$\alpha_4$-Adrenoceptor subtypes in the rat isolated epididymal vas deferens, spleen and human prostate and their signal transduction mechanisms

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Abstract

The aims of this investigation were (1) to functionally characterize the $\alpha_\text{1}^-$ adrenoceptor mediating contractions of the rat isolated epididymal vas deferens, spleen and human prostate using $\alpha_\text{1}^-$adrenoceptor subtype selective antagonists, and (2) to study the possible mechanisms of contraction to $\alpha_\text{1}^-$adrenoceptor stimulation in these tissues.

Contractions mediated by $\alpha_\text{1}^-$adrenoceptors in the rat epididymal vas deferens and human prostate were antagonized by subtype selective competitive antagonists with $pA_2$ values consistent with their affinities in binding studies on tissue $\alpha_{1\text{a}}^-$adrenoceptors and correlated best with the antagonists published affinities for the expressed $\alpha_{1\text{a}}^-$adrenoceptor clone. For the rat spleen $\alpha_\text{1}^-$adrenoceptor mediated contractions the antagonist $pA_2$ values were consistent with their affinities in binding studies on tissue $\alpha_{1\text{b}}^-$adrenoceptors and correlated best with the antagonists published affinities for the expressed $\alpha_{1\text{b}}^-$adrenoceptor clone. It was concluded that the functional $\alpha_\text{1}^-$adrenoceptor in the rat epididymal vas deferens and human prostate was the $\alpha_{1\text{a}}^-$subtype and this is the same as the expressed $\alpha_{1\text{a}}^-$clone, while in the rat spleen it was the $\alpha_{1\text{b}}^-$subtype and this is the same as the expressed $\alpha_{1\text{b}}^-$clone.

The $\alpha_{1\text{a}}^-$adrenoceptor mediated contraction of the rat epididymal vas deferens appeared to depend upon activation of protein kinase C by diacylglycerol, resulting in the influx of extracellular $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels. The $\alpha_{1\text{b}}^-$adrenoceptor mediated contraction of the rat spleen consisted of an initial phasic contraction due to release of intracellular $Ca^{2+}$ and a larger tonic contraction due to capacitative $Ca^{2+}$ influx through non-voltage-gated $Ca^{2+}$ channels and which may involve a tyrosine kinase. The $\alpha_{1\text{a}}^-$adrenoceptor mediated contraction in the human prostate was dependent on both influx of extracellular $Ca^{2+}$ through a partially nifedipine sensitive channel and a component which may possibly be due to either release of $Ca^{2+}$ from ryanodine sensitive intracellular stores or a $Ca^{2+}$ independent mechanism.
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Abbreviations

Ca$^{2+}$ calcium ions
$[\text{Ca}^{2+}]_i$ cystolic intracellular Ca$^{2+}$ concentration
cAMP cyclic adenosine monophosphate
cDNA complementary DNA
CIF Ca$^{2+}$ influx factor
Da daltons
DAG diaclylglycerol
DMSO dimethyl sulphoxide
DNA deoxyribonucleic acid
DR dose ratio
EGTA ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid
g grams
GDP guanosine diphosphate
GTP guanosine triphosphate
IP$_3$ inositol 1,4,5-trisphosphate
IP$_4$ inositol 1,3,4,5-tetrakisphosphate
K$^+$ potassium ions
M molar
mRNA messenger ribonucleic acid
mM millimolar
PDBu phorbol-12,13-dibutyrate
PIP$_2$ phosphatidylinositol 4,5-bisphosphate
PKC protein kinase C
PLA$_2$ phospholipase A$_2$
PLC phospholipase C
PLD phospholipase D
R 59022 [6-(2-((4-((p-fluorophenyl)phenyl)methylene)-1-piperidinyl)ethyl)-7-methyl-5H-thiazolo(3,2-a)pyrimidine-5-one]
RNA ribonucleic acid
s.e.mean standard error of the mean
U-57,908 [1,6-bis-(cyclohexyloximinocarbonylamino)-hexane]
Chapter 1.

Introduction

1.1 The adrenoceptors

In 1895 it was observed by Oliver and Schafer that crude extracts from the adrenal medulla produced an increase in blood pressure and that the effects of this extract paralleled those of sympathetic nerve stimulation (Langley, 1901). The active compound in this extract was isolated and purified by Takamine (1902) and termed adrenalin. The idea that an adrenalin-like substance is released from sympathetic nerve terminals when stimulated was first proposed by Elliot (1905) and it was later shown that noradrenaline was the substance released upon sympathetic nerve stimulation (Peart, 1949).

In 1905 Langley proposed that tissues being stimulated by adrenalin had excitatory or inhibitory receptive substances on their cells to explain the effects of nerve stimulation. Dale (1906) also referred to a “receptive mechanism for adrenalin” and showed that ergot could selectively block the effects of adrenalin on blood pressure but not those on heart rate, thus demonstrating the selective antagonism of what would become known as α- and β- adrenoceptors.

1.1.1 α- and β- Adrenoceptor subtypes

Ahlquist (1948) described two types of adrenoceptor which could not be simply classified as inhibitory or excitatory. He measured the rank order of potency for several catecholamines in functional tests involving adrenoceptor mediated responses and found two different orders of potency. One order, with noradrenaline being more potent than isoprenaline, was found for predominantly excitatory effects (apart from in the intestine). The other order, with isoprenaline being more potent than noradrenaline, was found for predominantly inhibitory effects (apart from in the heart). Ahlquist interpreted these findings to represent
different types of adrenoceptor. He termed the mainly excitatory receptors α-adrenoceptors and the mainly inhibitory receptors β-adrenoceptors.

The existence of both α- and β- adrenoceptors received firmer experimental evidence with the development of α- and β- antagonists. Ergot (Dale, 1906) and dibenamine (Nickerson, 1949) were subsequently shown to block α-adrenoceptor mediated but not β-adrenoceptor mediated responses. Dichloroisoproterenol was later found to block β-adrenoceptors in the heart but not α-adrenoceptors (Moran & Perkins, 1958; Powell & Slater, 1958).

1.1.2 β₁- and β₂- Adrenoceptors

β-Adrenoceptors were further classified into β₁- and β₂- adrenoceptors again based on rank orders of potency for catecholamines on a number of different β-adrenoceptor mediated responses (Lands et al., 1967). Evidence showing not only the existence of both α- and β-adrenoceptors but possible differences in β-adrenoceptors was also shown by Furchgott (1967) using the β-adrenoceptor antagonist pronethalol.

1.1.3 α₁- and α₂- Adrenoceptors

The subclassification of α-adrenoceptors took longer due partly to their existence both pre- and post- synaptically. Inhibitory presynaptic α-adrenoceptors were demonstrated by Starke et al. (1971) showing that tritiated noradrenaline release from nerve terminals was enhanced by phentolamine. Starke (1972) and Langer (1974) then classified α-adrenoceptors based on anatomical location, with postsynaptic receptors, thought to be excitatory in function, termed α₁-adrenoceptors, and presynaptic receptors, thought to be inhibitory in function, termed α₂-adrenoceptors. It was noted by Berthelsen & Pettinger (1977) however that some postsynaptic α-adrenoceptor mediated responses were inhibitory and also had the same order of agonist potency as found for the presynaptic receptor, which was different from the postsynaptic excitatory receptors. They therefore proposed a functional classification with the
inhibitory functions being mediated by $\alpha_\text{2}$-adrenoceptors. The two $\alpha$-adrenoceptor subtypes were finally characterized pharmacologically using the selective $\alpha_\text{2}$-adrenoceptor agonist clonidine and the selective $\alpha_\text{1}$-adrenoceptor antagonist phenoxybenzamine (Dubocovich & Langer, 1974; Starke et al., 1975). The functional classification of $\alpha$-adrenoceptors was later found to be flawed by the discovery of postjunctional $\alpha_\text{2}$-adrenoceptor mediated vasoconstriction (Drew & Whiting, 1979). $\alpha_\text{1}$-Adrenoceptors are now most commonly characterized by the high affinity of the antagonist prazosin for them (Cambridge et al., 1977; Doxey et al., 1977) and their stimulation by the agonists phenylephrine and methoxamine (Wilson et al., 1991). $\alpha_\text{2}$-Adrenoceptors are characterized by the high affinity of the antagonists rauwolscine and idazoxan and their stimulation by the agonists clonidine and UK-14,304 (Wilson et al., 1991).

1.2 $\alpha_\text{1}$-Adrenoceptor Subtypes

1.2.1 Early observations

Subdivision of $\alpha_\text{1}$-adrenoceptors was first proposed by McGrath (1982) based on differing effects of structurally unrelated $\alpha$-adrenoceptor agonists in the rat anococcygeus and rat vas deferens. In the rat anococcygeus phenylethanolamines produced biphasic concentration-response curves, while non-phenylethanolamines produced monophasic concentration-response curves. In the rat epididymal vas deferens oxymetazoline had a similar potency to noradrenaline while in the rat prostatic vas deferens it was much less potent compared to noradrenaline. The receptors mediating responses to lower concentrations of phenylethanolamines and non-phenylethanolamines were termed $\alpha_\text{1A}$-adrenoceptors and those to higher concentrations of phenylethanolamines were termed $\alpha_\text{1B}$-adrenoceptors.

1.2.2 $\alpha_\text{1H}$- and $\alpha_\text{1L}$-classification

Another subclassification of $\alpha_\text{1}$-adrenoceptors was proposed by Flavahan & Vanhoutte (1986) based on work by Holck et al. (1983) which showed varying affinities for prazosin at $\alpha_\text{1}$-adrenoceptors in functional studies. The $\alpha_\text{1}$-
adrenoceptors with a higher affinity for prazosin were termed the $\alpha_{\text{HI}}$-subtype and those with a lower affinity for prazosin were termed the $\alpha_{\text{IL}}$-subtype.

This classification gained support by the identification of high and low prazosin $\alpha_1$-adrenoceptor affinities in functional and binding experiments from dog, rat and rabbit tissues (Muramatsu et al., 1990b). They also used the terms $\alpha_{\text{HI}}$ and $\alpha_{\text{IL}}$ to describe the proposed $\alpha_1$-adrenoceptor subtypes corresponding to high and low affinities for prazosin. A third subtype was also proposed based on a difference in affinity for the antagonist HV 723 at $\alpha_1$-adrenoceptors found to have a low affinity for prazosin. The $\alpha_1$-adrenoceptors with a low affinity for prazosin and a high affinity for HV 723 were termed $\alpha_{\text{IL}}$-adrenoceptors. These subtypes have been further characterized in rabbit tissues (Muramatsu et al., 1990a; Oshita et al., 1993) and rat tissues (Oshita et al., 1991; Ohmura et al., 1992). However there is currently no evidence at the molecular level for $\alpha_1$-adrenoceptors with different affinities for prazosin.

1.2.3 $\alpha_{\text{IA}}$, $\alpha_{\text{IB}}$ and $\alpha_{\text{II}}$ classification

The majority of evidence for the existence of $\alpha_1$-adrenoceptor subtypes has come from differing affinities of a range of antagonists other than prazosin for $\alpha_1$-adrenoceptors in tissues. Biphasic displacement of $[^{3}H]$-prazosin by WB 4101 and phentolamine in rat cerebral cortex suggested there were high and low affinity $\alpha_1$-adrenoceptor binding sites for these antagonists (Battaglia et al., 1983). High and low affinity $\alpha_1$-adrenoceptor binding sites for WB 4101 were also found in rat brain by Morrow & Creese (1986). Functional and binding studies found that the reactive derivative of clonidine, chlorethylclonidine, alkylated some populations of $\alpha_1$-adrenoceptors to a much greater extent than others (Han et al., 1987a,b; Johnston & Minneman, 1987). These chlorethylclonidine sensitive $\alpha_1$-adrenoceptors were found to have a lower affinity for the competitive antagonist WB 4101 compared with the chlorethylclonidine insensitive receptors (Han et al., 1987b; Minneman et al., 1988). Receptors with a higher affinity for WB 4101 were termed the $\alpha_{\text{IA}}$-subtype while those being more sensitive to alkylation by chlorethylclonidine were termed the $\alpha_{\text{IB}}$-subtype.
5-Methyl urapidil was found to have high and low affinity $\alpha_1$-adrenoceptor binding sites in various rat tissues, with the high affinity sites corresponding to the $\alpha_{1A}$-subtype (Gross et al., 1988; Hanft & Gross, 1989), as did (+)-niguldipine (Boer et al., 1989), oxymetazoline (Hanft & Gross, 1989) and benoxathian (Han et al., 1987a). All these observations were made in binding studies in rat tissues but high and low $\alpha_1$-adrenoceptor binding sites for 5-methyl urapidil were also found in human brain (Gross et al., 1989).

The $\alpha_{1A}$- and $\alpha_{1B}$- subtypes were identified in tissues and later identified at the molecular level (see below), however a third $\alpha_1$-adrenoceptor was also cloned which did not correspond to any known subtype at the time. The discovery of a highly selective ligand for this subtype, BMY 7378 (Saussy et al., 1994), has now however revealed the presence of this subtype, called the $\alpha_{1D}$-subtype, in the rat aorta (Saussy et al., 1994; Kenny et al., 1995).

1.2.4 Pharmacology of expressed cloned $\alpha_1$-adrenoceptors

Functionally defined receptors are now denoted by upper case and cloned receptors by lower case letters (Bylund et al., 1994). Two $\alpha_1$-adrenoceptors have been cloned from a rat cerebral cortex cDNA library (using a probe based on a hamster $\alpha_{1E}$-adrenoceptor, Cotecchia et al., 1988), which appeared to have pharmacological profiles in cell lines where they had been expressed corresponding to the $\alpha_{1A}$- and $\alpha_{1B}$- subtypes (Lomasney et al., 1991). However a near identical clone to the one thought to correspond with the $\alpha_{1A}$-subtype was cloned by Perez et al. (1991). When expressed in cell lines this clone had a different pharmacological profile to the $\alpha_{1A}$-subtype as it was found to be partially chlorethylclonidine sensitive and had a lower affinity for 5-methyl urapidil compared with tissue $\alpha_{1A}$-adrenoceptors. It was therefore termed the $\alpha_{1D}$-subtype and the two near identical clones were then sometimes referred to as the $\alpha_{1A/D}$- subtype. Schwinn and Lomasney (1992) subsequently agreed with Perez et al. (1991), showing that their clone when expressed in cell lines also had a low affinity for 5-methyl urapidil and benoxathian. This clone is now referred to as the $\alpha_{1D}$-subtype (Hieble et al., 1995).
A third $\alpha_2$-adrenoceptor has also been cloned, initially from a bovine brain cDNA library which when expressed had a similar pharmacology to the $\alpha_{1\alpha}$-subtype except that it was also partially chlorethylclonidine sensitive (Schwinn et al., 1990) and was not thought to be expressed in the rat as it could not be detected by Northern blot analysis (Schwinn et al., 1991). This clone was therefore thought to represent a new subtype and was termed $\alpha_2c$- (Schwinn et al., 1990). However a homologous clone of this subtype has been found in the rat (Laz et al., 1993; Perez et al., 1994) and shown to be expressed in several rat tissues using RNAs protection assays (Perez et al., 1994; Rokosh et al., 1994). It appears that the rat $\alpha_{1c}$-clone is quite insensitive to chlorethylclonidine compared with the bovine clone (Laz et al., 1993; Forray et al., 1994a) and binding studies have shown tissue $\alpha_{1\alpha}$-adrenoceptors and the expressed cloned $\alpha_{1c}$-adrenoceptor to be pharmacologically very similar (Laz et al., 1994; Perez et al., 1994). Based on this evidence the $\alpha_{1c}$-clone is now thought to correspond to the tissue $\alpha_{1\alpha}$-adrenoceptor and has now been renamed the $\alpha_2$-clone (Hieble et al., 1995), as it will be referred to in the remainder of the thesis. All three subtypes have now also been cloned from human tissue (Bruno et al., 1991; Ramarao et al., 1992; Hirasawa et al., 1993).

The affinities of a range of different antagonists have been measured for the three expressed $\alpha_2$-clones in binding studies using membranes from cells transfected with the corresponding cDNA (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Saussy et al., 1994; Testa et al., 1994 and Goetz et al., 1995). Prazosin was found to be non-selective between the subtypes, while WB 4101, 5-methyl urapidil, phentolamine, benoxathian and indoramin were found to have highest affinity for the $\alpha_{1\alpha}$-subtype (as they do for the $\alpha_{1\alpha}$-subtype found in tissues) and spiperone had a slightly higher affinity for the $\alpha_{1b}$-subtype. BMY 7378 has recently been shown to be very selective for the $\alpha_{1d}$-subtype (Saussy et al., 1994) which until recently had not been identified in any tissue (Goetz et al., 1995).
1.2.5 Distribution of α-adrenoceptor subtypes in rat tissues

All three α-adrenoceptor subtype clones have been found to be expressed in various rat tissues. At first, using Northern blot analysis, it was thought that the α, subtype clone was not expressed in any rat tissue (Schwinn et al., 1990), however using the more sensitive RNase protection assay it has been found to be expressed in cerebral cortex, submaxillary gland, lung, heart, kidney, aorta, vena cava and vas deferens (Perez et al., 1994; Price et al., 1994a; Rokosh et al., 1994). The αia-subtype clone was found to be expressed in cerebral cortex, heart, liver, kidney, lung and spleen and the αia-subtype clone to be expressed in brain, lung, heart and vas deferens (Price et al., 1994a).

Binding studies have shown both αia- and αias- subtypes to exist in the rat brain (Morrow & Creese, 1986), heart (Michel et al., 1994), kidney (Michel et al., 1993) and vas deferens (Gross et al., 1988). A single population of α-adrenoceptors shown to be of the αia-subtype has been identified in rat submaxillary gland (Michel et al., 1989). Some caution is needed with early characterization studies which were interpreted before the existence of the αia-subtype was appreciated. These relied, at least in part, on a high affinity for WB 4101 to indicate the presence of the αia-subtype, although it is now clear that the affinity of WB 4101 for the αia-subtype is nearly the same. For example, all three subtypes were shown in rat lung but only 5-methyl urapidil could distinguish between the αia- and αias- subtypes (Hiramatsu et al., 1994). A single population of αias-adrenoceptors has been characterized in the rat liver (Garcia-Sainz et al., 1992), spleen (Veenstra et al., 1992), white fat cells (Torres-Marquez et al., 1992) and vena cava (Sayet et al., 1993). The αias-subtype has been identified in the rat aorta (Saussy et al., 1994), although this does not appear to be a homogeneous population of α-adrenoceptors. Again some caution is needed with the conclusions reached by early characterization studies as identification of the αias-subtype may have relied on susceptibility to alkylation by chlorehthylclonidine to which the αias-subtype is also now known to be sensitive, as in rat aorta (Aboud et al., 1993; Saussy et al., 1994).
Functional evidence has suggested that $\alpha_{1a}$-adrenoceptors mediate contractions of the rat vas deferens (Han et al., 1987b; Aboud et al., 1993), portal vein (Lepretre et al., 1994), renal artery (Han et al., 1990) and vasoconstrictor responses of the perfused mesentery (Williams & Clarke, 1995) and kidney (Blue et al., 1995). $\alpha_{1}$-Adrenoceptor contractions of the rat spleen (Han et al., 1987b; Aboud et al., 1993) and vena cava (Sayet et al., 1993) have been suggested to be mediated by the $\alpha_{1a}$-subtype, while contraction of the rat aorta to noradrenaline appears to be mediated predominantly by the $\alpha_{1d}$-subtype (Saussy et al., 1994; Kenny et al., 1995).

1.2.6 Distribution of $\alpha_{1}$-adrenoceptor subtypes in human tissues

Expression of all three cloned $\alpha_{1}$-adrenoceptor subtypes has been found in human tissues by RNAse protection assays. The $\alpha_{1a}$-subtype was found in the prostate, liver, heart, cerebellum and cerebral cortex. The $\alpha_{1b}$-subtype was found in the prostate, spleen, kidney, cerebellum and cerebral cortex, and the $\alpha_{1d}$ subtype in the prostate, heart, spleen, aorta and cerebral cortex (Price et al., 1993; Price et al., 1994b). Binding studies have identified both $\alpha_{1a}$- and $\alpha_{1b}$- subtypes in human brain (Gross et al., 1989). Both WB 4101 and 5-methyl urapidil bound with high and low affinity in prostate suggesting the presence of the $\alpha_{1a}$-subtype and also either the $\alpha_{1b}$- or $\alpha_{1d}$- subtypes or possibly all three (Lepor et al., 1993a).

1.2.7 $\alpha_{1}$-Adrenoceptor antagonists and benign prostatic hyperplasia

Benign prostatic hyperplasia is frequent in elderly males with a prevalence of 43% in those over 65 displaying some symptoms (Garraway et al., 1991). Bladder outlet obstruction caused by this condition has two components: static, related to cellular mass and dynamic, related to prostatic smooth muscle tone. One pharmacological approach in treatment is to relax prostatic smooth muscle by antagonizing $\alpha_{1}$-adrenoceptors.

It has been known for many years that stimulation of the pre-sacral sympathetic nerves in man causes the prostate to contract (Learmonth, 1931). Subsequent work has shown that $\alpha$-adrenoceptors mediated contraction of the
prostate (Caine et al., 1975) and ligand binding experiments revealed both \( \alpha_r \)- and \( \alpha_\tau \)-adrenoceptors, the former predominating in the stroma (Chapple et al., 1989; James et al., 1989). Experiments on isolated prostatic tissue showed that it was only the \( \alpha_\tau \)-adrenoceptors which mediated contraction of the tissue (Hieble et al., 1985; Chapple et al., 1989). These studies provided a scientific basis for the use of selective \( \alpha_r \)-adrenoceptor antagonists e.g. prazosin, in the treatment of benign prostatic hyperplasia (e.g. Chapple et al., 1990) after initial trials with phenoxybenzamine (Caine et al., 1978). While prazosin may provide symptomatic relief including improved urinary flow rate, there is a therapeutic ceiling to the dose which can be employed due to side effects e.g. hypotension, which also arise from \( \alpha_r \)-adrenoceptor antagonism. However if the \( \alpha_\tau \)-adrenoceptor subtype mediating contraction in the prostate is different to those in the vasculature then an antagonist selective for the prostatic \( \alpha_r \)-adrenoceptor subtype might have a higher therapeutic ceiling with reduced cardiovascular side effects.

1.3 Molecular biology of \( \alpha_r \)-adrenoceptors

1.3.1 Isolation of the \( \alpha_r \)-adrenoceptor and cloning of its cDNA

The first \( \alpha_r \)-adrenoceptor cDNA to be cloned was isolated from a hamster smooth muscle DDT\(_1\)-MF\(_2\) cell line (Cotecchia et al., 1988). \( \alpha_r \)-Adrenoceptors were first purified by detergent extraction from cell membranes, affinity chromatography using an immobilized prazosin analogue, wheat germ lectin chromatography and size exclusion high performance liquid chromatography. The isolated glycoprotein had a molecular weight of 80,000 Da. Three peptides were recovered from reverse phase high performance liquid chromatography after cleavage of the purified receptor at methionine residues. An oligonucleotide probe based on one of the peptides amino acid sequence was labelled and used to screen a genomic library. A single clone was isolated that hybridized to the probe. However this proved to be incomplete as only the sequence for two of the three peptides isolated could be found. A cDNA library constructed from DDT\(_1\)-MF\(_2\) mRNA was then probed using the genomic fragment isolated. From this a single clone was isolated which coded for all three peptides. The deduced protein
coded for by the clone consisted of 515 amino acids and a molecular weight of 56,000 Da. This suggested that 30% of the receptors molecular weight is carbohydrate and four potential glycosylation sites were identified. Hydrophobicity analysis predicted the isolated protein to have seven putative transmembrane-spanning domains as for the rhodopsin receptor (Applebury & Hargrave, 1986), typical for a G-protein-linked receptor. The cDNA was inserted into an expression vector which was used to transiently transfect COS-7 cells. The cells expressed the $\alpha_r$-adrenoceptor that had been cloned and pharmacological characterization using binding studies showed it to be the $\alpha_{1b}$-subtype (Cotecchia et al., 1988).

1.3.2 Cloning of different $\alpha_r$-adrenoceptor subtypes

Using the hamster $\alpha_{1b}$-adrenoceptor as a probe, the cDNA for two $\alpha_r$-adrenoceptors was cloned from a rat brain cDNA library (Lomasney et al., 1991) which were eventually shown to be the rat $\alpha_{1b}$- and $\alpha_{1d}$-adrenoceptor subtypes. The cDNA for the $\alpha_{1a}$-subtype was cloned from bovine brain again using the hamster clone (Schwinn et al., 1990) and the corresponding rat $\alpha_{1a}$-subtype was then cloned using a probe based on the bovine $\alpha_{1a}$-clone (Laz et al., 1994). Hydrophobicity analysis again predicted all of these expressed clones to have seven putative transmembrane-spanning domains typical of G-protein-linked receptors.

