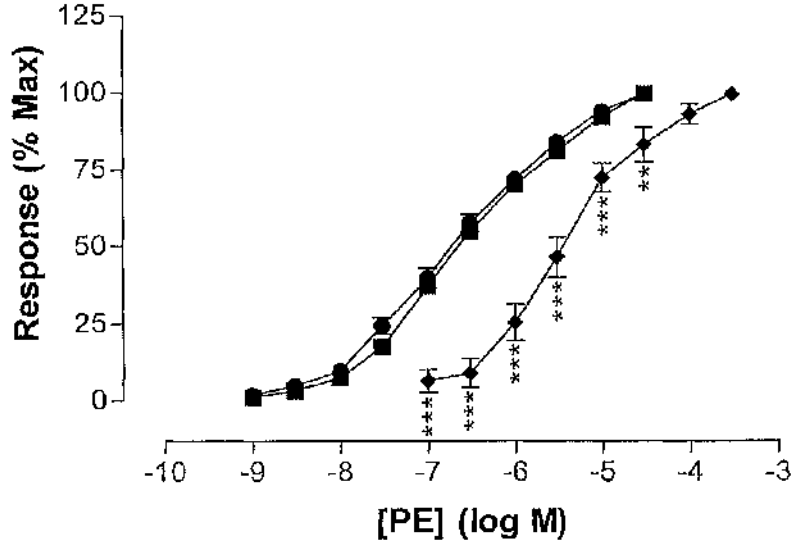


Figure 4-11. Comparison of the PE-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae (■ WT, n = 12; ● α_{1B}-KO, n = 6; ◆ α_{1D}-KO, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (**p<0.01, ***p<0.001 against WT: two-way-ANOVA, Bonferroni).

A

	n	E_{max} (grams)
WT	26	1.01 ± 0.05
α_{1B}-KO	16	0.97 ± 0.09
α_{1D}-KO	6	0.64 ± 0.12*

B

	pEC_{50}	Hill slope
WT	6.66 ± 0.07	0.59 (*0.44-0.74*)
α_{1B}-KO	6.73 ± 0.08	0.55 (*0.45-0.66*)
α_{1D}-KO	5.52 ± 0.13***	0.85 (*0.57-1.13*)

Table 4-11. Comparison of the PE-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae.

The '*n*' number, maximum responses generated, expressed as grams force (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*p<0.05, ***p<0.001: one-way-ANOVA, Bonferroni post-test).

