A COMBINED PHARMACOLOGICAL/KNOCKOUT APPROACH TO SUBTYPING α_1-ADRENOCEPTORS IN MURINE TISSUES

by

Clare Deighan

A thesis presented for the degree of Doctor of Philosophy in Faculty of Medicine, University of Glasgow.

September 2001

Lab 440
West Medical Building
University of Glasgow
Glasgow
G12-8QQ

SUMMARY

The characterisation of adrenoceptor subtypes has traditionally been achieved using pharmacological methods. However, in recent years molecular biology and biochemical methods have become increasingly popular. Transgenic technology has provided a merging of pharmacology and molecular biology, whereby molecular biology is used to identify and manipulate the gene of interest whilst pharmacology is used to investigate the functional consequences of these genetic manipulations.

The process of subtyping α_1-adrenoceptor subtypes is often made more complicated by a lack of reliable subtype selective drugs, in particular for the α_{1B}-adrenoceptor, for which there are no such compounds. The α_{1B}-adrenoceptor KO mouse created by Cavalli *et al* (1997) provides a unique environment in which to study α_1-adrenoceptor subtypes. The study of these KO mice may provide a valuable insight into the functional role of individual α_1-adrenoceptor subtypes. For this reason my project investigated the functional consequences of deleting the α_{1B}-adrenoceptor using traditional pharmacological methods including radioligand binding and wire myography and the more recently developed method of confocal microscopy.

In Chapter 2, I describe the effects of deletion of the α_{1B}-adrenoceptor from the brain and livers of WT and KO mice. Using five subtype selective ligands (prazosin, RS100329, (R)-A-61603, L765,314 and BMY7378), radioligand binding was used to characterise the α_1-adrenoceptor subtypes in WT and KO brain and liver tissue. The WT brain was found to contain a heterogeneous population of α_1-adrenoceptors, consisting of α_{1A}- and α_{1B}-adrenoceptors, whilst the KO brain had a smaller receptor

number compared to the WT and a pure population of α_{1A}-adrenoceptors. In the WT liver (4 month old) a pure population of α_{1B}-adrenoceptors was determined to be present, consistent with previous reports (Cavalli *et al*, 1997; Yang *et al*, 1998). The KO liver (4 month old) was unexpectedly found to contain a population of α_1-adrenoceptors: characterised to be α_{1A}-adrenoceptors. Preliminary results from studies of 3 month old KO livers revealed that there are negligible amounts of α_1-adrenoceptors present at this age, whereas in 3 month old WT liver α_1-adrenoceptor density is higher compared with 4 month old livers. This study demonstrates the loss (without apparent replacement) of the α_{1B}-adrenoceptor from the KO brain and the substitution of the α_{1A}-adrenoceptor to compensate for the loss of the α_{1B}-adrenoceptor in the KO liver. This upregulation of the α_{1A}-adrenoceptor appears to occur between 3 and 4 months of age.

Chapter 3 describes a general pharmacological characterisation of the mouse carotid artery and a detailed investigation into the characterisation of the α_1-adrenoceptor subtype mediating contraction in WT and KO carotid arteries. The general pharmacology of the carotid artery was studied using a number of vasoactive compounds. Surprisingly, very few were capable of producing a response in this tissue. The agonists which contracted the tissue were NA, PE, (R)-A-61603 and 5-HT with a rank order of potency of:-

NA > (R)-A-61603 = 5-HT > PE in the WT carotid artery and

NA > PE = 5-HT = (R)-A-61603 in the KO carotid artery.

The pEC_{50} for phenylephrine was significantly higher in the KO compared with the WT carotid artery. Noradrenaline produced biphasic curves in WT and KO carotid arteries

The rank order of potency for those agents which relaxed the tissue were:-

ACh > ISO > UK14304

The relaxant properties were only studied in WT arteries.

Prazosin, 5-methylurapidil, L765,314 and BMY7378 were used to characterise the α_1-adrenoceptor subtype mediating contraction to phenylephrine in WT and KO carotid arteries. From these results the WT and KO carotid artery appear to mediate contraction predominantly through the α_{1D}-adrenoceptor although there is evidence that a component of the contraction in the WT carotid artery may be mediated by the α_{1B}-adrenoceptor.

In a separate series of experiments, concentration response curves to phenylephrine in carotid and mesenteric arteries from mice that either overexpressed the α_{1B}-adrenoceptor or expressed a constitutively active α_{1B}-adrenoceptor, displayed no significant differences in sensitivity compared with control mice, providing no evidence for an enhanced α_{1B}-adrenoceptor component to these transgenic models.

Adventitial fibroblasts have recently been shown to express α_1-adrenoceptors (Faber *et al*, 2001) and the adventitia has been implicated in a number of pathological conditions e.g. atherosclerosis, restenosis. Chapter 4 describes a study investigating the effects which deletion of the α_{1B}-adrenoceptor has on the structure of the adventitia of WT and KO carotid arteries. The number and density of adventitial cells in the KO carotid artery was found to be significantly smaller than in the WT carotid artery. The implications of this are not yet certain, but the α_{1B}-adrenoceptor has been implicated in growth (Chen *et*

al, 1995; Zhang and Faber, 2001) which may account for the observed differences in the KO carotid artery compared with the WT.

The results presented in this thesis have gone some way to furthering our knowledge of the α_{1B}-adrenoceptor and demonstrate that the effects of deleting the α_{1B}-adrenoceptor depend not only on the tissue in which it is expressed, but also on the cell type. The merging of transgenic technologies with pharmacological techniques (pharmacogenomics) provides the pharmacologist with information which would not be possible with drugs alone and looks set to be a way forward in clarifying the confusing pharmacology arising from the use of inadequate drugs.

CONTENTS

LIST OF FIGURES

Chapter 1 – General Introduction

Chapter 2 – Characterisation of the α_1-adrenoceptor subtypes in brain and liver tissue from WT and KO mice

Chapter 3 – Functional characterisation of the murine carotid artery and classification of the α_1-adrenoceptor subtype mediating contraction to phenylephrine in WT and KO murine carotid arteries.

Chapter 4 – Investigation into the structural differences in WT and KO murine carotid arteries using confocal laser scanning microscopy.

ACKNOWLEDGEMENTS

Sincere thanks to Professor Ian M^{c}Grath for his supervision throughout my PhD. His constant help and guidance (which were much needed) has taught me to think more like a scientist and his enthusiasm for his research has now infected me. I can only hope that one day I will know as much about α_1-adrenoceptors as he does!

Thank you to Susanna Cotecchia of the University of Lausanne, Switzerland, without her gift of the α_{1B}-KO mice none of this would have been possible.

I owe a special debt of thanks to Janet Mackenzie, Craig Daly, Simon M^{c}Grory and Joyce MacMillan who always answered my persistent questions to the best of their ability. I have learnt a lot from them.

To all in lab 440 (past and present) who made the bad times bearable and the good times so much more enjoyable, with special thanks to Ann, Angela, Anne, Craig, Darren, Jillian, Melissa and Simon who have all become my good friends.

A huge big thank you to Alison, who over the past three years has become one of my closest friends. We've been through so much together and my life would be a duller place without her.

To all my friends and family, I would like to say thank you for always being there for me, it means a lot. I would like to express my profound gratitude and admiration for my parents whose continual love and support throughout these past three years and indeed

throughout my life has made this possible and shaped me into the person I am today. Thank you for putting up with me and for always believing in me. And Dad, thanks for the help with my maths, after 4 years I found something you could help me with!!! To my brother, Edward and my sister, Monica who are not just my siblings but my friends as well, thanks for putting up with my many moods and always knowing how to make me smile! And special thanks to Carol, who helped keep me sane and along with Alison, is one of my most treasured friends...you're a star!!!

Finally, a special word of thanks to my fiancé James, whose love and support have never wavered for the 8 years we have been together. Thank you for always believing I was capable of this and for putting up with me over the last 3 years, especially these last few months. I love you very much and I hope I've made you proud.

This thesis is dedicated to my grandparents, John and Logie Stevenson, who unwittingly inspired me to follow a career in science and supported me fully throughout my education. I only wish you could be here today.

DECLARATION

I declare that the work presented in this thesis was carried out by myself, with the exception of the radioligand binding which used the compound L765,314. This was carried out by David Slattery, an Honours student who worked in our lab in 1998. The work was carried out at the Autonomic Physiology Unit, Division of Neuroscience and Biomedical Systems, West Medical Building, University of Glasgow, Glasgow, G12-8QQ.

Part of the work presented in this thesis has been published as follows:

Zuscik, M. J., Chalothorn, D., Hellard, D., Deighan, C., M^{c}Gee, A., Daly, C. J., Waugh, D. J. J., Ross, S. A., Gaivin, R. J., Morehead, A. J., Thomas, J. D., Plow, E. F., M^{c}Grath, J. C., Piascik, M. T. and Perez, D. M. (2001) Hypotension, autonomic failure and cardiac hypertrophy in transgenic mice overexpressing the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **276(17)**, 13738-13743.

M^{c}Grath, J. C., Arribas, S. M., Deighan, C., M^{c}Grory, S. P., Mackenzie, J. F., Macmillan, J. B., M^{c}Gee, A., Pediani, J., Woollhead, A. and Daly, C. (2001) Remodelling of resistance artery adventitia is ubiquitous to hypertensive and heart failure models and is under adrenergic influence. *8th International Symposium on Mechanisms of Vasodilation.*

Deighan, C. and M^{c}Grath, J. C. (2000) The characterisation of α_1-adrenoceptors in murine carotid artery using α_{1B}-adrenoceptor knockout mice. *Basic Res. Cardiol.* **95(6)**, 33 (Abstract).

Deighan, C., Slattery, D. A., Mackenzie J. F., Cotecchia, S. and M^{c}Grath, J. C. (1999) The characterisation of α_1-adrenoceptors in murine liver using radioligand binding and transgenic mice. *Brit. J. Pharmacol.* **128**, 91P.

ABBREVIATIONS

A-61603	(N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide
ACh	acetylcholine
ATP	adenosine tris phosphate
B_{max}	maximal specific binding, expressed in fmol of radioligand/mg protein
BMY7378	(dihydrochloride 8-[2-[4-(2-methoxyphenyl)-1-piperozynl]ethyl]-8-azaspiro(4,5}decone-7,9-dione
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium
cAMP	adenosine 3'-5'cyclic monophosphate
cDNA	single stranded DNA
CEC	chloroethylclonidine
CI	confidence intervals
CLSM	confocal laser scanning module
cpm	counts per minute
CRC	concentration response curve
DAG	diacylglycerol
DR	dose ratio
EC_{50}/pEC_{50}	The molar concentration of an agonist that produces 50% of the maximum response of that agonist/ negative logarithm to base 10 of EC_{50}
eNOS	endothelial NOS
g	gravity
GDP	guanosine diphosphate
G protein	GTP-dependent regulatory proteins
GPCR	G protein coupled receptor

GRK	G protein receptor coupled kinase
GTP	guanosine trisphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
5-HT	5-hydroxytryptamine
iNOS	inducible NOS
IP_3	inositol-1,4,5-trisphosphate
ISO	isoprenaline
kb	kilobase
K_B/pK_B	equilibrium dissociation constant of an antagonist/ negative logarithm to base 10 of K_B
K_D	concentration of radioligand which occupies 50% of receptors at equilibrium
kDa	kilo Daltons
K_i/pK_i	concentration of competitor that will bind to 50% of receptors in the absence of radioligand or any other competitor at equilibrium/ negative logarithm to base 10 of K_i
KO	knockout/α_{1B} knockout
l, ml, μl	litre, millilitre, microlitre
L765,314	(4-amino-2-[4-[1-(benzyloxycarbonyl)-2-(S)-[[(1,1 dimethylethyl)amino]carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline
L-NAME	N^w-nitro-L-arginine methyl ester hydrochloride
M (mM, μM, nM)	Molar (moles per litre; millimolar, micromolar, nanomolar)
MAPK	mitogen activated protein kinase
mRNA	messenger RNA
NA	noradrenaline
NOS	nitric oxide synthase

pA_2	negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original response
PE	phenylephrine
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
RT-PCR	reverse transcriptase polymerase chain reaction
s.e.m.	standard error of the mean
TMD	transmembrane domain
μm	micrometre
WT	wildtype

CHAPTER 1 – GENERAL INTRODUCTION

1.1. INTRODUCTION

Adrenoceptors mediate the central and peripheral actions of the primary sympathetic neurotransmitter, noradrenaline and the primary adrenal medullary hormone (and central neurotransmitter), adrenaline. Adrenoceptors are present in both the periphery and the central nervous system where they are found on many neuronal populations as well as in tissues (Bylund *et al*, 1994). α_1-Adrenoceptors, which will be the focus of this thesis, participate in many essential physiological processes such as sympathetic neurotransmission, modulation of hepatic metabolism, control of vascular tone, cardiac contraction, and the regulation of smooth muscle activity in the genitourinary tract (Garcia-Sainz *et al*, 1999).

1.2. HISTORICAL PERSPECTIVE

1.2.1. Classification of adrenoceptors

The classification of adrenoceptors has been an ongoing process for over a century. It began in its simplest terms in 1896 when Oliver and Schafer demonstrated that injection of extracts of the adrenal gland caused a rise in arterial pressure. It wasn't until 1913 that Dale showed that not only could adrenaline (which had been isolated and identified as the active component by this time) produce a rise in arterial blood pressure through vasoconstriction, but that it could also produce vasodilation. The vasoconstrictor effect predominates and this was the effect observed by Oliver and Schafer. Dale avoided interpreting this result in terms of a distinction between categories of receptor (Rang and Dale, 1995) and 35 years passed before Ahlquist (1948) proposed the existence of two adrenoceptor subtypes based on different rank orders of potency within a series of

structurally related natural and synthetic agonists. He called them α- and β-adrenoceptors and their relative potencies were found to be as follows:-

α:- Noradrenaline > Adrenaline > Isoprenaline

β:- Isoprenaline > Adrenaline > Noradrenaline

This quantitative approach used by Ahlquist was in contrast to the qualitative analysis his peers were using which tended to place too much emphasis on the nature of the response rather than the potency of the drugs producing the response. As a result of this there were attempts to classify the effects of sympathomimetic amines as 'excitatory' or 'inhibitory' and to assign these responses separate second messengers (sympathin E and sympathin I), which were activated by one adrenoceptor (Cannon and Rosenbleuth, 1933). This distinction proved to be useless in the identification of adrenoceptor subtypes. Ahlquist's nomenclature was universally accepted and was supported by the discovery of highly selective and potent antagonists for the different subtypes (Ruffolo *et al*, 1991). But the use of these antagonists suggested the existence of further subdivisions of both α- and β-adrenoceptors. The subclassification of β-adrenoceptors is beyond the scope of this thesis and from this point this thesis is concerned only with α-adrenoceptors, in particular α_1-adrenoceptors.

The subclassification of α-adrenoceptors was proposed by Langer in 1974 but the starting point for this chain of events was a paper by Brown and Gillespie (1957) which reported that the α-adrenoceptor antagonists, dibenamine and phenoxybenzamine, increased the release of noradrenaline by sympathetic nerve stimulation from the cat spleen. Initially this phenomenon was not explained in the context of subtypes of α-adrenoceptors. Brown and Gillespie explained it as being a result of blockade of

postjunctional α-adrenoceptors, preventing the released noradrenaline from acting on the α-adrenoceptors on the effector cells, leading to an increase in noradrenaline overflow. Thoenen *et al* (1964), who also showed an increased overflow of noradrenaline when α-antagonists were used, believed it resulted from inhibition of neuronal uptake (Ruffolo *et al* 1991). It was only when Starke *et al* (1971) demonstrated that the effect was independent of uptake blockade and that α-agonists were capable of reducing the stimulation-evoked release of noradrenaline from nerves (Docherty, 1989) that it seemed more likely that this effect was being mediated by prejunctional α-adrenoceptors. As the evidence for prejunctional α-adrenoceptors grew, Langer (1974) proposed the first subdivision of α-adrenoceptors based on their anatomical location. The postjunctional α-adrenoceptor, which mediates the effector organ's response, was to be termed α_1 and the prejunctional α-adrenoceptor, which regulates neurotransmitter release, was to be termed α_2. Studies of the interactions of agonists and antagonists with these α-adrenoceptors extended this subclassification scheme to a functional subdivision (Berthelsen and Pettinger, 1977) which was finally replaced by a pharmacological subclassification after the identification of potent and highly selective α_1- and α_2-adrenoceptor agonists and antagonists (Bylund *et al*, 1994). Therefore an α-adrenoceptor which was activated by methoxamine, cirazoline or phenylephrine and blocked by prazosin, WB4101 or corynanthine was an α_1-adrenoceptor. Whereas an α-adrenoceptor which was activated by α-methylnoradrenaline, UK-14,304, B-HT 920 or B-HT 933 and blocked by yohimbine, rauwolscine or idazoxan was an α_2-adrenoceptor.

1.2.2. α_1-Adrenoceptor heterogeneity

Initially, Langer's subdivision of α-adrenoceptors into α_1 and α_2 appeared to hold true. However it soon became apparent that the situation was not as simple as first presumed, as an increasing amount of data was published which didn't appear to fit convincingly into either subdivision. For example, M^{c}Grath (1982) showed that the contractile response of the *in situ* anococcygeus was shown to have a prazosin-resistant response, but no part of the response could be ascribed to α_2-adrenoceptors. pA_2 Values were obtained using the agonists, amidephrine and xylazine (α_1- and α_2- agonists respectively) and the antagonists, corynanthine and rauwolscine (α_1- and α_2- antagonists respectively). The pA_2 values were consistent with the relative potency of the antagonists against the α_1-agonists and revealed no evidence for a postjunctional α_2-adrenoceptor (M^{c}Grath, 1982). Where did this response fit into the α_1/α_2 subclassification?

It was also apparent that results from radioligand binding studies and physiological studies didn't add up. For example, binding studies by Wood *et al* (1979) showed that methoxamine and phenylephrine, which in functional studies were more potent in contracting postsynaptic smooth muscle than in inhibiting presynaptic noradrenaline release did not seem to follow the same trend in radioligand binding studies. They also found that uterus tissue which had been previously classified as containing mainly α_1-adrenoceptors was found to have high affinity for the α_2-selective agonist, α-methylnoradrenaline. Wood *et al* (1979) recognised that heterogeneity existed within each α-adrenceptor subclass and that the α_1/α_2 designation described by Langer served only as a starting point from which further subclassification was necessary.

M°Grath (1982) was the first to propose a subdivision of α_1-adrenoceptors when he classified them into α_{1a}- and α_{1b}-adrenoceptors based on data from the rabbit basilar artery and the rat anococcygeus. In both these tissues phenylethanolamines produced dose response curves with a 'shoulder' indicative of a response with two components. In contrast the 'non-phenylethanolamine' agonists produced virtually no response in the rabbit basilar artery and a monophasic curve in rat anococcygeus. M°Grath suggested that the 'low concentration component' of the response to phenylethanolamines and the response to 'non-phenylethanolamines' was mediated by one type of α_1-adrenoceptor which he designated α_{1a} while the response to high concentrations of phenylethanolamines was mediated by a second subtype which he designated α_{1b}. The rabbit basilar artery, it was suggested, has a 'low dose component' of the response to noradrenaline which corresponds more closely to the 'α_{1b}-' than the 'α_{1a}-'adrenoceptor in rat anococcygeus which would explain the lack of response of the tissue to 'non-phenylethanolamines'.

Attempts to discover any antagonists capable of distinguishing between α_{1a} and α_{1b}-adrenoceptors had limited success, warning against the premature suggestion of receptor subtypes. Subsequent evidence suggested that the results, instead of being a consequence of α_1-subtypes, may actually be produced by a single type of α_1-adrenoceptor which was able to mediate more than one response, possibly at the second messenger level (Wilson *et al*, 1991).

Despite general reservations about the concept of further subdivision of α_1-adrenoceptors, the amount of published data which could be not be classified into simply α_1 or α_2 was mounting. Holck *et al* (1983) demonstrated that clonidine and

methoxamine produced differential interactions at postjunctional α_1-adrenoceptors of the rabbit pulmonary artery suggesting that there were multiple subtypes. Medgett and Langer (1984) using the superfused tail artery found that two populations of α_1-adrenoceptors existed. One had high affinity for prazosin and the other had low affinity. Medgett and Langer interpreted their high and low affinities for prazosin as an indication that prazosin, which is regarded as being highly selective for α_1-adrenoceptors, was differentiating between two subtypes. Drew (1985) and Flavahan and Vanhoutte (1986) separately reviewed the literature on the affinity values of prazosin and demonstrated that prazosin had a range of affinities on smooth muscle that spread over two orders of magnitude, which they independently interpreted as an indication that further classification of α-adrenoceptors was required.

In 1986, Morrow and Creese provided evidence that α_1-adrenoceptor subtypes existed. The two subtypes, which were identified by binding studies in the rat brain were found to have very different rank orders of antagonist affinities. Half of the sites exhibited the pharmacological profile:-

WB4101 > prazosin > phentolamine > indoramin > dihydroergocryptine

and they called this site α_{1A}.

In contrast the second subtype demonstrated the rank order of:-

prazosin > indoramin > dihydroergocryptine > WB4101 > phentolamine

and this was called α_{1B}.

Although prazosin was able to label the two binding sites it couldn't distinguish between them, whereas [^{3}H]-WB4101 was only able to label one of these sites, the α_{1A} and was found to have a much lower affinity for the other binding site, the α_{1B} (Morrow and Creese, 1986).

After this initial subclassification many studies were conducted into the characterisation of these subtypes. Han *et al* (1987a) produced results in accordance with Morrow and Creese. They also found that prazosin had similar affinities for both subtypes but that WB4101 and benoxathian bound with 2 separate affinities. α_{1A}-Adrenoceptors had high affinities for the two drugs and α_{1B}-adrenoceptors were found to have low affinities. Han *et al* not only looked at the affinities of antagonists at α_1-adrenoceptors but also looked at their mechanism for increasing intracellular calcium in smooth muscle. Their results showed that the α_{1B} subtype regulates inositol phospholipid hydrolysis and gives rise to contractions which are independent of extracellular calcium while the α_{1A} subtype appears to control the opening of dihydropyridine-sensitive membrane channels to allow influx of extracellular calcium (Han *et al*, 1987a). Jagadeesh and Deth (1987) also found that WB4101 distinguished between subtypes of α_1-adrenoceptors in bovine aortic plasma membranes but additionally found that prazosin identified two sites, a contradiction to Morrow and Creese's results. Gross *et al* (1988) demonstrated that a derivative of urapidil, 5-methylurapidil, bound to two sites in the rat hippocampus and the vas deferens, displaying approximately 50-fold selectivity for the high affinity site over the low affinity site. In agreement with Morrow and Creese, prazosin was unable to distinguish the two sites.

Shortly after Morrow and Creese's initial identification of two α_1-adrenoceptor subtypes, Han *et al* (1987b), who were working with an alkylating analogue of clonidine, chloroethylclonidine (CEC), demonstrated that it was able to reduce the α_1-adrenoceptor population in rat liver and spleen by 70-80% whereas it had no significant effect in rat kidney, hippocampus, heart and vas deferens. A follow on paper from this (Minneman *et al*, 1988) which investigated the α_1-adrenoceptor subtypes distinguished

by WB4101 and CEC showed that the low affinity sites identified by WB4101 were the same sites which were sensitive to CEC inactivation, suggesting that these two drugs were distinguishing the same subpopulation of α_1-adrenoceptor. These and many other papers were in agreement with Morrow and Creese's initial findings, providing conclusive evidence that there were in fact two subtypes of α_1-adrenoceptor, the α_{1A} and α_{1B}.

The emerging pharmacological properties of the subtypes at this time were that :-

1. Prazosin was unable to distinguish between the two subtypes,
2. WB4101 and 5-methylurapidil bound with high affinity to the α_{1A}-subtype and with low affinity to the α_{1B}-subtype,
3. CEC was able to inactivate the α_{1B}-subtype, but that the α_{1A}-subtype was relatively insensitive to CEC inactivation.

About the same time as the α_{1A}/α_{1B} classification scheme was being developed, Flavahan and Vanhoutte (1986) reviewed the literature that was available at the time and came up with a different subclassification based on the wide range of affinities which both prazosin and yohimbine demonstrated in vascular smooth muscle. They subdivided α_1-adrenoceptors into α_{1H} and α_{1L}-adrenoceptors. α_{1H}-Adrenoceptors were defined as having a high affinity for prazosin (pA_2 values above 9.0) and yohimbine (pA_2 values above 6.4) whereas the α_{1L}-adrenoceptor has lower affinity for both antagonists (pA_2 values below 9.0 and 6.2 respectively). In 1990 Muramatsu *et al* added weight to this subclassification based on data using a range of antagonists in various blood vessels from different species. They found that the results fell into three groups. Group I tissues (canine saphenous vein, mesenteric artery and vein) were potently

inhibited by HV723 and WB4101 more so than prazosin, group II (canine carotid artery and rat thoracic aorta) were potently inhibited by prazosin more so than HV723 and WB4101 and group III (guinea-pig thoracic aorta, rabbit thoracic aorta, carotid artery and mesenteric artery) were inhibited by the three antagonists with the same potency. Groups II and III fitted in with Flavahan and Vanhoutte's subclassification with group II being assigned as α_{1H}-adrenoceptors and group III as α_{1L}-adrenoceptors. This left group I, which Muramatsu proposed to consist of tissues containing a novel subtype, the α_{1N}. This subtype has a relatively low affinity for prazosin, a higher than expected affinity for yohimbine and a high affinity for HV732.

An important aspect of this proposed subclassification is that it takes into account the wide range of affinity values for prazosin, first highlighted by Drew in 1985, whereas in Morrow and Creese's subclassification prazosin fails to distinguish between the subtypes. Muramatsu ascribed these subtypes to the α_{1H} group since they all have high affinity for prazosin.

1.3. α_1-ADRENOCEPTOR SUBTYPE-SELECTIVE COMPOUNDS

With the discovery of α_1-adrenoceptor subtypes it became clear that there was a limited number of compounds which exhibited selectivity for a given subtype and even today, 15 years on from their discovery, the choice of compounds which are convincingly selective is surprisingly sparse. This is generally regarded as the Achilles heel of those interested in pharmacologically defining α_1-adrenoceptor subtypes. Confusing and ambiguous results abound and much of the interpretation of this functional data relies on the individual experimenter's faith in their choice of compounds. What follows is a

brief overview of some of the compounds available and their selectivities for the α_1-adrenoceptor subtypes, with particular reference to the compounds used in this project.

WB4101 and phentolamine were the antagonists originally found to discriminate between α_{1A}- and α_{1B}-adrenoceptors in rat brain (Morrow and Creese, 1986). WB4101 has since been shown to have high affinity for the $\alpha_{1D/d}$-adrenoceptor subtype (Perez *et al*, 1991; Weinberg *et al*, 1994; Kenny *et al*, 1995 and Schwinn *et al*, 1995) and is used to distinguish $\alpha_{1A/a}$- and $\alpha_{1D/d}$-adrenoceptors from $\alpha_{1B/b}$-adrenoceptors; for which it has approximately 10-fold lower affinity (Perez *et al*, 1991; Weinberg *et al*, 1994 and Schwinn *et al*, 1995). Phentolamine has also been shown to have higher affinity at $\alpha_{1A/a}$- and $\alpha_{1D/d}$-adrenoceptors. However its selectivity is less than WB4101 and is somewhat variable (Muramatsu *et al*, 1990; Perez *et al*, 1991; Weinberg *et al*, 1994; Kenny *et al*, 1995 and Schwinn *et al*, 1995). As a result of this it is not used to select between subtypes but is often the drug of choice in radioligand binding, where it is used to define non-specific binding.

5-Methylurapidil, a derivative of urapidil, was first shown to discriminate between α_1-adrenoceptor subtypes in 1988 (Gross *et al*, 1988). It displayed high and low affinity binding in rat hippocampus, heart and vas deferens, whilst in rat liver and spleen it displayed monophasic binding of low affinity. The high affinity site was the α_{1A}-adrenoceptor, for which 5-methylurapidil has approximately 100 fold higher affinity, compared with the low affinity site, now recognised as the α_{1B}-adrenoceptor. This selectivity for the α_{1A}-adrenoceptor has also been demonstrated for all the cloned subtypes (Schwinn *et al*, 1995) and also in functional and binding studies in tissues proposed to contain the α_{1A}-adrenoceptor (Kong *et al*, 1994; Yang *et al*, 1998 and

Argyle and M^{c}Grath, 2000). Its affinity for $\alpha_{1D/d}$-adrenoceptors is between that for the $\alpha_{1A/a}$- and $\alpha_{1B/b}$-adrenoceptors (Perez *et al*, 1991; Schwinn *et al*, 1995; Kenny *et al*, 1995).

Tamsulosin has been used in the treatment of benign prostatic hypertrophy, to relieve urinary flow obstruction, which is believed to be caused by an increase in tone in prostate smooth muscle as a result of α_1-adrenoceptor activation. The α_1-adrenoceptor involved in prostate smooth muscle contraction is believed to be the α_{1A}-adrenoceptor (Forray *et al*, 1994) for which tamsulosin has been shown to have selectivity (Michel *et al*, 1993; Michel and Insel, 1994). Although selective for the α_{1A}-adrenoceptor its degree of uroselectivity appears to be very slight, so that not only does it inhibit the α_1-adrenoceptors in the prostate but it also potently decreases blood pressure (Martin *et al*, 1997), leading to side effects such as postural hypotension.

Prompted by this, a number of studies have been undertaken aimed at producing α_{1A}-adrenoceptor antagonists, which are also uroselective. One such study produced two α_1-adrenoceptor antagonists, Ro70-0004 and RS-100329. Both compounds showed greater than 50-fold selectivity for α_{1a}-adrenoceptors over α_{1b}- and α_{1d}-adrenoceptors at cloned human α_1-adrenoceptors. This selectivity was also demonstrated in functional studies by their ability to antagonise the contractions produced in human lower urinary tract tissues and rabbit bladder neck with nanomolar affinities (Williams *et al*, 1999). The degree of uroselectivity of these compounds in an *in vivo* situation has yet to be assessed. However their ability to discriminate $\alpha_{1A/a}$-adrenoceptors from $\alpha_{1B/b}$- and $\alpha_{1D/d}$-adrenoceptors makes them valuable tools in pharmacological research.

The $\alpha_{1A/a}$-adrenoceptor, as well as having a relatively large number of selective antagonists, in comparison to the other subtypes, can also boast of selective agonists. Oxymetazoline, which is a partial agonist at α_1-adrenoceptors, has been shown to possess an approximately 10-fold higher affinity at $\alpha_{1A/a}$-adrenoceptors than $\alpha_{1B/b}$- or $\alpha_{1D/d}$-adrenoceptors (Minneman *et al*, 1994; Knepper *et al*, 1995 and Schwinn *et al*, 1995). A-61603, which was developed by Abbott Laboratories is a full agonist at α_1-adrenoceptors and has also shown selectivity for the $\alpha_{1A/a}$-adrenoceptor, demonstrating a 15-20 fold higher affinity at this subtype than at the α_{1b}- and α_{1d}-adrenoceptors (Knepper *et al*, 1995). Its usefulness as an α_{1A}-adrenoceptor agonist has been demonstrated in functional studies (Knepper *et al*, 1995; Smith *et al*, 1997, Hrometz *et al*, 1999; Argyle and M^{c}Grath, 2000).

The number of selective compounds available for the $\alpha_{1B/b}$- and $\alpha_{1D/d}$-adrenoceptors is far fewer than those available for the $\alpha_{1A/a}$-adrenoceptor. Chloroethylclonidine (CEC) was believed to be an irreversible competitive antagonist for the $\alpha_{1B/b}$-adrenoceptor based on its ability to inactivate the α_{1B}-adrenoceptor selectively in various rat tissues (Morrow and Creese, 1986; Han *et al*, 1987b; Minneman *et al*, 1988). However the $\alpha_{1D/d}$-adrenoceptor has since been shown to be capable of inactivation by CEC (Perez *et al*, 1991). Further to this Hirasawa *et al* (1997) demonstrated that CEC is capable of inactivating α_{1a}- and α_{1b}-adrenoceptor membrane preparations, whilst in whole cells it can inactivate only the α_{1b}-adrenoceptor with any significance. They concluded that this discrepancy was due to the subcellular localisation of the α_1-adrenoceptors. Using GFP-tagged α_1-adrenoceptors they were able to show that α_{1a}-adrenoceptors were mostly intracellular, whilst α_{1b}-adrenoceptors were found on the cell surface. They proposed

that the highly hydrophilic CEC inactivates the α_1-adrenoceptor present on the cell surface irrespective of subtype. Studies such as these have placed doubts on the reliability of the information that CEC provides.

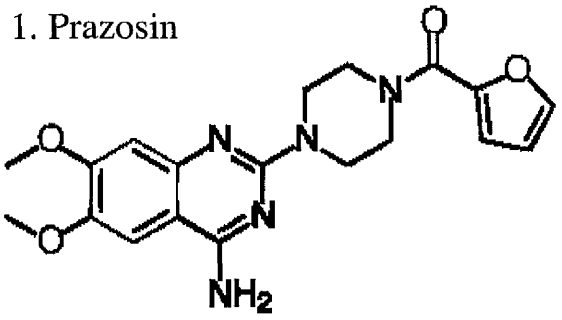
Spiperone was proposed to be an α_{1B}-adrenoceptor antagonist with 10-fold selectivity for the α_{1B}-adrenoceptor (Michel *et al*, 1989). However, subsequent studies showed that it was unable to discriminate between subtypes (Schwinn *et al*, 1995) and as a result it is not widely used. Cyclazosin, is another such compound proposed to be selective for α_{1B}-adrenoceptors. Binding studies displayed 90-130 fold selectivity for the α_{1B}-adrenoceptor (Giardina *et al*, 1996). But functional studies in the mouse spleen, which is regarded as having a homogeneous population of α_{1B}-adrenoceptors (Eltze, 1996), found it to have a low affinity and its action was not competitive (Stam *et al*, 1998) placing doubts on its usefulness as an α_{1B}-adrenoceptor selective antagonist.

In 1998, Patane *et al* described the synthesis and pharmacological evaluation of a structural analogue of prazosin, L765,314, which they deemed to be a potent α_{1B}-adrenoceptor selective antagonist. This was based on findings that at rat and human recombinant α_1-adrenoceptors L765,314 had 10 and 100 fold higher affinity for the α_{1b}-adrenoceptor compared with the α_{1d}- and α_{1a}-adrenoceptors, respectively. This selectivity was mirrored in native α_1-adrenoceptors. Despite these encouraging results, work in our laboratory utilising this compound on recombinant α_1-adrenoceptors, has failed to achieve the same degree of selectivity (Mackenzie, unpublished observations). Hence, to date, there are no convincing selective antagonists or agonists for the $\alpha_{1B/b}$-adrenoceptor.

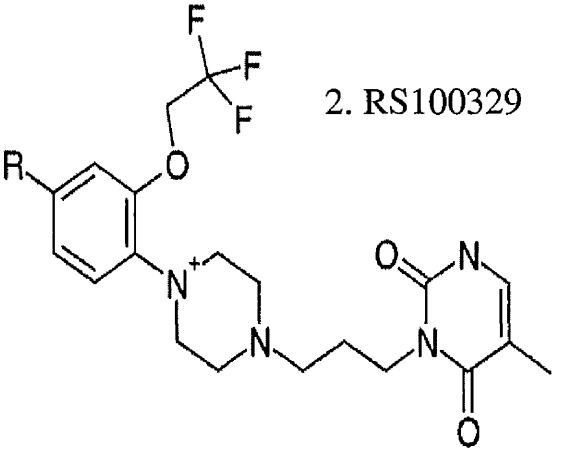
BMY7378 is the only widely accepted competitive antagonist, so far, for the $\alpha_{1D/d}$-adrenoceptor, for which it has been shown to have nanomolar affinity (Saussy *et al*, 1994; Goetz *et al*, 1995). Its affinity at $\alpha_{1A/a}$- and $\alpha_{1B/b}$-adrenoceptors is similar and is approximately 100 times less than at $\alpha_{1D/d}$-adrenoceptors. Through the use of this compound the contraction in the rat aorta was characterised as being mediated by the α_{1D}-adrenoceptor, answering the long running question as to its identity (Kenny *et al*, 1995).

Although there are no officially recognised selective $\alpha_{1D/d}$-adrenoceptor agonists, phenylephrine has been shown on a number of occasions to display a higher affinity at the $\alpha_{1D/d}$-adrenoceptor. Knepper *et al* (1995) found that phenylephrine had a 10-fold higher affinity at α_{1d}-adrenoceptors compared with the other subtypes. In native α_1-adrenoceptors it was 10 and 80 fold more potent in contracting the native α_{1D}-adrenoceptors in rat aorta, than at α_{1A}-adrenoceptors in the rat vas deferens, or α_{1B}-adrenoceptors in the rat spleen, respectively (Knepper *et al*, 1995). Minneman *et al* (1994) demonstrated a selectivity at recombinant α_1-adrenoceptors which matched that of Knepper *et al* (1995) but found that phenylephrine had similar potencies at all three subtypes in stimulating inositol phosphate formation.

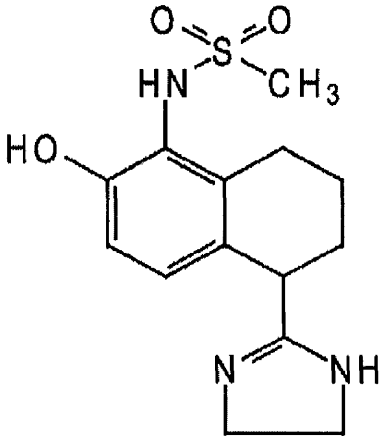
1. Prazosin



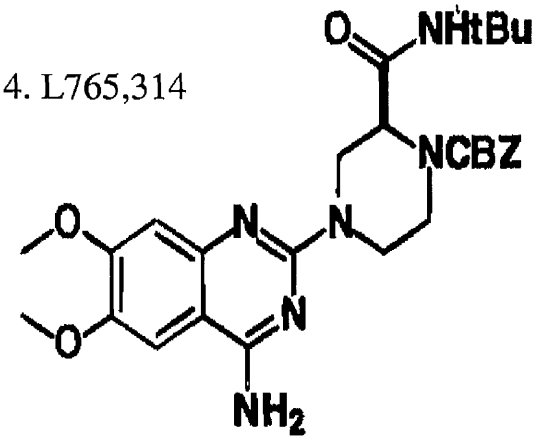
2. RS100329



3. (R)-A-61603



4. L765,314



5. BMY7378

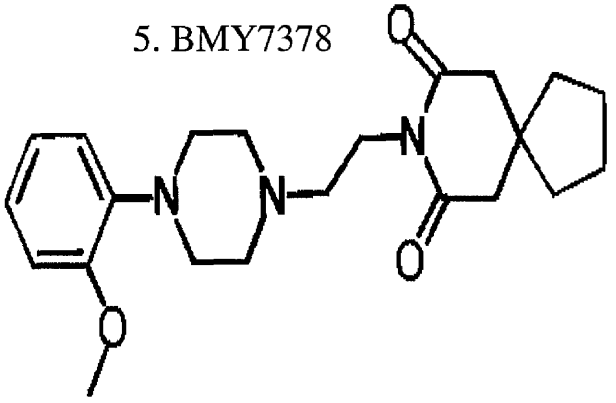


Figure 1.1:- The chemical structures of selective and non-selective ligands described in the text and used in the radioligand binding studies in Chapter 2. For RS100329 R=H.

1.4.MOLECULAR BIOLOGY OF α_1-ADRENOCEPTORS

Morrow and Creese's paper in 1986 is regarded as being the first paper to clearly show the presence of two α_1-adrenoceptor subtypes in native tissues. But it was a further 2 years before the first α_1-adrenoceptor was cloned.

1.4.1. Cloning of the α_{1b}-adrenoceptor

In 1988, Cotecchia *et al* described the cloning and sequencing of a purified α_1-adrenoceptor isolated from a hamster cDNA library. The isolated clone was found to have a deduced amino acid sequence of 515 amino acids with a molecular weight of 56kDa, in agreement with the apparent molecular mass of the deglycosylated α_1-adrenoceptor (Sawutz *et al*, 1987). Hydrophobicity analysis revealed 7 clusters of hydrophobic residues, 20-25 residues in length, consistent with previously cloned G protein coupled receptors (GPCRs). Potential sites for N-linked glycosylation were found in the amino terminus, in agreement with previous observations that the α_1-adrenoceptor is glycosylated with complex N-type linked oligosaccharides (Sawutz *et al*, 1987).

The isolated clone was found to share identity with previously cloned human adrenoceptors, particularly in the putative transmembrane domains (TMD) where the identities were found to be:- α_2: 44%, β_1:45% and β_2: 42%. Several threonine and serine residues were found in the second and third cytoplasmic loops representing potential sites for protein kinase C (PKC) phosphorylation. A consensus site for protein kinase A (PKA) phosphorylation was found in the third cytoplasmic loop, in the same position as

in the α_2-adrenoceptor. These potential phosphorylation sites suggested that this clone could be regulated by phosphorylation.

At this stage it appeared that the clone which had been isolated was indeed an α_1-adrenoceptor and to confirm this the function of the clone when expressed in cells was examined. It was found to bind the α_1-adrenoceptor antagonist [^{125}I]-HEAT with high affinity and was potently inhibited by prazosin, to a greater extent than either yohimbine or isoprenaline. In addition noradrenaline was found to dramatically increase the incorporation of ^{32}P into phosphatidylinositol. This was indeed proof that the isolated hamster clone was an α_1-adrenoceptor and in light of the pharmacological developments at the time the binding of WB4101 and phentolamine was investigated to determine which subtype of α_1-adrenoceptor had been identified. (These were the 2 antagonists which Morrow and Creese found were able to distinguish between subtypes of α_1-adrenoceptor). The hamster α_1-adrenoceptor was found to bind with low affinity to both WB4101 and phentolamine and it was concluded that the clone isolated from the hamster smooth muscle cell line was the α_{1b}-adrenoceptor.

Using a cDNA probe derived from the hamster α_{1b}-adrenoceptor, Voigt *et al* (1990) identified the rat α_{1b}-adrenoceptor and demonstrated that its pharmacological profile was similar to the hamster α_{1b}-adrenoceptor; the two clones shared >98% amino acid identity. Northern analysis of rat tissues showed mRNA expression for this clone in a variety of tissues which were expected to express the α_{1b} subtype, namely the heart, liver, spleen and cerebral cortex (Bylund *et al*, 1994). In 1992 the human α_{1b}-adrenoceptor was cloned and shown to be virtually identical in its amino acid sequence

and pharmacology to the hamster and rat α_{1b}-adrenoceptor clones (Ramarao *et al*, 1992). The close correspondence between the pharmacological properties of the expressed α_{1b}-adrenoceptor cDNA from the rat, hamster or human clones with those recognised for the α_{1B}-adrenoceptor indicates that the protein encoded by this clone represents the α_{1B}-adrenoceptor identified by functional and radioligand binding studies in native tissues (Morrow and Creese, 1986).

In 1995 the International Union of Pharmacology decided that all native receptors would be described by upper case subscripts and all recombinant clones would be described by lower case subscripts (Hieble *et al*, 1995). Therefore the α_{1B}-adrenoceptor is the native receptor and the α_{1b}-adrenoceptor is the recombinant receptor. This nomenclature is used throughout to distinguish between recombinant and native subtypes.

1.4.2. Cloning of the α_{1a}-adrenoceptor

A further 2 years on from the cloning of the α_{1b}-adrenoceptor, a second α_1-adrenoceptor was isolated and cloned, this time from a bovine brain cDNA library (Schwinn *et al*, 1990) using a probe derived from the hamster α_{1b}-adrenoceptor. Within the open reading frame of the clone a peptide was found from which the first 43 residues were 67% identical with the hamster α_1-adrenoceptor's putative 7th TMD and the beginning of the carboxyl terminus. The open reading frame encodes a polypeptide of 466 amino acids with a calculated molecular mass of 51kDa. The bovine clone was found to contain a striking similarity to other GPCRs that had been previously cloned, in particular the hamster α_{1b}-adrenoceptor, sharing an identity of 72% with this receptor.

When Schwinn *et al* compared the bovine clone they had isolated with the hamster α_{1b}-adrenoceptor isolated by Cotecchia *et al*, they found that the clones had 27% amino acid identity at the amino terminus, 12% amino acid identity at the carboxy terminus and 50% amino acid identity in the third cytoplasmic loop. Although the bovine clone and the hamster α_{1b}-adrenoceptor shared a relatively high identity it seemed doubtful that the clone isolated was the bovine homologue of the hamster α_{1b}-adrenoceptor since it was found that the hamster, rat and human α_{1b}-adrenoceptors were 98% identical (Ramarao *et al*, 1992). Additionally, the gene corresponding to the hamster α_{1b}-adrenoceptor is found on human chromosome 5 whereas the gene corresponding to the bovine clone is found on human chromosome 8 (Hieble *et al*, 1995).

However, many of the features of the hamster α_{1b}-adrenoceptor were shared with this bovine clone. For example, there were potential sites for N-linked glycosylation in the amino terminus, threonine and serine residues were again found in the second and third cytoplasmic loops and a consensus sequence for PKA phosphorylation was found to be in an analogous position to the hamster α_{1b}-adrenoceptor, suggesting that this clone could also be regulated by phosphorylation.

As with the hamster α_{1b}-adrenoceptor, the information obtained from the amino acid sequence of the bovine clone was consistent with that of an α_1-adrenoceptor and when the clone was expressed in cells it was found to bind [^{125}I]-HEAT with high affinity, similar to the α_{1b}-adrenoceptor. Competition assays were carried out to a range of α-adrenoceptor antagonists and agonists including WB4104 and phentolamine, the antagonists used on the hamster α_{1b}-adrenoceptor. However the K_i values for the bovine

α_1-adrenoceptor were quite different from the hamster α_{1b}-adrenoceptor. The bovine α_1-adrenoceptor was found to have a 10-fold higher affinity for WB4101 and phentolamine as well as for corynanthine, indoramin, oxymetazoline and methoxamine, when compared with the hamster α_{1b}-adrenoceptor. These affinity values for the bovine α_1-adrenoceptor were in agreement with those previously described for the native α_{1A}-adrenoceptor. The bovine clone was found to be less sensitive to CEC inactivation than the α_{1B}-adrenoceptor and more sensitive to CEC inactivation than the α_{1A}-adrenoceptor, when compared with previous reports (Han *et al*, 1987b; Minneman *et al*, 1988).
In addition to this binding data, Northern blot analysis of various rat and bovine tissues was performed to determine in which tissues this α_1-adrenoceptor was expressed. Surprisingly, no expression could be detected in any of the tissues examined which included rat cerebral cortex, hippocampus, liver, heart, lung, kidney, spleen, aorta, adipose tissue, skeletal muscle and vas deferens as well as bovine cerebral cortex, liver, heart, kidney, lung and adrenal gland.

So despite the bovine α_1-adrenoceptor clone displaying the pharmacological characteristics of the α_{1A}-adrenoceptor, no measurable expression levels could be detected in tissues which had been previously described as containing the α_{1A}-adrenoceptor, specifically rat vas deferens and hippocampus (Gross *et al*, 1988). On this basis Schwinn *et al* concluded that they had isolated a novel α_1-adrenoceptor subtype which they appropriately named the α_{1c}-adrenoceptor.

When the human homologue of the α_{1c}-adrenoceptor was identified (Hirasawa *et al*, 1993) it was found to have 92% identity with the bovine α_{1c}-adrenoceptor and a similar pharmacological profile i.e. high affinity for oxymetazoline, WB4101, phentolamine

and additionally 5-methylurapidil and it was extensively inactivated by CEC. In humans, the tissue distribution in brain, heart and liver, was contrary to the lack of expression seen in rat tissues. In 1994, a rat homologue of the bovine α_{1c}-adrenoceptor was cloned which displayed a similar pharmacological profile to the native α_{1A}-adrenoceptor (Laz *et al*, 1994). Further studies followed, which demonstrated that the affinities of an extensive series of competitive antagonists to the expressed α_{1c}-adrenoceptor correlated highly with their affinities in native tissues that were known to possess the α_{1A}-adrenoceptor (Langer *et al*, 1994; Testa *et al*, 1995a; Pimoule *et al*, 1995). This was considered strong evidence that the recombinant α_{1c}-adrenoceptor and the native α_{1A}-adrenoceptor were identical.

However a number of anomalies still remained, the first of which was to explain the difference in the observed sensitivity of both the recombinant and native receptor to CEC. It was proposed that this discrepancy resulted from differences in experimental conditions, the character of the membrane in which the receptor is expressed and/or species differences in the sensitivity of the receptors to CEC (Hieble *et al*, 1995). The final discrepancy was the apparent lack of tissue expression of the α_{1c}-adrenoceptor clone. In the original description of the bovine recombinant α_{1c}-adrenoceptor, its apparent lack of expression of mRNA when Northern analysis was used in rat tissues and its surprising presence in rabbit liver were among the main reasons that it was believed to be distinct from the native α_{1A}-adrenoceptor. However, Northern blot analysis using rat α_1-adrenoceptor probes (Faure *et al*, 1994) and RNase protection assays using rat probes (Price *et al*, 1994a; Rokosh *et al*, 1994) has since demonstrated the presence of the α_{1c}-adrenoceptor in every rat tissue originally described as containing the α_{1A}-adrenoceptor.

Based on this evidence the International Union of Pharmacology decided that the designation α_{1c} be discontinued and that the native and recombinant receptors be referred to as α_{1A}- and α_{1a}-adrenoceptors respectively (Hieble *et al*, 1995). The rat and human α_{1c}-adrenoceptor isolated by Laz *et al* (1994) and Hirasawa *et al* (1993) respectively, were soon renamed α_{1a}-adrenoceptors, in recognition that they were the rat and human homologue of the bovine α_{1a}-adrenoceptor. The gene encoding the mouse α_{1a}-adrenoceptor was not isolated or cloned until 1998 (Xiao *et al*, 1998) demonstrative of the indifference shown to studying the mouse genome until genetic engineering became popular.

1.4.3. Cloning of the α_{1d}-adrenoceptor

The third and final α_1-adrenoceptor to be cloned was isolated from the rat brain by Perez *et al* in 1991 and was found to encode a protein of 560 amino acids. Hydropathy analysis was consistent with a putative 7 TMD structure indicative of a GPCR and as with the previous α_1-adrenoceptors the highest concentration of identity was in these TMD. The percentages of identity were as follows: hamster α_{1b}-adrenoceptor; 71%, bovine α_{1c}-adrenoceptor; 64%, rat β_1-adrenoceptor; 40% and rat M_1 acetylcholine receptor; 30%.

This rat clone was found to have potential sites for N-linked glycosylation in the amino terminus and potential sites for PKC phosphorylation, consistent with the two previously cloned α_1-adrenoceptors, but unlike them this clone had no consensus site for PKA phosphorylation.

When the clone was expressed in cells prazosin was found to inhibit [^{3}H]-prazosin binding with an affinity consistent with an α_1-adrenoceptor. When competitive binding was performed using subtype-selective α_1-antagonists the results were unusual. The affinities for WB4101 and 5-methylurapidil were higher than expected at an $\alpha_{1B/b}$-adrenoceptor but lower than expected at an $\alpha_{1A/a}$-adrenoceptor. The clone also demonstrated that it could be extensively inactivated by CEC although not to the same extent as the $\alpha_{1B/b}$-adrenoceptor. Hence the results from the pharmacological analysis of the rat α_1-adrenoceptor appeared to indicate that it was neither an α_{1A}-adrenoceptor or an α_{1B}-adrenoceptor. Northern blot analysis on the other hand revealed tissue expression in hippocampus, cerebral cortex and vas deferens which had been shown previously to contain the α_{1A}-adrenoceptor (Gross *et al*, 1988). However, on the basis of the binding data, Perez *et al* concluded that their rat α_1-adrenoceptor was a novel clone and as such named it the α_{1d}-adrenoceptor.

At the same time as Perez *et al* identified their rat α_1-adrenoceptor clone, another group reported the isolation of a cDNA sequence which they claimed encoded the putative rat α_{1a}-adrenoceptor (Lomasney *et al*, 1991). The DNA sequence they published was identical to that of Perez *et al* except for 2 amino acids of a difference. So where was the discrepancy? The differing conclusion as to the identity of the isolated clone seems to have arisen as a consequence of the ligands used for the pharmacological evaluation of the clones. Perez *et al* used ligands which were selective for the α_{1A}-adrenoceptor, whilst Lomasney *et al* used mainly non-selective ligands. Perez *et al* could therefore identify their clone as novel from the use of these ligands because the affinities were too low to be the pharmacologically defined α_{1A}-adrenoceptor and too high to be the α_{1B}-

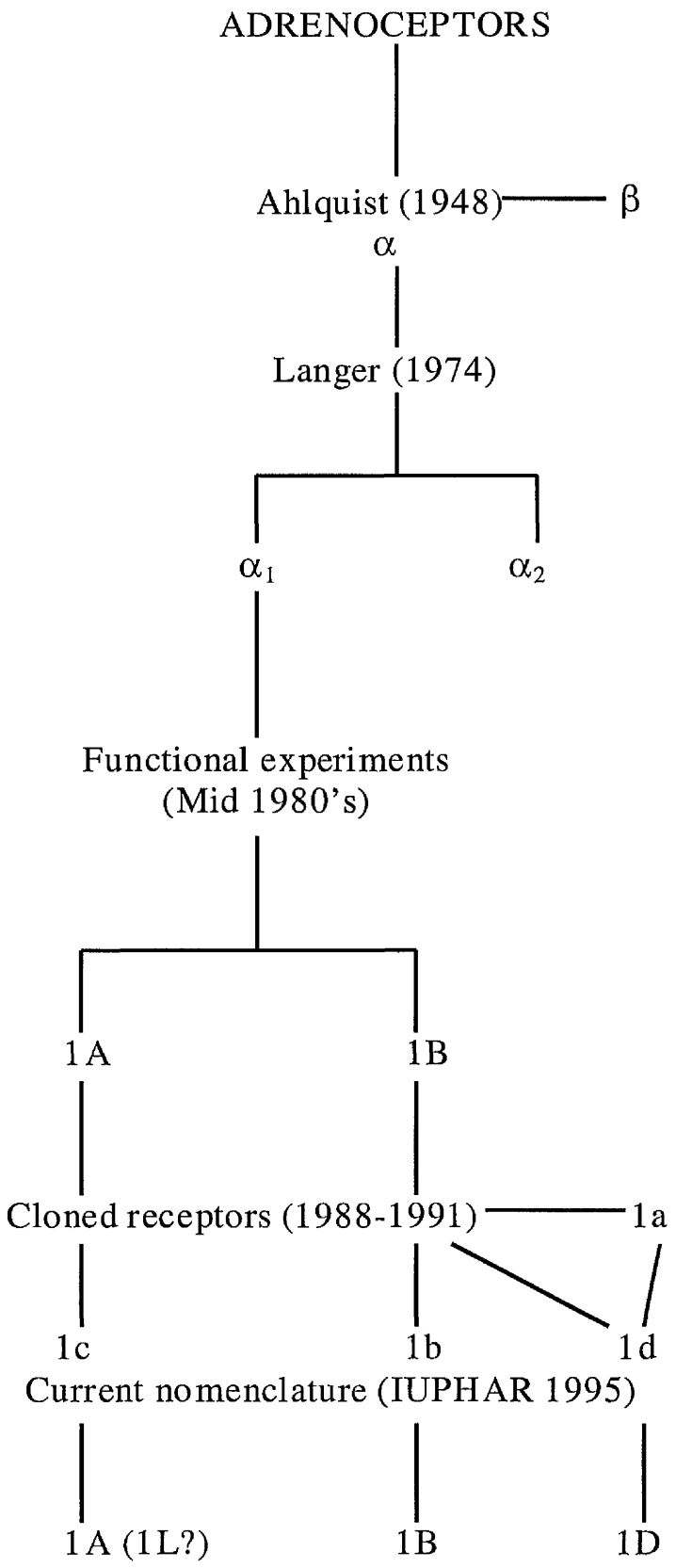


Figure 1.2:- A simplified diagrammatic representation of the history of the subdivision of α_1-adrenoceptors. The status of the α_{1L}-adrenoceptor is still unclear but may be a pharmacological phenotype of the α_{1A}-adrenoceptor .

adrenoceptor. It is now accepted that both groups had isolated the same novel clone which codes for the native α_{1D}-adrenoceptor, since been shown to be present in the rat aorta (Van der Graaf, 1993, Kenny *et al*, 1995). In 1995 the International Union of Pharmacology assigned the novel clone the nomenclature, α_{1d}-adrenoceptor.

The human α_{1d}-adrenoceptor was isolated and cloned by two different groups at approximately the same time (Weinberg *et al*, 1994; Schwinn *et al*, 1995). Weinberg *et al* found their clone to have 82% sequence identity with the rat clone, whilst Schwinn's clone had approximately 87% sequence homology with the rat α_{1d}-adrenoceptor and both had virtually identical pharmacological properties.

1.5. SIGNAL TRANSDUCTION OF α_1-ADRENOCEPTORS

α_1-Adrenoceptors belong to the superfamily of receptors known as G protein coupled receptors (GPCR) which, as their name suggests, are receptors which couple to G proteins to mediate their effects. G proteins have a heterotrimeric structure consisting of an α subunit and β and γ subunits which are closely associated with each other. So far at least 21 α subunits, 5 β subunits and 9 γ subunits have been discovered (Ulloa-Aguirre *et al*, 1999). Many of the functional differences between G proteins are found in the α subunit which therefore defines the G protein, and it is probably as a consequence of this that there are a far greater number of α subunits than β or γ subunits. Gα subunits have been grouped into 4 subfamilies according to their sequence and function:-

Gα_s – hormonal stimulation of adenylyl cyclases e.g. β-adrenoceptors

G$\alpha_{i/o}$ – hormonal inhibition of adenylyl cyclases e.g. α_2-adrenoceptors

$G\alpha_q$ – couples receptors to phospholipase C e.g. α_1-adrenoceptors

$G\alpha_{12}$ – the function of this elusive family of G proteins remains unknown.

Regardless of their function, all G proteins operate via the same basic mechanism. In the basal state, G proteins exist as heterotrimers and have guanosine diphosphate (GDP) bound to the catalytic site of an intrinsic guanosine trisphosphatase (GTPase) present on the α subunit. Activation of the receptor promotes the exchange of GDP for guanosine trisphosphate (GTP) which, when bound to the α subunit, induces the dissociation of the receptor α subunit and $\beta\gamma$ complex. The $G\alpha$-GTP complex and the $\beta\gamma$ complex are now able to regulate effectors. Hydrolysis of GTP to GDP by the α subunit's GTPase results in reassociation with the $\beta\gamma$ complex, which can then reenter the cycle (Offermanns, 1999; Summers and M^{c}Martin, 1993).

α_1-Adrenoceptor subtypes couple to the pertussis toxin-insensitive family of G proteins, G_q, which regulate phospholipase C (PLC) activity. The G_q family consists of 4 members whose α subunits are expressed by individual genes with different expression patterns. $G\alpha_q$ and $G\alpha_{11}$ are the most widely expressed and can be found coexpressed in most cells. $G\alpha_{14}$, the murine G protein $G\alpha_{15}$ and its human counterpart $G\alpha_{16}$ are more restricted in their expression. $G\alpha_{14}$ can be found in the kidney, lung, spleen and testis and $G\alpha_{15/16}$ has only been found in haematopoietic cells (Offermanns, 1999). Despite their specific patterns of expression, which may imply cell type specific functions, no phenotypic changes were observed in mice carrying inactivating mutations of the $G\alpha_{14}$ and $G\alpha_{15}$ genes (Offermanns, 1999).

$G\alpha_q$ and $G\alpha_{11}$ can potently mediate the activation of PLC induced by any of the 3 α_1-adrenoceptor subtypes whereas $G\alpha_{14}$ and $G\alpha_{15/16}$ are much less potent (Wu *et al*, 1992). A level of subtype specificity appears to exist among the α_1-adrenoceptor subtypes in their ability to couple to members of the G_q family. The α_{1B}-adrenoceptor couples efficiently with all 4 members i.e. $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15/16}$; the α_{1A}-adrenoceptor couples with $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$, whereas the α_{1D}-adrenoceptor only couples efficiently to $G\alpha_q$ and $G\alpha_{11}$ (Wu *et al*, 1992).

The G_q family of G proteins specifically activate the β isoform of PLC (Wu *et al*, 1992) to catalyse the hydrolysis of phosphatidylinositol-4,5-bisphosphate with the subsequent formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts to mediate intracellular calcium ($[Ca^{2+}]_i$) release from intracellular stores such as the sarcoplasmic reticulum and DAG activates PKC which phosphorylates specific target proteins (Minneman, 1988), including Ca^{2+} channels that may regulate intracellular Ca^{2+} or activate transcription of genes (Graham *et al*, 1996).

Although α_1-adrenoceptors mediate their actions predominantly through the G_q/PLC pathway there is a substantial amount of evidence demonstrating their ability to utilise other signal transduction pathways (Zhong and Minneman, 1999a). Burch *et al* (1986) has shown that α_1-adrenoceptors can stimulate arachidonic acid release in a rat thyroid cell line. Perez *et al* (1993) also demonstrated arachidonic acid release by activation of α_{1b}- and α_{1d}-adrenoceptors expressed in COS-7 and CHO cells. They showed that in COS-7 cells (where expression levels of α_1-adrenoceptors are high) arachidonic acid was released as a consequence of indirect phospholipase A_2 (PLA_2) activation, involving a pertussis toxin-sensitive G protein and requiring Ca^{2+} influx through

dihydropyridine-sensitive Ca^{2+} channels. However, in CHO cells (where expression levels of α_1-adrenoceptors approach *in vivo* levels) there appeared to be a different pathway stimulating the arachidonic acid release. Perez *et al* showed that CHO cells do not have voltage-sensitive Ca^{2+} channels but instead it appeared that the α_1-adrenoceptors were coupling directly to PLA_2 activation. This was the first time that a single α_1-adrenoceptor had been shown to activate multiple, distinct signal transduction pathways in different cell lines.

In rat-1 fibroblasts the release of arachidonic acid by phenylephrine-stimulated α_1-adrenoceptors appears to be due to phospholipase D (PLD) activation rather than PLA_2 (Ruan *et al*, 1998). All 3 α_1-adrenoceptor subtypes were shown to increase PLD activity, with the α_{1a}-adrenoceptor coupling more efficiently than either the α_{1b}-adrenoceptor or the α_{1d}-adrenoceptor. Accumulation of adenosine 3'-5'cyclic monophosphate (cAMP) was also seen in all 3 subtypes, a phenomenon which has been previously demonstrated in other cell types (Schwinn *et al*, 1991; Perez *et al*, 1993), where it was reported that the increase in cAMP accumulation was not due to a direct receptor-mediated activation of adenylyl cyclase but rather due to PKC activation. However, Ruan *et al* (1998) showed that neither the PKC inhibitor, bisindolylmaleimide, nor the PKC activator, phorbol 12-myristate 13-acetate, had any effect on the phenylephrine induced increase in cAMP level. Instead they provided evidence for a direct receptor activation of adenylyl cyclase, by demonstrating that the increase in cAMP accumulation could be potentiated by forskolin, an activator of adenylyl cyclase and attenuated by SQ22536, an inhibitor of adenylyl cyclase (Ruan *et al*, 1998). It is possible, given the evidence of the α_1-adrenoceptor's potential for

promiscuity with second messenger pathways that both PKC activation and adenylyl cyclase activation could be responsible for the increase in cAMP accumulation.

α_1-Adrenoceptors may also couple directly to Ca^{2+} influx. In 1987, shortly after Morrow and Creese confirmed the existence of α_1-adrenoceptor subtypes, Han *et al* proposed that α_{1A}-adrenoceptors increased $[Ca^{2+}]_i$ by influx of extracellular Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels. The stimulation of inositol phosphates was proposed to be the mechanism through which α_{1B}-adrenoceptors mediate the release of $[Ca^{2+}]_i$ (Han *et al*, 1987a). Although it is now agreed that this is not a subtype specific process it demonstrates that in certain tissues (in this case rat hippocampus and vas deferens) α_1-adrenoceptors can increase $[Ca^{2+}]_i$ by a PLC-independent mechanism.

1.6. STRUCTURE OF α_1-ADRENOCEPTORS

1.6.1. General

α_1-Adrenoceptors are single polypeptide chains ranging from 429-561 amino acids in length (Cotecchia *et al*, 1988; Schwinn *et al*, 1990; Perez *et al*, 1991; Ramarao *et al*, 1992; Hirasawa *et al*, 1993 and Xiao *et al*, 1998) that are posttranslationally modified by the attachment of oligosaccharides, as well as being phosphorylated (Graham *et al*, 1996). They have the same basic structure as all GPCRs i.e. an extracellular amino terminal, an intracellular carboxy terminal and 7 TMD connected by 3 intracellular and 3 extracellular loops. The amino termini of the original cloned α_1-adrenoceptors were all shown to contain consensus sites for modification by N-linked glycosylation as well as having several threonines and serines in the second and third intracellular loops representing potential sites for PKC phosphorylation. A consensus sequence for PKA

phosphorylation was found to be present in the hamster α_{1b}-adrenoceptor and the bovine α_{1a}-adrenoceptor but not in the rat α_{1d}-adrenoceptor (Cotecchia *et al*, 1988; Schwinn *et al*, 1990; Perez *et al*, 1991). The amino termini are found to vary considerably in length, with the terminus for the α_{1d}-adrenoceptor being much longer (approximately 90 amino acids) than the terminus for the α_{1a}-adrenoceptor (25 amino acids) or the α_{1b}-adrenoceptor (42 amino acids) (Graham *et al*, 1996). It has been suggested that this longer amino terminus of the α_{1d}-adrenoceptor may limit efficient translation or membrane insertion (Graham *et al*, 1996).

The first and second extracellular loops each contain a single cysteine residue and analogous cysteines are highly conserved in all GPCRs. In the β-adrenoceptor and rhodopsin these cysteines are essential for the correct folding of the proteins, for maturational glycosylation and for expression in the plasma membrane. This is due to the involvement of these cysteines in a disulphide bond (Graham *et al*, 1996). The α_{1B}-adrenoceptor has also been shown to contain a disulphide bond. Parini *et al* (1987) demonstrated that this disulphide bond was inaccessible to solvents because dithiothreitol alone was found to have no effect on the activity of the receptor but instead denaturation was required before any evidence for a disulphide bond could be detected.

1.6.2. Important amino acid residues

Since the discovery of the gene products of α_1-adrenoceptors a number of studies have concentrated on determining the amino acids responsible for agonist and antagonist binding and receptor activation. An elegant study carried out by Hwa *et al* (1995)

identified 2 amino acids as being critical to the different binding affinities observed by agonists at α_{1a}- and α_{1b}-adrenoceptors. A double mutant was created by site-directed mutagenesis which replaced Leu^{314} and Ala^{204} in the α_{1b}-adrenoceptor to the corresponding amino acids found in the WT α_{1a}-adrenoceptor, methionine and valine respectively. Binding analysis was carried out on the mutated α_{1b}-adrenoceptor, which was found to show a binding profile more consistent with an α_{1a}-adrenoceptor. When the double mutation was reversed (Val^{185} to Ala and Met^{293} to Leu), the binding profile was also reversed, back to an α_{1b}-adrenoceptor-like pharmacology. Some residual differences remained with methoxamine and cirazoline indicating that there may still be some minor conformational differences in the agonist ligand binding pocket of the α_{1a}-adrenoceptor. However this study demonstrated that Leu^{314} and Ala^{204} in the α_{1b}-adrenoceptor and their corresponding residues in the α_{1a}-adrenoceptor are the most critical determinants of the differences observed in agonist binding between these 2 subtypes (Hwa *et al*, 1995).

The two or three serines found in TMD 5 have been shown to be highly conserved in GPCRs that bind catecholamines with high affinity. The α_{1a}-adrenoceptor has two conserved serines (Ser^{188} and Ser^{192}) one of which has been shown to be important for receptor activation (Ser^{188}) (Hwa *et al*, 1996). The analogous serine to this in the α_{1b}-adrenoceptor (Ser^{207}) has, in contrast, been shown to be involved in ligand binding. It was discovered that when Ser^{207} was mutated to Ala the binding affinities of adrenaline and noradrenaline at the α_{1b}-adrenoceptors decreased by 250- and 350-fold respectively (Cavalli *et al*, 1996). The other two conserved cysteine residues of the α_{1b}-adrenoceptor (Ser^{208} and Ser^{211}) when expressed as a double mutant (both Ser residues were replaced

with Ala) completely impaired the ability of the α_{1b}-adrenoceptor to be activated by adrenaline. Yet, when expressed as single mutants, they had no effect on receptor activation, indicating that the integrity of at least one of these serines was required for receptor activation. Molecular dynamic analysis revealed that Ser^{208} and Ser^{211} are directed towards TMD 6 and TMD 4 respectively and may exert a structural/functional role, thus stabilising the active conformation of the α_{1b}-adrenoceptor, confirming the experimental findings. Therefore, despite the fact that these serines are highly conserved, the role that they carry out in α_{1b}-adrenoceptors is quite different from other catecholamine receptors, including the closely related α_{1a}-adrenoceptor.

In addition to deducing the role of the conserved serines in the α_{1b}-adrenoceptor, Cavalli *et al* (1996) also showed that the aspartate at position 125 is essential for both agonist and antagonist binding. When they mutated this residue to alanine the mutated α_{1b}-adrenoceptor was incapable of binding [^{125}I]-HEAT or producing adrenaline-induced stimulation of inositol phosphate.

Site directed mutagenesis is a common technique used to identify critical amino acid residues and their functions, sometimes with surprising results. A substitution mutation (Ala^{293} was substituted with Glu) in the C-terminal portion of the third intracellular loop of the α_{1b}-adrenoceptor resulted in a mutated receptor that was constitutively active. When this residue was systematically mutated by substituting all of the 19 amino acids in turn, it was discovered that all the mutated receptors were constitutively active to varying degrees (Kjelsberg *et al*, 1992).