The rat $\alpha_{1b}$-clone encoded for a 515 amino acid protein with 98.1% sequence homology with the hamster $\alpha_{1b}$-clone. The rat $\alpha_{1d}$-clone encoded for a 560 amino acid protein with 73% amino acid sequence homology with the rat $\alpha_{1b}$-clone and 63% with the bovine $\alpha_{1a}$-clone for the seven hydrophobic regions. Two potential glycosylation sites in the NH$_2$ terminus were found. The bovine $\alpha_{1a}$-clone encoded for a 466 amino acid protein with about 72% sequence homology within the putative seven transmembrane-spanning region with the hamster $\alpha_{1b}$-clone and 52% overall homology. Three potential glycosylation sites in the NH$_2$ terminus were found. The rat $\alpha_{1a}$-clone encoded for a 466 amino acid protein and has 91% homology with the bovine $\alpha_{1a}$-clone and 49% and 53% with the rat $\alpha_{1d}$-
and \( \alpha_{ia} \)-clones respectively. The rat \( \alpha_{ia} \)-clone had four potential glycosylation sites.

1.3.3 Cloning of the human \( \alpha_i \)-adrenoceptor subtypes

The cDNA for the human \( \alpha_{ia} \)-clone, (referred to as the \( \alpha_{ia} \)-subtype by Bruno et al.) was cloned from a human hippocampus cDNA library (Bruno et al., 1991). It encoded for a 501 amino acid protein with 78% homology with the rat \( \alpha_{id} \)-clone and 95% within the putative transmembrane-spanning domains. However, there were errors in this clone with substantial differences in the 5' end of the rat and human \( \alpha_{ia} \)-clones. Its cDNA has now been deduced from the two exons of a genomic sequence for this receptor (Weinberg et al., 1994). This clone encoded for a protein with 82% homology with the rat \( \alpha_{id} \)-clone with two potential glycosylation sites. The human \( \alpha_{ia} \)-gene was cloned by screening a human genomic library with a probe based on the hamster \( \alpha_{ia} \)-clone (as a cDNA was not obtained by screening a cDNA library) and the two exons of the gene were then spliced together (Ramarao et al., 1992). This resulted in a clone that encoded for a 517 amino acid protein with 98% homology with the expressed hamster \( \alpha_{ia} \)-clone. Four potential glycosylation sites were also identified. The human \( \alpha_{ia} \)-clone was isolated from a human prostate cDNA library and encoded for a 466 amino acid protein (Hirasawa et al., 1993). It has 92% homology with the expressed bovine \( \alpha_{ia} \)-clone and 58% homology with the expressed human \( \alpha_{id} \)-clone. Three potential glycosylation sites were also identified. All the expressed human \( \alpha_{i} \)-adrenoceptor clones were predicted to have seven putative transmembrane-spanning domains typical of G-protein-linked receptors.

1.3.4 \( \alpha_i \)-Adrenoceptor gene structure

The genes encoding for all three human \( \alpha_i \)-adrenoceptor subtypes contain an intron within the sixth transmembrane domain (Weinberg et al., 1994). The hamster \( \alpha_{ia} \)- and bovine \( \alpha_{ia} \)-adrenoceptor genes also contain an intron (Cotecchia et al., 1988; Schwinn et al., 1990). Alternative splicing of the exons producing multiple splice variants could therefore result in the existence of different \( \alpha_i \)-subtypes. The two exon structure is probably characteristic of all \( \alpha_i \)-adrenoceptor
genes. In contrast, all of the genes encoding for $\alpha_\text{r}$- and $\beta$- adrenoceptors do not contain introns.

1.3.5 Phosphorylation of $\alpha_\text{r}$-adrenoceptors

Several threonines and serines were found in the second and third cytoplasmic loops representing potential protein kinase C (PKC) phosphorylation sites on the expressed hamster $\alpha_{1b}$-, rat and bovine $\alpha_{1a}$- and rat $\alpha_{1d}$- clones. In the third cytoplasmic loop there is also a site for protein kinase A phosphorylation on the expressed hamster $\alpha_{1b}$- and the bovine and rat $\alpha_{1a}$- clones but not for the rat $\alpha_{1d}$-clone. Phosphorylation has been shown to be a mechanism of regulation for the $\alpha_\text{r}$-adrenoceptors (Bouvier et al., 1987; Leeb-Lundberg et al., 1987). G-protein linked receptors are thought to interact with G-proteins through the second and third cytoplasmic loops and so phosphorylation of this region might prevent this interaction and so be a mechanism for receptor desensitization. As stimulation of $\alpha_\text{r}$-adrenoceptors can lead to increased PKC activity, this could result in a feedback mechanism for $\alpha_\text{r}$-adrenoceptor regulation. The three human $\alpha_\text{r}$-adrenoceptor subtypes also contain sites for potential phosphorylation in the cytoplasmic loops (Weinberg et al., 1994).

1.4 $\alpha_\text{r}$-Adrenoceptor signal transduction mechanisms

1.4.1 Ca$^{2+}$ and smooth muscle contraction

Contraction of smooth muscle is regulated primarily by the [Ca$^{2+}$], and stimuli which induce contraction such as receptor agonists or cell membrane depolarization usually bring about a rise in [Ca$^{2+}$]. The rise in [Ca$^{2+}$], may be brought about either by an influx of extracellular Ca$^{2+}$ or by mobilization of Ca$^{2+}$ from intracellular stores thought to be within the sarcoplasmic reticulum. When [Ca$^{2+}$], is raised, Ca$^{2+}$ is thought to bind to calmodulin, which then interacts with myosin light chain kinase. This results in the activation of myosin light chain kinase leading to phosphorylation of myosin and contraction (Walsh, 1991).
Contraction can also be regulated by increased Ca\(^{2+}\) sensitivity of the smooth muscle cells. This is due to changing activities of the phosphorylating and dephosphorylating enzymes involved in myosin light chain phosphorylation, resulting in generation of force without a change in \([\text{Ca}^{2+}]\), (Somlyo & Somlyo, 1994). Arachidonic acid has been shown to be a Ca\(^{2+}\) sensitizing agent, increasing myosin light chain phosphorylation and causing smooth muscle contraction at constant \([\text{Ca}^{2+}]\), (Gong et al., 1992).

\(\alpha_\text{r-Adrenoceptors}\) generally mediate their cellular functions such as smooth muscle contraction when stimulated by producing a rise in \([\text{Ca}^{2+}]\), (Minneman, 1988). Contraction of the rat vas deferens to noradrenaline has been shown to be dependent on extracellular Ca\(^{2+}\), while in the rat spleen it was only partially dependent on extracellular Ca\(^{2+}\) (Han et al., 1987b). The influx of extracellular Ca\(^{2+}\) to noradrenaline stimulation has also been shown to be through a nifedipine sensitive (which selectively blocks voltage-gated Ca\(^{2+}\) channels) pathway in the rat vas deferens and an insensitive pathway in the rat spleen (Han et al., 1987b). In the rat aorta \(\alpha_\text{r-adrenoceptor}\) mediated contractions have been shown to be dependent on both influx of extracellular Ca\(^{2+}\) and mobilization of intracellular Ca\(^{2+}\) in functional studies (Oriowo & Ruffolo, 1992) and by intracellular Ca\(^{2+}\) measurements (Chiu et al., 1986). Contraction of the rabbit aorta to noradrenaline is also dependent on intracellular and extracellular Ca\(^{2+}\) (Suzuki et al., 1990). \(\alpha_\text{r-Adrenoceptor}\) stimulation has also been shown to mobilize intracellular Ca\(^{2+}\) in rat portal vein myocytes (Lepretre et al., 1994). It has been suggested that the initial phasic contractions in vascular smooth muscle are less dependent on influx of extracellular Ca\(^{2+}\) compared with the more slowly developing tonic contraction (Bevan, 1981).

1.4.2 G-proteins and receptors

\(\alpha_\text{r-Adrenoceptors}\) have been shown to belong to the G-protein coupled family of receptors. G-proteins have a heterotrimeric structure and their function is in coupling a large family of cell surface receptors (G-protein-linked receptors) to effector molecules. G-proteins are composed of three subunits, \(\alpha, \beta, \text{ and } \gamma\).
β and γ, the β and γ subunits are tightly associated whereas the α subunit readily dissociates from the β and γ subunits. Inactive G-proteins exist as a heterotrimer with GDP tightly bound to the α subunit (Spiegel, 1992).

G-protein-linked receptors, including the adrenoceptors, have a predicted structure containing seven putative transmembrane-spanning domains, highly suitable for detecting extracellular signals and transducing their presence into a cytoplasmic signal (Spiegel, 1992). These receptors when in their inactive conformation interact most efficiently with the heterotrimeric form of the G-protein, located on the cytoplasmic surface of the plasma membrane. When the receptor is changed to its active conformation, for example by agonist binding, conformational changes in the intracellular loops of the receptor are thought to catalyze the exchange of the tightly bound GDP for GTP (Strader et al., 1989). In the GDP-bound state the α subunit is in an inactive conformation and binding of GTP brings about a conformational change to the active state (Bourne et al., 1991). This leads to reduced affinity for the βγ subunit heterodimer, from which it dissociates and the activated α subunit can then interact with effector molecules. The α subunit only remains in the active state transiently however as it has intrinsic GTPase activity and upon hydrolysis of the GTP to GDP it reassociates with the βγ subunit heterodimer (Bourne et al., 1990).

Several different G-proteins have been identified both in function and at the molecular level, with different isoforms of the α subunit preferentially interacting with different effector molecules or having opposing effects on the same molecule (Simon et al., 1991). The Gq-protein mediates stimulation of adenylate cyclase leading to synthesis of cAMP, while Gs-proteins inhibit this enzyme and Gr-proteins may be involved in modulating K+ channels (Gilman, 1987). The Gq-protein mediates stimulation of phosphatidylinositol-specific phospholipase C (PLC), which has been shown using purified Gq-proteins (Smrcka et al., 1991; Taylor et al., 1991). Several other subclasses of G-proteins such as Gα-proteins have been identified by their molecular structure. Pertussis
toxin ADP-ribosylates the α subunit of G_{\gamma}- and G_\varepsilon- proteins inactivating them, while cholera toxin ADP-ribosylates the α subunit of the G_{\gamma} subunit resulting in constitutive activation. These toxins have been used to identify pathways involving G_{\gamma}, G_\varepsilon- and G_\zeta- proteins. However pathways involving the G_{\gamma}-subclass are insensitive to these toxins. G_{\gamma}-protein involvement can now be shown using antibodies directed at these proteins (Cerione et al., 1988). The involvement of G-proteins can also be shown using non-hydrolyzable analogues of GTP which leave the α subunit in a permanently activated state, causing persistent activation.

α_i-Adrenoceptor mediated responses have been shown to involve G_{\gamma}-proteins in rat liver cells (Uhing et al., 1986). Cells transfected with all three cloned α_i-adrenoceptor subtypes have been shown to be linked to inositol phosphate formation via a G_{\gamma}-protein (Wu et al., 1992). α_i-Adrenoceptors have also been shown to couple to G_\varepsilon-proteins and stimulation of PLC (Baek et al., 1993). α_i-Adrenoceptors have also been shown to be linked to phospholipase A_2 (PLA_2) via a pertussis toxin sensitive pathway (Burch et al., 1986; Nebigil & Malik, 1992; Perez et al., 1993). In contrast, α_i-adrenoceptors have been shown to couple to G_\varepsilon-proteins and inhibition of adenylate cyclase or G_\varepsilon-proteins and K^+ channel modulation and β-adrenoceptors have been shown to couple to G_\zeta-proteins and stimulation of adenylate cyclase (Gilman, 1987). Further support for the link between α_i-adrenoceptors and G-proteins was shown by mutation of an intracellular region of the α_{in}-adrenoceptor to produce constitutive activation of the receptor resulting in constant G-protein activation and increased inositol phosphate formation in the absence of agonist (Kjelsberg et al., 1992).

1.4.3 Phospholipases and the generation of second messengers

Many Ca^{2+} mobilizing agonists such as the α_i-adrenoceptors initiate their cellular responses by activation of various membrane bound phospholipases such as PLC, phospholipase D (PLD) and PLA_2 via G-proteins (Billah & Anthes, 1990; Lee & Severson, 1994). Activation of phosphatidylinositol-PLC results in
the hydrolysis of the cell membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂), to release the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Berridge, 1993; Lee & Severson, 1994). Michell (1975) provided the first evidence of the physiological significance for phosphoinositide breakdown by agonists showing that it was accompanied with mobilization of intracellular Ca²⁺. IP₃ is released into the cytosol and has been shown to release Ca²⁺ from IP₃ sensitive intracellular Ca²⁺ stores (Irvine, 1992; Berridge, 1993) and DAG remains membrane bound and has been shown to activate PKC (Lee & Severson, 1994). IP₃ can also be phosphorylated to inositol 1,3,4,5-tetrakisphosphate (IP₄) (Irvine, 1992). Activation of PLD or phosphatidylcholine-specific PLC results in hydrolysis of phosphatidylcholine, producing DAG but not IP₃ and PLA₂ hydrolyses phospholipids to liberate arachidonic acid (Billah & Anthes, 1990; Exton, 1990). Phosphatidylcholine is by far the most abundant phospholipid whereas the phosphoinositides comprise less than 0.1% of the total (Billah & Anthes, 1990). The phosphoinositides are resynthesized into the membrane following their breakdown. DAG derived from PIP₂ or phosphatidylcholine can be metabolized to phosphatidic acid by DAG kinase or to arachidonic acid by DAG lipase (Billah & Anthes, 1990; Lee & Severson, 1994).

Most evidence suggests that α₁-adrenoceptors produce a rise in inositol phosphates, suggesting they are linked to activation of PLC via a toxin insensitive G-protein (Fox et al., 1985; Uhing et al., 1986; Minneman, 1988; Wilson & Minneman, 1990). α₁-Adrenoceptors have also been shown to be linked to phosphatidylcholine hydrolysis by either activation of phosphatidylcholine-specific PLC (Slivka et al., 1988; Rapoport & Campbell, 1991) or PLD (Lláhi & Fain, 1992). α₁-Adrenoceptor mediated formation of arachidonic acid by PLA₂ via a pertussis toxin sensitive pathway has also been measured (Burch et al., 1986; Nebigil & Malik, 1992; Perez et al., 1993).

Several subclasses of phosphatidylinositol-specific PLC (α, β, γ, δ, ε) have been identified by protein purification and subsequent cDNA cloning. Evidence suggests that PLC γ is stimulated by a tyrosine kinase and probably
accounts for increased inositol phosphate production via stimulation of tyrosine kinase receptors. In contrast, the PLC β isoforms are regulated by G-proteins accounting for increased inositol phosphate production via stimulation of G-protein-coupled receptors (Berridge, 1993). The G_q protein has greatest efficacy for activation of PLC β_1 and PLC β_3 compared with PLC β_2. Cells transfected with all three cloned α_1-adrenoceptor subtypes have been shown to be linked to PLC β_1 via a G_q protein (Wu et al., 1992).

1.4.4 Protein kinase C (PKC)

Activation of PKC is involved in contraction of some smooth muscle tissues, producing a rise in [Ca^{2+}]_i in most cases, which is dependent on extracellular Ca^{2+} (Lee & Severson, 1994). PKC was first discovered by Inoue et al., (1977) and was then shown to require both Ca^{2+} and phospholipid for activation (Takai et al., 1979). Its structure consists of a regulatory domain with binding sites for Ca^{2+} and lipids and a kinase domain. Ten isoforms of PKC have now been identified some of which do not require Ca^{2+} for activation, but all require phosphatidylycerine. The “classical” Ca^{2+} dependent isoforms includes the α, β_1/β_2 and γ forms, and also require DAG for activation. The “new” Ca^{2+} independent isoforms include the δ, ε, η and θ forms and also require DAG for activation while the “atypical” isoforms ζ and ι do not require Ca^{2+} and also do not have a DAG binding site (Bell & Burns, 1991; Lee & Severson, 1994). The α, β, ε and ζ forms have all been shown to be present in vascular smooth muscle (Singer et al., 1992; Stauble et al., 1993).

The involvement of PKC in smooth muscle contraction can be demonstrated both using inhibitors of this enzyme and PKC activators such as phorbol esters. These methods have suggested that α_1-adrenoceptor mediated contractions in some tissues involves PKC activation (Shimamoto et al., 1993; Yang & Black, 1995).
1.4.5 Inositol phosphates and Ca\textsuperscript{2+} mobilization

IP\textsubscript{3} leads to a rise in [Ca\textsuperscript{2+}], which is not dependent on the presence of extracellular Ca\textsuperscript{2+} as it can release Ca\textsuperscript{2+} into the cytosol from intracellular stores (Streb et al., 1983; Ferris & Snyder, 1992; Berridge, 1993). These intracellular stores are thought to be contained within the sarcoplasmic reticulum and Ca\textsuperscript{2+} is mobilized from them via IP\textsubscript{3} receptors on the stores (Meldolesi et al., 1990). The rise in [Ca\textsuperscript{2+}], due solely to mobilization of Ca\textsuperscript{2+} from intracellular stores by IP\textsubscript{3}, is transient, lasting only a few minutes as the stores have a limited capacity and the plasma membrane Ca\textsuperscript{2+} pump soon restores [Ca\textsuperscript{2+}]\textsubscript{i} to resting levels (Meyer & Stryer, 1990). However the rise in [Ca\textsuperscript{2+}], associated with mobilization of Ca\textsuperscript{2+} from intracellular stores is usually larger and more sustained due to a linked influx of extracellular Ca\textsuperscript{2+} (Berridge, 1990). IP\textsubscript{3} can also be phosphorylated to IP\textsubscript{4} and it has been suggested that IP\textsubscript{4} may enable entry of extracellular calcium in some cells when IP\textsubscript{3} is also present (Morris et al., 1987; Irvine, 1991; Irvine, 1992; Luckhoff & Clapham, 1992).

1.4.6 Capacitative Ca\textsuperscript{2+} Influx

In some cells the influx of extracellular Ca\textsuperscript{2+} associated with mobilization of Ca\textsuperscript{2+} from intracellular stores does not appear to require either the presence of IP\textsubscript{3} or IP\textsubscript{4} once the intracellular Ca\textsuperscript{2+} stores have been depleted (Putney, 1986; 1990; Irvine, 1992). This form of Ca\textsuperscript{2+} influx was called capacitative Ca\textsuperscript{2+} entry by Putney (1986). At first it was thought that extracellular Ca\textsuperscript{2+} could in some way enter the lumen of the endoplasmic reticulum first before being released into the cytosol. However it is now generally agreed that Ca\textsuperscript{2+} directly enters the cytosol across the cell membrane, although the mechanism by which the Ca\textsuperscript{2+} content of the stores controls this remains unclear (Irvine, 1992; Fasolato et al., 1994).

Stimulation of the influx of extracellular Ca\textsuperscript{2+} as a consequence of the depletion of intracellular Ca\textsuperscript{2+} stores has been demonstrated in cells by measuring [Ca\textsuperscript{2+}], (or Mn\textsuperscript{2+} as a surrogate ion) in two ways. Firstly, cells have been stimulated by an agonist in the absence of extracellular Ca\textsuperscript{2+}, which is then washed out. The intracellular Ca\textsuperscript{2+} stores should remain depleted after washout
as there was no influx of extracellular Ca\(^{2+}\) to allow them to refill but inositol phosphate levels should return to resting levels. Then, upon addition of extracellular Ca\(^{2+}\), a rise in [Ca\(^{2+}\)], has been recorded (Jacob, 1990). Secondly, compounds such as cyclopiazonic acid or thapsigargin that do not produce inositol phosphates but deplete intracellular Ca\(^{2+}\) stores by inhibiting the endoplasmic reticulum Ca\(^{2+}\)-ATPase pump (Seidler et al., 1989; Deng & Kwan, 1991) stimulate influx of extracellular Ca\(^{2+}\) (Jacob, 1990). Capacitative Ca\(^{2+}\) influx can be reduced by non-specific Ca\(^{2+}\) channel blockers but not nifedipine (Hoth & Penner, 1992).

1.4.7 Ryanodine sensitive intracellular Ca\(^{2+}\) stores

Apart from IP\(_3\) sensitive intracellular Ca\(^{2+}\) stores there are also IP\(_3\) insensitive stores, which have become known as ryanodine sensitive stores due to the modulation of Ca\(^{2+}\) release from them by the plant alkaloid ryanodine (Ehrlich et al., 1994). Ryanodine receptors have been identified through which Ca\(^{2+}\) is thought to be released from these stores (Sorrentino & Volpe, 1993). Some cells contain only ryanodine sensitive Ca\(^{2+}\) stores such as skeletal muscle, while atrial cells and vascular smooth muscle cells contain both IP\(_3\)- and ryanodine-sensitive stores.

A number of mechanisms exist for activating the ryanodine receptor including Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release (McPherson & Campbell, 1993). In this case Ca\(^{2+}\) either entering the cell across the plasma membrane or released from IP\(_3\) sensitive intracellular Ca\(^{2+}\) stores is the stimulus, forming a positive feedback mechanism. In cardiac cells a small influx of Ca\(^{2+}\) through voltage-operated Ca\(^{2+}\) channels triggers release of Ca\(^{2+}\) from the sarcoplasmic reticulum. In skeletal muscle electromechanical coupling between the sarcoplasmic reticulum and the dihydropyridine receptor of the t-tubulular membrane opens the ryanodine receptor (Tanabe et al., 1990). Recently cADP ribose has been shown to activate the ryanodine receptor and has been proposed to be a novel second messenger (Lee et al., 1989; Galione et al., 1991; Galione, 1993).
The mechanism by which ryanodine interacts with ryanodine receptors remains uncertain. It is thought to open the channels at low concentrations and block them at higher concentrations, although the inhibitory actions may be due to a depletion of the Ca\(^{2+}\) stores. In functional studies ryanodine has been shown to inhibit contractions to noradrenaline in rat aorta (Julou-Schaeffner & Freslon, 1988) rabbit ear artery (Kanmura et al., 1988) and rabbit mesenteric artery (Itoh et al., 1992).
1.5 Aims of the present study

Functional studies using chlorethylclonidine and WB 4101 have suggested that the $\alpha_\text{\textsubscript{\textgamma}}$-adrenoceptor subtype mediating contraction of the rat vas deferens and spleen are the $\alpha_\text{\textsubscript{\textalpha}}$- and $\alpha_\text{\textsubscript{\textbeta}}$- subtypes respectively (Han et al., 1987b). However the cloning and pharmacological characterization of the $\alpha_\text{\textsubscript{\textbeta}}$-subtype has made the use of these antagonists unreliable as criteria for identification as this subtype is also sensitive to alkylation by chlorethylclonidine and has a high affinity for WB 4101 (Chapter 1.2). Therefore the aim of this study in Chapters 3 and 4 was to characterize the $\alpha_\text{\textgamma}$-adrenoceptor subtype mediating contraction of the rat isolated epididymal vas deferens and spleen respectively in functional studies using a range of $\alpha_\text{\textgamma}$-adrenoceptor subtype selective antagonists. Their affinities for the subtypes in the rat tissues were then correlated with the antagonists published affinities for the expressed cloned $\alpha_\text{\textgamma}$-adrenoceptor subtypes.

$\alpha_\text{\textgamma}$-Adrenoceptor antagonists are used in the treatment of benign prostatic hyperplasia (Chapter 1.2.7). The development of a selective antagonist for the $\alpha_\text{\textgamma}$-adrenoceptor subtype mediating contraction of the human prostate might have increased efficacy and reduced cardiovascular side effects. Therefore the aim of the study in Chapter 5 was to characterize the $\alpha_\text{\textgamma}$-adrenoceptor subtype mediating contraction of human isolated prostatic smooth muscle in functional studies using the same range of antagonists used on the rat tissues. Their affinities for the subtype in the human prostate were also correlated with the antagonists published affinities for the expressed cloned $\alpha_\text{\textgamma}$-adrenoceptor subtypes.

$\alpha_\text{\textgamma}$-Adrenoceptors are generally thought to mediate contraction of smooth muscle by increasing $[\text{Ca}^{2+}]$, via the stimulation of PLC and production of inositol phosphates and DAG (Chapter 1.4). The aims of the study in Chapters 6, 7 and 8 were therefore to study the possible mechanisms of contraction to stimulation of the $\alpha_\text{\textgamma}$-adrenoceptors in the rat epididymal vas deferens, spleen and human prostate respectively in functional experiments.
Chapter 2.

Methods and Materials

2.1 Preparation of Tissues For Isometric Tension Recordings

2.1.1 Rat tissues

Male Sprague Dawley rats between 350-450g were stunned and killed by cervical dislocation. The vasa deferentia were removed, associated blood vessels and mesentery were dissected away and were then bisected so that only the epididymal portion (15-20mm in length) was used. The spleen was also removed and bisected longitudinally into two strips. All the tissues were suspended in 5ml tissue baths containing Krebs solution of the following composition (mM): Na⁺143, K⁺5.9, Ca²⁺2.5, Mg²⁺1.2, Cl⁻128, HCO₃⁻25, HPO₄⁻²1.2, SO₄²⁻1.2 and D-glucose 11, (pH 7.4) at 37°C and bubbled with 95% O₂/5% CO₂. The vas was placed under 0.5g resting tension and equilibrated for 1 hour. The spleen was placed under 1.0g resting tension and equilibrated for 1.5 hours. Changes in isometric tension were measured using Grass FT.03 transducers and recorded by Biopac Systems Inc. MP100WS for Windows®. This consisted of the transducers being connected via Biopac amplifiers to the MP100 data acquisition unit which converted signals into digital form which were then sent to a Dell 486D/66 PC through an ISA card. The data was then displayed on screen and simultaneously recorded to disk by AcqKnowledge for Windows software.