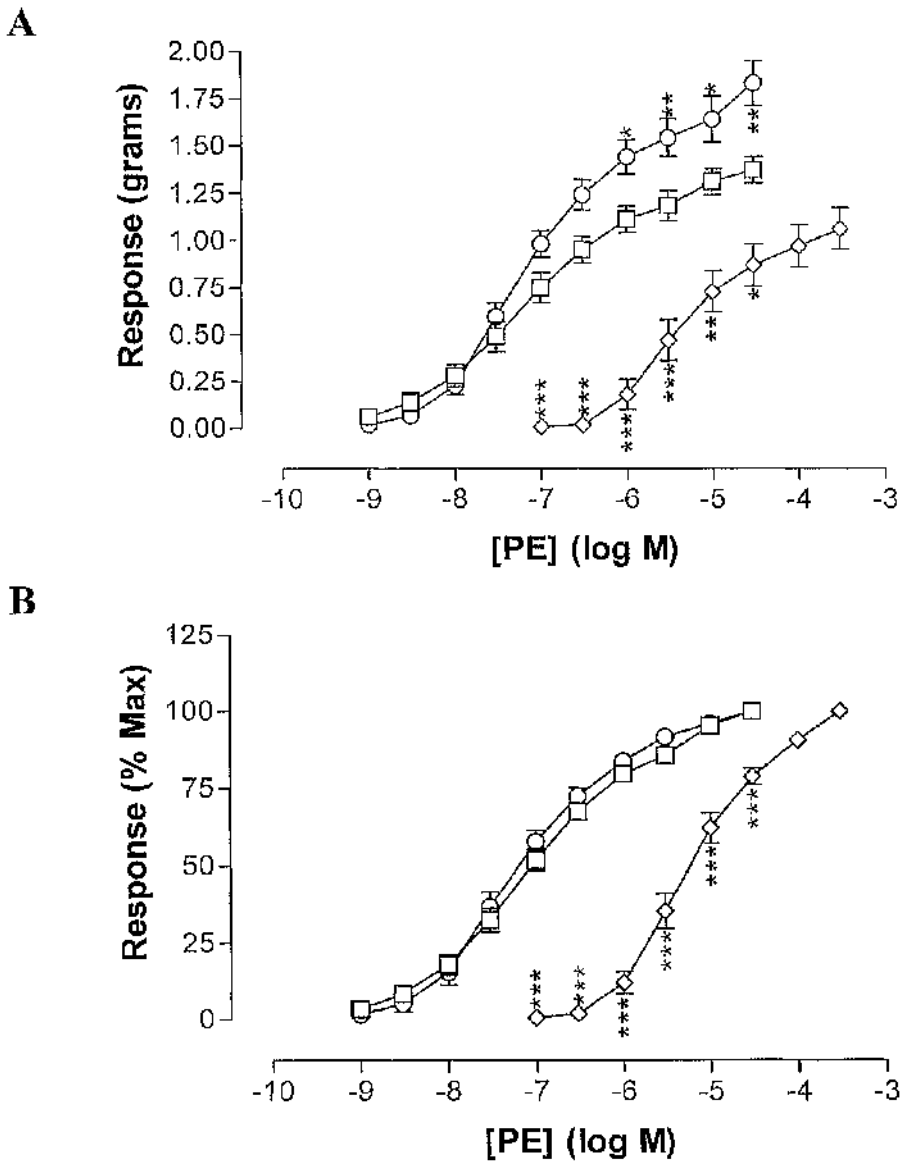


Figure 4-12. Comparison of the PE-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae in the presence of 100μM L-NAME (□ WT, $n = 12$; ○ α_{1B}-KO, $n = 6$; ◇ α_{1D}-KO, $n = 6$).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001 against WT: two-way-ANOVA, Bonferroni).

A

	n	E_{max} (grams)
WT	21	1.37 ± 0.07
α_{1B}-KO	9	1.73 ± 0.11*
α_{1D}-KO	7	0.98 ± 0.09*

B

	pEC_{50}	Hill slope
WT	7.11 ± 0.11	0.55 (*0.42-0.67*)
α_{1B}-KO	7.21 ± 0.11	0.67 (*0.51-1.82*)
α_{1D}-KO	5.29 ± 0.13***	0.86 (*0.57-1.17*)

Table 4-12. Comparison of the PE-induced contractile responses of WT, α_{1B}-KO & α_{1D}-KO aortae in the presence of 100μM L-NAME.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (*$p<0.05$, $p<0.001$: one-way-ANOVA, Bonferroni post-test).

