STUDIES ON α_2-ADRENOCEPTOR SUBTYPES, IMIDAZOLINE BINDING SITES AND COUPLING TO FUNCTIONAL RESPONSES

by
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To Mum and Dad

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others

signed:

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SUMMARY

It was the aim of this thesis to characterise α_2-adrenoceptor subtypes using radiolabelled agonist and antagonist ligands in a variety of tissue preparations. RS-15385-197 is a high affinity and selective α_2-adrenoceptor antagonist. [^{3}H]-RS-15385-197 was shown to label α_{2A}- and α_{2B}-adrenoceptor subtypes in human platelet and rat neonatal lung membranes, and a subtype in rat cortex which showed greatest similarity with the α_{2D}-adrenoceptor subtype. Thus the receptor in rat brain was shown to form a distinct subtype from that previously described. Differentiation of the α_2-adrenoceptor into 2A and 2B subtypes could not be demonstrated however with the agonist ligand, [^{3}H]-adrenaline, under normal assay conditions, suggesting that characterisation of receptor subtypes is best carried out using both an agonist and an antagonist radioligand.

The functional consequences of α_{2A}- and α_{2B}-adrenoceptor activation was addressed in a model of α_2-adrenoceptor mediated inhibition of cAMP accumulation. Human platelet α_{2A}-adrenoceptors are negatively coupled to adenylate cyclase, and the α_2-adrenoceptor agonists UK 14304, clonidine and oxymetazoline were shown to inhibit cAMP levels in whole cells and platelet membranes. In the neonatal rat lung preparation, which binding studies showed to contain α_{2B}-adrenoceptors, no inhibition of cAMP levels or adenylate cyclase activity was observed in response to the α_2-adrenoceptor agonists. It is suggested that the α_{2B}-adrenoceptor may not couple efficiently to adenylate cyclase.

[^{3}H]-idazoxan an α_2-adrenoceptor antagonist with an imidazoline structure, in addition to labelling α_2-adrenoceptors was also shown to label a population of imidazoline binding sites in rat kidney which were not adrenoceptors based on the low affinity of noradrenaline and RS-15385-197. Characterisation of these sites with [^{3}H]-idazoxan and another imidazoline ligand [^{3}H]-*p*-aminoclonidine suggested that the imidazoline sites labelled by these ligands were heterogeneous and were located in discrete areas of rat brain. As a direct consequence of this work RS-45041-190 was identified as a high affinity imidazoline compound which has greater than 10,000 fold selectivity for imidazoline sites over α_2-adrenoceptors. In the hamster adipocyte, a tissue which was shown previously to contain both α_2-adrenoceptors

and imidazoline binding sites, the inhibition of glycerol release by UK 14304 was reversed by compounds showing selectivity for α_2-adrenoceptors and not by imidazoline selective agents, suggesting that imidazoline sites are not involved in the UK 14304 mediated inhibition of lipolysis in this tissue.

The functional role of α_2-adrenoceptor subtypes and imidazoline binding sites are discussed.

List of Publications arising from this thesis

Published Abstracts:

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CONTENTS

ABBREVIATIONS

The abbreviations used in this thesis are in accordance with the guidelines set out in the British Journal of Pharmacology Instructions to authors. Those not defined in the above publication are listed below.

Ca^{2+}, Na^{+}, Mg^{2+}, Cl^{-}, K^{+} refer to the ionic species of calcium, sodium, magnesium, chloride and potassium respectively.

8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)-tetralin hydrobromide
ACTH	adrenocorticotrophic hormone
ADH	anti-diuretic hormone
ADP	adenosine 5′ diphosphate
ATP	adenosine 5′ triphosphate
BHT 920	6-allyl-2-amino-5,6,7,8-tetrahydro-4*H*-thiazolo-[4,5-d] azepine hydrochloride
BRL 44409	2-[5-chloro-1, 3-dihydro-2*H*-isoindol-2-yl) methyl]-4,5-dihydro-1*H*-imidazole dihydrochloride
cAMP	adenosine 3′ 5′ cyclic monophosphate
CDS	clonidine displacing substance
cGMP	guanosine 3′ 5′ cyclic monophosphate
CH-38083	7,8-(methylenedioxi)-14a-hydroxyalloberbane HCl
dor.	dorsal
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid (free acid)
ET-1	endothelin 1
FMRF-NH_2	phenylalanine-methionine-arginine-phenylalanine-amide
G-protein	guanyl nucleotide binding protein
GHRH	growth hormone releasing hormone
Gpp(NH)p	5′-guanylimidophosphate
GTP	guanosine 5′ triphosphate
HT29	human colonic adenocarcinoma cell line
IBMX	isobutyl methyl xanthine
L-654,284	(2R, 12bS)-N-(1,3,4,6,7,12b-hexahydro-2*H*-