2.1.2 Human tissue

Prostatic chips taken from patients undergoing transurethral resection for benign prostatic hyperplasia (age 60-85) were collected in Tyrodes solution and stored overnight at 4°C for experimental use the next day. Prostatic chips (about 20mm x 4mm x 2mm) were selected which contained the most smooth muscle. They were suspended in Tyrodes solution of the following composition (mM): Na⁺149, K⁺2.7, Mg²⁺0.5, Ca²⁺1.8, Cl⁻141, HCO₃⁻12, HPO₄⁻²0.3 and D-glucose 5.6, (pH 7.4) at 37°C in 5ml tissue baths and bubbled with 95%O₂ / 5%CO₂. The strips
were placed under 1g resting tension, and equilibrated for 1 hour. Changes in isometric tension were measured using Grass FT.03 transducers and recorded by Biopac Systems Inc. MP100WS for Windows® as described above.

2.2 Drugs and solutions

All the compounds used are listed in Table 2.1 and were made up in the following way:

1Dissolved in distilled water and made up fresh each day.
2Dissolved in distilled water and stored frozen.
3Dissolved in DMSO and stored frozen.
4Stock solution dissolved in DMSO, and then diluted to working concentrations in distilled water and stored frozen.
5Dissolved in ethanol and then diluted to working concentrations in distilled water and made up fresh each day.
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<tr>
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Chapter 3.

Characterization of the $\alpha_1$-adrenoceptor subtype mediating contraction of the rat epididymal vas deferens.

3.1 Introduction

Using RNase protection assays the $\alpha_{1a}$, $\alpha_{1b}$, and $\alpha_{1d}$ clones have all been found to be expressed in the rat vas deferens (Perez et al., 1994; Price et al., 1994a; Rokosh et al., 1994). Functional experiments have shown that contractions to noradrenaline in the whole rat vas deferens were unaffected by chloroethylclonidine and had a relatively high affinity for WB 4101, suggesting that only the $\alpha_{1a}$-adrenoceptor mediates the contraction (Han et al., 1987b; Aboud et al., 1993).

The affinities of several different antagonists have been measured in binding studies using membranes from cells transfected with the cDNA for the three subtypes (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Saussy et al., 1994; Testa et al., 1994 and Goetz et al., 1995). While prazosin was shown to be non-selective, WB 4101, 5-methyl urapidil, phentolamine, benoxathian, spiperone, indoramin and BMY 7378 all showed some degree of selectivity. In particular BMY 7378 has recently been shown to be very selective for the $\alpha_{1a}$-subtype (Saussy et al., 1994; Goetz et al., 1995).

The aim of this investigation therefore was to functionally characterize the $\alpha_1$-adrenoceptor mediating contractions in the rat epididymal vas deferens to noradrenaline using the antagonists mentioned above and to see how well their affinities correlated with those that had previously been obtained on the three cloned subtypes. Only the epididymal portion of the rat vas deferens was used as it has been suggested that the contraction to noradrenaline of the rat prostatic and epididymal vas deferens may be mediated via different $\alpha_1$-adrenoceptor subtypes (McGrath 1982).
3.2 Experimental Protocol

Epididymal portions of the rat vas deferens were set up as described in Chapter 2. Non-cumulative additions of noradrenaline were used as the contraction was phasic (Figure 3.1). Contractions to increasing concentrations of noradrenaline were measured with a separation of 10 minutes between doses. The concentration-contraction curve was then either repeated or repeated in the presence of cocaine and β-oestradiol (both $10^{-6}$M) or a concentration-effect curve to another agonist was constructed. When oxymetazoline was used as the agonist, a separation of 20 minutes between doses was used as this agonist was harder to wash out. In other tissues cocaine and β-oestradiol were always present and after an initial curve to noradrenaline, this was either repeated or a concentration-effect curve to another agonist was measured or the noradrenaline curve was repeated in the presence of an antagonist (equilibrated for 30 minutes).

The effect of prazosin was also measured on the contractions to oxymetazoline to show whether these contractions were mediated by $\alpha_1$-adrenoceptors. In some tissues the second curve to noradrenaline was constructed in the presence of DMSO (0.01%) which was the highest concentration of DMSO produced in the tissue bath due to addition of antagonists that had it included in stock solutions. When chlorethylclonidine ($10^{-4}$M) was the antagonist it was incubated with the tissues for 30 minutes and then washed out for 30 minutes before another concentration-effect curve was established. A second curve to noradrenaline was also constructed after 60 minutes without chlorethylclonidine treatment and used as a control. Chlorethylclonidine ($10^{-4}$M) was used as it has previously been shown to have selectivity at this concentration (Han et al., 1987b). As the potency of partial agonists can be dependent upon receptor reserve in a tissue the effect of reducing the number of $\alpha_1$-adrenoceptors in the vas was assessed by controlled alkylation of receptors using phenoxybenzamine. After the first curve to noradrenaline, tissues were incubated with phenoxybenzamine for 30 minutes, then washed for 15 minutes before either a second curve to noradrenaline or a concentration-response curve to oxymetazoline.
3.2.1 Data analysis

All the responses were calculated as a percentage of the maximum response to noradrenaline in the initial concentration-effect curve and plotted as the mean of at least 4 separate experiments with vertical bars representing s.e.mean. When error bars cannot be seen on Figures this is because they are smaller than the symbols. Curve fitting by non-linear regression was performed on individual concentration-response curves for the calculation of EC₅₀ values. When concentration-response curves in the presence of an antagonist did not reach a maximum this was fixed at 100%, i.e. the control maximum. Where agonist potencies have been given as a pEC₅₀ values this was equal to - log of the EC₅₀ value for the agonist. For the competitive antagonists prazosin, WB 4101, 5-methyl-urapidil, phen tolamine, benoxathian, spiperone, indoramin and BMY 7378, Schild plots were constructed where the x axis intercept is equal to the pA₂ (Arunlakshana & Schild, 1959). Concentration-ratios were calculated using the second concentration-response curve in the absence of an antagonist as the control curve and the second concentration-response curve in the presence of an antagonist. Linear regression was used for calculation of pA₂ values with each point on the Schild plot calculated from a single concentration-ratio, of which there were 4 for each concentration of antagonist. All points on the plot may not be visible however when they lie very close together. For pK₈ values calculated using a single concentration of antagonist this was equal to log (dose ratio -1) - log [antagonist]. Linear regression was also used to correlate the pA₂ values for the antagonists with average pK₈ values for each cloned subtype. Curve-fitting and linear regression were performed using InPlot (GraphPAD Software, San Diego, Calif., USA). Statistical significance of differences between EC₅₀ values or maximum responses was tested for using a paired t test. A P value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPAD Software, San Diego, Calif., USA).

3.3 Results

Noradrenaline produced a phasic contraction of the rat epididymal vas deferens (Figure 3.1), as did the selective α₁-adrenoceptor agonists phenylephrine
and methoxamine. The first and second curves to noradrenaline were not significantly different (pEC\textsubscript{50} 5.6±0.1 and 5.6±0.1 respectively). The first and second curves to noradrenaline in the presence of cocaine and β-oestradiol (both 10\textsuperscript{-6}M) were also not significantly different (pEC\textsubscript{50} 7.0±0.1 and 7.0±0.1 respectively). The second curve to noradrenaline was not affected by 0.01% DMSO, the highest concentration resulting from addition of antagonists that had it included in stock solutions. The potency of noradrenaline in the epididymal vas deferens was compared with that of phenylephrine, methoxamine and oxymetazoline. In the absence of uptake blocking drugs oxymetazoline was 20 fold more potent than noradrenaline and phenylephrine and 40 fold more potent than methoxamine (pEC\textsubscript{50} 6.9±0.1; 5.6±0.1; 5.6±0.1 and 5.3±0.1 respectively, Figure 3.2a). In the presence of cocaine and β-oestradiol, both at 10\textsuperscript{-6}M, the noradrenaline and phenylephrine concentration-effect curves were shifted to the left but the oxymetazoline and methoxamine curves remained the same. Under these conditions noradrenaline was about equipotent with oxymetazoline and 3 and 50 fold more potent than phenylephrine and methoxamine respectively (pEC\textsubscript{50} 7.0±0.1; 6.9±0.1; 6.5±0.1 and 5.3±0.1 respectively, Figure 3.2b). All subsequent experiments with noradrenaline were carried out in the presence of the uptake inhibitors. Phenylephrine and methoxamine were both full agonists with respect to the maximum contraction evoked by noradrenaline (2.77±0.18g). Phenylephrine reached a significantly higher maximum response compared with noradrenaline in the absence and presence of cocaine and β-oestradiol, (108±1% maximum response to noradrenaline (P<0.05) with cocaine and β-oestradiol). The reason for this is not clear. Oxymetazoline was a partial agonist reaching 83±1% of the maximum response to noradrenaline. The contractions to oxymetazoline were antagonized by prazosin (10\textsuperscript{-8}M) p\textsubscript{K\text{a}} 9.5±0.1, (Figure 3.3).

The contractions to noradrenaline were shifted nearly 6 fold to the right by phenoxybenzamine (3x10\textsuperscript{-6}M) with no reduction in maximum response, while (1x10\textsuperscript{-6}M) produced a further shift and a 30±8% reduction in maximum (Figure 3.4a). The maximum response to oxymetazoline was reduced by 71±2% with a 100 fold shift to the right by phenoxybenzamine (1x10\textsuperscript{-6}M) and completely abolished at 3x10\textsuperscript{-6}M (Figure 3.4b).
Prazosin was a competitive antagonist of the noradrenaline contractions (pA2 9.2 slope 1.02±0.03, Figure 3.5). Chlorethylclonidine (10^8 M) had no effect on contractions to noradrenaline in the vas deferens (Figure 3.6). The following were all competitive antagonists of the noradrenaline contractions: WB 4101 (pA2 9.6, slope 1.00±0.08, Figure 3.7), 5-methyl urapidil (pA2 8.7, slope 1.04±0.07, Figure 3.8), phentolamine (pA2 8.3, slope 1.02±0.03, Figure 3.9), benoxathian (pA2 9.4, slope 1.05±0.09, Figure 3.10), spiperone (pA2 7.5, slope 1.02±0.03, Figure 3.11), indoramin (pA2 8.4, slope 0.97±0.05, Figure 3.12) and BMY 7378, (pA2 6.7, slope 1.02±0.04, Figure 3.13).

Table 3.1 compares the average affinities of antagonists from binding studies on cell lines expressing either the cloned α1a-, α1b- or α1d- adrenoceptors (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995) with their pA2 values in the rat epididymal vas deferens obtained in this study. The pK5 values on each subtype for the antagonists have been plotted against their pA2 values on the vas deferens (Figure 3.14). Correlation values (r), and slopes for each of these plots are shown in Table 3.2. These show that the pA2 values on the rat epididymal vas deferens correlate most closely with the cloned α1a-adrenoceptor.
Figure 3.1. Typical recordings of contractions to noradrenaline (in the presence of cocaine $10^{-3}$M and $\beta$-oestradiol $10^{-3}$M) in the rat epididymal vas deferens.
Figure 3.2. Non-cumulative concentration-effect curves for noradrenaline (●), phenylephrine (▲), methoxamine (■) and oxymetazoline (▲), in rat epididymal vas deferens (a) without uptake blockade and (b) in the presence of cocaine (10^{-3} M) and β-oestradiol (10^{-6} M). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 3.3. Antagonism of contractions to oxymetazoline in rat epididymal vas deferens by prazosin. Control (●), + prazosin 1×10⁻⁶M (▼). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 3.4. The effect of phenoxybenzamine (30 minutes incubation followed by 15 minutes washout) on contractions in the rat epididymal vas deferens to (a), noradrenaline and (b), oxymetazoline. Control (●), + phenoxybenzamine, 1x10⁻⁹M (▲), 3x10⁻⁹M (●), 1x10⁻⁸M (▲), 3x10⁻⁸M (♦) and 1x10⁻⁷M (●). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 3.5. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by prazosin. Control (●), + prazosin $3 \times 10^{-9} \text{M}$ (▼), $1 \times 10^{-9} \text{M}$ (■), $3 \times 10^{-9} \text{M}$ (▲), and $1 \times 10^{-7} \text{M}$ (♦). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for prazosin using dose ratios from (a).
Figure 3.6. The effect of chlorehylclonidine (30 minutes incubation followed by 30 minutes washout) on contractions in the rat epididymal vas deferens to noradrenaline, control (●), + chlorehylclonidine $10^4$M (▼). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 3.7. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by WB 4101. Control (•), + WB 4101 $1 \times 10^5$M (▼), $3 \times 10^5$M (■), and $1 \times 10^7$M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for WB 4101 using dose ratios from (a).
Figure 3.8. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by 5-methyl urapidil. Control (●), + 5-methyl urapidil 1x10^−4M (▲), 3x10^−5M (■), and 1x10^−7M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for 5-methyl urapidil using dose ratios from (a).
Figure 3.9. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by phentolamine. Control (○), + phentolamine 1x10^-7 M (▼), 3x10^-7 M (■), and 1x10^-6 M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for phentolamine using dose ratios from (a).
Figure 3.10. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by benoxathian. Control (●), + benoxathian 1x10⁻⁴M (▼), 3x10⁻⁴M (■), and 1x10⁻³M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for benoxathian using dose ratios from (a).
Figure 3.11. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by spiperone. Control (○), + spiperone $1 \times 10^{-7}$M (▼), $3 \times 10^{-7}$M (■), and $1 \times 10^{-6}$M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for spiperone using dose ratios from (a).
Figure 3.12. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by indoramin. Control (●), + indoramin $1\times10^{-6}$M (▼), $3\times10^{-6}$M (■), and $1\times10^{-7}$M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for indoramin using dose ratios from (a).
Figure 3.13. (a), Antagonism of contractions to noradrenaline in rat epididymal vas deferens by BMY 7378. Control (●), + BMY 7378 3x10⁻⁷M (▼), 1x10⁻⁸M (■), 3x10⁻⁹M (▲), and 1x10⁻⁹M (♦). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for BMY 7378 using dose ratios from (a).
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Table 3.1.

Comparison of $pA_2$ values for the antagonists with their published $pK_i$ on cloned subtypes.

*Data are mean±s.e.mean for values from Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995 (no s.e.m. for compounds with only one or two values). In each study the bovine $\alpha_{\text{I}_\text{W}}$, hamster $\alpha_{\text{I}_\text{b}}$ and rat $\alpha_{\text{I}_\text{d}}$ clones were used except for Forray et al. (1994b) and Goetz et al., (1995) where the three human $\alpha_i$-subtype clones were used.
Figure 3.14. Correlation of average pKₐ values for the displacement of [³H] prazosin on cloned α₁-adrenoceptor subtypes from Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995, with pA₂ values for the antagonists prazosin (1), WB 4101 (2), 5-methyl urapidil (3), phentolamine (4), benoxathian (5), spiperone (6), indoramin (7) and BMY 7378 (8) against rat epididymal vas deferens noradrenaline contractions. The solid line is a linear regression fit through all the points and the dashed line has a slope equal to unity, passing through the origin.
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<td>0.33±0.34</td>
</tr>
<tr>
<td>$\alpha_{ld}$</td>
<td>0.09</td>
<td>0.09±0.42</td>
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Table 3.2.
Correlation values and slopes of the correlations for the pA$_2$ values of the competitive antagonists on the rat epididymal vas deferens with their pK$_i$ values on the expressed $\alpha_\text{-}$ subtype clones as shown in Table 3.1.

3.4 Discussion

The $\alpha_\text{-}$-adrenoceptor mediating contraction of rat epididymal vas deferens has been characterized using antagonists which have been found to show some subtype specificity when used in binding experiments on membranes from cells transfected with either the cloned $\alpha_{la}$- or $\alpha_{lb}$- or $\alpha_{ld}$-adrenoceptor.

When uptake mechanisms were blocked in the epididymal vas deferens the order of potency for the agonists was noradrenaline $\geq$ oxymetazoline $>$ phenylephrine $>$ methoxamine. Oxymetazoline was antagonized by prazosin with an affinity consistent with $\alpha_\text{-}$-adrenoceptors. The order of affinity of these agonists measured in binding studies on membranes from rat 1 fibroblast cells expressing the three cloned $\alpha_\text{-}$-adrenoceptor subtypes (Lomasney et al., 1991) was oxymetazoline $>$ noradrenaline $>$ phenylephrine $>$ methoxamine for the $\alpha_{la}$- and $\alpha_{lb}$- subtypes and noradrenaline $>$ oxymetazoline $>$ phenylephrine $>$ methoxamine for the $\alpha_{ld}$-subtype. Thus, as the relative order of potency for noradrenaline, phenylephrine and methoxamine is the same for all three subtypes, comparison of their relative potencies is not very helpful in distinguishing between subtypes and as potency is a combination of affinity and efficacy it is not directly comparable, although they were at least all full agonists.
Oxymetazoline was about equipotent with noradrenaline, which relative to the potency of the other agonists again is not helpful for subtype characterization. However the effect of phenoxybenzamine on the oxymetazoline contractions suggests that it is a partial agonist with respect to noradrenaline in this tissue, as phenoxybenzamine at a concentration which did not reduce the maximum response to noradrenaline completely abolished the contractions to oxymetazoline. Also the maximum response to oxymetazoline was smaller than that to noradrenaline. This might suggest that as oxymetazoline was equipotent with noradrenaline it must have a high affinity for the $\alpha_1$-adrenoceptors in this tissue as its efficacy appears to be much lower, oxymetazoline having the highest affinity for the cloned $\alpha_{1A}$-adrenoceptor. The potency of oxymetazoline would also however depend on the size of the receptor reserve in a tissue as it is a partial agonist so caution would be needed in comparing its potency relative to noradrenaline between tissues. The epididymal vas deferens probably has a large receptor reserve as receptor alkylation with phenoxybenzamine produced over a three fold shift to the right in the concentration-effect curve with no reduction in maximum, in agreement with Diaz-Toledo & Marti, (1988).

The competitive antagonism of noradrenaline contractions by prazosin in the vas deferens was consistent with that expected for $\alpha_1$-adrenoceptors. The concentration-response curve to noradrenaline was not altered by the alkylating agent chlorehylclonidine which shows that the contractions are unlikely to be mediated either via an $\alpha_{1B}$- or possibly an $\alpha_{1D}$-adrenoceptor subtype. WB 4101, 5-methyl-urapidil and benoxathian were all competitive antagonists with affinities similar to other reported values in this tissue (e.g. Aboud et al., 1993). The high affinities of WB 4101, benoxathian, 5-methyl-urapidil and phentolamine and the low affinity of spiperone are consistent with the affinities of these antagonists measured in binding studies on $\alpha_{1A}$-adrenoceptors using tissues expressing either just the $\alpha_{1A}$-adrenoceptor e.g. guinea pig liver (Garcia-Sainz & Romero-Avila, 1993) or only the $\alpha_{1A}$-adrenoceptor after chlorehylclonidine treatment e.g rat hippocampus (Testa et al., 1993) and rat cerebral cortex (Kenny et al., 1994a). Taken together these results show that the contractions to noradrenaline in the
epididymal portion of the rat vas deferens are mediated by pharmacologically defined $\alpha_{\text{IA}}$-adrenoceptors. This conclusion is in line with that of a number of other workers (Han et al., 1987b; Hanft & Gross, 1989; Aboud et al., 1993).

The nomenclature used so far to describe the $\alpha_i$-subtypes is that most commonly employed (Bylund et al., 1994) although one based on different affinities for prazosin and HV723 in the vasculature has been described (Muramatsu et al., 1990b). This group has recently reported that contractions to exogenous noradrenaline in the rat epididymal vas deferens are predominantly mediated via an $\alpha_{\text{II}}$-subtype which is characterised by low affinities for prazosin, WB 4101 and HV723 (Ohmura et al., 1992). However their results differ from the present results and those of other workers who have not found the lower affinity of WB 4101, prazosin and benoxathian (Table 3.1). In addition the $\alpha_{\text{IA}}$- and $\alpha_{\text{IB}}$-subtypes have been classified by Muramatsu as part of the $\alpha_{\text{IH}}$ rather than $\alpha_{\text{IL}}$ group and thus their conclusion (Ohmura et al., 1992) is incompatible with that of the present results and that of other workers.

Using cell membranes from cells transfected with the cDNA for either the $\alpha_{\text{IA}}$, $\alpha_{\text{IB}}$- or $\alpha_{\text{ID}}$-subtype, $pK_i$ values for different antagonists at these $\alpha_i$-adrenoceptors have been determined in several binding studies (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995). While prazosin appears to have equal affinity for the three subtypes, other compounds show some subtype selectivity. In particular, 5-methyl urapidil and indoramin have a higher affinity for the $\alpha_{\text{IA}}$-subtype and BMY 7378 has a much higher affinity for the $\alpha_{\text{IB}}$-subtype. The average $pK_i$ value for these antagonists from the above studies were plotted against their $pA_2$ values on the epididymal vas deferens to see how well they correlated with each cloned subtype. A correlation value of one and slope of unity would result from the $pK_i$ and $pA_2$ values for each antagonist being identical and therefore pharmacologically the same receptor. It is clear from the correlations that the functional response in the vas deferens is unlikely to be mediated via a receptor similar to either the $\alpha_{\text{IB}}$-clone or $\alpha_{\text{IA}}$-clone. However there is a very good correlation between the $pA_2$,
values and the pKᵢ values at the αᵢ₄-clone (r 0.97, slope 0.91±0.09). This suggests that the functional αᵢ₄-adrenoceptor in the vas deferens and the expressed αᵢ₄-clone are the same.

Due to initial reports showing the bovine αᵢ₄-clone to be chlorethylclonidine sensitive and not expressed in the rat by Northern blot analysis (Schwinn et al., 1991) the αᵢ₄-adrenoceptor clone was originally thought not to correspond to the αᵢ₄-adrenoceptor found in rat tissues and so was referred to as the αᵢ₄-clone. However the αᵢ₄ subtype has now been found to be expressed in the rat by the more sensitive RNAse protection assay (Perez et al., 1994; Rokosh et al., 1994) and this subtype may not be eliminated from a rat tissue by chlorethylclonidine treatment (Laz et al., 1993; Forray et al., 1994a). Binding studies have also shown tissue αᵢ₄-adrenoceptors and the expressed cloned αᵢ₄-adrenoceptor to be pharmacologically very similar (Laz et al., 1994; Perez et al., 1994) and so the αᵢ₄-clone has now been renamed the αᵢ₄-clone (Hieble et al., 1995). The finding in this study that the functional αᵢ₄-adrenoceptor in the vas deferens and the expressed αᵢ₄-clone are the same subtype therefore also supports the renaming of the αᵢ₄-clone as the αᵢ₄-clone.

In binding studies on the cloned αᵣ-adrenoceptors expressed in cell lines the αᵢ₄-subtype was most sensitive to alkylation and the αᵢ₄-subtype was least sensitive (Schwinn et al., 1990; Lomasney et al., 1991; Perez et al., 1991; Marshall et al., 1992; Forray et al., 1994a,b). As chlorethylclonidine did not affect the contractions to noradrenaline in the epididymal vas deferens this corresponds most closely with its alkylation of the αᵣ-subtype.

In conclusion, contractions of the rat epididymal vas deferens to noradrenaline are mediated by a receptor similar to the classical αᵣ₄-adrenoceptor as previously defined in tissues. The αᵣ₄-functional receptor also correlated very well pharmacologically with the expressed αᵣ₄-clone and appears to be the same subtype. The rat epididymal vas deferens therefore is a useful tissue in which to study functional αᵣ₄-adrenoceptors.
Chapter 4.

Characterization of the $\alpha_\mathrm{I}$-adrenoceptor subtype mediating contraction of the rat spleen.

4.1 Introduction

Using RNAse protection assays the $\alpha_{\mathrm{A}}$- and $\alpha_{\mathrm{B}}$- clones have been found to be expressed in the rat spleen (Perez et al., 1994; Price et al., 1994a; Rokosh et al., 1994). Functional experiments have shown that contractions to noradrenaline in the rat spleen were sensitive to chlorehthylclonidine suggesting that the $\alpha_{\mathrm{B}^{-}}$-subtype could mediate at least part of this contraction (Han et al., 1987b; Aboud et al., 1993). However, recent results suggest that the functional $\alpha_{1}$-adrenoceptors in the rat aorta are predominantly of the $\alpha_{\mathrm{B}^{-}}$-subtype (Goetz et al., 1995; Kenny et al., 1995), which have similar sensitivity to chlorehthylclonidine (Aboud et al., 1993). Therefore, the aims of this investigation were to functionally characterize the $\alpha_{1}$-adrenoceptor mediating contractions in the rat spleen using the same antagonists as were used to characterize the $\alpha_{1}$-adrenoceptor mediating contractions in the rat vas deferens (Chapter 3) and again to see how well their affinities correlated with those that had previously been obtained on the three cloned subtypes.

4.2 Experimental Protocol

Rat splenic strips were set up as described in Chapter 2. An initial contraction to a concentration of noradrenaline ($1\times10^{-5}$M) which gave a maximum response was first measured in all the tissues followed one hour later by a cumulative concentration-effect curve to noradrenaline or phenylephrine or methoxamine or oxymetazoline. It was possible to construct cumulative curves as the contractions to these agonists were well maintained. The curves were then repeated either as repeat control curves or in the presence of cocaine and $\beta$-oestradiol (both $10^{-5}$M) or in the presence of an antagonist (equilibrated for 30 minutes). When chlorehthylclonidine was the antagonist it was incubated with the
tissues for 30 minutes and then washed out for 30 minutes. In all experiments the repeat noradrenaline, methoxamine and oxymetazoline curves were carried out one hour after the first curve. However, the repeat phenylephrine curve was begun two hours after the first curve because contractions to phenylephrine took much longer to return to baseline during the washout period. In some tissues the second curve to noradrenaline or phenylephrine was measured in the presence of DMSO (0.01%) which was the highest concentration of DMSO produced in the tissue bath due to addition of antagonists that had it included in stock solutions.