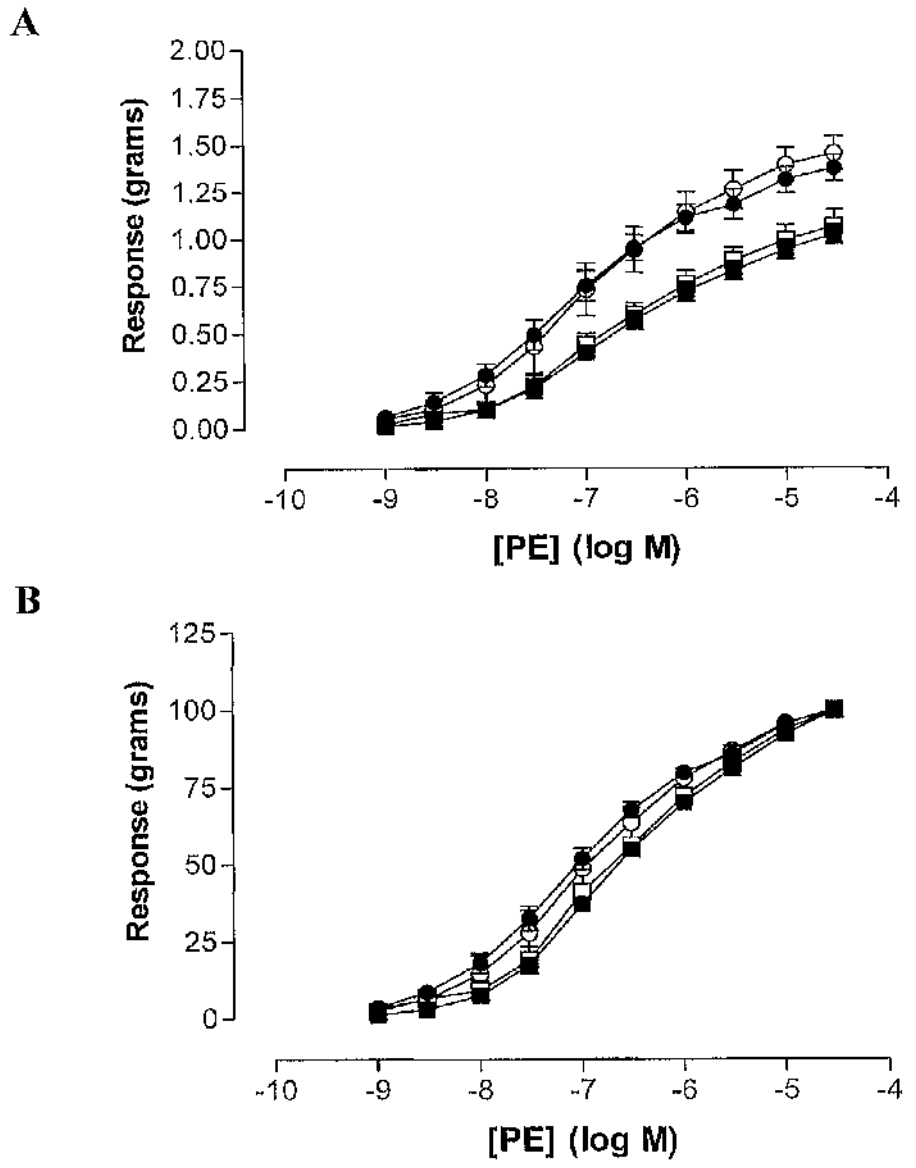


Figure 4-13. The effect of 30nM rauwolscine on the PE-induced contractile responses of WT both in the control and 100μM L-NAME treated rings (■ control, n = 12; □ + rauwolscine, n = 12; ● L-NAME control, n = 6; ○ L-NAME + rauwolscine, n = 6).

Responses are shown both in grams (**A**) and normalised, expressed as percentage of maximum response (**B**). Data points are the mean ± S.E.M. (No No statistical significance against controls: two-way-ANOVA, Bonferroni).

A

		n	E_{max} (grams)
Normal	**Control**	26	1.01 ± 0.05
	+ rauwol.	8	1.06 ± 0.09
+ L-NAME	**Control**	21	1.37 ± 0.07
	+ rauwol.	7	1.45 ± 0.09

B

		pEC50	Hill slope
Normal	**Control**	6.67 ± 0.07	0.59 (*0.44-0.74*)
	+ rauwol.	6.71 ± 0.08	0.60 (*0.43-0.77*)
+ L-NAME	**Control**	7.11 ± 0.11	0.54 (*0.41-0.67*)
	+ rauwol.	6.98 ± 0.19	0.58 (*0.47-0.69*)

Table 4-13. The effect of 30nM rauwolscine on the PE-induced contractile responses of WT both in the control and 100μM L-NAME treated rings.

The '*n*' number, maximum responses generated, expressed as grams (**A**) and agonist sensitivity, expressed as a pEC_{50} value (**B**) are shown as the mean ± S.E.M. Hill slopes (**B**) are shown as the mean with the 95% confidence intervals shown in parenthesis (No statistical significance against either normal control or L-NAME treated control: Student's t-test).

Discussion- Chapter 4

NO production and L-NAME

Palmer *et al.* (1988) discovered that NO is liberated during the catalytic conversion of L-arginine into L-citrulline by the nitrogen oxide synthase (NOS) family of enzymes. Three subsets of NOS have been described (reviewed by Ricciardolo *et al.*, 2004): endothelial NOS (eNOS or NOS III), inducible NOS (iNOS or NOS II), and neuronal NOS (nNOS or NOS I).

iNOS is generally involved in inflammatory responses and is produced *de novo* as a result of pre-transcriptional regulation. nNOS and eNOS are constitutive forms of NOS (cNOS) who are dependent on the level of intracellular calcium ($[Ca^{2+}]_i$). Thus, cNOS activity is controlled by Ca^{2+} /calmodulin (Busse & Mulch, 1990) and as a result, elevation in $[Ca2+]_i$ leads to an increased eNOS activity and NO liberation.