	benzo[b]-furo[2,3-a] quinolizone-2-yl)-N-methyl-2-hydroxyethanesulphonamide
L-657,743 (MK-912)	(2S, 12bS) 1′,3′dimethylspiro (1,3,4,5′,6,6′,7,12b-octahydro-2*H*-benzo[b]furo[2,3-a]quinazoline)-2,4′-pyrimidin-2′-one
lat.	lateral
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
MDL 72222	1αH,3αH,5αH-tropan-3-yl 3,5 dichlorobenzoate methane sulphonate
med.	medial
MK 912 (L-657,743)	(2S, 12bS) 1′,3′dimethylspiro (1,3,4,5′,6,6′,7,12b-octahydro-2*H*-benzo[b]furo[2,3-a]quinazoline)-2,4′-pyrimidin-2′-one
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormone
NG108-15	neuroblastoma x glioma hybrid cell line
NMDG-Cl	N-methyl-D-glucamine chloride
NRL	nucleus reticularis lateralis
NSB	non specific binding
NTS	nucleus of the solitary tract
nuc.	nucleus
O.D.	optical density
PK 11195	1-2(chlorophenyl-N-methyl-N-(1-methylpropyl))-3-isoquinolinecarboxamide
PMSF	phenyl-methyl-sulphonyl-fluoride
PRP	platelet rich plasma
RU 24969	5-methoxy-3-(1,2,3,6, tetrahydropyridin-4-yl)-1*H*-indole
RX 821002	2-[2-(2-methoxy-1,4-benzodioxanyl) imidazoline hydrochloride
SKF 104078	6-chloro-9-(3-methyl 2 butenyl) oxyl-3-methyl-1*H*-2,3,4,5-tetrahydro-3-benzazepine
RS-15385-197	(8aR, 12aS, 13aS)-5,8,8a,9,10,11,12,12a,13, 13a-decahydro-3-methoxy-12-(methylsulfonly)-

	6*H*-isoquino [2,1-g][1,6]-naphthyridine
subs. nigra	substantia nigra
TRH	thyrotropin releasing hormone
trig.	trigeminal
UK 14304	5-bromo-6[2-imidazoline-2yl amino] quinoxaline
VIP	vasoactive intestinal polypeptide
WB 4101	2-[2,6-dichloro(N-beta chloroethyl-N-methyl)-4-methyl amino] phenylimino-2-imidazoline dihydrochloride
WY 26703	W-methyl-N-(1,3,4,6,7,11b-hezahydro-2*H*-benzo-αaβ-quinolizin-2-yl)-s-butane-sulphonamide

CHAPTER ONE

GENERAL INTRODUCTION

1.1 HISTORICAL PERSPECTIVES

The actions of adrenaline, an adrenal hormone and a central neurotransmitter, and noradrenaline, a peripheral and central neurotransmitter, are mediated through α- and β-adrenoceptors. The initial subclassification of adrenergic receptors into the alpha and beta subtypes was done by Alquist (1948) on the basis of their pharmacology. He studied the effect of five catecholamines on eight different physiological functions and showed that the order of potency for the first five functions was markedly different from the remaining three functions. He attributed this difference to an actual difference in the receptors involved and termed these receptors α- and β-adrenergic receptors. At that time the adrenoceptor antagonists available, phentolamine and phenoxybenzamine, appeared to block only α–mediated responses. Specific β-antagonists were not developed for another 10 years. The subdivision of β-adrenoceptors into β_1 and β_2 was carried out by Lands *et al.* (1967), where β_1-adrenoceptors were defined as having equal affinity for adrenaline and noradrenaline, whereas β_2-adrenoceptors had higher affinity for adrenaline. The subclassification has been substantiated by the development of subtype specific antagonists and by direct binding studies (Minneman *et al.*, 1979).