As the potency of partial agonists can be dependent upon receptor reserve in a tissue the effect of reducing the number of $\alpha_1$-adrenoceptors in the spleen was assessed by controlled alkylation of receptors using phenoxybenzamine. 75 minutes after the first curve to phenylephrine, tissues were incubated with phenoxybenzamine for 30 minutes, then washed for 15 minutes before a second concentration-response curve to phenylephrine was measured.

4.2.1 Data analysis

The responses were calculated either as a percentage of the initial response to noradrenaline (10$^{-7}$M) or as a percentage of the maximum in the first concentration-effect curve and plotted as the mean of at least 4 separate experiments with vertical bars representing s.e.mean. When error bars cannot be seen on Figures this is because they are smaller than the symbols. Curve fitting by non-linear regression was performed on individual concentration-response curves for the calculation of EC$_{50}$ values. When concentration-response curves in the presence of an antagonist did not reach a maximum this was fixed at 100%, i.e. the control maximum. Where agonist potencies have been given as a pEC$_{50}$ value this was equal to - log of the EC$_{50}$ value for the agonist. For the competitive antagonists prazosin, WB 4101, 5-methyl-urapidil, phentolamine, benoxathian, spiperone, indoramin and BMY 7378, Schild plots were constructed where the x axis intercept is equal to the pA$_2$ (Arunlakshana & Schild, 1959). Concentration-ratios were calculated using the second concentration-response curve in the absence of an antagonist as the control curve and the second concentration-response curve in the presence of an antagonist. Linear regression was used for
calculation of pA₂ values with each point on the Schild plot calculated from a single concentration-ratio, of which there are 4 for each concentration of antagonist. All points on the plot may not be visible however when they lie very close together. For pKᵦ values calculated using a single concentration of antagonist this was equal to log (dose ratio -1) - log [antagonist]. Linear regression was also used to correlate the pA₂ values for the antagonists with average pKᵦ values for each cloned subtype. Curve-fitting and linear regression were performed using InPlot (GraphPAD Software, San Diego, Calif., USA). Statistical significance of differences between EC₅₀ values or maximum responses was tested for using a paired t test. A P value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPAD Software, San Diego, Calif., USA).

4.3 Results

Noradrenaline produced a well maintained tonic contraction of the rat spleen, as did phenylephrine (Figure 4.1) and so cumulative concentration-effect curves for the agonists could be constructed (Figure 4.1). The first and second curves to noradrenaline were not significantly different (pEC₅₀ 5.2±0.1 and 5.2±0.1 respectively). The first and second curves to phenylephrine were also not significantly different (pEC₅₀ 4.8±0.1 and 4.8±0.1 respectively). The potency of noradrenaline in the spleen was compared with that of phenylephrine, methoxamine and oxymetazoline. Noradrenaline (pEC₅₀ 5.2±0.1, maximum tension 0.35g±0.02) was the most potent, followed by phenylephrine (pEC₅₀ 4.8±0.1, which reached 95±2% of the maximum response to noradrenaline and was not significantly different to this) and methoxamine (pEC₅₀ 3.3±0.1 but which had not reached a maximum at the highest concentration used, 73±3% of noradrenaline maximum at 3x10⁻⁷M) (Figure 4.2a). Oxymetazoline had no contractile effect up to 10⁻⁷M. The addition of both cocaine and β-oestradiol (each 10⁻³M) had no effect on the noradrenaline, phenylephrine or methoxamine curves and oxymetazoline still did not produce a contraction (Figure 4.2b). However oxymetazoline (10⁻⁶M) antagonized the contractions to noradrenaline and phenylephrine (pKᵦ 6.2±0.1 against both agonists, results not shown). The second curve to noradrenaline or phenylephrine was not affected by 0.01% DMSO, the
highest concentration resulting from addition of antagonists that had it included in stock solutions.

The contractions to phenylephrine were shifted nearly 2 fold to the right by phenoxybenzamine (3x10^−6M) which also reduced the maximum response by 17±2%, while (1x10^−6M) produced nearly a 4 fold shift and a 28±3% reduction in maximum (Figure 4.3).

Prazosin at concentrations of 3x10^−6M and 1x10^−6M produced relatively small rightward shifts in the noradrenaline curve in an apparently competitive manner but at higher concentrations (3x10^−5M and 1x10^−5M) prazosin was not a competitive antagonist, with a slope of 0.71±0.08 for the Schild plot using all four concentrations (Figure 4.4). Prazosin also produced a rightward shift of the phenylephrine curve and this time it acted as a competitive antagonist up to 1x10^−7M, the highest concentration used (pA_2 9.2, slope 1.06±0.08, Figure 4.5).

Chlorethylclonidine (1x10^−4M) produced a 30-fold shift to the right in the noradrenaline control curve in the spleen (Figure 4.6). However chlorethylclonidine (1x10^−4M) produced a 300 fold shift to the right in the phenylephrine control curve in the spleen (Figure 4.6), ten times greater than that produced against noradrenaline.

WB 4101 was a competitive antagonist of the phenylephrine contractions in the rat spleen (pA_2 8.1, slope 0.95±0.07, Figure 4.7), as were 5 methyl-urapidil (pA_2 7.1, slope 1.08±0.08, Figure 4.8), phentolamine (pA_2 7.3, slope 0.98±0.09, Figure 4.9), benoxathian (pA_2 7.4, slope 0.96±0.07, Figure 4.10), spiperone (pA_2 7.9, slope 0.96±0.05, Figure 4.11), indoramin (pA_2 7.5, slope 0.94±0.08, Figure 4.12) and BMY 7378 (pA_2 7.4, slope 0.93±0.08, Figure 4.13).

Table 4.1 compares the average affinities of antagonists from binding studies on cell lines expressing either the cloned α_1a-, α_1b-, or α_1d- adrenoceptors (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995) with their pA_2 values in the rat spleen obtained in this study.
The pKᵢ values on each subtype for the antagonists have been plotted against their pA₂ values on the rat spleen (Figure 4.14). Correlation values (r), and slopes for each of these plots are shown in Table 4.2. These show that the pA₂ values on the rat spleen correlate most closely with the cloned α₈-adrenoceptor.
Figure 4.1. Typical recordings of (a), a contraction to a single addition of noradrenaline or cumulative concentration-effect curves to (b), noradrenaline and (c), phenylephrine in the rat spleen.
Figure 4.2. Cumulative concentration-effect curves for noradrenaline (●), phenylephrine (▼), methoxamine (■), and oxymetazoline (▲), in rat spleen (a) without uptake blockade and (b) in the presence of cocaine (10⁻⁶M) and β-oestradiol (10⁻⁶M). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 4.3. The effect of phenoxybenzamine (30 minutes incubation followed by 15 minutes washout) on contractions to phenylephrine in the rat spleen. Control (●), + phenoxybenzamine, 3x10⁻⁷M (▼), 1x10⁻⁷M (■), 3x10⁻⁸M (▲), and 1x10⁻⁹M (♦). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 4.4. (a), Antagonism of contractions to noradrenaline by prazosin in rat spleen. Control (●), + prazosin 3x10⁻⁶M (▲), 1x10⁻⁶M (■), 3x10⁻⁷M (▲), and 1x10⁻⁷M (●). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for prazosin using dose ratios from (a).
Figure 4.5. (a), Antagonism of contractions to phenylephrine in rat spleen by prazosin. Control (●), + prazosin 1x10^4M (▲), 3x10^4M (■), and 1x10^7M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for prazosin using dose ratios from (a).
Figure 4.6. The effect of chlorehyclonidine (30 minutes incubation followed by 30 minutes washout) on contractions in the rat spleen to noradrenaline, control (●), + chlorehyclonidine 10⁻⁶M (▼) and phenylephrine, control (○), + chlorehyclonidine 10⁻⁴M (▼). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 4.7. (a), Antagonism of contractions to phenylephrine in rat spleen by WB 4101. Control (●), + WB 4101 3x10⁻⁴M (▲), 1x10⁻⁷M (■), and 3x10⁻⁷M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for WB 4101 using dose ratios from (a).
Figure 4.8. (a), Antagonism of contractions to phenylephrine in rat spleen by 5-methyl urapidil. Control (○), + 5-methyl urapidil 1x10⁻⁷M (▼), 3x10⁻⁷M (■), and 1x10⁻⁶M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for 5-methyl urapidil using dose ratios from (a).
Figure 4.9. (a), Antagonism of contractions to phenylephrine in rat spleen by phentolamine. Control (●), + phentolamine 3x10⁻⁷M (▼), 1x10⁻⁴M (■), and 3x10⁻⁶M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for phentolamine using dose ratios from (a).
Figure 4.10. (a), Antagonism of contractions to phenylephrine in rat spleen by benoxathian. Control (●), + benoxathian 3x10^{-8}M (▲), 1x10^{-7}M (■), and 3x10^{-7}M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for benoxathian using dose ratios from (a).
Figure 4.11. (a), Antagonism of contractions to phenylephrine in rat spleen by spiperone. Control (●), + spiperone 1x10^-7M (▼), 3x10^-7M (■), and 1x10^-6M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for spiperone using dose ratios from (a).
Figure 4.12. (a), Antagonism of contractions to phenylephrine in rat spleen by indoramin. Control (○), + indoramin 1x10⁻⁷M (▼), 3x10⁻⁷M (■), and 1x10⁻⁶M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for indoramin using dose ratios from (a).
Figure 4.13. (a), Antagonism of contractions to phenylephrine in rat spleen by BMY 7378. Control (●), + BMY 7378 1x10⁻⁷M (▼), 3x10⁻⁷M (■), and 1x10⁻⁸M (▲). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for BMY 7378 using dose ratios from (a).
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**Table 4.1.**

Comparison of $pA_2$ values for the antagonists with their published $pK_i$ on cloned subtypes.

*Data are mean±s.e.mean for values from Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995 (no s.e.m. for compounds with only one or two values). In each study the bovine $\alpha_{1a}$, hamster $\alpha_{1b}$ and rat $\alpha_{1d}$ clones were used except for Forray et al. (1994b) and Goetz et al., (1995) where the three human $\alpha_i$-subtype clones were used.
Figure 4.14. Correlation of average pK₁ values for the displacement of [³H] prazosin on cloned α₁-adrenoceptor subtypes from Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995, with pA₂ values for the antagonists prazosin (1), WB 4101 (2), 5-methyl urapidil (3), phentolamine (4), benoxathian (5), spiperone (6), indoramin (7) and BMY 7378 (8) against rat spleen phenylephrine contractions. The solid line is a linear regression fit through all the points and the dashed line has a slope equal to unity, passing through the origin.
Table 4.2.
Correlation values and slopes of the correlations for the pA₂ values of the competitive antagonists on the rat spleen with their pKᵢ values on the expressed αᵣ-subtype clones as shown in Table 4.1.

4.4 Discussion

The αᵣ-adrenoceptor mediating contraction of the rat spleen has been characterized using antagonists which have been found to show some subtype specificity when used in binding experiments on membranes from cells transfected with either the cloned αₓ- or the αᵣ- or the αᵢ₉-adrenoceptor.

The order of potency for the agonists on the spleen was noradrenaline > phenylephrine > methoxamine and were not affected by blockade of uptake mechanisms. The order of affinity of these agonists measured in binding studies on membranes from rat 1 fibroblast cells expressing the three cloned αᵣ-adrenoceptor subtypes (Lomasney et al., 1991) was the same for all 3 subtypes and the same as the functional order of potency in the spleen (and vas, Figure 3.2). Thus this comparison is not helpful in distinguishing between subtypes and as potency is a combination of affinity and efficacy it is not directly comparable.

Oxymetazoline, although having a higher affinity for the αₓ- and αᵣ-subtypes than the other three agonists and only noradrenaline having a higher affinity at the αᵢ₉-subtype, did not produce a contraction in the spleen. Oxymetazoline was found to have affinity for the αᵣ-adrenoceptors in the spleen.
as it antagonized the contractions to phenylephrine with a pKₘ value similar to that which would be expected for either the α₉⁻ or α₁⁻ adrenoceptor subtypes (Lomasney et al., 1991). One possible reason for oxymetazoline not being an agonist in the spleen is that although having a high affinity for α₁-adrenoceptors, it has a low efficacy and is a partial agonist (as was found in epididymal vas deferens, Chapter 3). Phenoxybenzamine reduced the maximum response to phenylephrine in the spleen with only small shifts to the right for the concentration-response curves which suggests a small receptor reserve in this tissue, and could therefore be one reason why oxymetazoline did not produce a contraction.

Prazosin caused a shift in the noradrenaline curve on the spleen but was a competitive antagonist only at concentrations which produced a relatively small shift to the right. At higher concentrations of prazosin the shift was no longer in a competitive manner. Prazosin is not a subtype selective α₁-antagonist so the response to noradrenaline in the rat spleen must contain a non α₁-adrenoceptor component, possibly mediated by α₂-adrenoceptors (Kenakin & Novak, 1987). To isolate only the α₁-adrenoceptor component of the contraction, phenylephrine was therefore tried as the agonist. In this case the phenylephrine contractions, unlike those to noradrenaline, were competitively antagonized by prazosin (pA₂ 9.2), up to 1×10⁻⁶M. This confirmed that the phenylephrine contractions were mediated by α₁-adrenoceptors with no non-α₁-adrenoceptor component.

The responses to noradrenaline in the spleen were antagonized by the α₁β alkylationg agent chlorehylclonidine (Taddei et al., 1993) at a concentration which had no effect on contractions to noradrenaline in the rat epididymal vas deferens (Chapter 3). This suggests that at least part of the response might be mediated by the α₁β-subtype. However the response was not antagonized as much as might be expected when compared with the ability of chlorehylclonidine to reduce the B_max of the α₁β-subtype in binding experiments, which was over 80% at 10⁻⁵M (Taddei et al., 1993). Chlorehylclonidine (1×10⁻⁴M) caused a much greater shift to the right of the control curve for phenylephrine than with noradrenaline. With the
selective \( \alpha_1 \)-adrenoceptor agonist the effect of chlorethylclonidine appeared to agree with that expected for the \( \alpha_{1\beta} \)-subtype when compared with binding data for reduction in \( B_{\text{max}} \) in tissues (Han et al., 1987a; Taddei et al., 1993). However recent evidence using competitive antagonists suggests that contractions of the rat aorta to noradrenaline are mediated mainly by \( \alpha_{1\beta} \)-adrenoceptors (Goetz et al., 1995) and these contractions are also sensitive to chlorethylclonidine (Aboud et al., 1993). The sensitivity of phenylephrine contractions in the spleen to chlorethylclonidine could also therefore be due to \( \alpha_{1\beta} \)-adrenoceptors.

The relatively low pA\(_2\) values for WB 4101, 5-methyl urapidil, phentolamine and benoxathian and relatively high pA\(_2\) value for spiperone that were measured against phenylephrine contractions in rat spleen (Table 4.1) are also consistent with the affinities of these antagonists measured in binding studies using tissues thought to express just \( \alpha_{1\beta} \)-adrenoceptors e.g. rat liver (Taddei et al., 1993; Kenny et al., 1994), rat white fat cells (Torres-Marquez et al., 1992) and rat spleen (Veenstra et al., 1992). The pA\(_2\) for BMY 7378 in the spleen also indicates that the phenylephrine contractions are not mediated by \( \alpha_{1\beta} \)-adrenoceptors when compared with its affinity for this subtype in the rat aorta (Goetz et al., 1995; Kenny et al., 1995). Taken together, these results show that the contractions to \( \alpha_1 \)-adrenoceptor agonists in the rat spleen are mediated by pharmacologically defined \( \alpha_{1\beta} \)-adrenoceptors, in agreement with the conclusions of other workers (Han et al., 1987b; Aboud et al., 1993). It is clear that at least two pharmacologically distinct \( \alpha_1 \)-adrenoceptors exist in the rat as the spleen results are markedly different to those for the epididymal vas deferens (Chapter 3).

Table 4.1 compares the average affinities of antagonists from binding studies on cell lines expressing either the cloned \( \alpha_{1\alpha} \)-, \( \alpha_{1\beta} \)- or \( \alpha_{1\delta} \)-adrenoceptors (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995) with their pA\(_2\) values in the rat spleen obtained in this study. As was done for the epididymal vas deferens in Chapter 3, the pK\(_S\) values on each subtype for the antagonists have been plotted against their pA\(_2\) values on the spleen (Figure 4.14). The correlations showed that the subtype mediating
contraction in the rat spleen was most similar to the $\alpha_{ib}$-clone ($r = 0.96$, slope $1.27 \pm 0.14$) and did not correlate well with the other two subtypes. This confirms that the expressed $\alpha_{ib}$-clone is the same as the $\alpha_{ib}$-adrenoceptor found in tissues. Although the slope of the correlation differed significantly from unity this is probably due to the narrow range of affinities for most of these antagonists on the rat spleen. This reflects the narrow range of affinities of these compounds for the expressed $\alpha_{ib}$-clone.

The effect of chlorethylclonidine on $B_{\text{max}}$ has also been measured in binding studies on the cloned receptors expressed in cell lines (Schwinn et al., 1990; Lomasney et al., 1991; Perez et al., 1991; Marshall et al., 1992; Forray et al., 1994a,b). It is clear that the cloned $\alpha_{ia}$-subtype is most sensitive to alkylation, in agreement with its effects both on $B_{\text{max}}$ in tissues and its effects on the functional response in the spleen. However the other two $\alpha_{i}$-clones are also partially sensitive to chlorethylclonidine, with the $\alpha_{ia}$-clone having an intermediate sensitivity, in agreement with its effect on noradrenaline contractions of the rat aorta (Aboud et al., 1993) which are mediated mainly by $\alpha_{ib}$-adrenoceptors (Goetz et al., 1995). Chlorethylclonidine is therefore most useful in identifying the $\alpha_{ia}$-subtype because of its relative insensitivity to alkylation but does not distinguish clearly between the $\alpha_{ia}$- and $\alpha_{iu}$- subtypes.

In conclusion, contractions to phenylephrine in the rat spleen are mediated by the $\alpha_{ib}$-adrenoceptor. The good correlation between the affinities of the selective competitive antagonists on the expressed $\alpha_{ib}$-cloned receptor and the $\alpha_{ib}$-adrenoceptor in the rat spleen show that these receptors are pharmacologically the same. The rat spleen is therefore a useful tissue in which to study functional $\alpha_{ib}$-adrenoceptors.
Chapter 5.

Characterization of the $\alpha_1$-adrenoceptor subtype mediating contraction of the human prostate.

5.1 Introduction

All three $\alpha_1$-adrenoceptor clones have been found to be expressed in the human prostate by RNAse protection assay and quantitative solution hybridization assays showed that the predominant subtype expressed was the $\alpha_1_2$-adrenoceptor. The mRNA for this subtype was also found to be predominantly expressed in the stromal (fibromuscular) compartment by in situ hybridization (Price et al., 1993). These findings raise the possibility that the $\alpha_1$-adrenoceptor subtype in the prostate might differ from that in the vasculature. Therefore the aim of the present experiments was to use drugs with known subtype selectivity to pharmacologically characterize the $\alpha_1$-adrenoceptor in the prostate as a step in the development of prostate selective $\alpha_1$-adrenoceptor antagonists to treat benign prostatic hyperplasia (Chapter 1.2.7).

5.2 Experimental Protocol

Strips of prostatic smooth muscle were set up as described in Chapter 2. Cumulative additions of noradrenaline were added to each tissue to produce concentration-response curves as the response to noradrenaline was maintained and the maximum response was not much different to a single dose of noradrenaline producing a maximal response. After 1 hour the curve was then either repeated, or repeated in the presence of cocaine and $\beta$-oestradiol (both $10^{-5}$ M), or in the presence of an antagonist (equilibrated with the tissue for 30 minutes). In some tissues a concentration-effect curve to another agonist was measured. The alkylating agent chlorethylclonidine was incubated with the tissue for 30 minutes and then washed out for 30 minutes. The effect of the highest concentration of DMSO (0.01%) resulting in the tissue bath due to the
addition of compounds that had it included in stock solutions was also measured on the noradrenaline repeat curve.

5.2.1 Data analysis

All the responses were calculated as a percentage of the maximum response to noradrenaline in the initial concentration-effect curve and plotted as the mean of 3 or 4 separate experiments with vertical bars representing s.e.mean. When error bars cannot be seen on Figures this is because they are smaller than the symbols. Curve fitting by non-linear regression was performed on individual concentration-response curves for the calculation of EC\(_{50}\) values. When concentration-response curves in the presence of an antagonist did not reach a maximum this was fixed at 100%, i.e. the control maximum. Where agonist potencies have been given as a pEC\(_{50}\) value this was equal to - log of the EC\(_{50}\) value for the agonist. For the competitive antagonists prazosin, WB 4101, 5-methyl urapidil, phentolamine, benoxathian, spiperone, indoramin and BMY 7378, Schild plots were constructed where the x axis intercept is equal to the pA\(_2\) (Arunlakshana & Schild, 1959). Concentration-ratios were calculated using the the second concentration-response curve in the absence of an antagonist as the control curve and the second concentration-response curve in the presence of an antagonist. Linear regression was used for calculation of pA\(_2\) values with each point on the Schild plot calculated from a single concentration-ratio, of which there are 3 or 4 for each concentration of antagonist. All points on the plot may not be visible however when they lie very close together. For pK\(_g\) values calculated using a single concentration of antagonist this was equal to log (dose ratio -1) - log [antagonist]. Linear regression was also used to correlate the pA\(_2\) values for the antagonists with average pK\(_g\) values for each cloned subtype. Curve-fitting and linear regression were performed using InPlot (GraphPAD Software, San Diego, Calif., USA). Statistical significance of differences between EC\(_{50}\) values was tested for using a paired \(t\) test. A \(P\) value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPAD Software, San Diego, Calif., USA).
For some of the competitive antagonists Schild plots were constructed for the
determination of $pA_2$ values with only two concentrations of antagonist. As
the slopes of these Schild plots were always close to unity, $pA_2$ values were
calculated rather than $pK_g$ values. While more antagonist concentrations would
be desirable, due to the difficulty in obtaining human tissue it was decided to use
a greater number of antagonists rather than more concentrations of fewer
agonists.

5.3 Results

Noradrenaline produced a well maintained contraction to noradrenaline
on the human prostate (Figure 5.1) and so cumulative additions of agonists could
be used to construct concentration-contraction curves (Figure 5.1). Repeat
concentration-effect curves were not significantly different to the initial curve
(first curve $pEC_{50} 5.5\pm0.1$, and second curve $pEC_{50} 5.5\pm0.1$, maximum response
$0.66\pm0.07g$, Figure 5.2). The responses to noradrenaline were not significantly
different in the presence of the highest concentration of DMSO (0.01%) resulting
in the tissue bath due to the addition of compounds that had it included in stock
solutions. When neuronal and extra neuronal uptake was blocked by cocaine and
$\beta$-oestradiol (both $10^{-4}M$) the concentration-effect curve to noradrenaline was not
significantly altered, $pEC_{50} 5.5\pm0.1$ in the absence and $5.6\pm0.1$ in the presence of
cocaine and $\beta$-oestradiol. Phenylephrine and methoxamine also dose
dependently contracted the prostate ($pEC_{50} 5.1\pm0.1$ and $4.4\pm0.1$ respectively,
Figure 5.2) but appeared to be partial agonists in this tissue (maximum response
compared to noradrenaline $66\pm1\%$ for phenylephrine and $56\pm1\%$ for
methoxamine). The maximum response and $pEC_{50}$ for phenylephrine and
methoxamine were not significantly different in the presence of cocaine and $\beta$-
oestradiol, $pEC_{50} 5.2\pm0.1$ and $4.3\pm0.1$ respectively, maximum response $67\pm1\%$ and
$55\pm1\%$ respectively. Oxymetazoline did not contract the prostate up to the
highest concentration used ($10^{-3}M$). However oxymetazoline ($10^{-3}M$) antagonized
the contractions to noradrenaline ($pK_g 7.4\pm0.1$, results not shown). The selective
$\alpha_2$-adrenoceptor agonist UK 14,304, had no contractile effect in the prostate up to
the highest concentration used ($10^{-7}$- $10^{-3}M$, results not shown).
Prazosin was a competitive antagonist of the contractions to noradrenaline, producing dose dependent rightward shifts in the concentration-effect curve (pA₂ 8.5, slope 0.94±0.07, Figure 5.3). Chlorehyliclonidine (1x10⁻⁴M, 30 minutes), produced about a 3 fold rightward shift in the concentration-effect curve to noradrenaline and a 31±1% decrease in the maximum (Figure 5.4). WB 4101 was a competitive antagonist producing dose dependent rightward shifts in the concentration-effect curve to noradrenaline (pA₂ 9.0, slope 0.91±0.11, Figure 5.5). The same was also true for 5-methyl urapidil (pA₂ 8.6, slope 0.99±0.09, Figure 5.6), phenotolamine (pA₂ 7.6, slope 1.06±0.06, Figure 5.7), benoxathian (pA₂ 8.5, slope 0.99±0.16, Figure 5.8), spiperone (pA₂ 7.3, slope 1.04±0.12, Figure 5.9), indoramin (pA₂ 8.2, slope 1.01±0.12, Figure 5.10) and BMY 7378, (pA₂ 6.6, slope 0.91±0.11, Figure 5.11).