Following on from this work, Cotecchia and colleagues have used this technique in combination with comparative molecular dynamic simulations of the WT and mutant receptors in an attempt to identify key residues involved in the activation of the α_{1b}-adrenoceptor. The residues Arg^{143} and Asp^{142} which can be found in the highly conserved DRY motif at the cytosolic end of TMD 3 (Scheer *et al*, 1999) were two of the amino acids they directed their efforts towards. Arg^{143} was found to be directed towards TMD1 and TMD 2 and to be involved in hydrogen bond interactions with Asp^{91}, an amino acid which is part of the highly conserved TMD 'polar pocket'. Constitutively active mutants carrying substitutions at Ala^{293} were found to 'push' Arg^{143} out of this 'polar pocket', with the extent of the 'push' being equivalent to the degree of constitutive activity of the mutants. When the aspartate of the DRY motif (Asp^{142}) was replaced with the non-polar amino acid alanine, the mutated α_{1b}-adrenoceptor had a high level of constitutive activity. Subsequent substitution mutations at this residue of all natural amino acids demonstrated that as with the Ala^{293}Glu mutations all mutants showed various levels of constitutive activity. From these results it appeared that the hydrophobic/hydrophilic character of Asp^{142} which could be regulated by protonation/deprotonation of this residue (depending on the substituted amino acid's overall charge) was an important modulator of the transition between the inactive and active state of the α_{1b}-adrenoceptor (Scheer *et al*, 1996; Scheer *et al*, 1997). When Asp^{142} is in its anionic form, the interaction of Arg^{143} with Asp^{91} is highly favoured and this maintains the arginine residue and several other amino acids of the second and third intracellular loops in a 'buried' condition with respect to the cytosol. When the receptor is activated, either by protonation of Asp^{142} or by constitutively active mutations, Arg^{143} is shifted out of the 'polar pocket'. The main role of Arg^{143} appears to be to mediate receptor activation, allowing several amino acids in the second

intracellular loop and both N-terminal and C-terminal portions of the third intracellular loop to have the right configuration for the formation of a site with docking complementarity with the G protein (Scheer *et al*, 1999). This agrees with experimental findings that mutations targeting Arg^{143} totally inactivate the α_{1b}-adrenoceptor (Scheer *et al*, 1996).

A number of other amino acid residues have been suggested to be involved in receptor activation. Porter *et al* (1996) proposed that the residues Asp^{125} and Lys^{331} had the potential to form a salt bridge, which may help to stabilise the receptor in the inactivated state. Therefore, if this salt bridge was disrupted by site directed mutagenesis with charged or neutral residues (positively charged residues in the case of Asp^{125} and negatively charged residues in the case of Lys^{331}) then this would result in a constitutively active receptor. Porter *et al* (1996) elegantly demonstrated that when the ionic bond between Asp^{125} and Lys^{331} was broken by amino acid substitution the mutant receptors generated were constitutively active, providing strong evidence for the existence of a salt bridge maintaining the receptor in its basal confirmation.

This section discusses only some of the amino acid residues which have been implicated in some aspect of receptor activation. These papers all show that a constitutively active mutant is relatively easy to produce if you know which amino acids to target. Therefore it would seem that maintaining the receptor in an inactive state is a more highly regulated process than its activation and further studies look set to concentrate on this aspect, rather than the easily attainable activation state.

1.6.3. Genomic organisation

α_1-Adrenoceptors are unusual amongst GPCRs and unique within the family of adrenergic receptors because they have been found to have a single large intron of 14-20kb (Ramarao *et al*, 1992; Weinberg *et al*, 1994; Perez *et al*, 1991; Graham *et al*, 1996). All α_1-adrenoceptor subtypes have been shown to contain two exons separated by a single large intron, which interrupts the coding region at the end of the putative sixth transmembrane domain (Graham *et al*, 1996). The exon/intron boundary follows the consensus splice sequence AG/GT and is situated after the first base of the codon indicating a type I splice phase (Ramarao *et al*, 1992). Exon 1 consists of the coding region that ends near the distal part of the putative sixth transmembrane domain and exon 2 contains the rest of the coding region as well as the 3' non-coding region (Ramarao *et al*, 1992).

1.6.4. Isoforms of the α_{1a}-adrenoceptor

The α_{1a}-adrenoceptor is unusual, in that it is the only α_1-adrenoceptor subtype for which several isoforms have been identified. In the process of cloning the human α_{1a}-adrenoceptor, Hirasawa *et al* (1995) identified two additional clones. Upon sequencing these clones it was found that they encoded part of the α_{1a}-adrenoceptor, whilst the 3' portion of these clones was found to be distinct from the full length α_{1a}-adrenoceptor clone (designated $\alpha_{1a\text{-}1}$). The human $\alpha_{1a\text{-}1}$-adrenoceptor was a protein of 466 amino acids, whilst the two novel clones (designated $\alpha_{1a\text{-}2}$ and $\alpha_{1a\text{-}3}$) were proteins of 499 and 429 amino acids respectively. The three α_{1a}-adrenoceptor isoform proteins have the same TMD part with isoform-specific C-terminals. Hirasawa *et al* demonstrated that

these three α_{1a}-adrenoceptor isoforms originated from a single gene and that they were generated by alternative RNA splicing. The three isoforms were expressed in all tissues examined (heart, liver, cerebellum, cerebrum and pituitary) with the $\alpha_{1a\text{-}1}$ isoform being most abundant. The binding and functional properties of the three isoforms were found to be similar, demonstrating that $\alpha_{1a\text{-}2}$ and $\alpha_{1a\text{-}3}$ produced functional proteins.

Further isoforms of the α_{1a}-adrenoceptor have since been identified. Chang *et al* (1998) identified four full length and four truncated isoforms. Three of the full length isoforms were identical to those isolated by Hirasawa *et al* (i.e. $\alpha_{1a\text{-}1}$, $\alpha_{1a\text{-}2}$ and $\alpha_{1a\text{-}3}$). However the fourth was a novel isoform which they named $\alpha_{1a\text{-}4}$. This isoform was identical to the three original isoforms up to position 1269 but was followed by a novel sequence. This is the same position that the previously published isoforms diverge, suggestive that this was indeed a novel isoform of the α_{1a}-adrenoceptor. The $\alpha_{1a\text{-}4}$-adrenoceptor is a protein of 455 amino acids. When expressed in COS-7 cells all four isoforms displayed similar affinities for the ligands tested. The truncated isoforms did not display any specific binding. However, it is not known if they are expressed in the plasma membrane following transient transfection and so it is not known if the generated products are non-functional. In functional studies $\alpha_{1a\text{-}4}$ exhibited lower affinities for prazosin and the $\alpha_{1A/a}$-antagonist, RS-17053, as did the $\alpha_{1a\text{-}1}$ isoform, indicative of the α_{1L}-adrenoceptor. The classical α_{1A}-adrenoceptor i.e. $\alpha_{1a\text{-}1}$, has previously been shown to display an α_{1A}-adrenoceptor pharmacology in binding studies but in functional experiments it resembled the α_{1L}-adrenoceptor (Ford *et al*, 1997). Recently, all four isoforms have been shown to display low affinities for $\alpha_{1A/a}$-adrenoceptor antagonists in functional studies, displaying α_{1L}-adrenoceptor pharmacology (Daniels *et al*, 1999). It

was hoped that with the discovery of isoforms of the α_{1a}-adrenoceptor that one of these would account for the α_{1L}-adrenoceptor. However, after the demonstration by Daniels *et al* that all four isoforms displayed an α_{1L}-adrenoceptor pharmacology in functional studies, it seems that no individual isoform represents the distinct pharmacological phenotype of the $\alpha_{1A/a}$- or the α_{1L}-adrenoceptor. The factors that determine this pharmacological pleiotropism remain unknown.

When Chang *et al* analysed the tissue expression of their novel isoform they discovered, surprisingly, that it was the most abundant isoform in the human prostate. It was from the human prostate that Hirasawa *et al* originally cloned the full length human α_{1a}-adrenoceptor (Hirasawa *et al*, 1993) and subsequently cloned α_{1a-2} and α_{1a-3} (Hirasawa *et al*, 1995). Why was it never discovered before?

To date the α_{1a-1}, α_{1a-2}, α_{1a-3} and α_{1a-4} isoforms are the only full length, functional isoforms which have been characterised although a number of truncated isoforms have been isolated in addition to those identified by Chang *et al* (1998). Coge *et al* (1999) cloned nine isoforms of the α_{1a}-adrenoceptor, three of which were the previously identified α_{1a-1}, α_{1a-2} and α_{1a-3}. The remaining isoforms were found to have lost transmembrane domain 7 and were not able to bind [^{3}H]-prazosin specifically. Coge *et al* failed to isolate α_{1a-4}, identified by Chang *et al*, which may be a result of using human liver to isolate their clones rather than the human prostate. However, Chang *et al* demonstrated that the α_{1a-4} isoform was present in the liver in greater amounts than in the prostate.

1.7. mRNA EXPRESSION AND DISTRIBUTION OF α_1-ADRENOCEPTORS

α_1-Adrenoceptors are important mediators of sympathetic nervous system responses and as such are found to be present in the majority of tissues. Mapping the distribution of α_1-adrenoceptor subtypes has been performed mainly by analysis of mRNA expression (e.g. reverse transcriptase polymerase chain reaction (RT-PCR), RNase protection assays, Northern blotting) because a lack of sufficiently selective antagonists makes qualitative analysis by radioligand binding methods difficult to interpret and until recently no subtype selective antibodies were available. However, antibodies specific to the α_1-adrenoceptor subtypes have since become commercially available and have been used to identify the expression and localisation of α_1-adrenoceptor protein. Studies utilising these antibodies have revealed that even expression of the protein of an α_1-adrenoceptor subtype does not guarantee that the subtype is functional (Piascik *et al*, 1997; Hrometz *et al*, 1999; M^{c}Cune *et al*, 2000).

Most of the mRNA work has been carried out in rats and humans, but as the mouse becomes a more popular choice of experimental animal, thanks to the advances in genetic engineering, the focus of this work is shifting and studies investigating the mRNA expression and distribution of murine α_1-adrenoceptors are beginning to emerge (Cavalli *et al*, 1997).

The data which has been collected, detailing the distribution of α_1-adrenoceptors, shows the existence of multiple subtypes in the majority of tissues examined, regardless of the species studied, with one subtype appearing to dominate (Faure *et al*, 1994; Price *et al*, 1994a and b; Schofield *et al*, 1995, Cavalli *et al*, 1997). Generally the mRNA analysis

carried out is non-quantitative. However Schofield *et al* (1995), devised a quantitative RT-PCR method, which relied on known amounts of a competing cRNA product to determine mRNA expression and distribution in 19 rat tissues. Their results were in agreement with the non-qualitative analyses which had already been published (Faure *et al*, 1994, Price *et al*, 1994a; Rokosh *et al*, 1994) but in addition to being able to tell where the specific α_1-adrenoceptor subtype mRNA was expressed Schofield *et al* were also able to determine how much mRNA was expressed. Most of the tissues examined were found to express relatively high levels of the α_{1A}-adrenoceptor mRNA, with the most significant expression being in the vas deferens, colon, stomach, cerebral cortex, heart, small intestine, testis and prostate. α_{1B}-adrenoceptor mRNA was found to have the highest levels of expression in the heart, cerebral cortex and liver with lower levels of expression being found in the rest of the tissues examined. The α_{1D}-adrenoceptor mRNA was expressed at its highest levels in the vas deferens, cerebral cortex, aorta, adrenal gland and, as with the other subtypes, lower levels of expression were found in the rest of the tissues; the exception being the liver where no α_{1D}-adrenoceptor mRNA could be detected (Schofield *et al*, 1995). This is similar to findings in the mouse where the expression of α_{1D}-adrenoceptor mRNA was also detected in all tissues examined except the liver (Cavalli *et al*, 1997). When the expression levels in rat tissues were expressed as a percentage of the total α_1-adrenoceptor mRNA, a dominant subtype was revealed in all tissues except the cerebral cortex. In the pancreas, prostate, small intestine, bladder, stomach, colon, kidney and testis, the dominant subtype was the α_{1A}-adrenoceptor. In the liver, heart and ureter it was the α_{1B}-adrenoceptor which dominated, whereas in the aorta, adrenal gland, trachea, spleen and vas deferens it was the α_{1D}-adrenoceptor (Schofield *et al*, 1995). A previous study has shown that in

addition to the rat aorta expressing α_{1D}-adrenoceptor mRNA it also expresses α_{1B}-adrenoceptor mRNA (Ping and Faber, 1993).

In human tissues, the distribution of α_1-adrenoceptor mRNA was found to be on the whole, similar, although expression of α_1-subtypes did differ in some tissues. The α_{1A}-adrenoceptor mRNA was found to be dominant in the heart, liver, cerebellum and cerebral cortex, (although quantification was in relative, not absolute terms) whilst α_{1B}-adrenoceptor mRNA was predominant in spleen and kidney and in the aorta and adrenal gland it was the α_{1D}-adrenoceptor mRNA which was found to be most abundant (Price *et al*, 1994b). When competitive RT-PCR was carried out, to allow for quantification of the levels of mRNA expression, the results were similar to those obtained by Price *et al* (1994b) who had used a qualitative method. The heart and liver expressed predominantly α_{1A}-adrenoceptor mRNA, spleen and liver were found to express α_{1B}-adrenoceptor mRNA and the aorta was predominantly α_{1D}-adrenoceptor mRNA (Faure *et al*, 1995).

The relationship between mRNA and protein expression does not seem to be an obvious one. The presence of mRNA in a tissue does not guarantee that the protein will be expressed. A prime example of this is the α_{1D}-adrenoceptor. Despite the presence of the mRNA for this subtype in a number of tissues (detailed above) the protein has still to be detected by radioligand binding in rat and mouse tissues (Yang *et al*, 1997; Yang *et al*, 1998; this thesis). However, there is evidence from functional studies that the α_{1D}-adrenoceptor is responsible for vascular contraction in the rat aorta, carotid, femoral and iliac arteries as well as the mouse aorta and carotid artery (Kenny *et al*, 1995; Nagadeh,

1996, University of Glasgow, PhD thesis; Piascik et al, 1997; Hrometz et al, 1999; Daly *et al*, unpublished observations; this thesis).

In combination with functional and radioligand binding experiments it appears that as well as possessing the mRNA for multiple subtypes, many tissues also express multiple α_1-adrenoceptor subtypes at the protein level. The liver appears to be one of the few tissues with a homogenous population of α_1-adrenoceptors, although the subtype expressed between species can differ. The rat, mouse and hamster appear to express the α_{1B}-adrenoceptor (Garcia-Sainz *et al*, 1994; this thesis), whereas the guinea-pig, rabbit, dog and human liver express the α_{1A}-adrenoceptor (Garcia-Sainz *et al*, 1992, Garcia-Sainz *et al*, 1995a and b).

The functional significance for this heterogeneity among species and even within tissues is unknown. It is also unclear whether tissue heterogeneity is a result of multiple cell types expressing a single subtype or if it is due to coexpression of subtypes by cells (Zhong and Minneman, 1999a). Clearly mRNA analysis on its own is insufficient to determine which α_1-adrenoceptors are important within a specified tissue. However, when combined with functional and radioligand binding experiments it can provide further insight into the expression, distribution and functionality of α_1-adrenoceptors.

1.8. VASCULAR α_1-ADRENOCEPTORS

α_1-Adrenoceptors are found throughout the arterial and venous circulation where they have an important function in the regulation and maintenance of vascular resistance and blood flow. In most mammalian species contraction of vascular smooth muscle is

predominantly mediated via α_1-adrenoceptors. Through the desire to characterise and subtype these vascular α_1-adrenoceptors our knowledge of which subtypes are present in a particular blood vessel and contributing to vascular contraction has deepened, although not without a certain amount of debate and discussion among groups. The presence of a subtype as identified by radioligand binding or mRNA analysis does not guarantee that it contributes to contraction. Therefore a functional analysis should always be carried out, preferably in conjunction with at least one of the aforementioned techniques. The process of subtyping α_1-adrenoceptors involved in vasoconstriction generally uses Schild analysis to produce a pA_2 value, representative of the affinity of an antagonist at the α_1-adrenoceptor in the vessel of interest.

The α_1-adrenoceptors in various vascular beds of the rat are well characterised, within the limitations of available antagonists. The α_1-adrenoceptor subtype(s) involved in vasoconstriction of all the major arteries have been identified. A lot of work has also been carried out on rabbit and dog blood vessels, with less being known about human vessels. Blood vessels from mice have largely been ignored because the mouse is such a small animal that most of the arteries have been considered too small to be studied. However this trend appears to be changing. As the mouse becomes popular through genetic engineering, there is a need for knowledge of its physiology and pharmacology, in order to identify the significance of changes brought about by manipulation of its genome.

What follows is a brief overview, detailing the subtypes of α_1-adrenoceptor proposed to be involved in vasoconstriction of some of the more frequently studied arteries.

1.8.1. Aorta

A lot of confusion and ambiguity surrounded the identity of the subtype(s) involved in vasoconstriction of the rat aorta, up until about 5 years ago. When α_1-adrenoceptor subtyping was in its infancy and the choice of subtype selective antagonists was few, the rat aorta was characterised as containing the α_{1B}-adrenoceptor, based on findings that CEC almost completely abolished the contractile response to noradrenaline, whilst nifedipine had no effect (Han *et al*, 1990). This was at a time when it was believed that α_{1A}-adrenoceptors were dependent on Ca^{2+} influx through dihydropyridine sensitive Ca^{2+} channels, therefore it was believed that nifedipine would block α_{1A}-mediated responses. Based on this train of thought it appeared that the α_{1B}-adrenoceptor mediated contraction in the rat aorta. However, Muramatsu *et al* (1991a) found the rat aorta to be sensitive to the effects of nifedipine and to produce high affinities for WB4101 and benoxathian and concluded that the α_1-adrenoceptor in the rat aorta was atypical.

Heterogeneity of the α_1-adrenoceptors in rat aorta was proposed by Van der Graaf *et al* (1993; 1994; 1996a) when they found that several of the antagonists they had used caused steepening of the noradrenaline concentration response curve and could be accounted for more accurately with a 2-site receptor model.

The barrage of conflicting results and conclusions continued when Aboud *et al* (1993) proposed that the rat aorta's α_1-adrenoceptor population was non-α_{1A}, non-α_{1B}. They found WB4101, benoxathian and 5-methylurapidil to bind with high affinity, suggestive of an α_{1A}-adrenoceptor. However they found the contractile response to be sensitive to CEC. These results were similar to Muramatsu's (1991a). Aboud *et al* did tentatively

suggest that the α_1-adrenoceptor was the α_{1D}-adrenoceptor, which had been cloned and found to be present in rat aorta by Perez *et al* (1991).

In 1995, Kenny *et al*, brought some clarity to the situation by using the recently identified $\alpha_{1D/d}$-adrenoceptor antagonist, BMY7378, in their analysis of the rat aorta. In their hands BMY7378 had a pK_B of 8.3, which in combination with their other antagonist data suggested the identity of this mysterious α_1-adrenoceptor to be the much sought after native α_{1D}-adrenoceptor. This has been confirmed by a number of other studies (Piascik *et al*, 1995; Testa *et al*, 1995b; Deng *et al*, 1996; Hussain and Marshall, 1997; Piascik *et al*, 1997; Guimaraes and Moura, 2001).

Recently the α_1-adrenoceptor population of the rat aorta has been proposed to be constitutively active (Gisbert *et al*, 2000). It was discovered that after depletion of intracellular Ca^{2+} stores and subsequent refilling of these stores by the addition of Ca^{2+}, there was an increase in the resting tone of the aorta that wasn't apparent in the tail artery when it was subjected to the same conditions. When these conditions were reproduced to investigate the accumulation of inositol phosphates, the basal level of inositol phosphate accumulation was significantly increased in the aorta, but not the tail artery. Both the increase in resting tone and the increased inositol phosphate accumulation could be inhibited by the α_1-antagonists, prazosin, BMY7378 and 5-methylurapidil. The subtype-selective antagonists produced pIC_{50}'s which correlated with binding affinities at α_{1D}-adrenoceptors, suggesting that it was the α_{1D}-adrenoceptor population of the rat aorta that was constitutively active. This is the first time that constitutive activity has been suggested for a functioning native receptor.

Functional analysis of the rabbit aorta reveals a role for a mixed population of α_1-adrenoceptors. Satoh and colleagues suggest the presence of α_{1A}- and α_{1B}-adrenoceptors, as revealed by sensitivity to CEC inactivation and a biphasic response to WB4101. They proposed that the high affinity site observed with WB4101 was the α_{1A}-adrenoceptor and the low affinity site was the α_{1B}-adrenoceptor (Takayanagi *et al*, 1991). This is consolidated by results obtained with BMY7378, which was found to have a low affinity in the rabbit aorta ($pA_2 = 6.6$) ruling out the possibility of a contribution to contraction from the α_{1D}-adrenoceptor (Satoh *et al*, 1999a; 1999b). Muramatsu and colleagues have also studied the rabbit aorta reaching a slightly different conclusion. They identified the presence of the α_{1L}-adrenoceptor in the rabbit aorta (Muramatsu *et al*, 1990; Oshita *et al*, 1993). This was one of the tissues used by Muramtasu when he expanded the α_{1H}-, α_{1L}-subclassification of α_1-adrenoceptors to include the α_{1N}-adrenoceptor. The rabbit aorta was shown to have low affinity for prazosin (Muramatsu *et al* 1990). A further study, by the same group, concentrating on the rabbit aorta revealed two distinct sites when radioligand binding with [^{3}H]-prazosin was performed on aortic membranes. When this was repeated after pre-treatment with CEC the data fitted better to a one-site model. In functional studies investigating the contractile response to noradrenaline, prazosin was again found to exhibit two distinct affinity values. The affinities of WB4101 and HV723 were approximately equal to the low affinity site for prazosin, providing no evidence for the α_{1A}- or α_{1N}-adrenoceptor (Oshita *et al*, 1993). From this data they concluded that the rabbit aorta contains the α_{1B}- and α_{1L}-subtypes of α_1-adrenoceptor.

1.8.2. Carotid artery

Despite the carotid artery being a major conduit vessel carrying blood to the brain, it has largely been overlooked when it comes to α_1-adrenoceptor subtyping. Instead, it is more commonly used as a model to study the effects of vascular remodelling. The pharmacology of the carotid artery is the basis for Chapter 3 of this thesis. Therefore our present knowledge of its pharmacology, with particular reference to α_1-adrenoceptors will be discussed in detail later.

1.8.3. Mesenteric artery

Mesenteric arteries are a widely used *in vitro* preparation because they provide an easily isolated supply of resistance arteries in which to study the maintenance and regulation of blood pressure. With regard to the subtyping of α_1-adrenoceptors, the mesenteric arteries are no different from other arteries in that there are always conflicting results as to the identity of the particular subtype, based on the drugs used and the experimenter's interpretation of the results.

The α_1-adrenoceptor subtype seems to differ between the superior and resistance mesenteric arteries. Kong *et al* (1994) found the superior to contain mainly α_{1B}-adrenoceptors based on a relative insensitivity to 5-methylurapidil and a potent inhibition by CEC, whilst in the perfused mesenteric vasculature, consisting of mainly resistance arteries, the opposing effects were found to be true. The resistance arteries were found to be highly sensitive to α_{1A}-antagonism by 5-methylurapidil and were much less sensitive to CEC, resulting in the conclusion that the resistance arteries consisted of mostly α_{1A}-adrenoceptors.

Piascik and colleagues (1997) also identified different subtypes in the superior and resistance mesenteric arteries of the rat in an elegant study using an antibody specific for the α_{1B}-adrenoceptor, in combination with receptor protection studies. The α_{1B}-adrenoceptor antibody stained the medial layer of all the vessels examined, which included both the superior and resistance arteries of the mesentery, demonstrating expression of the α_{1B}-adrenoceptor protein. In functional studies, Piascik *et al* used BMY7378 and A-61603 to protect α_{1D}- and α_{1A}-adrenoceptors respectively, from alkylation by either phenoxybenzamine or CEC. BMY7378 was found to protect the superior mesenteric artery from irreversible alkylation by phenoxybenzamine and CEC suggesting that contraction in this artery is due to activation of α_{1D}-adrenoceptors. On the other hand, the resistance arteries of the mesentery were not protected by either BMY7378 or A-61603 suggesting that it is the α_{1B}-adrenoceptor through which this artery mediates contraction. Therefore, as with mRNA expression it seems that protein expression of a subtype does not guarantee a functional role. Hussain and Marshall (2000) have recently characterised the rat mesenteric artery as mediating contraction through the α_{1B}- and α_{1D}-adrenoceptors when they found that all subtype selective antagonists used produced shallow Schild slopes. 5-methylurapidil was found to have a low pK_B and BMY7378 a high pK_B in this artery.

To add to the confusion, a role for the α_{1L}-adrenoceptor in the resistance vessels of the rat mesentery has also been proposed, when Van der Graaf *et al* (1996b) found prazosin to have a low affinity in this tissue. The same group in a later paper found that the rat mesenteric artery was insensitive to CEC and produced low pA_2 values with BMY7378 and RS17053 arguing against the involvement of α_{1B}, α_{1D} or α_{1A}-adrenoceptors

respectively (Stam *et al*, 1999). These results led to the conclusion that the α_{1L}-adrenoceptor is the predominant α_1-adrenoceptor subtype in the rat mesenteric artery.

The apparent confusion over which subtypes are present in the rat mesenteric artery may be a result of vessels from different areas in the mesentery being used because not all groups stated from which part of the mesentery they had isolated their vessels.

The α_{1L}-adrenoceptor has also been proposed to be responsible for vasoconstriction in the rabbit mesenteric artery by two separate groups. Muramatsu *et al* (1990) found it to have low affinity for prazosin and yohimbine, satisfying two of the criteria for an α_{1L}-adrenoceptor (Flavahan and Vanhoutte, 1986) and Van der Graaf *et al* (1997) found the rabbit mesentery to have low affinity for prazosin and a number of selective α_{1A}-antagonists, such as 5-methylurapidil, WB4101 and RS-17053. Involvement of the α_{1D}-adrenoceptor was ruled out by a low affinity for BMY7378.

Muramatsu and colleagues have characterised the canine mesenteric artery as belonging to the α_{1N}-subtype of α_1-adrenoceptors. HV723 has been proposed to be an α_{1N}-selective antagonist and it potently inhibits the contractions of the dog mesenteric artery. In addition to this it has low affinity for prazosin, WB4101 and 5-methylurapidil (Muramatsu *et al*, 1990; Kohno *et al*, 1994).

More recently, Daniel *et al* (1999) found evidence for the involvement of the α_{1A}-adrenoceptor in mediating contraction in the dog mesenteric artery. They showed that a number of selective α_{1A}-antagonists potently inhibited contraction in the canine mesentery when the concentrations of antagonist used were low. But as the concentration increased the pK_B decreased, to values expected of an α_{1L}-adrenoceptor.

They concluded that the canine mesenteric artery contains α_{1A}-adrenoceptors with the possible presence of an α_{1L}-adrenoceptor.

1.8.4. Miscellaneous

In addition to the blood vessels of the mesentery (discussed above), Piascik *et al* (1997) also studied the caudal, femoral, iliac and renal arteries in an attempt to correlate the expression of α_1-adrenoceptors on vascular smooth muscle with the regulation of contraction in these blood vessels. Using the same techniques as described previously for the mesenteric arteries, the α_{1B}-adrenoceptor antibody was found to stain the medial layer of all the arteries mentioned above. In the receptor protection studies, BMY7378 (at a concentration which would occupy more than 90% of the α_{1D}-adrenoceptor population, whilst occupying less than 6% of either the α_{1A}- or α_{1B}-adrenoceptors), protected the femoral and iliac arteries but had no protective effect on the caudal or renal arteries. In contrast, when A-61603 was used, it showed protective activity in the caudal and renal arteries whilst no effect was seen in the femoral and iliac arteries. From this, Piascik *et al* concluded that despite the evidence of expression of the α_{1B}-adrenoceptor protein in these vessels it played no part in the regulation of contraction. Rather, it was the α_{1D}-adrenoceptor that produced a contraction in the femoral and iliac arteries and the α_{1A}-adrenoceptor in the caudal and renal arteries. The involvement of these subtypes in the contraction of the femoral and renal arteries has since been confirmed (Hrometz *et al*, 1999).

1.9. α_1-ADRENOCEPTORS AS MITOGENS

Traditionally, mitogenic responses were believed to be mediated primarily by peptide growth factors such as epidermal growth factor and nerve growth factor. Mitogenic responses are caused by a conserved cascade of events including receptor phosphorylation, binding of adaptor proteins (e.g. Shc and Grb2) and the subsequent recruitment and activation of the small molecular weight G protein, Ras (Zhong and Minneman, 1999a). Ras activates Raf which then activates downstream mitogen activated protein kinase (MAPK) cascades (Zhong and Minneman, 1999a). It is now apparent that GPCRs are capable of rapidly activating MAPK pathways and that this activation plays an important role in the regulation of cell proliferation and growth. Despite intensive study the mechanisms underlying these effects remains unknown.

α_1-Adrenoceptors are able to activate mitogenic responses in a variety of cell types and would appear, increasingly, to play an important role in regulating cell growth and proliferation. α_1-Adrenoceptor agonists stimulate hypertrophy in cultured neonatal rat ventricular myocytes (La Morte *et al*, 1994) and overexpression of a constitutively active mutant of the α_{1B}-adrenoceptor in cardiac myocytes induces myocardial hypertrophy in transgenic animals (Milano *et al*, 1994). More recently a transgenic mouse, which overexpresses the α_{1B}-adrenoceptor in all tissues which naturally express the α_{1B}-adrenoceptor has been shown to display early signs of cardiac hypertrophy (Zuscik *et al*, 2001). A role for α_1-adrenoceptors in the regulation of vascular smooth muscle growth was revealed in rat aortic smooth muscle cells where a CEC-sensitive α_1-adrenoceptor was shown to mediate proliferation-independent hypertrophy whilst non-CEC-sensitive α_1-adrenoceptors opposed this effect. These actions were attributed

to the α_{1B}- and α_{1A}-adrenoceptors respectively, based on the α_{1B}-adrenoceptor's sensitivity to CEC and the α_{1A}-adrenoceptor's high affinity for 5-methylurapidil (Chen *et al*, 1995).

α_1-Adrenoceptors activate MAPK pathways with coupling efficiencies similar to those observed for second messenger responses, in that the α_{1a}-adrenoceptor couples most efficiently followed by the α_{1b}-adrenoceptor, with the α_{1d}-adrenoceptor coupling least effectively of the three (Zhong and Minneman, 1999b). Activation of the α_{1a}-adrenoceptor has recently been found to cause a much wider array of transcriptional responses than growth factor receptor activation (Minneman *et al*, 2000), which is surprising given the traditional view that mitogenic signalling is mediated by growth factor receptors.

1.10. DESENSITISATION AND INTERNALISATION

Many receptor-coupled cellular responses become desensitised as the result of agonist-induced changes in the chemical and physical properties of their signal transduction pathways (Cowlen and Toews, 1988). Desensitisation is a loss of functional response from the receptor and is an adaptive mechanism thought to facilitate responsiveness of the cell to successive multiple extracellular stimuli over time (Chuang *et al*, 1996). The loss of functional response can be short term (seconds or minutes) or long term (hours).

For GPCRs desensitisation is a multistep phenomenon. The first step is the uncoupling of the phosphorylated receptor from the G protein resulting in desensitisation of receptor function. The receptor is then sequestered into an intracellular compartment

which is believed to be a mechanism that allows dephosphorylation (by specific phosphatases) and resensitisation of the receptor. If the stimulation is chronically persistent then the receptor is downregulated. The decrease in receptor number is a result of the internalised receptors being degraded, rather than being recycled to the plasma membrane and reduction of the steady-state mRNA which is mostly due to a decrease in the stability of the mRNA (Chuang *et al*, 1996).

There are 2 different classes of desensitisation, homologous and heterologous. Homologous desensitisation is an agonist-specific process. The decreased responsiveness is observed exclusively in the agent (in this case the agonist) that originally stimulated the response e.g. neurotransmitter and hormone receptors (Garcia-Sainz *et al*, 2000). With heterologous desensitisation the decreased responsiveness is observed in an agent or agents unrelated to the initial stimulus e.g. second messengers (Garcia-Sainz *et al*, 2000). This classification scheme is purely an operational one so it is feasible that both processes occur simultaneously in cells.

Homologous desensitisation involves receptor phosphorylation by G protein receptor coupled kinases (GRKs). GRKs are a family of at least 6 serine/threonine protein kinases that phosphorylate GPCRs only in the agonist-bound state (Garcia-Sainz *et al*, 2000). This strict agonist-binding requirement for GRK-mediated phosphorylation makes this mechanism strictly homologous. The GRK family includes GRK 1 (rhodopsin kinase), GRK 2 (β-adrenergic receptor kinase-1), GRK 3 (β-adrenergic receptor kinase-2), GRK 4, GRK 5 and GRK 6 (Grady *et al*, 1997). When a receptor is occupied by an agonist the activated G protein releases the Gβγ complex. This membrane bound Gβγ heterodimer and phosphatidylinositol bisphosphate bind to the

carboxy terminal domain of the GRK which targets the kinase to the receptor (Garcia-Sainz *et al*, 2000). These enzymes then phosphorylate the receptors which markedly increases the affinity of the receptor for arrestin, the functional cofactor of the GRKs. Without binding of arrestin minimal desensitisation occurs but once arrestin has bound desensitisation reaches a maximum (Chuang *et al*, 1996).

The majority of what we know about desensitisation and internalisation of α_1-adrenoceptors has come about through studying the $\alpha_{1B/b}$-adrenoceptor, purely because it was the first α_1-adrenoceptor to be cloned for which it was agreed, early on, that the native and cloned receptors were identical.

Noradrenaline has been shown to markedly increase receptor phosphorylation of the hamster α_{1b}-adrenoceptor (Lattion *et al*, 1994). This can be inhibited by truncation of the carboxy terminal of the receptor, implying that this part of the receptor plays an important part in receptor phosphorylation and ultimately desensitisation. Further studies by Cotecchia and colleagues implicated GRK 2 and 3 as playing a role in agonist-induced phosphorylation of the α_{1b}-adrenoceptor. When GRK 2 and 3 were overexpressed with the α_{1b}-adrenoceptor there was a decrease in the sensitivity of the receptor for the agonist and a decrease in its ability to reach the maximum response (classical signs of desensitisation) as well as an increase in agonist-induced phosphorylation of the receptor (Diviani *et al*, 1996). In the same study GRK 5 and GRK 6 were shown to be capable of increasing the phosphorylation of the α_{1b}-adrenoceptor, although only GRK 6 was able to increase agonist-induced phosphorylation, whilst GRK 5 could increase only basal phosphorylation levels. Using site-directed mutagenesis, the phosphorylation sites involved in GRK-mediated

desensitisation have been identified as being 3 serine residues (Ser^{404}, Ser^{408} and Ser^{410}) which are located in the carboxy terminal (Diviani *et al*, 1997).

The consequence of desensitisation of the receptor is its internalisation into the cell. Agonist-promoted internalisation of the α_{1b}-adrenoceptor is sensitive to cytochalasin D (an actin depolymerisation agent) but insensitive to hypertonic treatment (may inhibit clathrin-mediated endocytosis) suggesting that the α_{1b}-adrenoceptor is endocytosed via a non-coated pathway (Hirasawa *et al*, 1998). Contradicting this are results from Fonseca *et al* (1995) who have proposed that α_{1b}-adrenoceptors are internalised in coated vesicles upon discovering that transferrin (a classic endosomal marker which is internalised in clathrin coated vesicles) colocalised with the α_{1b}-adrenoceptor after 1 hour of noradrenaline exposure. They also found that internalisation could be blocked by hypertonic treatment. These differences could indicate that the mechanism of internalisation is dependent on cell type; Hirasawa used CHO cells whereas Fonseca used HEK293 cells.

Many GPCRs are desensitised via feedback regulation by second messenger-stimulated kinases, such as PKA and PKC. This type of desensitisation can be classed as heterologous, since in theory, any stimulant that can increase cAMP or DAG has the potential to induce the phosphorylation and desensitisation of any GPCR containing the consensus phosphorylation sites for PKA or PKC (Garcia-Sainz *et al*, 2000). Phosphorylation by PKA and PKC can lead to a significant (40-50%) loss of receptor function (Chuang *et al*, 1996).

Originally, it was in rat hepatocytes that activation of PKC with phorbol esters was shown to block the action of the α_{1b}-adrenoceptor (Corvera and Garcia-Sainz, 1984). Then Bouvier *et al* (1987) observed that PKC induced receptor phosphorylation and that this phosphorylation was further increased by noradrenaline *in vitro*. Recently mouse fibroblasts expressing the human α_{1b}-adrenoceptor have been shown to be phosphorylated in response to activation of PKC by phorbol esters (Garcia-Sainz *et al*, 2000). The phosphorylation was shown to be associated with blockade of α_{1b}-adrenoceptor mediated increases in cytosolic Ca^{2+} and phosphoinositide hydrolysis.

The action of phorbol esters on α_{1b}-adrenoceptors seems to involve several processes. Blockade of receptor function is very rapid, which is consistent with receptor phosphorylation and uncoupling from G proteins (Garcia-Sainz *et al*, 2000). Receptor internalisation in cells endogenously expressing α_{1B}-adrenoceptors has also been observed with activation of PKC e.g. hepatocytes (Beeler and Cooper, 1995) and also in transfected cells e.g. HEK293 cells (Fonseca *et al*, 1995).

α_{1b}-Adrenoceptor phosphorylation has also been observed via receptors coupled to G_q, G_i and receptors with endogenous tyrosine kinase activity, for a detailed review see Garcia-Sainz *et al* (2000).

1.11. TRANSGENIC TECHNIQUES AND THEIR APPLICATION TO α_1-ADRENOCEPTORS

Over the past decade genetically altered animals have become routine in many areas of biomedical research. The animal of choice for such procedures has, on the whole, been

the mouse, although larger animals have been used with some success. The ability to manipulate gene expression *in vivo* has led to a greater understanding of the physiological functions conveyed by genes and in some cases revealed the presence of redundant or compensatory signalling systems. A number of transgenic techniques have been developed, which include:-

Pronuclear microinjection of DNA:- The DNA of interest is injected into fertilised mouse eggs. Integration of the DNA vector into the genome is random. Injected eggs are then implanted into pseudopregnant females and following birth and weaning the pups are screened to see which have acquired the transgene.

Inducible gene expression system:- This requires 2 separately encoded transgenes, one which codes for the protein of interest which is under the control of an inducible promoter and the other encodes for a transactivator that is able to modulate expression of the receptor in an inducer-specific fashion. This approach provides the experimenter with the ability to switch the transgene on and off, as and when required. This avoids potential problems associated with transgene expression at unwanted times during development. E.g. The tetracycline-inducible Tet transactivator, where the presence or absence of tetracycline switches the transgene off or on.

Gene targeting:-

1. 'Knockout' strategy:- An endogenous genetic locus can be specifically altered by disruption of the normal expression pattern or by replacing the endogenous gene with a modified version. A common approach is to inactivate the gene of interest by insertion of a reporter or drug resistance gene into the desired locus, via homologous

recombination in embryonic stem cells. The targeted embryonic stem cells are microinjected into blastocysts and allowed to invade and populate the host embryo, which is then implanted into a pseudopregnant female. Chimeric pups are identified by mixed coat colour since the host embryos and targeted embryonic stem cells are derived from mouse strains differing in coat colour.

2. 'Hit and Run' strategy:- This procedure attempts to alter genes in a more subtle fashion than the 'knockout' strategy, which theoretically results in complete loss of gene function. The targeting vector containing a mutated sequence is firstly inserted to generate a duplication of a portion of the gene. Then, the normal sequence is excised. This technique allows for very subtle mutations to be engineered into the targeting vector and hence into the genome.

3. 'Knock-in' strategy:- This technique replaces one coding sequence with that of another, while maintaining the genomic sequence context of the original gene.

All definitions are from Rohrer and Kobilka, 1998 and Wei, 1997.

The application of transgenic techniques to α_1-adrenoceptors has concentrated on the α_{1b}-adrenoceptor. Cardiac-specific overexpression of a constitutively active mutant of the α_{1b}-adrenoceptor was initially carried out in mice to study the well-known phenomenon of α_1-adrenoceptor mediated cardiac hypertrophy (Milano *et al*, 1994). This mutant had been previously shown to possess increased agonist affinity and receptor/G_q coupling in the absence of any agonist, when expressed in cells (Cotecchia *et al*, 1990). In this instance it was used to explore the role of α_1-adrenoceptor signalling

and hypertrophic responses in the absence of any underlying hypertension or increased afterload (Rohrer and Kobilka, 1998). These animals were found to have elevated DAG levels (indicative of PLC activation), increased myocyte cross-sectional area, increased heart weight/body weight ratio and an increase in atrial natriuretic factor (ANF) mRNA (a well known marker for cardiac hypertrophy) (Milano *et al*, 1994).

The same group produced transgenic mice with cardiac-specific overexpression of the WT α_{1b}-adrenoceptor (Akhter *et al*, 1997). Total cardiac α_1-adrenoceptor was approximately 40-fold higher than in WT animals, DAG content and ANF mRNA were also increased. In fact the increase in ANF mRNA was double that which was seen with the constitutively active α_{1b}-adrenoceptor mouse. Despite the high levels of ANF mRNA the animals showed no signs of cardiac hypertrophy (Akhter *et al*, 1997). Both mouse models show signs of increased signalling but the reasons behind why only one of them displays signs of cardiac hypertrophy remains to be explained.

As well as creating mice which overexpress the α_{1B}-adrenoceptor, gene targeting has been used to create a mouse with disrupted expression of the α_{1B}-adrenoceptor, which results in inactivation of the α_{1B}-adrenoceptor gene (Cavalli *et al*, 1997). The pressor response to phenylephrine has been shown to be decreased in this mouse, although the basal blood pressure is unaltered. Isolated aortic rings were shown to have a decreased responsiveness to α_1-adrenoceptor agonists, although results from our own group find the opposite to be true. In isolated aortic and carotid rings the sensitivity to phenylephrine in these α_{1B}-adrenoceptor knockout mice is increased. The α_1-adrenoceptor pharmacology of this mouse model is the basis of this thesis and so will

not be discussed in any more detail here. From this point, KO will be used to refer to the α_{1B}-adrenoceptor knockout mouse and WT to the non-knockout mouse.

Towards the end of my project two strains of mice were developed, one which overexpressed the α_{1B}-adrenoceptor and the other which expressed a constitutively active α_{1B}-adrenoceptor (Zuscik *et al*, 2001). The expression of these mutated α_{1B}-adrenoceptors was under the control of the mouse promoter, which meant that these receptors would only be expressed in cells that naturally expressed the α_{1B}-adrenoceptor. The *in vitro* responses of mesenteric and carotid arteries of these mice to phenylephrine are studied in Chapter 3 of this thesis. This was due to initial findings that these mice were hypotensive (Zuscik *et al*, 2001) and the group who originally designed the mice wanted to know if this was due to the mutated α_{1B}-adrenoceptors.

CHAPTER 2 – CHARACTERISATION OF THE α_1-ADRENOCEPTOR SUBTYPES IN BRAIN AND LIVER TISSUES FROM WT AND KO MICE

2.1. INTRODUCTION

2.1.1. α_1-Adrenoceptors in the brain

α_1-Adrenoceptors are present throughout the brain with their distributions varying among species. In the rat brain the highest density of α_1-adrenoceptors is found in the olfactory bulb, particularly in the neocortex and thalamus, with a similar distribution being found in the mouse and guinea-pig (Palacios *et al*, 1987). In the rhesus monkey and human the distribution is somewhat different, with the highest densities being present in the hippocampus, which in the rat, mouse and guinea-pig had the lowest distribution of α_1-adrenoceptors (Palacios *et al*, 1987).

With respect to α_1-adrenoceptor subtypes, the brain is typical of most organs in that it contains a mixed population of α_1-adrenoceptors. It was in rat brain that the presence of α_1-adrenoceptor subtypes was first discovered when WB4101 was found to bind with high and low affinities (Morrow and Creese, 1986). These high and low affinity sites were designated α_{1A}- and α_{1B}-adrenoceptors respectively. The heterogeneity of α_1-adrenoceptors in the rat brain has been confirmed in further studies; most groups concur with Morrow and Creese that the rat brain contains a mixed population of α_{1A}- and α_{1B}-adrenoceptors (Han *et al*, 1987b; Minneman *et al*, 1988; Terman *et al*, 1990; Yang *et al*, 1997). However, Oshita *et al* (1991) detected the presence of three α_1-adrenoceptor subtypes in the rat cerebral cortex. Saturation binding with [^{3}H]-prazosin revealed two sites and inhibition binding with phentolamine and WB4101 showed that the high affinity site detected in saturation studies was inhibited biphasically by phentolamine and WB4101, as well as there being high and low affinity sites for prazosin. They

concluded that there were three α_1-adrenoceptors present, α_{1A}-, α_{1B}- and α_{1L}-adrenoceptors (Oshita *et al*, 1991).

Although there is no radioligand binding data to support the presence of the α_{1D}-adrenoceptor in the rat brain, the mRNA for this α_1-adrenoceptor has been shown to be present (Schofield *et al*, 1995). In addition to this, the α_{1D}-adrenoceptor protein has been detected by Western blotting in the neonate and adult rat brain (Shen *et* al, 2000). This provides more evidence for the unusual nature of the $\alpha_{1D/d}$-adrenoceptor. Despite the presence of the mRNA for this subtype in the majority of tissues examined, there are only a handful of rat tissues known to elicit a functional response through the α_{1D}-adrenoceptor. These include the aorta (Kenny *et al*, 1995; Piascik *et al*, 1995; Deng *et al*, 1996), the carotid artery (Nagadeh, 1996, University of Glasgow, PhD thesis), the iliac artery (Piascik *et al*, 1995) and the femoral artery (Hrometz *et al*, 1999). It has been suggested that the $\alpha_{1D/d}$-adrenoceptor is poorly expressed in the plasma membrane, since studies looking at rat-1 fibroblasts transfected with the α_{1d}-adrenoceptor found it to be mainly located intracellularly (M^{c}Cune *et al*, 2000).

The α_1-adrenoceptors in the pig and mouse brain appear to have a pharmacological profile similar to the rat. The pig cerebral cortex expresses both α_{1A}- and α_{1B}-adrenoceptors with a ratio of 64%:36% in favour of the α_{1A}-adrenoceptor (Wikberg-Matsson *et al*, 1998). The mouse brain also contains α_{1A}- and α_{1B}- adrenoceptors. However the ratio of subtypes differs from the pig. Cavalli *et al* (1997) report that only 42% of the total α_1-adrenoceptors are of the α_{1A}-subtype with the remaining 58% being

made up of the α_{1B}-subtype, whilst Yang *et al* (1998) report a proportion of 30%:70%, in favour of the α_{1B}-adrenoceptor.

A lack of antagonists, which are both subtype-selective and able to penetrate the blood brain barrier, makes it virtually impossible to allocate a particular central effect to a specific subtype. However, transgenic mice which either overexpress the WT α_{1B}-adrenoceptor or a constitutively active α_{1B}-adrenoceptor have provided a means of characterising the central effects mediated by α_{1B}-adrenoceptors. At 3 months of age these mice showed hindlimb dysfunction which manifested itself in abnormal gait, sprawling and dragging of hindlimbs, a lack of spontaneous locomotion and a prevalence of tremor at rest (Zuscik *et al*, 2000). This locomotor phenotype was reported to resemble Parkinsonism, which is in agreement with Cash *et al* (1984) who found that the density of α_1-adrenoceptors was increased in Parkinson patients who also had dementia. In addition to the locomotor impairment, 12 month old transgenic mice were found to suffer from grand mal-type seizures characterised by behavioural arrest, facial twitching, loss of balance, forelimb automatism, whole-body jerking and hypersalivation (Zuscik *et al*, 2000). The percentage of mice showing seizures correlated with the level of α_{1B}-adrenoceptor overstimulation i.e. constitutively active mice had a higher percentage of seizures than overexpressed mice. Both the impaired locomotor function and the seizures could be reversed by terazosin, an α_1-adrenoceptor antagonist. These mice also showed signs of neurodegeneration which began in areas that express a high density of α_{1B}-adrenoceptors such as the cortex, hypothalamus and cerebellum, progressing with age to encompass all brain areas (Zuscik *et al*, 2000). The central effects of deleting the α_{1B}-adrenoceptor from the KO mouse created by Cavalli *et al* (1997) has not been studied. However, in the light of these findings by Zuscik *et al*

(2000) it would be of interest to determine if the central effects in this mouse are the opposite of what Zuscik *et al* (2000) observed in mice with overstimulation of the α_{1B}-adrenoceptor.

2.1.2. α_1-Adrenoceptors in the liver

The presence of α_1-adrenoceptors in the liver is a well-documented phenomenon and is hardly surprising given that α_1-adrenoceptors are found throughout the body. What is unusual is that the liver is one of the few organs that has been found to contain a relatively pure population of one α_1-adrenoceptor subtype, in practically every species investigated. The specific subtype differs between species but the overall homogeneity appears to be a characteristic of hepatic α_1-adrenoceptors.

Garcia-Sainz and colleagues (1992-1996) have contributed substantially to our knowledge of α_1-adrenoceptors in the liver, identifying and characterising the α_1-adrenoceptor subtype in the liver of no fewer than ten species, ranging from the mouse and hamster to the rhesus monkey and human. The methodology used was similar for each species with slight differences in the antagonists used depending on the popular subtype-selective antagonists at the time. CEC was used to determine the percentage of receptors susceptible to inactivation and binding was carried out using agonists and antagonists in a bid to determine orders of potency. The agonists used throughout were adrenaline, noradrenaline, methoxamine and oxymetazoline. Surprisingly, the order of potency for the agonists was the same, regardless of the subtype concluded to be expressed. The order of potency was:-

Oxymetazoline > adrenaline > noradrenaline > methoxamine

The only difference between species was in the absolute affinity values. For example, in livers which were subtyped as α_{1A} (cat, dog and human (Garcia-Sainz *et al*, 1996b; 1995a; 1995b)) oxymetazoline produced nanomolar affinity values, whereas in α_{1B}-expressing livers (mouse, rat and hamster (Garcia-Sainz *et al*, 1992; 1994)) it had micromolar affinity values.

The antagonists used for the earlier competitive binding studies (Garcia-Sainz *et al*, 1992; 1994; 1995a; 1995b) were as follows: prazosin, WB4101, phentolamine, benoxathian, 5-methylurapidil and (+)-niguldipine. Prazosin and phentolamine were relatively unselective, whilst the remaining antagonists showed selectivities expected of α_{1A}-selective antagonists. The later studies on rhesus monkey and cat liver utilised additional antagonists, which had not been available for the other studies, i.e. tamsulosin (α_{1A}-selective) and BMY7378 (α_{1D}-selective) for the rhesus monkey (Garcia-Sainz *et al*, 1996a) and spiperone (α_{1B}-selective) and BMY7378 for the cat liver (Garcia-Sainz *et al*, 1996b).

The conclusions reached regarding the α_1-adrenoceptor subtype expressed in the liver of each species are summarised in tabular form in Figure 2.1

An interesting pattern is revealed when the results are presented like this. When species such as human, rhesus monkey, cat and dog are used in experiments they tend to be further on in their life cycle than animals such as mice, rats and hamsters. These are also the species which express α_{1A}-adrenoceptor's whereas the mice, rats and hamsters all express the α_{1B}-adrenoceptor. An age-related change in the α_1-adrenoceptors of the rat aorta has been documented by Gurdal *et al* (1995). 1 Month old rats were shown to

SPECIES	CONCLUSION
Mouse	$1B^{1}$
Hamster	$1B^{1}$
Rat	$1B^{1,2}$
Guinea-pig	$1A^{2}$
Rabbit	$1A^{2}$
Cat	$1A^{3}$
Dog	$1A^{4}$
Rhesus monkey	$1A/1B^{5}$
Human	$1A^{6}$

Figure 2.1:- Conclusions reached regarding the identity of the α_1-adrenoceptor subtype in the livers of these species. [1]Garcia-Sainz *et al*, 1994; [2]Garcia-Sainz *et al*, 1992; [3]Garcia-Sainz *et al*, 1996; [4]Garcia-Sainz *et al*, 1995; [5]Garcia-Sainz *et al*, 1996; [6]Garcia-Sainz *et al*, 1995

express a mixture of α_{1B}- and α_{1D}-adrenoceptors. However by 6 months of age the level of expression of α_{1B}-adrenoceptors had decreased whilst the level of expression of α_{1A}-adrenoceptors had increased. The rat liver is also well known for changing the balance of its adrenoceptor populations with age (Rossby and Cornett, 1991). Therefore the variation seen between species could be due to an age-related difference in the α_1-adrenoceptor expressed rather than a definitive species difference in receptor.

Although Garcia-Sainz and colleagues have made a major contribution to subtyping hepatic α_1-adrenoceptors a number of other groups have also characterised α_1-adrenoceptors in the livers of various species. Whether it be for purely pharmacological desires to subtype the α_1-adrenoceptors (Taddei *et al*, 1993; Yang *et al*, 1998) or to test the selectivity of an α_1-adrenoceptor antagonist (Gross *et al*, 1988; Hanft and Gross, 1989; Deng *et al*, 1996), there is a seemingly endless supply of papers looking at various aspects of hepatic α_1-adrenoceptors. The reason the liver is such a popular choice is due to its perceived homogeneity of α_1-adrenoceptors, which provides an environment in which to study native α_1-adrenoceptors without the complications associated with heterogeneity of subtypes. Much of the work has been carried out in rats. In fact, the first evidence that the α_1-adrenoceptors of rat liver were α_{1B}-adrenoceptors came, not from Garcia-Sainz, but from Minneman *et al* (1988). They showed that WB4101 bound with low affinity to hepatic α_1-adrenoceptors and that CEC inactivated these receptors, providing preliminary evidence for a homogeneous population of α_{1B}-adrenoceptors. It is now universally agreed that α_{1B}-adrenoceptors are the only α_1-adrenoceptor subtype present in the rat liver, many papers attesting to this

(Terman *et al*, 1990; Han and Minneman, 1991; Taddei *et al*, 1993; Ohmura and Muramatsu, 1995; Buscher *et al*, 1996; Yang *et al* 1997; Shen *et al*, 2000).

The α_1-adrenoceptor subtype in pig liver has been characterised to be a homogeneous population of α_{1A}-adrenoceptors (Wikberg-Matsson *et al*, 1998), which is in agreement with the observation that larger/older mammals contain hepatic α_1-adrenoceptors of the α_{1A}-adrenoceptor subtype.

Ohmura and Muramatsu identified two α_1-adrenoceptor subtypes in rabbit liver when they used 0.3μM prazosin to define non-specific binding, whilst they could identify only one subtype when they used 10μM phentolamine (traditionally used to define non-specific binding). Their basis for using prazosin was that phentolamine at a concentration of 10μM was insufficient to completely inhibit the binding of [^{3}H]-prazosin to α_1-adrenoceptors (Ohmura and Muramatsu, 1995). The two sites which they had identified appeared to represent the α_{1A}-adrenoceptor (high affinity for antagonists used) and the α_{1L}-adrenoceptor (low affinity for antagonists used, in particular prazosin, $pK_i = 8$). The low affinity site appeared to dominate, representing approximately 70-80% of the total binding sites. This was in direct contrast to Garcia-Sainz *et al* (1992) who also used prazosin to define the non-specific binding, but could identify only one subtype, the α_{1A}. This was the same group who identified three binding sites in the rat cerebral cortex where all other groups had identified only two (Oshita *et al*, 1991).

The α_1-adrenoceptor subtype in the mouse liver was characterised as being the α_{1B}-subtype by Garcia-Sainz *et al* as far back as 1992 (prior to the nomenclature devised by

IUPHAR in 1995) and it has been only in recent years that any further characterisation, with more subtype specific drugs has occurred. Targeted disruption of the α_{1B}-adrenoceptor gene resulted in a mouse that failed to express the α_{1B}-adrenoceptor protein (Cavalli *et al*, 1997). Binding studies revealed that there was a 98% loss of α_1-adrenoceptors in the liver of this KO mouse compared to the WT mouse, indicating a homogeneous population of α_{1B}-adrenoceptor in mouse liver. Confirmation of this was provided by Yang *et al* (1998) who reported that a series of α_1-adrenoceptor ligands, including 5-methylurapidil and BMY7378, bound monophasically and with low affinity to the hepatic α_1-adrenoceptors of mice, indicative of an α_{1B}-adrenoceptor population.

The initial aim of this study was to use radioligand binding to confirm the previously published reports detailing the presence of a mixed population of α_{1A}- and α_{1B}-adrenoceptors in the WT murine brain and a homogeneous population of α_{1B}-adrenoceptors in the WT murine liver. Using the α_{1B}-adrenoceptor KO mouse, a further aim was to demonstrate the loss of the native α_{1B}-adrenoceptor in the KO mouse brain and liver. The results from this preliminary investigation were surprising with regard to the KO liver and so a more detailed pharmacological analysis was carried out in both WT and KO brain and liver tissue, in an attempt to characterise changes present in the KO tissue, which had not been previously identified for this particular mouse. This may provide an insight into the role of the α_{1B}-adrenoceptor subtype.

2.2 METHODS

2.2.1 Solutions and drugs used

All binding experiments were carried out in assay buffer of the following composition (mM):- NaCl (150), Tris-HCl (50), EDTA (5), $MgCl_2$ (10) and 10% glycerol – pH 7.4. When preparing plasma membranes 100mls of this assay buffer was used to which protease inhibitors were added in the following concentrations (μg/ml):- soybean trypsin inhibitor (500), leupeptin (1), bacitracin (1), pepstatin A (1), antipain (1) and 10mM benzamidine. The wash buffer used to terminate the binding reaction and wash the filters was a 50mM Tris-HCl solution – pH 7.4

The following compounds were used:-

(R)-A-61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide, a gift from Dr. Hancock, Abbott Laboratories, USA), **antipain** (Sigma), **BMY7378** (dihydrochloride 8-[2-[4-(2-methoxyphenyl)-1-piperozynl]ethyl]-8-azaspiro(4,5}decone-7,9-dione, Research Biochemicals International), **bacitracin** (Sigma), **benzamidine** (Sigma), **GTP-γ-S** (Guanosine 5'-O-(3-thiotriphosphate) tetralithium salt, Sigma), **L765,314** (4-amino-2-[4-[1-(benzyloxycarbonyl)-2-(S)-[[(1,1 dimethylethyl)amino]carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline, a gift from Dr. Patane, Merck and Company, USA), **leupeptin** (Sigma), **pepstatin A** (Sigma), **phentolamine hydrochloride** (Sigma), **polyethylenimine** (Sigma), **[^{3}H]-prazosin hydrochloride** (Amersham), **prazosin hydrochloride** (Sigma) **RS100329** (a gift from Dr Michelson, Roche Bioscience, USA) and **soybean trypsin inhibitor** (Sigma).

2.2.2 Animals used/tissue preparation

Unless otherwise stated the mice tissues used in the age studies were treated in an identical fashion to those used for the subtyping studies.

Wildtype and knockout male C57-Black mice were obtained from the Central Research Facility at the University of Glasgow. Mice were killed by cervical dislocation (or by overdose of carbon dioxide if carotid arteries were being removed). All mice used were ~4 months old, weighing 30-40g with the exception of mice used in the age studies which were only 3 months old (20-30g). The brain and liver tissues were rapidly removed (only liver tissue was collected from 3 month old mice), washed in assay buffer and then snap frozen in liquid nitrogen. The tissues were stored at -80°C until required for analysis.

2.2.3 Isolation of plasma membranes

Brain and liver tissues were pulverised under liquid nitrogen using a stainless steel percusson mortar, followed by a pestle and mortar. Tissues were suspended in assay buffer containing protease inhibitors. The volume in which the tissues were suspended was dependent on the number of brains and livers used in each assay (generally 5-10mls). The tissue suspensions were homogenised in a pre-cooled polytron, on ice, for 3/4, 15s bursts at setting 6. The homogenate was filtered through two layers of muslin and centrifuged at 1200 x g for 5 minutes at 4°C. The supernatant was removed and kept on ice and the pellet was resuspended in approximately 5mls of buffer. When isolating plasma membranes using liver tissue this first supernatant was discarded due to the high

concentration of red blood cells present in this tissue which, if not removed, would produce high amounts of non-specific binding when the radioligand binding assay was performed. The resuspended pellet was homogenised using the polytron (setting 6) and then centrifuged as before. This homogenisation/centrifugation step (which endeavours to wash away endogenous agonists and mediators (e.g. guanine nucleotides) which may interfere with the radioligand binding assay) was repeated 3 times in total (4 for the liver) and the supernatants pooled together. The pooled supernatants were centrifuged at high speed (56,000 x g for 30 minutes at 4°C) in an ultracentrifuge. The supernatant was discarded and the pellet was washed and resuspended in assay buffer and ultracentrifuged again at the same settings. The resultant pellet was resuspended in 1-2 mls of assay buffer and homogenised in a glass homogeniser until smooth and then aliquoted into eppendorfs and stored at -80°C until needed. Plasma membranes were usually used within a week of isolation and were never kept for longer than 4 weeks.

2.2.4 Determination of protein concentration

A Pearce Bicinchoninic Acid (BCA) Protein Assay kit was used to determine the concentration of plasma membrane protein that had been isolated. The protein standard used was bovine serum albumin (BSA) which came in a stock solution of 2mg/ml. This was diluted using deionised water to give working concentrations of 1, 0.5, 0.25, 0.125 and 0.0625mg/ml from which the protein content of the plasma membranes could be calculated. 25µl of either the BSA dilutions or the membrane samples (diluted to a 1:10 solution in order to minimise the amount of membranes used) were pipetted into a 96-well plate. The BSA samples were incubated in duplicate and the membrane samples in quadruplicate. 200µl of the BCA working reagent was added to each sample. The BCA

working reagent was made up with 50 parts of Reagent A (a solution of sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarate in 0.2M sodium hydroxide) and 1 part of reagent B (a solution containing 4% cupric sulphate). The reaction was allowed to proceed for 30 minutes at 37°C after which the reaction was terminated when the samples were removed from the incubator and left to stand for 5-10 minutes at room temperature (22°C). The protein present in the samples reduces Cu^{2+} to Cu^{1+}, which then goes on to react with the BCA to produce a colour change. This water soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations and so after termination of the reaction the absorbance of the samples was measured at 562nm using a Dynatech plate reader to quantify the colour change. A graph was plotted using the values obtained for the BSA standards, which was then used to calculate the actual concentration of membranes. The membranes were diluted using assay buffer to give a working concentration of 2.5mg/ml.

2.2.5 Saturation experiments

The affinities (K_D) and maximal receptor densities (B_{max}) of expressed α_1-adrenoceptors in WT and KO brains and livers were determined using saturation binding assays. Brain and liver membranes (0.5mg/ml) were incubated in duplicate with [^{3}H]-prazosin (0.025-5nM, 75Ci/mmol) made up to a total volume of 0.5mls with assay buffer. Non specific binding was determined in the presence of 10μM phentolamine at each concentration of [^{3}H]-prazosin. After equilibrium (30 minutes at 22°C) bound ligand was separated from free by vacuum filtration over GF/C filters (pre-soaked in assay buffer containing 0.3% aqueous polyethylenimine to reduce non specific binding) which were washed three times with ice cold wash buffer using a Brandell cell harvester. Each filter was placed in

3mls of scintillant and left overnight after which time the filter had dissolved and the radioactivity could be measured in a Beckman LS5000TD liquid scintillation counter.

2.2.6 Competition experiments

To determine the α_1-adrenoceptor subtypes present in the brains and livers of WT and KO mice competition binding assays were performed using various subtype selective ligands. The ligands used and their subtype selectivities are as follows:- Prazosin (non-selective α_1-adrenoceptor antagonist), (R)-A-61603 (α_{1A}-selective agonist), RS100329 (α_{1A}-selective antagonist), L765, 314 (α_{1B}-selective antagonist) and BMY7378 (α_{1D}-selective antagonist).

Competition binding assays were performed by incubating WT and KO brain and liver membranes with 0.5nM [^{3}H]-prazosin in the presence or absence of the competing ligands. When deciding on the concentration of [^{3}H]-prazosin to use 2 factors are taken into consideration. In general a concentration of [^{3}H]-prazosin approximately equal to its K_D is preferred. However the concentration used has to be sufficient that the counts per minute are large enough to be measurable, and so a further factor in deciding the concentration to be used is whether or not the counts per minute will be detected reliably by the scintillation counter. A concentration of [^{3}H]-prazosin that produces total counts of approximately 1000 is deemed to be sufficient. In KO livers this was found to occur at a concentration of 0.5nM. However 0.2nM was found to be adequate to produce counts of 1000 in the other tissues used (WT and KO brains and WT livers). It was decided that 0.5nM would be used throughout the study for all tissues examined in

order to provide accurate counts for all tissues used and to enable a valid comparison to be made between tissues.

The concentrations of the competing ligands used in the assay were dependent on the specific ligand's affinity range for the three subtypes of α_1-adrenoceptor. Preliminary experiments were carried out for each of the competitors over a wide range of concentrations increasing in log increments in order to determine the affinity range of each ligand. This allowed for the determination of a suitable range of concentrations for each competing ligand, which had to include a plateau at the top and bottom of the concentration range in order to perform non-linear curve fitting analysis. From this data 17 concentrations were chosen which increased in half-log increments. The concentrations for each ligand were:- Prazosin:-1pM – 0.1mM, RS100329:- 0.1pM – 10μM, L765,314:- 1pM – 0.1mM, BMY7378:- 10pM – 1mM and (R)-A61603:- 10pM – 1mM. Non specific binding was determined in the presence of 10μM phentolamine as for saturation assays. The incubations were allowed to proceed for 30 minutes at 22°C until equilibrium was reached whereupon the reaction was terminated and counted by the same method employed for the saturation assays.

All experiments using (R)-A-61603 were carried out both in the presence and absence of 100μM GTPγS (a non-hydrolysable analogue of GTP). The reason for this is that in the hands of this laboratory (R)-A-61603 exhibits a biphasic curve due to its interaction as an agonist with G proteins. This occurs because the binding of (R)-A-61603 to α_1-adrenoceptors will result in a proportion of the receptors interacting with G proteins, while the rest of the receptors do not. When an agonist-receptor complex interacts with the α-subunit of a G protein there is a decrease in the affinity of the nucleotide binding

site for GDP and a ternary complex forms where the α subunit is bound to the receptor and the β and γ subunits are empty. If GTP is absent the activated receptor is unable to dissociate from the α subunit. When this happens it increases the stability of the agonist-receptor complex which increases the affinity of the agonist for the receptor (Bockaert *et al*, 1997). When the G protein concentration is limiting (as is the case when using isolated plasma membranes) there will be a percentage of the receptors which have a higher than normal affinity for the agonist.

In a study such as this, that relies on fitting data to one-site or two-site binding isotherms, it is imperative that any heterogeneity that was observed was as a result of there being two binding sites, and not due to experimental conditions which would not be present physiologically. Experiments were carried out in the presence and absence of a non-hydrolysable analogue of GTP, GTPγS, to allow the biphasic nature of (R)-A-61603 to be demonstrated and to show that the presence of GTP could abolish this.

2.2.7 Analysis of results

The average counts per minute (cpm) for each antagonist in WT and KO tissues is presented in tabular form in appendix A1-A12. All data was analysed using GraphPad Prism 3.01 (Institute for Scientific Information, San Diego, California, USA). All equations and nomenclature used in the text are taken from GraphPad Prism 3.01. Statistical comparison of WT and KO data was performed using an unpaired t test. A p value of less than 0.05 was considered to be significant.

2.2.7.1 Data analysis for saturation curves

Specific binding in saturation experiments was calculated by subtracting non-specific binding from total binding for each concentration of [^{3}H]-prazosin. Non-specific binding was analysed using linear regression. Total and specific binding were analysed using non-linear regression, comparing the data to a one-site or a two-site model. For saturation experiments the data was consistently found to fit more accurately to a one-site model. All saturation assays were carried out in duplicate, each experiment was repeated a minimum of three times and the results from each pooled and expressed as mean ± s.e.m. The K_D (which is the equilibrium dissociation constant i.e. the concentration of ligand which occupies half of the receptors at equilibrium) and the B_{max} (the maximal receptor density) are calculated using the following equation:-

$$\text{Specific binding} = \frac{B_{max}.[RL]}{K_D + [RL]}$$

This equation describes a rectangular hyperbola where [RL] is the concentration of free radioligand.

2.2.7.2 Data analysis for competition curves

As with saturation curves, experiments were carried out in duplicate, repeated a minimum of three times and the results from each pooled and expressed as mean ± s.e.m. For competition curves specific binding was determined by a different method from that used with saturation curves. Non-specific binding (determined in the presence

of 10μM phentolamine) was defined as 0% and total binding (in the absence of any competitor) was defined as 100%. Specific binding is then calculated accordingly and expressed as % specific binding of maximum.

In the simplest case competitive binding can be described by the equation:-

$$Y = NSB + \frac{(Total-NSB)}{1+10^{(\log[D]-\log[IC_{50}])}}$$

Y = binding in the presence if the competitor

Log[D] = logarithm of the concentration of the competitor

NSB = non-specific binding in the presence of a saturating concentration of D

Total = binding in the absence of a competitor

$[\log IC_{50}]$ = logarithm to the base 10 of the concentration of the competitor which inhibits the binding of the radioligand by 50%

This equation describes a situation in which the radiolabel and the competing ligand compete for only 1 receptor type. However in many tissues there are 2 classes of binding sites, which may represent 2 subtypes of receptor. This situation can also be described by an equation, albeit a more complicated one:-

$$Y = NSB + (Total-NSB).\left[\frac{F}{1+10^{\log[D]-\log(IC_{50})_1}} + \frac{1-F}{1+10^{\log[D]-\log(IC_{50})_2}}\right]$$

Where Y, NSB, Total and [D] are as for the previous equation and F = fraction of binding to receptors of the first type of receptor.

Data from competition experiments were analysed using both the one-site and two-site models. Results of these were compared to determine which gave the more accurate fit. This was done in a number of ways. Firstly the graphs were compared visually to assess which equation looked to be fitting the data better, after which a number of statistical parameters were looked at, which are provided by Graph Pad when performing nonlinear regression. (All definitions are taken from the GraphPad guide to Nonlinear Regression by Harvey Motulsky, 1996).

95% Confidence Intervals:- if all the assumptions of non-linear regression are true then there is a 95% chance that the true value of the variable lies within the interval. Therefore the narrower the confidence interval, the better the fit.

Sum of Squares (SS):- the sum of squares is defined as the sum of the square of the vertical distances of the points from the curve. So the smaller the value of SS then the closer the points are to the curve and the more accurate the fit.

r^2:- a measure of the goodness of the fit. It is a fraction between 0 and 1 and has no units. When $r^2 = 0$ the best-fit curve fits the data no better than a horizontal line going through the mean of all the y values, i.e. knowing x does not help you predict y. When $r^2 = 1$ all points lie exactly on the curve with no scatter i.e. if you know x you can predict y.

Prism also performs a test called **the F test** which directly compares the fits of 2 equations and reports which is statistically better suited to the data. The results are presented in the form of an **F ratio** which is calculated as shown below:-

$$F = \frac{(SS1-SS2)/SS2}{(DF1-DF2)/DF2}$$

SS = sum of squares

DF = degrees of freedom

If the simpler equation (i.e. the one-site model) is correct then $F \approx 1$ because the relative increase in the sum of squares is approximately equal to the relative increase in the degrees of freedom. However if F is much greater than 1 then the more complicated equation is correct because the relative increase in the sum of squares (going from the complicated to the simpler model) is greater than the relative increase in the degrees of freedom.

For the purposes of this study a two-site model was accepted only when the F ratio was found to be much greater than 1 and produced a p value of < 0.05.