As such, pharmacological agents to block NO production are commercially available. L-NAME is such an agent. It is a competitive eNOS blocker (Rees *et al.*, 1990) whose effects can be overcome by increasing L-arginine availability, but can be used in vascular preparations to inhibit NO production.

ACh-induced relaxations

The discovery of the role of endothelium-derived relaxing factor (EDRF) was originally due to apparent inconsistencies of the vascular effects of ACh in rabbit aorta (Furchgott & Zawadzki, 1980). They explained these inconsistencies by reporting the vasorelaxant properties of ACh were dependent on the presence of an intact endothelium. Palmer *et al.* (1987) identified EDRF as the simple molecule nitric oxide (NO).

Thus, it has become the norm in vascular preparations to test for endothelial function by testing for ACh-induced vasodilatation. The results show ACh relaxatory responses were significantly smaller in those vascular rings that had been treated with L-NAME. Thus, L-NAME has inhibited the production and therefore the effects of NO in the mouse aorta.

However L-NAME only inhibits NO production. Endothelial cells (ECs) are able to release a host of other vasoactive agents such as the relaxants, prostaglandin and EDHF (endothelium-derived hyperpolarising factor), or vasoconstrictors, such as endothelin (Rang *et al.* 1999). The effect of these agents has not been taken into account as this was out-with the scope of the thesis.

To compliment the use of L-NAME, attempts were made to denude the mouse aorta rings of their endothelium. Initial attempts were often unsuccessful, resulting in variable responses. When the endothelium was successfully removed, no ACh-induced relaxation was observed but the maximum contractile responses to KCl, PE and 5-HT were significantly reduced (data not shown), suggesting that, as well as endothelial removal, damage to the vascular smooth muscle (medial) layer was being done. No further attempts to denude the endothelium were made.

An interesting note was that although the α_{1B}-KO and WT aortae were not significantly different the α_{1D}-KO had a significantly larger relaxation. However this difference could be attributed to experimental design. The relaxatory response to 3μM ACh was tested on tone raised by addition of 10μM PE in all strains. Since the α_{1D}-KO was significantly less sensitive than the WT to PE the tone was significantly less, and the effect of 3μM ACh was notably greater.

KCl responses

In the mouse aorta, KCl responses were significantly increased in the presence of L-NAME, confirming that in control vessels, KCl exposure was either resulting in NO liberation or there was constitutive release of NO. Either way, the observed contractile response of the control rings was the summation of simultaneous contractile and relaxatory responses. The contractile response was due to VSMC depolarisation and the relaxant response due to NO liberation.

The effect of L-NAME on the KCl responses was probably due to KCl activity at ECs. 125mM KCl PSS is a depolarising solution, hence, when used in a vessel with intact endothelium, ECs are depolarised along with VSMCs. The depolarisation of ECs results in an increased $[Ca^{2+}]_i$ and hence, an increase in the production and release of NO.

Therefore, the KCl response of control vessels was not a true representation of the maximal contractile response of the vessel as the response was blunted by NO. Instead, the KCl-induced contraction in the presence of L-NAME is more representative of the absolute maximum contractile response of the mouse aorta, although the release of other substance from ECs has not been taken into account.

Furthermore, the use of KCl results in the depolarisation of cells within the adventitia, which is now becoming increasingly implicated in vascular function (reviewed by Faber *et al.*, 2001) and has not been considered.

In general, KCl contractile responses were significantly increased by L-NAME due to the loss of NO liberation from ECs.

Serotonergic responses and L-NAME

5-HT is a potent vasoconstrictor in the mouse thoracic aorta (Russell &Watts, 2000) mediating its contractile response through 5-HT_{2A} receptors (McKune & Watts, 2001). L-NAME treatment in the mouse aorta did not affect the maximum responses to 5-HT but resulted in an increased sensitivity in WT, α_{1B}-KO and α_{1D}-KO aortae.

Cocks & Angus (1983) demonstrated, in dog coronary arteries, that both NE and 5-HT were more potent and efficacious vasoconstrictors in the absence of endothelium. Maclean *et al.* (1994) reported that 5-HT responses of bovine pulmonary arteries were enhanced by endothelium removal and by L-NAME treatment confirming 5-HT also has NO-mediated endothelium-dependent vasorelaxant properties. Generally, the vasorelaxant response of 5-HT are associated with the activation of 5-$HT_{1\text{-like}}$ receptor subtypes (reviewed by Saxena & Villalon, 1991).