Table 5.1 compares the average affinities of antagonists from binding studies on cell lines expressing either the cloned α₁a-, α₁b- or α₁d- adrenoceptors (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995) with their pA₂ values in the human prostate obtained in this study. The pKᵢ values on each subtype for the antagonists have been plotted against their pA₂ values on the prostate (Figure 5.12). Correlation values (r), and slopes for each of these plots are shown in Table 5.2. These show that the pA₂ values on the human prostate correlate most closely with the cloned α₁a- adrenoceptor.
Figure 5.1. Typical recordings of (a), a contraction to a single addition of noradrenaline and (b), a cumulative concentration-effect curve to noradrenaline in the human prostate.
Figure 5.2. Cumulative concentration-effect curves for noradrenaline (●), phenylephrine (▼), methoxamine (■), and oxymetazoline (▲), in human prostate. Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 5.3. (a), Antagonism of contractions to noradrenaline in human prostate by prazosin. Control (○), + prazosin $1 \times 10^4 \text{M}$ (▼), $3 \times 10^4 \text{M}$ (■), $1 \times 10^7 \text{M}$ (▲). Each plot represents the mean with s.e.mean of at least 4 separate. (b), Schild plot for prazosin using dose ratios from (a).
Figure 5.4. The effect of chlorethylclonidine on contractions to noradrenaline in human prostate. Control (○), + chlorethylclonidine 10⁻⁴M (▼). Each plot represents the mean with s.e.mean of at least 4 separate experiments.
Figure 5.5. (a), Antagonism of contractions to noradrenaline in human prostate by WB 4101. Control (●), + WB 4101 1x10^-5M (▼), 3x10^-5M ( ■ ), 1x10^-7M ( ▲ ). Each plot represents the mean with s.e.mean of at least 4 separate experiments. (b), Schild plot for WB 4101 using dose ratios from (a).
Figure 5.6. (a), Antagonism of contractions to noradrenaline in human prostate by 5-methyl urapidil. Control (●), + 5-methyl urapidil 1x10⁶M (▼), 3x10⁴M (■), 1x10³M (▲). Each plot represents the mean with s.e.mean of at least 3 separate experiments. (b), Schild plot for 5-methyl urapidil using dose ratios from (a).
Figure 5.7. (a), Antagonism of contractions to noradrenaline in human prostate by phentolamine. Control (●), + phentolamine 3×10⁻⁷M (▼), 1×10⁻⁷M (■). Each plot represents the mean with s.e.mean of at least 3 separate experiments. (b), Schild plot for phentolamine using dose ratios from (a).
Figure 5.8. (a), Antagonism of contractions to noradrenaline in human prostate by benoxathian. Control (●), + benoxathian 1x10⁻⁶M (▼), 3x10⁻⁶M (■). Each plot represents the mean with s.e.mean of at least 3 separate experiments. (b), Schild plot for benoxathian using dose ratios from (a).
Figure 5.9. (a), Antagonism of contractions to noradrenaline in human prostate by spiperone. Control ( ● ), + spiperone 3x10^-7M ( ▼ ), 1x10^-7M ( ■ ). Each plot represents the mean with s.e.mean of at least 3 separate experiments. (b), Schild plot for spiperone using dose ratios from (a).
Figure 5.10. (a), Antagonism of contractions to noradrenaline in human prostate by indoramin. Control (○), + indoramin 3×10⁻⁶M (▼), 1×10⁻⁷M (■). Each plot represents the mean with s.e.mean of at least 3 separate experiments. (b), Schild plot for indoramin using dose ratios from (a).
Figure 5.11. (a), Antagonism of contractions to noradrenaline in human prostate by BMY 7378. Control (●), + BMY 7378 3x10^{-6}M (▼), 1x10^{-5}M (■). Each plot represents the mean with s.e.mean of at least 3 separate experiments. (b), Schild plot for BMY 7378 using dose ratios from (a).
<table>
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<th>Antagonist</th>
<th>pK&lt;sub&gt;i&lt;/sub&gt; on cloned α&lt;sub&gt;i&lt;/sub&gt;-adrenoceptors expressed in cells&lt;sup&gt;*&lt;/sup&gt;</th>
<th>pA&lt;sub&gt;2&lt;/sub&gt; human prostate</th>
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</thead>
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<td></td>
<td>α&lt;sub&gt;ia&lt;/sub&gt;</td>
<td>α&lt;sub&gt;ib&lt;/sub&gt;</td>
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<tr>
<td>Prazosin</td>
<td>9.2±0.2</td>
<td>9.6±0.2</td>
</tr>
<tr>
<td>WB4101</td>
<td>9.5±0.3</td>
<td>8.2±0.1</td>
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<tr>
<td>5-methyl urapidil</td>
<td>8.8±0.1</td>
<td>6.8±0.3</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>8.1±0.3</td>
<td>7.3±0.2</td>
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<td>Benoxathian</td>
<td>9.0</td>
<td>7.8</td>
</tr>
<tr>
<td>Spiperone</td>
<td>7.9±0.3</td>
<td>8.3±0.2</td>
</tr>
<tr>
<td>Indoramin</td>
<td>8.2±0.3</td>
<td>7.3±0.1</td>
</tr>
<tr>
<td>BMY 7378</td>
<td>6.6</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Table 5.1.

Comparison of pA<sub>2</sub> values for the antagonists with their published pK<sub>i</sub> on cloned subtypes.

*Data are mean±s.e.mean for values from Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995 (no s.e.m. for compounds with only one or two values). In each study the bovine α<sub>ia</sub>, hamster α<sub>ib</sub> and rat α<sub>id</sub> clones were used except for Forray et al. (1994b) and Goetz et al. (1995) where the three human α<sub>i</sub>-subtype clones were used.
Figure 5.12. Correlation of average pKᵢ values for the displacement of [³H] prazosin on cloned α₁-adrenoceptor subtypes from Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995, with pA₂ values for the antagonists prazosin (1), WB 4101 (2), 5-methyl urapidil (3), phentolamine (4), benoxathian (5), spiperone (6), indoramin (7) and BMY 7378 (8) against human prostate noradrenaline contractions. The solid line is a linear regression fit through all the points and the dashed line has a slope equal to unity, passing through the origin.
Table 5.2.
Correlation values and slopes of the correlations for the pA₂ values of the competitive antagonists on the human prostate with their pKᵣ values on the expressed αᵣ-subtype clones as shown in Table 5.1.

<table>
<thead>
<tr>
<th>αᵣ-subtype</th>
<th>correlation (r)</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>αᵣa</td>
<td>0.96</td>
<td>1.11±0.13</td>
</tr>
<tr>
<td>αᵣb</td>
<td>0.26</td>
<td>0.29±0.44</td>
</tr>
<tr>
<td>αᵣd</td>
<td>-0.04</td>
<td>-0.05±0.52</td>
</tr>
</tbody>
</table>

5.4 Discussion

The human prostate was shown to contract to noradrenaline, with its potency and maximal response being unaffected by neuronal and extra-neuronal uptake blockade by cocaine and β-oestradiol, which were therefore not included in further experiments. The αᵣ-adrenoceptor agonists phenylephrine and methoxamine also contracted the prostate, indicating that αᵣ-adrenoceptors mediate at least part of the response to noradrenaline. The most potent of the three agonists was noradrenaline with phenylephrine and methoxamine being about 3 and 10 fold less potent respectively. Phenylephrine and methoxamine also appeared to be partial agonists with respect to the maximum response to noradrenaline. Although they are usually considered to be full agonists, it may be that they have a slightly lower efficacy compared with noradrenaline and this is not noticeable in other tissues where there is a larger receptor reserve.

Oxymetazoline did not produce a contraction of the prostate despite having a higher affinity than phenylephrine and methoxamine for all the cloned αᵣ-adrenoceptor subtypes and it causing contraction of the rat epididymal vas deferens (Chapter 3). Oxymetazoline was found to have affinity for the αᵣ-adrenoceptors in the prostate as it antagonized the contractions to noradrenaline with a pKᵣ value closest to that which would be expected for the αᵣ-adrenoceptor
subtype (Lomasney et al., 1991). However oxymetazoline was a partial agonist in the rat vas, which also probably has a large receptor reserve (Chapter 3). Therefore oxymetazoline may not contract the prostate because there is not a large enough receptor reserve. The effect of the alkylating agent chlorethylclonidine on the prostate is in line with this suggestion as there was a reduction in maximum response with only a small shift to the right in the noradrenaline concentration-response curve.

Another possible reason for the maximum response to noradrenaline being greater than that of the other agonists in this tissue is that it may also be mediated by $\alpha_2$-adrenoceptors. However the full $\alpha_4$-adrenoceptor agonist UK-14,304 did not have any contractile effect (in agreement with Chappie et al., 1989) and prazosin had a pA$_r$ value against the noradrenaline contraction consistent for an $\alpha_1$-adrenoceptor. Therefore the response to noradrenaline is mediated by $\alpha_1$-adrenoceptors, as stated by Chappie et al. (1989) and was therefore a suitable agonist to study the $\alpha_1$-adrenoceptor subtype(s) in this tissue. Human hyperplastic prostatic tissue shows no relaxation to isoprenaline in in vitro functional studies (Caine et al., 1975; Kitada 1983). Therefore it was considered unnecessary to include a $\beta$-adrenoceptor antagonist in these experiments.

Chlorethylclonidine alkylates tissue $\alpha_{1b}$-adrenoceptors (e.g. in rat spleen, Figure 4.6) and the expressed $\alpha_{1b}$-clone (Perez et al., 1991) rather than tissue $\alpha_{1a}$-adrenoceptors (e.g. lack of effect in rat vas deferens, Figure 3.6) or the expressed $\alpha_{1a}$-clone (Perez et al., 1991; Forray et al., 1994a). The degree of sensitivity to chlorethylclonidine of the $\alpha_{1a}$-clone may however be species dependent to some extent (Forray et al., 1994a). The $\alpha_{1a}$-clone has an intermediate sensitivity (Perez et al., 1991; Forray et al., 1994a) and $\alpha_{1d}$-adrenoceptor mediated contractions of the rat aorta (Goetz et al., 1995) are also chlorethylclonidine sensitive (Aboud et al., 1993). The response to noradrenaline in the prostate was antagonized by chlorethylclonidine but its effect was much less than that observed on the rat spleen (Figure 4.6) or rat aorta (Aboud et al., 1993) where it caused about a 300 fold shift in the $\alpha_1$-adrenoceptor mediated contractions compared with about a 3
fold shift in the prostate. So contraction of the prostate is unlikely to be mediated solely by \( \alpha_{\text{A}_1} \) or \( \alpha_{\text{A}_2} \) adrenoceptors but could be mediated by \( \alpha_{\text{A}_a} \)-adrenoceptors as the corresponding expressed human clone for this subtype (\( \alpha_{\text{A}_a} \)), has been shown to be partially chlorethylclonidine sensitive.

Due to the problems of interpreting the effects of chlorethylclonidine in some cases the affinities of subtype selective competitive antagonists may give more reliable information. Table 5.1 shows the average affinities of antagonists from binding studies on cell lines expressing either the cloned \( \alpha_{\text{A}_1} \)- \( \alpha_{\text{A}_2} \)- or \( \alpha_{\text{A}_e} \)-adrenoceptors (Faure et al., 1994; Forray et al., 1994b; Kenny et al., 1994a,b; Testa et al., 1994 and Goetz et al., 1995) with their \( pA_2 \) values in the human prostate obtained in this study. As was done for the rat epididymal vas deferens in Chapter 3 and rat spleen Chapter 4, the \( pK_i \) values on each subtype for the antagonists have been plotted against their \( pA_2 \) values on the prostate (Figure 5.12). The correlations show that the subtype in the prostate is unlike either the \( \alpha_{\text{A}_2} \)- or \( \alpha_{\text{A}_e} \)-subtypes but correlates very well with the \( \alpha_{\text{A}_a} \)-subtype (\( r 0.96 \)).

In conclusion, the \( \alpha_{\text{A}_1} \)-adrenoceptor mediating contractions to noradrenaline in the human prostate was only partially sensitive to chlorethylclonidine and the affinities of subtype selective antagonists for this functional receptor correlated very well with the expressed \( \alpha_{\text{A}_a} \)-subtype clone. On this evidence the \( \alpha_{\text{A}_1} \)-adrenoceptor mediating the contraction is the same as the expressed \( \alpha_{\text{A}_a} \)-subtype clone, which corresponds well with this subtype having the highest expression in the prostatic stroma (Price et al., 1993). As the expressed \( \alpha_{\text{A}_a} \)-subtype clone corresponds to the tissue \( \alpha_{\text{A}_a} \)-adrenoreceptor (Chapter 3; Laz et al., 1993; Perez et al., 1994), the subtype mediating contraction of the human prostate is the \( \alpha_{\text{A}_a} \)-adrenoceptor. This conclusion is therefore consistent with the findings that \( \alpha_{\text{A}_1} \)-adrenoceptors in human prostate were similar to tissue \( \alpha_{\text{A}_a} \)-adrenoceptors in binding studies (Testa et al., 1993) and similar to the expressed bovine \( \alpha_{\text{A}_e} \)-clone in functional studies (Lepor et al., 1993b). An antagonist selective for the \( \alpha_{\text{A}_a} \)-subtype may therefore be of benefit in the treatment of benign prostatic hyperplasia.
Chapter 6

The role of diacylglycerol and activation of protein kinase C in the $\alpha_{1A}$-adrenoceptor mediated contraction to noradrenaline of the rat epididymal vas deferens.

6.1 Introduction

Stimulation of $\alpha_1$-adrenoceptors can mediate contraction of smooth muscle by raising $[Ca^{2+}]_i$ (Minneman & Esbenshade, 1994). These G-protein coupled receptors are linked to the activation of PLC which hydrolyses PIP$_2$ to IP$_3$ and DAG. IP$_3$ can mobilize Ca$^{2+}$ from intracellular stores (Berridge, 1993), while DAG can activate PKC (Lee & Severson, 1994).

The aim of the present experiments was to investigate the role of DAG, PKC activation, IP$_3$ and capacitative Ca$^{2+}$ influx (Chapter 1.4) in the $\alpha_{1A}$-adrenoceptor mediated contraction of the rat epididymal vas deferens to noradrenaline (Chapter 3; Aboud et al., 1993).

6.2 Experimental Protocol

The epididymal portion of the rat vas deferens was set up as described in Chapter 2. In all tissues a contraction to noradrenaline ($10^{-6}$M) was measured followed by a non-cumulative concentration-effect curve to noradrenaline ($10^{-8}$M-$10^{-4}$M). In some tissues this was then followed 30 min later by a second concentration-effect curve, either as a control or under appropriate experimental conditions. In other tissues, after the first concentration-effect curve to noradrenaline, responses to noradrenaline at either $1\times10^{-6}$M or $3\times10^{-6}$M or $3\times10^{-5}$M were then repeated at either 30 minute or 60 minute intervals until responses were reproducible. Only one concentration and one time interval was used for each tissue. A final response to noradrenaline was then measured either as a control or under appropriate experimental conditions. The effect of prazosin ($10^{-7}$M) on contractions to K$^+$, 60mM (by addition of KCl solution to the tissue bath),
which produced a contraction similar in magnitude to the noradrenaline contractions, was measured in some tissues. Contractions to K⁺ (60mM) were then measured, always in the presence of prazosin (10⁻⁶M), at 30 minute or 60 minute intervals until the responses were reproducible (within ±2%). They were then used as controls where appropriate for the experiments involving noradrenaline. Control responses to noradrenaline (3x10⁻⁶M and 3x10⁻⁵M) and K⁺ (60mM) were also measured in the presence of DMSO (0.1%), which was used to dissolve staurosporine, calphostin C, R 59022, U-57,908 and cyclopiazonic acid.

The contraction to noradrenaline (10⁻⁶M) (a concentration which produced a submaximal response so that any potentiation in response could be observed) was measured either in the presence of the DAG kinase inhibitor R 59022 (3x10⁻⁷M, 30 minute incubation) or the DAG lipase inhibitor U-57,908 (10⁻⁶M, 30 minute incubation). Preliminary experiments showed that at higher concentrations R 59022 and U-57,908 had inhibitory effects on the contractions. Contractions to K⁺ (60mM) were also measured in the presence of R 59022 (3x10⁻⁷M, 30 minute incubation). Contractions to noradrenaline (3x10⁻⁶M and 3x10⁻⁵M) and the contraction to K⁺ (60mM) were measured either in the presence of the non-selective protein kinase inhibitor staurosporine (10⁻⁶M, 30 minute incubation) or the selective PKC inhibitor calphostin C (10⁻⁶M, 1 hour incubation). Preliminary experiments showed these conditions for calphostin C at inhibiting functional responses were close to maximum without affecting contractions to K⁺. Calphostin C was used in a bright light environment as this is essential to its activity (Bruns et al., 1991).

The phorbol ester phorbol-12,13-dibutyrate (PDBu), was added to some tissues (10⁻⁷M-10⁻⁸M, one concentration per tissue) in normal Krebs solution 40 minutes after the initial noradrenaline concentration-effect curve. The response to PDBu (10⁻⁷M, a concentration which produced a submaximal response to PDBu) was also measured in the presence of calphostin C (10⁻⁶M, 1 hour incubation). PDBu (3x10⁻⁶M) was pre-incubated with some tissues for 2 hours before a repeat response to either noradrenaline (10⁻⁶M) or K⁺ (60mM) was measured.
Contractions induced by non-cumulative additions to noradrenaline ($10^{-8}$M-$10^{-7}$M) were measured in Ca$^{2+}$ free Krebs solution containing EGTA (1mM). After the initial concentration-effect curve (in normal Krebs solution) the tissue was allowed to recover for 15 minutes in normal Krebs solution followed by 15 minutes equilibration in Ca$^{2+}$ free Krebs solution (containing EGTA, 1mM) before the start of the second curve with concentrations given in ascending order. In other tissues the effect of a single addition of noradrenaline ($10^{-4}$M) was measured immediately following the 15 minute equilibration in Ca$^{2+}$ free Krebs solution (containing EGTA, 1mM) to see if the response to a high concentration of noradrenaline was affected by a gradual depletion of intracellular Ca$^{2+}$ which could not be replenished during the non-cumulative additions of noradrenaline. A concentration-effect curve to noradrenaline (in normal Krebs solution) was also measured in the presence of nifedipine ($10^{-6}$M, 20 minute equilibration, a concentration which should block voltage-gated Ca$^{2+}$ channels). Some responses to PDBu ($10^{-6}$M) were also measured either in Ca$^{2+}$ free Krebs solution (containing EGTA, 1mM) or in the presence of nifedipine ($10^{-4}$M, 20 minute equilibration in normal Krebs solution).

To see if any influx of extracellular Ca$^{2+}$ was stimulated by depletion of intracellular Ca$^{2+}$ stores, 20 minutes after the single addition of noradrenaline ($10^{-4}$M) in Ca$^{2+}$ free Krebs solution the agonist was washed out for another 40 minutes still in Ca$^{2+}$ free Krebs solution (containing EGTA, 1mM) so that the concentrations of any second messengers generated by $\alpha$-adrenoceptor stimulation would probably no longer be raised. The intracellular Ca$^{2+}$ stores should remain depleted however as there was no extracellular Ca$^{2+}$ available to enter and refill them. EGTA was removed from the Krebs solution for the last 20 minutes. Ca$^{2+}$ (2.5mM) was then added to the Krebs solution and any response to this was measured. The endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor cyclopiazonic acid ($10^{-5}$M, a concentration which produced a maximum contraction in the rat spleen, Chapter 7) was also added to some tissues in normal Krebs solution 40 minutes after the initial concentration-effect curve to noradrenaline.
In other tissues the effect of cyclopiazonic acid (10⁻⁶M, 30 minute incubation) or ryanodine (10⁻⁴M, 30 minute incubation, a concentration shown to abolish increases in [Ca²⁺], produced by noradrenaline in rabbit mesenteric artery, Itoh et al., 1992) was measured on the contraction to noradrenaline (3x10⁻⁷M) in normal Krebs solution. The effect of pre-incubating some tissues with cyclopiazonic acid (10⁻⁵M) for either one or three minutes was also measured on the response to PDBu (10⁻⁶M).

6.2.1 Data analysis

The results were calculated as percentage maximum response of the initial concentration-effect curve to noradrenaline. Responses were then plotted as the mean of at least four separate experiments with vertical bars representing s.e.mean. Error bars appear on figures only when they exceed the symbol size. Curve fitting for the calculation of pEC₅₀ values by non linear regression was performed using InPlot (GraphPAD Software, San Diego, Calif., USA). Where potencies have been given as a pEC₅₀ value this was equal to - log of the EC₅₀ value. Statistical significance of differences between control and test means was tested for on raw data (g/tension) using a paired t test except for comparison of means for the PDBu responses where an unpaired t test was used. A P value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPAD Software, San Diego, Calif., USA).

6.3 Results

Noradrenaline (10⁻⁶M) produced a maximal contraction in the rat epididymal vas deferens (2.72±0.12g, n=8, mean±s.e.mean, Figure 6.1) and the non-cumulative concentration-effect curves were reproducible (pEC₅₀ 5.6±0.1 for the first and second curve). Contractions to K⁺ (60mM) were reduced by prazosin (10⁻⁷M) from 53±2% to 40±2% indicating some neuronal release of noradrenaline and therefore subsequently prazosin (10⁻⁷M) was added prior to all other additions of high K⁺ solution. DMSO (0.1%) had no significant effect on the contractions to noradrenaline or K⁺.
Contractions to noradrenaline at $10^6$M were potentiated by the DAG kinase inhibitor R 59022 ($3 \times 10^7$M) from 49±4% to 63±3% maximum response ($P<0.05$) (Figure 6.2) and the time taken from initiation of contraction to the maximum response was reduced from 16±2 seconds to 9±1 seconds ($P<0.05$) (Figure 6.2). Contractions to K+ (60mM) were not potentiated by R 59022, $3 \times 10^7$M (control, 38±2% maximum response to noradrenaline and 35±2% maximum response in the presence of R 59022, representing a significant reduction, $P<0.05$, Figure 6.3) and the time taken from initiation of contraction to the maximum response was not significantly different (6.0±0.6 seconds control and 5.8±0.5 seconds in the presence of R 59022, Figure 6.3). Contractions to noradrenaline were not significantly potentiated by the DAG lipase inhibitor U-57,908, $10^5$M (51±2% control and 53±4% in the presence of U-57,908) and the time taken from initiation of contraction to the maximum response was not significantly different (17±1 seconds control and 16±1 seconds in the presence of U-57,908) (Figure 6.4). Higher concentrations of U-57,908 reduced the noradrenaline contractions.

The control contraction to noradrenaline at $3 \times 10^5$M was 68±2% maximum response and was reduced to 28±2% of the maximum ($P<0.05$) by staurosporine ($10^7$M) and to 20±2% of the maximum ($P<0.05$) by calphostin C ($10^4$M) (Figure 6.5a). The control contraction to noradrenaline at $3 \times 10^5$M was 94±2% maximum response and was reduced to 50±2% of the maximum ($P<0.05$) by staurosporine ($10^7$M) and to 44±2% of the maximum ($P<0.05$) by calphostin C ($10^4$M) (Figure 6.5b). The control contraction to K+ (60mM) was 40±2% maximum response to noradrenaline and was 35±2% of the maximum ($P<0.05$) in the presence of staurosporine ($10^7$M) and 43±3% of the maximum in the presence of calphostin C ($10^4$M) (Figure 6.6).

The phorbol ester PDBu produced a phasic contraction of the rat epididymal vas deferens returning to baseline within 90 seconds (Figure 6.1). The PDBu contraction was also concentration dependent ($10^7$M-$10^4$M) and was 41±2% of the maximum response to noradrenaline at $10^4$M PDBu (Figure 6.7). The response to PDBu was not reproducible due to desensitization and so only one addition of PDBu was made to each tissue. The submaximal contraction to
PDBu (10^5M) was significantly reduced by the selective PKC inhibitor calphostin C (10^6M) from 33±5% to 4±1% (P<0.05) maximum response to noradrenaline (Figure 6.8a). The response to noradrenaline 10^-4M, was reduced when the tissue was pre-incubated with PDBu (3x10^-5M) for two hours (PDBu contraction returned to baseline within 90 seconds) from 100% to 25±2% maximum response (Figure 6.8b) but the response to K^- 60mM was not significantly affected by this treatment (control, 44±3% maximum response to noradrenaline and 42±2% after PDBu treatment).

The contractions to noradrenaline (10^-8M-10^-4M) were abolished in Ca^{2+} free Krebs solution containing EGTA (1mM) (Figure 6.9a), as was the response to a single addition of noradrenaline (10^-6M) in Ca^{2+} free Krebs solution (results not shown). The responses to noradrenaline were also greatly reduced in the presence of nifedipine (10^-8M) (Figure 6.9a). The contraction to PDBu (10^-5M) was abolished in Ca^{2+} free Krebs solution containing EGTA (1mM) or by the presence of nifedipine (10^-4M) in normal Krebs solution (Figure 6.9b).

Following the single addition of noradrenaline (10^-4M) in Ca^{2+} free Krebs solution containing EGTA (1mM), when the agonist had been washed out and the EGTA removed from the Krebs solution, the readdition of Ca^{2+} to the Krebs solution did not produce a response. Cyclopiazonic acid (10^-6M) also failed to alter the resting tension in normal Krebs solution.