The K_i or inhibition constant is defined as the concentration of the unlabelled drug that will bind half the receptors at equilibrium, in the absence of radioligand or any other competitors and can be calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):-

$$K_i = \frac{IC_{50}}{1+\frac{[RL]}{K_D}}$$

For each competition experiment a K_i was calculated and then converted to a pK_i value, which is the negative logarithm to the base 10 of the K_i.

However, these three equations are based on assumptions, which if not met, deem the equations no longer accurate. One of these assumptions is that only a small fraction of the added ligand actually binds. Therefore the assumption being made is that the concentration of free ligand in solution is the same as the concentration of ligand added. This can only be upheld when less than 10% of the ligand binds. In cases where more than 10% binds any data analysis carried out would have to take account of this ligand depletion. To determine if ligand depletion had taken place in any of the competition experiments carried out for this study the percentage of ligand bound was calculated:-

$$\% \text{ Bound} = \frac{\text{Total Binding}}{\text{Total RL added}} \times 100\%$$

This phenomenon was only found to occur in WT tissues. In the WT liver it occurred when using RS100329 and BMY7378 and in the WT brain it occurred with BMY7378. This meant that the equations describing competitive binding to one or two sites could not be used to fit the data, nor could the Cheng Prusoff equation be used to calculate the K_i's. Fortunately Graph Pad has an equation that can be used in a more complicated situation such as this:-

$$Y = \frac{(-1.b + \sqrt{(b.b-4.a.c)})}{2a}$$

$K_dCPM = K_dnM.SpAct.V.1000$

$R=NSB+1$

$S=[1+10^{(X-LogKi)}].K_dCPM+Hot$

$a=-1.R$

$b=R.S+NSB.Hot + B_{max}$

$c= -1.Hot.(S.NSB + B_{max})$

where:-

SpAct = specific activity of radioligand

V = volume

Hot = amount of labelled ligand added to each tube

This equation accounts for the fact that the free concentration of radioligand is less than the concentration added and was used to fit data in any situation where bound ligand was found to account for more than 10% of ligand added.

Non linear regression was also carried out using the Hill equation in order to determine the Hill slope, which allows for the quantification of the steepness of the slope.

$$Y = \frac{NSB + (Total-NSB)}{1+10^{(\log IC_{50}-\log[RL]).slope\ factor}}$$

A one-site competitive binding curve that follows the law of mass action will have a slope of –1 (it is negative because the curve goes downhill). If the slope differs significantly from -1 then the binding does not follow the law of mass action. One of the possible explanations for such an event would be that there is a heterogeneous population of receptors for which the competing drug has different affinities or it could be due to heterogeneity in the receptor coupling to other molecules such as G proteins. A Hill slope was calculated for each competition experiment in order to provide further information about the nature of the binding.

2.3 RESULTS

2.3.1 Binding studies in brains from WT and KO mice

2.3.1.1 Saturation studies

[^{3}H]-prazosin demonstrated specific, saturable binding of high affinity in brains from both WT and KO mice and was found to fit to a one-site binding isotherm (Figure 2.2). K_D's were calculated as being 0.6 ± 0.08nM for WT brain and 0.8 ± 0.2nM for KO brain, confirming the presence of α_1-adrenoceptors in the murine brain. The difference in the affinity of [^{3}H]-prazosin between WT and KO was not significant. However, when the total receptor number was calculated there was found to be a smaller number of receptors in KO brains (60.33 ±5.0 fmol/mg), when compared with brains from WT mice (87.5 ± 3.8fmol/mg) ($p< 0.05$).

2.3.1.2. Competition studies

With the presence of α_1-adrenoceptors in the murine brain confirmed, the subtype(s) that made up this population were investigated. A number of selective and non-selective antagonists and a selective agonist were utilised in order to determine the α_1-adrenoceptor subtype(s) present in the murine brain. The antagonists used were prazosin (non-selective α_1-antagonist), RS100329 (an α_{1A}-selective antagonist), L765,314 (an α_{1B}-selective antagonist), BMY7378 (an α_{1D}-selective antagonist) and (R)-A-61603 (an α_{1A}-selective agonist).

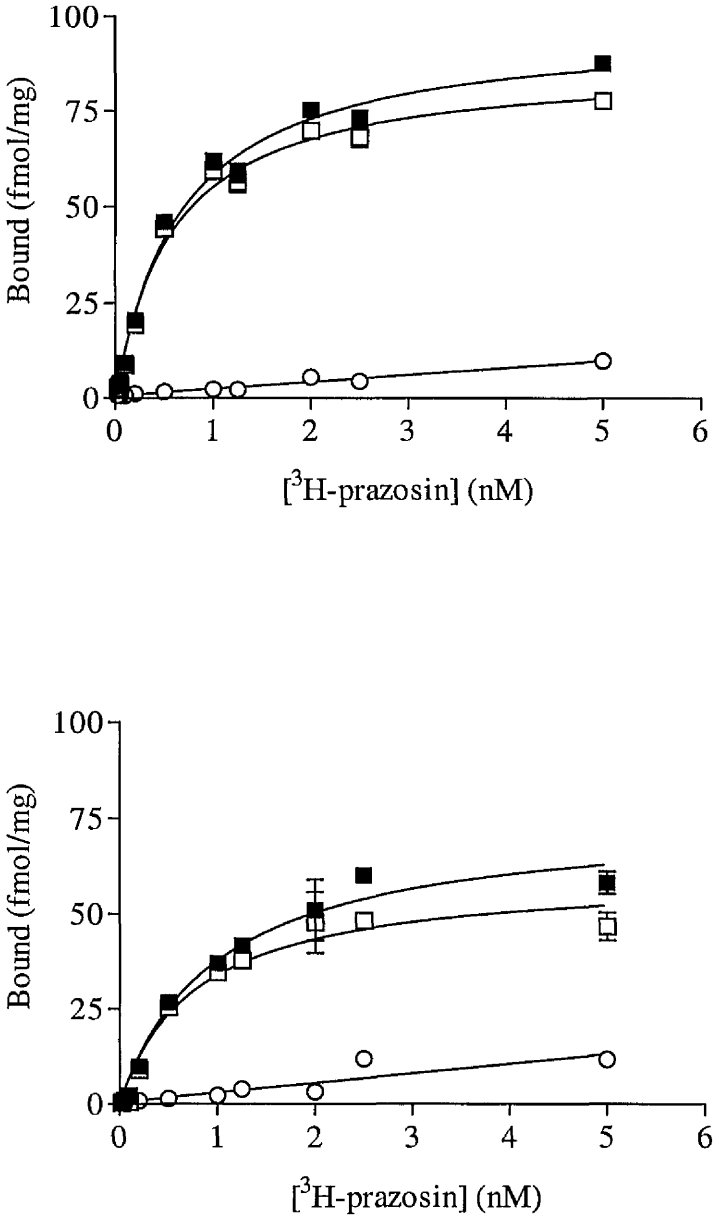
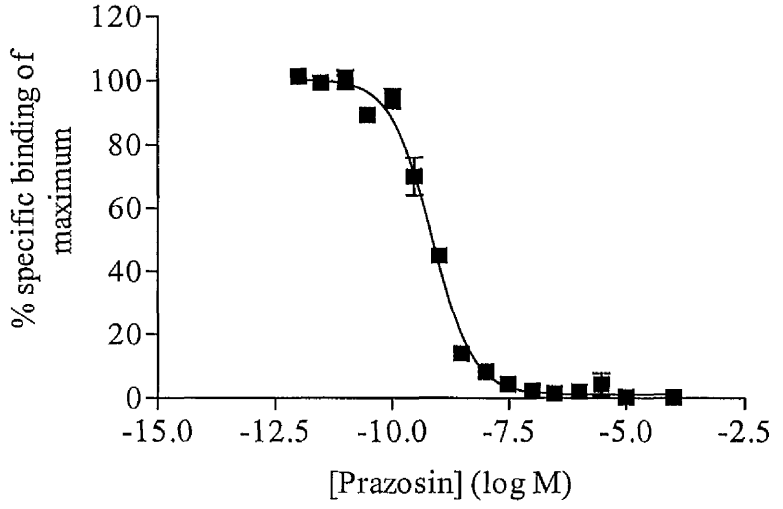


Figure 2.2:-Saturation curves showing [^{3}H]-prazosin binding (0.025-5nM) in WT (top) and KO (bottom) brain membranes. WT brain produced a K_D of 0.6±0.08nM and a B_{max} of 87.5±3.8fmol/mg, whilst KO brain produced a K_D of 0.8±0.2nM and a B_{max} of 60.33±5.0fmol/mg Total (■), specific (□) and non-specific (o) binding. Each data point is expressed as mean ± s.e.m (n=4).

The competition curves for the ligands used are shown in Figures 2.3 – 2.7 and a table with their pK_i values and Hill slopes is given in Figure 2.8.

Prazosin inhibited [^{3}H]-prazosin binding in WT and KO brain (Figure 2.3) resulting in pK_i values of 9.3±0.05 and 9.2±0.06 respectively, which correlate well with values expected at α_1-adrenoceptors (Bylund *et al*, 1994). In addition to this high affinity binding site (which accounted for 89±4% of the total number of binding sites) the KO brain also had a low affinity site with a pK_i of 7.4±0.45. The Hill slope in the KO brain reflects the biphasic nature of prazosin's binding in that it is significantly different from negative unity (-0.85: 95% CI = -0.99 to -0.72). However the graph in Figure 2.3 does not necessarily reflect this biphasic nature as a result of the data plotted being the mean data rather than single experiments. When single experiments were analysed the graph was more obviously biphasic than when plotted as mean data. The Hill slope in the WT brain is indicative of competitive binding to a single site, in that it is not significantly different to negative unity (-1.1: 95% CI = -1.4 to -0.9).

RS100329, an $\alpha_{1A/a}$-selective antagonist with approximately 120 fold selectivity over the α_{1b}-adrenoceptor and 50 fold selectivity over the α_{1d}-adrenoceptor (Williams *et al*, 1999), demonstrated a one-site binding curve in the KO brain, with a pK_i of 9.3±0.03 and a Hill slope not significantly different from negative unity of -1.0 (95% CI = -1.2 to -0.8). However, in the WT brain the situation was not suggestive of one-site binding but showed instead that RS100329 was binding with two separate affinities. The high affinity site of 9.5±0.1 is comparable to the value of 9.3±0.03 in the KO brain and makes up 36±2.5% of the binding sites. However, there is an additional second site found to be present in the WT which is not present in the KO brain. This second site in



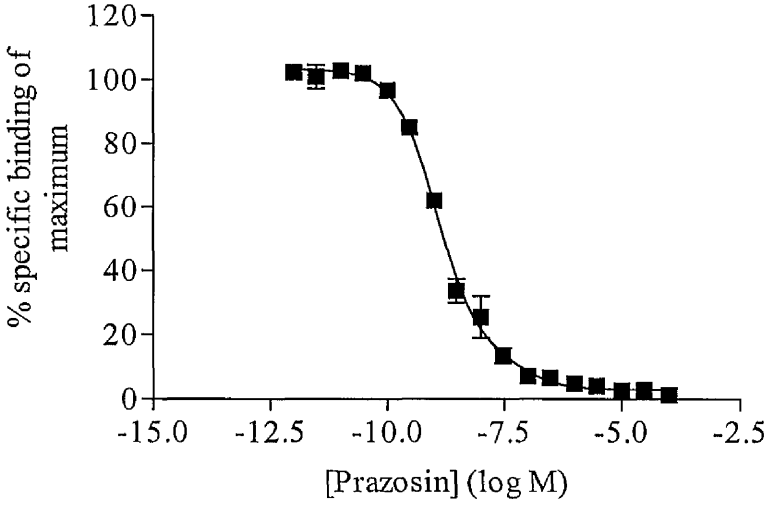


Figure 2.3:-Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) brain membranes by increasing concentrations of prazosin (1pM-0.1mM). Data points are expressed as mean ± s.e.m. (n≥3)

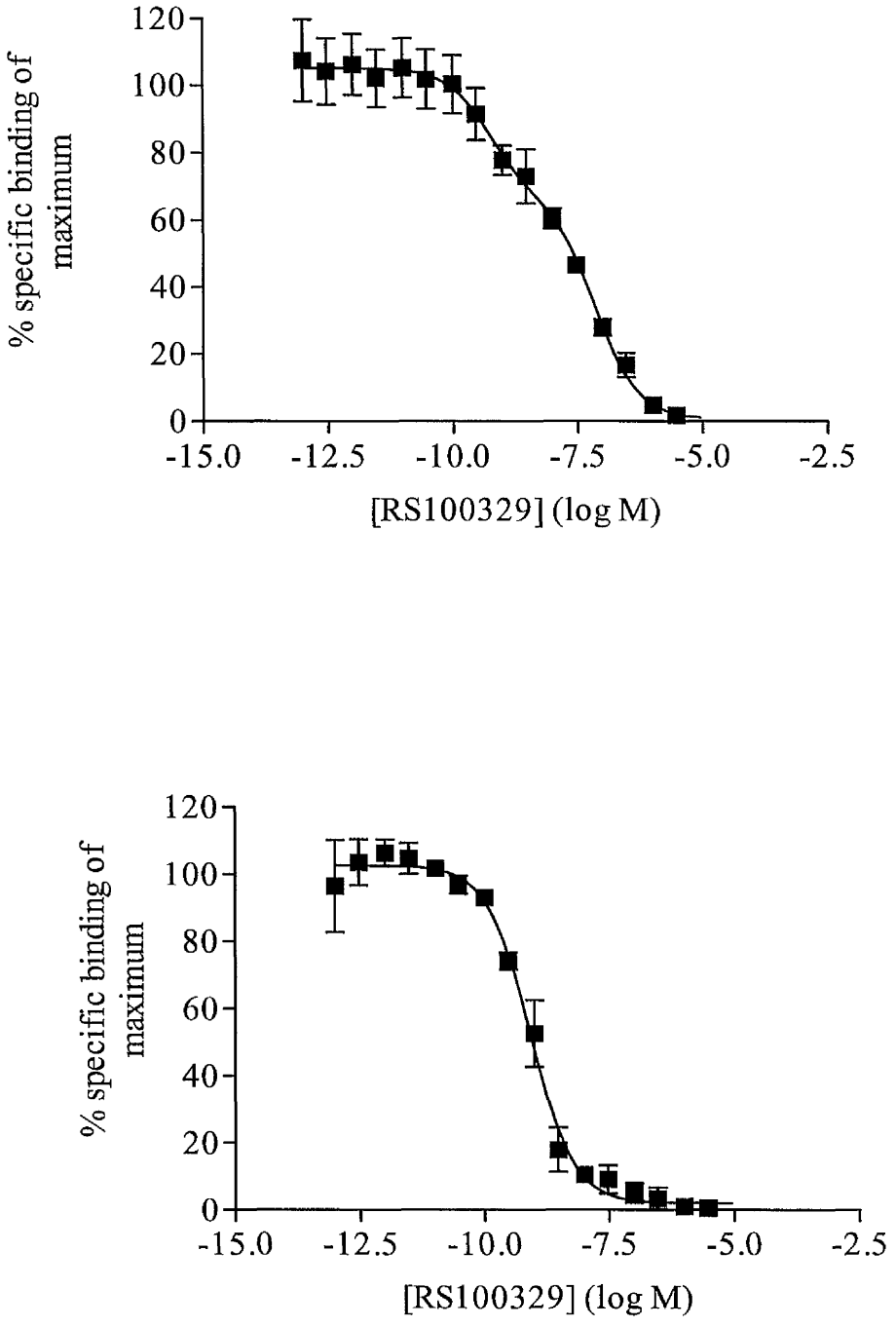


Figure 2.4:-Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) brain membranes by increasing concentrations of RS100329 (0.1pM-10μM). Data points are expressed as mean ± s.e.m.(n=3)

the WT brain has 100 fold lower affinity than the first site with a pK_i value of 7.4±0.07. The shallow Hill slope (-0.46: 95% CI = -0.53 to -0.38) confirms the biphasic nature of the binding of RS100329 in WT brain (Figure 2.4).

The selective agonist used in this study, (R)-A-61603, demonstrated two-site binding both in the presence and absence of GTPλS when used in the WT brain. The pK_i values were identical under both conditions (6.7±0.16 and 5.2±0.13) and the Hill slopes were both shallow and significantly different from negative unity (-0.64 (95% CI = -0.78 to -0.47) without GTPλS and -0.66 (95% CI = -0.77 to -0.55) in the presence of GTPλS). (R)-A-61603 behaved in a similar manner in the KO brain, in that it exhibited two-site binding both in the presence and absence of GTPγS, with similar affinities to the WT brain. When GTPγS was absent the pK_i values were 7.0±0.07 and 5.5±0.2 with a Hill slope which was significantly different from negative unity (0.73: 95% CI = -0.81 to –0.65). The presence of GTPγS doesn't appear to affect the binding curve, resulting in pK_i's which are almost identical to those without GTPγS: 6.8±0.06 and 5.3±0.4. There is a slight steepening of the Hill slope going from 0.73 in the absence of GTPγS to 0.84 when GTPγS is present. However the Hill slope is still significantly different from negative unity with 95% CI = -0.95 to –0.73. Although the pK_i values for WT and KO brain are virtually identical there is a significant difference ($p<0.05$) in the fraction of high affinity sites present in the WT compared with the KO brain. In the WT brain the high affinity site corresponds to 45±6.3% of the total binding sites whereas in the KO brain the high affinity site accounts for 86±5.3% of the total (Figure 2.5).

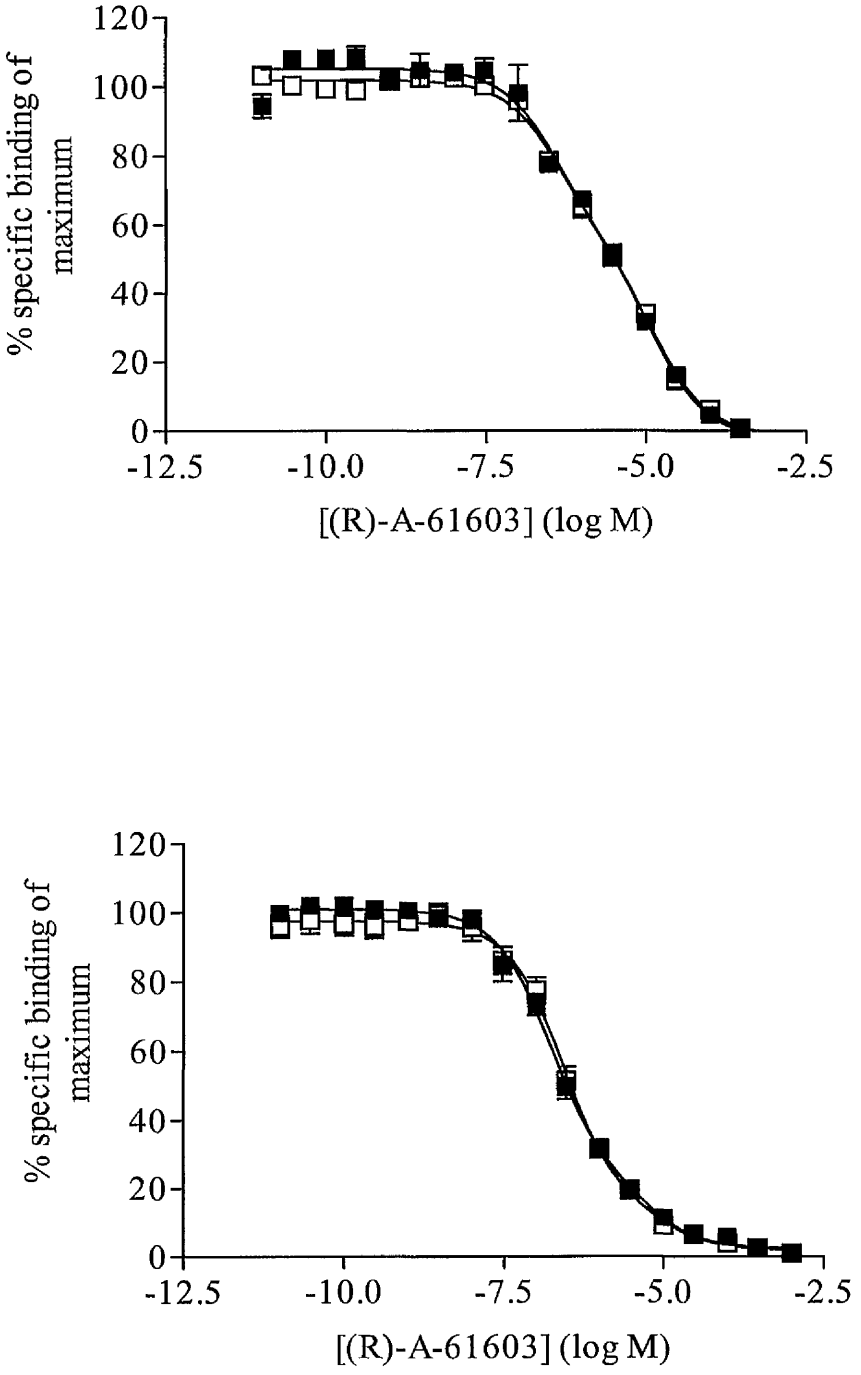


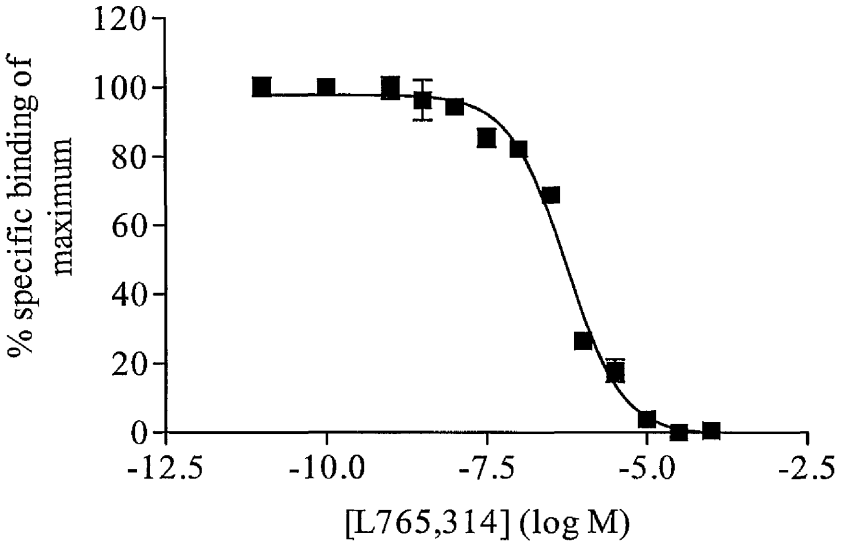
Figure 2.5:- Displacement of 0.5nM[^{3}H]-prazosin from WT (top) and KO (bottom) brain membranes by increasing concentrations of (R)-A-61603 (10pM-1mM) in the presence (□) and absence (■) of GTPγ S. Data points are expressed as mean ± s.e.m. (n≥3)

L765,314 inhibited [^{3}H]-prazosin binding in WT brain with a pK_i value of low affinity (6.4±0.07) and a Hill slope of -1.0, (95% CI = -1.3 to -0.66) suggestive of monophasic binding. The KO brain was found to produce a similar pK_i value of 6.3±0.09 with a steep Hill slope which was not significantly different from negative unity (-0.96: 95% CI = -1.3 to -0.65) (Figure 2.6).

BMY7378, which is the only antagonist to date with significant selectivity for the α_{1D}-adrenoceptor over the other two subtypes (Saussy *et al*, 1994) was found to bind in a monophasic manner and with low affinity in the WT brain resulting in a pK_i of 6.0±0.03 and producing a Hill slope of -1.0 (95% CI = -1.4 to -0.84). The KO brain was also found to bind BMY7378 with low affinity (pK_i = 6.1±0.06) which resulted in a steep Hill slope which was not significantly different from negative unity (-0.87: 95% CI = -1.1 to -0.65) (Figure 2.7).

2.3.1.3 Correlations

Figures 2.9 and 2.10 show binding correlations for the ligands used in WT and KO brain respectively, plotted against the published binding affinities for these ligands at the three cloned α_1-adrenoceptors. For WT brain the values plotted for RS100329 and (R)-A-61603 are the high affinity pK_i's when plotted against the α_{1a}-adrenoceptor values but the low affinity pK_i values were used when plotted against the α_{1b}-adrenoceptor values. For KO brain the high affinity prazosin site was used throughout. All (R)-A-61603 data was plotted as the pK_i values in the presence of GTPγS. The published pK_i values used for A-61603 are the affinities of the racemic mixture at the cloned subtypes, whereas the experiments carried out here used the R-enantiomer of A-



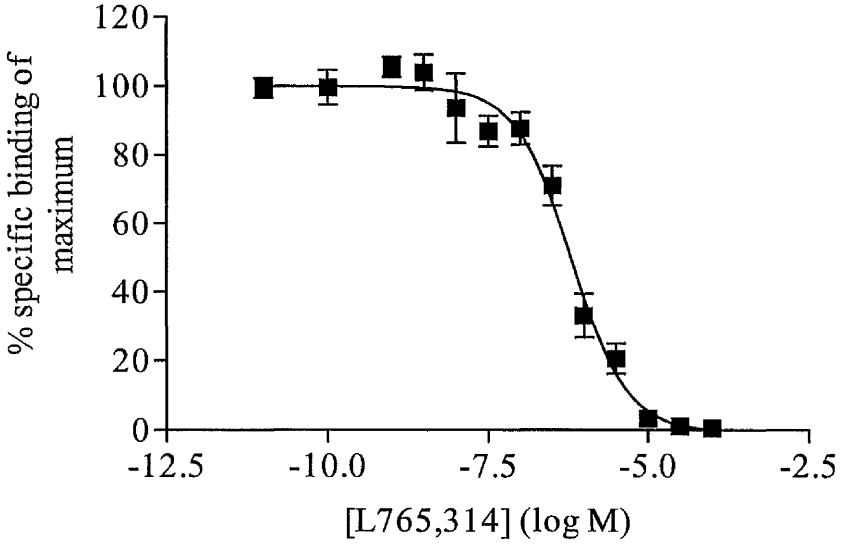
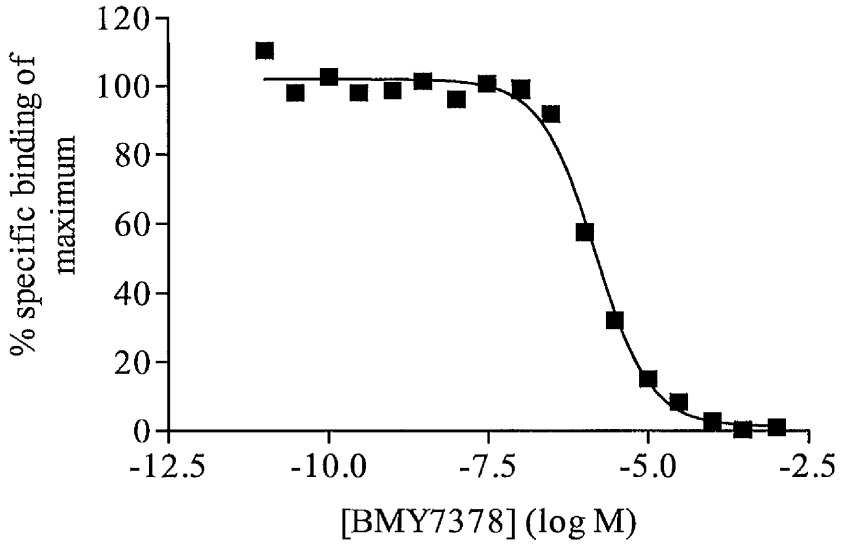


Figure 2.6:-Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) brain membranes by increasing concentrations of L765,314 (1pM-0.1mM). Data points are expressed as mean ± s.e.m. (n≥3)



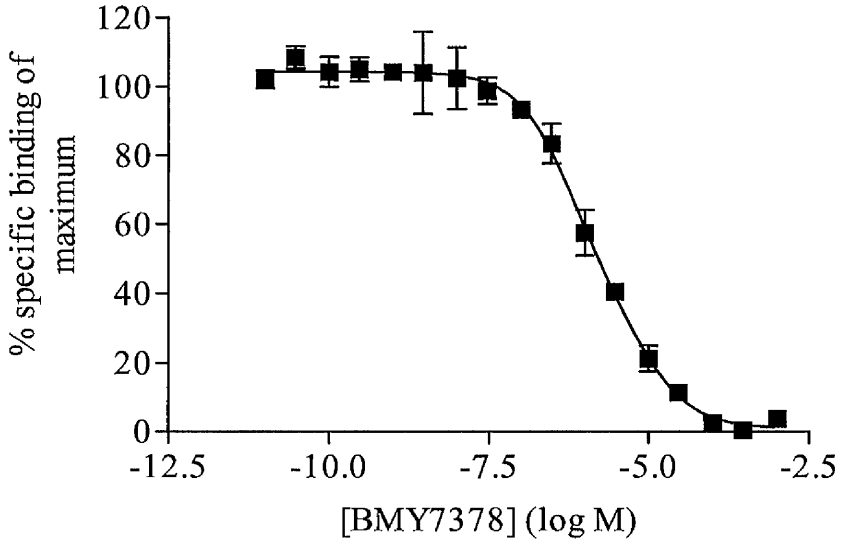
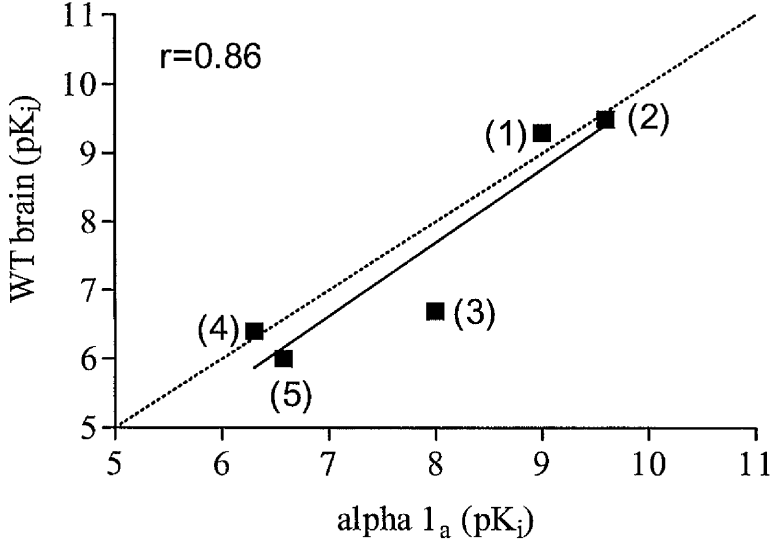
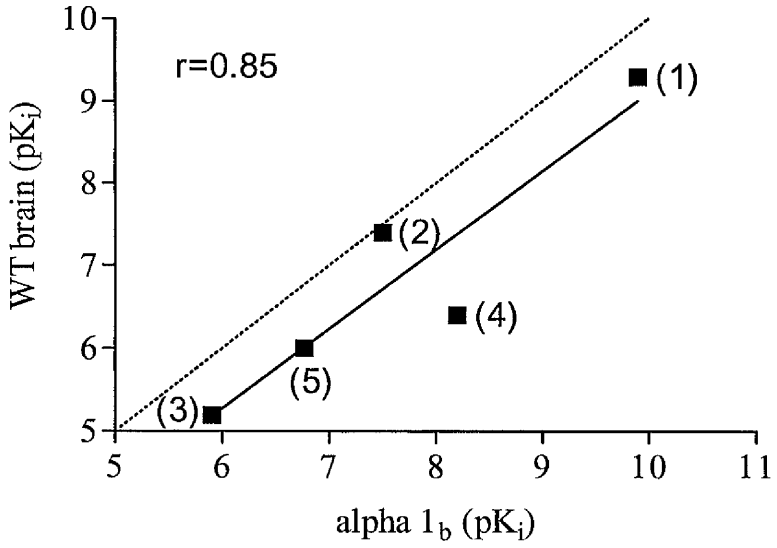


Figure 2.7:-Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) brain membranes by increasing concentrations of BMY7378 (10pM-1mM). Data points are expressed as mean ± s.e.m. (n≥3)

		WT BRAIN		KO BRAIN	
		pK_i	- nH	pK_i	- nH
Prazosin	(H) (L)	9.3±0.05	1.1±0.1	9.2±0.06, 7.4±0.45	0.85±0.06*
(R)-A-61603	(H) (L)	6.7±0.14, 5.2±0.13	0.64±0.05*	7.0±0.07 5.5±0.2	0.73±0.03*
(R)-A-61603 +GTPγS	(H) (L)	6.7±0.16, 5.2±0.13	0.66±0.05*	6.8±0.06 5.3±0.4	0.84±0.03*
RS100329	(H) (L)	9.5±0.1 7.4±0.07	0.46±0.03*	9.3±0.03	1.0±0.1
L765,314		6.4±0.07	1.0±0.1	6.3±0.09	0.96±0.1
BMY7378		6.0±0.03	1.0±0.07	6.1±0.06	0.87±0.1

Figure 2.8:- Inhibition affinities in WT and KO brain membranes expressed as pK_i values ± s.e.m. Hill slopes (nH) are expressed as the negative of the hill slope ± s.e.m.(H) = high affinity site and (L) = low affinity site.
* indicates a Hill slope which is significantly different from negative unity.





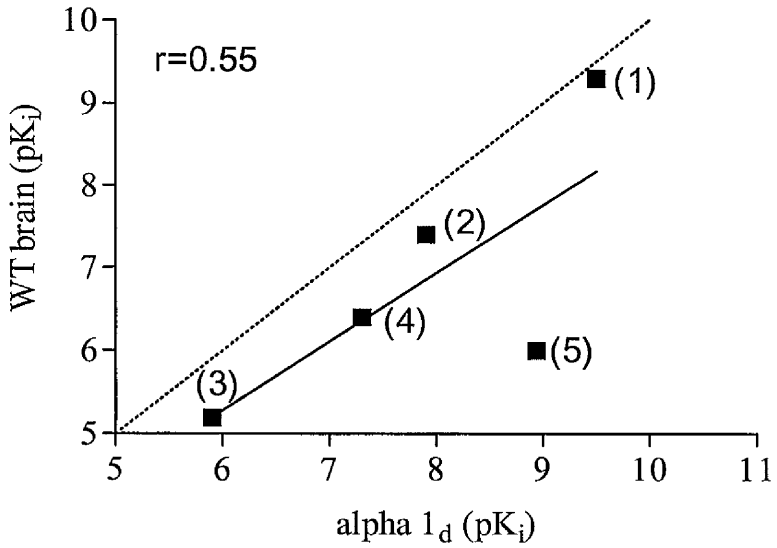


Figure 2.9:- Correlation of potencies of ligands used on WT brain membranes with published potencies of the same ligands at cloned α_1-adrenoceptor subtypes. (1):- prazosin, (2):- RS100329, (3):- (R)-A61603, (4):- L765,314 and (5):- BMY7378. (Williams *et al*, 1999, Knepper *et al*, 1995,Patane *et al*, 1998 and Yang *et al*, 1997). The dotted line shows the line of unity.

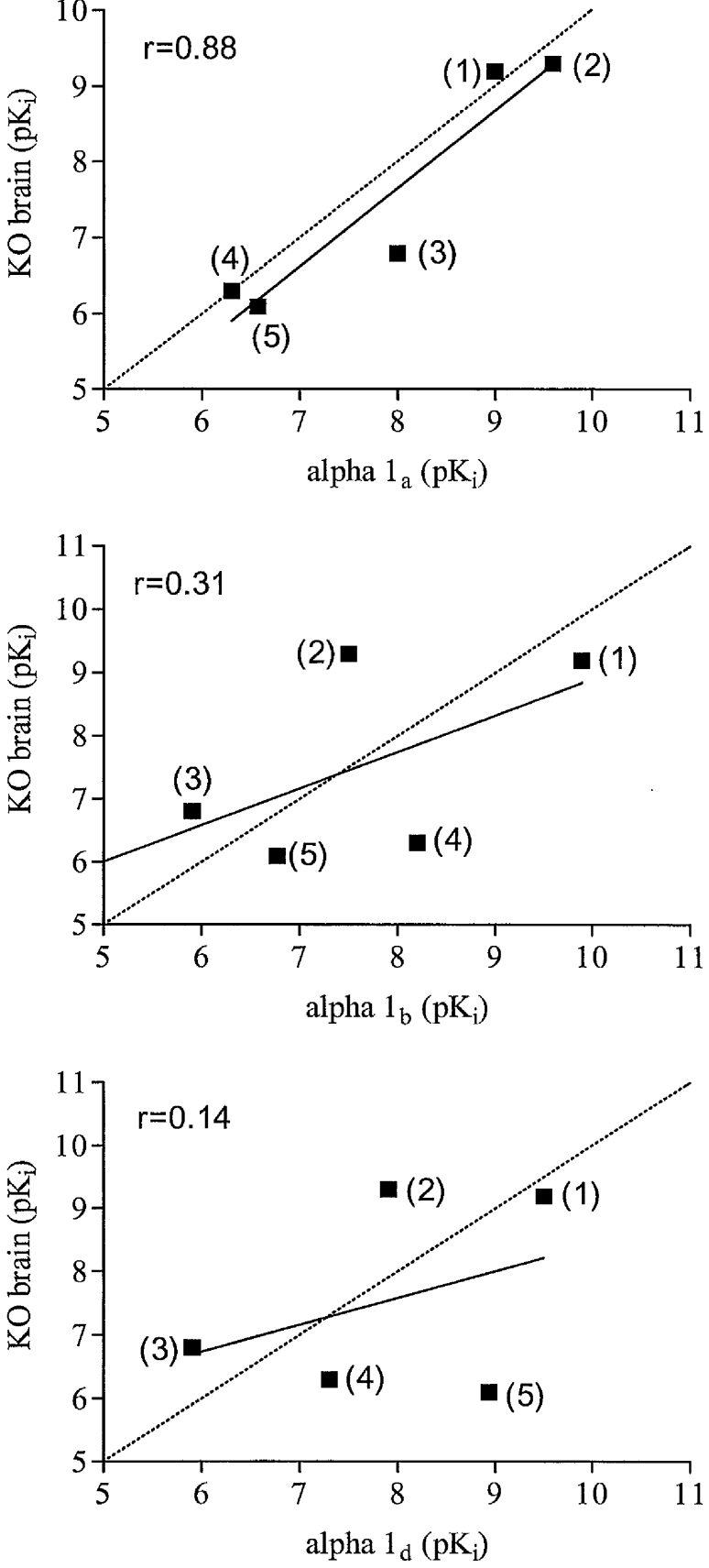


Figure 2.10:- Correlation of potencies of ligands used on KO brain membranes with published potencies of the same ligands at cloned α_1-adrenoceptor subtypes. (1):- prazosin, (2):- RS100329, (3):- (R)-A61603, (4):- L765,314 and (5):- BMY7378. (Williams *et al*, 1999, Knepper *et al*, 1995, Patane *et al*, 1998 and Yang *et al*, 1997). The dotted line shows the line of unity.

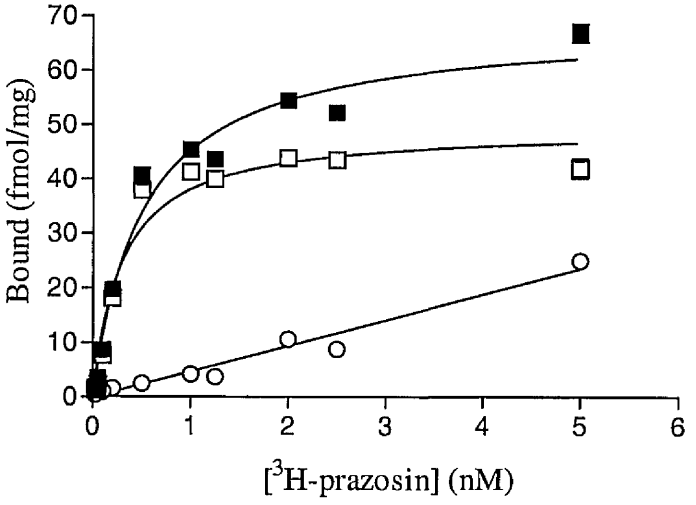
61603. However, only data at α_{1A}-adrenoceptors has been published for the R-enantiomer so to enable correlations to be carried out it was decided to use the values quoted for the racemic mixture.

There appears to be a weak correlation between the affinities for the ligands in the WT brain and for cloned α_{1d}-adrenoceptors (r = 0.55). However the correlation is much stronger when the affinities in the WT brains are plotted against the affinities in either α_{1a}-adrenoceptors or α_{1b}-adrenoceptors. Both correlations give very similar r values of 0.86 at α_{1a}-adrenoceptors and 0.85 at α_{1b}-adrenoceptors but the plot with α_{1a}-adrenoceptors most closely followed the line of unity. In KO brain there is a very good linear correlation with α_{1a}-adrenoceptors (r = 0.88) which closely resembles the line of unity. The correlations with α_{1b}-adrenoceptors and α_{1d}-adrenoceptors are poor with r values of 0.31 and 0.14 respectively.

2.3.2 Binding studies in livers from wildtype and knockout mice

2.3.2.1 Saturation studies

As was the case with the murine brain, the liver was found to display specific, saturable binding of high affinity to [^{3}H]-prazosin (Figure 2.11) and as with the brain was found to fit consistently to a one-site binding isotherm. The WT liver was found to have an affinity of 0.3 ± 0.08nM whilst KO liver had an affinity of 0.15 ± 0.04nM, indicating a population of α_1-adrenoceptors present in both WT and KO liver. The B_{max} was found to be significantly lower in the KO (30.0 ± 2.0fmol/mg) when compared to the WT liver (50.0 ± 3.1fmol/mg), which mirrors the findings in the brain. Murine liver has been proposed to contain a pure population of α_{1B}-adrenoceptors (Yang *et al*, 1998). We



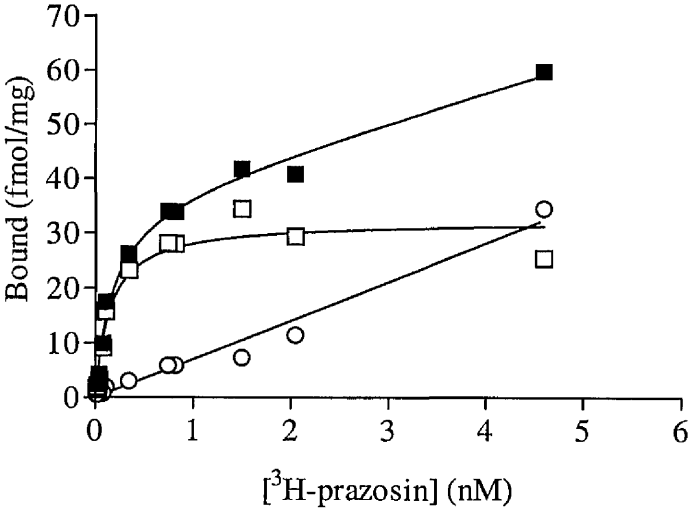


Figure 2.11:- Saturation curves showing [^{3}H]-prazosin binding (0.025-5nM) in WT (top) and KO (bottom) liver membranes. WT liver produced a K_D of 0.3±0.08nM and a B_{max} of 50±3.1fmol/mg, whilst KO liver produced a K_D of 0.15±0.04nM and a B_{max} of 30±2.0fmol/mg. Total (■), specific (□) and non-specific (○) binding. Each data point is expressed as mean ± s.e.m. ($n \geq 3$).

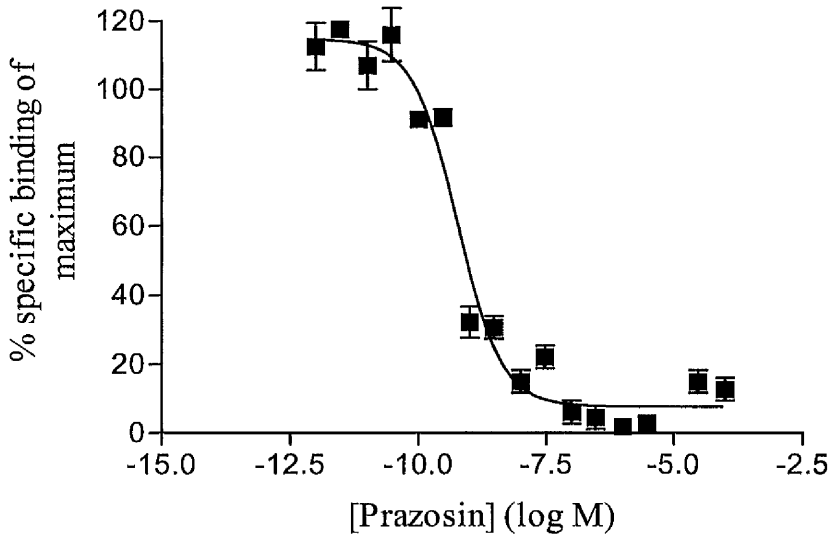
therefore expected to find no specific binding with [^{3}H]-prazosin in the KO liver as a direct consequence of the deleted α_{1B}-adrenoceptor. Not only is there specific binding but this binding is of high affinity and well within the range of pK_i values that prazosin has been shown to exhibit at α_1-adrenoceptors (Bylund *et al*, 1994 and Garcia Sainz *et al*, 1994).

2.3.2.2 Competition studies

The competition curves for the ligands used in this set of experiments are shown in Figures 2.12 – 2.16 with the table of their respective pK_i values given in Figure 2.17.

When prazosin was used as the competing ligand, as well as for the radioligand, the KO liver was found to produce a two-site binding curve with a Hill slope significantly different from negative unity (-0.5: 95% CI = -0.8 to –0.2), despite saturation studies with [^{3}H]-prazosin displaying one-site binding in KO liver. The pK_i values were calculated to be 9.0 ± 0.5 and 7.0 ± 0.23 with the high affinity site being present in much greater numbers than the low affinity site, making up almost 60% of the total binding sites (57±6.3%). This is a similar situation to that seen in the KO brain where prazosin also demonstrated two-site binding. Conversely, in WT liver prazosin binds monophasically producing a Hill slope of exactly -1.0 (95% CI = -1.1 to –0.9) and a pK_i of 9.7 ± 0.11 indicating binding to a homogeneous population of α_1-adrenoceptors (Figure 2.12).

Although the use of prazosin as a competing ligand confirms that α_1-adrenoceptors are present in the WT and KO liver it does not confirm the identity of the subtype(s)



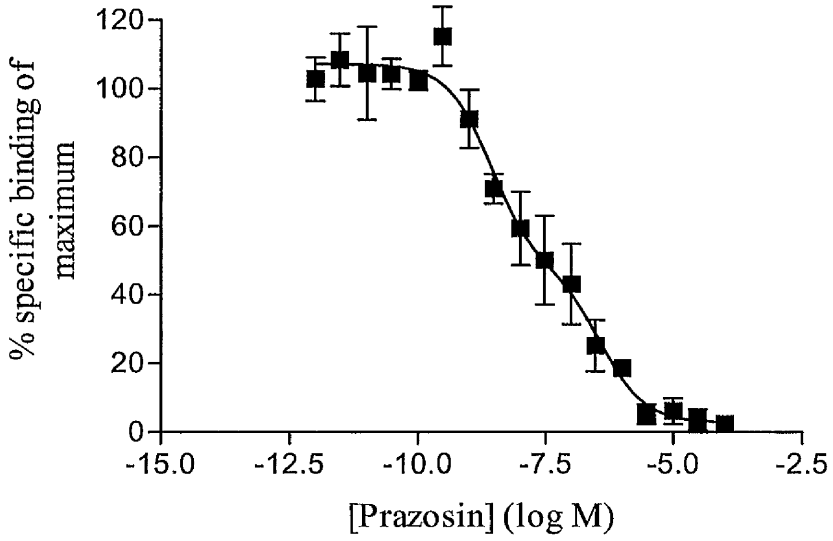


Figure 2.12:- Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) liver membranes by increasing concentrations of prazosin (1pM-0.1mM). Data points are expressed as mean ± s.e.m. (n≥3)

present. In order to determine this we used the same subtype selective ligands as those used in the brain.

In the WT liver RS100329 bound with low affinity, producing a pK_i value of 7.8±0.02 with a Hill slope which was not significantly different from negative unity (-1.0: 95% CI = -1.1 to –0.9), indicative of competitive binding to a homogeneous population of receptors. The KO liver on the other hand was found to bind RS100329 with greater affinity producing a pK_i value of 9.3±0.07 and this binding appeared to be to only one-site due to the Hill slope of the curve being not significantly different from negative unity (-0.82: 95% CI = -1.03 to –0.61) (Figure 2.13).

In WT liver tissue (R)-A-61603 produced a biphasic curve with affinities of 7.4 ± 0.4 and 4 ± 0.06 and a shallow Hill slope of -0.7 (95% CI = -0.9 to –0.5) when no GTPγS was present. This high affinity site was only 7±2% of the total number of binding sites and upon addition of GTPγS this site disappeared producing a monophasic curve of low affinity (pK_i = 4.2 ± 0.08) with a Hill slope not different from negative unity (-1.1 : 95% CI = -1.3 to –0.8). The binding of (R)-A-61603 in the KO liver was unaffected by the presence of GTPγS. When GTPγS was absent, (R)-A-61603 bound with a high affinity (7.9±0.15) which appeared to be competitive due to the steep Hill slope which was not significantly different from negative unity (-1.3: 95% CI = -2.2 to –0.5). In the presence of GTPγS the same was found to be true: (R)-A-61603 bound with high affinity producing a pK_i of 8.2±0.13 with a steep Hill slope (-0.75: 95% CI = -1.1 to –0.4) (Figure 2.14).

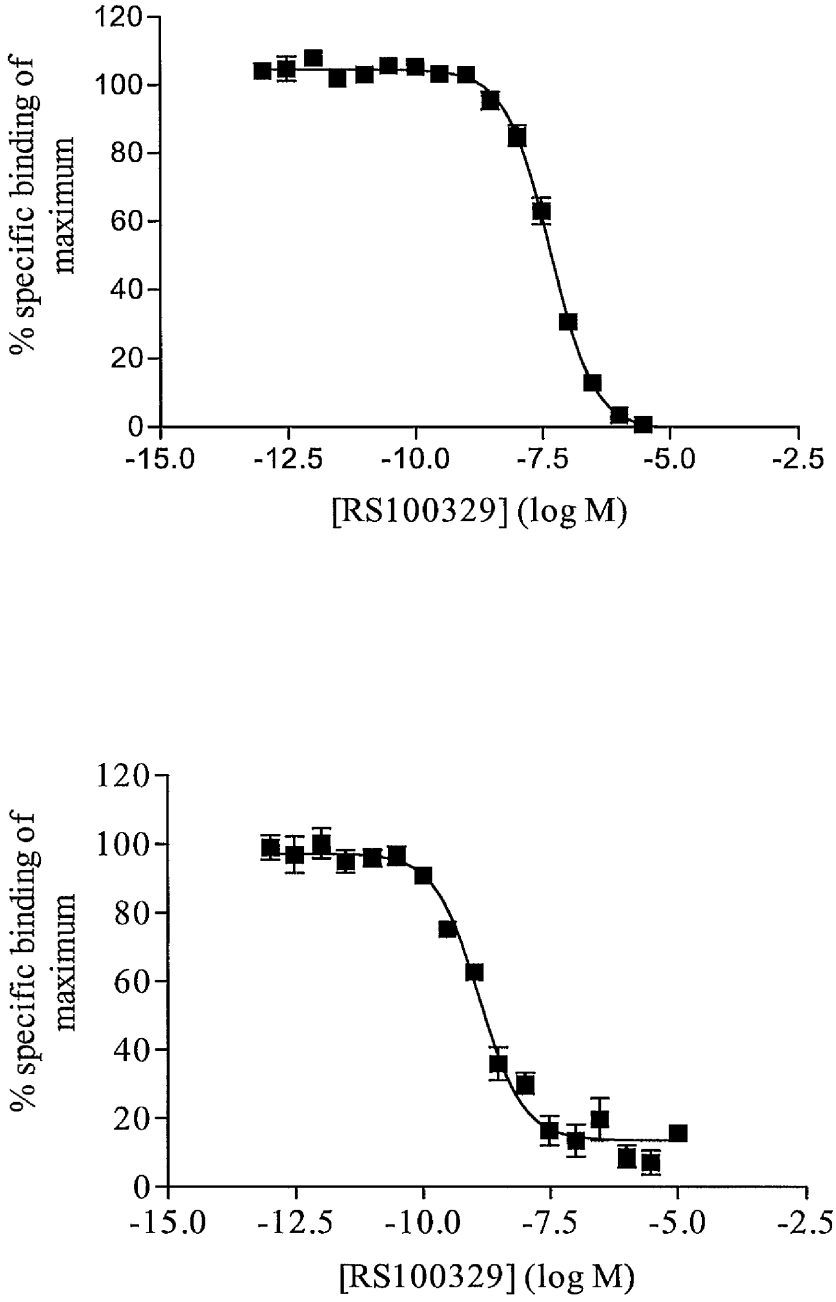
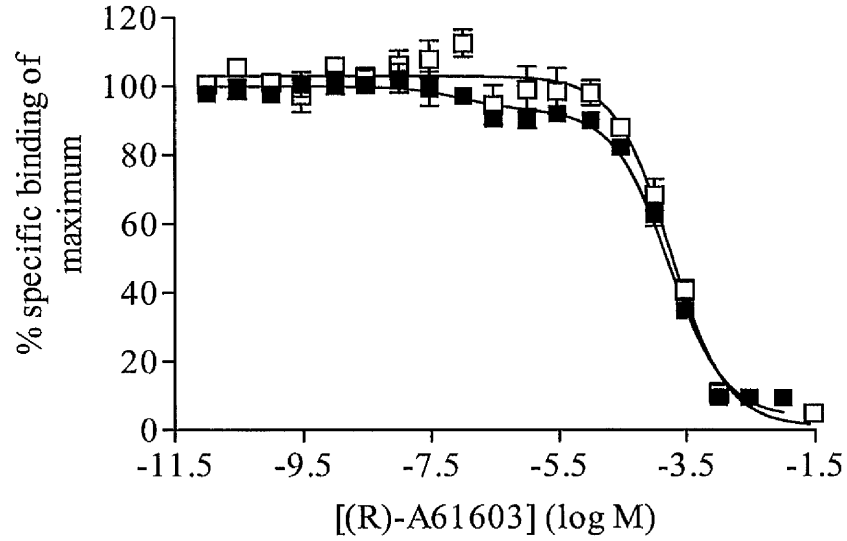


Figure 2.13:- Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) liver membranes by increasing concentrations of RS100329 (0.1pM-10μM). Data points are expressed as mean ± s.e.m.(n≥3)



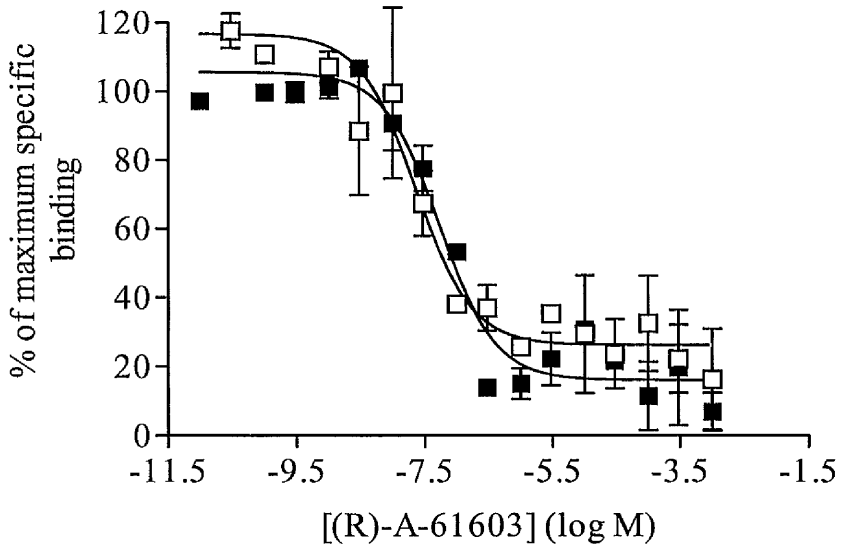


Figure 2.14:- Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) liver membranes by increasing concentrations of (R)-A-61603 (10pM-1mM) in the presence (□) and absence (■)of GTPγ S. Data points are expressed as mean ± s.e.m. (n≥3)

L765,314, which has been reported in the literature to be selective for α_{1B}-adrenoceptors (Patane *et al*, 1998) bound monophasically and with relatively low affinity in both WT and KO liver membranes resulting in pK_i values of 7.6±0.03 and 7.8±0.1, respectively. The Hill slopes for both curves were not significantly different from negative unity with the WT liver producing a slope of -1.1 (95% CI = -1.3 to -0.9) and the KO liver a slope of -0.8 (95% CI = -1.1 to -0.5) (Figure 2.15).
In WT and KO liver, BMY7378 inhibited [^{3}H]-prazosin binding with low affinity to produce virtually identical pK_i values of 6.3±0.02 and 6.2±0.09 respectively, with similar Hill slopes of -1.0 (95% CI = -1.16 to –0.94) and –0.9 (95% CI = -1.19 to-0.54) respectively, which were not significantly different from negative unity, indicating competitive one-site binding (Figure 2.16).

2.3.2.3. Correlations

The correlations for the ligand's affinities in WT and KO liver and their affinities at cloned α_1-adrenoceptors are shown in Figures 2.18 and 2.19 respectively. For KO liver the high affinity pK_i for prazosin was used throughout. All (R)-A-61603 data was plotted as pK_i values in the presence of GTPγS. As with correlations in brain the affinities for A-61603 are for the racemic mixture at the three recombinant subtypes.

In the WT liver the strongest correlation is evident with cloned α_{1b}-adrenoceptors, which produced an r value of 0.90, whereas there was only a weak correlation with α_{1d}-adrenoceptors (r = 0.58) and virtually no correlation with α_{1a}-adrenoceptors

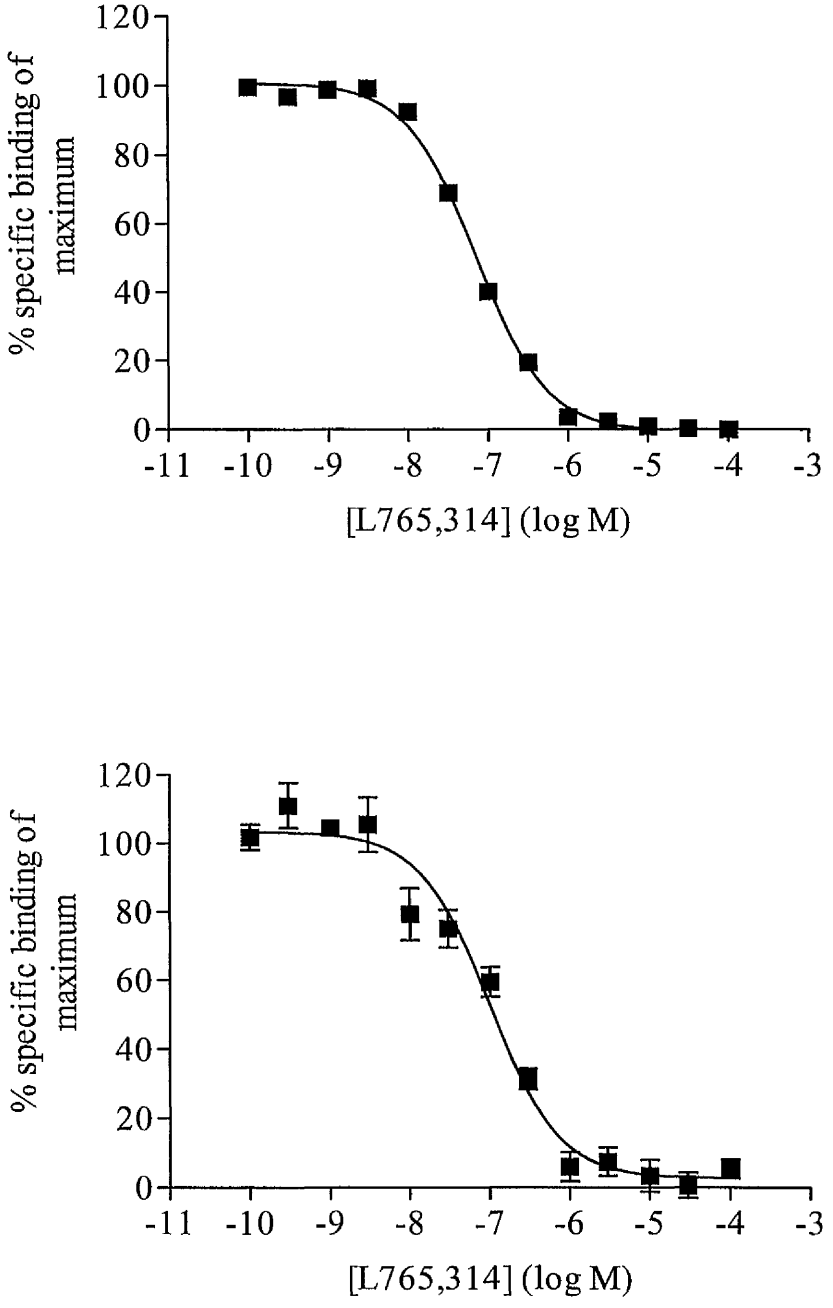


Figure 2.15:- Displacement of 0.5nM [^{3}H]-prazosin from WT (top) and KO (bottom) liver membranes by increasing concentrations of L765,314 (0.1nM-0.1mM). Data points are expressed as mean ± s.e.m. (n≥3)

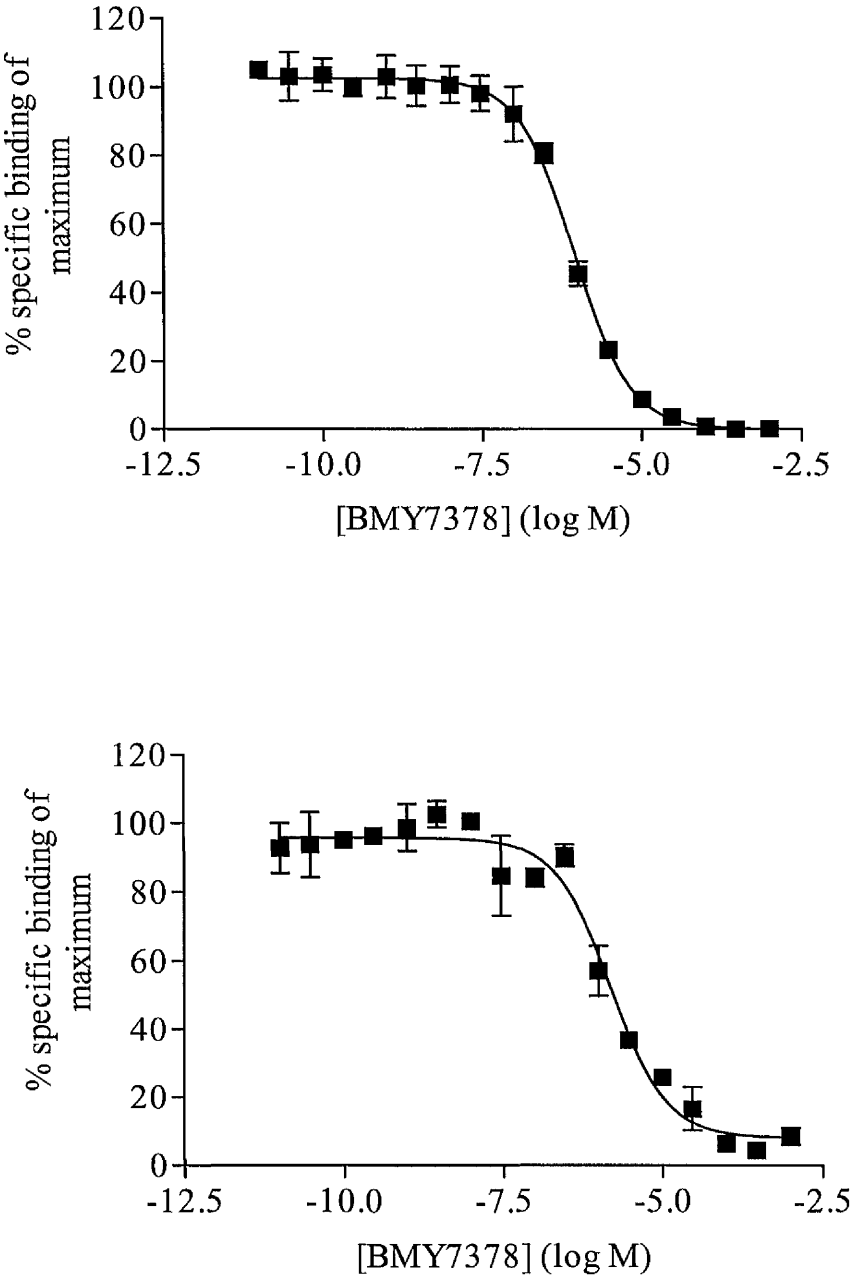


Figure 2.16:- Displacement of 0.5nM [^{3}H]-prazosin from WT (top)and KO (bottom) liver membranes by increasing concentrations of BMY7378 (10pM-1mM). Data points are expressed as mean ± s.e.m. (n≥3)

	WT LIVER		KO LIVER	
	pK_i	- nH	pK_i	- nH
Prazosin (H) (L)	9.7±0.11	1.0±0.25	9.0±0.5, 7.0±0.23	0.50±0.08*
(R)-A-61603 (H) (L)	7.4±0.4, 4.0±0.06	0.70±0.12*	7.9±0.15	1.3±0.4
(R)-A-61603 +GTPγS	4.2±0.08,	1.1±0.2	8.2±0.14	0.75±0.17
RS100329	7.8±0.02	1.0±0.04	9.3±0.07	0.82±0.1
L765,314	7.6±0.03	1.1±0.07	7.8±0.1	0.8±0.14
BMY7378	6.3±0.02	1.0±0.05	6.2±0.09	0.9±0.15

Figure 2.17:- Inhibition affinities in WT and KO liver membranes expressed as pK_i values ± s.e.m. Hill slopes (nH) are expressed as the negative of the hill slope ± s.e.m. (H) = high affinity site and (L) = low affinity site. * indicates a Hill slope which is significantly different from negative unity.

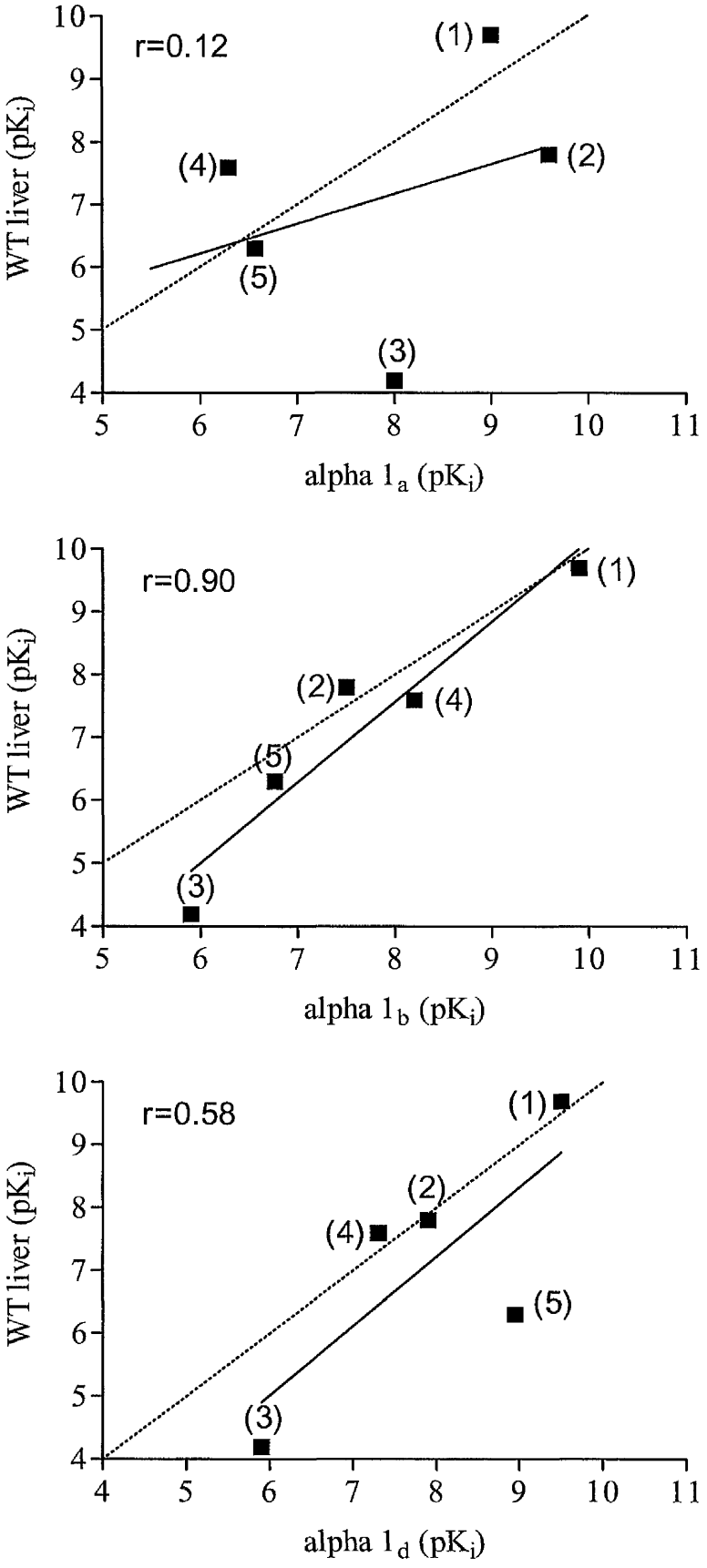


Figure 2.18:- Correlation of potencies of ligands used on WT liver with published potencies of the same ligands at cloned α_1-adrenoceptor subtypes. (1):- prazosin, (2):- RS100329, (3):- (R)-A61603, (4):- L765,314 and (5):- BMY7378. (Williams *et al*, 1999, Knepper *et al*, 1995, Patane *et al*, 1998 and Yang *et al*, 1997). The dotted line shows the line of unity.