In the mouse aorta no evidence is yet available of the involvement of 5-$HT_{1\text{-like}}$ receptors in vasoconstriction or dilation. However, the data indicates that there is NO-dependent attenuation of the serotonergic response. However, this cannot confidently be attributed to the activation of 5-HT receptor on the endothelium.

The presence and role of myoendothelial gap junctions is a novel and growing area of study (reviewed by Dhein, 2004a; Dhein & Jongsma, 2004; Griffith *et al.*, 2004; Griffith, 2004). Gap junctions allow the transmission of both chemical and electrical

signals between cells. Thus, alterations in the membrane potential or $[Ca^{2+}]_i$ of VSMCs can affect ECs and vice versa. As a result, the effect of 5-HT on VSMCs may have been transmitted through these gap junctions on VSMCs.

Furthermore, cNOS is a constitutive enzyme that is $[Ca^{2+}]_i$-dependent, therefore, there may have been constitutive basal release of NO, which would attenuate the 5-HT response. If NO was constitutively released, the attenuation would have been constant i.e. concentration independent. Careful analysis of the curve expressing the 5-HT responses in grams (Figures 4-3[A], 4-4[A] and 4-5[A] for WT, α_{1B}-KO and α_{1D}-KO respectively), reveals that the effect of L-NAME seems to have been constant over the entire curve except towards the maximum. At this point the curves tended to converge and this may have been because the maximal 5-HT response is the tissue maximal response and L-NAME treatment could not increase it further. Therefore it appears there may have been constitutive basal release of NO, attenuating the control 5-HT responses.

However, the sensitivity increase of the serotonergic response was present in all three strains of mice studied (WT, α_{1B}-KO and α_{1D}-KO). Thus, the effect of L-NAME, on the serotonergic response was α_1-AR subtype independent. Furthermore the comparison of WT, α_{1B}-KO and α_{1D}-KO 5-HT responses both in the absence (Figure 4.6; Table 4.6) and the presence (Figure 4.7; Table 4.7) of L-NAME shows that the serotonergic responses are not significantly different between mouse strains.

In the previous chapter (Chapter 3) the involvement of α_{1D}-ARs in the 5-HT-induced response was discussed. The effect of 5-HT at α_{1D}-ARs was amplified by a synergistic interaction with 5-HT_{2A} receptors on VSMCs. Further on in this discussion, the involvement of α_{1D}-ARs in vasorelaxant responses is examined. However, the effect of 5-HT at α_{1D}-ARs resulting in vasodilatation can by discounted as no evidence for 5-HT_{2A} receptors causing vasodilatation is available and, thus, it is unlikely there was a synergistic interaction (discussed in Chapter 3) causing NO dependent vasodilatation.

α_1-ARs and L-NAME

L-NAME treatment resulted in an increased maximal response for PE induced contractions in all three strains studied. However, it resulted in increased PE sensitivity in only WT and α_{1B}-KO aortae. In α_{1D}-KO aortae, the response in the presence of L-

NAME was not significantly altered. The difference is therefore due to the lack of functional α_{1D}-ARs in the α_{1D}-KO aorta.

In all strains, the effect of L-NAME on the response appeared to be PE-concentration dependent (Figures 4-8[A], 4-9[A] & 4-10[A] for WT, α_{1B}-KO and α_{1D}-KO respectively), i.e. the higher the PE concentration, the greater the increase in response in the presence of L-NAME, indicating a vasorelaxant response of PE may be involved. This evidence combined with the lack of sensitivity increase in the α_{1D}-KO appeared to point to a role for α_{1D}-ARs in vasodilatation.

α_{1D}-ARs and the endothelium

The role of α_1-ARs in endothelium-dependent relaxant responses has been shown in rabbit bronchial arteries (Zschauer *et al.*, 1997) and rat muscular arterioles (Tuttle & Falcone, 2001). Recently, Fillipi *et al.* (2001) demonstrated that the α_{1D}-ARs are involved in endothelium-dependent vasodilatation in the rat mesenteric vascular bed. They also demonstrated α_{1D}-ARs, on bovine coronary venular postcapillary endothelial cells, could raise IP_1 levels (an IP_3 metabolite) and, hence, NOS activity.