The response to noradrenaline at 3x10^-4M (67±2% maximum response) was not significantly affected when the tissues were pre-incubated for 30 minutes with either cyclopiazonic acid 10^-5M (65±2% maximum response) or ryanodine 10^-4M (68±2% maximum response) (Figure 6.10a). The time for noradrenaline to reach a maximal effect at 3x10^-4M (17±1 seconds) was also not significantly affected by the 30 minute pre-incubation with either cyclopiazonic acid or ryanodine (18±1 seconds and 17±1 seconds respectively). The contraction to PDBu (10^-5M, 33±5% maximum response to noradrenaline) was not increased when cyclopiazonic acid was added to the tissues either 1 or 3 minutes prior to addition of PDBu (31±2% and 32±2% maximum response to noradrenaline respectively, Figure 6.10b).
Figure 6.1. Recordings of a contraction to noradrenaline (NA, $10^{-4}$M) and a contraction to PDBu ($10^{-7}$M) in the rat epididymal vas deferens.
Figure 6.2. The effect of pre-treatment with the DAG kinase inhibitor R 59022 on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. Control response to noradrenaline $10^{-6}$M (○), + R 59022, $3 \times 10^{-7}$M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa represents the time in seconds from initiation of the contraction.
Figure 6.3. The effect of pre-treatment with the DAG kinase inhibitor R 59022 on contractions to K⁺ (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. Control response to K⁺ 60mM, in the presence of prazosin, 10⁻⁷M (●), + R 59022, 3x10⁻⁷M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa represents the time in seconds from initiation of the contraction.
Figure 6.4. The effect of pre-treatment with the DAG lipase inhibitor U-57,908 on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. Control response to noradrenaline $10^{-4}\text{M}$ (●), + U-57,908, $10^{-5}\text{M}$ (▼). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa represents the time in seconds from initiation of the contraction.
Figure 6.5. The effect of staurosporine and calphostin C on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a), Control response to noradrenaline 3x10⁻⁷M (□), + staurosporine 10⁻⁷M (×□), + calphostin C 10⁻⁷M (■). (b), Control response to noradrenaline 3x10⁻⁷M (□), + staurosporine 10⁻⁷M (×□), + calphostin C 10⁻⁷M (■). Each bar represents the mean with s.e.mean of 4 separate experiments.
Figure 6.6. The effect of staurosporine and calphostin C on contractions to K⁺ (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. Control response to K⁺ 60mM, in the presence of prazosin, 10⁻⁷M (□), + staurosporine 10⁻⁷M (△), + calphostin C 10⁻⁴M (■). Each bar represents the mean with s.e.mean of 4 separate experiments.
Figure 6.7. Concentration-response curve to non-cumulative additions of PDBu (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens (○). The plot represents the mean with s.e.mean of 4 separate experiments for each of the 7 concentrations of PDBu (28 tissues).
Figure 6.8. The effect of calphostin C on the contraction to PDBu and the effect of a 2 hour pre-incubation with PDBu on the response to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a), Control response to PDBu 10^-6 M (▲), + calphostin C 10^-6 M (■). (b), Control response to noradrenaline 10^-6 M (▲), + PDBu 3x10^-6 M (■). Each bar represents the mean with s.e.mean of 4 separate experiments.
Figure 6.9. The effect of nifedipine and Ca\(^{2+}\) free Krebs solution containing EGTA (1mM) on contractions to noradrenaline and PDBu (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a), Control concentration-response curve for non-cumulative additions of noradrenaline in normal Krebs solution (●), in the presence of nifedipine 10\(^{-7}\)M (▼), or in Ca\(^{2+}\) free Krebs solution containing EGTA (1mM) (■). (b), Control contraction to PDBu 10\(^{-5}\)M (□□), in the presence of nifedipine 10\(^{-7}\)M (□□), or in Ca\(^{2+}\) free Krebs solution containing EGTA (1mM) (■■). Each plot or bar represents the mean with s.e.mean of 4 separate experiments.
Figure 6.10. (a), The effect of 30 minute pre-incubation with either cyclopiazonic acid or ryanodine on contractions to noradrenaline in the rat epididymal vas deferens. Control response to noradrenaline $3 \times 10^{-7}$M (■), + cyclopiazonic acid $10^{-6}$M (××), + ryanodine $10^{-6}$M ( ). (b), The effect of cyclopiazonic acid ($10^{-5}$M) on the contraction to PDBu in the rat epididymal vas deferens. Control response to PDBu $10^{-5}$M, ( ), + cyclopiazonic acid 1 minute prior to PDBu (××), + cyclopiazonic acid 3 minutes prior to PDBu ( ). Each bar represents the mean with s.e.mean of at least 4 separate experiments.
6.4 Discussion

The potential role of DAG and IP₃ in the contraction to noradrenaline of the rat epididymal vas deferens, mediated by $\alpha_{IA}$-adrenoceptors (Chapter 3; Aboud et al., 1993), has been studied in functional experiments. Similar experiments were performed on contractions to high K⁺ as a control where appropriate, as it was assumed that these contractions were the direct result of membrane depolarization leading to the opening of voltage-gated Ca²⁺ channels and Ca²⁺ influx.

$\alpha_{IA}$-Adrenoceptor stimulation has been shown to increase the formation of inositol phosphates in various tissues including the rat vas deferens (Fox et al., 1985) and this should therefore be accompanied by DAG formation. DAG can be metabolized in cells via two main pathways, being converted either to phosphatidic acid by DAG kinase (Bishop & Bell, 1986; Kanoh et al., 1993) or to arachidonic acid by DAG lipase (Severson & Hee-Cheong, 1986). The activity of one or both of these enzymes is therefore at least partly responsible for attenuating PKC activity in response to DAG formation. To see if the PKC activity involved in the noradrenaline contraction of this tissue was stimulated by formation of DAG the effect of inhibitors of DAG kinase and DAG lipase were investigated. The DAG kinase inhibitor R 59022 (de Chaffoy de Corcelles et al., 1985) significantly increased the magnitude and rate of the noradrenaline contraction but not the K⁺ contraction (on which a statistically significant reduction was observed), while the DAG lipase inhibitor U-57,908 (Yang et al., 1991) had no significant effect. This suggests that the PKC activity involved in the noradrenaline contraction is stimulated by production of DAG, which in this tissue is metabolized by DAG kinase but not DAG lipase, although it is possible that U-57,908 was not an effective inhibitor of DAG lipase in this tissue. In some tissues DAG can also be produced from the hydrolysis of phosphatidylcholine by either phosphatidylcholine-specific PLC or PLD (Billah & Anthes, 1990). The species of DAG derived from PIP₂ hydrolysis is rich in arachidonic acid compared with that derived from phosphatidylcholine. It has been shown that a membrane bound form of DAG kinase preferentially metabolizes arachidonoyl-DAG and so the increase in DAG formed from PIP₂ is
more transient compared with DAG derived from phosphatidylcholine, which is more sustained (MacDonald et al., 1988a,b). The potentiation of the noradrenaline contraction by the DAG kinase inhibitor is therefore consistent with a rise in DAG derived from PIP$_2$. This is in contrast to vascular smooth muscle where a sustained rise in DAG derived from phosphatidylcholine has been measured (Grillone et al., 1988; Gu et al., 1992), and DAG seems to be mainly hydrolysed by DAG lipase (Severson & Hee-Cheong, 1989; Chuang et al., 1990). However as the PDBu contraction in the vas was also phasic (see below) this suggests that other mechanisms are involved in attenuating the noradrenaline response as well as DAG metabolism.

As DAG can stimulate PKC, the role of this enzyme was investigated. The PKC inhibitor calphostin C (over 100 fold selectivity for PKC over other protein kinases, Kobayashi et al., 1989) significantly reduced contractions to noradrenaline in the epididymal vas deferens at a concentration which did not inhibit the contractions to K$^+$. This suggests that PKC activation is involved in the contraction of the rat epididymal vas deferens to noradrenaline. The relatively non-selective protein kinase inhibitor staurosporine (Tamaoki et al., 1986) significantly reduced the noradrenaline contractions and also significantly reduced the K$^+$ contractions but to a lesser extent. This is in agreement with Shimamoto et al., (1993), who reported that contractions in the rat aorta to both phenylephrine and K$^+$ were reduced by staurosporine while only those to phenylephrine were inhibited by calphostin C. Abraham & Rice (1992) found the protein kinase inhibitor iso-H7 reduced both noradrenaline and K$^+$ induced contractions in the rat vas deferens. They suggested that PKC was therefore a common site of activation in both contractions resulting from the increased [Ca$^{2+}$], in both responses. However the results with calphostin C (see above) suggests the effect of iso-H7 on contractions to K$^+$ in the rat vas deferens (Abraham & Rice, 1992) was not due to PKC inhibition.

Phorbol esters are known to activate PKC by binding to the DAG binding site (Castagna et al., 1982). The effect of the phorbol ester PDBu was therefore studied in the rat epididymal vas deferens. PDBu produced a
concentration dependent contraction of the tissue which was phasic, similar to
the noradrenaline response, but produced a smaller maximum response
(41±2%) compared with noradrenaline. The contraction to PDBu was also
significantly reduced by calphostin C confirming that the effect of the phorbol
ester is mediated by stimulation of PKC. Abraham & Rice (1992) found that
phorbol-12,13-diacetate and PDBu produced contractions less than 6%
maximum response to noradrenaline in the rat vas deferens. The reason for this
difference is not clear although it is not stated whether they used the whole vas
or just the epididymal or prostatic portion. The magnitude of the responses to
noradrenaline reported by Abraham & Rice (1992) however suggests a
predominantly prostatic portion was used.

Incubation of tissues with phorbol esters can also cause down-regulation
of PKC (Merkel et al., 1991) and the response to noradrenaline in the rat
epididymal vas deferens was significantly reduced by pre-treatment with PDBu
in the present experiments, without affecting the K+ contraction. This is also
consistent with PKC activity being involved in the noradrenaline contraction.

As hydrolysis of PIP_2 by PLC also produces IP_3, the possibility that
release of Ca^{2+} from intracellular stores also contributes to the noradrenaline
contraction in the rat epididymal vas deferens was studied. Non-cumulative
contractions to noradrenaline in this tissue were completely abolished in Ca^{2+} free
Krebs solution and almost completely abolished in the presence of nifedipine in
agreement with Han et al., (1987b). A single high concentration of noradrenaline
in Ca^{2+} free Krebs solution also failed to produce a response showing that the lack
of response to noradrenaline was not due to a gradual depletion of Ca^{2+} from
intracellular stores (by individual doses of increasing concentration) which could
not be replenished and therefore could not initiate contraction. Therefore the
contraction appears completely dependent on the influx of extracellular Ca^{2+}
through voltage-gated Ca^{2+} channels and mobilization of intracellular Ca^{2+} does
not contribute directly to the contraction. The contraction to PDBu was also
completely abolished in Ca^{2+} free Krebs solution and in the presence of nifedipine,
confirming that activation of PKC mediates a contraction via influx of
extracellular Ca²⁺ through voltage-gated Ca²⁺ channels. In porcine carotid artery activation of PKC produces contraction without a change in [Ca²⁺], (Chatterjee & Tejada, 1986), but this is not the case in the rat epididymal vas deferens. PKC has been shown to translocate to the cell membrane upon stimulation in some cells (Haller et al., 1990), where it would be able to facilitate influx of extracellular Ca²⁺ in this tissue, possibly by phosphorylation of an ion channel.

It might be possible however that Ca²⁺ release from intracellular stores by IP₃ in this tissue, while not being sufficient alone to initiate contraction, might still stimulate capacitative Ca²⁺ influx which could then be partially responsible for the contraction. If this were the case then when the tissue had been stimulated by noradrenaline (and then washed out) in Ca²⁺ free Krebs solution the intracellular stores should remain depleted so that upon addition of extracellular Ca²⁺, capacitative Ca²⁺ influx would still occur resulting in a contraction. However there was no response in this tissue to the readdition of Ca²⁺ showing that this mechanism of Ca²⁺ influx does not exist in the response to noradrenaline. This is unlike the rat spleen where readdition of Ca²⁺ after stimulation of α₁β₂ adrenoceptors with phenylephrine did produce a contraction (Chapter 7). Also, cyclopiazonic acid, which can deplete Ca²⁺ from intracellular stores by inhibiting the sarcoplasmic reticulum Ca²⁺-ATPase (Seidler et al., 1989; Deng & Kwan, 1991) and therefore stimulate capacitative Ca²⁺ influx in tissues where this mechanism exists, had no effect in the rat epididymal vas deferens at a concentration which produced a maximal contraction in the rat spleen (Chapter 7). This confirmed that capacitative Ca²⁺ influx cannot be stimulated in the rat epididymal vas deferens.

Both Ca²⁺ dependent and independent isoforms of PKC have been identified (Lee & Severson, 1994). It is possible therefore that following hydrolysis of PIP₂, IP₃ which is released into the cytosol, raises [Ca²⁺], producing a translocation of PKC to the membrane (Haller et al., 1990) where DAG, which remains membrane bound, can then also bind to PKC resulting in its full activation. (This mechanism might occur even though noradrenaline in Ca²⁺ free Krebs solution failed to produce a response as the rise in [Ca²⁺], may not be great
enough to initiate contraction). This could also be a reason why activation of PKC by PDBu could not produce the same maximal response as that to noradrenaline. Some tissues were therefore pre-incubated with cyclopiazonic acid for 30 minutes before addition of noradrenaline. This treatment should deplete Ca\(^{2+}\) from intracellular stores and therefore this would not be available for release by IP\(_3\) in the noradrenaline response. The length of the incubation time for cyclopiazonic acid with the tissue should ensure that any rise in [Ca\(^{2+}\)], produced by this compound had returned to resting levels before addition of the noradrenaline. However pre-incubation with cyclopiazonic acid did not affect either the magnitude or rate of rise of the noradrenaline contraction in this tissue. Other tissues were pre-incubated with cyclopiazonic acid for either one or three minutes prior to the addition of PDBu, when any rise in [Ca\(^{2+}\)], produced by cyclopiazonic acid might still exist. This did not however potentiate the PDBu contraction. Some cells have also been shown to have intracellular Ca\(^{2+}\) stores which are insensitive to IP\(_3\) but are sensitive to ryanodine (Sorrentino & Volpe, 1993; Ehrlich et al., 1994) and ryanodine binding sites have been demonstrated in the rat vas deferens (Bourreau et al., 1991). However ryanodine at a concentration shown to abolish increases in [Ca\(^{2+}\)], produced by noradrenaline in rabbit mesenteric artery (Itoh et al., 1992), had no effect on the noradrenaline contraction in the rat epididymal vas deferens. These results suggest that release of intracellular Ca\(^{2+}\) by IP\(_3\) (or by a ryanodine sensitive mechanism) is not required for the PKC activity involved in the contraction to noradrenaline. This could mean that the PKC isoform involved in the contraction of the rat epididymal vas deferens belongs to the Ca\(^{2+}\) independent group. Alternatively, DAG/phorbol ester binding to PKC might increase the affinity of the enzyme for Ca\(^{2+}\) and therefore a Ca\(^{2+}\) dependent isoform could be involved which does not require a rise in [Ca\(^{2+}\)] for activation (Castagna et al., 1982). If IP\(_3\) is not required for the contraction it would also be possible that the DAG produced in response to noradrenaline in this tissue is not a product of PIP\(_2\) hydrolysis but is formed via PLC or PLD mediated hydrolysis of phosphatidylcholine. However, as mentioned earlier, the potentiation of the noradrenaline contraction by DAG kinase inhibition suggests involvement of arachidonyl-DAG formed from PIP\(_2\) hydrolysis and secondly,
noradrenaline has been shown to stimulate inositol phosphate formation in the rat vas deferens (Fox et al., 1985).

It has been suggested by Bultmann et al. (1993) that the adrenergic component of neurogenic contractions in the rat vas deferens is mediated in part by mobilization of \([Ca^{2+}]\), in the smooth muscle as these responses are inhibited in the presence of ryanodine. However ryanodine did not reduce contractions to exogenous noradrenaline in these experiments, in agreement with Bourreau et al., (1991). The reason for this difference is not clear.

The maximum response to PDBu being less than that to noradrenaline could be due to the time PDBu takes to diffuse into the tissue, or PDBu might be a “partial agonist” compared with DAG for the PKC isoform involved. Different phorbol esters can produce varying biological responses in tissues suggesting that they do have some isoform selectivity (Ryves et al.,1991) and different species of DAG may also have PKC isoform selectivity (Nishizuka, 1986; Ford et al., 1989).

Figure 6.11 is a schematic diagram showing the cellular mechanisms proposed to be involved in the contraction of the rat epididymal vas deferens to noradrenaline. It shows the \(\alpha_{1A}\)-adrenoceptor mediated contraction is dependent on activation of PKC by DAG produced from PLC mediated hydrolysis of PIP\(_2\), which results in an influx of extracellular \(Ca^{2+}\) through voltage-gated \(Ca^{2+}\) channels. No evidence for inositol trisphosphate involvement in the contraction was found.
**Figure 6.11.** Schematic diagram of the cellular mechanisms proposed to be involved for the $\alpha_{IA}$-adrenoceptor mediated contraction to noradrenaline (NA) in the rat epididymal vas deferens, which also shows the sites of action for compounds used in this study. DAG: diacylglycerol, PA: phosphatidic acid, DK: DAG kinase, PKC: protein kinase C, GDP: guanosine diphosphate, GTP: guanosine trisphosphate, IP$_3$: inositol 1,4,5-trisphosphate, NA: noradrenaline, PIP$_2$: phosphatidylinositol 4,5-bisphosphate, PLC: phospholipase C, membr.: cell membrane, extra.: extracellular, intra.: intracellular. $\alpha$ and $\beta/\gamma$ are the three subunits of a heterotrimeric G-protein.
Chapter 7

The role of capacitative Ca\textsuperscript{2+} influx in the $\alpha_{\text{1b}}$-adrenoceptor mediated contraction to phenylephrine in the rat spleen.

7.1 Introduction

$\alpha_{\text{1}}$-Adrenoceptors mediate cellular responses such as contraction of smooth muscle by raising $[\text{Ca}^{2+}]_i$ (Minneman & Esbenshade, 1994). They are generally linked upon stimulation to activation of PLC, which hydrolyses the lipid precursor PIP\textsubscript{2} to IP\textsubscript{3} and DAG. IP\textsubscript{3} can mobilize Ca\textsuperscript{2+} from intracellular stores, raising the cytosolic $[\text{Ca}^{2+}]_i$, while DAG can stimulate PKC (Berridge, 1993).

The aim of the present experiments was to investigate the role of DAG, PKC activation, IP\textsubscript{3} and capacitative Ca\textsuperscript{2+} influx (Chapter 1.4) in the $\alpha_{\text{1b}}$-adrenoceptor mediated contraction of the rat spleen to phenylephrine (Chapter 4; Aboud et al., 1993).

7.2 Experimental Protocol

The rat spleen was set up as described in Chapter 2. A contraction to phenylephrine ($3\times10^{-4}$M) which produced a maximal response was measured in each tissue. All tissues were then left for two hours before any further responses were measured due to the long recovery time of this tissue following a contraction to phenylephrine. When Ca\textsuperscript{2+} free Krebs solution was used this contained 1mM EGTA unless stated otherwise. When cumulative additions of either phenylephrine ($10^{-7}$M-$10^{-3}$M) or K\textsuperscript{+} ($2\times10^{-5}$M-$10^{-3}$M) were used a concentration effect curve was recorded 2 hours after the initial contraction to phenylephrine ($3\times10^{-4}$M) and then repeated after a further 2 hours as either a control or under the relevant conditions for the experiment.
Contractions induced by cumulative additions of phenylephrine (10\(^{-7}\)M-10\(^{-3}\)M) were measured in the presence of the selective PKC inhibitor calphostin C (10\(^{-6}\)M, 1 hour incubation). In some experiments, after the initial contraction to phenylephrine, responses to phenylephrine (10\(^{-5}\)M, which produced a contraction about 60% maximum response) were measured at 1 hour intervals until they became reproducible (within ±2%). The final response to phenylephrine (10\(^{-5}\)M) was then measured in the presence of the DAG kinase inhibitor R 59022 (3×10\(^{-5}\)M, 30 minute incubation). Calphostin C was used in a bright light environment as this is essential to its activity (Bruns et al., 1991). Some control responses were measured in the presence of DMSO (1%) which was used to dissolve the calphostin C.

Contractions induced by cumulative additions of phenylephrine (10\(^{-7}\)M-10\(^{-3}\)M) were measured in Ca\(^{2+}\) free Krebs solution. In other tissues the response to a single addition of phenylephrine (3×10\(^{-5}\)M) was measured either in normal Krebs solution or in Ca\(^{2+}\) free Krebs solution, in which case the tissue was first allowed to recover for 90 minutes in normal Krebs solution following the initial phenylephrine contraction before being equilibrated for 30 minutes in Ca\(^{2+}\) free Krebs solution.

To see if influx of extracellular Ca\(^{2+}\) was stimulated by depletion of the intracellular Ca\(^{2+}\) stores, 20 minutes after the addition of phenylephrine in Ca\(^{2+}\) free Krebs solution the agonist was washed out for another 120 minutes (still in Ca\(^{2+}\) free Krebs solution) so that the concentrations of any second messengers generated by α\(_i\)-adrenoceptor stimulation were unlikely to remain raised. The intracellular Ca\(^{2+}\) stores should remain depleted however as there was no extracellular Ca\(^{2+}\) to enter and refill them. EGTA was removed from the Krebs solution for the last 30 minutes. Ca\(^{2+}\) (2.5mM) was then added to the Krebs solution and the response to this measured. As a control, in other tissues 20 minutes after the addition of phenylephrine in normal Krebs solution the agonist was washed out for 90 minutes in normal Krebs solution and then equilibrated for 30 minutes in Ca\(^{2+}\) free Krebs solution with the EGTA being removed for the
last 15 minutes. $\text{Ca}^{2+}$ (2.5mM) was then added to the Krebs solution and the response to this measured.

The endoplasmic reticulum $\text{Ca}^{2+}$-ATPase inhibitor cyclopiazonic acid was added to some tissues in normal Krebs solution ($10^7 \text{M}-3 \times 10^7 \text{M}$, one concentration per tissue). In other tissues the effect of cyclopiazonic acid ($10^5 \text{M}$) was then measured in $\text{Ca}^{2+}$ free Krebs solution. One hour after the end of the contraction to cyclopiazonic acid in $\text{Ca}^{2+}$ free Krebs solution and with the EGTA removed for the final 30 minutes, $\text{Ca}^{2+}$ (2.5mM) was added to the Krebs solution and the response to this measured.

To see if contractions to phenylephrine and cyclopiazonic acid in $\text{Ca}^{2+}$ free Krebs solution might be due to release of intracellular $\text{Ca}^{2+}$ from the same store, some tissues were incubated with cyclopiazonic acid ($10^5 \text{M}$) in $\text{Ca}^{2+}$ free Krebs solution for 30 minutes and the response to the subsequent addition of phenylephrine ($3 \times 10^4 \text{M}$) was then measured.

Contractions induced by cumulative additions of phenylephrine ($10^7 \text{M}-10^5 \text{M}$) or the contraction to cyclopiazonic acid ($10^5 \text{M}$) were measured in the presence of the $\text{Ca}^{2+}$ channel blockers nifedipine ($10^4 \text{M}$, 20 minutes equilibration) or SK&F 96365 ($3 \times 10^5 \text{M}$, 20 minutes equilibration).

The effect of the tyrosine kinase inhibitors genistein $3 \times 10^5 \text{M}$, or tyrphostin 23 $10^6 \text{M}$, (either incubated with the tissue for 1 hour) were measured on contractions to cumulative additions of phenylephrine or $K^+$ and also a contraction to cyclopiazonic acid ($3 \times 10^4 \text{M}$). Control responses were measured in the presence of DMSO (0.5%) which was used to dissolve the genistein and tyrphostin 23. Preliminary experiments showed that higher concentrations of these inhibitors caused a reduction in the contractions to $K^+$.

7.2.1 Data analysis

The results were calculated as percentage maximum response of the initial phenylephrine contraction or as percentage maximum response of the first

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cumulative concentration-effect curve for either phenylephrine or K⁺ when one of these was used. Responses were then plotted as the mean of at least four separate experiments with vertical bars representing s.e.mean. Error bars appear on Figures only when they exceed the symbol size. Curve fitting for the calculation of pEC₉₀ values by non-linear regression was performed using InPlot (GraphPad Software, San Diego, Calif., USA). Where potencies have been given as a pEC₉₀ value this was equal to - log of the EC₉₀ value. Statistical significance of differences between control and test means was tested for on raw data (g/tension) using a paired t test. A P value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPad Software, San Diego, Calif., USA).

7.3 Results

Phenylephrine (3x10⁻⁴M) produced a maximal response in the rat spleen (0.27±0.02g). The cumulative contractions to phenylephrine were reproducible (pEC₉₀ 4.8±0.1, 96±2% maximum response compared with the initial phenylephrine contraction), as were those to increasing [K⁺] (115±4% maximum response to phenylephrine, pEC₉₀ 1.4±0.1 for the first curve and 114±3% maximum response to phenylephrine, pEC₉₀ 1.4±0.1 for the second curve).