1.2. α-ADRENOCEPTOR CLASSIFICATION

1.2.1. α_1/α_2 classification

It had been known for many years that α-adrenoceptor antagonists increased the flow of noradrenaline evoked by sympathetic nerve stimulation. Brown & Gillespie (1957) suggested that the effect of the antagonists was to bind to postjunctional α-adrenoceptors, thus preventing the interaction of these receptors with released transmitter and thereby increasing transmitter overflow. Other suggestions were that the effect was due to neuronal (Thoenen *et al.*, 1964) or extraneuronal (Langer, 1970) uptake. The concept of a presynaptic receptor which could modulate the release of noradrenaline was put forward in 1971 by two groups (Starke *et al*, 1971; Langer *et al.*, 1971). The subsequent finding that clonidine could discriminate between pre- and postjunctional adrenoceptors (Starke *et al.*, 1974) led

Langer (1974) to propose that the postjunctional receptor that mediates the response in the effector organ be termed α_1, whereas the prejunctional receptor that regulates neurotransmitter release be termed α_2.

The anatomical classification holds true in several instances, however, it became clear that some postjunctionally mediated events such as the inhibition of melanin granule dispersion in frog skin (Pettinger, 1977), and the inhibition of isoprenaline-induced increases in lipolysis and glycerol release from isolated hamster adipocytes (Schimmel, 1976), were produced by agonists that were highly selective for α_2-adrenoceptors. In addition, α_2-adrenoceptors were reported to exist postjunctionally in the vasculature due the selectivity of yohimbine in reversing pressor responses to noradrenaline in the pithed rat (Drew & Whiting, 1979). Therefore, a new method of classification was developed based on the relative affinities of selective antagonists and sometimes agonists. Thus, a response that was activated by methoxamine or phenylephrine and competitively blocked by prazosin or WB4101 was classified as being mediated via an α_1-adrenoceptor, whilst a response evoked by clonidine, UK-14304 or BHT 920 and competitively blocked by yohimbine, rauwolscine or idazoxan was classified as an α_2-adrenoceptor mediated response. Since the initial classification, newer antagonists have been developed which show greater affinity and/or selectivity for the α_2-adrenoceptor than yohimbine, rauwolscine or idazoxan. These include L-654,284 (Pettibourne *et al.*, 1986), L-657,743 (Pettibourne *et al.*, 1987), CH-33083 (Vizi *et al.*, 1986) and RX 821002 (Stillings *et al.*, 1985; Langin *et al.*, 1989).

1.2.2. Characterisation of α_2-adrenoceptor subtypes in radioligand binding studies

Direct measurement of α-adrenoceptors *in vitro* was acheived in 1976 with the development of [^{3}H]-dihydroergotryptine (DHE, William & Lefkowitz, 1976). Although [^{3}H]-DHE was the first radioligand used to label α_2-adrenoceptors, it binds with equal affinity at both α_1- and α_2-adrenoceptors. However, its use in conjunction with selective competitors provided compelling evidence for the existence of unique α_1- and α_2-adrenoceptors (Miach *et al.*, 1978). Since then, selective radiolabelled agonists and antagonists has been used to reversibly label

α_2-adrenoceptor subtypes. Table 1.1. shows some of the radioligands which have been used.

According to Hoffman &Lefkowitz (1980), the α_2-adrenoceptor, like the β-adrenoceptor exists in two conformational states; antagonists recognise both states of the α_2-adrenoceptor with equal affinity, whereas agonists have high affinity for only one of the states and show biphasic inhibition curves against labelled antagonists. A labelled agonist at low concentrations would be expected to bind to the high affinity (H) state of the receptor, and with increasing concentrations to identify an increasing amount of the low affinity (L) state. Thus, saturation transformations of radioligand binding over a wide concentration range would be curvilinear, but would be linear over a more restricted range.

Early studies with labelled agonists were carried out with [^{3}H]-catecholamines. [^{3}H]-Adrenaline was used in preference to [^{3}H]-noradrenaline due to the higher affinity of the former compound. Initial studies were limited by the use of the low specific activity racemic mixture of [^{3}H]-adrenaline (U'Prichard & Snyder, 1977). [^{3}H]-adrenaline binding was limited compared to that with [^{3}H]-imidazolines such as clonidine, due to expense and technical difficulties. Care had to be taken to prevent oxidation and degradation and binding to other adrenoceptors present in the membrane preparation. However, the human platelet was found to be an ideal tissue to study α_2-adrenoceptors as this tissue contained a very low density of α_1- and β-adrenoceptors (Bylund & U'Prichard, 1983).