Thus, the presence of α_{1D}-ARs on the endothelium in the mouse aorta could explain why the α_{1D}-KO did not exhibit an increased sensitivity to PE in the presence of L-NAME. The findings in the mouse aorta, demonstrate that there is a fundamental difference in the effect of L-NAME in α_{1D}-KO mice compared with WT (and α_{1B}-KO), and this was due to the lack of functional α_{1D}-AR with the ability to cause NO-dependent vasodilatation as well as vasoconstriction.

PE responses of WT, α_{1B}-KO and α_{1D}-KO compared: The effect of L-NAME

Comparison of the PE responses of α_{1B}-KO and α_{1D}-KO aortae with the WT aortae (Figure 4.11; Table 4.11) reveals that the α_{1B}-KO and WT were not significantly different, but PE was less sensitive and had a lower maximum response in the α_{1D}-KO.

However, in the presence of L-NAME (Figure 4.12; Table 4.12) the differences between the strains appear to be accentuated. The α_{1B}-KO has a significantly larger maximum response although no significant difference in sensitivity. The α_{1D}-KO has a

significantly lower maximum response similar to the difference in WT and α_{1D}-KO in the absence of L-NAME. However, the sensitivity was 66-fold less sensitive in the presence of L-NAME compared with only 14-fold less sensitive in the absence of L-NAME.

The difference between WT and α_{1B}-KO responses uncovered by L-NAME could be due to compensatory mechanisms. McBride *et al.* (2003) reported a BMY 7378-sensitive (α_{1D}-AR selective antagonist: Saussy *et al.*, 1994), 5-MU-resistant (α_{1A}-AR selective antagonist: Gross *et al.*, 1988; Schwinn *et al.*, 1995) contractile component in α_{1B}-KO mesenteric arteries, that was not observed in the WT. The authors concluded that there was an apparent up-regulation of α_{1D}-ARs as a result of the loss of α_{1B}-ARs. Furthermore, in some vessels from the α_{1B}-KO, the response to PE was significantly larger than the WT (Daly *et al.*, 2002).

Thus, a systemic up-regulation of α_{1D}-ARs not only in VSMCs but also in ECs could explain the uncovered difference by L-NAME. It may be that in the α_{1B}-KO, the contraction to PE is actually larger than in the WT but this contraction is attenuated by a greater degree, due to an increased role for α_{1D}-AR mediated NO release from endothelial cells.

Furthermore, the difference between WT and α_{1D}-KO is enhanced in the presence of L-NAME, not due to an alteration of the PE response in α_{1D}-KO, but due to the increased sensitivity of WT aortae to PE. Thus the α_{1D}-KO is involved with NO-dependent vasodilatation in the mouse aorta. This is consistent with the findings reported by Fillipi *et al.* (2001), but further studies involving removal of the endothelium of the mouse aorta are required to confirm that the α_{1D}-AR relaxant effect is endothelium-dependent.

Involvement of α_2-ARs in the PE response

Notably, in the α_{1D}-KO, L-NAME treatment, although not affecting sensitivity, resulted in an increased maximum response to PE. α_2-ARs involvement with relaxatory response was originally described by Cocks & Angus (1983) in porcine and dog coronary arteries. Vanhoutte & Miller (1989) performed a study in selected arteries from dog rat and pig and associated endothelium dependent relaxations with NO release. Thus, the

increased maxima of PE responses in α_{1D}-KO aorta as a result of L-NAME treatment may have been due to the involvement of endothelial α_2-ARs. Therefore, the sensitivity of the PE responses to rauwolscine (α_2-antagonist: Perry & U'Prichard, 1981) was tested in the WT to determine the involvement of α_2-ARs in the PE response.

No significant effect of rauwolscine in the mouse aortae was observed either in the absence or presence of L-NAME. The experiments were performed in both the presence and absence of L-NAME in order to determine the involvement of α_2-ARs at both endothelial sites and VSMCs. It was hypothesised that α_2-ARs may be involved in both vasoconstriction and vasodilatation, thus if no effect of rauwolscine was observed in control tissue, the effect of rauwolscine in L-NAME treated tissues would be to decrease sensitivity or efficacy of PE.

We did not observe any effect of rauwolscine, therefore there was no role for α_2-ARs in mediating the PE response. However, these experiments were carried out in the WT thus the involvement of α_{1D}-ARs made the analysis more difficult as α_{1D}-AR-mediated NO-dependent vasodilatation had to be taken into account.