The contractions to cumulative additions of phenylephrine in the presence of calphostin C (10⁻⁶M) were not significantly different to those in the presence of DMSO (1%, for which the maximum response was reduced by 13±5%) (Figure 7.1a). The contraction to phenylephrine (10⁻⁵M) in the presence of R 59022 (3x10⁻⁷M) was not significantly different to the control contraction (57±4% and 61±4% maximum response to phenylephrine respectively, Figure 7.1b). The time taken to reach the maximum response from initiation of the contraction was also not significantly different (control, 82±3 seconds and in the presence of R 59022, 80±3 seconds).

The contraction to a single addition of phenylephrine (3x10⁻⁴M) in normal Krebs solution consisted of an initial phasic contraction and a more slowly developing tonic contraction (Figure 7.2). The contractions to cumulative
additions of phenylephrine were completely abolished in Ca\textsuperscript{2+} free Krebs solution (results not shown). However a single addition of phenylephrine (3x10\textsuperscript{-4}M) in Ca\textsuperscript{2+} free Krebs solution produced a phasic contraction (46±3% maximum response), returning to baseline in 5 minutes (Figure 7.2). When Ca\textsuperscript{2+} (2.5mM) was added to the Krebs solution two hours after the phenylephrine had been washed out following the contraction in Ca\textsuperscript{2+} free Krebs solution, a slowly developing tonic contraction was produced in each tissue reaching 97±4% maximum response (Figure 7.2). However after the contraction to phenylephrine in normal Krebs solution and following 90 minutes washout with normal Krebs solution and then 30 minutes equilibration in Ca\textsuperscript{2+} free Krebs solution, when Ca\textsuperscript{2+} (2.5mM) was added, no response was observed (Figure 7.2).

Cyclopiazonic acid produced a concentration-dependent slowly developing tonic contraction in normal Krebs solution (10\textsuperscript{-7}-10\textsuperscript{-5}M, pEC\textsubscript{50} 5.7±0.1, Figure 7.3). The maximum response to cyclopiazonic acid was reached at 10\textsuperscript{-5}M, 35 minutes after addition (202±8% maximum response to phenylephrine, Figure 7.4). The response to cyclopiazonic acid (10\textsuperscript{-5}M) in Ca\textsuperscript{2+} free Krebs solution was much smaller and not as well maintained (30±4% maximum response to phenylephrine), returning to baseline after 20 minutes (Figure 7.4). When Ca\textsuperscript{2+} (2.5mM) was added to the Krebs solution one hour after the end of the contraction in Ca\textsuperscript{2+} free Krebs solution and with the EGTA removed, a tonic contraction developed in the tissue (263±12% maximum response to phenylephrine, Figure 7.4).

When tissues were pre-incubated with cyclopiazonic acid (10\textsuperscript{-5}M) in Ca\textsuperscript{2+} free Krebs solution for 30 minutes the subsequent contraction to phenylephrine (3x10\textsuperscript{-4}M) was reduced to 9±2% maximum response to phenylephrine (Figure 7.5).

Nifedipine (10\textsuperscript{-6}M) had no significant effect on either the contractions to cumulative additions of phenylephrine or the contraction to cyclopiazonic acid (10\textsuperscript{-5}M) (Figure 7.6). SK&F 96365 (3x10\textsuperscript{-5}M) reduced the maximum response for the cumulative additions of phenylephrine to 35±1% (Figure 7.7a) and the contraction to cyclopiazonic acid (10\textsuperscript{-5}M) from 202±8% to 108±8% maximum.
response to phenylephrine (Figure 7.7b). In 3 out of 12 tissues SK&F 96365 (3x10^{-5} M) produced a contraction (up to 30% of the phenylephrine maximum response) and so the responses in these tissues were not included in the results.

Genistein (3x10^{-5} M) and tyrphostin 23 (10^{-4} M) reduced the maximum response for the cumulative additions of phenylephrine to 51±4% and 44±5% respectively (Figure 7.8a). DMSO (0.5%, which was used to dissolve these inhibitors) had no significant effect on the contractions to phenylephrine. Genistein (3x10^{-5} M) and tyrphostin 23 (10^{-4} M) also reduced the maximum response for the contraction to cyclopiazonic acid (3x10^{-5} M) from 132±6% to 82±5% and 80±7% maximum response to phenylephrine respectively (Figure 7.8b). At these concentrations genistein and tyrphostin 23 had little or no effect on the contractions to increasing [K^+] when compared to those in the presence of DMSO, 0.5%. This concentration of DMSO had no significant effect on the K^+ pEC_{50} but reduced the maximum response by 10±2%. Control pEC_{50} 1.4±0.1, with genistein pEC_{50} 1.4±0.1, with tyrphostin pEC_{50} 1.4±0.1, (Figure 7.9).
Figure 7.1. (a), The effect of calphostin C on contractions to cumulative additions of phenylephrine in the rat spleen. Control in the presence of DMSO (●), + calphostin C 10⁻⁷M (▼). (b), the effect of R 59022 on a contraction to phenylephrine (10⁻⁶M) in the rat spleen. Control (●), + R 59022 3x10⁻⁷M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 7.2. The effect of removing extracellular Ca\(^{2+}\) on the contraction to phenylephrine in the rat spleen and the response to readdition of Ca\(^{2+}\) after washout. Contraction to phenylephrine (3x10^{-4}M) in normal Krebs solution (●), in Ca\(^{2+}\) free Krebs solution containing EGTA 1mM (▼), and the following contraction to the addition of Ca\(^{2+}\) 2.5mM, 2 hours after the phenylephrine has been washed out and the EGTA has been removed from the Krebs solution (■). The effect of readdition of Ca\(^{2+}\) 2.5mM after a contraction to phenylephrine (3x10^{-4}M) in normal Krebs solution, followed by 90 minutes washout in normal Krebs solution and then 30 minutes equilibration in Ca\(^{2+}\) free Krebs solution (▲). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa follows time in seconds after the addition of phenyleprine or the readdition of Ca\(^{2+}\).
Figure 7.3. Contraction of the rat spleen to non-cumulative additions of cyclopiazonic acid in normal Krebs solution (●). The plot represents the mean with s.e.mean of 4 separate experiments (one concentration per tissue) for each of the 6 concentrations of cyclopiazonic acid.
Figure 7.4. The effect of removing extracellular Ca\(^{2+}\) on the contraction to CPA in the rat spleen and the following response to the readdition of Ca\(^{2+}\). Contraction to CPA (10\(^{-3}\)M) in normal Krebs solution (●), in Ca\(^{2+}\) free Krebs solution containing EGTA 1mM (▲). The contraction to the addition of Ca\(^{2+}\) (2.5mM) 1 hour after the end of the response to CPA in Ca\(^{2+}\) free Krebs solution with the EGTA removed from the Krebs solution (■). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa follows time in minutes after the addition of CPA or the readdition of Ca\(^{2+}\).
Figure 7.5. The effect of cyclopiazonic acid pre-treatment on the contraction to phenylephrine in Ca²⁺ free Krebs solution in the rat spleen. Control contraction to phenylephrine (3x10⁴M) in Ca²⁺ free Krebs solution containing EGTA 1mM (●), and after 30 minutes pre-incubation with cyclopiazonic acid 10⁻⁵M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 7.6. The effect of nifedipine in the rat spleen on (a), contractions to cumulative additions of phenylephrine and (b), a contraction to cyclopiazonic acid (10⁻⁶M). Control (●), and in the presence of nifedipine 10⁻⁶M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 7.7. The effect of the non-selective Ca\textsuperscript{2+} channel blocker SK\&F 96365 in the rat spleen on (a), contractions to cumulative additions of phenylephrine and (b), a contraction to cyclopiazonic acid (10\textsuperscript{-5}M). Control (●), and in the presence of SKF 96365 3\times10\textsuperscript{-5}M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 7.8. The effect of the tyrosine kinase inhibitors genistein and tyrphostin 23 on (a), contractions to cumulative additions of phenylephrine in the rat spleen and (b), a contraction to cyclopiazonic acid (3x10^{-5}M) in the rat spleen. Control (●), in the presence of genistein 3x10^{-5}M (▲), and in the presence of tyrphostin 23 10^{-4}M (■). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 7.9. The effect of the tyrosine kinase inhibitors genistein and tyrphostin 23 on contractions to cumulative additions of $K^+$ in the rat spleen. Control (●), in the presence of genistein $3 \times 10^3 \text{M}$ (▼), and in the presence of tyrphostin 23 $10^5 \text{M}$ (■). Each plot represents the mean with s.e.mean of 4 separate experiments.
7.4 Discussion

The mechanism of contraction to phenylephrine in the rat spleen which is mediated by α\textsubscript{ill}-adrenoceptors (Chapter 4; Aboud et al., 1993) has been investigated using functional studies. α\textsubscript{il}-Adrenoceptors are G-protein linked receptors which generally mediate their cellular responses via stimulation of PLC and production of inositol phosphates and DAG (Minneman & Esbenshade, 1994). As in the previous chapter, some experiments were performed on contractions to high K\textsuperscript{+} as a control, again it was assumed that these contractions were the direct result of membrane depolarization leading to the opening of voltage-gated Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+} influx.

The DAG kinase inhibitor R 59022 (de Chaffoy de Courcelles et al., 1985), which inhibits metabolism of DAG to phosphatidic acid, did not potentiate the contraction to phenylephrine in the spleen and the selective PKC inhibitor calphostin C (Kobayashi et al., 1989) did not reduce the contraction. R 59022 has been shown to potentiate contractions to noradrenaline (Chapter 6) and neurokinin A (Burt et al., 1993) in the rat epididymal vas deferens and calphostin C to inhibit them. The effects of R 59022 and calphostin C suggests that DAG and PKC are not involved in the contraction of the rat spleen to phenylephrine.

IP\textsubscript{3} can mobilize Ca\textsuperscript{2+} from intracellular stores causing a transient rise in [Ca\textsuperscript{2+} ], (Irvine, 1992; Berridge, 1993). The effect of removing Ca\textsuperscript{2+} from the Krebs solution was therefore studied to see if the contraction to phenylephrine in the spleen was dependent upon influx of extracellular Ca\textsuperscript{2+}. In normal Krebs solution the maximal response to phenylephrine consisted of an initial phasic contraction and a larger fairly well maintained tonic contraction which lasted over 20 minutes. In Ca\textsuperscript{2+} free Krebs solution a contraction to the same concentration of phenylephrine was reduced by over 50% and consisted of an initial phasic contraction which disappeared within 5 minutes. This is consistent with the contraction to phenylephrine in the rat spleen consisting of an initial phasic contraction due to the release of Ca\textsuperscript{2+} from intracellular stores by IP\textsubscript{3} and a larger tonic contraction due to the influx of extracellular Ca\textsuperscript{2+}. When cumulative additions of phenylephrine were made in Ca\textsuperscript{2+} free Krebs solution no response
was observed. This is probably due to the intracellular Ca\(^{2+}\) stores being depleted gradually and so not causing a large enough rise in [Ca\(^{2+}\)] at any time to initiate contraction.

The possibility that the influx of extracellular Ca\(^{2+}\) in the contraction of the spleen to phenylephrine might be stimulated by the depletion of Ca\(^{2+}\) from intracellular stores was then investigated. After the contraction to phenylephrine in Ca\(^{2+}\) free Krebs solution the phenylephrine was removed and the tissues were allowed to recover for 2 hours (in Ca\(^{2+}\) free Krebs solution). After this period any rise in concentration of second messengers produced by the stimulation of the \(\alpha_1\)-adrenoceptors or the rise in [Ca\(^{2+}\)] should have returned to resting levels. However intracellular Ca\(^{2+}\) stores should remain depleted as there has been no influx of extracellular Ca\(^{2+}\) to allow them to be refilled. Therefore influx of Ca\(^{2+}\) should still occur when extracellular Ca\(^{2+}\) is made available if the depletion of intracellular Ca\(^{2+}\) stores is the stimulus for Ca\(^{2+}\) influx. Upon addition of Ca\(^{2+}\) to the Krebs solution after this 2 hour period a well maintained tonic contraction developed in the tissue, equivalent in size to the phenylephrine contraction in normal Krebs solution but without the initial phasic component. This suggests that influx of extracellular Ca\(^{2+}\) in the spleen is stimulated as a consequence of the depletion of intracellular Ca\(^{2+}\) stores and that this is the mechanism by which the tonic component of the contraction to phenylephrine in the spleen is produced. It was observed that the contraction to the addition of Ca\(^{2+}\) to the Krebs solution after the 2 hour recovery period was better maintained than the tonic contraction to phenylephrine in the spleen. Therefore second messengers (other than IP\(_3\)) produced by the stimulation of the \(\alpha_1\)-adrenoceptors may be involved in the restoration of [Ca\(^{2+}\)] to resting levels. When intracellular Ca\(^{2+}\) stores were allowed to refill after a contraction to phenylephrine by washing the tissue in normal Krebs solution, no response was observed to the addition of Ca\(^{2+}\) after equilibrating the tissue in Ca\(^{2+}\) free Krebs solution. This was consistent with the contraction to Ca\(^{2+}\) following the phenylephrine contraction in Ca\(^{2+}\) free Krebs solution being stimulated by depletion of intracellular Ca\(^{2+}\) stores and was not an effect of equilibrating the tissue in Ca\(^{2+}\) free Krebs solution.
Another way to investigate the effects of depleting intracellular Ca\(^{2+}\) stores in tissues is to use compounds that deplete Ca\(^{2+}\) from these stores by inhibiting the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase such as cyclopiazonic acid (Deng & Kwan, 1991) without causing a rise in inositol phosphate levels (Demaurex et al., 1992). The effects of cyclopiazonic acid on the spleen were therefore studied. Cyclopiazonic acid produced a concentration dependent tonic contraction of the spleen with a maximal effect at 10\(^{-5}\)M. To see if the tonic contraction to cyclopiazonic acid was dependent upon influx of extracellular Ca\(^{2+}\), the contraction to cyclopiazonic acid (10\(^{-5}\)M) in normal Krebs solution was compared to the response to cyclopiazonic acid in Ca\(^{2+}\) free Krebs solution. Removal of extracellular Ca\(^{2+}\) reduced the size of the contraction to cyclopiazonic acid and this was not well maintained. However, when the tissue was given time to recover after the contraction to cyclopiazonic acid in Ca\(^{2+}\) free Krebs solution, a large tonic contraction was still produced in the tissue when Ca\(^{2+}\) was added to the Krebs solution. This is consistent with a small component of the contraction to cyclopiazonic acid in the spleen being due to the release of intracellular Ca\(^{2+}\), with a much larger tonic component of the contraction being due to influx of extracellular Ca\(^{2+}\) stimulated by depletion of intracellular Ca\(^{2+}\) stores. These results therefore further support this mechanism of Ca\(^{2+}\) influx existing in the rat spleen and its involvement in the contraction of the spleen to phenylephrine.

The contraction to cyclopiazonic acid was different in some respects compared with the phenylephrine contraction but this can be explained by the difference in the way the two compounds deplete intracellular Ca\(^{2+}\) stores rather than different cellular mechanisms being involved. Firstly unlike phenylephrine, the cyclopiazonic acid contraction did not have an initial phasic component possibly due to the cyclopiazonic acid having to first enter the cell and then deplete the Ca\(^{2+}\) stores by inhibiting the Ca\(^{2+}\)-ATPase. In this case the Ca\(^{2+}\) release from intracellular stores by cyclopiazonic acid is probably more gradual compared with that following addition of phenylephrine. Another difference is that the maximum contraction to cyclopiazonic acid was about twice the size of that to phenylephrine. This might be because cyclopiazonic acid is more efficient at depleting intracellular Ca\(^{2+}\) stores, resulting in a larger stimulus for Ca\(^{2+}\) influx.
Thirdly, the contraction to cyclopiazonic acid was also better maintained than the phenylephrine contraction. However this is similar to the contraction to Ca\(^{2+}\) in the spleen after the the contraction to phenylephrine in Ca\(^{2+}\) free Krebs solution and the 2 hour recovery period. So in both cases this might be explained by second messengers produced upon stimulation of the \(\alpha\)-adrenoceptors being involved in the restoration of [Ca\(^{2+}\)] to resting levels. Fourthly, the contraction to the addition of Ca\(^{2+}\) after the contraction to cyclopiazonic acid in Ca\(^{2+}\) free Krebs solution was even larger than the contraction to cyclopiazonic acid in normal Krebs solution. While it is not suprising that this contraction is more rapid, due to the cyclopiazonic acid having already depleted the intracellular Ca\(^{2+}\) stores, there is no obvious reason why this contraction should be relatively so large.

There is evidence to suggest that in some cells there are IP\(_3\) sensitive and IP\(_3\) insensitive (e.g. ryanodine sensitive) intracellular Ca\(^{2+}\) stores (Sorrentino & Volpe 1993; Ehrlich et al., 1994). The effect of cyclopiazonic acid (10\(^{-6}\)M) on the contraction to phenylephrine in Ca\(^{2+}\) free Krebs solution was therefore investigated to see if they were releasing Ca\(^{2+}\) from the same intracellular stores. The cyclopiazonic acid caused a large reduction in the phasic contraction to phenylephrine in Ca\(^{2+}\) free Krebs solution suggesting that they both release Ca\(^{2+}\) from the same intracellular store, although it is still possible that cyclopiazonic acid additionally releases Ca\(^{2+}\) from an IP\(_3\) insensitive Ca\(^{2+}\) store.

The first electrophysiological evidence that depletion of intracellular Ca\(^{2+}\) stores activated a Ca\(^{2+}\) current was shown in rat mast cells by Hoth & Penner (1992), which they named \(I\text{\textsubscript{CRAC}}\) (Ca\(^{2+}\) release activated Ca\(^{2+}\) current). \(I\text{\textsubscript{CRAC}}\) has been shown not to involve voltage-gated Ca\(^{2+}\) channels (Hoth & Penner, 1992; Fasolato et al., 1994). However the non-selective Ca\(^{2+}\) channel blocker SK&F 96365 (Merritt et al., 1990) does inhibit Ca\(^{2+}\) influx stimulated by intracellular Ca\(^{2+}\) store depletion (Demaurex et al., 1992; Wayman et al., 1995). The voltage-gated Ca\(^{2+}\) channel blocker nifedipine was found to have no significant effect on contractions to either phenylephrine or cyclopiazonic acid in the spleen but SK&F 96365 did inhibit both contractions. This suggests that the influx of extracellular Ca\(^{2+}\) stimulated by intracellular Ca\(^{3+}\) store depletion in the rat spleen is also through
non-voltage-gated Ca\textsuperscript{2+} channels. It is not certain why SK&F 96365 at 3x10^{-5}M produced a contraction in a few tissues (which were not included in the results). However SK&F 96365 has been shown to cause intracellular Ca\textsuperscript{2+} release from IP\textsubscript{3} insensitive stores in some cells (Merritt et al., 1990; Hughes & Schachter 1994).

Depletion of intracellular Ca\textsuperscript{2+} stores has been shown to lead to a rise in tyrosine phosphorylation in human platelets (Sargeant et al., 1994). Also, influx of extracellular Ca\textsuperscript{2+} stimulated by depletion of intracellular Ca\textsuperscript{2+} stores has been shown to be reduced by tyrosine kinase inhibitors in rat pancreatic acinar cells (Yule et al., 1994) and human lymphocytes (Tepel et al., 1994). The two tyrosine kinase inhibitors genistein (Akiyama et al., 1987) and tyrphostin 23 (Gazit et al., 1989) were found to reduce the contractions to phenylephrine and cyclopiazonic acid at concentrations which had no significant effect on contractions to increasing [K\textsuperscript{+}] in the rat spleen. This suggests that a tyrosine kinase is also involved in the influx of extracellular Ca\textsuperscript{2+} stimulated by depletion of intracellular Ca\textsuperscript{2+} stores in the rat spleen.

The mechanism by which tyrosine kinase activity might be increased in the rat spleen upon depletion of the intracellular Ca\textsuperscript{2+} stores cannot be deduced from these results. However it has been reported that a small anion, Ca\textsuperscript{2+} influx factor (CIF), is produced in human tumour lymphocytes upon depletion of intracellular Ca\textsuperscript{2+} stores which can itself stimulate influx of extracellular Ca\textsuperscript{2+} (Randriamampita & Tsien, 1993). It is possible that CIF stimulates a tyrosine kinase which directly or indirectly causes the opening of a Ca\textsuperscript{2+} channel in the plasma membrane. Alternatively CIF might inhibit a tyrosine phosphatase revealing basal tyrosine kinase activity in the cell, as capacitative Ca\textsuperscript{2+} influx in Xenopus oocytes has been shown to involve a phosphatase (Parekh et al., 1993). Another possibility is that a tyrosine kinase is involved in the production of CIF.

Figure 7.10 is a schematic diagram showing the cellular mechanisms proposed to be involved in the contraction of the rat spleen to phenylephrine based on these results. It shows the contraction consisting of an initial phasic component due to release of intracellular Ca\textsuperscript{2+} and a tonic component due to
capacitative Ca\(^{2+}\) influx through non-voltage-gated Ca\(^{2+}\) channels which may involve a tyrosine kinase.

**Figure 7.10.** Schematic diagram of the cellular mechanisms proposed for the α\(_{1B}\) adrenoceptor mediated contraction to phenylephrine in the rat spleen. DAG: diacylglycerol, GDP: guanosine diphosphate, GTP: guanosine triphosphate, IP\(_3\): inositol 1,4,5-trisphosphate, PHE: phenylephrine, PIP\(_2\): phosphatidylinositol 4,5-bisphosphate, PLC: phospholipase C, SR: sarcoplasmic reticulum, TK: tyrosine kinase, membr.: cell membrane, extra.: extracellular, intra.: intracellular. α and β/γ are the three subunits of a heterotrimeric G-protein. Although this scheme shows the increased tyrosine kinase activity in the cytosol this may occur in the membrane.
Chapter 8

No evidence for the involvement of PKC or capacitative Ca\textsuperscript{2+} influx in the $\alpha_{1A}$-adrenoceptor mediated contraction to noradrenaline of the human prostate.

8.1 Introduction

Stimulation of $\alpha_{1}$-adrenoceptors has been shown to produce a rise in IP\textsubscript{3} in several tissues and also in cells expressing the cloned $\alpha_{1}$-adrenoceptor subtypes (Minneman & Esbenshade, 1994). This rise in IP\textsubscript{3} is presumably accompanied with a rise in DAG (Berridge, 1993). IP\textsubscript{3} can mobilize Ca\textsuperscript{2+} from intracellular stores, raising the cytosolic [Ca\textsuperscript{2+}], while DAG can stimulate PKC (Berridge, 1993). Functional evidence suggested that the $\alpha_{1A}$-adrenoceptor mediated contraction of the rat epididymal vas deferens involved DAG and activation of PKC (Chapter 6), while capacitative Ca\textsuperscript{2+} influx appeared to be involved in the $\alpha_{1B}$-adrenoceptor mediated contraction of the rat spleen (Chapter 7).

The aim of these experiments was to investigate the possible involvement of PKC activation and / or capacitative Ca\textsuperscript{2+} influx (Chapter 1.4) in the $\alpha_{1A}$-adrenoceptor mediated contraction of the human prostate to noradrenaline (Chapter 5).

8.2 Experimental Protocol

Human prostate was set up as described in Chapter 2. In some tissues contractions to cumulative additions of noradrenaline (10^{-6}M-3x10^{-4}M) were measured. These were then either repeated as control curves or under relevant experimental conditions after 60 or 90 minutes. In other tissues a contraction to noradrenaline (10^{-4}M) which produced a maximum response was measured and this was either repeated as a control or under relevant experimental conditions.
after 90 minutes. When Ca\(^{2+}\) free Tyrodes solution was used this contained 1mM EGTA unless stated otherwise.

Contractions induced by cumulative additions of noradrenaline were measured in the presence of the selective PKC inhibitor calphostin C (10\(^{-6}\)M, 1 hour incubation). Calphostin C was used in a bright light environment as this is essential to its activity (Bruns et al., 1991). Some control responses were measured in the presence of DMSO (0.1%) which was used to dissolve the calphostin C. The PKC activator PDBu (10\(^{-6}\)M) was added to other tissues 1 hour after the initial concentration-effect curve to noradrenaline. This concentration produced a near maximum contraction to PDBu in the rat epididymal vas deferens (Figure 6.1).

Contractions induced by cumulative additions of noradrenaline were measured in Ca\(^{2+}\) free Tyrodes solution. After the initial concentration effect curve to noradrenaline the tissues were allowed to recover in normal Tyrodes solution for 60 minutes before being equilibrated in Ca\(^{2+}\) free Tyrodes solution for 30 minutes before the second curve. Contractions induced by cumulative additions of noradrenaline were also measured in the presence of nifedipine (10\(^{-4}\)M, equilibrated for 30 minutes) in normal Tyrodes solution. This concentration of nifedipine should block voltage-gated Ca\(^{2+}\) channels.

In some tissues the effect of Ca\(^{2+}\) free Tyrodes solution was measured on the response to a single addition of noradrenaline. In this case a contraction to noradrenaline (10\(^{-5}\)M) was measured first, tissues were then allowed to recover for 1 hour in normal Tyrodes solution and then equilibrated for 30 minutes in Ca\(^{2+}\) free Tyrodes solution before the addition of noradrenaline (10\(^{-4}\)M).