The development of an assay buffer system to prevent oxidation of [^{3}H]-adrenaline for up to 4 hours (Sènard *et al.*, 1988) has improved the selective labelling of α_2-adrenoceptors by [^{3}H]-adrenaline, allowing the characteristics of the receptor to be examined using the endogenous ligand. The (H) state of the receptor has also been labelled with [^{3}H]-clonidine, [^{3}H]-*p*-aminoclonidine and [^{3}H]-UK 14304 (for references see Table 1.1.) and in general showed very similar characteristics to the site labelled with [^{3}H]-adrenaline; however, these ligands have also been shown to label non-adrenergic binding sites in many tissues (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989; Michel & Insel, 1989; Langin *et al.*, 1990). The radiolabelled antagonists most frequently used have been the alkaloid isomers yohimbine and rauwolscine, and for a while these have been the most selective and potent α_2-antagonists available. Binding of

these radioligands has been carried out in human platelets (Motulsky *et al.*, 1980; Limbird *et al.*, 1982), human brain (Petrash & Bylund, 1986), human spleen (Dickinson *et al.*, 1986), rat brain (Cheung *et al.*, 1982; Rouot *et al.*, 1982; Bylund, 1985; Brown *et al.*, 1990a,b), rat kidney (Schmitz *et al.*, 1981; Woodcock & Johnston, 1982), neonatal rat lung (Latifpour *et al.*, 1982), and rabbit spleen (Michel *et al.*, 1989b), see also references cited in Regan (1988). However, there are instances where the presence of α_2-adrenoceptors have been irrefutably proven in functional studies and in binding studies with [^{3}H]-clonidine and [^{3}H]-UK14304, but where no specific [^{3}H]-yohimbine binding has been detected. Such a tissue is the adult rat submandibular gland which contains a high density of [^{3}H]-clonidine binding sites (150 fmoles/mg protein), but contains no specific [^{3}H]-yohimbine binding (Bylund & Martinez, 1981). Similarly, in rabbit adipocytes, a tissue which contains functional α_2-adrenoceptors (Lafontan & Berlan 1980), [^{3}H]-yohimbine failed to bind to α_2-adrenoceptors (Langin & Lafontan, 1989) whereas [^{3}H]-UK 14304 labelled the high affinity state and [^{3}H]-RX 821002 labelled the whole population of α_2-adrenoceptors (B_{max} = 289 fmoles/mg protein, Langin *et al.*, 1990). The inability of [^{3}H]-yohimbine to label α_2-adrenoceptors in these studies was explained by its relatively low affinity in these tissues, and the high level of non-specific binding.

Amongst the newer α_2-adrenoceptor antagonist ligands, [^{3}H]-idazoxan has been employed to label α_2-adrenoceptors in rat cerebral cortex (Langer *et al.*, 1983; Doxey *et al.*, 1983b; Lane *et al.*, 1983) and human platelets (Elliot & Rutherford, 1983). These studies showed that [^{3}H]-idazoxan had high affinity and labelled sites with the characterisics of the α_2-adrenoceptor, but demonstrated a high level of non-specific binding and was more sensitive to buffer composition than [^{3}H]-yohimbine. Recent studies from this laboratory (MacKinnon *et al.*, 1989; Brown *et al.*, 1990a) and others (Yablonsky *et al.*, 1988; Michel & Insel, 1989) have demonstrated an additional non-adrenergic site which [^{3}H]-idazoxan labels with high affinity. Unless binding to this additional site is excluded (which may not be possible due to the lack of selectivity of available compounds), the use of [^{3}H]-idazoxan as a selective marker for α_2-adrenoceptors is limited, and at present offers no particular advantage over [^{3}H]-yohimbine or [^{3}H]-rauwolscine in the measurement of α_2-adrenoceptor affinity. As shown in Table 1.1.