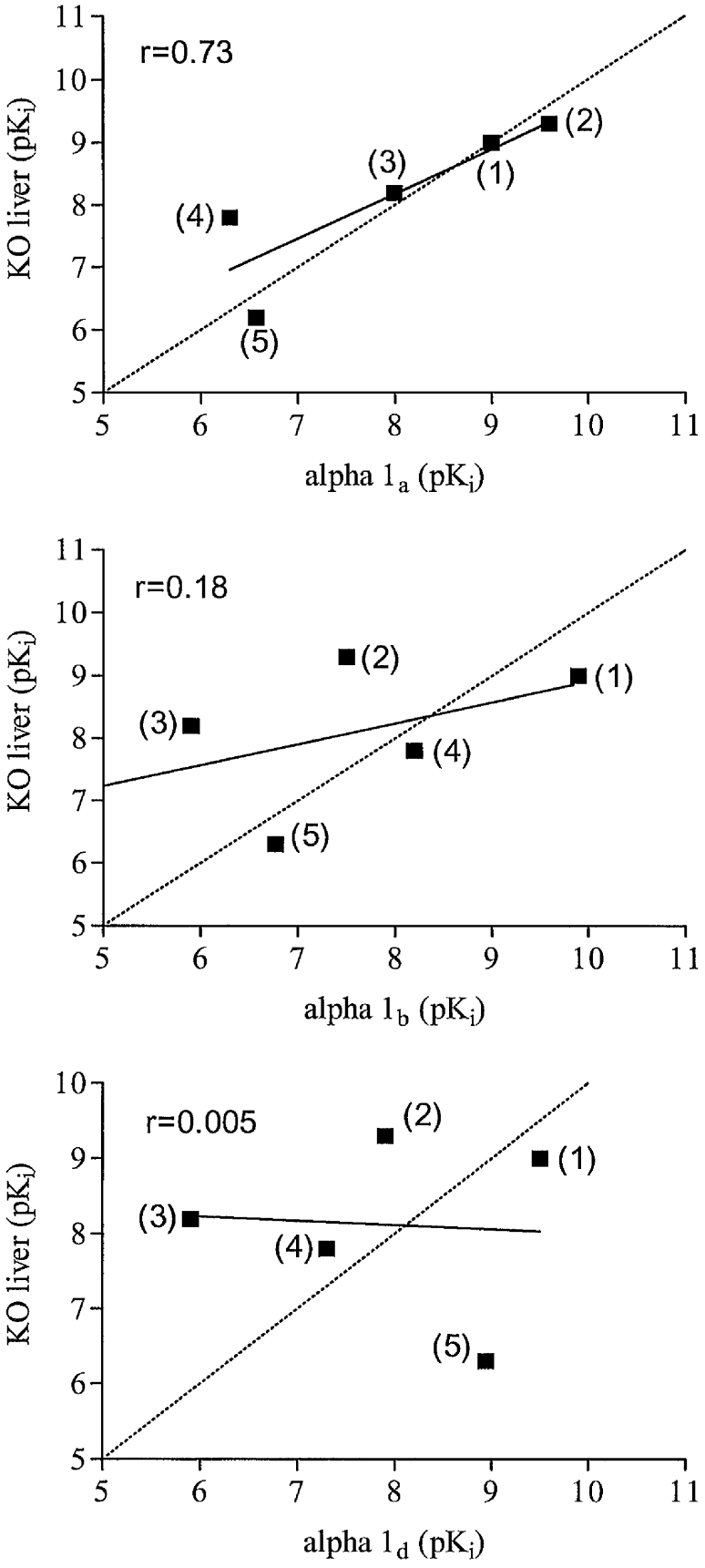
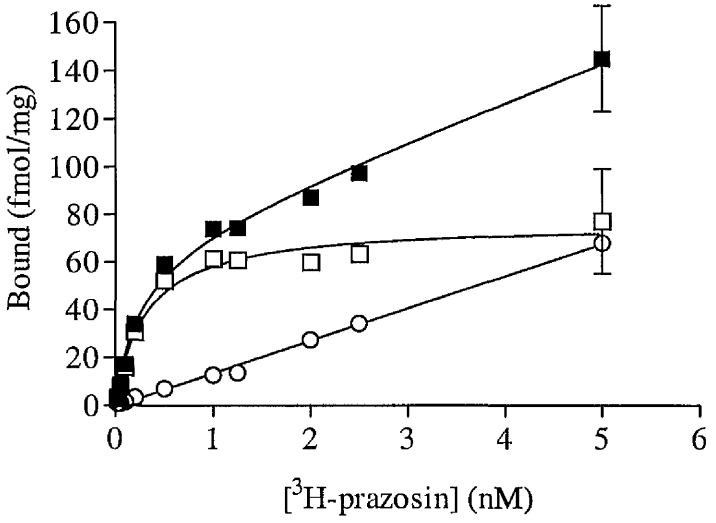


Figure 2.19:- Correlation of potencies of ligands used on KO liver with published potencies of the same ligands at cloned α_1-adrenoceptor subtypes. (1):- prazosin, (2):- RS100329, (3):- (R)-A61603, (4):- L765,314 and(5):- BMY7378. (Williams *et al*, 1999, Knepper *et al*, 1995, Patane *et al*, 1998 and Yang *et al*, 1997). The dotted line shows the line of unity.

(r = 0.12). The KO liver displayed a different correlation to that of the WT liver by showing its strongest correlation with α_{1a}-adrenoceptors (r = 0.73) with no correlation evident with either α_{1b}-adrenoceptors (r = 0.18) or α_{1d}-adrenoceptors (r = 0.005).

2.3.3. Preliminary age studies

A chance observation in 3 month old KO mice showed that there are negligible α_1-adrenoceptors present in KO liver at this age. Saturation studies revealed a B_{max} of 7.4±0.73fmol/mg and a K_D of 1.0±0.27nM (Figure 2.20), compared with livers from 4 month old KO livers (Figure 2.11) (which were used for the subtyping experiments): as previously reported, these had a B_{max} of 30±2.0fmol/mg and a K_D of 0.15±0.04nM. The B_{max} and K_D in 3 month old KO livers was significantly different compared with 4 month old KO livers. Initially it was thought that this discrepancy in the density of α_1-adrenoceptors in this tissue was a result of using immature mice. However, when saturation studies were performed on livers from 3 month old WT mice the α_1-adrenoceptor population was found to be present in densities similar to that seen in livers from 4month old WT mice. 3 month old WT mice had a B_{max} of 76±3.3fmol/mg and a K_D of 0.3±0.05nM (Figure 2.20), whilst 4 month old WT mice had a B_{max} of 50±3.1fmol/mg and a K_D 0.3±0.08nM (Figure 2.11). In fact the B_{max} from 3 month old WT mice is significantly greater ($p<0.05$) than that from 4 month old WT mice, with the K_D remaining the same. Therefore it would appear that this phenomenon seen in the liver is unique to the KO mouse.



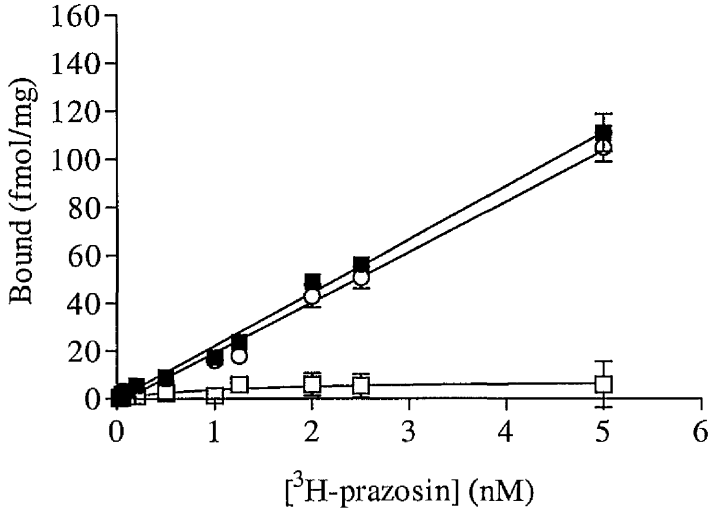


Figure 2.20:- Saturation curves showing [^{3}H]-prazosin binding (0.025-5nM) in 3 month old WT (top) and KO (bottom) liver membranes. WT liver produced a K_D of 0.3±0.05nM and a B_{max} of 76±3.3fmol/mg, whilst KO liver produced a K_D of 1.0±0.27nM and a B_{max} of 7.4±0.73fmol/mg Total (■), specific (□) and non-specific (○) binding. Each data point is expressed as mean ± s.e.m. (n=3 for WT and KO).

2.4. DISCUSSION

Radioligand binding is a popular technique with which to characterise α_1-adrenoceptor subtypes because it can provide direct measurements of drug-receptor dissociation constants that are independent of the character of the drug. Radioligand binding studies, using purified plasma membranes, avoid the problems associated when obtaining drug affinities in tissues e.g. transport of the drug to its site of action, neuronal or extraneuronal uptake of the drug, the drug's metabolism and desensitisation of the response. Therefore radioligand binding may provide the most accurate measurement of a drug's true binding affinity. If an antagonist binds to two groups with different affinities this constitutes strong evidence that the receptor populations are different, hence radioligand binding becomes a valuable tool with which to identify and characterise α_1-adrenoceptor subtypes.

Radioligand binding studies were undertaken on plasma membranes isolated from the brains and livers of WT and KO mice in order to confirm previous findings that the WT brain has a mixture of α_{1A}- and α_{1B}-adrenoceptors (Cavalli *et al*, 1997; Yang *et al*, 1998), whilst the WT liver contains α_{1B}-adrenoceptors (Cavalli *et al*, 1997; Yang et al, 1998). A further aim was to ascertain if there was a reduced number of receptors in the KO tissues as a result of deleting the α_{1B}-adrenoceptor.

2.4.1. Brain

The presence of α_1-adrenoceptors in WT and KO brain was confirmed by saturation studies using [^{3}H]-prazosin. The calculated K_D's of 0.6nM (WT) and 0.8nM (KO) are

within the range of K_D values which have been quoted for [^{3}H]-prazosin binding to α_1-adrenoceptors in the brains of rats, mice, pigs and humans (50pM – 2nM) (Han *et al*, 1987b; Palacios *et al*, 1987; Minneman *et al*, 1988; Oshita *et al*, 1991; Cavalli *et al*, 1997 and Wikberg-Matsson *et al*, 1998). The number of receptors in the KO brain was significantly decreased compared with the WT brain (30% reduction). If the WT brain does indeed contain a mixed a population of α_{1A}- and α_{1B}-adrenoceptors then a reduction in α_1-adrenoceptor density in the KO brain is to be expected.

When unlabelled prazosin was used to compete with [^{3}H]-prazosin, both WT and KO brains exhibited a high affinity site with a pK_i greater than 9.0, characteristic of prazosin binding to α_1-adrenoceptors (Bylund *et al*, 1994). In addition to this high affinity site the KO brain had a second low affinity site, with a pK_i of 7.4. The putative α_{1L}-adrenoceptor has been defined as having a low affinity for prazosin (Drew, 1985; Flavahan and Vanhoutte, 1986; Muramatsu *et al*, 1990) and although, to date, it has not been identified genetically there is an increasing amount of evidence which points to its existence functionally (Muramatsu *et al* 1990; Ford *et al*, 1997; Van der Graaf *et al*, 1997; Daniels *et al*, 1999), whether it be as a subtype in its own right or as an isoform of the α_{1A}-adrenoceptor. Although the affinities obtained for prazosin at the α_{1L}-adrenoceptor can sometimes be as low as ~8.0 (Kohno *et al*, 1994; Van der Graaf *et al*, 1997) it is very rare to see affinities of less than 8.0. Therefore it is unlikely that the low affinity site for prazosin observed in the KO brain can be attributed to the α_{1L}-adrenoceptor. It seems more likely that the second site represents prazosin binding to α_2-adrenoceptors. The initial identification of α_2-adrenoceptor subtypes was based on differential affinity for prazosin. The α_{2B}- and α_{2C}-adrenoceptors have relatively high affinities for prazosin, whilst the α_{2A}- and α_{2D}-adrenoceptors have relatively low

affinities for prazosin (Bylund *et al*, 1994). The affinity of prazosin at the α_{2B}- or α_{2C}-adrenoceptor is approximately 7-7.5 (Bylund *et al*, 1994), which correlates well with the second site prazosin identified in the brain.

The α_{1A}-selective antagonist, RS100329, bound consistently to two sites in the WT brain, of high and low affinity. The pK_i values of 9.5 and 7.4 are consistent with binding to α_{1A}- and α_{1B}-adrenoceptors respectively (Williams *et al*, 1999). The ratio for the two sites as a percentage of the total binding was 36%:64% in favour of the lower affinity site (α_{1B}). This is similar to the ratios of α_{1A}:α_{1B}-adrenoceptors reported by Cavalli *et al* (1997) and Yang *et al* (1998) (58% and 70% respectively of the total binding was attributed to the α_{1B}-adrenoceptor). In the KO brain, where this second low affinity site is absent, RS100329 demonstrates competitive binding to a single high affinity site comparable to the high affinity site in the WT brain, representative of the α_{1A}-adrenoceptor.

The agonist used in this study, (R)-A-61603, is regarded in the literature as being α_{1A}-selective (Knepper *et al*, 1995; Ford *et al*, 1997). Experiments were conducted in the presence and absence of GTPγS (a non-hydrolysable analogue of GTP). When plasma membranes are used the concentration of GTP is limiting. Therefore if an agonist is used as a competing ligand without the addition of GTP or a non-hydrolysable analogue (such as that used here), two-site binding is more likely to occur. A more detailed explanation of this phenomenon is given in the methods section of this chapter. If two-site binding is a result of a limiting concentration of GTP, then addition of GTPγS should abolish this and only one-site will be detected.

The WT and KO brain both exhibit two-site binding to (R)-A-61603 of similar affinities regardless of whether GTPγS is present. This would suggest that the two-site binding is due to the presence of two receptor populations rather than a deficiency of GTP. The low affinity site in the WT brain seems likely to be the α_{1B}-adrenoceptor as this correlates with the affinity quoted for the racemic mixture of A-61603 at $\alpha_{1B/b}$-adrenoceptors (Knepper *et al*, 1995). The high affinity site of 6.7 although too low to be an α_{1A}-adrenoceptor is too high to be an α_{1D}-adrenoceptor, if the published pK_i values are used as a guideline (Knepper *et al*, 1995). It should be noted here that the present study used the R-enantiomer of A-61603, which appears to confer potency at α_{1A}-adrenoceptors (Knepper *et al*, 1995). However its potency at α_{1B}- and α_{1D}-adrenoceptors has not been determined.

The pK_i values in KO brain are also confusing. The low affinity site could be either an α_{1B}- or an α_{1D}-adrenoceptor. However, the α_{1B}-adrenoceptor cannot be present in the KO brain because its deletion has been confirmed both by the group who created the mouse (Cavalli *et al*, 1997) and also by our own laboratory (unpublished observations). The affinity of A-61603 (the racemic mixture) at α_{1b}- and α_{1d}-adrenoceptors is similar, whilst in tissues it is 10 times more potent at α_{1B}-adrenoceptors than at α_{1D}-adrenoceptors (Knepper *et al*, 1995). Therefore, the low affinity site is more likely to be the α_{1D}-adrenoceptor, whilst the high affinity site is most probably the α_{1A}-adrenoceptor, despite its relatively low affinity at this subtype. If this is the case then logically the high affinity site in the WT brain is also an α_{1A}-adrenoceptor, due to the similar affinity values of these two sites. In WT brain the proportion of the two sites is similar to the proportions found with RS100329, with the low affinity site, likely to be the α_{1B}-adrenoceptor, constituting 55% of the total α_1-adrenoceptor population. In the

KO brain this proportion has shifted in favour of the high affinity site, likely to be the α_{1A}-adrenoceptor, accounting for 86% of α_1-adrenoceptors in the brain. The remaining 14% could be α_{1D}-adrenoceptors (but see discussion of BMY7378 data). Alternatively it could be an experimental observation, for which there is no explanation at this point.

From work in our own laboratory, this unexpected behaviour of (R)-A-61603 does not seem to be limited to native α_1-adrenoceptors. In cell lines expressing recombinant α_{1a}-adrenoceptors, (R)-A-61603 has been shown to be unaffected by GTPγS, producing biphasic curves whether GTPγS is present or not. However, when phenylephrine was used in binding studies, using the same cell line, biphasic curves were produced in the absence of GTPγS, becoming monophasic in the presence of GTPγS. It is possible that (R)-A-61603 is resistant to the effects of GTPγS or may respond in a different manner to that of other agonists, such as phenylephrine, when it is present. This may be related to its selectivity at $\alpha_{1A/a}$-adrenoceptors. (R)-A-61603 has an affinity at α_{1a}-adrenoceptors that is approximately 30 fold higher than phenylephrine's affinity at this receptor and in tissues expressing α_{1A}-adrenoceptors this selectivity is increased; (R)-A-61603 is 160-330 times more potent than phenylephrine. Could the higher affinity and selectivity of (R)-A-61603 at $\alpha_{1A/a}$-adrenoceptors be responsible for its unusual behaviour when GTPγS is present?

During my project a new α_1-adrenoceptor antagonist, L765, 314, became available to me (a kind gift from Dr. Patane at Merck) which had been proposed to be selective for the α_{1B}-adrenoceptor (Patane *et al*, 1998). Binding studies at cloned receptors had shown that L765,314 possessed a 10-fold selectivity at the α_{1b}-adrenoceptor over the

α_{1d}-adrenoceptor and 100-fold selectivity over the α_{1a}-adrenoceptor (Patane *et al*, 1998). Binding in native tissues expressing either a homogeneous population of α_{1A}- or α_{1B}-adrenoceptors i.e. rat prostate and rat spleen showed that L765,314 had approximately 50 fold selectivity at α_{1B}-adrenoceptors over α_{1A}-adrenoceptors. L765,314's affinity in tissues expressing α_{1D}-adrenoceptors was not determined (Patane *et al*, 1998). The possibility that a drug had been developed which showed selectivity for the α_{1B}-adrenoceptor was exciting because to date there have been no examples of a convincingly selective α_{1B}-adrenoceptor antagonist. I hoped that L765,314 would highlight a clear distinction between the α_1-adrenoceptor subtypes of WT and KO tissues.

In WT and KO brain L765,314 bound competitively to one site. Having only the affinity values quoted by Patane *et al* (1998) for comparison the data would suggest that in both WT and KO brain L765,314 is binding to α_{1A}-adrenoceptors. This is not only unexpected but is inconsistent with my data using other competitors and with the literature. If the WT brain contains a mixture of α_{1A}-and α_{1B}-adrenoceptors as has been previously proposed (Cavalli *et al*, 1997; Yang *et al*, 1998) and if L765,314 is selective for the α_{1B}-adrenoceptor then I would have expected L765,314 to either exhibit two-site binding in WT brain or to bind selectively to α_{1B}-adrenoceptors producing a pK_i similar to that reported by Patane *et al* (1998). However, two-site binding is not apparent in the WT brain and the pK_i is lower than expected for L765,314 to be acting at α_{1B}-adrenoceptors. This could be accounted for if the α_{1B}-adrenoceptor was present in a proportionately smaller amount than the α_{1A}-adrenoceptor resulting in the α_{1A}-adrenoceptor masking the presence of the α_{1B}-adrenoceptor. However, the results with

RS100329 and (R)-A-61603 contradict this by showing that the α_{1B}-adrenoceptor makes up a substantial portion of the α_1-adrenoceptors in the WT brain, enough to demonstrate two-site binding with α_{1A}-selective ligands. An alternative explanation may be that L765,314 is not as selective at the α_{1B}-adrenoceptor as has been proposed by Patane *et al* (1998). When we carried out radioligand binding on all three cloned α_1-adrenoceptor subtypes using L765,314 as a competitor, no selectivity was observed amongst any of the subtypes (Mackenzie, unpublished observations).

The final antagonist used was BMY7378, an α_{1D}-adrenoceptor antagonist which displays nanomolar affinity at the α_{1D}-adrenoceptor (Kenny *et al*, 1995; Saussy *et al*, 1994; Goetz *et al*, 1995). BMY7378 bound competitively with low affinity to a single site in both WT and KO brains. This low affinity suggests that the α_{1D}-adrenoceptor is not present in either the WT or KO brain. Although the low affinity site for (R)-A-61603 seemed to suggest an α_{1D}-adrenoceptor population, the results with BMY7378 overrule this because BMY7378 has been more widely characterised with regard its affinity values at the three α_1-adrenoceptor subtypes. In addition to this, being an antagonist it provides more accurate affinity values without the complications associated with using an agonist. With this in mind I was confident that the data obtained with BMY7378 provided evidence against the presence of an α_{1D}-adrenoceptor in either the WT or KO brain. This is consistent with the results of Cavalli *et al* (1997) and Yang *et al* (1998) who found no evidence for the functional presence of the α_{1D}-adrenoceptor in any of the tissues they examined despite the presence of the α_{1D}-adrenoceptor mRNA (Cavalli *et al*, 1997). The low affinity 'single site' binding of BMY7378 in both WT and KO brains is likely a reflection of BMY7378's inability to

distinguish between α_{1A}- and α_{1B}-adrenoceptors and is consistent with its known affinity at these receptors (Saussy *et al*, 1994; Goetz *et al*, 1995). Thus it binds to α_{1A}-adrenoceptors in the KO brain and α_{1A}- and α_{1B}-adrenoceptors in the WT brain with similar affinity.

The correlations for the WT and KO brain show that the WT brain correlates to the same extent with α_{1a}- and α_{1b}-adrenoceptors, indicative of a mixed population whereas the KO brain clearly correlates better with the α_{1a}-adrenoceptors. The saturation and competition studies suggest that there has been an upregulation of the α_{1A}-adrenoceptor in the KO brain. The saturation studies reveal that approximately a third of the binding appears to be due to α_{1B}-adrenoceptors, whereas the competition studies with RS100329 and (R)-A-61603 suggest that approximately 60% of the binding can be attributed to α_{1B}-adrenoceptors. Therefore it appears that the brain attempts to compensate for the reduction in α_1-adrenoceptor density.

Defining the role of α_1-adrenoceptors in the central nervous system and in particular the brain is problematic due to the problems associated with drugs crossing the blood brain barrier and a lack of subtype selective antagonists. The activation of central α_1-adrenoceptors has been shown to enhance locomotor activity and arousal (Sirvio and MacDonald, 1999). However, results obtained from transgenic mice, which either overexpress the α_{1B}-adrenoceptor or express a constitutively active form of the receptor, contradict the view that central α_1-adrenoceptors have a stimulatory effect. Zuscik *et al* (2000) reported that these mice had a locomotor dysfunction, suffered grand mal-type seizures and were subject to neurodegeneration that worsened with age. In the light of

these findings it would be of interest to examine the central effects of deleting the α_{1B}-adrenoceptor using the KO mice used in this study.

To summarise, these binding studies confirm the presence of a mixed population of α_{1A}- and α_{1B}-adrenoceptors in the WT brain with a ratio of 36%:64% respectively and the absence of the α_{1B}-adrenoceptor from the KO brain, which now contains a pure population of α_{1A}-adrenoceptors. In addition, deletion of the α_{1B}-adrenoceptor from the KO brain appears to have unmasked a population of α_2-adrenoceptors that is not evident from binding studies in the WT brain. The possible reasons for this will be discussed in more detail in the discussion for the liver results.

2.4.2. Liver

The radioligand binding studies in the brain produced results that were, on the whole, anticipated. However results from the liver studies were unexpected. It was predicted that the KO liver would not possess an α_1-adrenoceptor population and therefore would display no specific binding, as a consequence of deleting the α_{1B}-adrenoceptor, the sole receptor subtype reported to be present in the murine liver (Cavalli *et al*, 1997; Yang *et al*, 1998). Cavalli *et al* (1997) found there to be a 98% decrease in α_1-adrenoceptor density in KO livers when compared to WT livers. In contrast to this I discovered that at 4 months old there was only a 40% decrease in α_1-adrenoceptor numbers in KO livers. The WT liver had a B_{max} of 50fmol/mg, which is identical to that described by Yang *et al* (1998). Cavalli *et al* reported a B_{max} of only 20fmol/mg in WT liver, which decreased to a negligible amount of protein in the KO liver. However, in the present study the B_{max} in the KO liver remained as high as 30fmol/mg. The calculated K_D values in WT and

KO liver were similar and comparable to those of Cavalli *et al* (1997) and Yang *et al* (1998). I believe the discrepancy between the results presented here and those published by Cavalli *et al* (1997) are due to methodological differences in the isolation of the plasma membranes used for these experiments. Cavalli *et al* used a centrifugation speed of 10,000 x g, which is not a sufficiently high enough speed to isolate plasma membranes, but will isolate only mitochondrial membranes (Evans, 1990). To isolate plasma membranes a centrifugal speed of at least 30,000 x g is required and a speed in excess of this was used for the isolation of plasma membranes in my experiments.

Although the presence of α_1-adrenoceptors in the KO liver was unexpected, a mechanism to compensate for the absence of the α_{1B}-adrenoceptor was not surprising. As well as determining the subtype present in the WT liver, an interesting aim of this study became the determination of the α_1-adrenoceptor subtype which had 'replaced' the α_{1B}-adrenoceptor in the KO liver.

When prazosin was used as the unlabelled competitor the WT liver displayed one-site binding and the KO liver displayed two-site binding, a similar scenario to that observed in the brains of WT and KO mice. The site in the WT liver and the high affinity site in the KO liver display pK_i's typical of prazosin binding to α_1-adrenoceptors (Bylund *et al*, 1994) with similar affinity values to the high affinity sites in the WT and KO brain. A further analogy with the KO brain and KO liver can be found in the low affinity site to prazosin, which appears to represent α_2-adrenoceptors. As was the case in the KO brain, a contribution from the putative α_{1L}-adrenoceptor in the KO liver can be ruled out as the pK_i is too low to be attributed to the α_{1L}-adrenoceptor. However, prazosin has been shown to have an affinity of 7-7.5 at the α_{2B}- and α_{2C}-adrenoceptor subtypes

(Bylund *et al*, 1994) similar to the affinity observed in the KO liver. Saturation studies would not identify this lower affinity site found in the KO brain and liver because the concentrations of [^{3}H]-prazosin used are not high enough to label this population. It is possible that this second site is observed only in KO tissues because there is a reduction in the density of α_1-adrenoceptors which alters the ratio of α_1/α_2-adrenoceptors to an extent that the α_2-adrenoceptor population is uncovered. Alternatively there could be an upregulation of α_2-adrenoceptors in the KO tissues to compensate for the loss of α_1-adrenoceptors as a result of deletion of the α_{1B}-adrenoceptor.

From the subtype selective antagonists used, the two α_{1A}-selective ligands, RS100329 and (R)-A-61603 showed the clearest distinction in affinity values between the WT and KO liver, providing evidence that the α_1-adrenoceptor population in the KO liver had altered. RS100329 had a low binding affinity in the WT liver, consistent with an α_{1B}-adrenoceptor, whilst the [^{3}H]-prazosin binding sites in the KO liver had a 100-fold greater affinity for RS100329 than those in the WT, with a pK_i value similar to its published pK_i values at $\alpha_{1A/a}$-adrenoceptors (Williams *et al*, 1999). This 100-fold difference in affinity provides good evidence that the α_1-adrenoceptor population in the KO liver is different from that of the WT liver and suggests that it is the α_{1A}-adrenoceptor.

The results with (R)-A-61603, the α_{1A}-selective agonist, appear to consolidate these findings. In the presence of GTPγS, (R)-A-61603 is 10,000 times more potent at the α_1-adrenoceptors in the KO liver than those in the WT liver. The pK_i value calculated for (R)-A-61603 in the KO liver correlates well with previous reports of its affinity at $\alpha_{1A/a}$-

adrenoceptors (Knepper *et al*, 1995; Ford *et al*, 1997; Hrometz *et al*, 1999; Argyle and M^{c}Grath, 2000; Mackenzie *et al*, 2000). When GTPγS is not present, (R)-A-61603 still displays one-site binding in KO liver to a high affinity site with a similar pK_i to that when GTPγS is present. A biphasic curve in the absence of GTPγS is not evident. The reasons for this are not clear. A possible explanation may be that the α_1-adrenoceptor subtype in the KO liver is continually in a high affinity state; therefore, the addition of GTPγS would make no difference to the binding affinity. Alternatively this may be an effect that is related to (R)-A-61603's selectivity for $\alpha_{1A/a}$-adrenoceptors (mentioned in discussion of brain results). The only situation in which (R)-A-61603, with and without GTPγS, behaves as expected is in the WT liver. When GTPγS is absent, high and low affinity sites are observed. The high affinity site only accounts for 7% of the total binding and is found to disappear when GTPγS is present, suggesting this site is a result of a limiting concentration of GTP. The low affinity site in WT liver suggests an α_{1B}- or α_{1D}-adrenoceptor population.

Neither L765,314 nor BMY7378 differentiate between the α_1-adrenoceptors of WT and KO livers. BMY7378 binds with low affinity to both WT and KO hepatic α_1-adrenoceptors, the WT pK_i being comparable to that reported by Yang *et al* (1998). The calculated pK_i's are similar to the affinity values published for BMY7378 at α_{1A}- and α_{1B}-adrenoceptors (Saussy *et al*, 1994; Goetz *et al*, 1995). Although it is not possible to determine which of these two subtypes BMY7378 is binding to, it is possible to exclude α_{1D}-adrenoceptors from being present in either the WT or KO liver. However, there is some evidence from experiments carried out by a fellow colleague on isolated hepatocytes, that after 24 hours in culture both WT and KO hepatocytes express α_{1A}- and α_{1D}-adrenoceptors (Woollhead, PhD thesis, 2001). Therefore, it would seem that

the liver is capable of expressing all three α_1-adrenoceptor subtypes, although the triggers which switch each subtype on, remains to be determined. L765,314 produces very similar pK_i values in both WT and KO liver which are almost identical to the affinity values published for L765,314 at α_{1B}-adrenoceptors (Patane *et al*, 1998). For the WT liver this corresponds with the low affinities observed for RS100329, (R)-A-61603 and BMY7378 which overall seem to point to a pure population of α_{1B}-adrenoceptors. However, the KO liver does not contain α_{1B}-adrenoceptors and the binding data reveals that the α_1-adrenoceptor pharmacology is more like that of the α_{1A}-adrenoceptor. Again, these results call into question the selectivity and validity of L765,314 as an α_{1B}-adrenoceptor antagonist.

When the binding data from all the ligands used in WT and KO livers is analysed, the pharmacology confirms previous reports that the WT murine liver contains a pure population of α_{1B}-adrenoceptors, whereas the KO liver appears to contain a pure population of α_{1A}-adrenoceptors. The correlations in Figures 2.18 and 2.19 show this convincingly.

Not only does the α_1-adrenoceptor pharmacology of the KO liver point to this conclusion, but in theory it seems one of the subtypes would have to 'replace' the α_{1B}-adrenoceptor if the liver requires an α_1-adrenergic component to function correctly. α_1-Adrenoceptors are required in the liver for essential functions such as glycogenolysis (Schwarz *et al*, 1984; Ishac *et al*, 1992; Thai *et al*, 1996) so if the naturally expressed α_1-adrenoceptor subtype fails to function then a replacement mechanism must be present or these mice would not be viable. The KO mice appear healthy and demonstrate no outward signs of abnormalities.

The α_{1A}-adrenoceptor would be the likely replacement bearing in mind that only a handful of native tissues have been shown to functionally express the α_{1D}-adrenoceptor in either mice (Cavalli *et al*, 1997; Yang *et al*, 1998) or rats (Yang *et al*, 1997) despite its presence (at least in rats) at the mRNA (Faure *et al*, 1994; Price *et al*, 1994a; Rokosh *et al*, 1994; Scofield et al, 1995) and protein (Shen *et al*, 2000) level. The tissues in which the α_{1D}-adrenoceptor appears to be functional is mainly blood vessels, namely the rat aorta (Kenny *et al*, 1995; Piascik *et al*, 1995; Testa *et al*, 1995b; Piascik *et al*, 1997), mouse aorta (Daly *et al*, unpublished), rat iliac and femoral artery (Piascik *et al*, 1995; Piascik *et al*, 1997), rat carotid artery (Nagadeh, 1996, University of Glasgow, PhD thesis) and the mouse carotid artery (see Chapter 3). There is no evidence of the α_{1D}-adrenoceptor being functional in liver tissue, not only in mice and rats, but also in hamsters, guinea-pigs, rabbits, cats, dogs and humans. Although there is evidence that cultured hepatocytes express α_{1D}-adrenoceptors (Woollhead, 2001, University of Glasgow, PhD thesis) there is as yet no evidence to show that these receptors are functional.

In mice, rats and hamsters the α_{1B}-subtype is the functional α_1-adrenoceptor in the livers of these animals, whereas the remaining animals all functionally express the α_{1A}-adrenoceptor. This species difference in the subtype of α_1-adrenoceptor expressed in the liver may be age-related, a suggestion which was alluded to in the introduction to this chapter. With the exception of the guinea-pig and rabbit, the animals studied early on in their life span i.e. mice, rats and hamsters, all appear to express the α_{1B}-adrenoceptor. In contrast, those species that are studied experimentally at a later point in their life cycle i.e. cat, dog and human express the α_{1A}-adrenoceptor.

The α_{1B}-adrenoceptor has been implicated as being important in the regulation of growth. Chen *et al* (1995) found that stimulation of an α_{1B}-like adrenoceptor mediated a growth-promoting effect, characterised by an increase in cell protein and total RNA. Overexpression of the α_{1B}-adrenoceptor has also been shown to cause cardiac hypertrophy (Zuscik *et al*, 2001). In addition, Faber *et al* (2001) found that noradrenaline-stimulated α_1-adrenoceptors in cultured adventitial fibroblasts promoted proliferation and protein synthesis. Although it was not determined which subtype mediated this effect, the α_{1A}-adrenoceptor was not considered to be responsible because α_{1A}-adrenoceptor mRNA declined to almost undetectable levels when adventitial fibroblasts were cultured. The same group working on balloon injured rat aorta discovered that the trophic effects which this insult had on the adventitia could be inhibited by an α_{1B}-adrenoceptor antagonist (Zhang and Faber, 2001). If the α_{1B}-adrenoceptor is responsible for growth regulation it seems highly probable that the α_{1B}-adrenoceptor would be functionally present at a young age with its importance decreasing over time.

As well as having the capacity to regenerate, the liver is also one of the few organs which has been well documented as being capable of altering its balance of α_1- and β-adrenoceptors with age. In the liver of newborn rats the density of α_1-adrenoceptors is low and that of β-adrenoceptors high (Rossby and Cornett, 1991). Over a period of 6 days, from postnatal day 21 to postnatal day 27 the density of α_1-adrenoceptors increased until they reached a steady state, which was maintained over the developmental period studied (birth-90 days). In contrast the number of β-adrenoceptor binding sites decreased at postnatal day 6 and remained low throughout the 90 day period studied (Rossby and Cornett, 1991). If the rat liver is capable of altering its α_1-

and β-adrenoceptor populations then it is possible that it may also be capable of changing the subtype of α_1-adrenoceptor expressed. To my knowledge this phenomenon has not been reported in the mouse liver. However, I plan to carry out experiments to investigate this phenomenon to determine if this change does indeed occur in the murine liver.

Preliminary experiments carried out on 3 month old WT and KO livers reveal that the B_{max} of the WT liver is significantly greater, compared with 4 month old WT livers, being respectively, 76fmol/mg and 50fmol/mg. The K_D was unaltered. Interestingly, the KO liver at 3 months was found to have virtually no α_1-adrenoceptors with a B_{max} of only 8fmol/mg compared with the 4 month old livers which had a B_{max} of 30fmol/mg ($p<0.05$). In addition to this altered B_{max}, the K_D was approximately 10 times higher at 3 months (K_D = 1.0nM) compared with the K_D at 4 months (K_D = 0.15nM) ($p<0.05$). The reasons for this are unclear. These preliminary studies of 3 month old livers demonstrate that upregulation of α_1-adrenoceptors in the KO liver does not occur prior to birth or even in the first few weeks of life (when the switch from β- to α_1-adrenoceptors takes place in the rat) but it would seem that the majority of this upregulation takes place between 3 and 4 months. It is also clear that the α_1-adrenoceptors of murine liver are capable of changing not only the subtype that is expressed, but also the density of α_1-adrenoceptors. It is possible that the α_{1B}-adrenoceptor is expressed early on in the life of WT mice but as these mice age the α_{1B}-adrenoceptor population decreases to be replaced with the α_{1A}-adrenoceptor. In the KO liver the α_{1B}-adrenoceptor is already absent and so the α_{1A}-adrenoceptor is evident at an earlier age. This may also explain why there is a reduced α_1-adrenoceptor density in 4 month old WT livers when compared with 3 month old WT livers. In order to prove or disprove this hypothesis the

α_1-adrenoceptors in the WT liver at 3 months would have to be characterised and subtyped. A further age point at a later stage in the mouse life cycle, e.g. 1 year, would also need to be studied, carrying out the same experiments with the same ligands as at 3 and 4 months.

In conclusion, the binding studies on WT murine brains and livers reveal that they express a mixed population of α_{1A}- and α_{1B}-adrenoceptors and a pure population of α_{1B}-adrenoceptors, respectively, confirming previous reports (Cavalli *et al*, 1997; Yang *et al*, 1998). Binding in KO brain displays the characteristics of a pure population of α_{1A}-adrenoceptors, as does the KO liver. Deletion of the α_{1B}-adrenoceptor from the brain and liver tissues of KO mice uncovered a putative α_2-adrenoceptor population that was not detected in the WT tissues examined. The α_{1A}-adrenoceptors of the KO liver may result from upregulation of this receptor subtype to compensate for the deletion of the α_{1B}-adrenoceptor. This upregulation takes place between 3 and 4 months of age because at 3 months of age there are virtually no hepatic α_1-adrenoceptors present in KO mice. Although the purpose of this study was purely to confirm the subtypes present in WT brains and livers and to verify the absence of the α_{1B}-adrenoceptor from the equivalent KO tissues, some interesting issues have been raised with regard to the regulation of α_1-adrenoceptor subtypes in the liver. Further study will be necessary to characterise the exact nature of this regulation.

CHAPTER 3 – FUNCTIONAL CHARACTERISATION OF THE MURINE CAROTID ARTERY AND CLASSIFICATION OF THE α_1-ADRENOCEPTOR SUBTYPE MEDIATING CONTRACTION TO PHENYLEPHRINE IN WT AND KO MURINE CAROTID ARTERIES.

3.1. INTRODUCTION

3.1.1. Models using the carotid artery

The carotid artery is commonly used in models of atherosclerosis, angioplasty-induced restenosis and vascular injury, as well as being the artery most commonly used for gene transfer into blood vessels. The carotid artery lends itself well to studies such as these because it can be manipulated with relative ease in an *in vivo* situation. These models have been heavily studied in rats, rabbits and pigs (Wilcox *et al*, 1996; Shi *et al*, 1996; Ooboshi *et al*, 1998; Faggin *et al*, 1999; Gutterman *et al*, 1999; Oparil *et al*, 1999). But it is only in recent years, with the continuing advances of transgenic technologies, that mouse models of these pathological conditions have begun to emerge, as the ability to introduce transgenes or to disrupt endogenous gene expression has made the mouse an attractive species with which to study the function of specific genes in vascular biology. Mice have been developed that are deficient in apolipoprotein E leading to the development of a severe form of atherosclerosis, similar to humans (Plump *et al*, 1992). A carotid artery ligation model has been designed in the mouse which results in the development of a neointima similar to that seen with restenosis and many other injuries to the vasculature (Kumar and Lindner, 1997). The development of these models makes it easier to manipulate these pathological conditions at a genetic level and work already carried out in rats and rabbits means that the gene targets have already been identified.

Models of atherosclerosis or restenosis have highlighted the complexities of these disease processes. Studies have shown that these are multifactorial diseases, and no one factor has been implicated as being wholly responsible for their pathogenesis. For

example, there is indirect evidence to suggest that stimulation of α_1-adrenoceptors increases smooth muscle cell growth in the growing and adult artery and worsens atherosclerosis and restenosis after balloon injury (Xin *et al*, 1999). Angiotensin IV has been demonstrated to have trophic effects, whereby it has been shown to increase DNA synthesis and is capable of modulating cellular proliferation and hypertrophy in cardiovascular tissues. Following balloon injury in rabbit carotid arteries, AT_4 receptors, which bind angiotensin IV, were found to increase to 230% of control arteries, 20 weeks after the balloon injury (Moeller *et al*, 1999). Atherosclerotic arteries have been shown to have impaired relaxant properties, which can be rescued by transfecting nitric oxide synthase into the adventitia (Ooboshi *et al*, 1998).

3.1.2. Pharmacology of the carotid artery

Despite the carotid artery being widely used to study atherosclerosis, restenosis etc. very little effort has been focused on its 'normal' pharmacology. Any knowledge we have has generally been obtained from comparison of control arteries with carotid arteries from models such as those described above. This section will concentrate on some of the literature that is available about the pharmacology of the carotid artery, with the following section concentrating on what is known about the α_1-adrenoceptor pharmacology of the carotid artery.

Phenylephrine and UK14304 have been shown to contract the rat carotid artery through stimulation of α_1- and α_2-adrenoceptors respectively (Nagadeh, 1996, University of Glasgow, PhD thesis; Fujimoto and Itoh, 1995). 5-Hydroxytryptamine (5-HT) is also capable of producing a contraction, which in rats, has been shown to be inhibited by

tyrosine kinase inhibitors, suggesting that tyrosine kinase activation may partially mediate contractility to 5-HT in arterial smooth muscle (Watts *et al*, 1996). Angiotensin II contracts the rat carotid via the AT_1 receptor (Caputo *et al*, 1995; Boulanger *et al*, 1995), but it is also capable of relaxing this artery through the same receptor, causing the release of nitric oxide, which, in turn, inhibits the contraction mediated by angiotensin II (Boulanger *et al*, 1995). In an *in vitro* system designed to mimic the *in situ* carotid artery, the rat carotid artery has been shown to release endothelin from endothelial cells, in response to stretch. The released endothelin exerts a tonic stiffening effect, mediated via ET_A receptors (Marano *et al*, 1999). Vasopressin is capable of relaxing the rat carotid artery through V_1 receptors as is isoprenaline through a mixture of classical (β_1/β_2) and atypical (β_3) –adrenoceptors respectively (Rutschmann *et al*, 1998; MacDonald *et al*, 1999).

The rabbit carotid artery can be contracted by endothelin via ET_A receptors (Maurice *et al*, 1997) and by 5-HT mediated through 5-HT_{2A} receptors (Black *et al*, 1981). Bradykinin is capable of relaxing the rabbit carotid artery but pre-incubation with bradykinin is required before the relaxation is evident. Its actions appear to be mediated through BK_1 receptors, which are coupled to the release of endothelium-derived nitric oxide (Pruneau and Belichard, 1993).

3.1.3. α_1-Adrenoceptor pharmacology of the carotid artery

The subtyping of α_1-adrenoceptors in the carotid artery, in comparison with other arteries such as the aorta and mesenteric arteries, has not been extensively studied.

However, a handful of publications have investigated the α_1-adrenoceptor subtypes mediating vasoconstriction in this artery.

The rabbit carotid artery was initially proposed to contain an α_{1L}-adrenoceptor population, based on a low affinity for prazosin (Muramatsu *et al*, 1990). However, when the same group repeated their experiments a year later the rabbit carotid artery was found to be biphasically inhibited by prazosin, producing two distinct pK_B values of high and low affinity, in addition to having low affinity for the α_{1N}-adrenoceptor antagonist, HV723. Based on these findings, it appeared that the rabbit carotid artery mediated contraction through α_{1H}- and α_{1L}-adrenoceptors (Muramatsu *et al*, 1991b). The α_{1H}-subtype was later characterised to be the α_{1B}-adrenoceptor (Muramatsu *et al*, 1995).

The dog carotid artery was proposed to contain α_{1H}-adrenoceptors when it was discovered to have high affinity for prazosin (Muramatsu *et al*, 1990; Muramatsu *et al*, 1991b). This α_{1H}-subtype was later characterised as the α_{1B}-adrenoceptor, when it was shown to have low affinity for the α_{1A}-antagonists, WB4101 and 5-methylurapidil as well as having low affinity for HV723, ruling out a contribution from the α_{1N}-subtype (Muramatsu *et al*, 1991a; Kohno *et al*, 1994).

The α_1-adrenoceptors of the rat carotid artery have been characterised by a number of groups as being of the α_{1D}-subtype. Villalobos-Molina and Ibarra (1996) used WB4101, 5-methylurapidil and BMY7378 to determine the subtype of α_1-adrenoceptor mediating contraction in the rat carotid artery. However, they discovered that 5-methylurapidil and WB4101 showed similar affinities in all the arteries they looked at (in addition to the

carotid artery they also studied the aorta, mesenteric and caudal arteries). As a result of this they did not use these antagonists to make their conclusions but based them solely on the affinity of BMY7378. In the carotid artery BMY7378 produced a pA_2 of 8.7 which they concluded was evidence that the α_1-adrenoceptors of the rat carotid artery were of the α_{1D}-subtype (Villalobos-Molina and Ibarra, 1996). Nagadeh (1996) used a variety of subtype selective antagonists including BMY7378, WB4101, 5-methylurapidil, tamsulosin, prazosin and HV723. 5-Methylurapidil and WB4101 produced high affinities in the rat carotid artery but the slopes of the Schild plots were shallow, indicating possible heterogeneity of receptors. However, the pA_2 values correlated better with affinities for the cloned α_{1d}- compared with α_{1a}- or α_{1b}-adrenoceptor clones, which in conjunction with a high affinity for BMY7378 was consistent with a functional α_{1D}-adrenoceptor population. Both Nagadeh (1996) and Villalobos-Molina and Ibarro placed a lot of faith in BMY7378 as being a selective $\alpha_{1D/d}$-adrenoceptor antagonist. This is often necessary if any conclusions are to be made about which subtype is present in the tissue because more often than not one or two of the antagonists will be contradictory to the general trend of the other antagonists used. The experimenter then has to make a decision about which antagonists he/she has the most faith in.

A study by de Oliveira *et al* (1998) which was investigating the effects of maturation and ageing on the responsiveness of the carotid artery to α_1-adrenoceptor stimulation reported a high affinity for WB4101 in all ages examined (3, 8, 17 and 29 weeks) and inhibition of the phenylephrine induced contraction by CEC. From this data they concluded that the rat carotid artery contained a population of α_{1D}-adrenoceptors in agreement with Villalobos-Molina and Ibarro (1996) and Nagadeh (1996).

The α_1-adrenoceptor subtype(s) mediating contraction in the mouse carotid artery had yet to be discovered and indeed there is still much to be learnt about the pharmacology of the mouse. The identification of functional α_1-adrenoceptor subtypes can be a difficult process due to the limited number of subtype-selective drugs (see Introduction, Section 1.3). The α_{1B}-adrenoceptor KO mouse described by Cavalli *et al* (1997) provides a new approach to tackling this problem. There are no well established $\alpha_{1B/b}$-adrenoceptor selective drugs that make it possible to definitively identify or rule out the α_{1B}-adrenoceptor from a particular tissue and much of the confusion of subtyping α_1-adrenoceptors arises from this problem. The α_{1B}-adrenoceptor KO mouse has the advantage in that the α_{1B}-adrenoceptor can be immediately excluded as a possible contributor to the functional response. This simplifies the situation to two subtypes for which there are well established selective antagonists and in theory should make the α_1-adrenoceptor subtyping of tissues a less daunting task.

The aim of this study was to use the α_{1B}-adrenoceptor KO mouse along with its WT counterpart to subtype the α_1-adrenoceptors mediating phenylephrine-induced contraction in the carotid arteries of these mice. As mentioned at the start of this introduction, the carotid artery is commonly used in models of atherosclerosis, angioplasty-induced restenosis and vascular injury. α_1-Adrenoceptors have been implicated in these pathophysiological conditions. Therefore, it is of interest to know which α_1-adrenoceptor subtypes are functional in this artery and contributing to contraction. Cavalli *et al* (1997) have suggested a role for the α_{1B}-adrenoceptor in the maintenance of vascular tone using the α_{1B}-adrenoceptor KO mouse. They demonstrated a decreased response to phenylephrine in the isolated aorta of KO mice, as

well as a decreased pressor response *in vivo*. By using the KO mouse this should allow the contribution of the α_{1B}-adrenoceptor to vasoconstriction in the murine carotid artery (and its contribution to the regulation of blood pressure) to be elucidated.

In addition to determining the identity of the α_1-adrenoceptor population responsible for vasoconstriction of the murine carotid artery, this study will also undertake a preliminary investigation of the general pharmacology of the WT carotid artery, similar to that which was published during the course of my project for the mouse aorta (Russell and Watts, 2000). This will enhance our knowledge and understanding of murine pharmacology, which is imperative if we are to understand the consequences of their genetic manipulation.

Towards the end of my project, two additional strains of mice became available to me, which overexpressed either the WT or a constitutively active form of the α_{1B}-adrenoceptor. Preliminary cardiovascular studies on these mice revealed that they were hypotensive (Zuscik *et al*, 2001). A final aim of this section of my thesis was to investigate the sensitivity of the carotid and mesenteric arteries of these mice to phenylephrine. The carotid artery was chosen to follow up on my work in WT and KO carotid arteries. The mesenteric artery was chosen, because, being a resistance vessel, it was hoped that if the α_{1B}-adrenoceptor had a role in the control of blood pressure, this would be evident in differing sensitivities to phenylephrine in the mesenteric arteries from the different strains of mice. The results from the mesenteric artery have been published in Journal of Biological Chemistry; Zuscik *et al*, 2001; 276(17):13738-13743.

3.2 METHODS

3.2.1 Solutions and drugs used

The Krebs-Hensleit solution used as the physiological salt solution in which all experiments were carried out was of the following composition (mM): NaCl (112), KCl (5.9), $MgCl_2$ (1.2), $CaCl_2$ (2), $NaHCO_3$ (25), $NaHPO_4$ (1.2), glucose (11.5) and Na_2EDTA (0.023).

The following compounds were used:-

(R)-A-61603 (Abbott laboratories, gift of Dr. Hancock), **L-arterenol** (noradrenaline, Sigma), **acetylcholine hydrochloride** (Sigma), **angiotensin II** (Sigma), **BMY7378** (dihydrochloride 8-[2-[4-(2-methoxyphenyl)-1-piperozynl]ethyl]-8-azaspiro(4,5}decone-7,9-dione, Research Biochemicals International) **endothelin** (Scientific Marketing Association), **histamine** (Sigma), **5-hydroxytryptamine** (Sigma), **isoprenaline** (Sigma), **L765,314** (4-amino-2-[4-[1-(benzyloxycarbonyl)-2-(S)-[[(1,1 dimethylethyl)amino]carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline, Merck and Company, a gift of Dr. Patane), **L-NAME** (N^{ϖ}-nitro-L-arginine methyl ester hydrochloride, Sigma) **5-methylurapidil** (Research Biochemicals International), **phenylephrine hydrochloride** (Sigma), **Prazosin hydrochlori**de (Sigma), **UK14304** (Research Biochemicals International), **vasopressin** (Blood Pressure Unit, University of Glasgow).

3.2.2 Animals used and set-up procedure

Wildtype and knockout male C57-Black mice were obtained from the Central Research Facility at the University of Glasgow. Control, over-expressed and constitutively active CBA mice were obtained from the Royal Infirmary. All mice were killed by lethal overdose of carbon dioxide. The common carotid arteries were removed from all mice and dissected free of connective tissue with the aid of a dissecting microscope. The mesentery was removed from control, over-expressed and constitutively active mice only. First order mesenteric arteries were isolated and dissected using a microscope to ensure as little connective tissue remained attached to the vessel as possible. All WT and KO mice were used at a similar age (~16 weeks) and weight (30-40g). Control, over-expressed and constitutively active mice were ~ 60 weeks of age and ~ 40-50g in weight.

Experiments were carried out in a four chamber Mulvany Halpern wire myograph (J.P. Trading. Aarhus, Denmark.) (Mulvany and Halpern, 1976). Arteries were cut into approximately 2mm lengths and mounted on two 40μm wires. One wire was attached to a fixed head while the other was attached to a head connected to a force transducer. The force transducer was in turn connected to a Linseis 4 channel pen recorder to allow recordings of the force achieved.

3.2.3 Determination of optimal resting tension

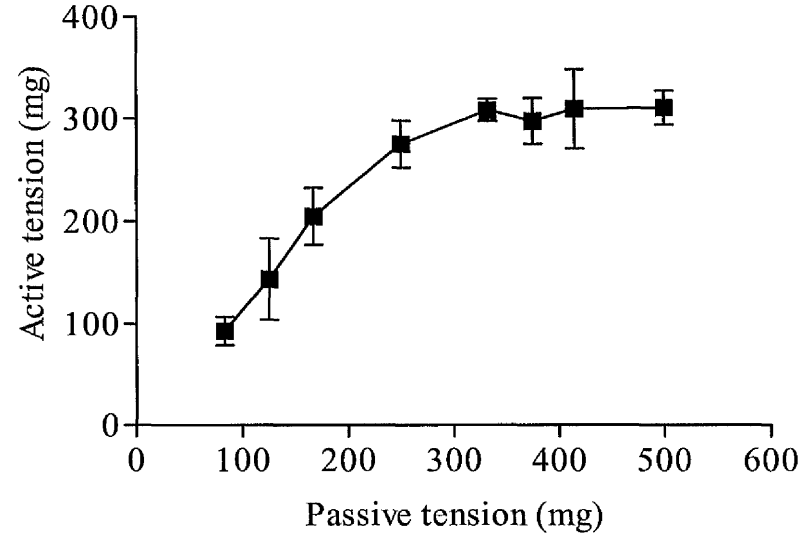
In order to determine the optimal resting tension for the murine carotid artery, arterial strips were placed under a tension of 83mg (equivalent to 5 small boxes on a Linseis 4

channel pen recorder) and allowed to equilibrate for 30 minutes. After this equilibration period the tissues were challenged with a near maximal concentration of phenylephrine (10μM) and the active force generated was recorded. This was repeated until reproducible responses of similar magnitude were achieved with 10 minute rest intervals between challenges with phenylephrine. This usually required 3 challenges with phenylephrine at each tension. Once a reproducible response had been obtained the passive tension was increased. This process was repeated several times over a tension range of 83-500mg in order to allow for the generation of a passive-active tension curve. This type of experiment has previously been carried out in rat carotid artery where a resting tension of 2500-3000mg was found to be optimal (Nagadeh, PhD thesis, 1996). For the mouse carotid artery the optimal tension was deemed to be 250mg (Figure 3.1). Chataigneau *et al* (1999) found the optimal tension to be approximately the same in their studies using the murine carotid artery. For all subsequent experiments the vessels were stretched to a resting tension of 250mg.

The optimal resting tension of the mesenteric arteries of the mouse has been previously shown by our laboratory to be 170mg (internal diameter ~200μm) (M^{c}Gee, unpublished observations) and all mesenteric arteries were placed under this tension for all experiments.

3.2.4 Start-up procedure

Vessels were allowed to equilibrate in Krebs (37°C and gassed with 95% O_2, 5% CO_2) for 15 minutes after which 250mg (carotid) or 170mg (mesenteric) of tension was placed on each artery. Arteries were allowed to equilibrate at this tension for 30-45



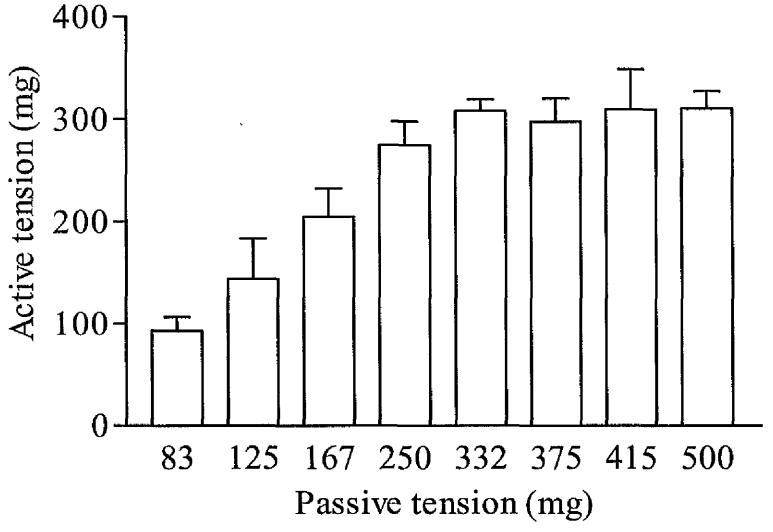


Figure 3.1:- Tension development curves of the effect of passive tension on the active contractile force of the mouse isolated common carotid artery in response to 10μM PE. Only WT vessels were used to determine the optimum tension under which to place the vessels for all subsequent experiments, which was determined to be 250mg. It was deemed that this tension produced a near maximum response without putting the arteries under any undue stress (n = 11).

minutes and were washed with Krebs every 15 minutes. Prior to the start of each experiment vessels were challenged with a sensitising concentration of phenylephrine ($3x10^{-7}$M). This concentration was chosen because it is approximately equal to the EC_{50} of phenylephrine in carotid arteries (see Results, Section 3.3.1). When the contraction had reached a plateau (usually after 3-5minutes) the vessels were washed until the tone had returned to baseline. This sensitising procedure was repeated until a reproducible response was maintained, with 10-15 minute rest intervals between concentrations. This served to confirm that vessels were functional and it was found that following this there was good reproducibility of up to 4 concentration response curves. On occasions when this was not carried out there was a tendency for the tension developed in the vessels to increase in magnitude with each curve and so this procedure was always performed at the start of each experiment.

3.2.5. Agonist profiles

To ascertain the contribution of α_1- and α_2-adrenoceptors to vasoconstriction in the murine carotid artery, agonist profiles were performed using a selection of adrenoceptor agonists with varying selectivities. Noradrenaline (1nM - 100μM) was used since it is the endogenous agonist and will act via α_1- and α_2-adrenoceptors, phenylephrine (1nM – 1mM) was used in its capacity as a selective α_1-agonist, UK14304 (1nM - 30μM) as a selective α_2-agonist and (R)-A-61603 (1nM - 100μM) as an α_{1A}-selective agonist. In addition, agonists were used which act on several important vasoactive systems to determine not just the α-adrenergic properties of this artery but also to investigate its general pharmacological properties. It should be noted that this was not meant to be an exhaustive study, in terms of investigating substances that can alter the tone of the

carotid artery, but rather was to serve as a starting point from which further studies could be undertaken, dependent on the results presented here. The agonists used for this purpose and the concentration range in which they were tested were as follows. Isoprenaline (1nM - 30μM), acetylcholine (1nM - 30μM), vasopressin (0.1pM – 300nM), endothelin (0.1pM – 300nM), histamine (1nM - 30μM), adenosine tris phosphate (ATP) (1nM - 30μM), bradykinin (1nM - 30μM), angiotensin II (10pM - 1μM) and 5-HT (1nM – 30μM). The ability of each of these agonists to produce a contraction in murine carotid arteries was tested and in addition to this several of the agonists were tested for relaxant properties in carotid arteries. These were UK14304, acetylcholine, isoprenaline, histamine, bradykinin and ATP. The concentrations used were the same as when testing these agonists for their contractile properties.

4-8 vessels were set up in parallel, as described previously and after the start-up procedure were allowed a 30 minute recovery period before the experiment started and between subsequent concentration response curves. Cumulative CRC were constructed using half-log increments. The CRC were repeated a total of 3 times in each vessel with a different agonist being used each time.

For relaxant agents the arteries were initially contracted with 10μM phenylephrine and the contraction was allowed to reach a plateau for approximately 10 minutes before the concentration response curve (CRC) was carried out to ensure that the contraction would be maintained throughout the experiment and that any relaxation was purely as a result of the compounds being tested. If relaxation was produced by any of the agonists the concentration response curve was repeated in the presence of 100μM L-NAME.

3.2.6. Antagonist profiles

As for agonist profiles, 4-8 vessels were set up in parallel and each vessel was randomly assigned to a competitive antagonist or designated as the time control. The time control serves to demonstrate the effect of time (if there is any) on the response from the blood vessels. An initial cumulative CRC was carried out to phenylephrine (1nM – 1mM) increasing in half-log increments, after which the vessels were washed and allowed to return to the baseline. Only when the vessels were at their resting tension were antagonists added to the bath and left for 30 minutes, after which time it was deemed that the antagonist would have reached equilibrium. A total of 4 CRC were carried out in each vessel:- a control CRC followed by three CRC carried out in the presence of increasing concentrations of antagonist. The antagonists, their α_1-adrenoceptor subtype selectivity and the concentrations used were as follows:- Prazosin (non-selective, 1nM, 10nM and 100nM), 5-methylurapidil (α_{1A}-selective, 0.1μM, 0.3μM and 1μM), L-765,314 (α_{1B}-selective, 10nM, 100nM and 1μM) and BMY7378 (α_{1D}-selective, 1nM, 10nM and 100nM).

3.2.7. Control, over-expressed and constitutively active mice – carotid and mesenteric artery - Sensitivity to PE

A single CRC to phenylephrine was carried out (1nM – 100μM) in carotid and mesenteric arteries of control, over-expressed and constitutively active mice, to determine the effect, if any, of the genetic manipulation of the α_{1B}-adrenoceptor.

3.2.8. Data Analysis

3.2.8.1 Agonist studies

3.2.8.1.1 Contractile agents

All agonist data obtained from WT and KO mice as well as from CBA control, overexpressed and constitutively active mice was analysed by the same method. Responses to agonists were expressed as developed tension in grams and were either plotted as such, as a percentage of their own maximum response or as a percentage of the maximum phenylephrine contraction. All data from CRC was curve fitted using GraphPad Prism 3.01 (San Diego, California, USA) using an equation which describes a one-site sigmoidal dose response curve of variable slope, with the exception of data from noradrenaline CRC (see below). This equation calculates the maximum response, pEC_{50} and the Hill slope of the curve as determined by the following equation:-

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

where:- E = response, α = maximum response, [A] = concentration of agonist, $[A]_{50}$ = concentration of agonist which produces an effect that is 50% of the maximum response (α) and nH = Hill coefficient.

The mean data from noradrenaline CRC did not fit satisfactorily to the equation used for all other CRC but instead non-linear regression was carried out using a 2-site model described by the following equation:-

$$E = \alpha 1/(1+10^{((\log[A]_{50}1-X).n_{H1})}) + \alpha 2/(1+10^{((\log[A]_{50}2-X).n_{H2})})$$

Where:- E, $[A]_{50}$ and nH are as before and X = logarithm to the base 10 of the agonist concentration. Numbers 1 and 2 denote the two sites. This equation was first described by Furchgott (1981) and further developed by Lemoine and Kaumann (1983) and Kenakin (1992) and has been previously used by a colleague in this laboratory, Dr. Sally Argyle. It was with her assistance that the equation was programmed into GraphPad Prism for use in this section of my thesis, for which I am very grateful.

The mean of the raw data was calculated and data expressed as mean ± s.e.m. This data was plotted in logarithmic space and the best fit curve superimposed onto the data. Statistical analysis in the form of an unpaired t test was performed on the pEC_{50}'s, Hill slopes and the maximum response to determine significant differences between the responses of WT and KO carotid arteries to agonists. A p value of less than 0.05 was considered to be significant.

3.2.8.1.2 Relaxant agents

Data from relaxant agents was analysed in the same way as for the contractile agents but was expressed differently, as a percentage relaxation.

3.2.8.2 Antagonist studies

The data from the antagonist studies was expressed as a percentage of the maximum response of the first CRC. As with agonist studies the data was curve fitted using GraphPad Prism 3.01 using an equation describing a sigmoidal dose response curve of variable slope. The pEC_{50} value and the Hill slope (as calculated by GraphPad Prism

3.01) and the maximum response of each CRC in both WT and KO tissue was analysed using a two-way ANOVA. This method of statistical analysis allows for the effect of the antagonist on the response **and** the effect of the mouse type (i.e. either WT or KO) on the response to be analysed. If the antagonist was shown to produce a significant effect on the response then a one-way ANOVA followed by a Bonferroni post test was also carried out which allows for analysis of the CRC at each concentration of antagonist. It was necessary to perform one-way ANOVA on WT and KO data separately because, although two-way ANOVA will determine if the effect of the antagonist on the response is significant, it doesn't provide any information on the individual CRC at each concentration of antagonist. Therefore performing a one-way ANOVA allows for a direct comparison of the CRC in the presence of the antagonist with the control CRC, thus enabling identification of the concentration(s) of antagonist at which the effect was significant.

The aim of the antagonist studies was to determine the affinities of the various antagonists in WT and KO carotid arteries. This was determined by Schild regression (Arunlakshana and Schild, 1959). From this regression a pA_2 or pK_B value can be derived which allows the potency of a given antagonist to be quantified. The definitions and circumstances of use for these values will be described shortly.

In order to carry out Schild regression, a range of 3 concentrations was employed for each antagonist studied. Dose ratios (DR) were calculated for each concentration of antagonist. The dose ratio is the ratio of the EC_{50} in the presence and absence of the antagonist. The EC_{50} values were derived from the curve fitting procedure for individual experiments.

A Schild plot was constructed using these DR values, by plotting log (DR-1) against the log of the antagonist concentration and linear regression was performed through the points. The X-intercept of this linear regression line is equal to the pA_2 value. In order for Schild regression to be an accurate measure of antagonist potency certain criteria have to be met. Firstly equilibrium conditions must be met before any measurements are made, if not then an underestimation of the antagonist potency would result. A range of antagonist concentrations must be used and finally increasing concentrations of the antagonist must produce parallel dextral displacement of the control curve without a decrease in the maximal response (Kenakin, 1982; Arunlakshana and Schild, 1959). Therefore all CRCs were expressed as a percentage of the maximum response of the control curve before Schild regression was carried out.

The pA_2 value, as calculated by linear regression, is defined as the negative logarithm to the base 10 of the molar concentration of antagonist that makes it necessary to double the concentration of agonist needed to elicit the original response (Schild, 1947). It is an empirical measure of the activity of an antagonist that is not dependent on how the antagonist acts (Jenkinson *et al*, 1995). The pA_2 value is calculated from the Schild plot where the slope of the line does not have to be constrained to unity. The pK_B is the negative logarithm to the base 10 of the equilibrium dissociation constant of the antagonist. It is calculated from the Schild equation (Arunlakshana and Schild, 1959; Jenkinson *et al*, 1995) which constrains the slope of the line to 1.

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

[B] = concentration of antagonist and K_B = equilibrium dissociation constant of antagonist. The pA_2 and pK_B values only coincide if the requirements for Schild regression are met and if the slope of the line is exactly equal to unity (Jenkinson *et al*, 1995). In some instances the slope of the Schild plot was significantly less than one or the antagonist produced a depression in the maximum response. Therefore, it was decided to calculate pK_B values, in addition to pA_2 values, for each antagonist using the above equation. The primary objective was to shed some light on the affinities of the antagonists that produced shallow slopes.

An unpaired t test was performed on all pA_2 values to determine significant differences between WT and KO carotid arteries. A p value of less than 0.05 was considered significant.

3.3. RESULTS

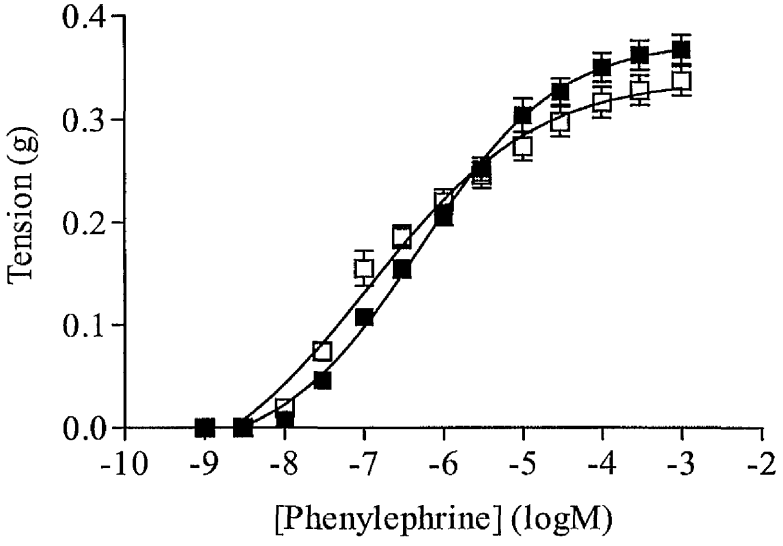
3.3.1. Agonist profiles

3.3.1.1. Contractile agents

Experiments were performed in the absence of any blocking agents. Out of 13 contractile agents tested for activity in WT and KO murine carotid arteries only 4 of these had the ability to produce a contraction. These were phenylephrine (Figure 3.2), noradrenaline (Figure 3.3), (R)-A-61603 (Figure 3.4) and 5-HT (Figure 3.5). Noradrenaline was found to produce a biphasic curve in both WT and KO carotid arteries. Figure 3.6 compares the concentration response curves for phenylephrine, noradrenaline, (R)-A-61603 and 5-HT expressed as a percentage of the maximum phenylephrine contraction. The remaining 9 agonists were shown to be ineffective as contractile agents. These were UK14304, isoprenaline, vasopressin, endothelin, angiotensin II, acetylcholine, ATP, histamine and bradykinin.

The pEC_{50} values produced by the agonists that elicited a response were - Noradrenaline:-7.97±0.04 and 5.12±0.04 (n=10), Phenylephrine:- 6.31 ± 0.06 (n=26), (R)-A61603:- 6.90 ± 0.09 (n=10) and 5-HT:- 6.88 ± 0.08 (n=9) in WT carotid arteries and the corresponding values in KO carotid arteries were – Noradrenaline:- 7.80±0.02 and 5.58±0.05 (n=7), Phenylephrine:- 6.95 ± 0.22 (n=24), (R)-A-61603:- 6.71 ± 0.08 (n=11) and 5-HT:- 6.77 ± 0.04 (n=6). Phenylephrine was the only agonist found to produce a significantly different pEC_{50} between WT and KO arteries.

The maximum responses obtained by each agonist were - Noradrenaline:- 0.23±0.02g, Phenylephrine:- 0.37±0.01g, (R)-A-61603:- 0.27±0.02g and 5-HT:- 0.27±0.04g for WT



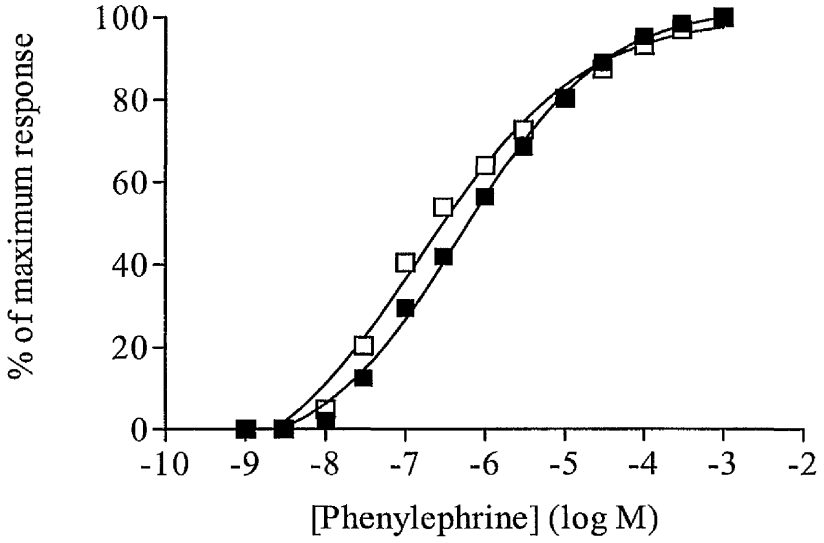
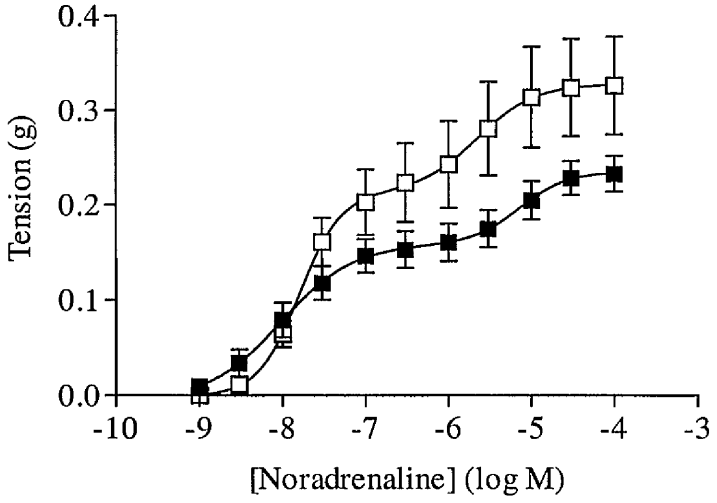


Figure 3.2:- Mean control concentration response data to phenylephrine in WT (■) and KO (□) murine carotid arteries plotted as tension in grams (top) or as a % of their own maximum response (bottom). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT and KO arteries n=26.