To see if influx of extracellular Ca\(^{2+}\) was stimulated by depletion of the intracellular Ca\(^{2+}\) stores, 20 minutes after the addition of noradrenaline (10\(^{-4}\)M) in Ca\(^{2+}\) free Tyrodes solution the agonist was washed out for another 120 minutes (still in Ca\(^{2+}\) free Tyrodes solution) so that the concentrations of any second messengers generated by \(\alpha_1\)-adrenoceptor stimulation were unlikely to remain
raised. The intracellular Ca\(^{2+}\) stores should remain depleted however as there was no extracellular Ca\(^{2+}\) to enter and refill them. EGTA was removed from the Tyrodes solution for the last 30 minutes. Ca\(^{2+}\) (1.8mM) was then added to the Tyrodes solution and the response to this measured. As a control, 20 minutes after the second addition of noradrenaline (10\(^{-4}\)M) in normal Tyrodes solution the agonist was washed out for 2 hours still in normal Tyrodes solution before a third addition of noradrenaline (10\(^{-4}\)M) to show the tissue was still responsive. The endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor cyclopiazonic acid (10\(^{-5}\)M) was added to some tissues in normal Tyrodes solution. This concentration produced a maximum contraction in the rat spleen (Figure 7.3).

Contractions to cumulative additions of noradrenaline were also measured in the presence of ryanodine (10\(^{-4}\)M, 30 minute incubation). This concentration has been shown to abolish increases in [Ca\(^{2+}\)], produced by noradrenaline in rabbit mesenteric artery, Itoh et al., 1992).

8.2.1 Data analysis

The results were calculated as a percentage maximum response of the first cumulative concentration-effect curve to noradrenaline or percentage maximum response of the initial contraction to a single addition of noradrenaline. Responses were then plotted as the mean of at least four separate experiments with vertical bars representing s.e.mean. Error bars appear on figures only when they exceed the symbol size. Where potencies have been given as a pEC\(_{50}\) value this was equal to \(-\log\) of the EC\(_{50}\) value. Curve fitting for the calculation of pEC\(_{50}\) values by non-linear regression was performed using InPlot (GraphPad Software, San Diego, Calif., USA). Statistical significance of differences between control and test means was tested for on raw data (g/tension) using a paired t test. A \(P\) value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPad Software, San Diego, Calif., USA).
8.3 Results

The cumulative contractions to noradrenaline were not significantly different in the presence of DMSO, 0.1% (pEC<sub>50</sub> 5.4±0.1 in the absence and presence of DMSO). The selective PKC inhibitor calphostin C (10<sup>-6</sup>M) produced less than a 2 fold shift to the right in the concentration-response curve to noradrenaline with no significant reduction in maximum response (95±3%, P>0.05) (Figure 8.1). PDBu (10<sup>-6</sup>M) did not alter the resting baseline.

Contraction of the prostate to cumulative additions of noradrenaline were reduced to 16±3% maximum response in Ca<sup>2+</sup> free Tyrodes solution containing EGTA (1mM) (Figure 8.2). These contractions were only partially sensitive to nifedipine (10<sup>-6</sup>M), being reduced to 61±2% maximum response (Figure 8.2). The contraction to a single addition of noradrenaline (10<sup>-4</sup>M) was reduced to 44±5% in Ca<sup>2+</sup> free Tyrodes solution (Figure 8.3). The maximum response to the single addition of noradrenaline was not as well maintained as the maximum response obtained following cumulative additions of noradrenaline.

When Ca<sup>2+</sup> (1.8mM) was added to the Tyrodes solution two hours after the noradrenaline (10<sup>-4</sup>M) had been washed out following the agonists addition in Ca<sup>2+</sup> free Tyrodes solution, no response was recorded (Figure 8.3). Tissues still contracted to noradrenaline (10<sup>-4</sup>M) in normal Tyrodes solution however 2 hours after the noradrenaline had been washed out following the addition of the agonist in normal Tyrodes solution, showing the tissues were still able to contract after this period of time. Cyclopiazonic acid (10<sup>-5</sup>M) did not alter the resting baseline.

The maximum response to cumulative additions of noradrenaline were reduced in the presence of ryanodine (10<sup>-4</sup>M) to 47±4% (Figure 8.4).
Figure 8.1. The effect of calphostin C on contractions to cumulative additions of noradrenaline in the human prostate. Control in the presence of DMSO (●), + calphostin C $10^{-7}$M (▲). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 8.2. The effect of nifedipine and Ca\(^{2+}\) free Tyrodes solution containing EGTA (1mM) on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the human prostate. Control concentration-response curve for cumulative additions of noradrenaline in normal Tyrodes solution (○), in the presence of nifedipine 10\(^{-6}\)M (▼), or in Ca\(^{2+}\) free Tyrodes solution containing EGTA (1mM) (■). Each plot represents the mean with s.e.mean of 4 separate experiments.
Figure 8.3. The effect of removing extracellular Ca\(^{2+}\) on the contraction to a single addition of noradrenaline in the human prostate and the response to readdition of Ca\(^{2+}\) after washout. Contraction to noradrenaline (10\(^{-4}\)M) in normal Tyrodes solution (●), in Ca\(^{2+}\) free Tyrodes solution containing EGTA 1mM (▲), and the following response to the addition of Ca\(^{2+}\) 1.8mM, 2 hours after the noradrenaline has been washed out and the EGTA has been removed from the Tyrodes solution (■). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa follows time in minutes after the addition of noradrenaline or the readdition of Ca\(^{2+}\).
Figure 8.4. The effect of ryanodine on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the human prostate. Control concentration-response curve for cumulative additions of noradrenaline (●), in the presence of ryanodine 10^{-4}M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments.
8.4 Discussion

The mechanism of contraction to noradrenaline in the human prostate mediated by \( \alpha_{1a} \)-adrenoceptors (Chapter 5) has been investigated in functional studies. \( \alpha_{1} \)-Adrenoceptors generally mediate their cellular responses via stimulation of PLC and production of inositol phosphates and DAG (Minneman & Esbenshade, 1994).

The selective PKC inhibitor calphostin C (Kobayashi et al., 1989) had very little effect on the noradrenaline contractions in the human prostate, particularly when compared with its effect on the noradrenaline contractions in the rat epididymal vas deferens (Chapter 6) where they were reduced by about 70%. The phorbol ester PDBu, an activator of PKC (Lee & Severson, 1994) also had no effect in this tissue, whereas in the rat vas it produced a contraction up to about 40% of the maximum contraction to noradrenaline. These results suggest that stimulation of PKC is not involved in the noradrenaline contraction of the prostate.

The contractions to cumulative additions of noradrenaline were greatly reduced in Ca\(^{2+} \) free Tyrodes solution but not abolished. The remaining contraction in Ca\(^{2+} \) free Tyrodes solution to a single addition of noradrenaline was much greater however, reaching over 40%. These results suggest that the contraction involves both influx of extracellular Ca\(^{2+} \) and either release of intracellular Ca\(^{2+} \) or a Ca\(^{2+} \) independent mechanism. There was no contraction to cyclopiazonic acid, which can deplete Ca\(^{2+} \) from intracellular Ca\(^{2+} \) stores by inhibiting the sarcoplasmic reticulum Ca\(^{2+} \)-ATPase (Seidler et al., 1989; Deng & Kwan, 1991). Therefore this indicates that the response in Ca\(^{2+} \) free Tyrodes does involve a mechanism not dependent on a rise in [Ca\(^{2+} \)]. However, cyclopiazonic acid might not cause a contraction as the intracellular Ca\(^{2+} \) stores may have been gradually depleted so that the [Ca\(^{2+} \)] does not rise enough to initiate contraction. Similarly, the reason why the maximum contraction to a single addition of noradrenaline in Ca\(^{2+} \) free Tyrodes solution was larger than for the cumulative additions in Ca\(^{2+} \) free Tyrodes solution might be that intracellular Ca\(^{2+} \) is released.
more gradually during the cumulative contractions and so the maximum rise in 
$[Ca^{2+}]_i$ is less.

The possibility that influx of extracellular Ca$^{2+}$ might be stimulated in the 
prostate by depletion of Ca$^{2+}$ from intracellular stores (capacitative Ca$^{2+}$ influx) 
was investigated. Tissues were stimulated by noradrenaline in Ca$^{2+}$ free Tyrodes 
solution which was then washed out. If noradrenaline mobilizes intracellular Ca$^{2+}$ 
then the intracellular Ca$^{2+}$ stores should be remain depleted after the 
noradrenaline was washed out as there was no influx of extracellular Ca$^{2+}$ to 
allow them to be refilled. However, upon addition of extracellular Ca$^{2+}$ there was 
no contraction. This suggests that the capacitative Ca$^{2+}$ influx mechanism does 
not operate in the response to noradrenaline. This is unlike the rat spleen where 
readdition of Ca$^{2+}$ after stimulation of $\alpha_{1b}$-adrenoceptors with phenylephrine did 
drive a contraction (Chapter 7). Also, cyclopiazonic acid, which can deplete 
Ca$^{2+}$ from intracellular stores (Seidler et al., 1989; Deng & Kwan, 1991) and 
therefore stimulate capacitative Ca$^{2+}$ influx in tissues where this mechanism exists, 
had no effect in the prostate at a concentration which produced a maximal 
contraction in the rat spleen (Chapter 7). This again suggested that capacitative 
Ca$^{2+}$ influx cannot be stimulated in the human prostate.

The cumulative contractions to noradrenaline were only partially sensitive 
to nifedipine at a concentration which almost completely abolished the 
contractions in the rat epididymal vas deferens (Chapter 6). Less than 20% of the 
maximum response to noradrenaline was not dependent on extracellular Ca$^{2+}$ for 
the cumulative concentration-response curve in the prostate. Therefore this 
component could not account entirely for the nifedipine insensitive part of the 
contraction, suggesting that the influx of extracellular Ca$^{2+}$ is through a channel 
which is only partially nifedipine sensitive and is unlike the voltage-gated Ca$^{2+}$ 
channel in the rat epididymal vas deferens.

In some cells evidence suggests that there are IP$_3$ sensitive and IP$_3$
insensitive (ryanodine sensitive) intracellular Ca$^{2+}$ stores (Sorrentino & Volpe 
1993; Ehrlich et al., 1994). They are so called due to the ability of ryanodine to
block the Ca\(^{2+}\) channels on these stores. Ryanodine, at a concentration shown to abolish increases in [Ca\(^{2+}\)], produced by noradrenaline in rabbit mesenteric artery (Itoh et al., 1992), reduced the maximum contraction to noradrenaline by over 50% in the prostate. This might suggest that ryanodine was inhibiting the release of Ca\(^{2+}\) from ryanodine sensitive intracellular Ca\(^{2+}\) stores. However there was no contraction to cyclopiazonic acid which should be able to release Ca\(^{2+}\) from both IP\(_3\) sensitive and ryanodine sensitive intracellular Ca\(^{2+}\) stores (although as stated earlier, this might be due to cyclopiazonic acid depleting the intracellular Ca\(^{2+}\) stores gradually so that the [Ca\(^{2+}\)], does not rise enough to initiate contraction). Another possibility is that ryanodine inhibited the contractions via another mechanism such as blocking the influx of extracellular Ca\(^{2+}\).

In conclusion, the \(\alpha_1\)-adrenoceptor mediated contraction to noradrenaline in the human prostate was dependent both on influx of extracellular Ca\(^{2+}\) through a channel which was only partially sensitive to nifedipine and also consisted of a component which may possibly be due to either release of Ca\(^{2+}\) from ryanodine sensitive intracellular stores or a Ca\(^{2+}\) independent mechanism. The contraction did not appear to involve activation of PKC or capacitative Ca\(^{2+}\) influx. It may be that \(\alpha_1\)-adrenoceptors in the human prostate are not linked to activation of PLC. Further investigation of the noradrenaline contraction in the human prostate is required.
Chapter 9.

General Discussion

9.1 Characterization of $\alpha_\text{1}$-adrenoceptor subtypes

Currently three $\alpha_\text{1}$-adrenoceptor subtypes have been characterized pharmacologically and their cDNAs have also been cloned. The $\alpha_\text{1}$-adrenoceptor subtypes mediating contraction of the rat isolated epididymal vas deferens, spleen and human prostate have been characterized in this study using functional experiments (Chapters 3, 4 and 5 respectively).

First, the responses to several $\alpha$-adrenoceptor agonists were studied. Blocking uptake mechanisms with cocaine and $\beta$-oestradiol increased the potency of noradrenaline and phenylephrine but not methoxamine or oxymetazoline in the rat epididymal vas deferens. This suggested that in this tissue uptake mechanisms affect the potency of those agonists which are a substrate for these processes. In the rat spleen and human prostate however cocaine and $\beta$-oestradiol had no effect on the agonist potencies, suggesting uptake mechanisms do not affect agonist potencies in these tissues.

In all three tissues in the presence of uptake blockers noradrenaline and the selective $\alpha_\text{1}$-adrenoceptor agonists phenylephrine and methoxamine had the same order of potency, which was noradrenaline$>$phenylephrine$>$methoxamine. This is the same order as their affinities for the three $\alpha_\text{1}$-adrenoceptor subtypes measured in binding studies and so comparison of the potencies of these agonists does not help in determining the $\alpha_\text{1}$-subtype in a tissue.

Oxymetazoline was found to be an agonist only in the rat epididymal vas deferens. Two possible reasons could account this. Firstly, oxymetazoline may be a subtype selective $\alpha_\text{1}$-adrenoceptor agonist, by not having efficacy at all three $\alpha_\text{1}$-subtypes. This would suggest that the $\alpha_\text{1}$-subtype in the vas is different to
those in the spleen and human prostate. Alternatively, oxymetazoline could be a partial agonist and only contracts the rat epididymal vas deferens because it has a larger receptor reserve compared with the other two tissues. Receptor alkylation experiments suggested that oxymetazoline is a partial agonist and the vas deferens does have a larger receptor reserve compared with the spleen and prostate. While this does not necessarily prove that oxymetazoline is not also a selective agonist for the $\alpha_1$-adrenoceptor subtype in the vas it does make it possible that this subtype could also mediate contraction of the spleen or prostate.

Antagonism of noradrenaline contractions by prazosin in the rat epididymal vas deferens and human prostate suggested that these responses were mediated solely by $\alpha_1$-adrenoceptors but in the rat spleen there appeared to be a non-$\alpha_1$-adrenoceptor mediated component of the contraction. For this reason the selective $\alpha_1$-adrenoceptor agonist phenylephrine was used in the rat spleen while noradrenaline was used in the other two tissues.

The alkylating agent chlorethylclonidine had no effect on the contractions to noradrenaline in the rat epididymal vas deferens but produced a 300 fold shift in the concentration-effect curve to phenylephrine in the rat spleen. The $\alpha_{1b}$-subtype clone has been shown to be most sensitive to chlorethylclonidine with the $\alpha_{1a}$-subtype clone having an intermediate sensitivity and the $\alpha_{1n}$-subtype clone being least sensitive. This suggested that the subtype mediating contractions in the rat epididymal vas deferens was the $\alpha_{1a}$-adrenoceptor, and was probably the $\alpha_{1b}$-adrenoceptor in the rat spleen. However $\alpha_{1b}$-adrenoceptor mediated contractions in the rat aorta (Goetz et al., 1995; Kenny et al., 1995) have a similar sensitivity to chlorethylclonidine (Aboud et al., 1993). Therefore the $\alpha_1$-adrenoceptor in the rat spleen could also be the $\alpha_{1b}$-subtype based on its sensitivity to chlorethylclonidine. In the human prostate chlorethylclonidine produced about a three fold shift in the contractions to noradrenaline and a decrease in the maximum response. This effect was much less than was observed in the rat spleen in this study or the rat aorta (Aboud et al., 1993) suggesting that $\alpha_{1a}$-adrenoceptors could mediate contractions in the human prostate.
Chlorethylclonidine may therefore be most useful in characterizing the $\alpha_{1a}$-adrenoceptor subtype when responses are relatively insensitive to this alkylation agent but cannot be used reliably to distinguish between the $\alpha_{1b}$- and $\alpha_{1d}$-subtypes.

Chlorethylclonidine did however have a greater effect in the human prostate than in the rat epididymal vas deferens, where it had no effect. One problem with the interpretation of the effects of chlorethylclonidine is that the degree to which it affects a functional response depends to some extent on the receptor reserve of the tissue. If a tissue has a large receptor reserve then a reduction in receptor density may result in a rightward shift in the concentration response curve without a reduction in the maximum response. On the other hand if a tissue has a small receptor reserve then an equivalent reduction in receptor density may result in a rightward shift of the concentration response curve and also a decrease in the maximum response.

The fact that a decrease in maximum response was observed in the prostate after chlorethylclonidine treatment with a relatively small rightward shift is consistent with a small receptor reserve in this tissue. The rat vas deferens does have a large $\alpha_{1}$-adrenoceptor reserve (Chapter 3; Diaz-Toledo & Marti, 1988) so a small shift in the noradrenaline curve in this tissue would probably not be accompanied by a reduction in maximum response. The reduced maximum response in the prostate would then only represent a difference in receptor reserve between the prostate and the vas deferens. Therefore it could be argued that the 3 fold shift for the noradrenaline curve in the prostate shows relatively little difference in the effects of chlorethylclonidine between the two tissues. The $\alpha_{1a}$-subtype such as that found in the rat vas deferens corresponds to the cloned $\alpha_{1a}$-adrenoceptor (Chapter 3; Laz et al., 1993; Perez et al., 1994) and this clone also shows some species differences in chlorethylclonidine sensitivity. Therefore the subtypes in the rat vas deferens and the human prostate could both be the $\alpha_{1a}$-subtype and the relatively small difference in chlorethylclonidine sensitivity may reflect a species difference. The 300 fold shift in the phenylephrine contractions of
the rat spleen by chlorehylclonidine could not however be explained away in terms of receptor reserve, species differences or experimental variability.

Due to the problems associated with functional characterization of receptor subtypes using agonists or non-competitive antagonists such as chlorehylclonidine, the affinities of a range of competitive \( \alpha_{1} \)-subtype selective antagonists for the \( \alpha_{1} \)-adrenoceptors in the three tissues was measured. Their affinities in the rat epididymal vas deferens and human prostate corresponded well with those for \( \alpha_{1A} \)-adrenoceptors found in other tissues and also correlated well with their affinities for the expressed \( \alpha_{1B} \)-subtype clone. The affinities of the same antagonists in the rat spleen corresponded well with those for \( \alpha_{1B} \)-adrenoceptors found in other tissues and also correlated well with their affinities for the expressed \( \alpha_{1B} \)-subtype clone. It was therefore concluded that \( \alpha_{1} \)-adrenoceptor mediated contractions in both the rat epididymal vas deferens and human prostate were mediated by the \( \alpha_{1A} \)-subtype while those in the rat spleen were mediated by the \( \alpha_{1B} \)-subtype. An \( \alpha_{1A} \)-adrenoceptor selective antagonist might therefore have reduced side effects in the treatment of benign prostatic hyperplasia. This will also depend however on the \( \alpha_{1} \)-adrenoceptor subtypes mediating vasoconstriction in the human vasculature not being predominantly the \( \alpha_{1A} \)-subtype. Characterization of \( \alpha_{1} \)-subtypes in human isolated blood vessels would therefore provide further information on \( \alpha_{1A} \)-subtype selective antagonists which might be used to treat benign prostatic hyperplasia.

As mentioned above, oxymetazoline was an agonist only in the rat epididymal vas deferens. The \( \alpha_{1} \)-subtype selective competitive antagonists suggested that contractions in both the rat epididymal vas deferens and human prostate are mediated via \( \alpha_{1A} \)-adrenoceptors. So it is possible that the reason for oxymetazoline only being an agonist in the vas deferens was due to the larger receptor reserve in this tissue and oxymetazoline being a partial agonist. However, it has now been shown that several imidazolines including oxymetazoline are \( \alpha_{1B} \)-adrenoceptor selective agonists. In cells transfected with the cDNA for the three \( \alpha_{1} \)-subtypes and which all had a similar receptor reserve,
measurement of increases in inositol phosphates or \([\text{Ca}^{2+}]\), suggested that oxymetazoline is a selective agonist for the \(\alpha_1\)-subtype (Minneman et al., 1994; Horie et al., 1995). If this is the case then the choice of agonist used in functional experiments to characterize \(\alpha_1\)-adrenoceptor subtypes may be important particularly in tissues with a mixed population of subtypes. However the present results show that even if oxymetazoline does not evoke a response in a tissue it cannot be concluded there are no functional \(\alpha_1\)-adrenoceptors present.

Although three \(\alpha_1\)-adrenoceptor subtypes have so far been characterized and their corresponding cDNA cloned, further subtypes might potentially exist which have not yet been detected pharmacologically due to the lack of a selective antagonist. Other \(\alpha_1\)-adrenoceptor subtypes may exist that are the result of a splice variant or cell-specific post-translational modification of one of the existing \(\alpha_1\)-subtype mRNAs. It has recently been reported that two isoforms of the human \(\alpha_1\)-subtype cDNA have been isolated which result from alternative splicing of \(\alpha_1\) mRNA (Tsujimoto, 1995). All three \(\alpha_1\)-isoforms were found to have similar ligand binding properties (which compounds were used was not stated) but other antagonists might identify differences. Characterization of \(\alpha_1\)-adrenoceptor subtypes in other tissues and the search for antagonists that may show differences between the same subtype in different tissues is therefore necessary to determine whether there are other \(\alpha_1\)-subtypes.

9.2 \(\alpha_1\)-adrenoceptor signal transduction mechanisms

The mechanism of contraction to \(\alpha_1\)-adrenoceptor stimulation was investigated in the rat epididymal vas deferens, spleen and human prostate using functional studies (Chapters 6, 7 and 8 respectively). The \(\alpha_{1A}\)-adrenoceptor mediated contraction of the rat epididymal vas deferens appeared to involve activation of PKC by DAG and influx of extracellular \(\text{Ca}^{2+}\) through voltage-gated \(\text{Ca}^{2+}\) channels. The \(\alpha_{1B}\)-adrenoceptor mediated contraction of the rat spleen involved mobilization of intracellular \(\text{Ca}^{2+}\) and capacitative \(\text{Ca}^{2+}\) influx through nifedipine insensitive \(\text{Ca}^{2+}\) channels and may involve a tyrosine kinase. There was no evidence for activation PKC in the spleen or capacitative \(\text{Ca}^{2+}\) influx in the
epididymal vas deferens. The \( \alpha_{1A} \)-adrenoceptor mediated contraction of the human prostate involved a component which was dependent on influx of extracellular Ca\(^{2+} \) through a pathway that was partially nifedipine sensitive and also a component which may possibly be due to either release of Ca\(^{2+} \) from ryanodine sensitive intracellular stores or a Ca\(^{2+} \) independent mechanism. The contraction in the prostate did not appear to involve either PKC activation or capacitative Ca\(^{2+} \) influx.

It seems unlikely therefore that each \( \alpha_{1} \)-adrenoceptor subtype is always linked to the same mechanism of contraction as this is different for the \( \alpha_{1A} \)-adrenoceptor mediated contractions of the rat epididymal vas deferens and human prostate. Also, \( \alpha_{4D} \)-adrenoceptor mediated contractions in the rat aorta (Goetz et al., 1995; Kenny et al., 1995) partly involve activation of PKC (Shimamoto et al., 1993). It may be that cellular processes involved after PIP\(_2\) hydrolysis are more tissue rather than receptor subtype dependent as the NK\(_1\) mediated contraction to neurokinin A in the rat epididymal vas deferens, like that to noradrenaline, may involve activation of PKC by DAG (Burt et al., 1993).

The investigation of the mechanisms likely to be involved in the \( \alpha_{1} \)-adrenoceptor mediated contractions of the rat epididymal vas deferens, spleen and human prostate in this study all involved indirect evidence based on certain assumptions. These were that \( \alpha_{1} \)-adrenoceptors produced contraction of smooth muscle upon stimulation by raising [Ca\(^{2+}\)] and are linked to the activation of PLC, producing IP\(_3\) which can mobilize intracellular Ca\(^{2+}\) and DAG which can stimulate PKC. The \( \alpha_{1} \)-adrenoceptor mediated contraction in all three tissues did seem to require a rise in [Ca\(^{2+}\)] as they all required to some degree the presence of extracellular Ca\(^{2+}\). In the vas deferens and spleen the results also suggested that DAG or IP\(_3\), respectively are involved, however there was no indication that either of these second messengers is involved in the noradrenaline contraction of the prostate.
It would clearly be valuable to substantiate these results with direct
measurements of changes in [Ca\(^{2+}\)], second messenger formation and PKC
activity. However one problem with simply recording changes in second
messenger concentrations is that this is no proof alone of their direct involvement
in a response. For example, although levels of IP\(_3\) may be raised in both the rat
epididymal vas deferens and spleen upon \(\alpha\)-adrenoceptor stimulation, it may
only be involved in the contraction of the spleen.

Further investigation of the contraction to noradrenaline in the human
prostate is needed as the results do not yet indicate which second messengers
may be involved here. Measurement of IP\(_3\) levels in human prostate stimulated
by noradrenaline would show if the \(\alpha_{1A}\)-adrenoceptors in this tissue are linked to
activation of PLC. It would also be of interest to determine the mechanism by
which PKC activation facilitates Ca\(^{2+}\) influx, presumably via membrane
depolarization, in the rat epididymal vas deferens. The mechanism by which
depletion of intracellular Ca\(^{2+}\) stores can stimulate influx of extracellular Ca\(^{2+}\), as in
the contraction of the rat spleen to \(\alpha\)-adrenoceptor stimulation, also requires
further investigation.
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