Table 1.1. [3H]-agonist and [3H]-antagonist ligands used for the identification of α_2-adrenoceptors.

compound	specific activity Ci/mmole	selectivity	references
agonist ligands			
[3H]-adrenaline	60 - 90	non-selective	*a b*
[3H]-clonidine	20 - 60	α_2	*c d*
[125I]-iodoclonidine	2200	α_2	*e*
[3H]-p-aminoclonidine	40 - 60	α_2	*f*
[3H]-guanfacine	24	α_2	*g*
[3H]-UK 14304	60 - 90	α_2	*h*
antagonist ligands			
[3H]-dihydroergotryptine	20 - 50	non-selective	*i*
[3H]-dihydroergonine	27	non-selective	*j*
[3H]-phentolamine	23	non-selective	*k*
[3H]-yohimbine	70 - 90	α_2	*l m*
[3H]-rauwolscine	70 - 90	α_2	*n*
[3H]-idazoxan	30 - 50	α_2	*o p*
[3H]-RX 821002	44	α_2	*q*
[3H]-L-654,284	58	α_2	*r*
[3H]-L-675,743	70	α_2	*s*

a. U'Prichard & Snyder, 1977, *b*. Garcia-Sevilla & Fuster, 1986, *c*. U'Prichard *et al*., 1977, *d*. Tanaka & Starke, 1979, *e*. Baron & Siegel, 1990, *f*. Rouot & Snyder, 1979, *g*. Timmermans *et al*., 1982, *h*. Neubig *et al*., 1985, *j*. Miach *et al*., 1978, *k*. Steer *et al*., 1979, *l*. Motulsky *et al*., 1980, *m*. Brown *et al*., 1990a, *n*. Cheung *et al*., 1982,*o*. Lane *et al*., 1983, *p*. Boyajian & Leslie, 1987, *q*. Langin & Lafontan 1989, *r*. Randall *et al*., 1988, *s*. Pettibourne *et al*., 1988.

newer, and more selective, antagonists have been employed as probes for the α_2-adrenoceptor, but due to lack of general availability of these ligands, their use for classification of α_2-adrenoceptor subtypes is limited.

1.2.3. Subclassification of α_2-adrenoceptors in ligand binding studies

Despite the limitations of using [^{3}H]-yohimbine and [^{3}H]-rauwolscine to reversibly label all α_2-adrenoceptors, these ligands have been used exclusively to subdivide α_2-adrenoceptors into 3 or perhaps more subtypes (Bylund, 1988,1992). Early studies demonstrated a clear difference between the characteristics of the α_2-adrenoceptor in rodent and non-rodent species (Cheung *et al.*, 1982, Kawahara & Bylund, 1985). The affinity for yohimbine and oxymetazoline was higher in non-rodent than in rodent species, while the α_1-adrenoceptor antagonist prazosin showed higher affinity in rodents. The species dependent differences have been substantiated in soluble receptor populations from a variety of tissues and species, suggesting that variations in receptor environment could not account for the heterogeneity observed in intact membranes (Cheung *et al.*, 1986; McKernan *et al.*, 1986).

The high affinity of prazosin for [^{3}H]-yohimbine binding in rodent tissues originally led to some hesitation in defining the rodent receptor as an α_2-adrenoceptor (Yamada *et al.*, 1980). However, the finding that yohimbine had very low affinity for [^{3}H]-prazosin binding, and that yohimbine and rauwolscine had much higher affinity than the stereoisomer corynanthine, suggested that they did comply with the definition of an α_2-adrenoceptor (Bylund *et al.*, 1988). In addition, selective α_2-adrenoceptor antagonists, WY 26703, idazoxan and BDF 6143, had high affinity for [^{3}H]-yohimbine binding in the rat cerebral cortex (Brown *et al.*, 1990b). True receptor heterogeneity, however, should be demonstrated in the same species, so Bylund (1985) showed, from careful analysis of prazosin inhibition curves to [^{3}H]-yohimbine in the rat cerebral cortex, that prazosin displayed high and low affinity components, with 50 fold separation between the sites (high affinity site 4 - 20 nM; low affinity site 0.2 - 1 μM). Biphasic inhibition curves were subsequently reported to exist in some areas of human brain (Petrash & Bylund, 1986). Therefore it was suggested, based on the affinity of prazosin, that the prazosin insensitive component be termed

α_{2A} (oxymetazoline > prazosin), and the site demonstrating high affinity for prazosin be termed α_{2B} (prazosin > oxymetazoline) (Nahorski *et al.*, 1985; Bylund, 1985). Examples of tissues reported to contain an homogeneous population of one subtype include the human platelet (α_{2A}), rabbit spleen (α_{2A}), neonatal rat lung (α_{2B}) rat kidney (α_{2B}) and selective cell lines HT29 (α_{2A}) and NG108-15 (α_{2B}) (Nahorski *et al.*, 1985; Bylund, 1985; Bylund *et al.*, 1988; Michel *et al.*, 1989b,1990).