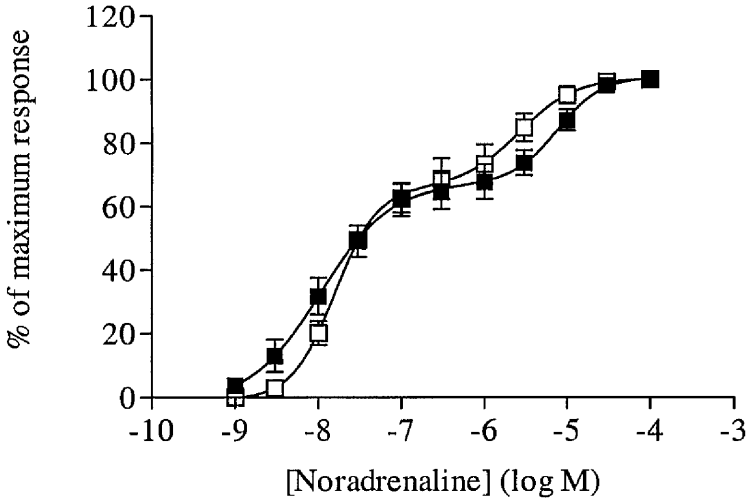
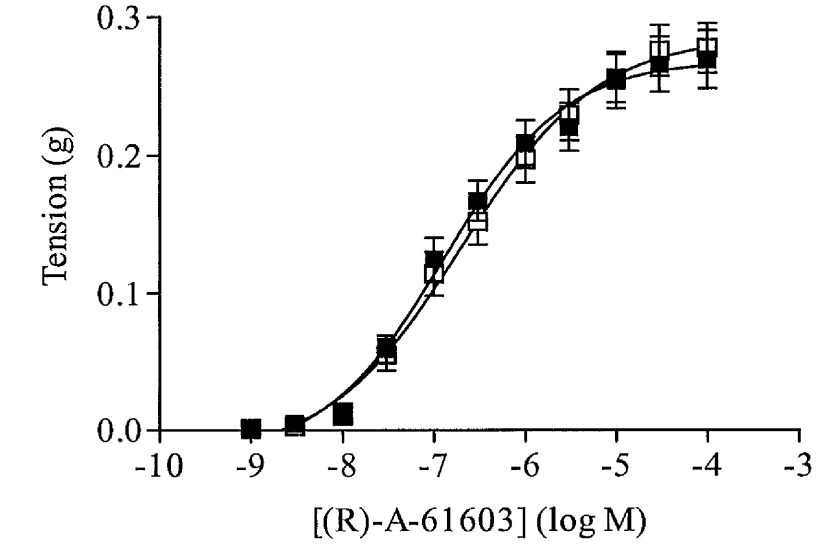


Figure 3.3:- Mean control concentration response data to noradrenaline in WT (■) and KO (□) murine carotid arteries plotted as tension in grams (top) or as a % of their own maximum response (bottom). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=8 and for KO arteries n=7.



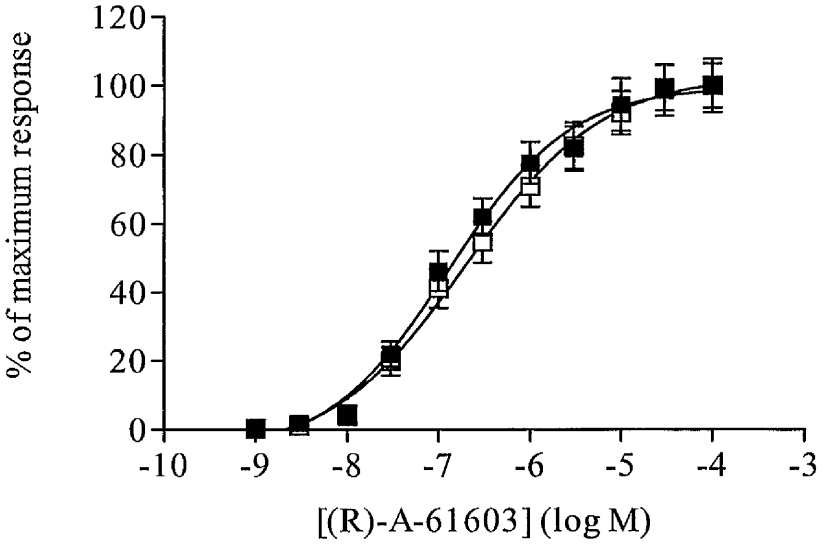
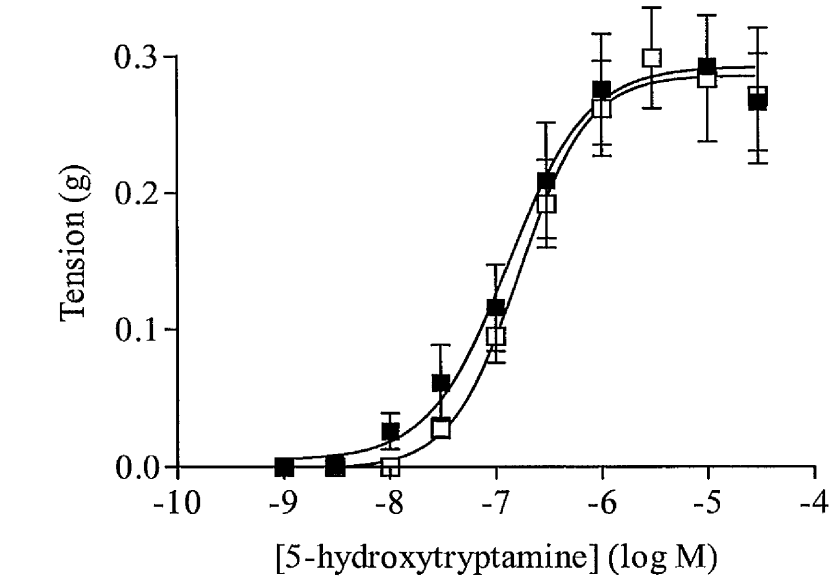


Figure 3.4:- Mean control concentration response data to (R)-A-61603 in WT (■) and KO (□) murine carotid arteries plotted as tension in grams (top) or as a % of their own maximum response (bottom). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=10 and for KO arteries n=11.



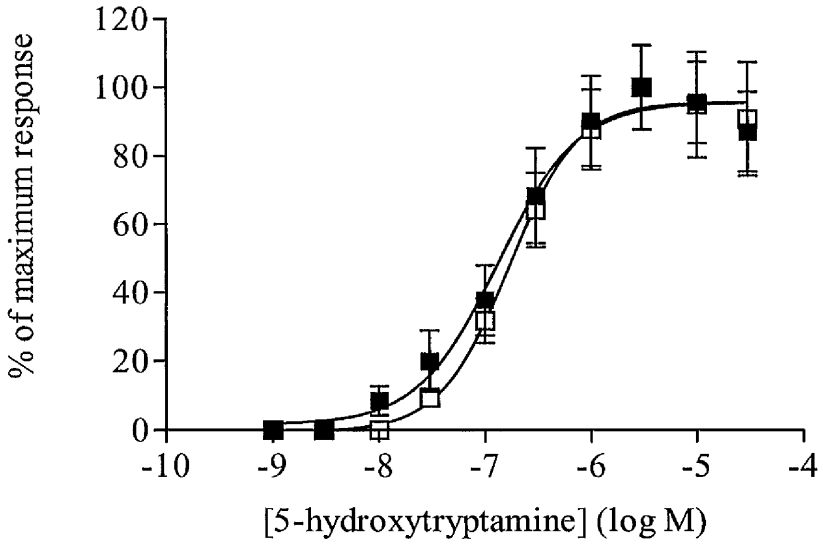


Figure 3.5:- Mean control concentration response data to 5-hydroxytryptamine in WT (■) and KO (□) murine carotid arteries plotted as tension in grams (top) or as a % of their own maximum response (bottom). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=9 and for KO arteries n=6.

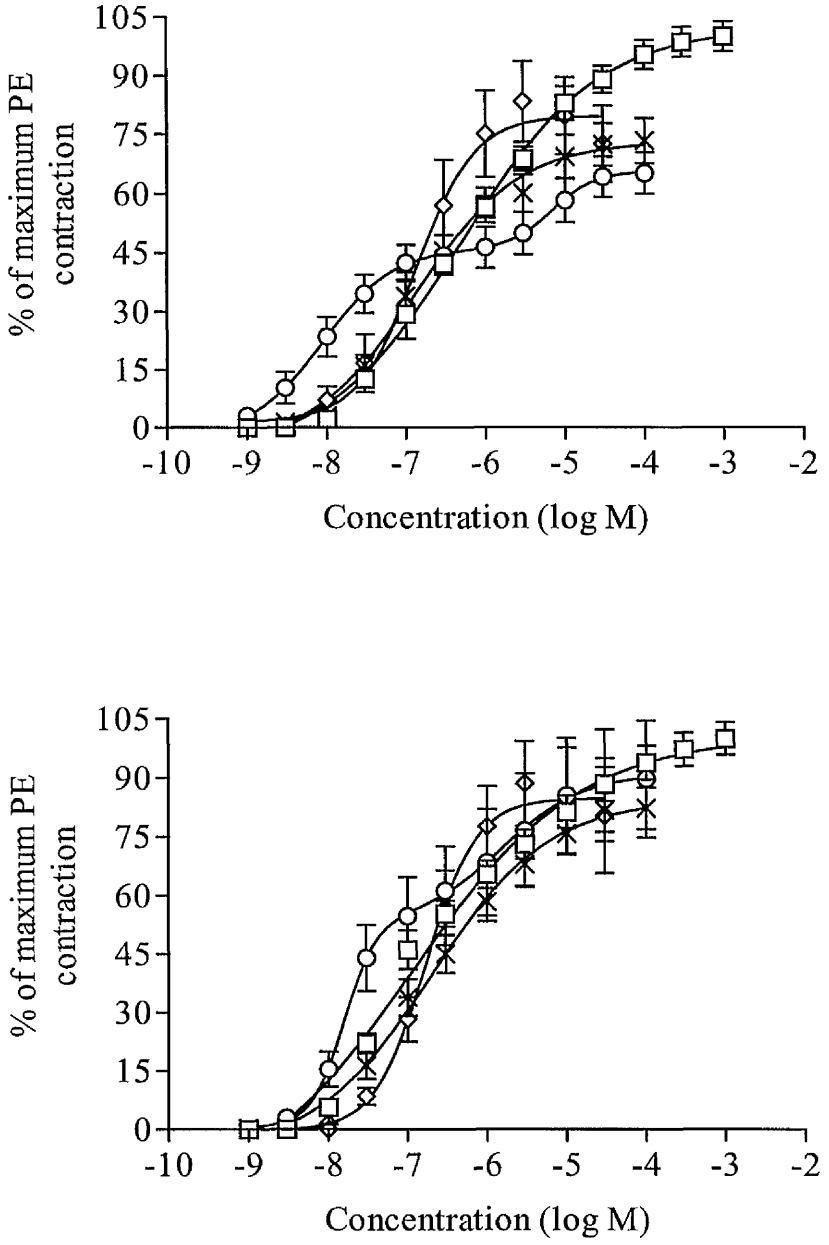


Figure 3.6:- Mean concentration response data for phenylephrine (□), noradrenaline (○), (R)-A-61603 (×) and 5-hydroxytryptamine (◇) in WT (top) and KO (bottom) carotid arteries expressed as a percentage of the maximum contraction to phenylephrine. Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed.

Agonist	pEC_{50}	Maximum response (g)	Hill slope
NA	7.97±0.04 5.12±0.05	0.23±0.02	1.27 (0.83-1.7) 1.47 (0.87-2.1)
PE	6.31±0.06	0.37±0.01	0.50 (0.41-0.59)$^{\#}$
(R)-A-61603	6.90±0.09	0.27±0.02	0.66 (0.43-0.88)$^{\#}$
5-HT	6.88±0.08	0.27±0.04	1.2 (0.6-1.7)

Agonist	pEC_{50}	Maximum response (g)	Hill slope
NA	7.80±0.02 5.58±0.05	0.33±0.05	1.7 (1.3-2.0)$^{\#}$ 1.25(0.69-1.80)
PE	6.95±0.22	0.34±0.01	0.41 (0.24-0.59)$^{\#}$
(R)-A-61603	6.71±0.08	0.28±0.02	0.59 (0.43-0.74)$^{\#}$
5-HT	6.77±0.04	0.27±0.05	1.35 (0.94-1.70)

Figure 3.7:- Comparison of pEC_{50} values, maximum responses and Hill slopes for agonists producing contractions in WT (top) and KO (bottom) mouse carotid arteries. The pEC_{50} values and maximum responses are given as mean ± s.e.m. and the hill slopes are given along with their 95% confidence intervals. * indicates $p<0.05$ compared to WT and $^{\#}$ indicates a Hill slope significantly different from unity.

carotid arteries and in KO carotid arteries the maximum responses achieved were – Noradrenaline:-0.33±0.05g, Phenylephrine:- 0.34±0.01g, (R)-A-61603:- 0.28±0.02g and 5-HT:- 0.27±0.05g. There was no significant difference in the maximum response between WT and KO carotid arteries for any of the agonists tested.

In both WT and KO carotid arteries only 5-HT exhibited binding to a single site of receptors producing Hill slopes of 1.2 (95% CI = 0.6-1.7) and 1.35 (95% CI = 0.94-1.7) respectively, which were not significantly different from unity. The adrenergic agonist, phenylephrine, demonstrated shallow Hill slopes of 0.50 (95% CI = 0.41-0.59) and 0.41 (95% CI = 0.24-0.59) in WT and KO carotid arteries respectively which were significantly different from unity. (R)-A-61603 was found to produce Hill slopes of 0.66 (95% CI = 0.43-0.88) and 0.59 (95% CI = 0.43-0.74) in WT and KO carotid arteries respectively. As with phenylephrine these Hill slopes were significantly different from unity.

The pEC_{50}'s, maximum responses and Hill slopes are presented in tabular form, see figure 3.7.

3.3.1.2 Relaxant agents

Of the 6 agonists tested for their ability to relax the mouse isolated carotid artery, only 3 were found to be able to produce a relaxation. These were acetylcholine, isoprenaline and UK14304 (Figure 3.8). Acetylcholine was capable of relaxing the contraction to phenylephrine by 76.5±0.99% with a pEC_{50} of 7.6±0.1. Isoprenaline was able to relax the tissue to a similar extent to acetylcholine (74.0±7.7%) but with a lower affinity of

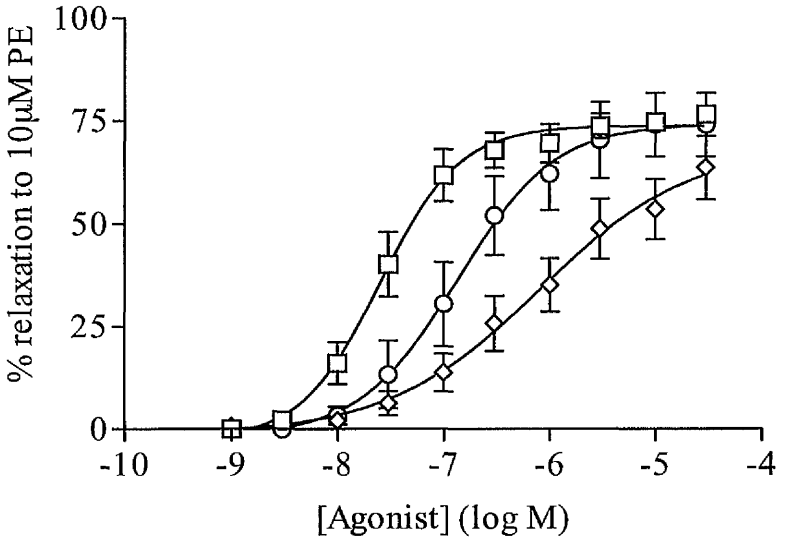


Figure 3.8:- Mean relaxation curves in the isolated mouse carotid artery to acetylcholine (□), isoprenaline (O) and UK14304 (◊). Mean curves were generated using non linear regression upon which the mean data ± s.e.m has been superimposed. Acetylcholine (n=6), UK14304 (n=10) and isoprenaline (n=7). Relaxation curves were only performed in WT carotid arteries.

Agonist	Pre contraction to 10μM PE (g)	pEC_{50}	Maximum relaxation (%)	Hill slope
Acetylcholine	0.63±0.11	7.6±0.04	76.5±0.99	1.2 (0.88-1.5)
UK14304	0.55±0.08	6.0±0.1	51.5±9.3	0.87 (0.51-1.23)
Isoprenaline	0.52±0.06	6.5±0.07	72.7±13.7	0.99 (0.63-1.34)

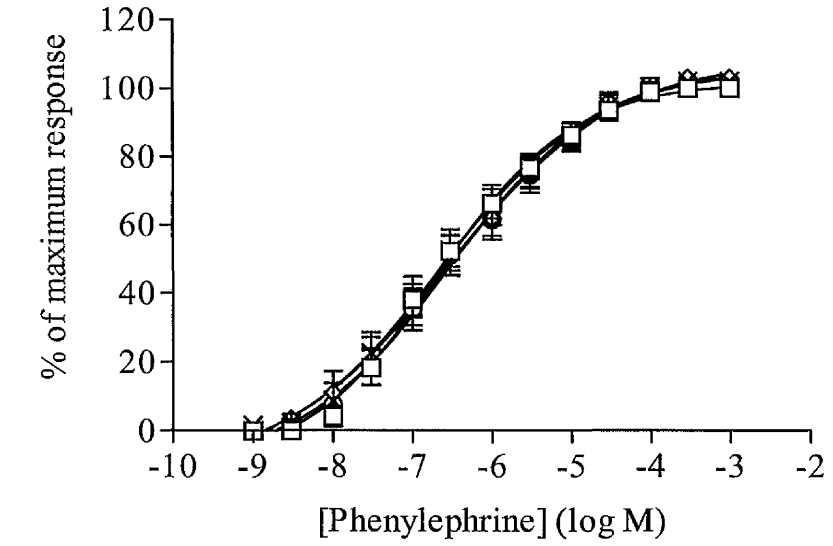
Figure 3.9:- Comparison of pEC_{50} values, maximum % relaxation and Hill slopes for agonists producing relaxation in WT mouse carotid arteries. The levels of pre contraction to PE ± s.e.m. are also given. The pEC_{50} values and maximum % relaxations are given as mean ± s.e.m. and the Hill slopes are given along with their 95% confidence intervals.
[#] indicates a Hill slope significantly different from unity.

6.9±0.1 and UK14304 produced a maximum relaxation of 63.6±7.7% with less potency than that of acetylcholine or isoprenaline (pEC_{50} = 6.1±0.1). Again these results are presented in tabular form, see figure 3.9. The relaxation produced by these agents was blocked by the nitric oxide synthase inhibitor, L-NAME.

3.3.2 Antagonist profiles

Time control CRC were carried out to ascertain that the CRC to phenylephrine was unaffected by time and that the observed effects were a consequence of the antagonist and not due to a deterioration in the vessel's functionality over time. All parameters i.e. maximum response, pEC_{50} and Hill slope remained the same throughout each experiment with no significant differences observed (Figure 3.10).

In the presence of increasing concentrations of prazosin the phenylephrine CRC of both WT and KO carotid arteries (Figure 3.11) were displaced to the right with a significant decrease in the **maximum response** but from the Bonferroni post test carried out after the one-way ANOVA this decreased maximum was found to occur at only the highest concentrations of prazosin used (100nM). One-way ANOVA (followed by a Bonferroni post test) of the **pEC_{50}** values in the presence of prazosin were significantly different from control values at 10nM and 100nM prazosin in WT arteries, whereas in KO arteries there was a significant difference in the pEC_{50} values at all concentrations of prazosin used when compared with the control CRC. Two-way ANOVA of the pEC_{50}'s obtained in WT and KO arteries revealed that there was a significant difference in the pEC_{50} values between the strains of mice. The **Hill slopes** from the WT carotid arteries in the presence of prazosin were found to be significantly different from control Hill



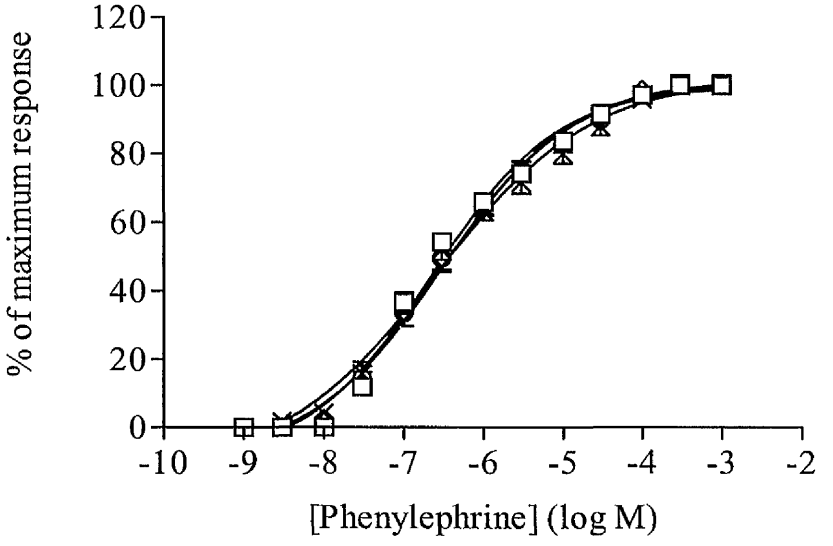


Figure 3.10:- Mean concentration response data for time controls to phenylephrine in WT (top) and KO (bottom) murine carotid arteries. 1st curve (□), 2nd curve (×), 3rd curve (◊) and 4th curve (○). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=7 and for KO arteries n=9.

slopes. A post test revealed that this was due to a steepening of the curve at 100nM which was not observed at any other concentration. In KO carotid arteries the situation was slightly different. The Hill slopes were again found to be significantly different from control values. In this case it was due to a steepening of the CRC at 10nM and 100nM and as a result of this two-way ANOVA revealed that the WT and KO Hill slopes when compared directly were significantly different. Schild regression of the WT data produced a pA_2 value of 9.6 for prazosin with a slope of 0.93 (95% CI = 0.77-1.08) whilst the KO data produced a significantly higher pA_2 of 10.3 ($p<0.05$) but with a similar slope factor of 0.92 (95% CI = 0.68-1.2) (Figure 3.15). The calculated pK_B's in the WT were 9.6±0.1 (1nM), 9.5±0.1 (10nM) and 9.4±0.1 (100nM) and in the KO were 10.1±0.2 (1nM), 10.2±0.2 (10nM) and 10.0±0.1 (100nM) (Figure 3.20). These values are similar to the pA_2 values calculated for prazosin in WT and KO carotid arteries and as such the KO pK_B values were found to be significantly different from WT values at all concentrations of prazosin.

5-Methylurapidil in increasing concentrations produced a rightward shift of the phenylephrine CRC with no decrease in the **maximum response** for either WT or KO carotid arteries (Figure 3.12). When the **pEC_{50}** values were analysed, using one-way ANOVA, significant differences were revealed for all concentrations of 5-methylurapidil, when compared with control values. This was true for both WT and KO arteries. However when WT and KO pEC_{50} values were compared using a two-way ANOVA no significant difference was observed. The **Hill slopes** for WT and KO carotid arteries when analysed separately revealed a significant difference to control values, which a post test revealed was due to an increased Hill slope at concentrations of 0.3μM and 1μM in WT arteries but only an increased Hill slope at 1μM 5-

methylurapidil in KO arteries. This difference in Hill slopes did not carry over into a direct difference between WT and KO carotid arteries because when a two-way ANOVA was carried out no significance was observed. Overall there were no significant differences in the maximum responses, pEC_{50}'s or Hill slopes in WT and KO arteries with 5-methylurapidil. This is reflected in the similar pA_2 values calculated for 5-methylurapidil in WT and KO arteries. The WT yielded a value of 7.5 and the KO a value of 7.6. The slopes for both were found to be identical and were not significantly different from unity (a slope of 1.1 for both WT and KO with 95% CI of 0.73 – 1.5 for WT and 0.77 – 1.5 for KO) (Figure 3.16). The pK_B values calculated for 5-methylurapidil in the WT carotid artery were 7.6±0.1 (0.1μM), 7.7±0.1 (0.3μM) and 7.7±0.2 (1μM) and in the KO carotid artery were 7.8±0.1 (0.1μM), 8.0±0.1 (0.3μM) and 7.9±0.1 (1μM) (Figure 3.20). These values are similar to the pA_2 values calculated for 5-methylurapidil in WT and KO carotid arteries and no significant differences were observed in these values between WT and KO.

L765,314 produced a rightward displacement of the phenylephrine CRC in both WT and KO carotid arteries without a decrease in the **maximum response** (Figure 3.13). Following one-way ANOVA the **pEC_{50}** values in the presence of L765,314 were found to be significantly different from control pEC_{50}'s, in both WT and KO carotid arteries, which a post test revealed was due to the highest concentration of antagonist used (1μM). The **Hill slopes** for both arteries were not found to change significantly from control values at any of the L765,314 concentrations used. Two-way ANOVA of the maximum response, pEC_{50} and Hill slope data from WT and KO arteries was not found to be significantly different indicating that there is no difference in these parameters between WT and KO mice. However, this was not reflected in the pA_2 values and slope

parameters calculated, which were quite different. The WT carotid artery had a pA_2 of 9.0 and a slope which was significantly different from unity (0.65, 95% CI = 0.4-0.9), whereas KO arteries were found to produce a pA_2 of 8.3 and a slope of 0.79 (95% CI = 0.46-1.1) which was not significantly different from unity (Figure 3.17). As a result of the shallow Schild slope calculated for L765,314 in the WT, pK_B values were calculated for WT and KO carotid arteries at the three concentrations of L765,314, in the hope that this would provide a further insight into its affinity in these arteries. For the WT carotid artery the pK_B's calculated were 8.6±0.09 (10nM), 8.4±0.15 (100nM) and 7.9±0.17 (1μM) and for the KO carotid artery were 8.3±0.12 (10nM), 7.9±0.15 (100nM) and 7.9±0.26 (1μM) (Figure 3.20). The pK_B's calculated at 100nM L765,314 were found to be significantly different between WT and KO carotid arteries ($p<0.05$).

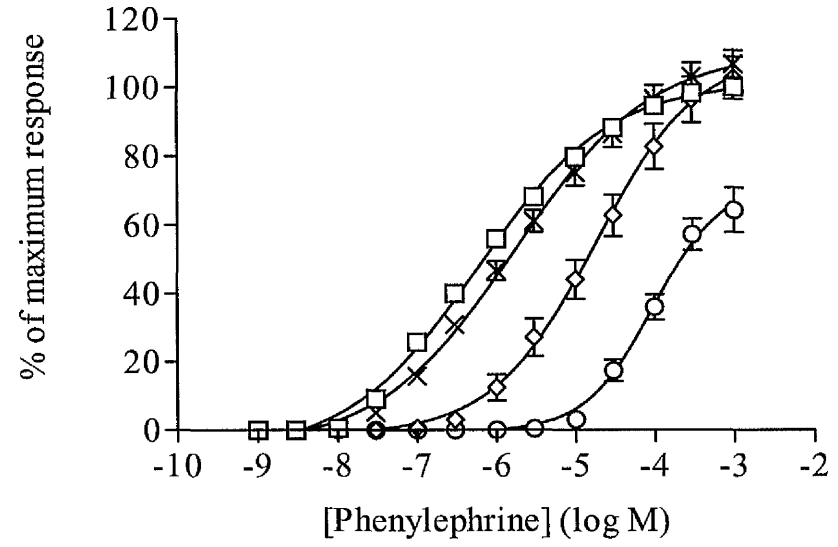
As with all other antagonists, BMY7378 shifted the CRC to phenylephrine to the right in a concentration-dependent manner (Figure 3.14). One-way ANOVA revealed that the **maximum response** achieved at all concentrations of BMY7378 in both WT and KO vessels was unchanged from the maximum response of the control CRC. However, the **pEC_{50} values** were significantly altered from control values in WT and KO arteries. In WT carotid arteries this was due to a significant difference between the control pEC_{50} and the pEC_{50} at 100nM BMY7378. This was also the case in KO carotid arteries. In addition, there was a significant difference between the pEC_{50} from the control CRC and the pEC_{50} at 10nM BMY7378. When a direct comparison of the pEC_{50} values for WT and KO arteries was made using a two-way ANOVA there was found to be a significant difference between mice. The **Hill slopes** in the presence of BMY7378 were significantly different from the control Hill slope when analysed separately using a one-way ANOVA and significantly different between mice, when directly compared using a

two-way ANOVA. When Schild regression was carried out, WT carotid arteries produced a pA_2 value of 9.7 with a shallow slope of 0.44, which was significantly different from unity (95% CI = 0.27-0.6). KO arteries produced a similar pA_2 of 9.6. However the slope calculated was 0.9, which was not significantly different from unity (95% CI = 0.66-1.1) (Figure 3.18). pK_B's were calculated for WT and KO carotid arteries at the three concentrations of BMY7378 for the same reasons as L765,314. For the WT carotid artery the calculated pK_B's were 9.5±0.12 (1nM), 8.5±0.07 (10nM) and 8.3±0.07 (100nM) and for the KO were 9.4±0.07 (1nM), 9.7±0.16 (10nM) and 9.2±0.18 (100nM) (Figure 3.20). Statistically significant differences were observed in the pK_B values calculated at 10nM and 100nM BMY7378 between WT and KO carotid arteries.

The pA_2 values and slope parameters calculated by linear regression are presented in tabular form in figure 3.19.

3.3.3. Correlations

Figures 3.21 and 3.22 show correlations between the antagonist pA_2 values in WT and KO carotid arteries with published values of the same antagonists in cloned α_1-adrenoceptors. The WT carotid artery (Figure 3.21) does not display great correlation with any of the subtypes but the best correlation is with α_{1d}-adrenoceptors (r = 0.6), whereas in the KO carotid artery this weak correlation in the WT with α_{1d}-adrenoceptors becomes much stronger, producing an r value of 0.94.



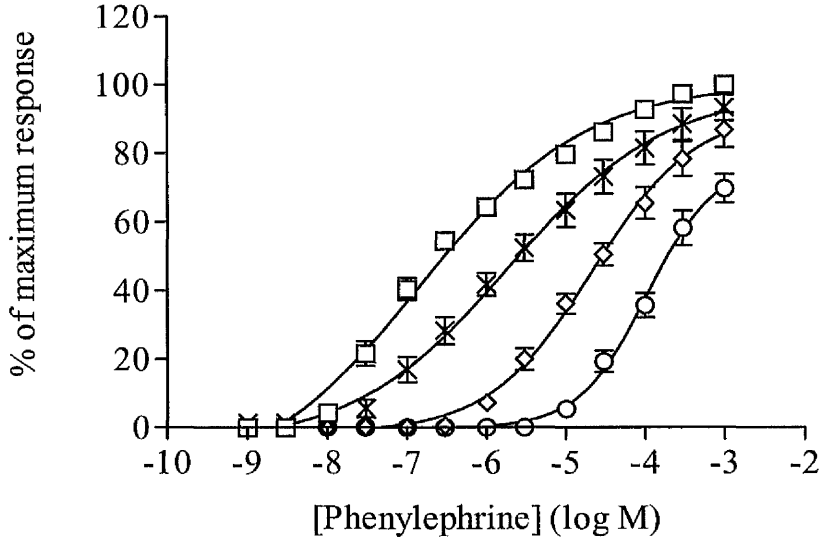
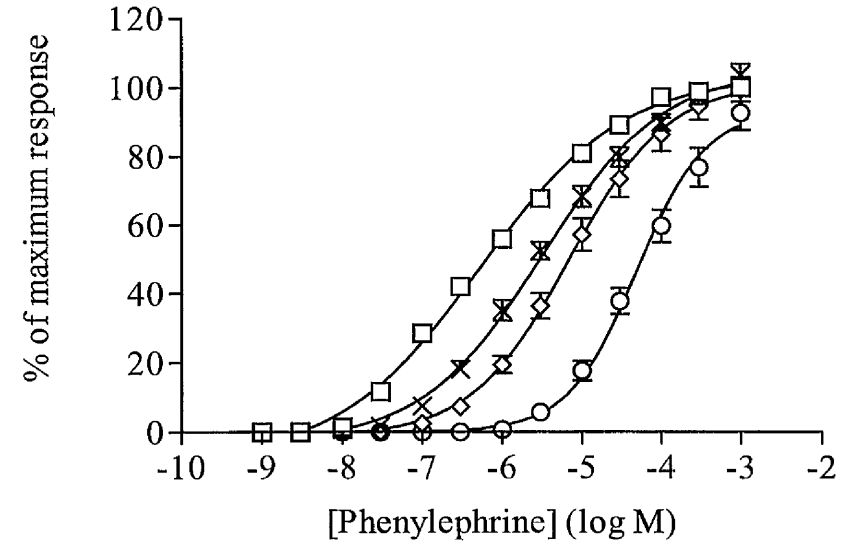


Figure 3.11:- Mean concentration response data to phenylephrine in the presence of increasing concentrations of prazosin in WT (top) and KO (bottom) murine carotid arteries. Control (□), 1 x 10^{-9}M (×), 1 x 10^{-8}M (◊) and1 x 10^{-7}M (○). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=12 and for KO arteries n=14.



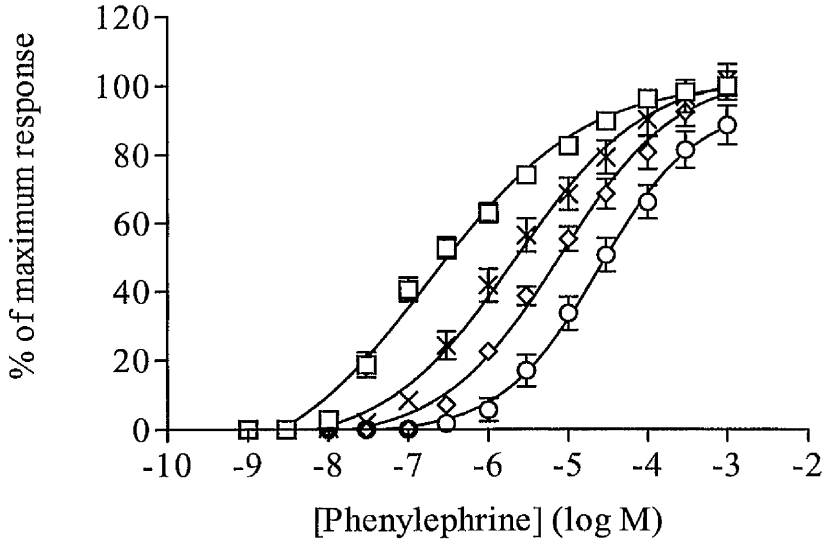


Figure 3.12:- Mean concentration response data to phenylephrine in the presence of increasing concentrations of 5-methylurapidil in WT (top) and KO (bottom) murine carotid arteries. Control (□), 1 x 10^{-7}M (×), 3 x 10^{-7}M (◊) and 1 x 10^{-6}M (○). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=13 and for KO arteries n=12.

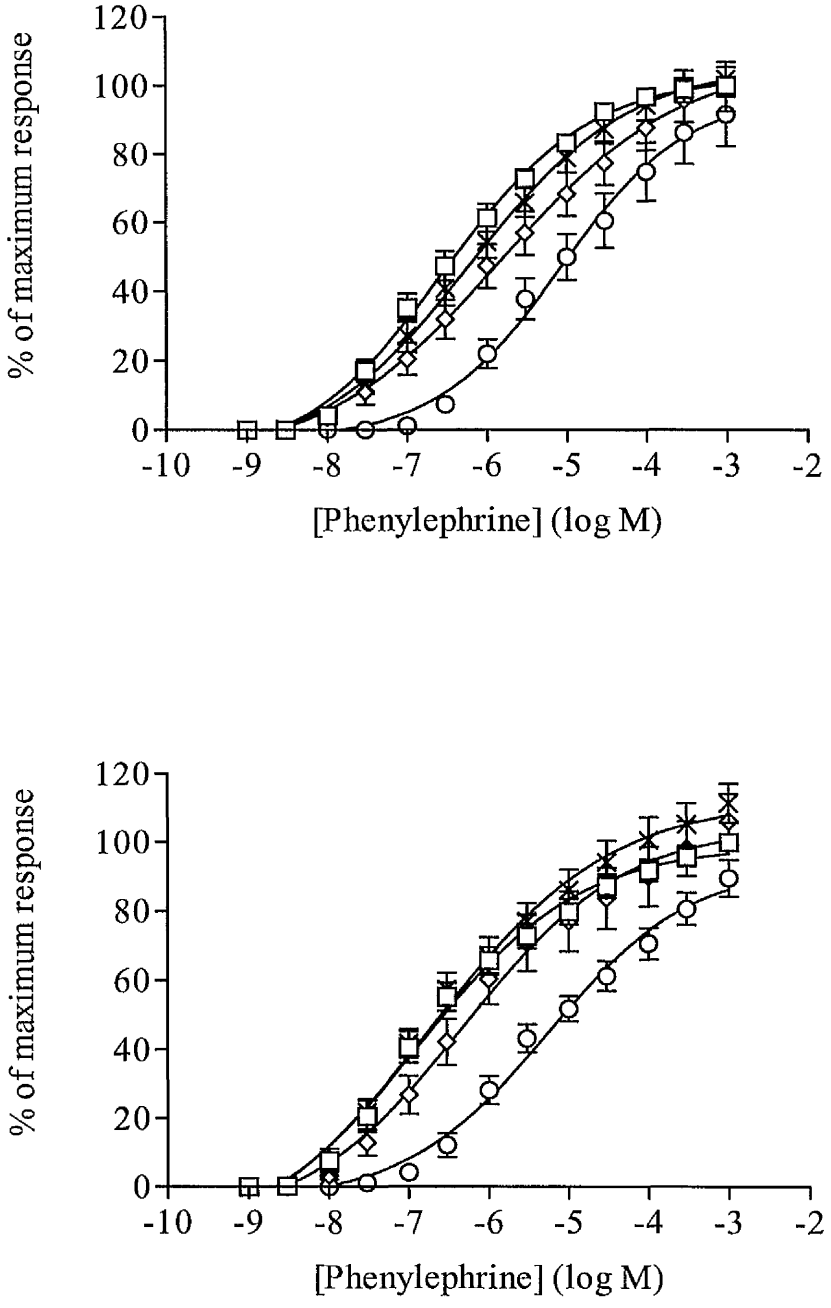


Figure 3.13:- Mean concentration response data to phenylephrine in the presence of increasing concentrations of L765,314 in WT (top) and KO (bottom) murine carotid arteries. Control (□), 1 x 10^{-8}M (×), 1 x 10^{-7}M (◊) and 1 x 10^{-6}M (○). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=14 and for KO arteries n=12.

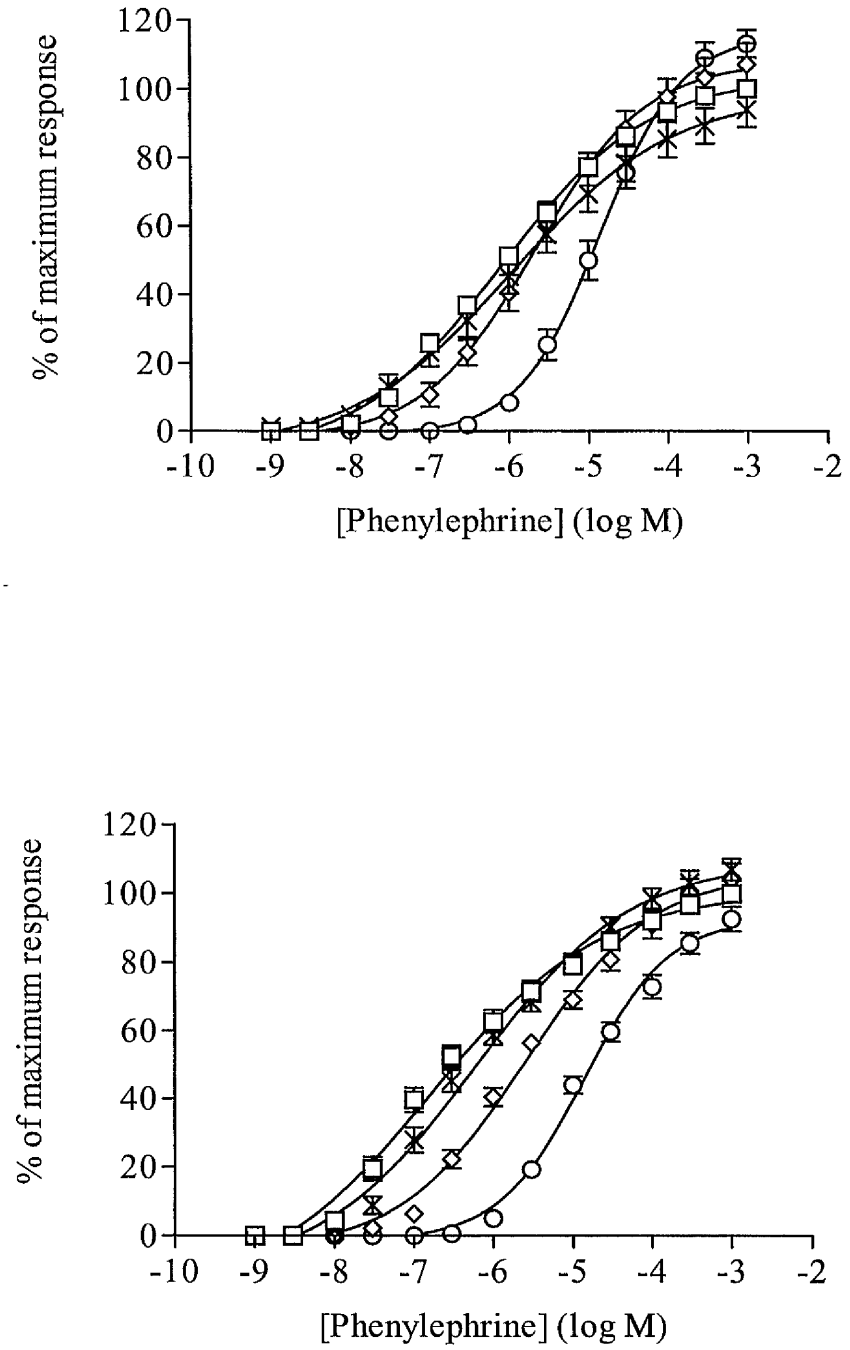


Figure 3.14:- Mean concentration response data to phenylephrine in the presence of increasing concentrations of BMY7378 in WT (top) and KO (bottom) murine carotid arteries. Control (□), 1 x 10^{-9}M (×), 1 x 10^{-8}M (◊) and 1 x 10^{-7}M (○). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. For WT arteries n=14 and for KO arteries n=14.

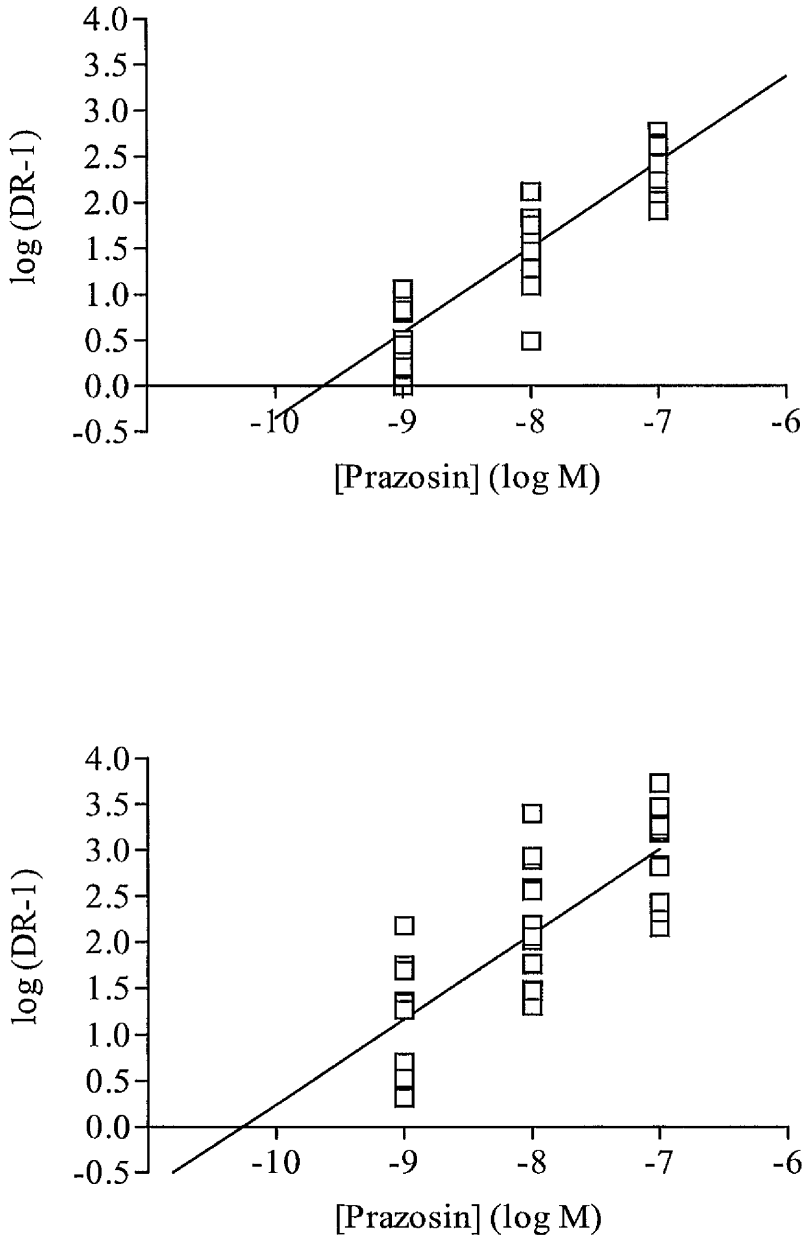


Figure 3.15:- Schild plots for prazosin in WT (top) and KO (bottom). Each point represents an individual experiment and linear regression was used to determine the line of best fit, the slope of this line and its x-intercept.

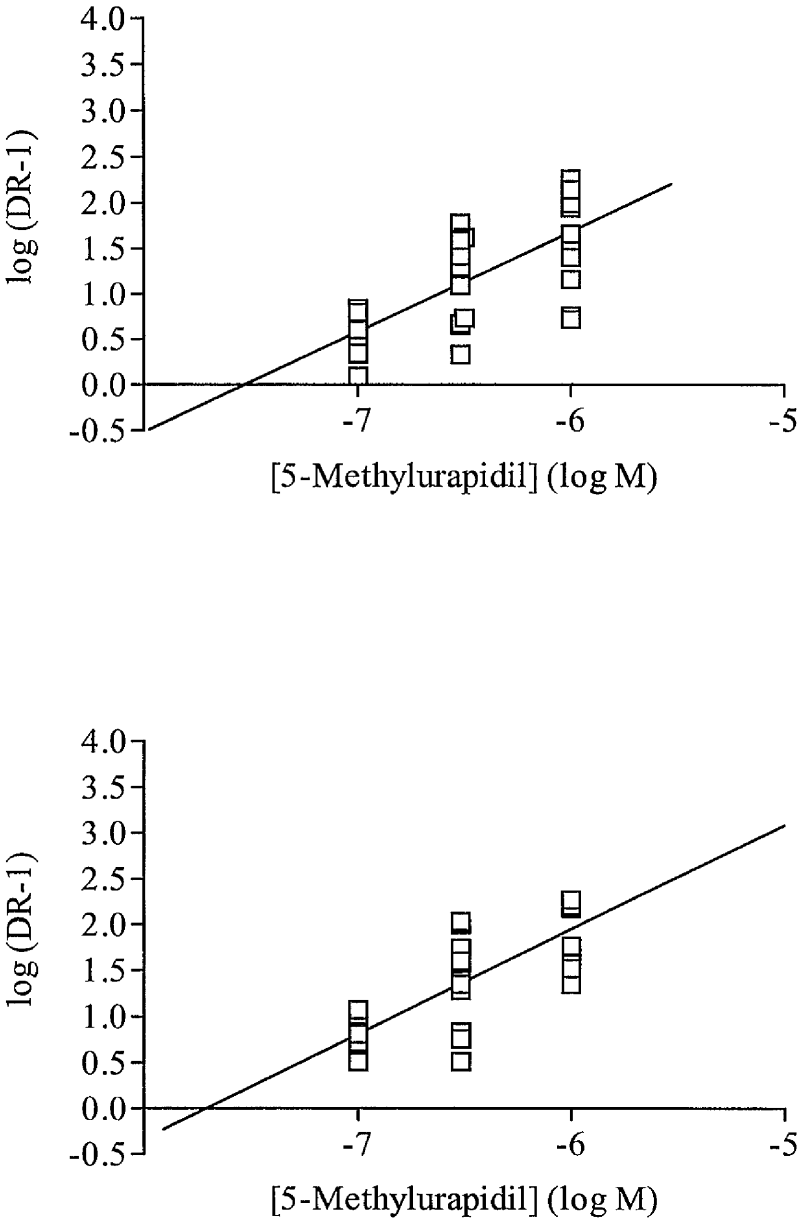


Figure 3.16:- Schild plots for 5-methylurapidil in WT (top) and KO (bottom) murine carotid arteries. Each point represents an individual experiment and linear regression was used to determine the line of best fit, the slope of this line and its x-intercept.

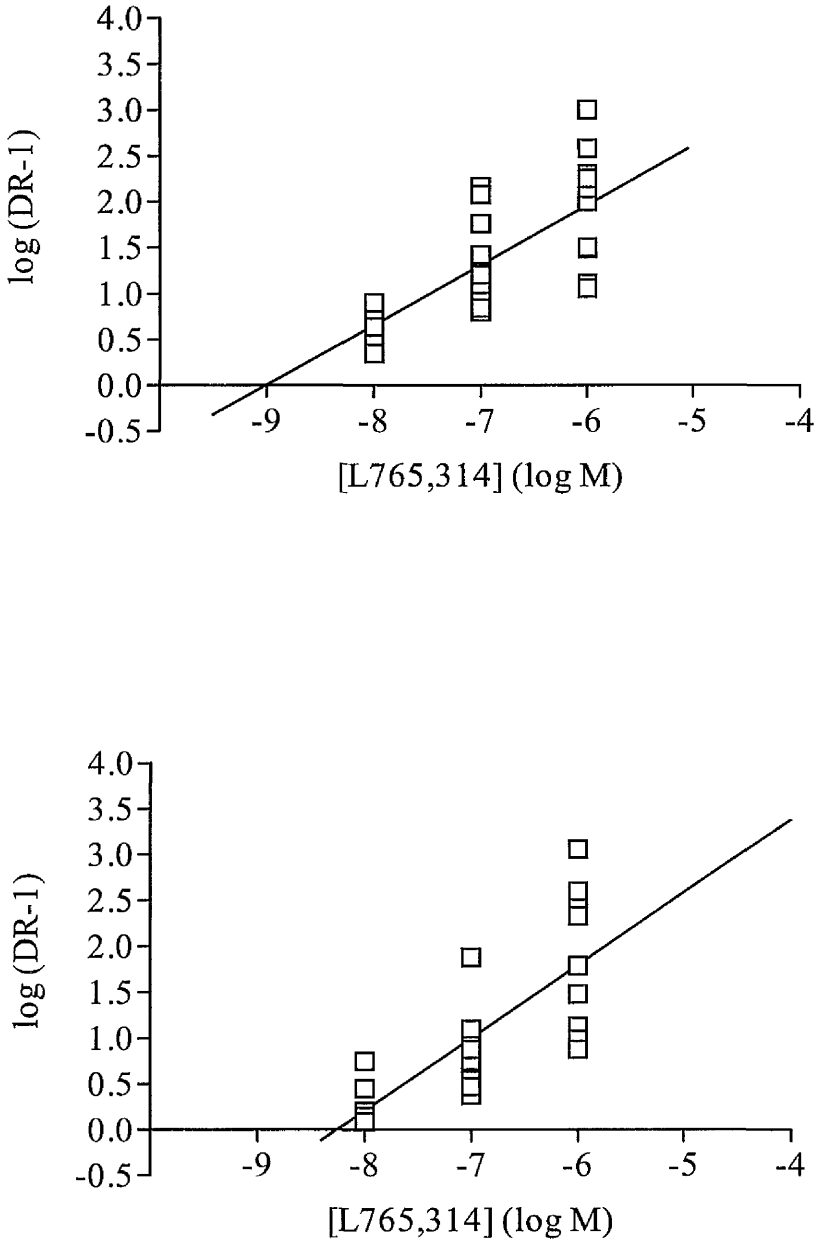


Figure 3.17:- Schild plots for L765,314 in WT (top) and KO (bottom) murine carotid arteries. Each point represents an individual experiment and linear regression was used to determine the line of best fit, the slope of this line and its x-intercept.

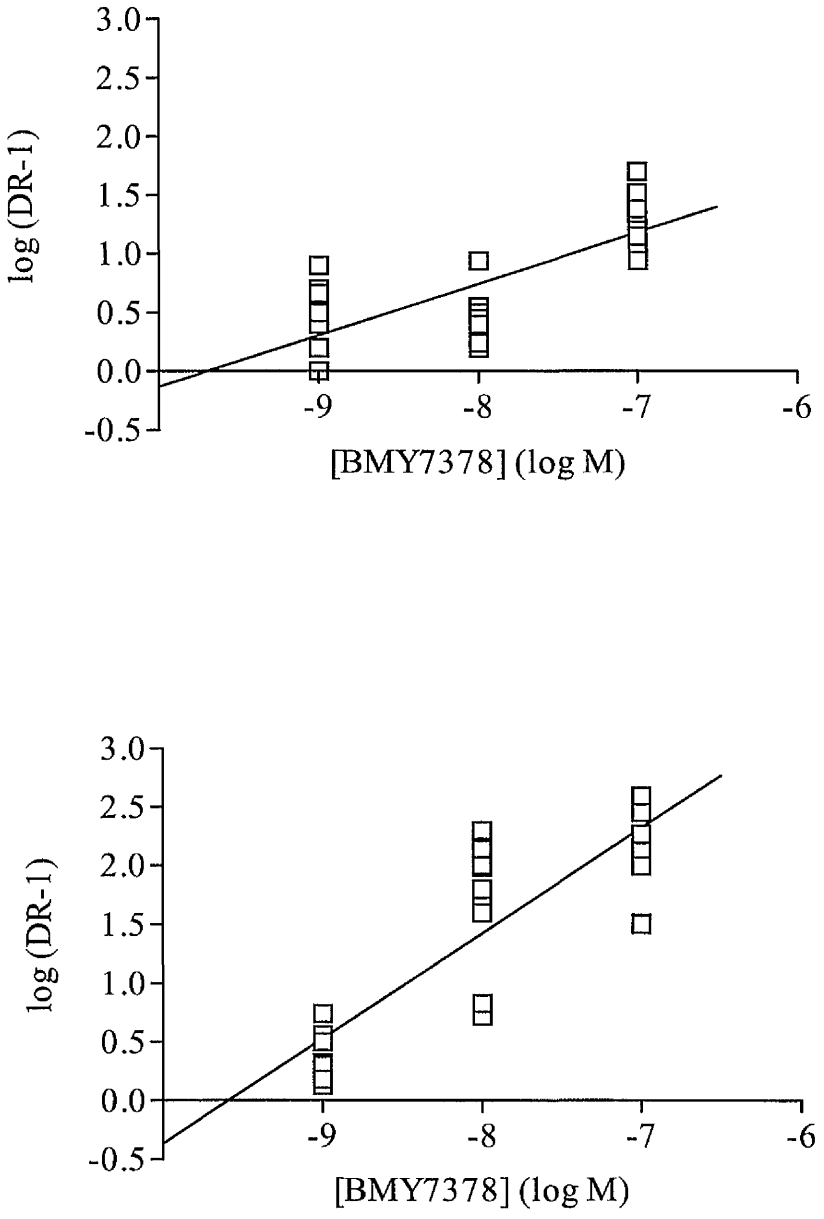


Figure 3.18:-Schild plots for BMY7378 in WT (top) and KO (bottom) murine carotid arteries. Each point represents an individual experiment and linear regression was used to determine the line of best fit, the slope of this line and its x-intercept.

	WT CAROTID ARTERY		KO CAROTID ARTERY	
	pA_2	Slope	pA_2	Slope
Prazosin	9.6	0.93 (0.77-1.08)	10.3	0.92 (0.68-1.2)
5-Methylurapidil	7.5	1.1 (0.73-1.5)	7.6	1.1 (0.77-1.5)
L765,314	9.0	0.65* (0.4-0.9)	8.3	0.79 (0.46-1.1)
BMY7378	9.7	0.44* (0.27-0.60)	9.6	0.90 (0.66-1.1)

Figure 3.19:- pA_2 values and slope parameters calculated from Schild regression of antagonists with phenylephrine in WT and KO carotid arteries. The slope value is given along with its 95% confidence intervals and an * indicates where the slope factor is significantly different from unity.

[PRAZOSIN]	WT CAROTID ARTERY (pK_B)	KO CAROTID ARTERY (pK_B)
1nM	9.6±0.1	10.1±0.2*
10nM	9.5±0.1	10.2±0.2*
100nM	9.4±0.1	10.0±0.1*
pA_2 **Slope**	**9.6** **0.93**	**10.3** **0.92**

[5-MEU]	WT CAROTID ARTERY (pK_B)	KO CAROTID ARTERY (pK_B)
100nM	7.6±0.1	7.8±0.1
300nM	7.7±0.1	8.0±0.1
1μM	7.7±0.2	7.9±0.1
pA_2 **Slope**	**7.5** **1.1**	**7.6** **1.1**

Figure 3.20:- Mean pK_B values ±s.e.m calculated for each concentration of prazosin (top) and 5-methylurapidil (5-MEU) (bottom) in WT and KO carotid arteries. The pA_2 value and slope as calculated by Schild regression are also given. * indicates $p<0.05$.

[L765,314]	WT CAROTID ARTERY (pK_B)	KO CAROTID ARTERY (pK_B)
10nM	8.6±0.1	8.3±0.1
100nM	8.4±0.2	7.9±0.1*
1μM	7.9±0.2	7.9±0.3
pA_2 **Slope**	**9.0** **0.65**	**8.3** **0.79**

[BMY7378]	WT CAROTID ARTERY (pK_B)	KO CAROTID ARTERY (pK_B)
1nM	9.5±0.1	9.4±0.1
10nM	8.5±0.1	9.7±0.2*
100nM	8.3±0.1	9.2±0.2*
pA_2 **Slope**	**9.7** **0.44**	**9.6** **0.90**

Figure 3.20 continued:- Mean pK_B values ±s.e.m calculated for each concentration of L765,314 (top) and BMY7378 (bottom) in WT and KO carotid arteries. The pA_2 value and slope as calculated by Schild regression are also given. * indicates $p<0.05$.

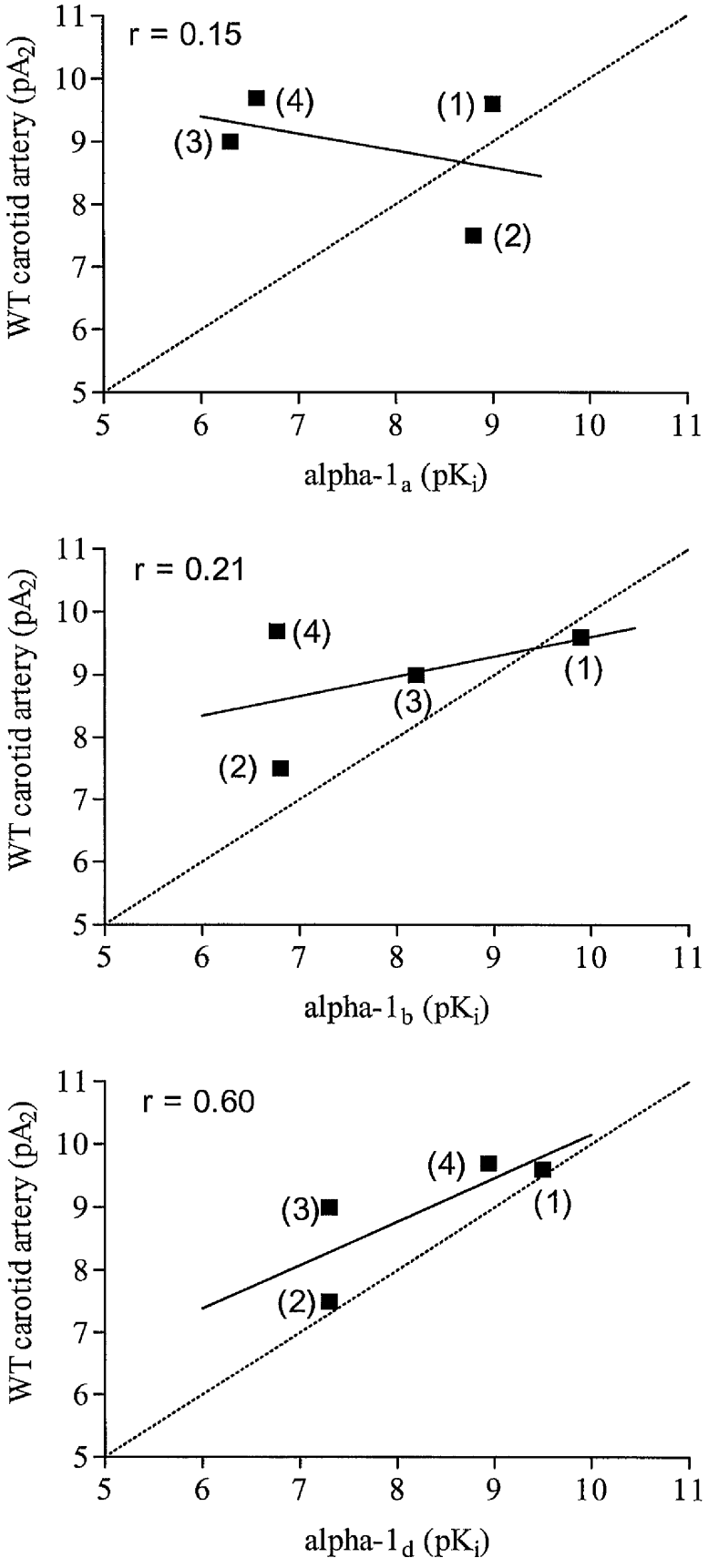


Figure 3.21:- Correlation of the potencies of antagonists used in WT carotid artery with their published potencies in cloned α_1-adrenoceptors. (1):- prazosin, (2):- 5-methylurapidil, (3):- L765,314 and (4):- BMY7378. (Williams *et al*, 1999, Burt *et al*, 1995, Patane *et al*, 1998, Yang *et al*, 1997). The dotted line shows the line of unity.

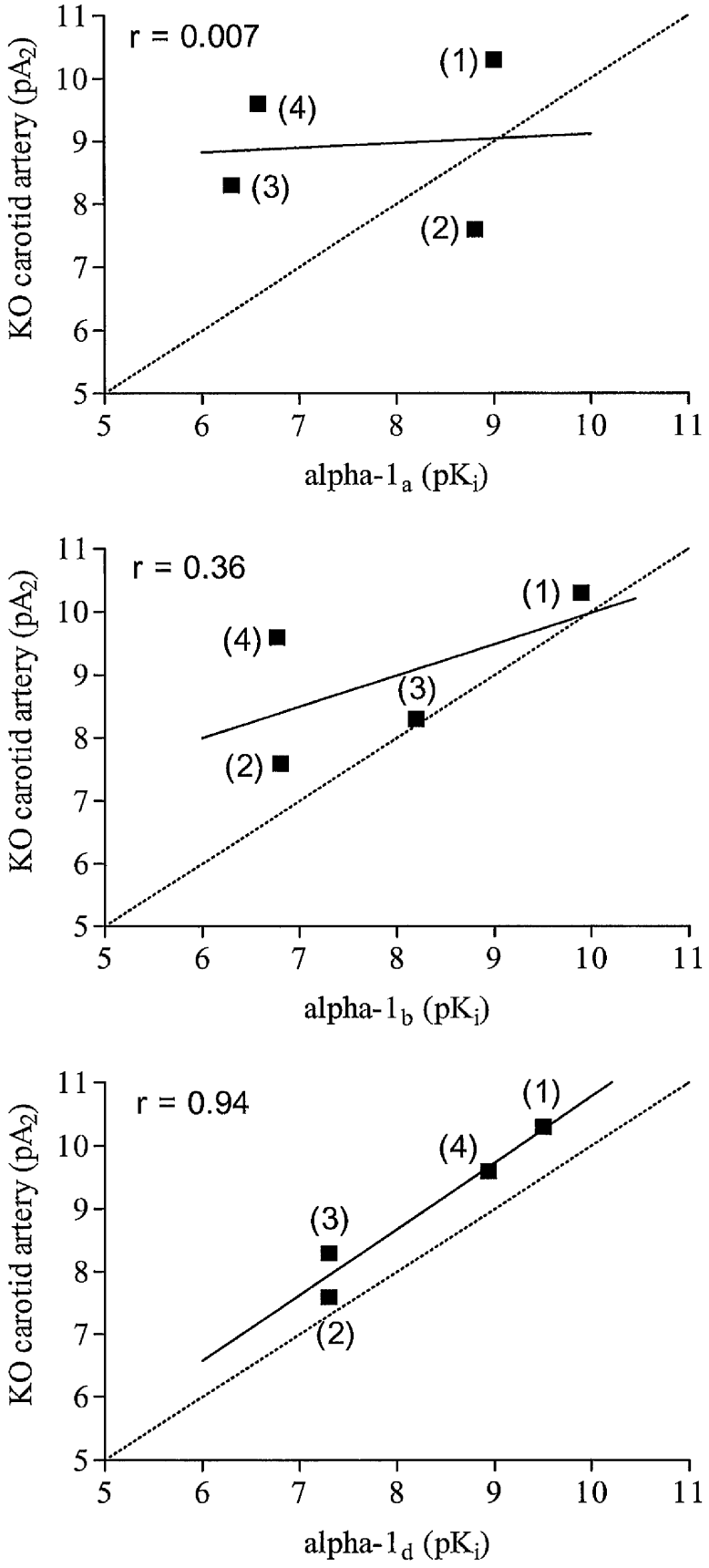
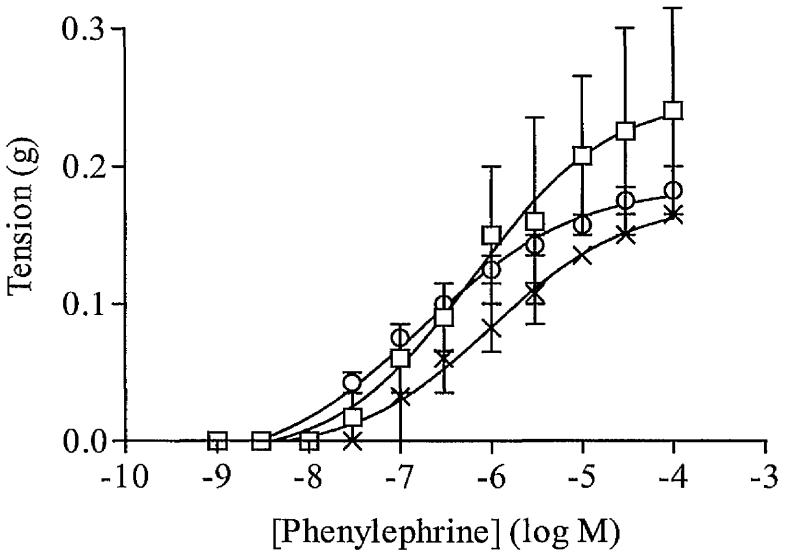


Figure 3.22:- Correlation of the potencies of antagonists used in KO carotid artery with their published potencies in cloned α_1-adrenoceptors. (1):- prazosin, (2):- 5-methylurapidil, (3):- L765,314 and (4):- BMY7378. (Williams *et al*, 1999, Burt *et al*, 1995, Patane *et al*, 1998, Yang *et al*, 1997). The dotted line shows the line of unity.

3.3.4. Control, over-expressed and constitutively active mice – carotid and mesenteric arteries - Sensitivity to PE

Figure 3.23 shows the CRC to phenylephrine obtained in the carotid arteries of the three strains of mice. The pEC_{50} values were 6.2±0.11 in control arteries (n=2), 6.0±0.12 in over-expressed arteries (n=2) and 6.8±0.15 in constitutively active arteries (n=2). The maximum responses produced were 0.24g±0.075 in control arteries, 0.165g in over-expressed arteries and 0.183g±0.017 in constitutively active arteries. The Hill slopes were found to be shallow and significantly different from unity in all arteries. Control arteries had a Hill slope of 0.59 (95% CI = 0.37-0.8), over-expressed arteries had a Hill slope of 0.58 (95% CI = 0.36-0.8) and constitutively active arteries had a Hill slope of 0.52 (95% CI = 0.26-0.77). Due to the low n number for these experiments meaningful statistical tests could not be carried out.

Figure 3.24 shows the CRC to phenylephrine obtained in the mesenteric arteries of the three strains of mice. The pEC_{50} values were 5.9±0.05 in control arteries (n=5), 6.2±0.03 in over-expressed arteries (n=5) and 5.9±0.04 in constitutively active arteries (n=4). The maximum responses produced were 0.44g±0.07 in control arteries, 0.27g±0.04 in over-expressed arteries and 0.31g±0.04 in constitutively active arteries. The Hill slopes for all arteries were similar and not significantly different from unity. These were 0.91 (95% CI = 0.72-1.1) for control arteries, 1.1 (95% CI = 0.88-1.26) for over-expressed arteries and 1.0 (95% CI = 0.79-1.2). One-way ANOVA revealed no significant differences in the pEC_{50}'s, maximum responses or Hill slopes.