Furthermore, the CCRC to PE in the WT was terminated at 30μM, whereas, in the α_{1D}-KO the effect of L-NAME was only observed at PE concentrations of 30μM, 100μM and 300μM. Therefore the effect of L-NAME observed in the α_{1D}-KO may have been due to α_2-AR activation but been missed in the study conducted in the WT, by prematurely ending the construction of the PE CCRC.

Thus the involvement of α_2-AR activation resulting in NO release cannot be completely ruled out in the α_{1D}-KO. Another explanation may be the constitutive release of NO from the endothelial cells since eNOS is a constitutive enzyme. Furthermore, bearing in mind, eNOS is Ca^{2+}-dependent for its activity, the role of ionic movement or electronic transmission via myoendothelial gap junctions cannot be ruled out.

Conclusions- Chapter 4

Conclusions

L-NAME treatment revealed that KCl, 5-HT and PE responses in the mouse aorta are attenuated by NO release. 5-HT and PE responses were significantly more sensitive in the presence of L-NAME with the exception of the PE response of α_{1D}-KO aortae which was not significantly more sensitive.

Thus the α_{1D}-AR is involved in both vasoconstriction and NO-dependent vasodilatation in the WT mouse aorta.

However, in α_{1D}-KO there remained an NO-dependent attenuation of the PE response that may have been one of the other α_1-ARs, α_2-ARs, basal NO release, or the result of ionic or electrical movement via gap junctions.

General Conclusions

General Conclusions

Conclusions

The evidence presented within this thesis has demonstrated the application of a pharmacological approach aided with the use of functional KOs of specific receptors.

In Chapter 1 the results showed that no significant age-related alterations in vasoactive responses occurred in the mouse aorta. It has also now been shown that the α_1-AR knockout mice used in the study maintained their contractile mechanisms, the phenotypic differences were receptor specific and maintained with age.

Furthermore the results demonstrated that the lack of functional α_{1B}-ARs in the mouse aorta, did not adversely affect adrenergic responses. Contrastingly, the $_{1D}$-KO had significantly reduced sensitivity to PE, at both age points studied confirming the α_{1D}-AR is the major adrenergic vasoconstrictor in the mouse aorta.

Chapter two provided further evidence confirming the α_{1D}-AR is the major adrenergic vasoconstrictor in WT aortae. In fact, α_{1D}-AR appears to be the sole α_1-AR involved in functional contractile responses. In the absence of α_{1D}-ARs the response was due to α_{1B}-ARs and in the absence of both α_{1B} and α_{1D}-ARs no adrenergic response was observed, suggesting the α_{1B}-AR has a minor vasoconstrictor role. However in the WT no functional contractile role for α_{1B}-AR could be observed thus the α_{1D}-AR is the sole contractile AR in the mouse aorta although it appears the α_{1B}-AR is held in reserve.

The involvement of the α_{1D}-AR in synergy with the serotonergic response of the mouse aorta was then reported in Chapter 3. The co-activation of the α_{1D}-AR along with the serotonergic vasoconstrictor, the 5-HT_{2A} receptor resulted in a potentiated response which was due to mutual effect amplification.

A minor role for α_{1D}-ARs in the serotonergic response was also reported in Chapter 3. The activity of 5-HT at both 5-HT_{2A} and α_{1D}-ARs, two receptors which potentiate one another's responses, somewhat explained the relatively high potency of 5-HT in the mouse aorta.

However, the lack of functional α_{1D}-ARs in the α_{1D}-KO did not significantly affect the serotonergic response, suggesting compensation by serotonergic receptors, i.e. 5-HT_{2A} receptors.

Finally in Chapter 4 the role of α_{1D}-AR activation in NO release was reported as the effect of L-NAME on α_{1D}-KO aortae was significantly different from the effect of L-NAME in α_{1D}-KO aortae. The results confirmed that the α_{1D}-AR is involved in the release of NO and hence vasodilatation of the mouse aorta.

Overall conclusion

The combined results indicate that the α_{1D}-AR is the sole adrenergic vasoconstrictor in the mouse aorta. Not only is the α_{1D}-AR responsible for mediating adrenergic responses it can also be activated by 5-HT and can synergistically interact with the serotonergic vasoconstrictor system. Furthermore, the α_{1D}-AR is not just a vasoconstrictor as it has a role in NO dependent vasodilatation. Thus the α_{1D}-AR has a complex and varied role in modulating the tone of WT mouse aorta.

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