A third subtype of α_2-adrenoceptor has been proposed to exist on a cell line from the opposum kidney (Murphy & Bylund, 1988). This subtype labelled by [^{3}H]-rauwolscine and [^{3}H]-yohimbine, had high affinity for prazosin and was initially termed α_{2B}-like, however, the ratio of yohimbine to prazosin affinity was closer to that described for the α_{2A}-subtype, and it was subsequently termed α_{2C}. There have been very few reports of receptors characteristic of the α_{2C} subtype in species other than the opposum, but in cultured smooth muscle cells of the rabbit aorta, prostaglandin synthesis elicited by adrenergic stimuli has been reported to be mediated via α_{1A}- and α_{2C}-adrenoceptors (Nebgil & Malik, 1992), suggesting that the α_{2C}-adrenoceptor may not merely be a species variant of the α_{2B}-adrenoceptor.

More recently, a fourth subtype has been proposed by Bylund and co-workers to exist in membranes prepared from bovine pineal gland (Simmoneaux *et al.*, 1991), which had similar characterisitics to that described by Michel *et al.* (1990) on rat submaxillary gland. This site did not show the characteristics of the α_{2A}, α_{2B} or α_{2C}-adrenoceptors when the affinities of 16 drugs were compared in correlation graphs and was proposed to be of a unique subtype, or a species variant of an existing subtype. The site was characterised as being a discrete subtype due to the low affinity of yohimbine and rauwolscine and the high affinity of phentolamine. This subtype also displayed a low affinity for SKF 104078 and was thus proposed to represent a prejunctional receptor (see following Section and Simmoneaux *et al.*, 1991).

The more recent identification of newer, and slightly more selective drugs for the α_2-adrenoceptor subtypes has helped somewhat in the distinction of separate subtypes, although a singularly selective drug for each subtype still remains to be identified. Therefore, to distinguish the presence of a subtype in radioligand binding experiments necessitates the comparison of several drugs. The matter is complicated

even more where two or more subtypes exist in the same tissue. This was highlighted by Uhlen & Wikberg (1991) who suggested that the rat kidney contained α_{2A}-adrenoceptors and two subtypes of α_{2B}-adrenoceptors, which could be delineated using guanoxabenz and ARC 239. Table 1.2. outlines the characterisitcs of the existing subtypes described to date.

As outlined previously the present definition of α_2-adrenoceptor heterogeneity lies solely on the characteristics of the receptor labelled with the [^{3}H]-antagonists, and more particularly with [^{3}H]-yohimbine and [^{3}H]-rauwolscine. However, an earlier study by Kahn *et al.* (1982) casts doubt on the use of [^{3}H]-antagonist binding to adequately describe the nature of the α_2-adrenoceptor on the NG 108-15 cell line, a cell line considered by Bylund *et al.* (1988) as being of the α_{2B}-subtype. When the receptor was labelled by the agonists [^{3}H]-*p*-aminoclonidine or [^{3}H]-adrenaline it demonstrated characteristics of the α_2-adrenoceptor but showed 10 - 100 fold lower affinity for antagonists, whereas [^{3}H]-yohimbine binding in the same tissue showed 20 fold lower affinity for agonists. Thus, the receptor labelled by the agonist ligands displayed high affinity for oxymetazoline and low affinity for prazosin, which was not consistent with the α_{2B}-adrenoceptor subtype and probably more consistent with the α_{2A}-subtype. This could provide evidence that agonists and antagonists label different sites, or that binding of an agonist results in a conformational change in the receptor which alters its ability to bind antagonists, i.e. different affinity states. The question then arises as to whether the receptor labelled by the agonist or by the antagonist is the functionally relevant receptor? Whatever the answer, it suggests that a receptor should be characterised in both [^{3}H]-agonist and [^{3}H]-antagonist binding studies, and that this would be more directly comparable to that employed in classical functional experiments. The difference between agonist and antagonist binding to α_2-adrenoceptor subtypes will be addressed later in this project.

1.2.4. Functional α_2-adrenoceptor subtypes

By far the majority of functional experiments with α_2-adrenoceptors have had the characterisitics of the α_{2A}-adrenoceptor subtype in that prazosin has had low affinity. A possible explanation for this is the greater pace of developments in the field of radioligand binding, coupled with fact that a high affinity for prazosin would have

Table 1.2. Affinity values (pK_i) for α_2-adrenoceptor subtypes.