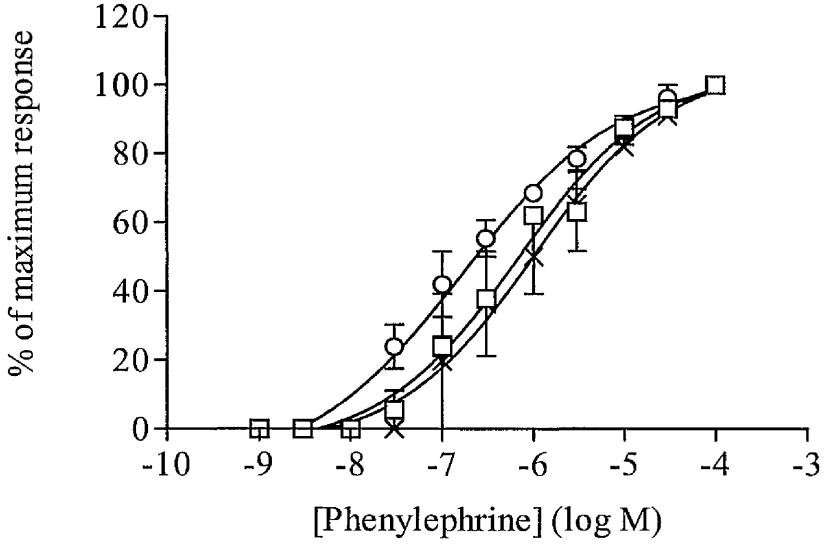


Figure 3.23:- Mean concentration response data to phenylephrine in control (□), over-expressed (×)and constitutively active (O) α_{1B}-adrenoceptor **carotid** arteries expressed as tension in grams (top) or as a percentage of their own maximum response (bottom). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. n=2 for control, overexpressed and constitutively active.

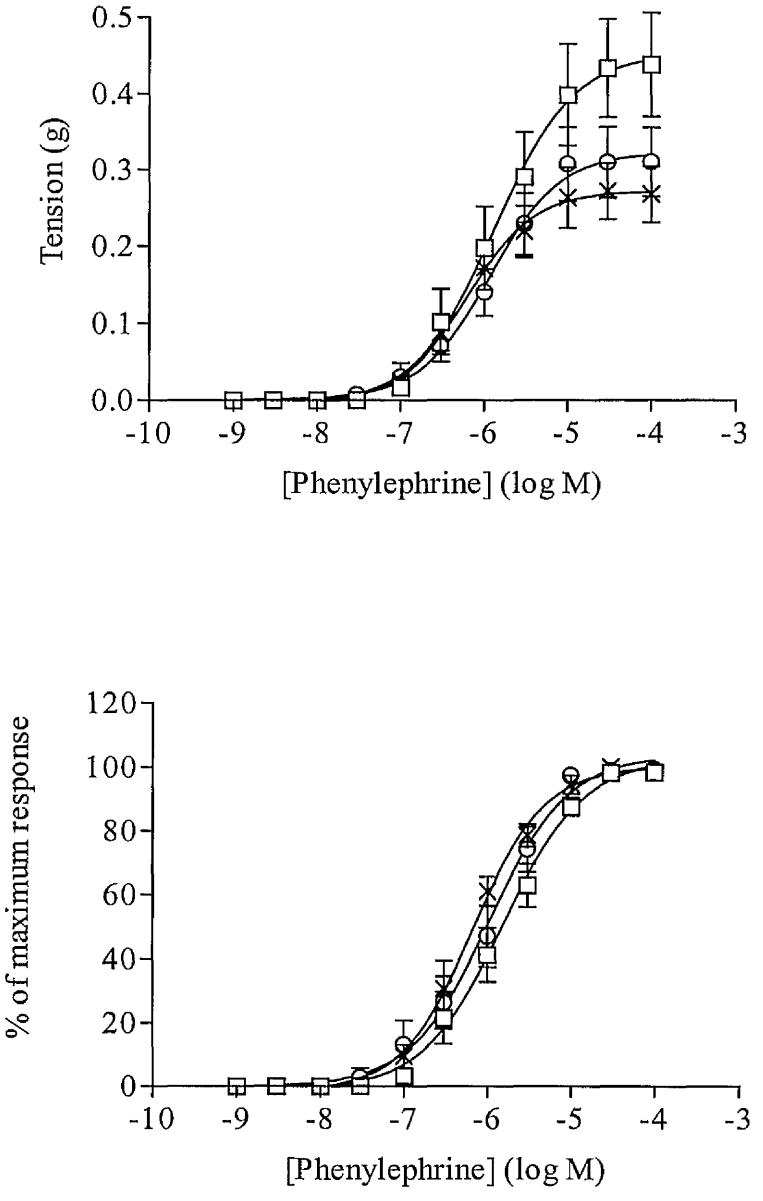


Figure 3.24:- Mean concentration response curves to phenylephrine in control (□), over-expressed (×) and constitutively active (○) α_{1B}-adrenoceptor **mesenteric** arteries expressed as tension in grams (top) or as a percentage of their own maximum response (bottom). Mean curves were generated using non-linear regression upon which the mean data ± s.e.m. has been superimposed. Control (n=5), over-expressed (n=5) and constitutively active (n=4).

3.4. DISCUSSION

Much of the physiology and pharmacology of vascular α_1-adrenoceptors has been carried out in rats, rabbits and dogs and until recently the mouse has been virtually ignored. However, the mouse is the only mammalian species to date, in which techniques to alter the genome of an animal, such as gene targeting have been successful. As these genetically engineered mice become a popular choice of laboratory animal it has become obvious that there is a gap in our knowledge of their physiology and pharmacology compared with our understanding of rats, rabbits and dogs. I undertook this study in an attempt to learn more about the α_1-adrenoceptor pharmacology and normal vascular function of the mouse carotid artery, neither of which had been studied.

3.4.1. Agonist profiles

Surprisingly, only a handful of agonists produced contraction in the mouse carotid artery. Of the agonists that produced a response (noradrenaline, phenylephrine, (R)-A-61603 and 5-HT), phenylephrine was the only agonist which was found to have differing sensitivities in WT and KO carotid arteries. There is some evidence from binding studies that phenylephrine may bind preferentially to the $\alpha_{1D/d}$-adrenoceptor (Minneman *et al*, 1994; Knepper *et al*, 1995; Yang *et al*, 1997). Knepper *et al* (1995) showed that phenylephrine was 15 times more potent in contracting the α_{1D}-adrenoceptors of the rat aorta than the α_{1A}-adrenoceptors of the rat vas deferens and 80 times more potent at contracting the α_{1B}-adrenoceptors of the rat spleen. The enhanced

sensitivity to phenylephrine observed in the KO carotid artery might be an indication of the presence of α_{1D}-adrenoceptors.

In both WT and KO carotid arteries, noradrenaline was found to produce a biphasic concentration response curve. The first component of the response produces a pEC_{50} that is typical of noradrenaline acting at an α_1-adrenoceptor (Nagadeh, 1996, University of Glasgow, PhD thesis; Russell and Watts, 2000). It is 15 times more potent than phenylephrine in WT carotid arteries and 10 times more potent than phenylephrine in KO carotid arteries. However, the basis for the second component of the response which is approximately 100 fold less sensitive to noradrenaline than the first component is not known and requires further investigation. Agents that block α_2- and β-adrenoceptors as well as neuronal and extraneuronal uptake of noradrenaline are normally employed when the α_1-adrenergic actions of noradrenaline are being investigated. These agents were not employed in this study because I was interested in how noradrenaline would act in the absence of the traditional blocking agents used. The biphasic response produced by noradrenaline may be due to its actions at α_2- or β-adrenoceptors. Alternatively, the biphasic response may result from the neuronal or extraneuronal uptake of noradrenaline or may arise through the release of mediators, such as nitric oxide. Further studies employing agents that block these receptors/processes may highlight the exact nature of this biphasic response.

(R)-A-61603, an α_{1A}-selective agonist synthesised by Abbot Laboratories, was equipotent to phenylephrine in the KO carotid artery whilst in the WT carotid artery it was found to be more potent than phenylephrine. This may suggest that the receptor in the WT carotid artery, which has a greater potency for (R)-A-61603 than for

phenylephrine is different from the receptor in the KO carotid artery where (R)-A-61603 is equipotent to phenylephrine. Noradrenaline was more potent than (R)-A-61603 in both WT and KO carotid arteries. The sensitivity (EC_{50} values) of the Abbott compound in native tissues representing each of the α_1-adrenoceptor subtypes has been published by Knepper *et al* (1995). The racemic mixture of A-61603 was found to be 330 times more potent in the rat vas deferens (α_{1A}) than phenylephrine and 200 times more potent than noradrenaline. In the rat spleen (α_{1B}) it was 40 times more potent than phenylephrine and 30 times more potent than noradrenaline. And in the rat aorta (α_{1D}), A-61603 was 30 and 510 times less potent than both phenylephrine and noradrenaline respectively. In the present study the R-enantiomer of A-61603 was used rather than the racemic mixture. The R-enantiomer appears to confer potency to the compound at α_{1A}-adrenoceptors. The potency of (R)-A-61603 was tested at the α_{1A}-adrenoceptors of the canine prostate by Knepper *et al* (1995) where it was found to be 590 times more potent than phenylephrine and 460 times more potent than noradrenaline. Unfortunately, (R)-A-61603's potency was not determined at α_{1B}- or α_{1D}-adrenoceptors but its sensitivity at α_{1A}-adrenoceptors is considerably higher than the sensitivity observed in either the WT or KO carotid artery, which may indicate that the α_{1A}-adrenoceptor does not mediate contraction in these arteries.

The concentration response curves to phenylephrine and (R)-A-61603 were both found to have shallow slopes that were significantly less than 1.0 in both the WT and KO carotid artery. This may indicate that these agonists are activating more than one population of receptors, since a shallow curve can result through the interaction of an agonist with more than one receptor.

The rank order of potency for those adrenergic agonists that elicited a response in the carotid artery, was:-

WT - Noradrenaline (1^{st} component) > (R)-A-61603 > Phenylephrine > Noradrenaline (2^{nd} component)

KO – Noradrenaline (1^{st} component) > Phenylephrine = (R)-A-61603 > Noradrenaline (2^{nd} component)

Interestingly, the rank order of potency of noradrenaline, phenylephrine and (R)-A-61603 in the KO carotid artery is similar to the rank order of potency observed by Knepper *et al* (1995) with the racemic mixture of A-61603, phenylephrine and noradrenaline at the α_{1D}-adrenoceptors of the rat aorta. The only difference is that in the present study phenylephrine was equipotent with (R)-A-61603 whereas Knepper *et al* found that phenylephrine was more potent than A-61603 (30-fold). This difference could be a result of this study using the R-enantiomer rather than the racemic mixture but the overall similarity might suggest that the KO carotid artery contains a population of α_{1D}-adrenoceptors.

A lack of a contractile response with the α_2-adrenoceptor agonist, UK14304 and the β-adrenoceptor agonist, isoprenaline coupled with the contractile response obtained using phenylephrine suggests that in mouse carotid artery the dominant adrenergic receptor subtype involved in mediating contraction is the α_1-adrenoceptor. (However, the dilator effects of UK14304 and isoprenaline, which are discussed below, could obscure any potential contractile effects.) This is similar to findings in the mouse aorta (Russell and Watts, 2000) and the rat carotid artery (Nagadeh, 1996, University of Glasgow, PhD

thesis). Although these studies reported that isoprenaline and UK14304 were capable of producing contractions in the mouse aorta and rat carotid artery respectively, these responses were found to be blocked by prazosin, indicating that they were mediated through α_1-adrenoceptors. The only other agonist found to be capable of contracting the mouse carotid artery in the present study was 5-HT whose sensitivity was similar to that of the mouse aorta (Russell and Watts, 2000). In addition to adrenergic and serotonergic receptors, Russell and Watts (2000) also found evidence for dopaminergic and prostaglandin receptors.

(U-46619, which is a thromboxane mimetic that stimulates thromboxane A_2 receptors was found to contract the WT mouse carotid artery, although CRC were not carried out to this agent due to time limitations.)

Relaxant agents were tested only on WT carotid arteries. The agents which produced relaxation were acetylcholine, UK14304 and isoprenaline with a potency order of Acetylcholine > Isoprenaline > UK14304. The relaxations produced by all three agents could be blocked by L-NAME indicating that they were mediated by nitric oxide. It is well documented that acetylcholine relaxes the mouse carotid artery via the actions of nitric oxide as this is often the artery studied when investigating the consequences of 'knocking out' the nitric oxide synthase (NOS) genes, such as inducible NOS (iNOS) or endothelial NOS (eNOS) (Chataigneau *et al*, 1999; Didion *et al*, 2000; Gunnett *et al*, 2000). The relaxation produced by isoprenaline has also been attributed to nitric oxide in the rat carotid artery (MacDonald *et al*, 1999; Nagadeh, 1996, University of Glasgow, PhD thesis). UK14304 has been shown to act as a partial α_1-agonist in the rat carotid artery (Nagadeh, 1996, University of Glasgow, PhD thesis). Although there was no evidence for this in the mouse carotid artery when the artery was at resting tension, a

partial agonistic effect may be revealed when the tissue is preconstricted with a non-adrenergic agonist such as U-46619. Further experiments are planned to investigate the receptors at which UK14304 acts to produce relaxation in the mouse carotid artery. Interestingly, UK14304 does not appear to produce relaxation in carotid arteries from D79N mice; transgenic mice whose $\alpha_{2A/D}$-adrenoceptor is uncoupled from its potassium channels resulting in it acting like a functional knockout. Time controls to relaxant curves were not carried out but would be useful to determine the effect of time on the contraction produced by phenylephrine.

The agents that did not produce any response, whether it be a contractile or relaxant effect, included such important vasoactive compounds as angiotensin II, endothelin, ATP, histamine, vasopressin and bradykinin. Receptors for these agents have been found previously in rat (Caputo *et al*, 1995; Marano *et al*, 1999; Rutschmann *et al*, 1998) and rabbit carotid artery (Maurice *et al*, 1997; Pruneau and Belichard, 1993). This study highlights the dangers in extrapolating information from one species of animal to the next. Although the mouse and rat are similar animals i.e. small rodents, it is clear that their vascular pharmacology is quite different. Whether this species difference in the expression of receptors is confined to the carotid artery or whether it will be observed in all arteries of these mammals remains to be determined.

3.4.2. Antagonist profiles

To determine the α_1-adrenoceptor subtype mediating contraction in WT and KO carotid arteries, four α_1-adrenoceptor antagonists with differing selectivities for the α_1-adrenoceptor subtypes were used. Prazosin produced a pA_2 value of 9.6 in WT carotid

artery and a significantly higher pA_2 of 10.3 in KO carotid artery. These high affinities indicate that the α_{1L}-adrenoceptor does not contribute to vasoconstriction in either WT or KO carotid arteries. The pA_2 in the KO is high for prazosin. However, this can be seen to arise as a result of a higher control sensitivity to phenylephrine in the KO. The pEC_{50}'s for phenylephrine in the control curves are found be significantly more sensitive to phenylephrine in the KO than in the WT, yet the subsequent curves in the presence of equal concentrations of prazosin are the same for both WT and KO carotid arteries. Therefore, the higher pA_2 value that was calculated does not appear to be due to prazosin having an absolute higher affinity in the KO carotid artery compared with the WT, but instead appears to be a result of phenylephrine having a higher sensitivity in the KO carotid artery (discussed above).

A pA_2 value of similar potency to that in the KO carotid artery has also been found in the KO mouse aorta (Daly *et al*, unpublished observations). The KO aorta has been shown to have an increased sensitivity to phenylephrine (Daly *et al*, unpublished observations), so it would be of interest to re-examine the pEC_{50}'s calculated in this artery to see if a similar situation also exists in the KO mouse aorta. This may be a characteristic of KO blood vessels or it may be a characteristic of the subtype of α_1-adrenoceptor expressed in the KO carotid artery and aorta. This latter suggestion seems more likely because results from the mesenteric and tail arteries of KO mice show that the sensitivity to phenylephrine is unchanged when compared with the equivalent WT vessels (Daly *et al*, unpublished observations). This is reflected in the pA_2 value for prazosin, which is identical in the WT and KO mesenteric artery (pA_2 values for prazosin have still to be calculated for tail arteries). Both the mesenteric and tail arteries are believed to mediate contraction through a mixture of α_{1A}- and α_{1B}-adrenoceptors in

the WT and through the α_{1A}-adrenoceptor in the KO (Daly *et al*, unpublished observations). The pharmacology of these vessels does not resemble the pharmacology of the mouse aorta or the mouse carotid artery (discussed in more detail below) suggesting that the α_1-adrenoceptor subtype mediating contraction in the mesenteric and tail arteries is different from that in the aorta or carotid artery. Therefore, the increased sensitivity to phenylephrine which results in an increased pA_2 for prazosin is more likely to be due to a characteristic of the α_1-adrenoceptor subtype found in the aorta and carotid artery or it may be a characteristic of large conducting arteries.

The α_{1A}-adrenoceptor antagonist, 5-methylurapidil, acted competitively in both WT and KO carotid arteries producing similar pA_2 values of low potency. At $\alpha_{1A/a}$-adrenoceptors, 5-methylurapidil has been shown to have pA_2 values of between 8.5 and 9.5 (Hanft and Gross, 1989; Kong *et al*, 1994; Schwinn *et al*, 1995; Vargas and Gorman, 1995; Daniel *et al*, 1999) whilst at $\alpha_{1B/b}$- and $\alpha_{1D/d}$-adrenoceptors its affinity is approximately 10-100 fold less (Hanft and Gross, 1989; Schwinn *et al*, 1995; Mackenzie *et al*, 2000). Therefore the pA_2 values calculated for WT and KO carotid arteries indicate that there is unlikely to be an α_{1A}-adrenoceptor component to the contraction in these arteries. It is more probable that the response involves α_{1B}- and/or α_{1D}-adrenoceptors. The α_{1B}-adrenoceptor cannot be a contributor to the contraction in the KO carotid artery and so it would seem likely that in both the WT and KO carotid artery, where similar pA_2 values were obtained, the α_{1D}-adrenoceptor is mediating contraction. However, the α_{1B}-adrenoceptor may be involved in the WT contraction because 5-methylurapidil does not distinguish between $\alpha_{1B/b}$- and $\alpha_{1D/d}$-adrenoceptors.

The results using L765,314 suggest that there is a contribution from the α_{1B}-adrenoceptor in the contraction of the WT carotid artery. The pA_2 for L765,314 is high but L765,314 is not acting competitively, producing a shallow Schild slope of 0.65 that was found to be significantly less than 1.0. This would be consistent with the presence of more than one receptor subtype distinguished by different sensitivities to L765,314. The shallow Schild slope would also result in a pA_2 that is artificially high. The high pA_2 value coupled with the shallow slope of the Schild plot may imply that there is a component of the contraction that is mediated by the α_{1B}-adrenoceptor and a component which is mediated by another α_1-adrenoceptor subtype. In the KO carotid artery, L765,314 is less potent than in the WT artery, although it appears to be acting competitively, producing a slope which is not significantly different from unity.

As a result of L765,314 producing a shallow Schild slope in the WT carotid artery pK_B's were calculated for each concentration of L765,314 used in the WT and KO carotid artery to determine whether these might shed some light on the affinity of this drug in the WT carotid artery. The pK_B's for L765,314 in the WT carotid artery were found to be smaller than the pA_2 value, whereas the KO pK_B's were similar to the pA_2 value (as expected given that L765,314 appears to be acting competitively in the KO). These pK_B values correlate better with L765,314's affinity at native and recombinant α_{1B}-adrenoceptors, than the pA_2 value in the WT carotid artery, which was about 10 times more potent than its published affinity at α_{1B}-adrenoceptors (Patane *et al*, 1998). The pK_B values and the pA_2 calculated for the KO are also similar to the affinity of L765,314 at $\alpha_{1B/b}$-adrenoceptors. The fidelity of the α_{1B}-adrenoceptor KO mouse has been proved by the receptor's absence in all of the major organs of the KO mouse (Cavalli *et al*, 1997; our own unpublished observations), so despite L765,314 displaying

an affinity in the KO carotid artery which is comparable to its affinity at $\alpha_{1B/b}$-adrenoceptors, this cannot be the subtype mediating contraction in this artery. The similarity of the pK_B values in the WT and KO carotid artery also imply that the $\alpha_{1B/b}$-adrenoceptor does not mediate contraction in the WT carotid artery either. However, the shallow Schild slope in the WT suggests heterogeneity of receptors and the presence of the α_{1A}-adrenoceptor has already been excluded because of the low pA_2 value for 5-methylurapidil. These results seem to place some doubt over the effectiveness of L765,314 as an α_{1B}-adrenoceptor antagonist.

The calculated pA_2 values for the $\alpha_{1D/d}$-adrenoceptor antagonist, BMY7378, were similar in WT and KO carotid arteries and were of high potency. However, the Schild plot in the WT carotid artery resulted in a shallow slope which was significantly different from unity, whilst the slope of the Schild plot in the KO carotid artery indicated competitive antagonism. This is similar to the circumstances presented with L765,314 where a shallow slope is evident in the WT carotid artery which is not observed in the KO carotid artery. As with L765,314, pK_B values were calculated for WT and KO arteries to gain a more accurate reflection of BMY7378's affinity in the WT carotid artery. The affinity of BMY7378 was reduced in the WT carotid artery when pK_B values were calculated, demonstrating that the pA_2 value was indeed artificially high, whilst the pK_B's in the KO carotid artery were similar to the pA_2. The affinity of BMY7378 was found to be significantly higher in KO arteries compared with WT arteries at concentrations of 10nM and 100nM. In the light of the findings with prazosin, the pEC_{50} values used in the Schild analysis for BMY7378 were examined. The trend was found to be the same as for prazosin. The control pEC_{50}'s were significantly different whereas the pEC_{50}'s in the presence of BMY7378 were the same

between WT and KO carotid arteries. Again this indicates that BMY7378 has a similar affinity at the α_1-adrenoceptors of WT and KO carotid arteries. The KO appears to have a higher affinity because it has an increased sensitivity to phenylephrine, which results in lower dose ratios being calculated for the KO, therefore higher pK_B's. This apparent difference in affinity is not observed in the pA_2 values because of the shallow Schild slope in the WT, producing a deceptively high affinity value. The affinities calculated for BMY7378 in WT and KO carotid artery indicate the presence of an α_{1D}-adrenoceptor population in both WT and KO carotid arteries, with the possibility of there being a second receptor subtype in the WT resulting in the shallow slope. BMY7378 does not discriminate between α_{1A}- and α_{1B}-adrenoceptors and its affinity at these receptors is approximately 100 fold less than its affinity at α_{1D}-adrenoceptors (Saussy *et al*, 1994; Goetz *et al*, 1995). There is no evidence for the presence of the α_{1A}-adrenoceptor from the agonist and antagonist studies, therefore the second subtype in the WT would appear to be the α_{1B}-adrenoceptor.

When correlations were performed, comparing the pA_2 values calculated here with published pK_i values for the same antagonists at cloned receptors, the WT carotid artery did not correlate particularly well with any of the subtypes but produced the best correlation with the α_{1d}-adrenoceptor ($r = 0.6$). In the KO carotid artery this correlation with the α_{1d}-adrenoceptor was improved ($r = 0.94$). These correlations confirm the involvement of the α_{1D}-adrenoceptor in the contractions of the WT and KO carotid arteries.

3.4.3. Control, over-expressed and constitutively active mice

The sensitivities of the control, over-expressed and constitutively active mice to phenylephrine were found to be unchanged in mesenteric arteries suggesting that the α_{1B}-adrenoceptor does not play a major role in contraction of this vessel. Interestingly, the carotid arteries of the constitutively active mice were found to have a pEC_{50} which was comparable to the KO carotid artery, whilst the control and over-expressed had similar values to the WT carotid artery. Due to the low n number for these experiments statistical analysis was not carried out. However, if these pEC_{50} values were found to remain at these values when the n number was increased it seems likely that there would be a significant difference between the constitutively active carotid arteries and the control and over-expressed arteries. This is an interesting point to note because after discovering an increased potency to phenylephrine in the KO carotid artery, it was anticipated that the opposite would be true in the constitutively active carotid arteries. Agonists have been shown to demonstrate increased affinity for constitutively active receptors (Cotecchia *et al*, 1990; Lefkowitz *et al*, 1993) which may explain the increased sensitivity observed in the constitutively active carotid arteries. The similarity in the pEC_{50} values in the KO and in these constitutively active arteries may not be related to an involvement of the α_{1B}-adrenoceptor in contraction but may result from both receptors being constitutively active. The first functional evidence for constitutively active receptors was presented last year when Gisbert *et al* (2000) proposed that the α_{1D}-adrenoceptors of the rat aorta were constitutively active. This would explain the increased sensitivity to phenylephrine observed in the KO carotid artery (which I am proposing to express the α_{1D}-adrenoceptor) and also observed in the KO aorta (also proposed to express the α_{1D}-adrenoceptor) (Daly *et al*, unpublished

observations). Another possible explanation could be if the α_{1D}-adrenoceptor of the KO carotid artery is upregulated in some way, which may manifest itself in an increased number of receptors or an increased efficiency of coupling of agonist and receptor. Either of these would result in an increased sensitivity to phenylephrine. However it should be noted that the WT and KO mice and the control, overexpressed and constitutively active mice are different strains of mice. The WT and KO mice are C57 Black 6J mice whereas the control, overexpressed and constitutively active mice are CBA mice. Different strains of mice may have differing sensitivities to agonists.

In conclusion, the data from this study provides evidence that the α_{1D}-adrenoceptor mediates contraction in both WT and KO murine carotid arteries. There is also evidence that a component of the contraction in the WT carotid artery is mediated through the α_{1B}-adrenoceptor, producing the shallow Schild slopes observed with L765,314 and BMY7378. The rat carotid artery has previously been characterised as having an α_{1D}-adrenoceptor population by two separate groups (Nagadeh, 1996, University of Glasgow, PhD thesis; Villalobos-Molina and Ibarra, 1996) based on high affinity for BMY7378. The rabbit carotid artery was found to have a low affinity for prazosin, prompting the suggestion that it contained the α_{1L}-adrenoceptor (Muramatsu *et al*, 1990), whilst the dog carotid artery has been claimed to mediate vasoconstriction through the α_{1B}-adrenoceptor on the basis of low affinities for 5-methylurapidil and WB4101 (Kohno *et al*, 1994). This species difference again highlights the pitfalls that exist if assumptions are made about the α_1-adrenoceptor expressed in different animals.

CHAPTER 4 – INVESTIGATION INTO THE STRUCTURAL DIFFERENCES IN WT AND KO MURINE CAROTID ARTERIES USING CONFOCAL LASER SCANNING MICROSCOPY

4.1. INTRODUCTION

4.1.1 Structure of blood vessels

All blood vessels, with the exception of the capillaries, share the same basic structural features. They are composed of three layers, or 'tunics'. The tunica intima consists of a layer of endothelial cells, which line the lumen of the vessel and which rest on a basal lamina. Beneath the endothelium is the subendothelial layer, consisting of loose connective tissue, which, in larger arteries, may contain occasional smooth muscle cells. The tunica media is primarily composed of concentric layers of smooth muscle cells oriented in a helix at an angle of 20°-40° to the longitudinal axis (Williams, 1998). Interspersed among these smooth muscle cells can be found elastic fibres and lamellae, collagen fibres and proteoglycans. In arteries, the media is separated from the intima by an internal elastic lamina which contains gaps (fenestrae) through which substances can diffuse in order to nourish cells deep in the vessel wall. The outermost layer of a blood vessel is the tunica adventitia which consists of an external elastic lamina, nerve fibres and surrounding connective tissue which contains collagen and elastic fibres, adventitial fibroblasts and macrophages (Gutterman, 1999).

4.1.2. Arteries

Arteries are classified according to their size:-
Arterioles:- Less than 500μm in diameter in humans and have relatively narrow lumens
Muscular arteries:- Medium-large diameters, most of the named arteries are of this type.

Large elastic arteries:- Includes aorta and its branches e.g. carotid. Have a yellowish colour due to the accumulation of elastin in the media.

These elastic arteries are also known as conducting arteries because their major function is to transport blood away from the heart. They also serve to smooth out the large fluctuations in pressure created by the heartbeat. The intima of these elastic arteries tends to be thicker than that of a muscular artery. The media consists of a series of concentrically arranged perforated elastic laminae whose number increase with age and whose thickness increase due to deposition of elastin. Interspersed between these elastic laminae are smooth muscle cells, collagen fibres and chondroitin sulphate. The tunica adventitia does not show an external lamina and is relatively underdeveloped.

4.1.3. Adventitia

Before 1980, study of the vasculature was primarily concerned with the tunica media of arteries, using pharmacological agents to examine vascular smooth muscle reactivity. With the discovery by Furchgott and Zawadzki (1980) that nitric oxide could be released from the endothelium, the emphasis shifted towards the study of the endothelium. 20 years on from this discovery, we now accept, that instead of simply acting as a semi-permeable barrier between the blood and the artery, the endothelium is capable of releasing a host of vasoactive substances through which it regulates vascular tone. Throughout this period the tunica adventitia received minor attention. Its importance was believed to be only that of a structural role. However, it is now becoming apparent that there is more to the adventitia than would first appear.

In recent years, the adventitia has become a popular target for gene transfer using adenovirus' which have met with varying success. In 1997, Chen *et al* introduced the endothelial nitric oxide synthase (eNOS) gene into adventitial fibroblasts of the canine basilar artery. Endothelial nitric oxide synthase (eNOS) is one of three isoforms of nitric oxide synthase, the enzyme which catalyses the formation of nitric oxide from L-arginine. Transfection of eNOS into canine basilar arteries resulted in an augmented relaxation response to bradykinin (Chen *et al,* 1997). Tsutsui *et al* (1998) also reported an enhanced relaxation response in basilar arteries transfected with eNOS which they discovered was unaffected by removal of the endothelium. A follow up paper revealed that the relaxation in these vessels could be inhibited by chemical (ethanol) or mechanical (rubbing) disruption of the adventitia indicating that the transfected adventitial fibroblasts were responsible for the increased relaxant response (Tsutsui *et al*, 1999).

The functional consequences of eNOS transfection do not seem to be limited to the basilar artery. The rabbit carotid artery was also found to produce an augmented relaxation to acetylcholine in both normal and atherosclerotic vessels transfected with eNOS (Ooboshi *et al*, 1998). The relaxation of atherosclerotic vessels is impaired, yet gene transfer of eNOS was able to restore a 'normal' relaxant response (Ooboshi *et al*, 1998). These studies suggest that adventitial fibroblasts are capable of releasing nitric oxide and acting as surrogate endothelial cells.

Data has also been presented to show that the adventitia of the rat aorta is capable of releasing nitric oxide when exposed to endotoxin. In fact, the adventitia was found to generate more nitric oxide than either the endothelium or the media (Zhang *et al*, 1999;

Kleschyov *et al*, 2000) demonstrating that the adventitia is capable of releasing nitric oxide without the need for gene transfer. These findings may impact on treatments in the future for vascular and inflammatory diseases.

There is mounting data to suggest that the adventitia may play an important role in the pathogenesis of atherosclerosis. Removal of the adventitia from large arteries of the rabbit was found to result in intimal and medial hyperplasia, characteristic of early atherosclerotic lesions (Barker *et al*, 1994). In the coronary circulation, adventitial mast cells were found to be present in higher concentrations in infarct-related coronary arteries, associated with coronary atherosclerosis, when compared with control arteries (Gutterman, 1999).

A role for the adventitia in postangioplasty restenosis has also been suggested. As with atherosclerosis the main focus of study for restenosis has been the medial and intimal layers of blood vessels, since this is the site of the lesion. However, there is now evidence to suggest that the adventitia is involved in the early proliferative phase prior to formation of the neointima. Wilcox *et al* (1996) found that 2-3 days after angioplasty, proliferation was primarily observed in the adventitia. A week after angioplasty the proliferation in the adventitia was reduced and was now concentrated in the neointima. These proliferating cells were found to include adventitial fibroblasts that had migrated from the adventitial layer to the site of the neointima. Intravascular irradiation was found to inhibit the growth of adventitial cells resulting in a decrease in the cellular proliferation in the neointima (Wilcox *et al*, 1996).

Laser scanning confocal microscopy revealed that the adventitia may contribute to the vascular remodelling observed in hypertension. The number of adventitial cells in mesenteric arteries was found to have almost doubled in SHRSP rats when compared with WKY controls (Arribas *et al*, 1997). Adventitial-like cells were also observed in the medial layer of SHRSP rats, implying migration of adventitial cells from the outermost part of the vessel into the media (Arribas *et al*, 1997). This is similar to the phenomenon observed in balloon injured arteries which may suggest that migration of adventitial cells is common to vascular remodelling.

This year the role of the adventitia was further questioned by the discovery that adventitial fibroblasts, isolated from the rat aorta, express α_1- and α_2-adrenoceptors and that the density of α_1-adrenoceptors was similar to that observed in the media (Faber *et al*, 2001). Using a combination of competitive RT-PCR and radioligand binding, adventitial fibroblasts were shown to express all three α_1-adrenoceptor subtypes and the α_{2D}-adrenoceptor. RT-PCR revealed that mRNA for the α_1-adrenoceptor subtypes was most abundant for the α_{1D}-adrenoceptor, followed by the α_{1A}- and then the α_{1B}-adrenoceptor. Radioligand binding contradicted these findings by revealing that the α_{1A}-adrenoceptor was present in the greatest density, followed by the α_{1B}- and α_{1D}-adrenoceptors. These experiments were also carried out on the medial layer of the rat aorta, where in contrast to the adventitia, results from RT-PCR and radioligand binding coincided. The α_{1D}-adrenoceptor was found to be the predominant subtype in terms of density, followed by the α_{1B}- and α_{1A}-adrenoceptors. These results are consistent with functional studies showing the α_{1D}-adrenoceptor to be responsible for the contraction of the rat aorta (Kenny *et al*, 1995; Piascik *et al*, 1995; Testa *et al*, 1995b; Deng *et al*, 1996; Hussain and Marshall, 1997; Piascik *et al*, 1997; Guimaraes and Moura, 2001).

The media and adventitia of the rat carotid artery were also found to express the same four α-adrenoceptor subtypes, (α_{1A}, α_{1B}, α_{1D} and α_{2D}) as the rat aorta, in similar amounts (Faber *et al*, 2001). An additional finding in this paper was that noradrenaline caused proliferation and protein synthesis in adventitial fibroblasts, a phenomenon which has been demonstrated previously in smooth muscle cells (Chen *et al*, 1995; Xin *et al*, 1997). The finding that adventitial fibroblasts express α_1-adrenoceptors is surprising, given the traditional view that fibroblasts are normally non-contractile and considered to be structural cells. However, this may go some way to explaining the discrepancies that surround attempts to correlate mRNA levels of α_1-adrenoceptors with the functioning subtype in intact arteries. Given the increasing amounts of data implicating the adventitia in vascular diseases, some of which has been reviewed here, it is possible that one or more of the α-adrenoceptor subtypes discovered on adventitial fibroblasts could be capable of signalling a trophic action of catecholamines on vascular wall growth and remodelling.

From the data obtained from functional studies in WT and KO murine carotid arteries, detailed in chapter 3 of this thesis, the contraction in the WT carotid artery appears to be mediated by the α_{1D}-adrenoceptor with a contribution from the α_{1B}-adrenoceptor, whilst the contraction in the KO carotid artery is mediated by the α_{1D}-adrenoceptor. With the recent discovery that the adventitia of the rat aorta and carotid artery express all three α_1-adrenoceptor subtypes (Faber *et al*, 2001) and the growing importance of the adventitia in the pathogenesis of vascular diseases, the possible structural and functional consequences of deleting the α_{1B}-adrenoceptor from the KO carotid artery was investigated with the focus being on the adventitia. It was hoped that such a study might reveal a role for the α_{1B}-adrenoceptor in the adventitia of these blood vessels. Ideally all

three layers of the arteries would have been studied in detail but due to technical limitations, both in the equipment available and the tissue used, it was only practical to study the adventitia in any detail.

4.2.METHODS

4.2.1. Solutions and drugs used

All vessels were fixed using neutral buffer formalin of the following composition:- 10% formaldehyde, sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$) – 135mM, sodium hydroxide (NaOH) – 105mM, pH – 7.4. The sodium borohydride solution was of the following composition:- sodium phosphate, dibasic (Na_2HPO_4) – 70mM, sodium borohydride ($NaBH_4$) – 13mM. 100ml of this solution was made up fresh on the day of the experiment and then discarded after use.

The following compound was used:-

Propidium iodide (Sigma).

4.2.2. Animals used

Wildtype and knockout male C57-Black mice were obtained from the Central Research Facility at the University of Glasgow. Mice were killed by lethal overdose of carbon dioxide and the common carotid arteries immediately removed by careful dissection using a microscope and placed in cold Krebs. The arteries were further dissected to ensure as little connective tissue remained attached to the vessel as possible. Where possible mice of a similar age (~16 weeks) and weight (30-40g) were used.

4.2.3. Pressurisation and fixation procedure

Vessels were mounted in Krebs at 37°C in a pressure myograph system by means of a glass cannula. One end of the vessel was secured to the cannula using fine ligatures, whilst the other end was left unattached. In order to produce a closed system in which pressure could be maintained the unattached end of the vessel was tied off with ligatures. The cannula was connected to a pressure-servo unit, which allowed the pressure of the system to be precisely controlled and altered at will. Pressure was applied to the vessel over a 15 minute period, increasing by 40mmHg every 5 minutes until the *in vivo* pressure of the artery was attained. This has been previously reported to be 120mmHg (Cavalli *et al*, 1997). The pressurised artery was allowed to equilibrate in Krebs for 15-20minutes to ensure that pressure was maintained. Towards the end of the equilibration period several measurements were taken from the artery, specifically, the internal diameter and the right and left wall thickness which allowed the external diameter to be calculated. This was achieved by carefully placing the perfusion myograph on the stage of a Zeiss light microscope and raising the stage until the tip of the objective (X10) was immersed in the Krebs solution. The artery was then brought into focus being careful not to allow the objective to touch either the artery or the cannula. A video dimension analyser was used to take the measurements from the vessel, which was visualised using a camera and monitor.

Once the equilibration period was over the Krebs solution was removed and replaced with neutral buffer formalin and the vessel was fixed overnight (a minimum of 18 hours and a maximum of 24 hours).

Following the fixation procedure the vessel was carefully removed, taken down from the cannula, placed in 70% ethanol and stored in the fridge until required. Vessels were normally used within a week to a month of being fixed.

4.2.4. Staining procedure

The arteries were removed from ethanol and washed once in distilled water. The end of the vessel, which had been tied off during the pressurisation procedure, was cut, proximal to where the vessel had been tied, in order to open up the lumen. This allowed the staining reagent access to the inside of the vessel resulting in better staining of the individual cells.

The arteries were placed in a solution of sodium borohydride prior to staining for 10 minutes, with gentle agitation every 2 minutes. The sodium borohydride prevents any non-specific histochemical reactions and binding of proteins by the free aldehyde groups introduced by the fixation procedure. After incubation with sodium borohydride the vessels were washed 4 times in distilled water.

The stain used throughout this set of experiments was the nuclear stain, propidium iodide (optimum excitation wavelength is 535nm and optimum emission wavelength is 617nm) (5μg/ml). All staining procedures were carried out in complete darkness. The arteries were stained for 1 hour and then washed 3-4 times in distilled water for 15 minutes each wash.

4.2.5. Mounting procedure

The vessels were mounted onto standard glass slides and were placed in a makeshift well constructed with thin streaks of a high vacuum silicone grease in the style of a noughts and crosses board. A drop of distilled water was placed in the well. This was enough to cover the vessel but not enough to allow it to move about on the slide. A glass cover slip (No. 1.5) was placed over the vessel and pressed down gently so as not to distort the 3-dimensional structure of the artery.

4.2.6. Confocal microscopy

Vessels were visualised with a Noran Odyssey Real Time Confocal Laser Scanning Module (CLSM) coupled to an upright Nikon Optiphot-2 microscope. The excitation wavelength used was the 514nm line of an argon ion laser with an emission wavelength bandpass filter of 550nm and a slit width of 15μm. Metamorph 4.5 (Universal Imaging Corporation) was used throughout to acquire images for all image analysis.

Brightfield was used in the first instance to locate the position of the artery on the slide, firstly with a x10 objective and then with a x40 water objective. The staining of the artery was confirmed using a fluorescence microscope through the use of a mercury lamp and only after this were the vessels visualised using the CLSM.

The 'standard' protocol in this laboratory when looking at arteries using the CLSM is to take a z-series through the artery using a x40 objective in 1μm (or less) steps. This is repeated a total of 3 times along the length of the vessel and then from this the number

of adventitial, smooth muscle and endothelial cell nuclei can be calculated, as well as the number of layers of each cell nuclei type in the artery wall and any other morphological features of interest to the experimenter. However, the mouse carotid artery, although a small artery in relative terms, is a large thick walled vessel (~ 50μm) in terms of murine anatomy. In addition to having thick walls, the carotid artery contains large amounts of elastin and collagen, which are known to autofluoresce. This made visualisation of the individual layers of cell nuclei almost impossible. In fact in most cases it proved difficult to see beyond the first layer of smooth muscle cell nuclei. Various staining techniques were used, with different stains, different concentrations and times of incubations tested to see if this aided in the visualisation of the vessel. After many permutations and combinations, none of which made any significant difference, it became clear that there were going to be limitations in the structural information obtained from the carotid artery using the technique of confocal microscopy. In the light of the recent interest in the adventitia as being more than just a structural component of the vessel wall, coupled with the fact that this was the only layer of artery that I could visualise, I decided to concentrate my efforts on studying the adventitial cell nuclei of WT and KO carotid arteries, in an attempt to determine the effects (if any) which deletion of the α_{1B}-adrenoceptor had on the adventitia. Two protocols were carried out to achieve this aim. The first protocol was devised early on in the study as an alternative to the standard protocol used in this laboratory and was designed with the sole purpose of providing an *estimate* of the number of adventitial cell nuclei present in WT and KO mouse carotid arteries. The second protocol was a shortened version of the standard protocol that I attempted after finishing the study using the first protocol because I felt confident that my staining method had improved and that I was more skilled at using the CLSM. I restained the vessels used for the first

protocol and performed z-series of these vessels through the adventitial layer. This allowed me to determine the thickness of the adventitial layer and from this I could calculate the density of adventitial cell nuclei. Knowing the thickness of the adventitia allowed me to calculate the cross sectional area of the adventitia and from this I could work out the number of adventitial cell nuclei in a 1mm length of artery.

4.2.7. FIRST PROTOCOL - Procedure for acquiring images of adventitial cell nuclei

Using a x40 water-immersion objective a focal plane within the adventitia was chosen which was deemed to contain (as near as possible) the first full layer of adventitial cell nuclei in the particular artery which was being examined. This was obviously different for each vessel examined. The depth at which the adventitia was imaged was approximately 5-10μm. Once the focal plane had been chosen this was the plane at which all subsequent images for that vessel were taken. Working from one end of the vessel to the other images were acquired for each section of the artery, being careful not to image the same nucleus twice, so that the images collected were representative of the number of adventitial cell nuclei present in the carotid artery at a particular focal point. Following this the artery was then examined to see if it was possible to visualise any smooth muscle or endothelial cell nuclei. If any were visible, images were acquired.

4.2.8. SECOND PROTOCOL - Procedure for acquiring images of adventitial cell nuclei

Using a x40 water-immersion objective, stacks of 1μm thick serial optical slices were taken in z-axis starting from the first adventitial cell nuclei until the first layers of smooth muscle were visible. This was repeated a total of 4 times along the length of the vessel.

4.2.9. Procedure for acquiring images for measurement of external diameter

The artery was imaged using a x10 objective. However, due to the size of the carotid artery the whole artery could not be visualised within the screen. To enable the external diameter to be calculated 2 images were taken of each section of the artery (top and bottom) using an easily recognisable region that was present in both images as a marker. This procedure was repeated a total of 3-4 times along the length of the vessel.

4.2.10. FIRST PROTOCOL - Image analysis of adventitial nuclei images

For each individual vessel there were between 20 and 50 frames of images dependent on the length of each vessel. In order to carry out image analysis a certain amount of image processing was necessary in order to get the images into a binary format from which the appropriate morphometric analysis could be carried out. The first stage in the image processing was to threshold the individual frames. Thresholding was carried out on individual frames because within each set of images and even within single frames there were marked differences in the intensity of the fluorescence. Thresholding was set

manually for each frame in accordance with certain criteria that were deemed to provide the most accurate thresholded images possible. These were:-

1. That the threshold identified as many individual objects as possible. In some cases when objects were in close proximity to one another the thresholding process would merge these objects together as one object. Therefore, a threshold was chosen which would minimise this effect.
2. That the threshold identified the correct boundary of as many objects as possible. 'Glare' and out of focus light can distort the true size and shape of an object and so the decision had to be made as to what was part of the object and what was an artefact of the imaging acquisition.

When a suitable threshold range had been identified i.e. it met either one or the other but preferably both the above criteria, the thresholded 8-bit image was converted into a binary image. All frames for each vessel were converted into a binary format and then subjected to integrated morphometry analysis (a function performed by Metamorph) which was used to measure a number of parameters. These were:-

1. Total area:- the total area of the object, including any holes present.
2. Perimeter:- the perimeter of the object as a measurement from the midpoints of each pixel around the border.
3. Fibre length:- the maximum length of the object.
4. Fibre breadth:- the maximum width of the object.

5. Shape factor:- this is a value from 0 to 1 representing how closely the object represents a circle. A straight line would have a shape factor of 0 and a perfect circle a shape factor of 1.
6. Elliptical form factor:- this is the ratio of the objects breadth to its length.

These measurements were used to determine suitable classifying parameters with which to identify and count the number of adventitial cell nuclei. From these measurements it was decided that the total area and shape factor would be the best parameters to use. One vessel was then chosen at random and the area and shape factor of each object was measured again along the length of the vessel, this time with a view to determining a range of values which appeared to consistently represent single adventitial cell nuclei. This was carried out in parallel with the original images, because it is much easier to identify an adventitial cell nuclei by sight rather than by a numerical method. On this basis, a total area classifier of between 300-4000 pixels and a shape factor classifier of 0.6-1.0 was used. If an object was smaller than 300 pixels it was assumed that it was (1) not an adventitial cell nuclei i.e. it was possibly debris or autofluorescence from collagen and elastin fibres or (2) it was a nuclei that was on a different plane from that which the image had been taken and only a small fraction of the nuclei was visible. Any object that was greater than 4000 pixels was generally more than one nucleus and so was not counted. The shape factor classifier was chosen because although adventitial cell nuclei are generally circular or round they are never a perfect circle and will invariably have a shape which is somewhere between that of a circle and an ellipse. In addition, the classifier, as it stands, rules out the possibility of including any stray smooth muscle cells in the count.

The data generated by both the measuring and classifying parameters was logged in Microsoft Excel 97. The mean and s.e.m. were calculated only for the classifying parameters (total area and shape factor) and then transferred into GraphPad Prism 3.01. to enable further analysis of the data (e.g. graphical representation of the data, statistical tests). The number of adventitial cell nuclei per mm^2 was calculated by dividing the number of adventitial cell nuclei counted by the area of artery from which the images had been taken. The area of vessel was calculated as follows:- each image is 512 x 512 pixels which, when using a x40 objective is equal to 100.4 x 100.4 μm (1μm = 5.1 pixels). Therefore the number of images acquired multiplied by 100.4μm is equal to the length of the vessel. This value, when multiplied by 100.4μm gives the area of the imaged artery. The values calculated from this method were entered into GraphPad Prism 3.01. to allow for further analysis.

4.2.11. SECOND PROTOCOL - Image analysis of adventitial cell nuclei images

To determine the density of adventitial cell nuclei in each of the vessels analysed the thickness of the adventitial layer had to be determined in addition to counting the number of adventitial cell nuclei. The adventitia was defined as beginning from the first adventitial cell nucleus at its maximum intensity and ending with the first smooth muscle cell nucleus at its maximum intensity. Metamorph has a tool which records the intensity of the fluorescence of individual cells thus allowing me to identify the individual frames which contained the first adventitial cell nucleus and first smooth muscle cell nucleus at their maximum intensities. Each image is captured at intervals of 1μm therefore the thickness of the adventitial layer can be determined. Once this had been calculated the adventitial cell nuclei were counted to this depth and the density

calculated by dividing the number of adventitial cell nuclei by the volume of the adventitia (the volume of the adventitia was calculated by area of adventitia imaged multiplied by the thickness of the adventitia) and was expressed as number of adventitial cell nuclei/mm^3 of artery. The cross sectional area (c.s.a.) of the adventitia was calculated using the equation:-

$c.s.a. = \pi.((D/2)^2 - (D/2-T)^2)$

which simplifies to:-

$c.s.a. = \pi.T(D-T)$

where D = external diameter (μm) and T = thickness of adventitial layer (μm).

The number of adventitial cell nuclei in a 1mm length of artery can then be calculated by multiplying the cross sectional area of the adventitia by the density of adventitial cell nuclei by 1mm. All data obtained using this protocol was analysed using Graph Pad Prism 3.01.

4.2.12. Image analysis for determination of external diameter

Images were analysed in their corresponding pairs, with one image showing the top of the vessel and the other the bottom. The marker was identified and using the single line tool a horizontal line was drawn through the centre of the marker region on the image which represented the top of the artery. 3 vertical lines were then drawn at separate intervals along the image, going from the horizontal line to the edge of the vessel, which was identified manually. The length of these vertical lines, in pixels, was measured. This process was repeated on the image representing the bottom of the artery, with the vertical lines from this image being drawn as close as possible to the vertical lines from

the image showing the top of the artery. Adding together the length of the two vertical lines from the top and bottom images of the artery gave the external diameter at that point. Three measurements of the diameter were taken from each section of artery which had been imaged therefore in total there were between 9 and 12 external diameters calculated for each vessel. These diameters were averaged and then converted from pixels into microns (1 pixel = 0.77μm) after which they were entered into GraphPad Prism 3.01.to allow for analysis of the data.

All WT and KO data was analysed for statistical significance using an unpaired t test. A p value of less than 0.05 was considered significant.

4.3. RESULTS

4.3.1. External diameters

The external diameters for WT and KO murine carotid arteries determined using the video dimension analyser were not significantly different. The WT carotid artery was found to have an external diameter of 541.8±23μm and the KO carotid artery had a diameter of 555.5±8.6μm (Figure 4.1). However, the external diameters determined from images taken with a x10 objective using a CLSM showed a significant difference between WT and KO carotid arteries. The WT had a diameter of 575±7.8μm, whilst the KO had a significantly smaller diameter of 513.8±14.3μm ($p<0.05$) (Figure 4.1). A comparison was made of the measurements taken in WT and KO arteries using both the video dimension analyser and the CLSM to determine if fixation of the artery affected its diameter and also to determine the accuracy of the video dimension analyser. It is assumed that images taken with the confocal microscope provide a more accurate measurement of the actual diameter of the vessel because the image is viewed in more detail than when using the video dimension analyser. Figure 4.2 shows the graphical representation of this comparison for WT and KO carotid arteries. For WT arteries there is no significant difference between the diameters obtained with the video dimension analyser than those obtained with the CLSM. However, the KO arteries were found to have a greater diameter when measured using the video dimension analyser than when using the CLSM ($p<0.05$).

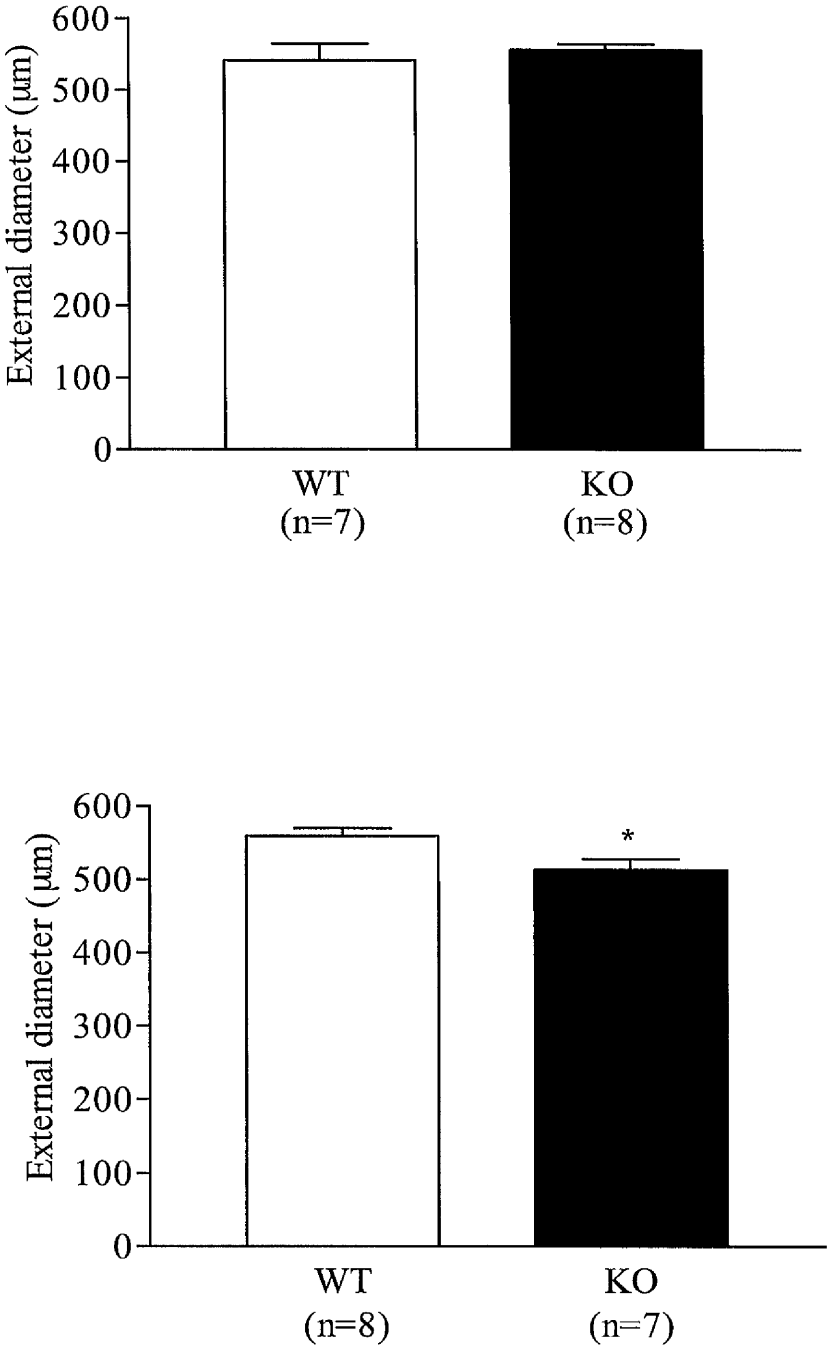


Figure 4.1:- External diameters of WT and KO murine carotid arteries, pressurised at 120mmHg and measured using the VDA prior to fixation of the arteries (top) and the CLSM after fixation (bottom). Data is presented as mean ± s.e.m for the number of arteries indicated in brackets. * indicates $p<0.05$.

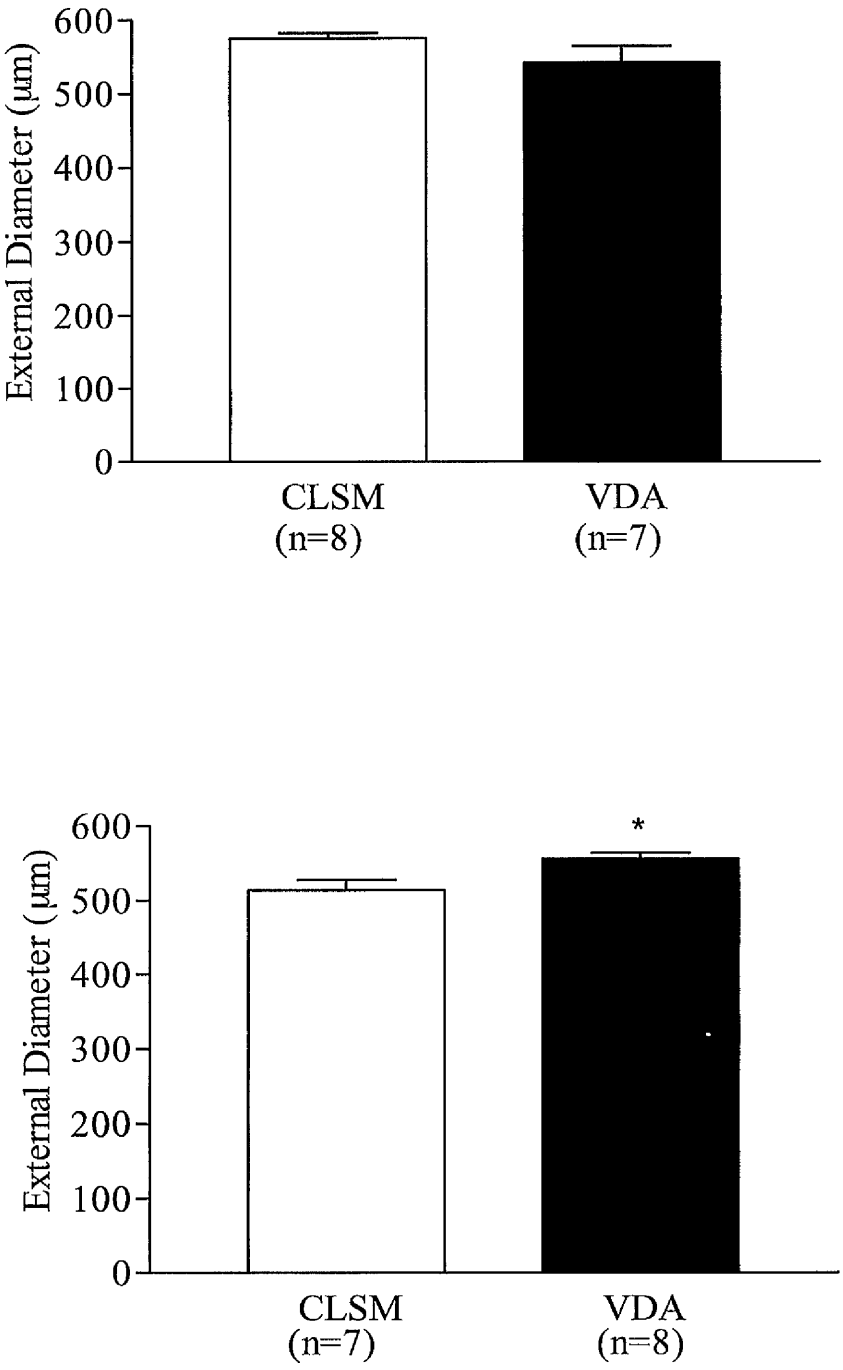


Figure 4.2:- A comparison of the external diameters calculated using the CLSM and the VDA in WT (top) and KO (bottom) murine carotid arteries. Data is presented as mean ± s.e.m for the number of arteries indicated in brackets. * indicates $p<0.05$.

4.3.2. FIRST PROTOCOL – Adventitial cell nuclei morphology

The shape factors of the adventitial nuclei from WT and KO mouse carotid arteries were virtually identical (Figure 4.3). WT adventitial nuclei were found to have a shape factor of 0.77±0.009, whilst KO adventitial nuclei had a shape factor of 0.76±0.004.

No differences were observed concerning the area of each adventitial nuclei. WT adventitial nuclei had an average area of 48.2±4.4μm^2, whilst KO adventitial nuclei averaged an area of 45.7±1.8μm^2 (Figure 4.3).

The number of adventitial nuclei occurring in a 1mm^2 area of adventitial tissue was calculated. No significant differences were observed in the number of adventitial nuclei in KO carotid arteries when compared with WT. In WT carotid arteries there were 796±54 adventitial nuclei/mm^2 of artery and in the KO carotid artery there was found to be 718±42 adventitial nuclei/mm^2 (Figure 4.3).

4.3.3. SECOND PROTOCOL – Adventitial thickness and cell nuclei density

There was no difference in the thickness of the adventitial layer of WT and KO carotid arteries. The WT adventitia had a thickness of 19.4±1.5μm, which was virtually identical to the thickness of the KO adventitia which was 19.8±1.2μm (Figure 4.4).

The cross sectional area of the adventitia, which is calculated from the external diameter and the thickness of the adventitia showed no significant differences between WT and KO carotid arteries. This is despite there being a significant difference in the external

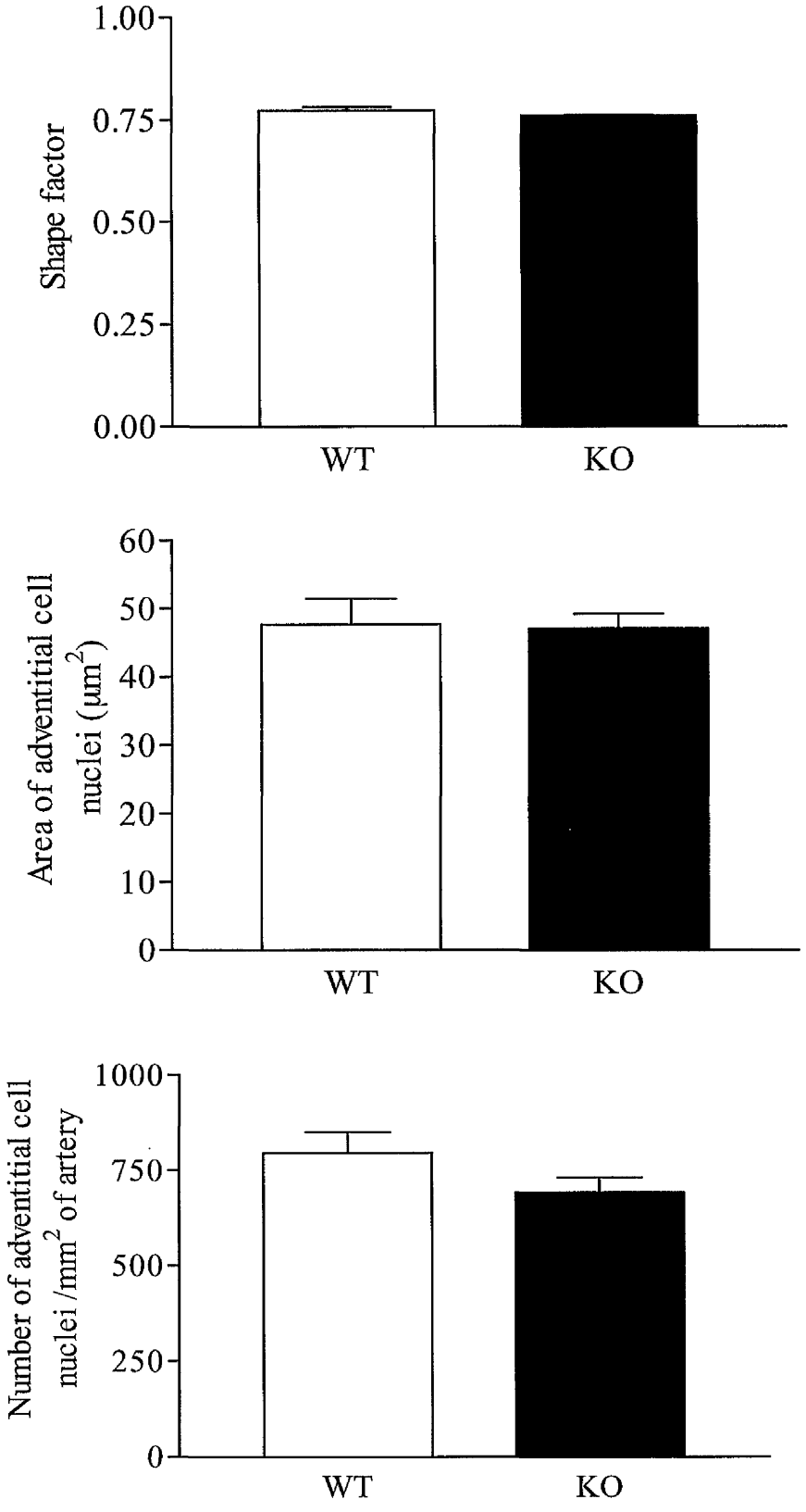


Figure 4.3:- Shape factor (top) and area (middle) of individual adventitial cell nuclei from WT and KO murine carotid arteries along with the number of adventitial cell nuclei per mm^2 of artery (bottom). A shape factor of 0 represents a straight line and a shape factor of 1 represents a perfect circle. Data is presented as mean ± s.e.m and n=7 for WT and KO. All arteries were pressurised and fixed at 120mmHg, stained with propidium iodide and imaged using a CLSM.

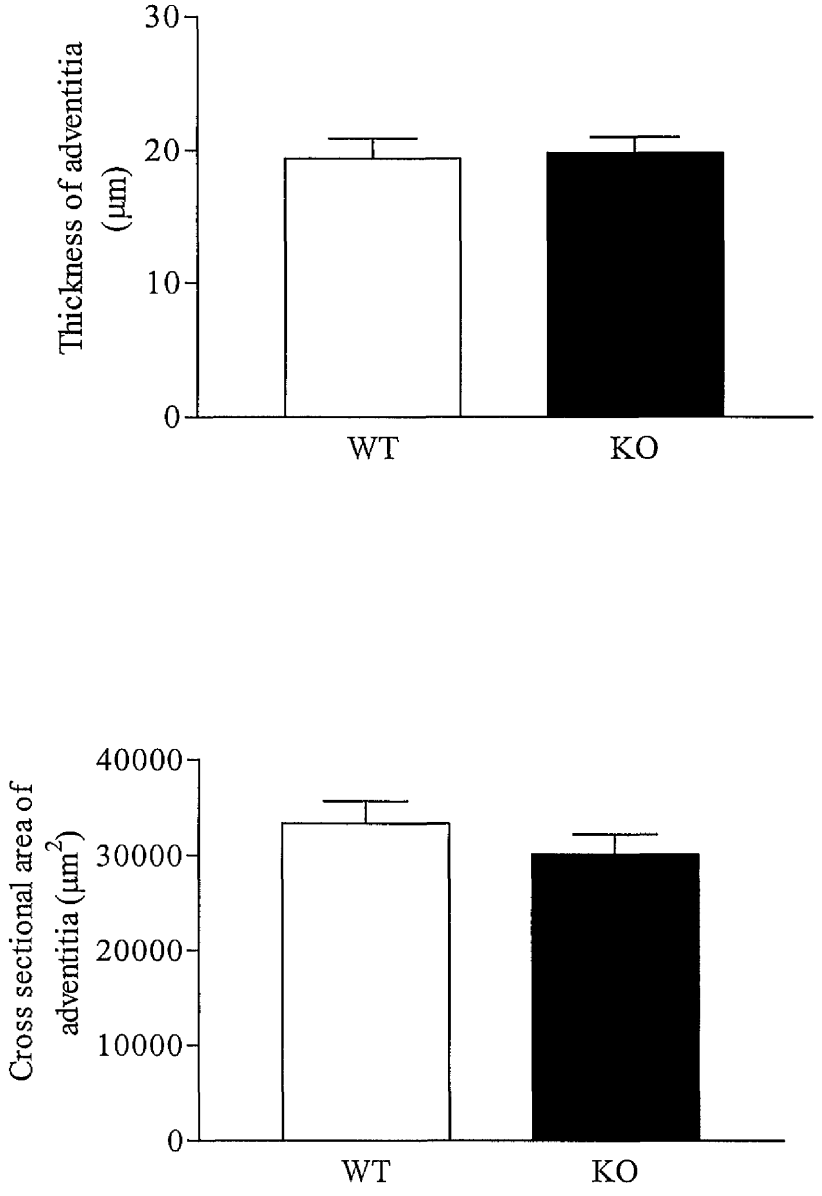


Figure 4.4:- Thickness (top) and cross sectional area of the adventitia (bottom) from WT and KO murine carotid arteries. All arteries were pressurised and fixed at 120mmHg, stained with propidium iodide and imaged using a CLSM. Data is presented as mean ± s.e.m for the number of arteries indicated in brackets. For WT n=6 and for KO n=7

diameter of the KO carotid artery compared with the WT, when measured using CLSM. The cross sectional area of the WT adventitia was 33,309±2345μm^2 while the KO adventitia had a cross sectional area of 30,093±2087μm^2 (Figure 4.4).

The density of adventitial nuclei in a 1mm^3 volume of adventitia was calculated. Although there was no difference in the thickness or cross sectional area of the adventitia between WT and KO carotid arteries there was a significant difference in the density of adventitial nuclei ($p<0.05$). The KO carotid artery was found to have a smaller density of adventitial nuclei (1.05±0.05 x10^5 adventitial nuclei/mm^3) in comparison to the WT carotid artery which was found to have1.24±0.09 x10^5 adventitial nuclei/mm^3 (Figure 4.5). The density of nuclei in the KO adventitia is 85% of the density of nuclei in WT adventitia.

The number of adventitial nuclei in a 1mm length of artery was also found to be significantly different between WT and KO arteries following a similar pattern to the density. As with the density of adventitial nuclei the actual number of nuclei in the KO adventitia was 85% of that in the WT. The number of nuclei was calculated as 3782±144 in the WT adventitia and 3213±191 in the KO adventitia ($p<0.05$) (Figure 4.5).

Figure 4.6 shows the data from both protocols in tabular form and Figures 4.7-4.12 are representative images of the adventitia, media and intima taken from WT and KO carotid arteries.

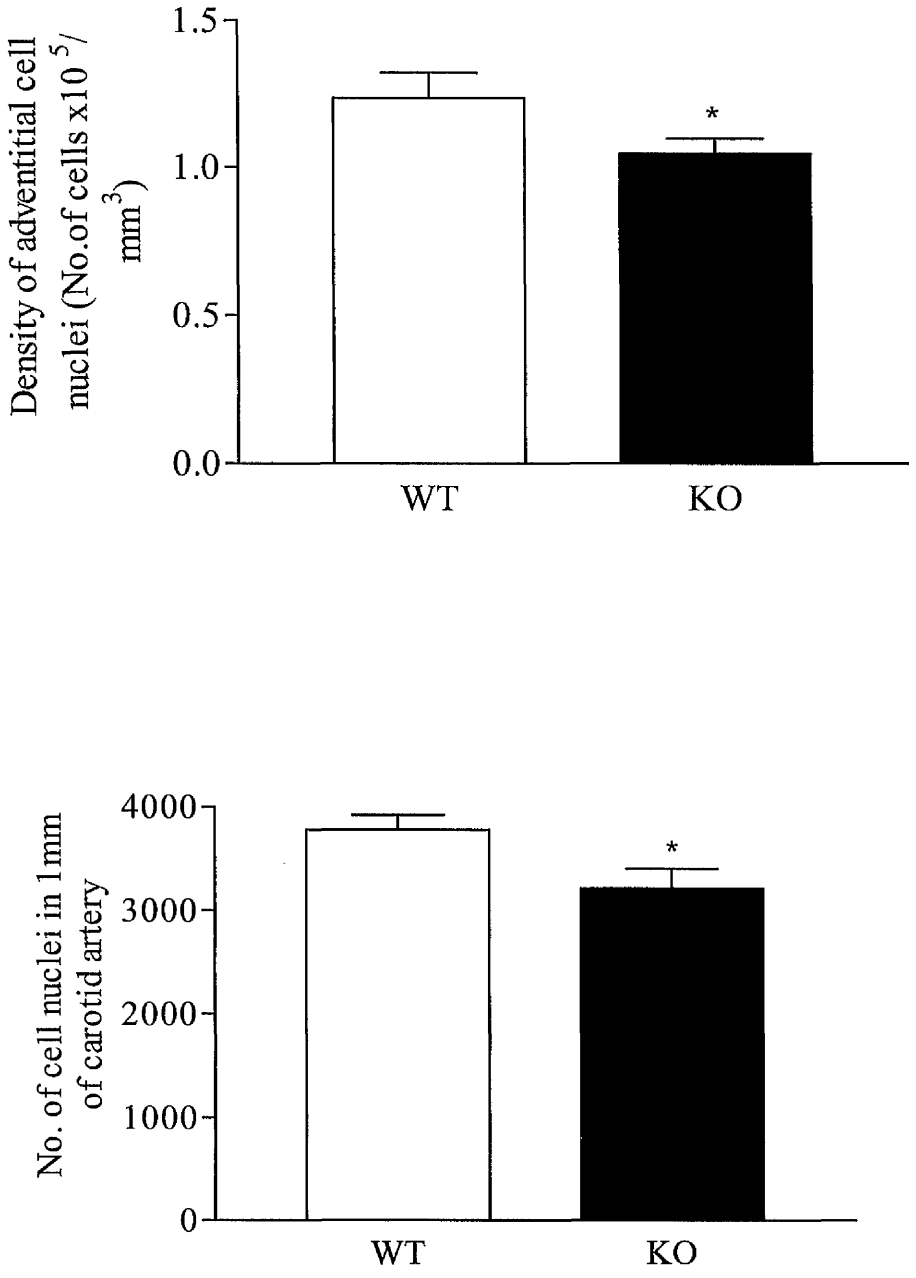


Figure 4.5:-Mean density of adventitial cell nuclei (top) and number of cell nuclei in a 1mm length of WT and KO murine carotid arteries (bottom). All arteries were pressurised and fixed at 120mmHg, stained with propidium iodide and imaged using a CLSM. Data is presented as mean ± s.e.m for the number of arteries indicated in brackets. For WT n=6 and for KO n=7. * indicates $p<0.05$.

	WT CAROTID ARTERY	KO CAROTID ARTERY	RATIO OF KO AS A % OF WT
VDA External Diameter (μm)	541.8±23	555.5±8.6	102.5
CLSM External Diameter (μm)	575±7.8	513.8±14.3*#	89
Ratio of VDA as a % of CLSM	*94*	*93*	*NA*
Shape Factor	0.77±0.009	0.76±0.004	99
Area of Adv. Cell Nuclei (μm^2)	48.2±4.4	45.7±1.8	95
No. Adv. Cell nuclei/ mm^2	796±54	718±42	90
Adv. Thickness (μm)	**19.4±1.5**	**19.8±1.2**	**102**
Cross sectional area (μm^2)	**33,309±2345**	**30,093±2087**	**90**
Density of Adv. Cell Nuclei x10^5/mm^3	**1.24±0.09**	**1.05±0.05***	**85**
No. of cells in 1mm of artery	**3782±144**	**3213±191***	**85**

Figure 4.6:- Tabular representation of structural data obtained from WT and KO carotid arteries using a VDA and a CLSM. Data is presented as mean ± s.e.m. Data in bold print was obtained from second protocol, all other data was obtained from first protocol. * indicates $p<0.05$ between WT and KO carotid arteries. # indicates $p<0.05$ between KO VDA external diameter and KO CLSM external diameter. NA = not applicable.

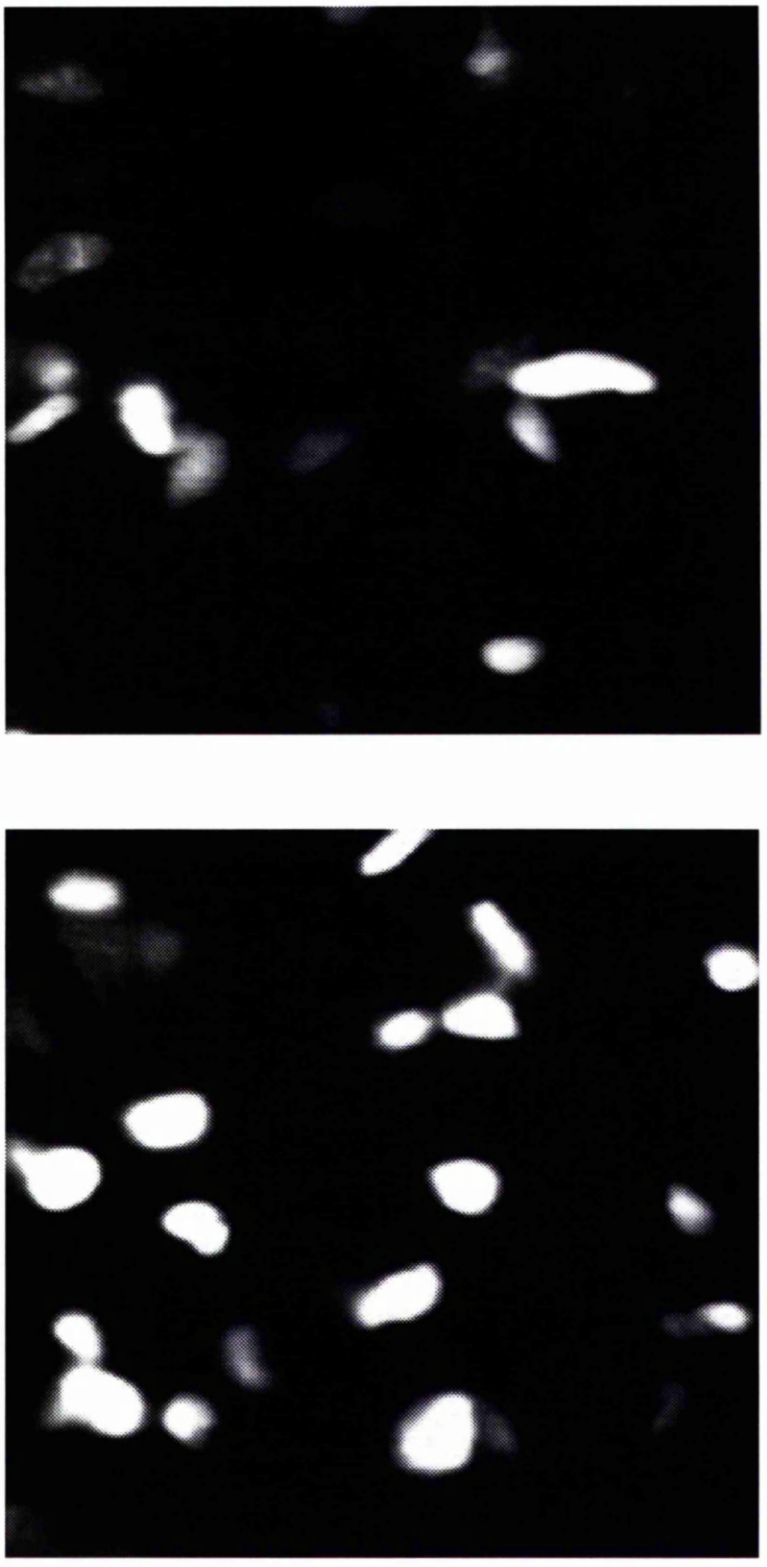


Figure 4.7:- Adventitial cell nuclei from WT murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Each image was captured from a different vessel - continued over the page.

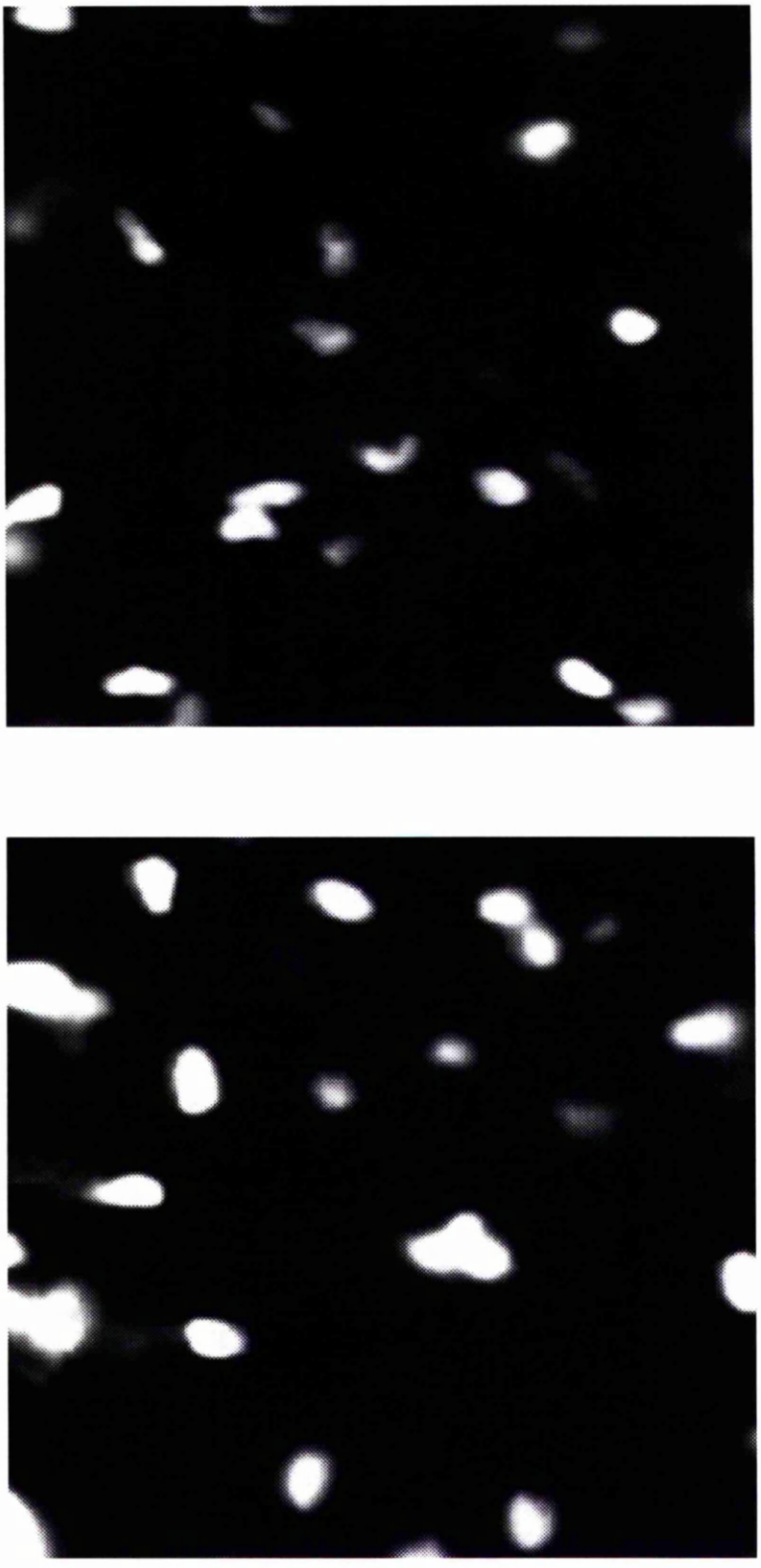


Figure 4.7 continued:- Adventitial cell nuclei from WT murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification.

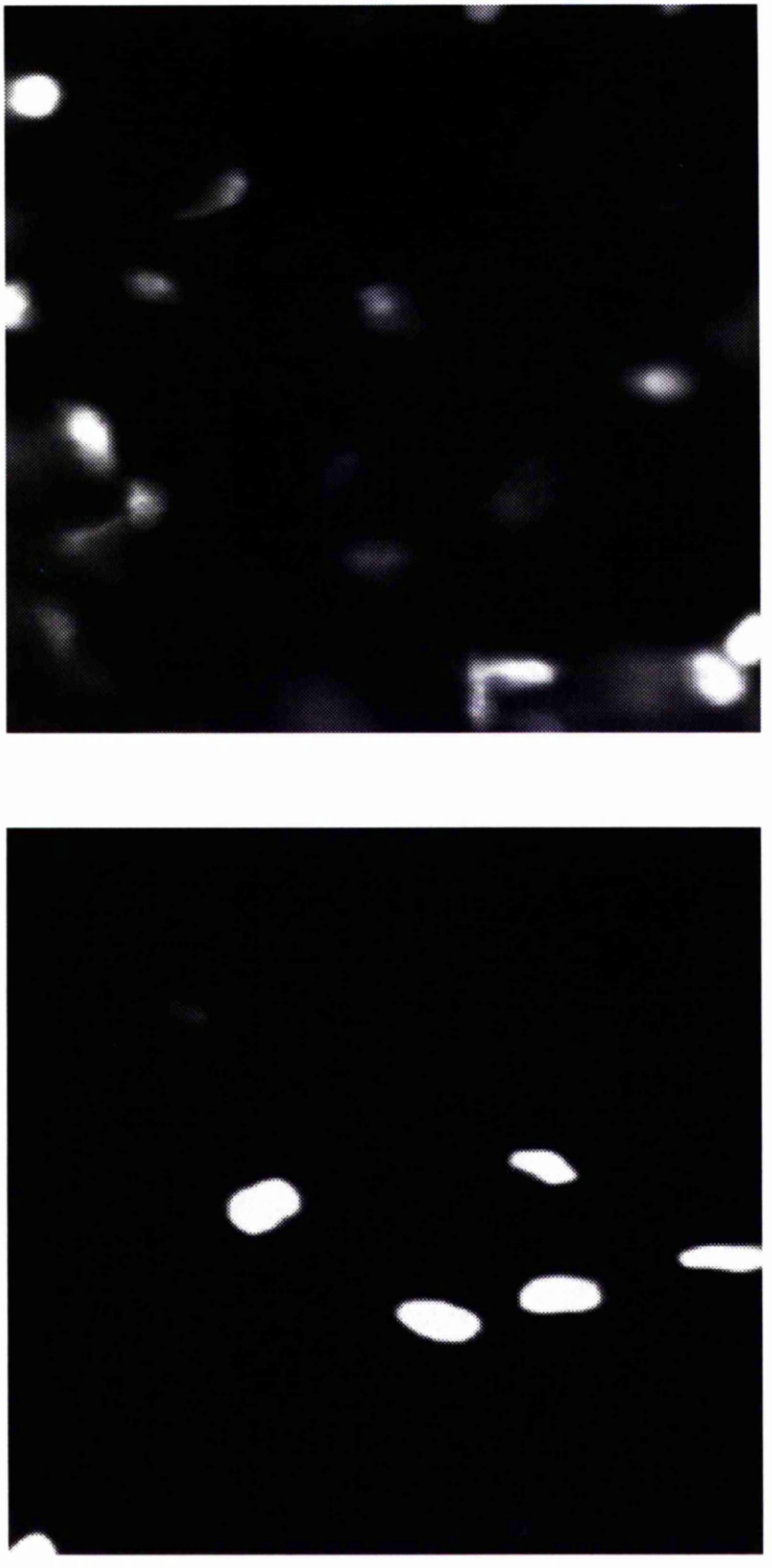


Figure 4.8:- Adentitial cell nuclei from KO murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Each image was captured from a different vessel - continued over the page.

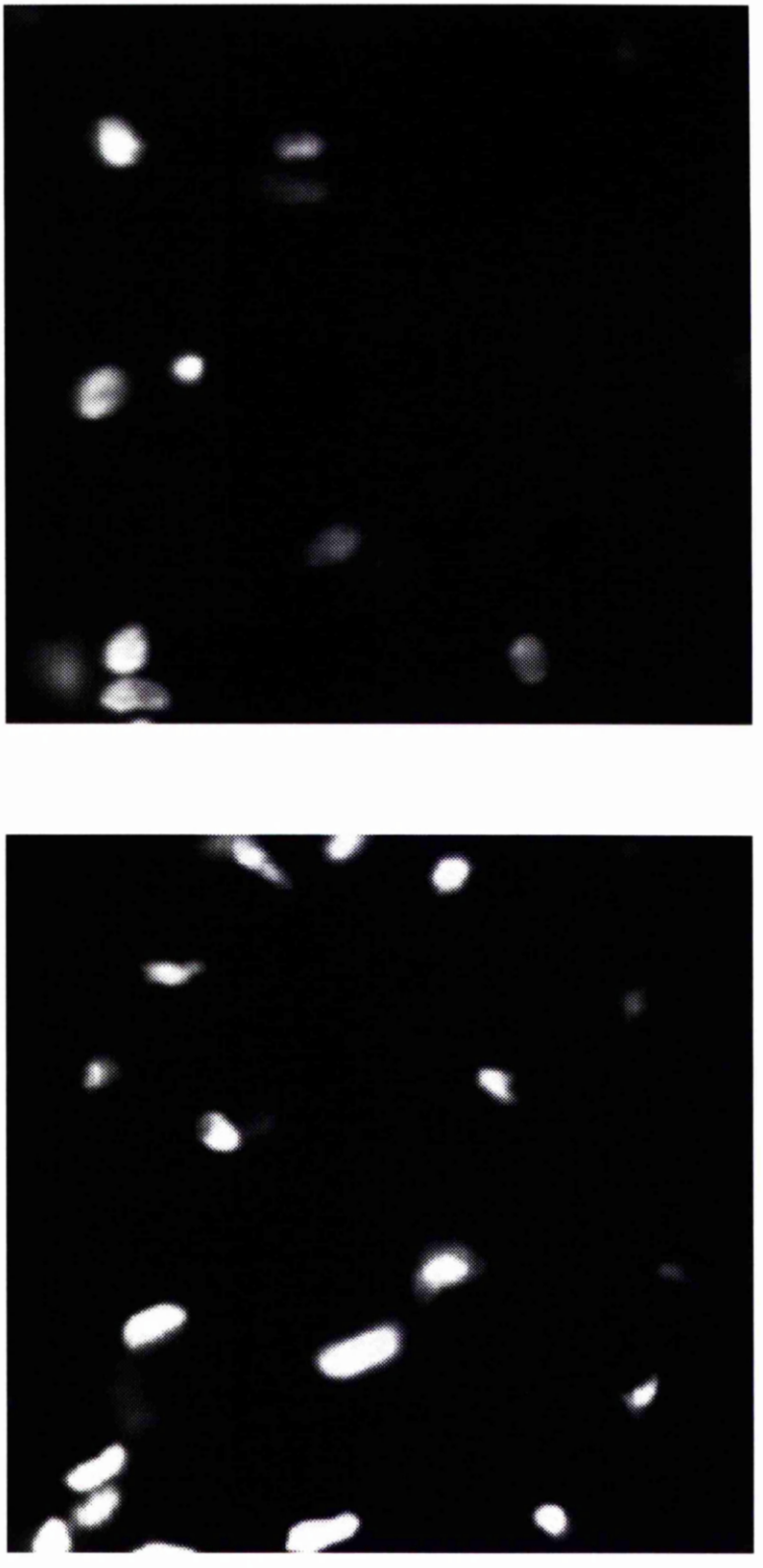


Figure 4.8 continued:- Adventitial cell nuclei from KO murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification.

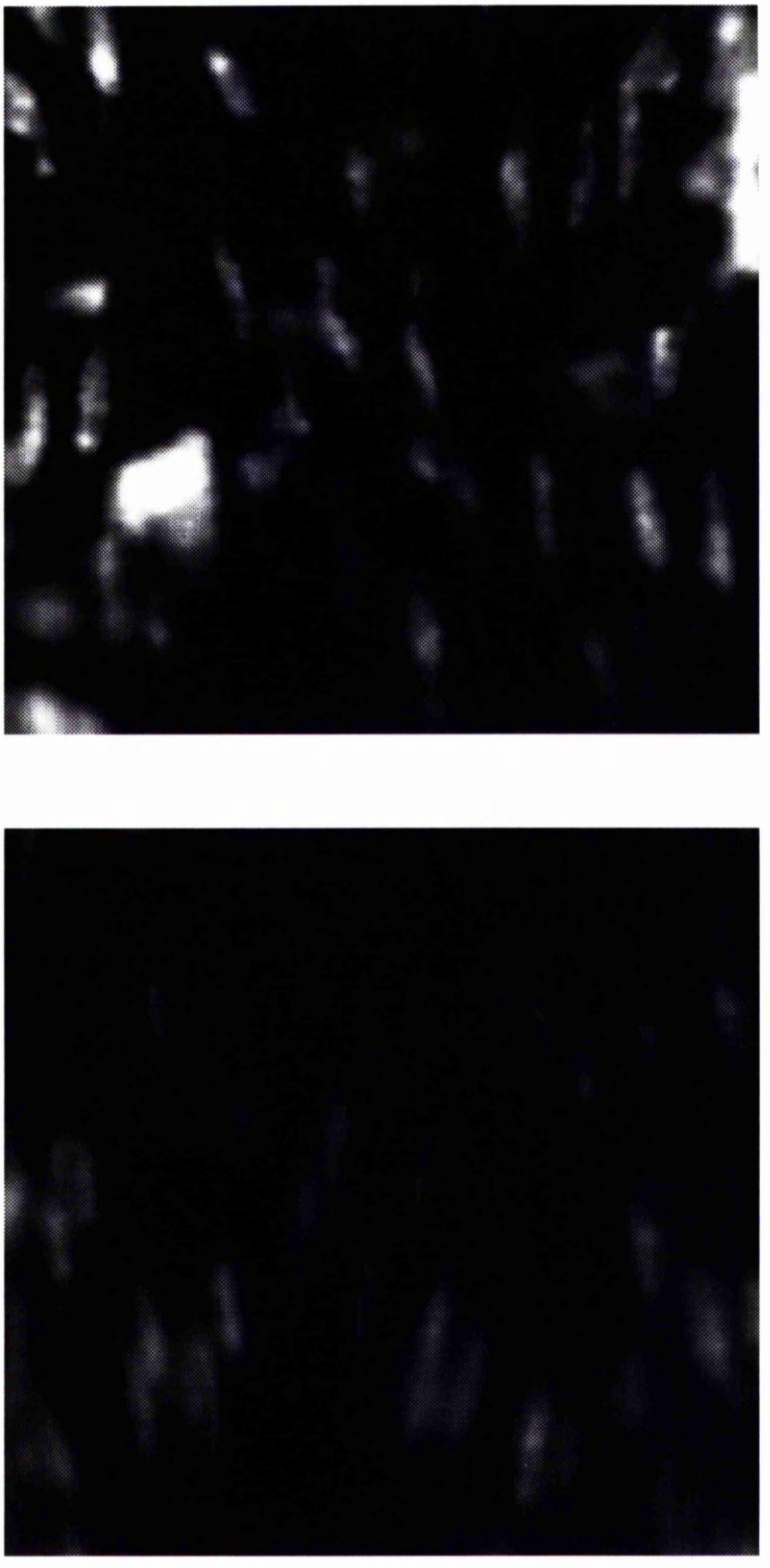


Figure 4.9:- Smooth muscle cell nuclei from WT murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Each image was captured from a different vessel - continued over the page.

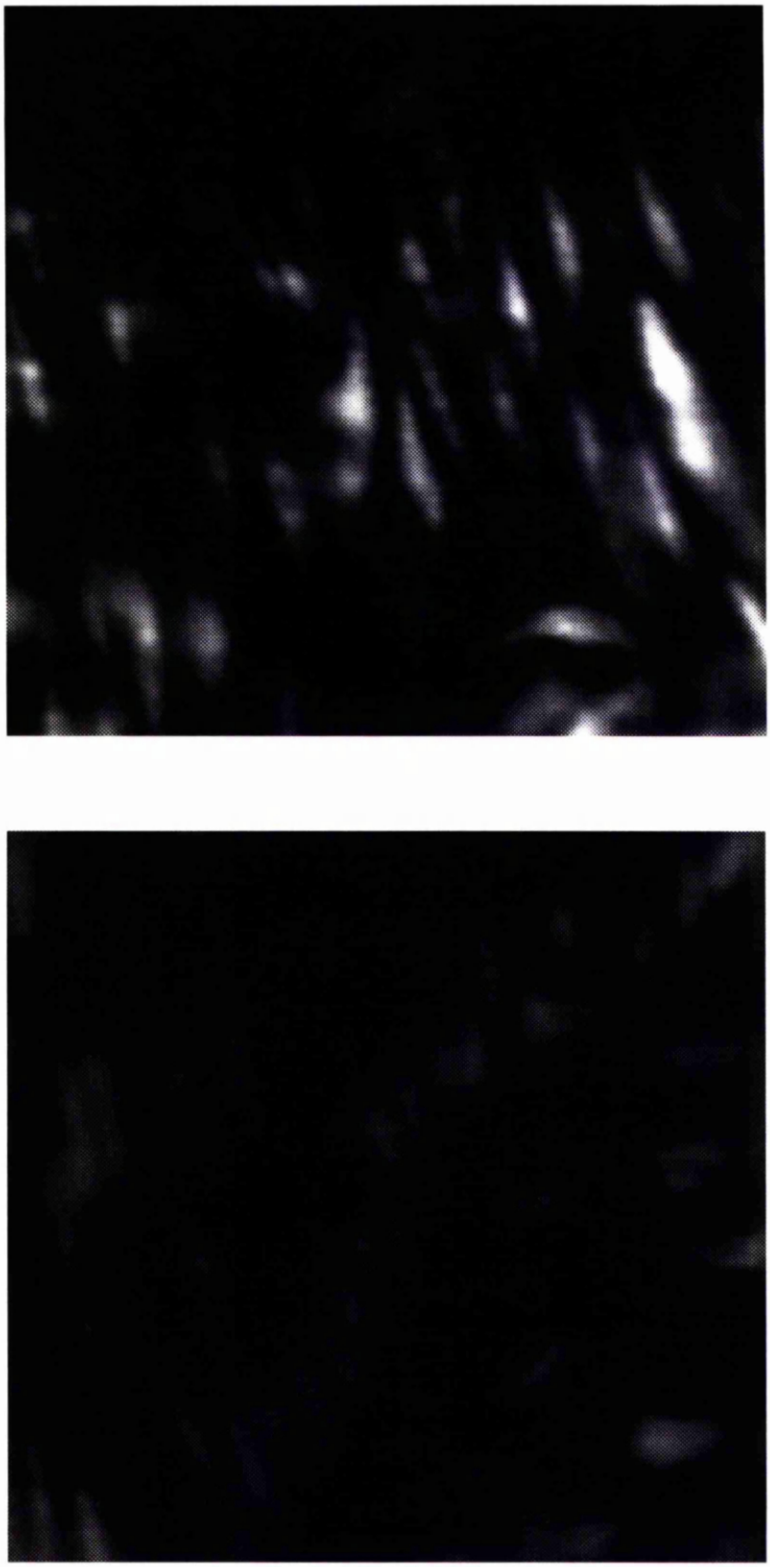


Figure 4.9 continued:- Smooth muscle cell nuclei from WT murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Some adventitial cell nuclei are visible.

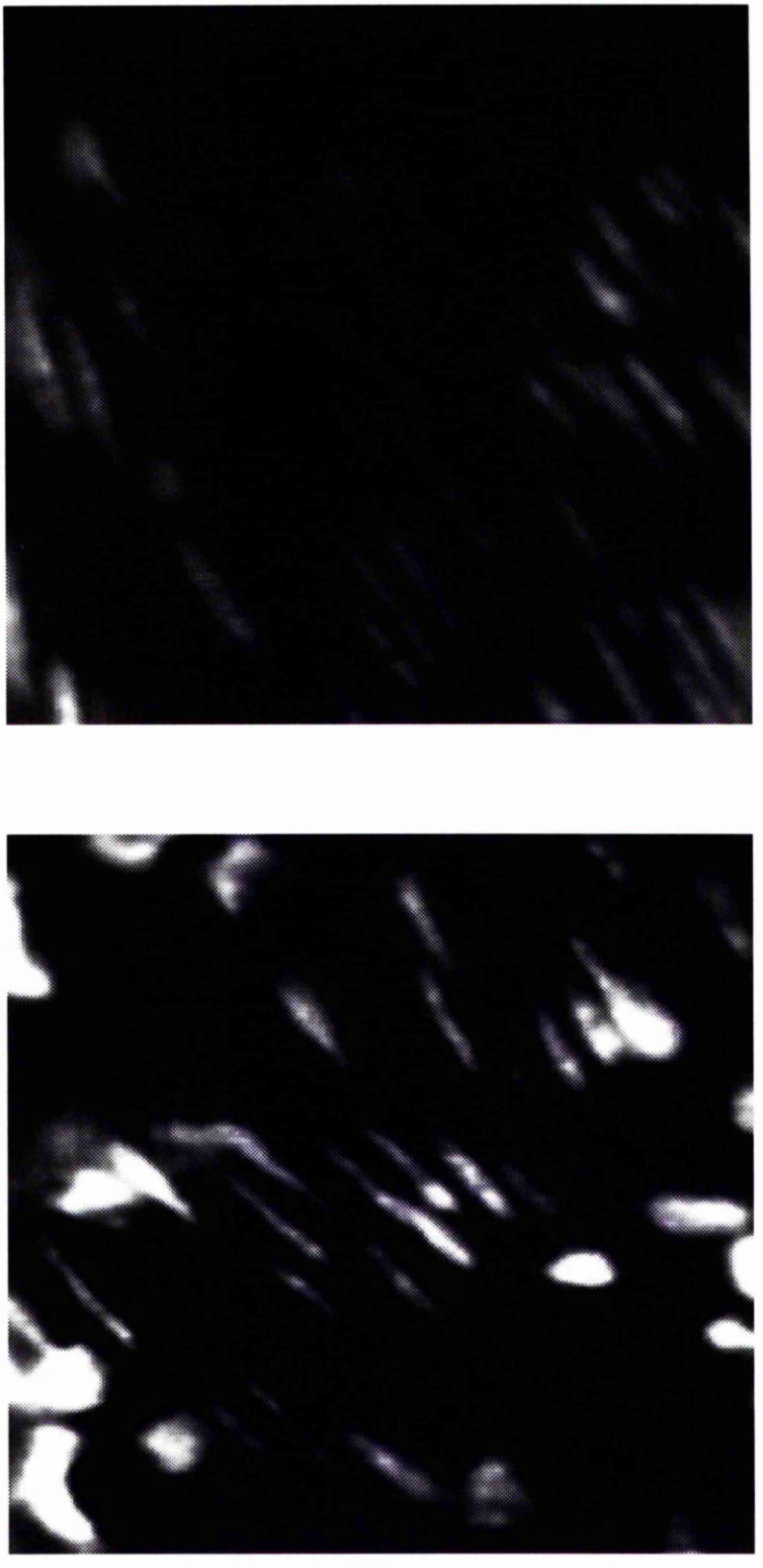


Figure 4.10:- Smooth muscle cell nuclei from KO murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Some adventitial cell nuclei are visible. Each image was captured from a different vessel - continued over the page.

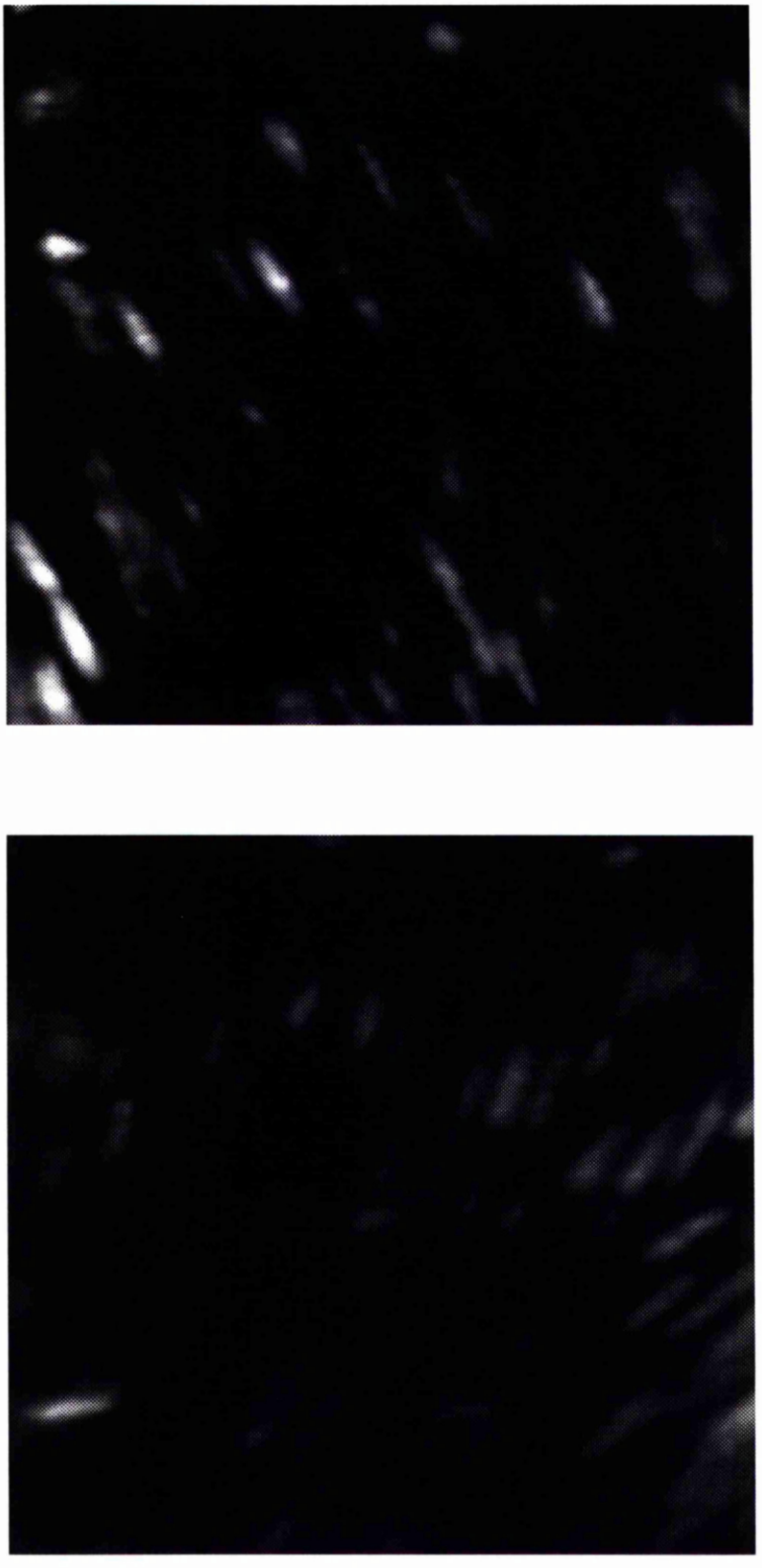


Figure 4.10 continued:- Smooth muscle cell nuclei from KO murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification.

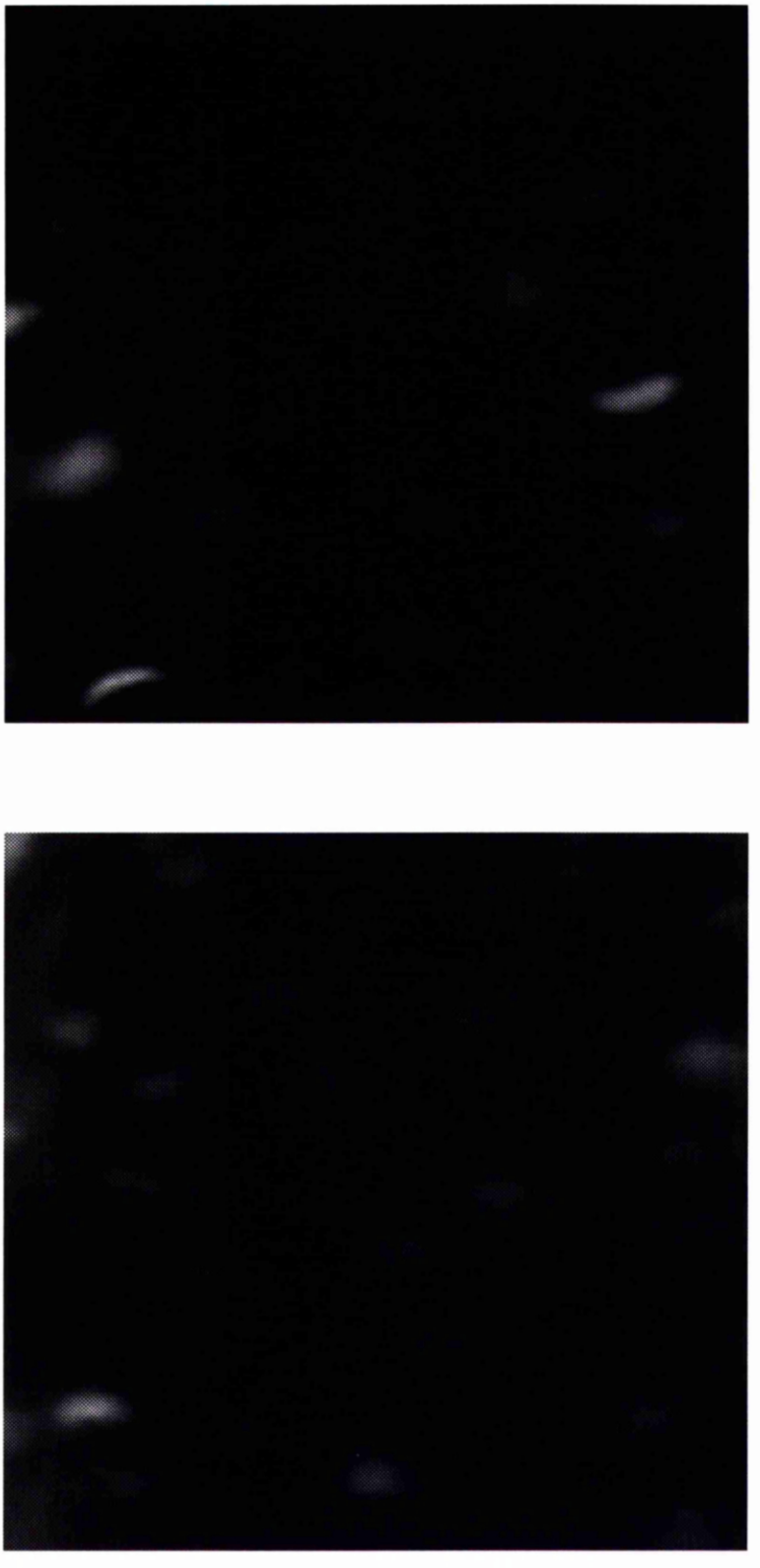


Figure 4.11:- Endothelial cell nuclei from WT murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Each image was captured from a different vessel.

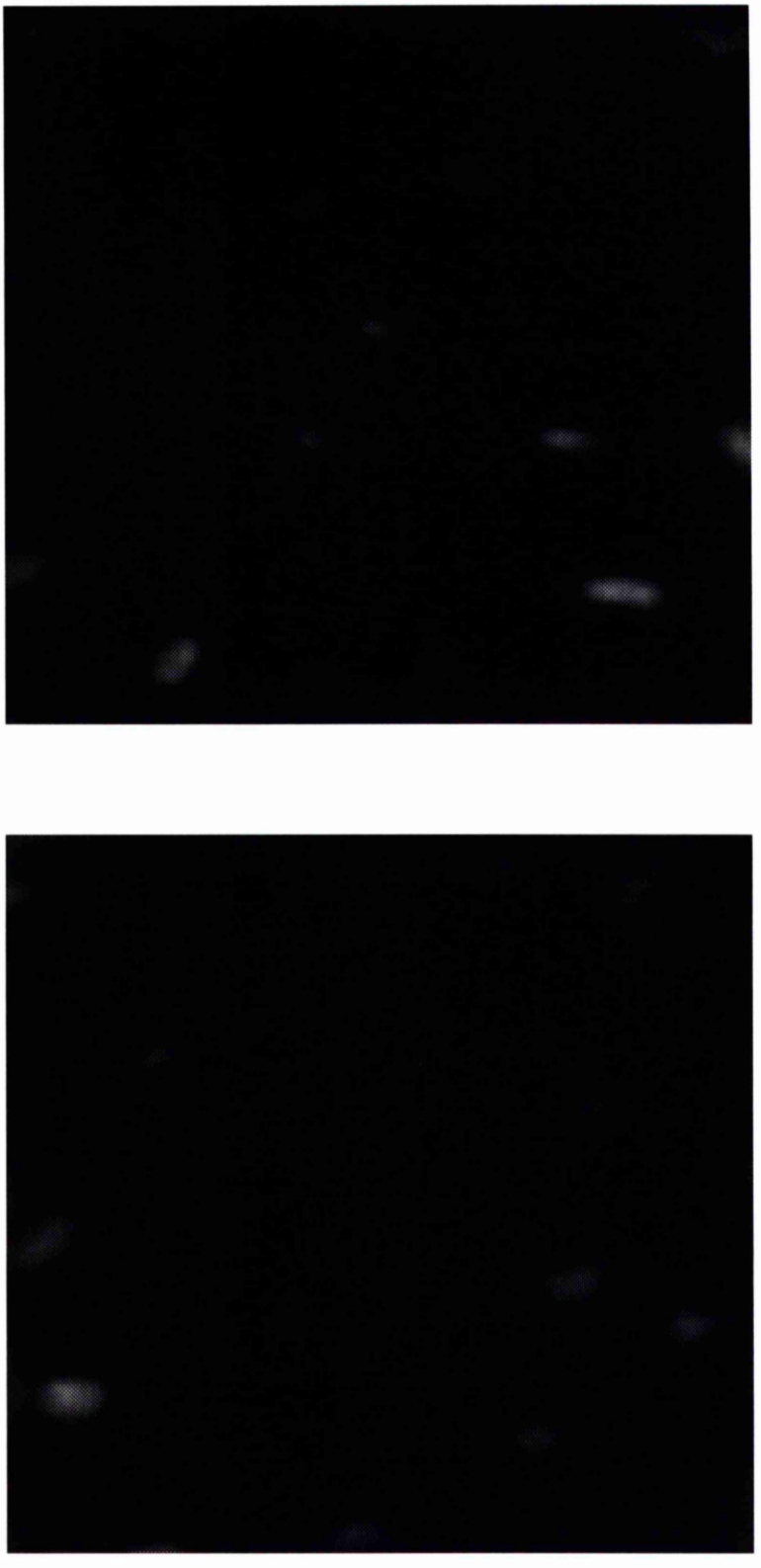


Figure 4.12:- Endothelial cell nuclei from KO murine carotid artery, stained with propidium iodide and imaged using CLSM at x40 magnification. Each image was captured from a different vessel.

4.4. DISCUSSION

The carotid artery of the mouse, despite being a major conduit vessel and one of the largest vessels found in the mouse vasculature, is relatively small compared to its equivalent in the rat. The external diameter of the rat carotid artery is approximately 950μm in WKY rats (Marano *et al*, 1999) whereas in the mouse this is approximately 550μm. The two methods used to measure the external diameter of the mouse carotid artery gave slightly different dimensions and one method, CLSM, revealed a difference between WT and KO. This difference (~10%) was substantial (WT = 575±7.8μm, KO = 513.8±14.3μm). No differences were observed in the weights of WT and KO mice used throughout this project excluding a simple proportionate difference based on the size of the mice. The basis for the difference between the two measurement techniques was that the WT dimensions were similar using both methods but the KO dimensions were smaller using the CLSM. Video dimension analyser measurement is made prior to fixation so the difference with CLSM may be a result of shrinkage during fixation. If so, it is not obvious why this should occur in the KO carotid artery but not in the WT. This may point to a structural difference between the two arteries. Another explanation for the differences observed using these two methods is that the video dimension analyser introduces more experimental error than the CLSM. Whilst both methods rely on the experimenter to determine the boundaries of the vessel wall the CLSM allows visualisation of the artery in more detail than the video dimension analyser making it easier to define the edge of the vessel. Therefore, the external diameters of WT and KO carotid arteries as measured using CLSM may be a truer representation of the actual diameter of these vessels.

The smaller external diameter of the KO carotid artery does not seem to be a consequence of the structural differences in the adventitia of this artery (discussed in detail below) which may indicate that there are also structural differences in the smooth muscle and endothelial layers of the artery. Due to the practical limitations of using CLSM to visualise the carotid artery, the images obtained of the smooth muscle and endothelial cell nuclei were not of a sufficient quality to allow any analysis to be carried out.

The Institute of Biophotonics within the Strathclyde Institute of Biomedical Science at Strathclyde University has developed lasers for dual photon confocal microscopy. This technique can produce images of better quality than those obtained using single photon confocal microscopy. Two photons of double the usual excitation wavelength, arriving close in time, excite the fluorophore. Since this occurs only at the point of focus, less unintended excitation occurs outside the plane of focus, making the image sharper. This is particularly suitable for visualisation of thick specimens such as intact blood vessels. I am hopeful that I will be able to get access to this microscope to allow me to visualise the WT and KO carotid arteries in more detail than was possible using the CLSM. Using this microscope I should be able to visualise the smooth muscle and endothelial layers of the vessels and so determine whether or not there are structural differences in these layers of the KO carotid artery, as well as in the adventitia.

The carotid arteries from WT and KO mice have been characterised pharmacologically in the previous chapter and were found to mediate contraction predominantly through the α_{1D}-adrenoceptor, although there was some evidence that the α_{1B}-adrenoceptor contributed to the contraction in the WT carotid artery. The α_{1B}-adrenoceptor has been

implicated in the regulation of growth of vascular smooth muscle cells (Chen *et al*, 1995) and in the trophic effects in the adventitia following balloon injury of the rat aorta (Zhang and Faber, 2001). In addition, preliminary findings from our own laboratory indicate a smaller density of adventitial cell nuclei in KO mouse tail arteries when compared with WT arteries, which may result from the deletion of the α_{1B}-adrenoceptor (Daly, unpublished observations). As a consequence of these studies I was interested in studying the adventitia of the carotid artery, to determine if deletion of the α_{1B}-adrenoceptor had any effects on the adventitia of KO carotid arteries.

Two protocols were used to investigate different aspects of the adventitia. The first protocol provided me with information on the shape factor and area of individual adventitial cell nuclei, as well as an estimate of the number of nuclei per mm^2 of vessel. The second protocol allowed me to measure the thickness of the adventitial layer and to calculate the cross sectional area of the adventitia, the density of adventitial cell nuclei per mm^3 and the number of adventitial cell nuclei in a 1mm length of artery.

The data obtained using the first protocol showed no significant differences in the shape, area or number of adventitial cell nuclei between WT and KO carotid arteries. However, a number of limitations exist with this protocol. A consequence of taking a series of images at one focal plane along the length of the vessel is that a percentage of the nuclei will not be completely within the focal plane. Alternatively two nuclei from different focal planes may overlap and appear as one nuclei, therefore a decision had to be made about whether or not to include these nuclei in the image analysis. To decide which nuclei were included and which were excluded, limits were set on the size and shape that a nuclei had to be if it was to be included in the image analysis. Therefore,

nuclei that were too small were those that were not sufficiently in the focal plane and those which were too large were classed as more than one nuclei and these were excluded from the analysis. This results in a skewed data analysis but was felt to be necessary if any meaningful information was to arise from this protocol.

The second protocol provided me with information on the thickness of the adventitia and the density of adventitial cell nuclei. The thickness' of the adventitia of WT and KO carotid arteries were virtually identical, approximately 19μm. This is a similar adventitial thickness to that reported for the second order mesenteric arteries of the rat, which was found to be approximately 15μm in WKY rats and approximately 21μm in SHRSP rats (Arribas *et al*, 1997). This is interesting given the different physiological roles that these vessels have and that these rat mesenteric vessels are smaller (external diameter ~ 250-300μm) than the mouse carotid artery. This may suggest that the composition of the adventitia is similar regardless of the size and physiological role of the individual vessels.

The density of adventitial cell nuclei and the number of cell nuclei in 1mm of vessel were found to be significantly smaller in KO carotid arteries compared with WT arteries. The difference between WT and KO vessels for these two parameters was similar in that the values in the KO artery were 85% of those in the WT. The smaller density that was observed in this study is similar to findings in the KO tail artery where the density of adventitial cell nuclei was also found to be smaller (Daly, unpublished observations). Adventitial fibroblasts from rat aorta and carotid artery have recently been shown to express the mRNA for all three α_1-adrenoceptor subtypes (Faber *et al*, 2001). At the protein level (as assessed by radioligand binding) the dominant subtypes

expressed on the adventitial fibroblasts from these vessels appear to be the α_{1A}- and α_{1B}-adrenoceptors (Faber *et al*, 2001). The functional significance of α_1-adrenoceptors being expressed on adventitial fibroblasts is at present unknown but stimulation with noradrenaline has been shown to induce proliferation and protein synthesis (Faber *et al*, 2001), similar to the effects observed when smooth muscle cells are stimulated with noradrenaline (Xin *et al*, 1997). This has led to suggestions that the α_1-adrenoceptors on adventitial fibroblasts may signal a trophic action of catecholamines on vascular wall growth and remodelling. The trophic effect of noradrenaline on the adventitia of the balloon injured rat aorta has been shown to be inhibited by the α_{1B}-adrenoceptor antagonist, AH11110A, suggesting that this action is mediated by the α_{1B}-adrenoceptor. α_{1A}- and α_{1D}-adrenoceptor antagonists had no effect (Zhang and Faber, 2001).

Although the presence of α_1-adrenoceptor subtypes on the adventitia of mouse blood vessels has yet to be demonstrated, it is possible that they too express all three α_1-adrenoceptor subtypes. If the α_{1B}-adrenoceptor's role is to mediate vascular growth, as has been suggested (Zhang and Faber, 2001), then it seems plausible that the decrease in the density and actual number of adventitial cell nuclei in the KO carotid artery is a direct result of deletion of the α_{1B}-adrenoceptor from this tissue. Although the KO carotid artery has a significantly smaller external diameter when measured using a CLSM this does not explain the smaller density of adventitial cell nuclei or the smaller number of cell nuclei. There is no difference in the cross sectional areas of WT and KO adventitia therefore there is a smaller number and density of cell nuclei in the KO carotid artery in the same volume of tissue as there is in the WT. A reduction in the number of cells might have been an adaptation to a smaller circumference but the reduced density reported here suggests a change beyond this.

Additional studies would be required to uncover the mechanism by which deletion of the α_{1B}-adrenoceptor results in a smaller density of adventitial cell nuclei. In the first instance the presence of α_1-adrenoceptors on adventitial cells should be investigated. This could be achieved by using a fluorescent analogue of prazosin, QAPB, which our laboratory has worked with for a number of years, in combination with confocal microscopy. If α_1-adrenoceptors are present on adventitial cells then QAPB will fluoresce when bound to these receptors allowing visualisation and localisation of the receptors. If experiments such as these provided evidence for α_1-adrenoceptors on the adventitial cells of mouse blood vessels a further question might be "do these receptors in any way contribute to modulation of contraction in the artery?" Taking the carotid artery as an example, if the adventitia of the WT carotid artery contains an α_{1B}-adrenoceptor population, it is possible that it could be these α_{1B}-adrenoceptors which are influencing the α_1-adrenoceptor pharmacology in functional studies, possibly through the release of a mediator. Therefore, would removal of the adventitia result in the WT having the same pharmacology as the KO carotid artery i.e. contraction mediated solely through the α_{1D}-adrenoceptor? There is some evidence to suggest that the adventitia can influence contraction. When the adventitia was removed from the rat carotid artery the response to noradrenaline was reduced, although it remains to be determined whether removal of the adventitia directly or indirectly affected contraction (Gonzalez *et al*, 2001). Alternatively the α_{1B}-adrenoceptors could be present on the smooth muscle cells, from where they exert an influence over the adventitia. Overall it seems more likely that α_{1B}-adrenoceptors will be found to be present on both the adventitia and the smooth muscle as Faber *et al* (2001) have reported for the rat aorta and carotid artery.

The adventitia has traditionally been regarded as nothing more than a structural support for the media. However as the evidence mounts, it seems that the adventitia has a more direct influence on vascular activity than it has been credited with. In addition to α_1-adrenoceptors the adventitia has also been shown to express α_{2D}-adrenoceptors (Faber *et al*, 2001) and has been shown to be capable of releasing nitric oxide in amounts comparable to that released from the endothelium, in response to endotoxin (Zhang *et al*, 1999; Kleschyov *et al*, 2000). Transfection of the endothelial nitric oxide synthase gene into the adventitia of blood vessels has been shown to improve relaxation of atherosclerotic arteries (Ooboshi *et al*, 1998) and to restore relaxation of arteries which have had their endothelium removed (Tsutsui *et al*, 1998). This ability of the adventitia to release nitric oxide and the success of adventitial transfections may have clinical implications for pathological conditions such as atherosclerosis where relaxation of blood vessels is impaired. The adventitia is also proving to be important in models of neointimal lesion formation caused by procedures such as angioplasty where studies have shown that one of the first stages in this process is proliferation of adventitial cells, with some groups also showing that adventitial cells migrate to the site of the neointima (Wilcox *et al*, 1996; Shi *et al*, 1996; Li *et al*, 2000). Studies such as these have, in the past, focused on the endothelium, but the emphasis is now shifting towards the adventitia as its importance in the pathology of these conditions is uncovered. Like the endothelium, which was once thought to be merely a barrier between the blood and blood vessels, it appears that the adventitia is directly involved in vascular biology and its importance in physiological and pathophysiological functions is certain to become a subject of great interest to researchers in the future.

CHAPTER 5 – GENERAL DISCUSSION

5. GENERAL DISCUSSION

Although pharmacology alone is not sufficient to clearly and confidently identify and subtype α_1-adrenoceptors, when it is combined with transgenic technologies the process can be made easier. Transgenic mice have been used in pharmacological research for a number of years (Plump *et al*, 1992; Rohrer and Kobilka, 1998) but it was only in 1997 that a transgenic mouse was designed which did not express the α_{1B}-adrenoceptor (Cavalli *et al*, 1997). Although transgenic mice had been produced previous to this, which either overexpressed the α_{1B}-adrenoceptor (Ahkter *et al*, 1997) or expressed a constitutively active α_{1B}-adrenoceptor (Milano *et al*, 1994), the expression of these receptors was generally confined to the myocardium. The mouse designed by Cavalli *et al* was novel in that the α_{1B}-adrenoceptor was not expressed in any of the tissues of the mouse.

Much of the confusion that arises when attempting to functionally define α_1-adrenoceptor subtypes is a result of there being no satisfactory drug to identify the α_{1B}-adrenoceptor. Its presence in native tissues has often been suggested purely on the basis of eliminating the other α_1-adrenoceptor subtypes from being present rather than a positive identification of the α_{1B}-adrenoceptor. (Testa *et al*, 1995c; Van der Graaf *et al*, 1996a; Smith *et al*, 1997). The α_{1B}-adrenoceptor KO mouse provides a unique opportunity in which to study the functional roles of the α_1-adrenoceptor subtypes, which provides, for the first time, an environment in which the functional presence of the α_{1B}-adrenoceptor can be discounted.

A novel α_1-adrenoceptor antagonist proposed to be selective for the α_{1B}-adrenoceptor (Patane *et al*, 1998) became available to me throughout the course of my project and it was hoped that this drug would succeed where many before have failed, in that it would be selective for the α_{1B}-adrenoceptor. The binding studies carried out using L765,314 by Patane *et al* (1998) looked promising. At α_{1b}-adrenoceptors L765,314 had 10-fold higher affinity than the α_{1d}-adrenoceptor and 100-fold higher affinity for the α_{1a}-adrenoceptor. L765,314 also appeared to be selective for native α_{1B}-adrenoceptors showing 35-65 fold differences in affinity at the α_{1B}-adrenoceptors in rat liver and spleen compared to the α_{1A}-adrenoceptors of rat and human prostate. However, my own experience of using this drug is that the selectivity claimed by Patane *et al* (1998) simply does not exist. The tissue in which I expected a significant difference in the pK_i values with this drug was in the WT and KO liver. Unfortunately, L765,314 was found to bind with similar affinity in both the WT and KO liver, similar affinities were also obtained in WT and KO brain tissue. This poor performance was witnessed once again in the functional studies in the WT and KO carotid artery. L765,314 does seem to be capable of binding to α_1-adrenoceptors with a high affinity but it fails to discriminate between subtypes. This is a similar story for many of the 'α_{1B}-adrenoceptor antagonists' discovered to date whereby the original paper shows evidence that the drug is selective for the $\alpha_{1B/b}$-adrenoceptor but future studies fail to repeat this.

Preliminary studies carried out by Cavalli *et al* (1997) investigating the functional consequences of deleting the α_{1B}-adrenoceptor revealed that the α_{1B}-adrenoceptor was absent from all the tissues in which it was believed to be expressed. In those tissues where the α_{1B}-adrenoceptor was believed to be expressed in conjunction with another subtype i.e. the brain, the α_1-adrenoceptor number was reduced whilst the liver, which

has been proposed by Cavalli *et al* (1997) and Yang *et al* (1998) to possess a pure population of α_{1B}-adrenoceptors, was found to have virtually no α_1-adrenoceptors in the KO. The binding studies in the brain presented in chapter 2 are in agreement with those of Cavalli *et al*. A reduced receptor number was reported in the KO brain compared with the WT and subtyping studies revealed that the WT brain contained a mixture of α_{1A}- and α_{1B}-adrenoceptors in a ratio of 36%: 64%, similar to the ratios reported by Cavalli *et al* (1997) and Yang *et al* (1998). The KO brain contained a pure population of α_{1A}-adrenoceptors.

The phenotypic changes associated with the loss of the α_{1B}-adrenoceptor from the brain were not investigated in this project. However, with the recent results of Zuscik *et al* (2000) demonstrating the neurodegenerative effects of overexpression of the α_{1B}-adrenoceptor, it would definitely be worthwhile examining the brains of KO mice for any physical manifestations of deletion of the α_{1B}-adrenoceptor.

The binding studies in the KO liver, which are presented in this thesis and those reported by Cavalli *et al* (1997) are different. Cavalli *et al* (1997) found there to be no α_1-adrenoceptors present in the livers of KO mice whereas my own studies found that there were α_1-adrenoceptors expressed in the liver which were subsequently subtyped to be α_{1A}-adrenoceptors. These differing results appear to be due to methodological differences that have been discussed in Chapter 2, Section 2.4.2.

The data obtained from the KO liver suggests that the mouse liver is capable of upregulating and expressing an alternative α_1-adrenoceptor subtype if the normal α_1-adrenoceptor subtype fails to function.

The liver is a unique organ. It is well known it has a massive regenerative potential. In fact, repopulation studies investigating the regeneration of the liver and replication of hepatocytes have discovered that the hepatocyte has a proliferative potential that may even exceed that of stem cells in haemopoietic tissues (Fausto, 1999). Another unusual characteristic of the liver, that has been well documented for the rat liver, is that there is an age-related changeover in the adrenoceptor that controls glucose metabolism. In the immature animal, glucose mobilisation is mediated through β_2-adrenoceptors whilst the mature animal mediates glucose metabolism through α_1-adrenoceptors (Rossby and Cornett, 1991; Thai *et al*, 1996). While it is possible that the mouse liver responds in a similar manner during maturation this has yet to be confirmed and would require further studies. However, my own studies show that not only is the liver able to change the α_1-adrenoceptor subtype it expresses but it seems that the murine liver does not require an α_1-adrenergic component in the first few months of life for it to function effectively; at 3 months of age there is a negligible amount of α_1-adrenoceptor protein in the KO liver. Does this mean that the β-adrenergic component is present in the mouse liver until 3 months of age? Or is this a consequence of the deletion of the α_{1B}-adrenoceptor? In rats, the density of β-adrenoceptors had fallen to a low level by postnatal day 6 (Rossby and Cornett, 1991). If this phenomenon is also observed in mice does the deletion of the α_{1B}-adrenoceptor delay this changeover from β to α_1?

The liver is a useful tissue in which to study α_1-adrenoceptors because it is one of the few tissues that appears to possess a homogeneous population of α_1-adrenoceptors and in the light of the findings in the KO liver may prove to be a valuable tissue in which to investigate the regulation of expression of α_1-adrenoceptor subtypes.

However most tissues, in particular blood vessels, express multiple subtypes, the functional significance of which remains unknown. In addition, there is a lot of confusion surrounding the identity of the subtypes which are expressed in blood vessels whereby different groups identify different subtypes as being functionally present in the same vessel. The use of a transgenic mouse, such as the α_{1B}-adrenoceptor KO mouse provides a means by which at least one subtype can be eliminated from the equation before any experiments are carried out and simplifies the daunting task of determining the subtypes responsible for contraction in blood vessels.

The pharmacological analysis of the α_1-adrenoceptor mediating contraction in the WT carotid artery was made easier because the experiments could be run in parallel with the KO carotid artery and comparisons made between the two arteries. The overall pharmacology of the two arteries was broadly similar. However, the KO carotid artery was found to have an increased sensitivity to phenylephrine, while the WT carotid artery appeared to express a heterogeneous population of α_1-adrenoceptors, producing shallow Schild slopes with L765,314 and BMY7378. The pharmacology of the KO carotid artery suggested that contraction was mediated by a pure population of α_{1D} adrenoceptors whereas the WT carotid artery appeared to mediate contraction predominantly through α_{1D}-adrenoceptors with a component mediated by the α_{1B}-adrenoceptor. The loss of the α_{1B}-adrenoceptor from the KO carotid artery did not alter the pharmacology of this artery substantially but eliminated the heterogeneity alluded to by the shallow Schild slopes in the WT carotid artery and resulted in an increase in the apparent affinity of BMY7378 in the KO carotid artery.

The functional role of the α_{1B}-adrenoceptor in the WT carotid artery appears to be minor, which corresponds with popular opinion at this time that the primary role of the α_{1B}-adrenoceptor is not to mediate contraction of blood vessels. The sensitivity of phenylephrine in mice that either overexpress the α_{1B}-adrenoceptor or have a constitutively active α_{1B}-adrenoceptor provides further evidence that the α_{1B}-adrenoceptor's contribution to contraction in blood vessels is minor. Mesenteric arteries from these mice were found to display no differences in sensitivity or size of response to phenylephrine providing no evidence for a role of the α_{1B}-adrenoceptor in these vessels. Cavalli *et al* (1997) proposed a role for the α_{1B}-adrenoceptor in the maintenance of blood pressure when they found that the KO mouse had a decreased pressor response to phenylephrine and a decreased response to phenylephrine in the mouse aorta *in vitro*. This was not reflected in the observations from mesenteric arteries, which might have been expected to reflect the *in vivo* pressor response. Further work is required to explain this discrepancy between *in vivo* and *in vitro* observations.

As the evidence increases it seems that the α_{1B}-adrenoceptor's role, rather than being one of contraction in blood vessels, may be in an involvement in the growth response of arteries. Chen *et al* (1995) demonstrated an α_{1B}-like adrenoceptor mediated hypertrophy in rat aortic smooth muscle cells while Zuscik *et al* (2001) reported that mice which overexpressed the α_{1B}-adrenoceptor showed early signs of cardiac hypertrophy. Zhang and Faber (2001) found that the trophic effects on the adventitia caused by balloon-injury of the rat aorta could be inhibited by an α_{1B}-adrenoceptor antagonist. Faber *et al* (2001) have also recently reported that the adventitial fibroblasts, as well as the smooth muscle cells, of the rat aorta express α_1-adrenoceptor subtypes and, when stimulated

with noradrenaline, were found to mediate proliferation, although the identity of the subtype was not determined.

The density and number of adventitial cell nuclei in the KO carotid artery were found to be significantly smaller when compared with the WT carotid artery. The mechanism underlying this reduction remains to be determined. However, the discovery that α_1-adrenoceptors are expressed on adventitial cells (Faber *et al*, 2001) raises the possibility that the decrease in the density of cells may be directly related to the deletion of the α_{1B}-adrenoceptor from adventitial cells. The presence of α_1-adrenoceptors on the adventitia of mouse blood vessels has not been confirmed but would seem likely given that they are expressed on the adventitia of rat blood vessels. Further studies are required to determine the mechanism through which the adventitia of the KO carotid artery has a smaller cell density and number. It would be of interest to examine the adventitia of carotid arteries from the mice that overexpress the α_{1B}-adrenoceptor and those that express a constitutively active α_{1B}-adrenoceptor to determine the density of adventitial cell nuclei in each of these arteries. Gonzalez *et al* (2001) describe a method to remove the adventitia from blood vessels which leaves the smooth muscle intact and functional. Using this method they reported that removal of the adventitia altered the pharmacology of the rat carotid artery. This technique could be applied to the carotid arteries of WT and KO mice to determine if removal of the adventitia affected the pharmacology of these vessels.

Our knowledge and understanding of the physiology and pharmacology of α_1-adrenoceptors has advanced considerably since the initial discovery of subtypes over 15 years ago. The discovery of the gene products for three α_1-adrenoceptor subtypes

confirmed what pharmacologists had been arguing about for years. Since then, a combination of molecular biology and pharmacology has brought us to a point at which it is now agreed that at least three subtypes of α_1-adrenoceptor exist and much of their biochemistry and pharmacology has been defined. However, the gap in our functional knowledge of these subtypes still remains, particularly with regard to the α_{1B}-adrenoceptor. This project, while far from filling the gap, has gone some way to advancing our understanding of the α_{1B}-adrenoceptor. I have demonstrated that the α_{1B}-adrenoceptor plays a minor but definite role in the contractile response of the murine carotid artery. This project also shows that the α_{1B}-adrenoceptor may have an influence on the density and number of adventitial cells in the WT carotid artery, which may have implications for the proposed role of the α_{1B}-adrenoceptor in the growth and development of blood vessels. Additionally, I have shown that the α_{1B}-adrenoceptor, although the α_1-adrenoceptor normally expressed in the liver at 4 months, is not indispensable to the mouse. If conditions are such that the α_{1B}-adrenoceptor is not expressed the liver is capable of upregulating another α_1-adrenoceptor subtype, presumably to compensate. This observation raises many questions. For example, is the liver the only organ capable of this upregulation or do all tissues have this ability? Would such a mechanism be present in tissues that express multiple subtypes? What are the factors which switch this upregulation off or on? There is evidence for an age-dependent change in the functional expression of α_1-adrenoceptor subtypes in blood vessels. Gurdal *et al* (1995) reported that expression of the α_{1A}-subtype increased with age in the rat aorta whereas the expression of the α_{1B}-subtype decreased while the α_{1D}-adrenoceptor was unchanged. In contrast to the findings of Gurdal *et al* (1995), an investigation into the alteration of α_1-adrenoceptor subtypes in the rat vasculature with

age found that the contribution of the α_{1D}-adrenoceptor increased with age (Ibarra *et al*, 1997). Therefore, it appears that blood vessels are also capable of altering the expression of α_1-adrenoceptor subtypes with age. It would be of interest to investigate this phenomenon in the WT and KO carotid artery at different age points to determine the effect of age on the expression of α_1-adrenoceptor subtypes.

Many fundamental questions have been given a new direction by the present observations and time will tell of the relevance of these observations to the physiological role of α_1-adrenoceptor subtypes. Studies such as this demonstrate the potential that transgenic animals provide to further the knowledge and understanding we have gained from more traditional pharmacological methods. I hope I have shown that in a complicated area of pharmacological research, such as that of α_1-adrenoceptors, KO mice can simplify the situation to one that is manageable and can often end up taking us in an unexpected direction, in our attempts to understand this field of research. Pharmacogenomics looks set to be the way pharmacological research is undertaken in the 21st century where mice such as the α_{1B}-KO and the recently developed α_{1D}-KO and $\alpha_{1B/C}$ KO's will bring us to an understanding of α_1-adrenoceptors that would have never been possible through pharmacology alone.

REFERENCES

6. REFERENCES

Aboud, R., Shafi, M. and Docherty, J. R. (1993) Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Brit. J. Pharmacol.* **109**, 80-87.

Ahlquist, R. P. (1948) A study of the adrenotropic receptors. *Am. J. Physiol.* **153**, 586-600.

Akhter, S. A., Milano, C. A., Shotwell, K. F., Cho, M-C., Rockman, H. A., Lefkowitz, R. J. and Koch, W. J. (1997) Transgenic mice with cardiac overexpression of α_{1B}-adrenergic receptors. *J. Biol. Chem.* **272(34)**, 21253-21259.

Argyle, S. A. and M^{c}Grath, J. C. (2000) An $\alpha_{1A/1L}$-adrenoceptor mediates contraction of canine subcutaneous resistance arteries. *J. Pharmacol. Exp. Ther.* **295**, 627-633.

Arribas, S. M., Hillier, C., Gonzalez, C., M^{c}Grory, S., Dominiczak, A. F. and M^{c}Grath, J. C. (1997) Cellular aspects of vascular remodelling in hypertension revealed by confocal microscopy. *Hypertension.* **30**, 1455-1464.

Arunlakshana, O. and Schild, H. O. (1959) Some quantitative uses of drug antagonists. *Brit. J. Pharmacol.* **14**, 48-58.

Barker, S. G. E., Tilling, L. C., Miller, G. C., Beesley, J. E., Fleetwood, G., Stavri, G. T., Baskerville, P. A. and Martin, J. F. (1994) The adventitia and atherogenesis: removal initiates intimal proliferation in the rabbit which regresses on generation of a 'neoadventitia'. *Atherosclerosis* **105**, 131-144.

Beeler, J. F. and Cooper, R. H. (1995) Regulation of hepatocyte plasma membrane α_1-adrenergic receptors by 4β-phorbol 12-myristate 13-acetate. *Biochem. J.* **305**, 73-79.

Berthelsen, S. and Pettinger, W. A. (1977) A functional basis for classification of α-adrenergic receptors. *Life Sciences.* **21**, 595-606.

Black, J. L., French, R. J. and Mylecharane, E. J. (1981) Receptor mechanisms for 5-hydroxytryptamine in rabbit arteries. *Brit. J. Pharmacol.* **74**, 619-626.

Bockaert, J., Brand, C. and Journot, L. (1997) Do recombinant receptor assays provide affinity and potency estimates. *Receptor Classification – Ann. N.Y. Acad. Sci.* **812**, 55-70.

Boulanger, C. M., Caputo, L. and Levy, B. I. (1995) Endothelial AT(1)-mediated release of nitric oxide decreases angiotensin –II contractions in rat carotid artery. *Hypertension.* **26(5)**, 752-757.

Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G. and Lefkowitz, R. F. (1987) Regulation of adrenergic receptor function by phosphorylation. II Effects of agonist occupancy on phosphorylation of α_1- and β_2-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **262 (7)**, 3106-3113.

Brown, G. L. and Gillespie, J. S. (1957) The output of sympathetic transmitter from the spleen of the cat. *J. Physiol.* **138**, 81-102.

Burch, R. M., Luini, A., Mais, D. E., Corda, D., Vanderhoek, J. Y., Kohn, L. D. and Axelrod, J. (1986) α_1-Adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. *J. Biol. Chem.* **261(24)**, 11236-11241.

Burt, R. P., Chapple, C. R. and Marshall, I. (1995) Evidence for a functional α_{1A}-(α_{1C}-) adrenoceptor mediating contraction of the rat epididymal vas deferens and an α_{1B}-adrenoceptor mediating contraction of the rat spleen. *Brit. J. Pharmacol.* **115(5)**, 467-475.

Buscher, R., Heeks, C., Taguchi, K. and Michel, M. C. (1996) Comparison of guinea-pig, bovine and rat α_1-adrenoceptor subtypes. *Brit. J. Pharmacol.* **117**, 703-711.

Bylund, D. B., Eikenberg, D.C., Hieble, J.P., Langer, S. Z., Lefkowitz, R. J., Minneman, K. P., Mollinof, P. B., Ruffolo, R. R. Jr and Trendelenburg, U. (1994) International Union of Pharmacology: nomenclature of adrenoceptors. *Pharmacol. Reviews*. **46** (2), 121-136.

Cannon, W. B. and Rosenbleuth, A (1933) Studies on conditions of activity in endocrine organs. xxix. Sympathin E and Sympathin I. *Am. J. Physiol.* **104**, 557-574.

Caputo, L., Benessiano, J., Boulanger, C. M. and Levy, B. I. (1995) Angiotensin-II increases cGMP content via endothelial angiotensin-II AT1 subtype receptors in the rat carotid artery. *Arterio. Thromb. Vasc. Biol.* **15(10)**, 1646-1651.

Cash, R., Ruberg, M., Raisman, R. and Agid, Y. (1984) Adrenergic receptors in Parkinson's disease. *Brain Research* **322**, 269-275.

Cavalli, A., Fanelli, F., Taddei, C., De Benedetti, P. and Cotecchia, S. (1996) Amino acids of the α_{1B}-adrenergic receptor involved in agonist binding: differences in docking catecholamines to receptor subtypes. *FEBS Letters.* **399**, 9-13.

Cavalli, A. Lattion, A-L., Hummler, E., Nenniger, M., Pedrazzini, T., Aubert, J-F., Michel, M. C., Yang, M., Lembo, G., Vecchione, C., Mostardinin, M., Scmidt, A., Beermann, F. and Cotecchia, S. (1997) Decreased blood pressure response in mice deficient of the α_{1b}-adrenergic receptor. *Proc. Natl. Acad. Sci. USA.* **94**, 11589-11594.

Chang, D. J., Chang, T. K., Yamanishi, S. S., Salazar, F. H. R., Kosaka, A. H., Khare, R., Bhakta, S., Jasper, J. R., Shieh, I-S., Lesnick, J. D., Ford, A. P. D. W., Daniels, D. V., Eglen, R. M., Clarke, D. E., Bach, C. and Chan, H. W. (1998) Molecular cloning, characterization and expression of novel human α_{1A}-adrenoceptor isoforms. *FEBS Letters.* **422**, 279-283.

Chataigneau, T., Feletou, M., Huang, P. L., Fishman, M. C., Duhault, J. and Vanhoutte, P. M. (1999) Acetylcholine-induced relaxation in blood vessels from enothelial nitric oxide synthase knockout mice. *Brit. J. Pharmacol.* **126**, 219-226.

Chen, A. F. Y., Jiang, S-W., Crotty, T. B., Tsutsui, M., Smith, L. A., O'Brien, T. and Katusic, Z. S. (1997) Effects of *in vivo* adventitial expression of recombinant endothelial nitric oxide synthase gene in cerebral arteries. *Proc. Natl. Acad. Sci. USA.* **94**, 12568-12573.

Chen, L., Xin, X., Eckhart, A. D., Yang, N. and Faber, J. E. (1995) Regulation of vascular smooth muscle growth by α_1-adrenoceptor subtypes *in vitro* and *in situ. J. Biol. Chem.* **270(52)**, 30980-30988.

Cheng, Y-C. and Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099-3108.

Chuang, T. T., Iacovelli, L., Sallese, M. and De Blasi, A. (1996) G-protein coupled receptors: heterologous regulation of homologous desensitization and its implications. *TIPS.* **17**, 416-421.

Coge, F., Guenin, S-P., Renouard-Try, A., Rique, H., Ouvry, C., Fabry, N., Beauverger, P., Nicolas, J-P., Galizzi J-P., Boutin, J. A. and Canet, E. (1999) Truncated isoforms inhibit [^{3}H]-prazosin binding and cellular trafficking of native human α_{1A}-adrenoceptors. *Biochem. J.* **343**, 231-239.

Corvera, S. and Garcia-Sainz, J. A. (1984) Phorbol esters inhibit α_1-adrenergic stimulation of glycogenolysis in isolated rat hepatocytes. *Biochem. Biophys. Res. Comm.* **119(3)**, 1128-1133.

Cotecchia, S., Schwinn, D. A., Randall, R. R., Lefkowitz, R. J., Caron, M. G. and Kobilka, B. K. (1988) Molecular cloning and expression of the cDNA for the hamster α_1-adrenergic receptor. *Proc. Natl. Acad. Sci. USA.* **85**, 7159-7163.

Cotecchia, S., Exum, S., Caron, M. G. and Lefkowitz, R. J. (1990) Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc. Natl. Acad. Sci. USA.* **87**, 2896-2900.

Cowlen, M. S. and Toews, M. L. (1988) Evidence for α_1-adrenergic receptor internalization in DDT_1 MF-2 cells following exposure to agonists plus protein kinase C activators. *Mol. Pharmacol.* **34**, 340-346.

Daniel, E. E., Brown, R. D., Wang, Y. F., Low, A. M., Lu-Chao, H. and Kwan, C-Y. (1999) α-Adrenoceptors in canine mesenteric artery are predominantly 1A subtype: pharmacological and immunochemical evidence. *J. Pharmacol. Exp. Ther.* **291(2)**, 671-679.

Daniels, D. V., Gever, J. R., Jasper, J. R., Kava, M. S., Lesnick, J. D., Meloy, T. D., Stepan, G., Williams, T. J., Clarke, D. E., Chang, D. J. and Ford, A. P. D. W. (1999) Human cloned α_{1A}-adrenoceptor isoforms display α_{1L}-adrenoceptor pharmacology in functional studies. *Eur. J. Pharmacol.* **370**, 337-343.

Deng, X. F., Chemtob, S. and Varma, D. R. (1996) Characterization of α_{1D}-adrenoceptor subtype in rat myocardium, aorta and other tissues. *Brit. J. Pharmacol.* **119**, 269-276.

Didion, S. P., Sigmund, C. D. and Faraci, F. M. (2000) Impaired endothelial function in transgenic mice expressing both human renin and human angiotensinogen. *Stroke.* **31**, 760-765.

Diviani, D., Lattion, A-L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L. and Cotecchia, S. (1996) Effect of different G-protein coupled receptor kinases on phosphorylation and desensitization of the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **271(9)**, 5049-5058.

Diviani, D., Lattion, A-L. and Cotecchia, S. (1997) Characterization of the phosphorylation sites involved in G-protein coupled receptor kinase-and protein kinase C-mediated desensitization of the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **272(45)**, 28712-28719.

Docherty, J. R. (1989) The pharmacology of α_1- and α_2-adrenoceptors: Evidence for and against a further subdivision. *Pharmacol. Ther.* **44**, 241-284.

Drew, G. M. (1985) What do antagonists tell us about α-adrenoceptors? *Clin. Sci.* **68 (Suppl 10)**, 15s-19s.

Eltze, M. (1996) Functional evidence for an α_{1B}-adrenoceptor mediating contraction of the mouse spleen. *Eur. J. Pharmacol.* **311**, 187-198.

Evans, W. H. (1990) Organelles and membranes of animal cells. Chapter 1 in Biological membranes: a practical approach. Edited by JBC Findlay and WH Evans. *Oxford University Press.*

Faber, J. E., Yang, N. and Xin, X. (2001) Expression of α-adrenoceptor subtypes by smooth muscle cells and adventitial fibroblasts in rat aorta and in cell culture. *J. Pharmacol. Exp. Ther.* **298**, 1-12.

Faggin, E., Puato, M., Zardo, L., Franch, R., Millino, C., Sarinella, F., Pauletto, P., Sartore, S. and Chiavegato, A. (1999) Smooth muscle-specific SM22 protein is expressed in the adventitial cells of balloon-injured rabbit carotid artery. *Arterioscler. Thromb. Vasc. Biol.* **19**, 1393-1404.

Faure, C., Pimoule, C., Arbilla, S., Langer, S. Z. and Graham, D. (1994) Expression of α_1-adrenoceptor subtypes in rat tissues: implications for α_1-adrenoceptor classification. *Eur. J. Pharmacol.* **268**, 141-149.

Faure, C., Gouhier, C., Langer, S. Z. and Graham, D. (1995) Quantification of α_1-adrenoceptor subtypes in human tissues by competitive RT-PCR analysis. *Biochem. Biophys. Res. Comm.* **213(3)**, 935-943.

Fausto, N. (1999) Mouse liver tumorigenesis: Models, mechanisms and relevance to human disease. *Seminars in Liver Disease.* **19(3)**, 243-252.

Flavahan, N. A. and Vanhoutte, P. M. (1986) α_1-Adrenoceptor subclassification in vascular smooth muscle. *TIPS.* **7(9)**, 347-349.

Fonseca, M. I., Button, D. C. and Brown, R. D. (1995) Agonist regulation of α_{1B}-adrenergic receptor subcellular distribution and function. *J. Biol. Chem.* **270(15)**, 8902-8909.

Ford, A. P. D. W., Daniels, D. V., Chang, D. J., Gever, J. R., Jasper, J. R., Lesnick, J. D. and Clarke, D. E. (1997) Pharmacological pleiotropism of the human recombinant α_{1A}-adrenoceptor: implications for α_1-adrenoceptor classification. *Brit. J. Pharmacol.* **121**, 1127-1135.

Forray, C., Bard, J. A., Wetzel, J. M., Chiu, G., Shapiro, E., Tang, R., Lepor, H., Hartig, P. R., Weinshank, R. L., Branchek, T. A. and Gluchowski, C. (1994) The α_1-adrenergic receptor that mediates smooth muscle contraction in human prostate has the pharmacological properties of the cloned human α_{1c}-subtype. *Mol. Pharmacol.* **45**, 703-708.

Fujimoto, S. and Itoh, T. (1995) Denopamine as an α_{1H}-adrenoceptor antagonist in isolated blood vessels. *Eur. J. Pharmacol.* **280(2)**, 143-147.

Furchgott, R. F. (1981) Adrenergic and dopaminergic peripheral receptors. *Proceedings of the 4th meeting on Adrenergic Mechanisms, Porto, Portugal.* 10-29 (Abstract)

Furchgott, R. F. and Zawadzki, J. V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 374-376.

Garcia-Sainz, J. A., Romera-Avila, M. T., Alcantara-Hernandez, R., Macias-Silva, M., Olivares-Reyes, A. and Gonzalez-Espinosa, C. (1992) Species heterogeneity of hepatic α_1-adrenoceptors: α_{1A}-, α_{1B}- and α_{1C}-subtypes. *Biochem. Biophys. Res. Comm.* **186(2)**, 760-767.

Garcia-Sainz, J. A., Casas-Gonzalez, P., Romero-Avila, M. T. and Gonzalez-Espinosa, C. (1994) Characterization of the hepatic α_{1B}-adrenoceptors of rats, mice and hamsters. *Life Sciences* **52(25)**, 1995-2003.

Garcia-Sainz, J. A., Romero-Avila, M. T. and Gonzalez-Espinosa, C. (1995a) Characterization of the α_1-adrenoceptors of the dog liver: predominance of the α_{1A}-subtype. *Eur. J. Pharmacol.* **272**, 139-143.

Garcia-Sainz, J. A., Romero-Avila, M. T. and Torres-Marquez, E. (1995b) Characterization of the human liver α_1-adrenoceptors: predominance of the α_{1A} subtype. *Eur. J. Pharmacol.* **289**, 81-86.

Garcia-Sainz, J. A., Romero-Avila, M. T. and Gonzalez-Espinosa, C. (1996a) Coexpression of α_{1A}- and α_{1B}-adrenoceptors in the liver of the rhesus monkey (*Macaca mulatta*). *Eur. J. Pharmacol.* **311**, 277-283.

Garcia-Sainz, J. A., Garcia-Caballero, A. and Gonzalez-Espinosa, C. (1996b) Characterization of the α_1-adrenoceptors of cat liver. Predominance of the α_{1A}-adrenergic subtype. *Life Sciences* **59(3)**, 235-242.

Garcia-Sainz, J. A., Vazquez-Prado, J. and Villalobos-Molina, R. (1999) α_1-Adrenoceptors: subtypes, signalling and roles in health and disease. *Arch. Med. Res.* **30**, 449-458.

Garcia-Sainz, J. A., Vazquez-Prado, J. and Del Carmen Medina, L. (2000) α_1-Adrenoceptors: function and phosphorylation. *Eur. J. Pharmacol.* **389**, 1-12.

Giardina, D., Crucianelli, M., Romanelli, R., Leonardi, A., Poggesi, E and Melchiorre C. (1996) Synthesis and biological profile of the enantiomers of [4-(4-amino-6,7-dimethoxyquinazolin-2-yl)-*cis*-octahydroquinoxalin-1-yl]furan-2-ylmethanone (cyclazosin), a potent competitive α_{1B}-adrenoceptor antagonist. *J. Med. Chem.* **39**, 4602-4607.

Gisbert, R., Noguera, M. A., Ivorra, M. D. and D'ocon, P. (2000) Functional evidence of a constitutively active population of α_{1D}-adrenoceptors in rat aorta. *J. Pharmacol. Exp. Ther.* **295**, 810-817.

Goetz, A. S., King, H. K., Ward, S. D. C., True, T. A., Rimele, T. J. and Saussy, D. L. (1995) BMY7378 is a selective antagonist of the D subtype of α_1-adrenoceptors. *Eur. J. Pharmacol.* **272**, R5-R6.

Gonzalez, M. C., Arribas, S. M., Molero, F. and Fernandez-Alfonso, M. S. (2001) Effect of removal of adventitia on vascular smooth muscle contraction and relaxation *Am. J. Physiol.* **280**, H2876-H2881.

Grady, E. F., Bohm, S. K. and Bunnett, N. W. (1997) Turning off the signal: mechanisms that attenuate signalling by G-protein coupled receptors. *Am J. Physiol.* **273(36)**, G586-G601.

Graham, R. M., Perez, D. M. and Hwa, J. (1996) α_1-Adrenergic receptor subtypes - molecular structure, function and signalling. *Circ. Res.* **78**, 737-749.

Gross, G., Hanft, G. and Rugevics, C. (1988) 5-Methylurapidil discriminates between subtypes of the α_1-adrenoceptor. *Eur. J. Pharmacol.* **151**, 333-335.

Guimaraes, S. and Moura, D. (2001) Vascular adrenoceptors: an update. *Pharmacol. Rev.* **53(2)**, 319-356.

Gunnett, C. A., Heistad, D. D., Loihl, A. and Faraci, F. M. (2000) Tumour necrosis factor-α impairs contraction but not relaxation in carotid arteries from iNOS-deficient mice. *Am. J. Physiol.* **279**, R1558-1564.

Gurdal, H., Tilakaratne, N., Brown, R. D., Fonseca, M., Friedman, E. and Johnson, M. D. (1995) The expression of α_1-adrenoceptor subtypes changes with age in the rat aorta. *J. Pharmacol. Exp. Ther.* **275**, 1656-1662.

Gutterman, D. D. (1999) Adventitia-dependent influences on vascular function. *Am. J. Physiol.* **277(4)**, H1265-H1272.

Han, C., Abel, P. W. and Minneman, K. P. (1987a) α_1-Adrenoceptor subtypes linked to different mechanisms of increasing intracellular Ca^{2+} in smooth muscle. *Nature.* **329**, 333-335.

Han, C., Abel, P. W. and Minneman, K. P. (1987b) Heterogeneity of α_1-adrenergic receptors revealed by chloroethylclonidine. *Mol. Pharmacol.* **32**, 505-510.

Han, C., Li, J. and Minneman, K. P. (1990) Subtypes of α_1-adrenoceptors in rat blood vessels. *Eur. J. Pharmacol.* **190**, 97-104.

Han, C. and Minneman, K. P. (1991) Interaction of subtype-selective antagonists with α_1-adrenergic receptor binding sites in rat tissues. *Mol. Pharmacol.* **40**, 531-538.

Hanft, G. and Gross, G. (1989) Subclassification of α_1-adrenoceptor recognition sites by urapidil derivatives and other selective antagonists. *Br. J. Pharmacol.* **97**, 691-700.

Hieble, J. P., Bylund, D. B., Clarke, D. E., Eikenberg, D. C., Langer, S. Z., Lefkowitz, R. J. and Minneman, K. P. (1995) International Union of Pharmacology X. Recommendation for nomenclature of α_1-adrenoceptors: consensus update. *Pharmacol. Reviews.* **47(2)**, 267-270.

Hirasawa, A., Horie, K., Tanaka, T., Murai, M., Yano, J. and Tsujimoto, G. (1993) Cloning, functional expression and tissue distribution of human cDNA for the α_{1c}-adrenergic receptor. *Biochem. Biophys. Res. Comm.* **195(2)**, 902-909.

Hirasawa, A., Shibata, K., Horie, K., Takei, Y., Obika, K., Tanaka, T., Muramoto, N., Takagaki, K., Yano, J. and Tsujimoto, G. (1995). Cloning, functional expression and tissue distribution of human α_{1C}-adrenoceptor splice variants. *FEBS Letters.* **363**, 256-260.

Hirasawa, A., Sugawara, T., Awaji, T., Tsumaya, K., Ito, H. and Tsujimoto, G. (1997) Subtype-specific differences in subcellular localization of α_1-adrenoceptors :chloroethylclonidine preferntially alkylates the accesible cell surface α_1-adrenoceptors irrespective of the subtype. *Mol. Pharmacol.* **52**, 764-770.

Hirasawa, A., Awaji, T., Sugawara, T., Tsujimoto, A. and Tsujimoto, G. (1998) Differential mechanism for the cell surface sorting and agonist-promoted internalization of the α_{1B}-adrenoceptor. *Brit. J. Pharmacol.* **124**, 55-62.

Holck, M. I., Jones, C. H. M. and Haeusler, G. (1983) Differential interactions of clonidine and methoxamine with the postsynaptic α-adrenoceptor of rabbit main pulmonary artery. *J. Cardiovasc. Pharmacol.* **5**, 240-248.

Hrometz, S. L., Edelmann, S. E., M^{c}Cune, D. F., Olges, J. R., Hadley, R. W., Perez, D. M. and Piascik, M. T. (1999) Expression of multiple α_1-adrenoceptors on vascular smooth muscle: correlation with the regulation of contraction. *J. Pharmacol. Exp. Ther.* **290**, 452-463.

Hussain, M. B. and Marshall I. (1997) Characterization of α_1-adrenoceptor subtypes mediating contractions to phenylephrine in rat thoracic aorta, mesenteric artery and pulmonary artery. *Brit. J. Pharmacol.* **122**, 849-858.

Hussain, M. B. and Marshall I. (2000) α_1-Adrenoceptor subtypes mediating contractions of the rat mesenteric artery. *Eur. J. Pharmacol.* **395**, 69-76.

Hwa, J., Graham, R. M. and Perez, D. (1995) Identification of critical determinants of α_1-adrenergic receptor subtype selective agonist binding. *J. Biol. Chem.* **270(39)**, 23189-23195.

Hwa, J. and Perez, D. (1996) The unique nature of the serine interactions for α_1-adrenergic receptor agonist binding and activation. *J. Biol. Chem.* **271(11)**, 6322-6327.

Ibarra, M., Terron, J. A., Lopez-Guerrero, J. J. and Villalobos-Molina, R. (1997) Evidence for an age-dependent functional expression of α_{1D}-adrenoceptors in the rat vasculature. *Eur. J. Pharmacol.* **322**, 221-224.

Ishac, E. J. N., Lazar-Wesley, E. and Kunos, G. (1992) Rapid inverse changes in α_{1B} and β_2-adrenergic receptors and gene transcripts in acutely isolated rat liver cells. *J. Cell. Physiol.* **152**, 79-86.

Jagadeesh, G. and Deth, R. C. (1987) Different affinity states of alpha-1 adrenergic receptors defined by agonists and antagonists in bovine aorta plasma membranes. *J. Pharmacol. Exp. Ther.* **243(2)**, 430-436.

Jenkinson, D. H., Barnard, E. A., Hoyer, D., Humphrey, P. P., Leff, P. and Shankley, N. P. (1995) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification IX: Recommendations on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **47(2)**, 255-266.

Kenakin, T. P. (1982) The Schild regression in the process of receptor classification. *Can. J. Physiol. Pharmacol.* **60**, 249-265.

Kenakin, T. P. (1992) Tissue response as a functional discriminator of receptor heterogeneity: Effects of a mixed receptor population on Schild regression. *Mol. Pharmacol.* **41**, 699-707.

Kenny, B. A., Chalmers, D. H., Philpott, P. C. and Naylor, A. M. (1995) Characterization of an α_{1D}-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. *Brit. J. Pharmacol.* **115**, 981-986.

Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G. and Lefkowitz, R. J. (1992) Constitutive activation of the α_{1B}-adrenergic receptor by all amino acid substitutions at

a single site – evidence for a region which constrains receptor activation. *J. Biol. Chem.* **267(3)**, 1430-1433.

Kleschyov, A. L., Muller, B., Keravis, T., Stoeckel, M-E. and Stoclet, J-C. (2000) Adventitia-derived nitric oxide in rat aortas exposed to endotoxin:cell origin and functional consequences. *Am. J. Physiol.* **279**, H2743-H2751.

Knepper, S. M., Buckner, S. A., Brune, M. E., DeBernardis, J. F., Meyer, M. D. and Hancock, A. A. (1995) A-61603, a potent α_1-adrenergic receptor agonist, selective for the α_{1A}-receptor subtype. *J. Pharmacol. Exp. Ther.* **274**, 97-103.

Kohno, Y., Saito, H., Takita, M., Kigoski, S. and Muramatsu, I. (1994) Heterogeneity of α_1-adrenoceptor subtypes involved in adrenergic contractions of dog blood vessels. *Brit. J. Pharmacol.* **112**, 1167-1173.

Kong, J-Q., Taylor, D. A. and Fleming, W. W. (1994) Functional distribution and role of alpha-1 adrenoceptor subtypes in the mesenteric vasculature of the rat. *J. Pharmacol. Exp. Ther.* **268(3)**, 1153-1159.

Kumar, A. and Lindner, V. (1997) Remodelling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscl. Throm. Vasc. Biol.* **17**, 2238-2244.

La Morte, V. J., Thorburn, J., Absher, D., Spiegel, A., Heller Brown, J., Chien, K. R., Feramisco, J. R. and Knowlton, K. U. (1994) G_q- and Ras-dependent pathways mediate hypertrophy of neonatal rat ventricular myocytes following α_1-adrenergic stimulation. *J. Biol. Chem.* **269(18)**, 13490-13496.

Langer, S. Z. (1974) Presynaptic regulation of catecholamine release. *Biochem. Pharmacol.* **23**, 1793-1798.

Langer, S. Z., Pimoule, C., Faure, C. and Graham, D. (1994) Evidence that the classical α_{1A}- and cloned α_{1c}-adrenoceptors are the same subtype. *Can. J. Physiol. Pharmacol.* **72(Suppl. 1)**, 554.

Lattion, A-L., Diviani, D. and Cotecchia, S. (1994) Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitisation of the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **269(3)**, 22887-22893.

Laz, T. M., Forray, C., Smith, K. E., Bard, J. A., Vaysse, P. J., Branchek, T. A. and Weinshank, R. L. (1994) The rat homologue of the bovine α_{1c}-adrenergic receptor shows the pharmacological properties of the classical α_{1A}-subtype. *Mol. Pharmacol.* **46**, 414-422.

Lefkowitz, R. J., Cotecchia, S., Samama, P. and Costa, T. (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *TIPS.* **14**, 303-307.

Lemoine, H. and Kaumann, A. J. (1983) A model for the interaction of competitive antagonists with two receptor-subtypes characterised by a Schild plot with apparent slope unity. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **322**, 111-120.

Li, G., Chen, S-J., Oparil, S., Chen, Y-F. and Thompson, J. A. (2000) Direct *in vivo* evidence demonstrating neointimal migration of adventitial fibroblasts after balloon injury of rat carotid arteries. *Circulation.* **101**, 1362-1365.

Lomasney, J. W., Cotecchia, S., Lorenz, W., Leung, W-Y., Schwinn, D. A., Yang-Feng, T. L., Brownstein, M., Lefkowitz, R. J. and Caron, M. G. (1991) Molecular cloning and expression of the cDNA for the α_{1A}-adrenergic receptor. *J. Biol. Chem.* **266(10)**, 6365-6369.

MacDonald, A., M^{c}Lean, M., MacAulay, L. and Shaw, A. M. (1999) Effects of propranolol and L-NAME on β-adrenoceptor-mediated relaxation in rat carotid artery. *J. Auton. Pharmacol.* **19**, 145-149.

Mackenzie, J. F., Daly, C. J., Pediani, J. D. and M^{c}Grath, J. C. (2000) Quantitative imaging in live human cells reveals intracellular α_1-adrenoceptor ligand-binding sites. *J. Pharmacol. Exp. Ther.* **294(2)**, 434-443.

Marano, G., Grigioni, M., Palazzesi, S. and Ferrari, A. U. (1999) Endothelin and mechanical properties of the carotid artery in Wistar-Kyoto and spontaneously hypertensive rats. *Cardiovasc. Res.* **41**, 701-707.

Martin, D. J., Lluel, P., Guillot, E., Coste, A., Jammes, D. and Angel, I. (1997) Comparative α_1-adrenoceptor subtype selectivity and functional uroselectivity of α_1-adrenoceptor antagonists. *J. Pharmacol. Exp. Ther.* **282**, 228-235.

Maurice, M-C., Gratton, J-P. and D'Orleans-Juste, P. (1997) Pharmacology of two novel mixed ET_A/ET_B receptor antagonists, BQ-928 and 238, in the carotid and pulmonary arteries and the perfused kidney of the rabbit. *Br. J. Pharmacol.* **120**, 319-325.

M^{c}Cune, D. F., Edelmann, S. E., Olges, J. R., Post, G. R., Waldrop, B. A., Waugh, D. J. J., Perez, D. M. and Piascik, M. T. (2000) Regulation of the cellular localization and signalling properties of the α_{1B}- and α_{1D}-adrenoceptors by agonists and inverse agonists. *Mol. Pharmacol.* **57**, 659-666.

M^{c}Grath, J. C. (1982) Evidence for more than one type of post-junctional α-adrenoceptor. *Biochem. Pharmacol.* **31**, 1277-1282.

Medgett, I. C. and Langer, S. Z. (1984) Heterogeneity of smooth muscle alpha adrenoceptors in rat tail artery *in vitro*. *J. Pharmacol. Exp. Ther.* **229(3)**, 823-830.

Michel, A. D., Loury, D. N. and Whiting R. L. (1989) Identification of a single α_1-adrenoceptor corresponding to the α_{1A}-subtype in rat submaxillary gland. *Br. J. Pharmacol.* **98**, 883-889.

Michel, M. C., Buscher, R., Kerker, J., Kraneis, H., Erdbrugger, W. and Brodde, O-E. (1993) α_1-Adrenoceptor subtype affinities of drugs for the treatment of prostatic hypertrophy: evidence for heterogeneity of chloroethylclonidine-resistant rat renal α_1-adrenoceptor. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **348**, 385-395.

Michel, M. C. and Insel, P. A. (1994) Comparison of cloned and pharmacologcially defined rat tissue renal α_1-adrenoceptor subtypes. *Naunyn-Schmiedebergs Arch. Pharmacol.* **350**, 136-142.

Milano, C. A., Dolber, P. C., Rockman, H. A., Bond, R. A., Venable, M. E., Allen, L. F. and Lefkowitz, R. J. (1994) Myocardial expression of a constitutively active α_{1B}-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc. Natl. Acad. Sci. USA.* **91**, 10109-10113.

Minneman, K. P., Han, C. and Abel, P. W. (1988) Comparison of α_1-adrenergic receptor subtypes distinguished by chloroethylclonidine and WB4101. *Mol. Pharmacol.* **33**, 509-514.

Minneman, K. P., Theroux, T. L., Hollinger, S., Han, C. and Esbenshade, T. A. (1994) Selectivity of agonists for cloned α_1-adrenergic receptor subtypes. *Mol. Pharmacol.* **46**, 929-936.

Minneman, K. P., Lee, D., Zhong, H., Berts, A., Abbott, K. and Murphy, T. J. (2000) Transcriptional responses to growth factor and G-protein coupled receptors in PC12 cells: comparison of α_1-adrenergic receptor subtypes. *J. Neurochem.* **74**, 2392-2400.

Moeller, I., Clune, E. F., Fennessy, P. A., Bingley, J. A., Albiston, A. L., Mendelsohn, F. A. O. and Chai, S-Y. (1999) Up regulation of AT_4 receptor levels in carotid arteries following balloon injury. *Regulatory Peptides.* **83**, 25-30.

Morrow, A. L. and Creese, I. (1986) Characterisation of α_1-adrenergic receptor subtypes in rat brain: a reevaluation of [^{3}H]WB4101 and [^{3}H]prazosin binding. *Mol. Pharmacol.* **29**, 321-330.

Motulsky, H. (1996) The GraphPad guide to nonlinear regression.

Muramatsu, I., Ohmura, T., Kigoshi, S., Hashimoto, S. and Oshita, M. (1990) Pharmacological subclassification of α_1-adrenoceptors in vascular smooth muscle. *Br. J. Pharmacol.* **99**, 197-201.

Muramatsu, I., Kigoshi, S. and Ohmura, T. (1991a) Subtypes of α_1-adrenoceptor involved in noradrenaline-induced contractions of rat thoracic aorta and dog carotid artery. *Japan. J. Pharmacol.* **57**, 535-544.

Muramatsu, I. (1991b) Relation between adrenergic neurogenic contraction and α_1-adrenoceptor subtypes in dog mesenteric and carotid arteries and rabbit carotid arteries. *Br. J. Pharmacol.* **102**, 210-214.

Muramatsu, I., Ohmura, T., Hashimoto, S. and Oshita, M. (1995) Functional subclassification of vascular α_1-adrenoceptors. *Pharmacol. Comm.* **6(1-3)**, 23-28

Nagadeh, M. M. (1996) Vasomodulator mechanisms in rat carotid artery and in vessels from an experimental model of heart failure. *PhD thesis.*

Offermanns, S. (1999) New insights into the *in vivo* function of heterotrimeric G-proteins through gene deletion studies. *Naunyn-Schmiedebergs Arch. Pharmacol.* **360**, 5-13.

Ohmura, M. and Muramatsu, I. (1995) Two distinct α_1-adrenoceptor subtypes in rabbit liver: a binding study. *Br. J. Pharmacol.* **116**, 2591-2596.

de Oliveira, A.M., Campos-Mello, C., Leitao, M. C. and Correa, F. M. A. (1998) Maturation and ageing-related differences in responsiveness of rat aorta and carotid arteries to α_1-adrenoceptor stimulation. *Pharmacology.* **57**, 305-313.

Ooboshi, H., Toyoda, K., Faraci, F. M., Lang, M. G. and Heistad, D. D. (1998) Improvement of relaxation in an atherosclerotic artery by gene transfer of endothelial nitric oxide synthase. *Arterio. Thromb. Vasc. Biol.* **18**, 1752-1758.

Oparil, S., Chen, S-J., Chen, Y-F., Durand, J. N., Allen, L. and Thompson, J. A. (1999) Estrogen attenuates the adventitial contribution to neointima formation in injured rat carotid arteries. *Cardiovasc. Res.* **44**, 608-614.

Oshita, M., Kigoshi, S. and Muramatsu, I. (1991) Three distinct binding sites for [^{3}H]-prazosin in the rat cerebral cortex. *Br. J. Pharmacol.* **104**, 961-965.

Oshita, M., Kigoshi, S. and Muramatsu, I. (1993) Pharmacological characterization of two distinct α_1-adrenoceptor subtypes in rabbit thoracic aorta. *Brit. J. Pharmacol.* **108**, 1071-1076.

Palacios, J. M., Hoyer, D. and Cortes, R. (1987) α_1-Adrenoceptors in the mammalian brain: similar pharmacology but different distribution in rodents and primates. *Brain. Research.* **419**, 65-75.

Parini, A., Homcy, C. J. and Graham, R. M. (1987) Structural properties of the α_1-adrenergic receptor: studies with membrane and purified receptor preparations. *Circ. Res.* **67(suppl I)**, I100-I104).

Patane, M. A., Scott, A. L., Broten, T. P., Chang, R. S> L., Ransom, R. W., DiSalvo, J., Forray, C. and Bock, M. G. (1998) 4-Amino-2-[4-[1-(benzyloxycarbonyl)-2(S)-[[(1,1-dimethylethyl)amino]carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline (L765,314): a potent and selective α_{1b}-adrenergic receptor antagonist. *J. Med. Chem.* **41(8)**, 1205-1208.

Perez, D. M., Piascik, M. T. and Graham, R. M. (1991) Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA. *Mol Pharmacol.* **40**, 876-883.

Perez, D. M., DeYoung, M. B. and Graham, R. M. (1993) Coupling of expressed α_{1B}- and α_{1D}-adrenergic receptors to multiple signalling pathways is both G protein and cell type specific. *Mol. Pharmacol.* **44**, 784-795.

Piascik, M. T., Guarino, R. D., Smith, M. S., Soltis, E. E., Saussy, D. L. Jr. and Perez, D. M. (1995) The specific contribution of the novel α_{1D}-adrenoceptor to the contraction of vascular smooth muscle. *J. Pharmacol. Exp. Ther.* **275**, 1583-1589.

Piascik, M. T., Hrometz, S. L., Edelmann, S. E., Guarino, R. D., Hadley, R. W. and Brown, R. D. (1997) Immunocytochemical localization of the α_{1B}-adrenergic receptor and the contribution of this and the other subtypes to vascular smooth muscle contraction: analysis with selective ligands and antisense oligonucleotides. *J. Pharmacol. Exp. Ther.* **283**, 854-868.

Pimoule, C., Langer, S. Z. and Graham, D. (1995) Further evidence that the classical α_{1A}- and cloned α_{1c}-adrenoceptors are the same subtype. *Eur. J. Pharmacol.* **290**, 49-53.

Ping, P. and Faber, J. E. (1993) Characterization of α-adrenoceptor gene expression in arterial and venous smooth muscle. *Am. J. Physiol.* **265(34)**, H1501-H1509.

Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J. G., Rubin, E. M. and Breslow, J. L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell.* **71**, 343-353.

Porter, J. E., Hwa, J. and Perez, D. (1996) Activation of the α_{1b}-adrenergic receptor is initiated by disruption of an interhelical salt bridge constraint. *J. Biol. Chem.* **271(45)**, 28318-28323.

Price, D. T., Chari, R. S., Berkowitz, D. E., Meyers, W. C. and Schwinn, D. A. (1994a) Expression of α_1-adrenergic receptor subtype mRNA in rat tissues and human SK-N-MC neuronal cells: Implications for α_1-adrenergic receptor subtype classification. *Mol. Pharmacol.* **46**, 221-226.

Price, D. T., Lefkowitz, R. J., Caron, M. G., Berkowitz, D. and Scwhinn, D. A. (1994b) Localization of mRNA for three distinct α_1-adrenergic receptor subtypes in human tissues: implications for human α-adrenergic physiology. *Mol. Pharmacol.* **45**, 171-175.

Pruneau, D. and Belichard, P. (1993) Induction of bradykinin-B1 receptor-mediated relaxation in the isolated rabbit carotid artery. *Eur. J. Pharmacol.* **239(1-3)**, 63-67.

Ramarao, C. S., Kincade Denker, J. M., Perez, D. M., Gaivin, R. J., Riek, R. P. and Graham R. M. (1992) Genomic organization and expression of the human α_{1B}-adrenergic receptor. *J. Biol. Chem.* **267(30)**, 21936-21945.

Rang, H. P., Dale, M. M. and Ritter J. M. (1995) Pharmacology (3rd edition). *Churchill Livingstone.*

Rohrer, D. K. and Kobilka, B. K. (1998) Insights from *in vivo* modification of adrenergic receptor gene expression. *Annu. Rev. Pharmacol. Toxicol.* **38**, 351-373.

Rokosh, D. G., Bailey, B. A., Stewart, A. F. R., Karns, L. R., Long, C. S. and Simpson, P. C. (1994) Distribution of α_{1C}-adrenergic receptor mRNA in adult rat tissues by

RNase protection assay and comparison with α_{1B} and α_{1D}. *Biochem. Biophys. Res. Comm.* **200(3)**, 1177-1184.

Rossby, S. P. and Cornett, L. E. (1991) Steady state levels of hepatic α_1- and β_2-adrenergic receptors and gene transcripts during development of the male rat. *J. Cell. Physiol.* **145**, 55-61.

Ruan, Y., Kan, H., Parmentier, J-H., Fatima, S., Allen, L. F. and Malik, K. U. (1998) α_{1a}-Adrenergic receptor stimulation with phenylephrine promotes arachidonic acid release by activation of phospholipase D in rat-1 fibroblasts: inhibition by protein kinase A. *J. Pharmacol. Exp. Ther.* **284**, 576-585.

Ruffolo, R. R. Jr., Nichols, A. J., Stadel, J. M. and Hieble, J. P. (1991) Structure and function of α-adrenoceptors. *Pharmacol. Reviews.* **43**(4), 475-505.

Russell, A. and Watts, S. (2000) Vascular reactivity of isolated thoracic aorta of the C57BL/6J mouse. *J. Pharmacol. Exp. Ther.* **294**, 598-604.

Rutschmann, B., Evequoz, D., Aubert, J. F., Brunner, H. R. and Waeber, B. (1998) Vasopressin dilates the rat carotid artery by stimulating V1 receptors. *J. Cardiovasc. Pharmacol.* **32(4)**, 637-641.

Satoh, M., Enomoto, K., Takayanagi, I and Koike, K. (1999a) Differences of antagonism for a selective α_{1D}-adrenoceptor antagonist BMY7378 in the rabbit thoracic aorta and iliac artery. *J. Smooth. Muscle. Res.* **34**, 151-158.

Satoh, M., Enomoto, K., Takayanagi, I and Koike, K. (1999b) Analysis of α_1-adrenoceptor subtypes in rabbit aorta and arteries: regional difference and co-existence. *Eur. J. Pharmacol.* **374**, 229-240.

Saussy, D. L. Jr., Goetz, A. S., King, H. K. and True, T. A. (1994) BMY7378 is a selective antagonist of α_{1D}-adrenoceptors:evidence that rat vascular α_1-adrenoceptors are of the α_{1D}-subtype. *Can. J. Physiol. Pharmacol.* **72(Suppl 1)**, 323.

Sawutz, D. G., Lanier, S. M. Warren, C. D. and Graham, R. M. (1987) Glycosylation of the mammalian α_1-adrenergic receptor by complex type N-linked oligosaccharides. *Mol. Pharmacol.* **32**, 565-571.

Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G. and Cotecchia, S. (1996) Constitutively active mutants of the α_{1B}-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *EMBO J.* **15(14)**, 3566-3578.

Scheer, A., Fanelli, F., Costa, T., De Benedetti, P.G. and Cotecchia, S. (1997) The activation process of the α_{1B}-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate. *Proc. Natl. Acad. Sci. USA.* **94**, 808-813.

Scheer, A., Fanelli, F., Diviani, D, De Benedetti, P. G. and Cotecchia, S. (1999) Structure-function relationships of the α_{1b}-adrenergic receptor. *Eur. Urol.* **36(Suppl 1)**, 11-16.

Schild, H. O. (1947) The use of drug antagonists for the identification and classification of drugs. *Brit. J. Pharmacol.* **2**, 251-258.

Schwarz, K. R., Lanier, S. M., Carter, E. A., Homcy, C. J. and Graham, R. M. (1984) Rapid reciprocal changes in adrenergic receptors in intact isolated hepatocytes during primary cell culture. *Mol. Pharmacol.* **27**, 200-209.

Schwinn, D. A., Lomasney, J. W., Lorenz, W., Szklut, P. J., Fremeau R. T. Jr., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J. and Cotecchia, S. (1990) Molecular cloning and expression of the cDNA for a novel α_1-adrenergic receptor subtype. *J. Biol. Chem.* **265(14)**, 8183-8189.

Schwinn, D. A., Page, S. O., Middleton, J. P., Lorenz, W., Ligget, S. B., Yamamoto, K., Lapetina, E. G., Caron, M. G., Lefkowitz, R. J. and Cotecchia, S. (1991) The α_{1C}-adrenergic receptor: characterization of signal transduction pathways and mammalian tissue heterogeneity. *Mol. Pharmacol.* **40**, 619-626.

Schwinn, D. A., Johnston, G. I., Page, S. O., Mosley, M. J., Wilson, K. H., Worman, N. P., Campbell, S., Fidock, M. D., Furness, M., Parry-Smith, D. J., Peter, B. and Bailey, S. (1995) Cloning and pharmacological characterization of human α_1-adrenergic receptors: sequence corrections and direct comparison with other species homologues. *J. Pharmacol. Exp. Ther.* **272**, 134-142.

Scofield, M. A., Liu, F., Abel, P. W. and Jeffries, W. B. (1995) Quantification of steady state expression of mRNA for α_1-adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. *J. Pharmacol. Exp. Ther.* **275(2)**, 1035-1042.

Shen, H., Peri, K. G., Deng, X-F., Chemtob, S. and Varma, D. R. (2000) Distribution of α_1-adrenoceptor subtype proteins in different tissues of neonatal and adult rats. *Can. J. Physiol. Pharmacol.* **78**, 237-243.

Shi, Y., Pieniek, M., Fard, A., O'Brien, J., Mannion, J. D. and Zalewski, A. (1996) Adventitial remodelling after coronary arterial injury. *Circulation.* **93**, 340-348.

Sirvio, J. and MacDonald, E. (1999) Central α_1-adrenoceptors: Their role in the modulation of attention and memory formation. *Pharmacol. And Ther.* **83**, 49-65.

Smith, K. M., Macmillan, J. B. and M^{c}Grath, J. C. (1997) Investigation of α_1-adrenoceptor subtypes mediating vasoconstriction in rabbit cutaneous resistance arteries. *Brit. J. Pharmacol.* **122**, 825-832.

Stam, W. B., Van der Graaf, P. H., Saxena, P. R. (1998) Functional characterisation of the pharmacological profile of the putative α_{1B}-adrenoceptor antagonist, (+)-cyclazosin. *Eur. J. Pharmacol.* **361**, 79-83.

Stam, W. B., Van der Graaf, P. H. and Saxena, P. R. (1999) Analysis of α_{1L}-adrenoceptor pharmacology in rat small mesenteric artery. *Brit. J. Pharmacol.* **127**, 661-670.

Starke, K., Montel, H. and Schumann, H. J. (1971) Influence of cocaine and phenoxybenzamine on noradrenaline uptake and release. *Naunyn-Schmiedebergs Arch. Pharmacol.* **270**, 210-214.

Summers, R. J. and M^{c}Martin, L. R. (1993) Adrenoceptors and their second messenger systems. *J. of Neurochem.* **60(1)**, 10-23.

Taddei, C., Poggesi, E., Leonardi, A. and Test, R. (1993) Affinity of different α_1-agonists and antagonists for the α_1-adrenoceptors of rabbit and rat liver membranes. *Life Sciences.* **53**, PL177-181.

Takayanagi, I., Harada, M., Koike, K and Satoh, M. (1991) Differences in α_1-adrenoceptor mechanisms for phenylephrine and tizanidine in rabbit thoracic aorta and common iliac artery. *Can. J. Physiol. Pharmacol.* **69**, 1819-1824.

Terman, B. I., Riek, R. P., Grodski, A., Hess, H-J. and Graham, R. M. (1990) Identification and structural characterization of α_1-adrenergic receptor subtypes. *Mol. Pharmacol.* **37**, 526-534.

Testa, R., Taddei, C., Poggesi, E., Destefani, C., Cotecchia, S., Hieble, J. P., Sulpizio, A. C., Naselsky, D., Bergsma, D., Ellis, C., Swift, A., Ganguly, S., Ruffolo, R. R. Jr. and Leonardi, A. (1995a) Rec15/2739 (SB216469): A novel prostate selective α_1-adrenoceptor antagonist. *Pharmacol. Comm.* **6(1-3)**, 79-86.

Testa, R., Destefani, C., Guarneri, L., Poggesi, E., Simonazzi, I., Taddei, C. and Leonardi, A. (1995b) The α_{1D}-adrenoceptor subtype is involved in the noradrenaline-induced contractions of rat aorta. *Life Sciences.* **57(13)**, PL159-163.

Testa, R., Guarneri L., Poggesi, E., Simonazzi, I., Taddei, C and Leonardi, A. (1995c) Mediation of noradrenaline-induced contractions of rat aorta by the α_{1B}-adrenoceptor subtype. *Brit. J. Pharmacol.* **114**, 745-750.

Thai, L., Galluzzo, J. M., M^{c}Cook, E. C., Seidler, F. J. and Slotkin, T. A. (1996) Atypical regulation of hepatic adenylyl cyclase and adrenergic receptors during a critical development period: agonists evoke supersensitivity accompanied by failure of receptor down-regulation. *Pediatric Res.* **39**, 697-707.

Tsutsui, M., Chen, A. F. Y., O'Brien, T., Crotty, T. B. and Katusic, Z. S. (1998) Adventitial expression of recombinant eNOS gene restores NO production in arteries without endothelium. *Arterioscler. Thromb. Vasc. Biol.***18**, 1231-1241.

Tsutsui, M., Onoue, H., Iida, Y., Smith, L., O'Brien, T. and Katusic, Z. S. (1999) Adventitia-dependent relaxations of canine basilar arteries transduced with recombinant eNOS gene. *Am. J. Physiol.* **276(45)**, H1846-H1852.

Ulloa-Aguirre, A., Stanislaus, D., Janovick, J. and Conn, P. M. (1999) Structure-activity relationships of G-protein coupled receptors. *Arch. Med. Res.* **30**, 420-435.

Van der Graaf, P. H., Welsh, N. J., Shankley, N. P. and Black J. W. (1993) Evidence of heterogeneity of α_1-adrenoceptors in the rat aorta. *Brit. J. Pharmacol.* **110**, 124P.
Van der Graaf, P. H., Welsh, N. J., Shankley, N. P. and Black, J. W. (1994) 'Functional inhibition curves' provide further evidence for heterogeneity of α_1-adrenoceptors in the rat aorta. *Brit. J. Pharmacol.* **112**, 216P.

Van der Graaf, P. H., Shankely, N. P. and Black, J. W. (1996a) Analysis of the activity of α_1-adrenoceptor antagonists in rat aorta. *Brit. J. Pharmacol.* **118**, 299-310.

Van der Graaf, P. H., Shankley, N. P. and Black, J. W. (1996b) Analysis of the effects of α_1-adrenoceptor antagonists on noradrenaline-mediated contractions of rat small mesenteric artery. *Brit. J. Pharmacol.* **118**, 1308-1316.

Van der Graaf, P. H., Deplanne, V., Duquenne, C. and Angel, I. (1997) Analysis of α_1-adrenoceptors in rabbit lower urinary tract and mesenteric artery. *Eur. J. Pharmacol.* **327**, 25-32.

Vargas, H. M. and Gorman, A. J. (1995) Vascular α_1-adrenergic receptor subtypes in the regulation of arterial pressure. *Life Sciences.* **57(25)**, 2291-2308.

Villalobos-Molina, R. and Ibarra, M. (1996) α_1-Adrenoceptors mediating contraction in arteries of normotensive and spontaneously hypertensive rats are of the α_{1D} or α_{1A} subtypes. *Eur. J. Pharmacol.* **298**, 257-263.

Voigt, M. M., Kispert, J. and Chin, H. (1990) Sequence of a rat brain cDNA encoding an α_{1B}-adrenergic receptor. *Nuc. Acids. Res.* **18(4)**, 1053.

Watts, S. W., Yeum, C. H., Campbell, G. and Webb, R. C. (1996) Serotonin stimulates protein tyrosyl phosphorylation and vascular contraction via tyrosine kinase. *J. Vasc. Res.* **33(4)**, 288-298.

Wei, L-N. (1997) Transgenic animals as new approaches in pharmacological studies. *Annu. Rev. Pharmacol. Toxicol.* **37**, 119-141.

Weinberg, D. H., Trivedi, P., Tan, C. P., Mitra, S., Perkins-Barrow, A., Borkowski, D., Strader C. D. and Bayne M. (1994) Cloning, expression and characterization of human α-adrenergic receptors: α_{1A}, α_{1B} and α_{1C}. *Biochem. Biophys. Res. Comm.* **201**, 1296-1304.

Wikberg-Matsson, A., Wikberg, J. E. S. and Uhlen, S. (1998) Characterization of α_1-adrenoceptors in the pig. *Eur. J. Pharmacol.* **347**, 301-309.

Wilcox, J. N., Waksman, R., King, S. B. and Scott, N. A. (1996) The role of the adventitia in the arterial response to angioplasty: the effect of intravascular radiation. *Int. J. Radiation. Oncology. Biol. Phys.* **36(4)**, 789-796.

Williams, B. (1998) Mechanical influences on vascular smooth muscle function. *J. Hypertension.* **16**, 1921-1929.

Williams, T. J., Blue, D. R., Daniels, D. V., Davis, B., Elwirthy, T., Gever, J. R., Kava, M. S., Morgans, D., Padilla, F., Tassa, S., Vimont, R. L., Chapple, C. R., Chess-Williams, R., Eglen, R. M., Clarke, D. E. and Ford, A. P. D. W. (1999) *In vitro* α_1-adrenoceptor pharmacology of Ro 70-0004 and RS-100329, novel α_{1A}-adrenoceptor selective antagonists. *Brit. J. Pharmacol.* **127**, 252-258.

Wilson, V. G., Brown, C. M. and M^{c}Grath, J. C. (1991) Are there more than two types of α-adrenoceptors involved in physiological responses. *Exp. Physiol.* **76**, 317-346.

Wood, C. L., Arnett, C. D. Clarke, W. R. Tsai, B. S. and Lefkowitz, R. J. (1979) Subclassification of α-adrenergic receptors by direct binding studies. *Biochem. Pharmacol.* **28**, 1277-1282.

Woollhead, A. M. (2001) Design of a molecular strategy to analyse the subcellular distribution of α_1-adrenoceptors in recombinant and native systems. *PhD thesis.*

Wu, D., Katz, A., Lee, C-H. and Simon, M. I. (1992) Activation of phospholipase C by α_1-adrenergic receptors is mediated by the α subunits of G_q family. *J. Biol. Chem.* **267(36)**, 25798-25802.

Xiao, L., Scofield, M. and Jeffries, W. B. (1998) Molecular cloning, expression and characterization of cDNA encoding a mouse α_{1a}-adrenoceptor. *Brit. J. Pharmacol.* **124**, 213-221.

Xin, X., Yang, N., Eckhart, D. and Faber, J. E. (1997) α_{1D}-adrenergic receptors and mitogen-activated protein kinase mediate increased protein synthesis by arterial smooth muscle. *Mol. Pharmacol.* **51**, 764-775.

Xin, X., Yang, N. and Faber, J. E. (1999) Platelet-derived growth factor inhibits α_{1D}-adrenergic receptor expression in vascular smooth muscle cells *in vitro* and *ex vivo*. *Mol Pharmacol.* **56**, 1143-1151.

Yang, M., Verfurth, F., Buscher, R. and Michel, M. C. (1997) Is α_{1D}-adrenoceptor protein detectable in rat tissues? *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355**, 438-446.

Yang, M., Reese, J., Cotecchia, S. and Michel, M. C. (1998) Murine α_1-adrenoceptor subtypes. I. Radioligand binding studies. *J. Pharmacol. Exp. Ther.* **286**, 841-847.

Zhang, H., Du, Y., Cohen, R. A., Chobanian, A. V. and Brecher, P. (1999) Adventitia as a source of inducible nitric oxide synthase in the rat aorta. *Am. J. Hypertens.* **12**, 467-475.

Zhang, H. and Faber, J. E (2001) Norepinephrine stimulation of injured aorta *ex vivo* increases growth of the intima-media via α_{1A} and the adventitia via α_{1B}-adrenoceptors. *FASEB J.* **15(5)**, A947.

Zhong, H. and Minneman, K. P. (1999a) α_1-Adrenoceptor subtypes. *Eur. J. Pharmacol.* **375**, 261-276.

Zhong, H. and Minneman, K. P. (1999b) Differential activation of mitogen-activated protein kinase pathways in PC12 cells by closely related α_1-adrenergic receptor subtypes. *J. Neurochem.* **72**, 2388-2396.

Zuscik, M. J., Sands, S., Ross, S. A., Waugh, D. J. J., Gaivin, R. J., Morilak, D. and Perez, D. M. (2000) Overexpression of the α_{1B}-adrenergic receptor causes apoptotic neurodegeneration: Multiple system atrophy. *Nature Medicine* **6(12)**, 1388-1394.

Zuscik, M. J., Chalothorn, D., Hellard, D., Deighan, C., M^{c}Gee, A., Daly, C. J., Waugh, D. J. J., Ross, S. A., Gaivin, R. J., Morehead, A. J., Thomas, J. D., Plow, E. F., M^{c}Grath, J. C., Piascik, M. T. and Perez, D. M. (2001) Hypotension, autonomic failure and cardiac hypertrophy in transgenic mice overexpressing the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **276(17)**, 13738-13743.

APPENDIX

APPENDIX - Counts per minute for binding experiments

Conc (log M)	CPM	S.E.M.
NSB	106.0000	15.0000
-4.0000	114.0000	25.0000
-4.5228	104.5000	7.5000
-5.0000	114.0000	3.0000
-5.5228	202.5000	76.5000
-6.0000	151.5000	37.5000
-6.5228	139.5000	0.5000
-7.0000	157.5000	1.5000
-7.5228	204.0000	3.0000
-8.0000	290.5000	2.5000
-8.5228	419.0000	32.0000
-9.0000	1116.5000	6.5000
-9.5228	1678.0000	133.0000
-10.000	2225.0000	68.0000
-10.5228	2109.5000	38.5000
-11.000	2355.5000	65.5000
-11.5228	2336.0000	34.0000
-12.000	2382.5000	16.5000
TOTAL	2349.5000	65.5000

WT BRAIN - PRAZOSIN

Conc (log M)	CPM	S.E.M.
NSB	154.6667	8.301271
-5.000	150.500	8.313643
-5.5228	197.6667	11.64951
-6.000	263.3333	33.02895
-6.5228	530.3333	81.27635
-7.000	783.8333	54.69517
-7.5228	1202.333	12.60071
-8.000	1514.333	69.07371
-8.5228	1796.333	180.3832
-9.000	1903.833	97.66386
-9.5228	2209.667	172.6628
-10.000	2411.667	194.6413
-10.5228	2444.000	199.0635
-11.000	2520.167	199.1374
-11.5228	2450.167	192.3219
-12.000	2542.833	205.4836
-12.5228	2497.000	223.4329
-13.000	2569.500	275.4347
TOTAL	2401.833	233.4673

WT BRAIN - RS100329

Conc (log M)	CPM	S.E.M.
NSB	208.500	1.500
-3.000	163.500	12.500
-3.5228	224.000	3.000
-4.000	301.500	15.500
-4.5228	531.000	32.000
-5.000	836.500	25.500
-5.5228	1219.000	16.000
-6.000	1550.500	3.500
-6.5228	1751.500	4.500
-7.000	2159.500	160.500
-7.5228	2288.000	67.000
-8.000	2280.500	21.500
-8.5228	2290.500	94.50001
-9.000	2242.500	50.500
-9.5228	2366.500	61.500
-10.000	2361.500	19.500
-10.5228	2357.000	37.000
-11.000	2088.500	66.500
TOTAL	2197.500	1.500

WT BRAIN - (R)-A-61603 (-GTP)

Conc (log M)	CPM	S.E.M.
NSB	166.5000	14.563080
-2.0000	178.7500	16.754970
-2.5228	176.0000	15.577760
-3.0000	163.0000	10.885770
-3.5228	181.7500	9.620941
-4.0000	293.5000	8.411302
-4.5228	472.2500	19.490920
-5.0000	861.7500	15.542280
-5.5228	1209.7500	10.403330
-6.0000	1503.2500	26.603180
-6.5228	1772.2500	17.307870
-7.0000	2135.0000	61.515580
-7.5228	2221.7500	46.087550
-8.0000	2269.2500	27.956440
-8.5228	2273.7500	52.950250
-9.0000	2251.0000	38.650140
-9.5228	2191.5000	45.651760
-10.0000	2205.7500	73.057480
-10.5228	2226.7500	53.965070
-11.0000	2281.5000	31.624090
TOTAL	2217.0000	50.518970

WT BRAIN - (R)-A-61603 (+GTP)

Conc (log M)	CPM	S.E.M.
NSB	79.100	4.600
-4.000	60.200	3.000
-4.5228	75.200	2.200
-5.000	138.400	6.500
-5.5228	348.700	18.600
-6.000	481.100	11.000
-6.5228	1125.700	39.700
-7.000	1331.200	57.100
-7.5228	1377.200	40.000
-8.000	1518.000	65.300
-8.5228	1539.700	20.000
-9.000	1596.900	47.600
-10.000	1604.000	74.800
-11.000	1609.800	105.800
TOTAL	1601.500	79.800

WT BRAIN - L765,314

Conc (log M)	CPM	S.E.M.
NSB	106.500	2.000
-3.000	122.250	3.750
-3.5228	129.8575	19.3575
-4.000	146.750	5.250
-4.5228	200.500	12.500
-5.000	297.750	0.250
-5.5228	537.250	21.250
-6.000	827.750	10.750
-6.5228	1164.250	110.250
-7.000	1315.250	50.750
-7.5228	1341.250	46.250
-8.000	1322.000	8.000
-8.5228	1382.750	15.250
-9.000	1318.500	45.500
-9.5228	1322.250	32.250
-10.000	1371.750	43.250
-10.5228	1332.500	22.000
-11.000	1424.250	89.250
TOTAL	1323.250	55.250

WT BRAIN - BMY7378

Conc (log M)	CPM	S.E.M.
NSB	119.125	5.429541
-4.000	134.875	5.005131
-4.5228	153.000	5.05682
-5.000	151.500	3.245877
-5.5228	168.375	4.161634
-6.000	177.125	6.682701
-6.5228	199.500	7.136426
-7.000	205.750	7.71073
-7.5228	280.500	16.23928
-8.000	418.875	39.47125
-8.5228	524.875	21.85458
-9.000	878.000	35.7686
-9.5228	1160.500	51.70348
-10.000	1303.250	52.97633
-10.5228	1370.875	67.91393
-12.000	1383.250	76.38081
-11.5228	1362.875	85.0908
-13.000	1376.250	71.16348
TOTAL	1344.250	62.96109

KO BRAIN - PRAZOSIN

Conc (log M)	CPM	S.E.M.
NSB	152.000	51.500
-5.000	133.500	35.000
-5.5228	153.750	47.250
-6.000	163.750	59.250
-6.5228	171.000	36.500
-7.000	188.250	46.750
-7.5228	223.250	54.750
-8.000	259.000	106.500
-8.5228	365.000	206.500
-9.000	631.250	211.250
-9.5228	883.500	391.000
-10.000	1089.750	518.750
-10.5228	1136.250	554.750
-11.000	1183.250	573.250
-11.5228	1224.500	612.500
-12.000	1238.500	615.000
-12.5228	1223.750	629.250
-13.000	1186.250	663.250
TOTAL	1154.250	543.250

KO BRAIN - RS100329

Conc (log M)	CPM	S.E.M.
NSB	183.1667	35.65124
-3.000	190.1667	34.71903
-3.5228	204.0833	32.18887
-4.000	238.0833	36.28232
-4.5228	248.000	44.19596
-5.000	296.1667	47.09205
-5.5228	376.9167	57.12419
-6.000	504.3333	78.42987
-6.5228	690.1667	113.2425
-7.000	932.000	160.3949
-7.5228	1034.000	159.771
-8.000	1176.333	192.6259
-8.5228	1175.250	182.2304
-9.000	1187.333	176.1287
-9.5228	1199.500	187.573
-10.000	1199.833	176.3132
-10.5228	1200.083	172.9198
-11.000	1186.167	184.5774
TOTAL	1184.083	179.7314

KO BRAIN - (R)-A-61603 (-GTP)

Conc (log M)	CPM	S.E.M.
NSB	200.4167	31.26217
-3.000	212.8333	33.75467
-3.5228	226.0833	28.71336
-4.000	237.000	22.17469
-4.5228	270.4167	36.62818
-5.000	298.500	41.01565
-5.5228	400.000	41.92911
-6.000	544.8333	76.48863
-6.5228	750.750	90.61243
-7.000	1037.667	125.9242
-7.5228	1138.000	151.6821
-8.000	1243.917	164.2207
-8.5228	1294.167	191.6507
-9.000	1270.833	182.9063
-9.5228	1251.000	175.9752
-10.000	1265.833	191.3732
-10.5228	1269.417	173.0185
-11.000	1255.417	183.9226
TOTAL	1292.583	177.6881

KO BRAIN - (R)-A-61603 (+GTP)

Conc (log M)	CPM	S.E.M.
NSB	58.800	4.600
-4.000	54.600	3.000
-4.5228	53.900	2.200
-5.000	76.900	6.500
-5.5228	169.700	18.600
-6.000	236.100	11.000
-6.5228	441.200	39.700
-7.000	512.500	57.100
-7.5228	507.200	40.000
-8.000	553.400	65.300
-8.5228	617.800	20.000
-9.000	646.200	47.600
-10.000	594.700	74.800
-11.000	594.500	105.800
TOTAL	597.700	79.800

KO BRAIN - L765,314

Conc (log M)	CPM	S.E.M.
NSB	188.500	13.500
-3.000	211.000	23.500
-3.5228	191.250	16.750
-4.000	204.000	27.500
-4.5228	253.250	25.750
-5.000	306.250	5.250
-5.5228	415.000	27.000
-6.000	515.000	84.000
-6.5228	660.000	94.50001
-7.000	712.500	74.500
-7.5228	744.750	92.750
-8.000	768.250	123.750
-8.5228	778.750	140.750
-9.000	773.000	72.000
-9.5228	779.750	94.250
-10.000	775.750	98.75001
-10.5228	799.000	95.00001
-11.000	759.750	58.250
TOTAL	749.250	71.750

KO BRAIN - BMY7378

Conc (log M)	CPM	S.E.M.
NSB	112.000	12.000
-4.000	138.250	18.750
-4.5228	158.250	6.750
-5.000	108.500	2.500
-5.5228	123.500	2.500
-6.000	120.250	4.750
-6.5228	125.500	2.500
-7.000	136.750	3.250
-7.5228	182.750	7.250
-8.000	169.250	4.250
-8.5228	224.500	4.500
-9.000	225.500	4.500
-9.5228	434.500	19.000
-10.000	432.750	9.250
-10.5228	519.750	37.250
-11.000	488.250	34.750
-11.5228	525.750	13.750
-12.000	507.750	34.250
TOTAL	463.750	10.250

WT LIVER - PRAZOSIN

Conc (log M)	CPM	S.E.M.
NSB	408.250	16.250
-5.000	409.250	16.250
-5.5228	430.250	5.750
-6.000	516.000	30.000
-6.5228	807.750	25.250
-7.000	1347.750	18.250
-7.5228	2337.500	86.500
-8.000	3013.000	58.000
-8.5228	3330.750	41.750
-9.000	3565.500	95.49999
-9.5228	3573.250	55.250
-10.000	3635.750	2.250
-10.5228	3647.000	30.000
-11.000	3566.750	32.250
-11.5228	3526.750	28.750
-12.000	3715.250	22.750
-12.5228	3618.500	69.500
-13.000	3599.000	17.500
TOTAL	3470.250	39.250

WT LIVER - RS100329

Conc (log M)	CPM	S.E.M.
NSB	314.500	19.500
-3.000	483.000	33.500
-3.5228	934.000	32.500
-4.000	1431.250	83.250
-4.5228	1776.500	52.500
-5.000	1913.500	25.500
-5.5228	1949.000	18.000
-6.000	1921.000	7.000
-6.5228	1924.500	52.500
-7.000	2043.750	65.750
-7.5228	2076.250	38.750
-8.000	2129.500	22.500
-8.5228	2098.000	24.000
-9.000	2107.000	6.000
-9.5228	2099.000	14.000
-10.000	2051.000	15.500
-10.5228	2074.500	1.000
-11.000	2052.750	27.750
TOTAL	2091.500	49.500

WT LIVER - (R)-A-61603 (-GTP)

Conc (log M)	CPM	S.E.M.
NSB	320.750	15.250
-2.000	363.000	22.000
-2.5228	387.500	12.500
-3.000	464.500	35.000
-3.5228	876.750	12.250
-4.000	1252.250	35.250
-4.5228	1531.750	153.250
-5.000	1661.500	97.000
-5.5228	1662.250	55.250
-6.000	1670.250	57.250
-6.5228	1610.000	62.000
-7.000	1860.250	116.750
-7.5228	1792.250	86.750
-8.000	1772.000	101.500
-8.5228	1723.000	114.000
-9.000	1771.250	139.750
-9.5228	1663.250	210.750
-10.000	1708.500	146.500
-10.5228	1767.250	137.250
-11.000	1698.750	134.750
TOTAL	1693.750	149.750

WT LIVER - (R)-A-61603 (+GTP)

Conc (log M)	CPM	S.E.M.
NSB	41.83333	4.094033
-4.000	42.515	3.214647
-4.5228	49.34667	1.331329
-5.000	64.19333	6.049585
-5.5228	94.445	5.749992
-6.000	125.4233	7.372498
-6.5228	494.2417	10.26778
-7.000	980.8117	9.726798
-7.5228	1658.153	31.20791
-8.000	2205.767	25.79542
-8.5228	2362.785	45.48272
-9.000	2351.620	17.67497
-10.000	2306.535	31.69616
-11.000	2372.653	30.16128
TOTAL	2381.739	35.06498

WT LIVER - L765,314

Conc (log M)	CPM	S.E.M.
NSB	110.500	2.500
-3.000	108.250	0.749
-3.5228	110.250	2.250
-4.000	142.500	2.500
-4.5228	257.250	8.250
-5.000	463.500	7.000
-5.5228	1047.750	17.750
-6.000	1935.500	73.00
-6.5228	3346.750	5.750
-7.000	3802.000	181.0
-7.5228	4049.250	60.250
-8.000	4153.750	63.750
-8.5228	4141.000	87.500
-9.000	4244.500	95.999
-9.5228	4119.250	55.750
-10.000	4267.750	35.750
-10.5228	4246.250	130.750
-11.000	4335.750	87.750
TOTAL	4126.500	149.000

WT LIVER - BMY7378

Conc (log M)	CPM	S.E.M.
NSB	103.750	11.250
-4.000	108.750	13.750
-4.5228	108.750	7.750
-5.000	118.500	23.000
-5.5228	116.000	20.500
-6.000	140.750	24.750
-6.5228	158.750	43.750
-7.000	180.750	18.250
-7.5228	193.750	20.750
-8.000	213.750	31.750
-8.5228	241.000	52.500
-9.000	290.000	92.000
-9.5228	337.750	109.250
-10.000	308.000	88.500
-10.5228	306.750	75.750
-11.000	319.750	111.250
-11.5228	323.500	102.500
-12.000	311.500	95.999
TOTAL	301.500	81.500

KO LIVER - PRAZOSIN

Conc (log M)	CPM	S.E.M.
NSB	210.000	6.000
-5.000	251.500	7.500
-5.5228	226.500	7.500
-6.000	230.500	0.500
-6.5228	235.500	6.500
-7.000	228.000	8.000
-7.5228	234.500	20.500
-8.000	259.500	6.500
-8.5228	292.500	6.500
-9.000	316.500	8.500
-9.5228	338.000	6.000
-10.000	374.000	8.000
-10.5228	365.000	15.000
-11.000	372.500	0.500
-11.5228	373.000	7.000
-12.000	379.000	3.000
-12.5228	366.500	13.500
-13.000	374.000	17.000
TOTAL	398.000	48.000

KO LIVER - RS100329

Conc (log M)	CPM	S.E.M.
NSB	524.250	1.750
-3.000	535.500	9.000
-3.5228	578.500	48.000
-4.000	556.000	27.500
-4.5228	571.250	13.750
-5.000	591.750	20.750
-5.5228	567.250	3.250
-6.000	554.000	1.000
-6.5228	554.000	6.000
-7.000	639.000	31.500
-7.5228	688.250	36.750
-8.000	716.000	43.000
-8.5228	757.500	75.500
-9.000	742.250	60.250
-9.5228	743.000	72.500
-10.000	742.000	69.500
-10.5228	781.500	62.000
-11.000	733.000	56.500
TOTAL	741.500	66.500

KO LIVER - (R)-A-61603 (-GTP)

Conc (log M)	CPM	S.E.M.
NSB	754.500	71.000
-3.000	834.250	142.250
-3.5228	863.000	119.000
-4.000	912.750	138.750
-4.5228	869.750	120.250
-5.000	897.750	154.250
-5.5228	926.500	62.500
-6.000	879.750	73.250
-6.5228	934.500	39.500
-7.000	939.750	60.750
-7.5228	1082.250	117.750
-8.000	1238.750	193.250
-8.5228	1184.250	18.750
-9.000	1275.500	94.50001
-9.5228	1352.000	101.000
-10.000	1293.500	53.000
-10.5228	1326.750	96.750
-11.000	1380.500	220.500
TOTAL	1240.750	72.250

KO LIVER - (R)-A-61603 (+GTP)

Conc (log M)	CPM	S.E.M.
NSB	110.000	10.000
-4.000	134.300	12.100
-4.5228	113.000	16.600
-5.000	125.400	20.900
-5.5228	143.900	18.600
-6.000	137.100	18.900
-6.5228	250.700	13.500
-7.000	377.100	19.500
-7.5228	447.100	24.900
-8.000	466.100	34.400
-8.5228	583.700	35.500
-9.000	579.400	8.800
-9.5228	608.800	29.300
-10.000	567.400	16.500
TOTAL	559.000	25.000

KO LIVER - L765,314

Conc (log M)	CPM	S.E.M.
NSB	108.000	7.000
-3.000	126.000	3.500
-3.5228	128.750	5.750
-4.000	117.750	2.750
-4.5228	139.750	0.250
-5.000	145.750	0.749
-5.5228	162.000	3.500
-6.000	204.250	2.750
-6.5228	240.750	17.750
-7.000	230.000	8.000
-7.5228	229.000	5.000
-8.000	248.750	24.250
-8.5228	256.750	10.250
-9.000	253.500	25.000
-9.5228	242.500	22.500
-10.000	247.000	16.000
-10.5228	246.750	27.750
-11.000	241.000	20.500
TOTAL	253.750	15.250

KO LIVER - BMY7378