	α_{2A} human platelet	α_{2A} rabbit spleen	α_{2B} neonate lung	α_{2B1} rat kidney	α_{2B2} rat kidney	α_{2C} OK cell	α_{2D} bovine pineal
yohimbine	9.26		9.00	8.17	7.68	9.73	8.44
rauwolscine	9.36	8.06	9.35	7.91	7.52	10.37	8.47
prazosin	6.47	5.33	8.27	7.45	7.14	7.82	6.98
oxymetazoline	9.20	8.11	7.28	5.91	5.83	7.16	8.84
ARC 239	6.74		8.80	8.21	7.42	7.89	6.83
BAM 1303			7.59			9.42	7.27
phentolamine	8.15	7.78	8.44			8.01	8.51
WB 4101	9.10	8.17	8.30	7.48	7.10	9.57	8.12
SKF 104078	7.74	7.00	7.31			7.37	6.40

Data from the human platelet from Bylund *et al.* (1988); the rabbit spleen from Michel *et al.* (1989b); rat kidney subtypes from Uhlen & Wikberg, (1991); all other values taken from Simonneaux *et al.*, (1991).

in the past been confused with an α_1-mediated event (see references cited in McGrath *et al.*, 1989; Wilson *et al.*, 1991a). Exceptions to this include the relatively high potency of prazosin in stimulating the release of noradrenaline in the rat submandibular gland (Turner *et al.*, 1984), the inhibition of neurotransmitter release in the rat atria (prazosin EC_{30} = 6.97, Smith *et al.*, 1992) and the high potency of prazosin (pA_2 = 7.2) in α_2-adrenoceptor mediated attenuation of cAMP formation in NG 108-15 cells (Bylund & Ray-Prenger, 1989). However, this must be taken into context with the report that adrenaline-induced Na+/H+ exchange in NG 108-15 cells was insensitive to prazosin (Isom *et al.*, 1987). Table 1.2. shows, that α_2-adrenoceptor subclassification is characterised by the differential affinities of a number of competing drugs. The demonstration of a functionally relevant α_{2B}-adrenoceptor cannot therefore be based solely on a relatively high affinity for prazosin, but must be characterised in conjunction with a number of other drugs. Functional definition of α_2-adrenoceptor subtypes therefore awaits clarification.

1.2.5. Pre/post junctional α_2-adrenoceptors

Early attempts failed to differentiate between pre- and postjunctional α_2-adrenoceptors. A good correlation of affinities was shown to exist between prejunctional α_2-adrenoceptors in the guinea pig atrium and postjunctional α_2-adrenoceptors in the cat saphenous vein (Hieble *et al.*, 1986) suggesting that there was no pre/postjunctional α_2-adrenoceptor heterogeneity. This held true for a number of antagonists including yohimbine, rauwolscine, idazoxan and phentolamine. Initial studies *in vivo* also provided evidence for no pre/postjunctional differentiation with the available antagonists. In 1981 however, de Jonge *et al.* (1981) demonstrated the prejunctional selectivity of 2,5-substituted imidazoline agonists. However, the interpretation of these data is complicated by the possible differences in efficacy of the agonists used and in the tissue receptor reserve. Evidence for the existence of heterogenous pre- and postjunctional α_2-adrenoceptors came with the identification of SKF 104078, an α_2-adrenoceptor antagonist of the 3-benzazepine class. This antagonist has been shown to have >100 fold selectivity for postjunctional α_2-adrenoceptors in the canine saphenous vein relative to the prejunctional receptor in the guinea pig atria (Ruffolo *et al.*, 1987). The ability of SKF 104087 to block

postjunctional α_2-adrenoceptors has been shown in several reports *in vitro* (Hieble *et al.*, 1988; Connaughton & Docherty, 1988; Kelly *et al.*, 1989). In addition SKF 104078 had little effect on noradrenaline overflow from rabbit aorta, guinea pig vas deferens and human saphenous vein. The ability of SKF 104078 to differentiate between pre- and postjunctional α_2-adrenoceptors was not species dependent, as selectivity for postjunctional receptors was demonstrated in the rabbit and dog saphenous vein (Hieble *et al.*, 1988). In addition, the selectivity has recently been demonstrated within a single tissue, the human saphenous vein (Hieble *et al.*, 1991).

The demonstration of the pre/postjunctional selectivity of SKF 104078, however, has been disputed (Connaughton & Docherty, 1988), and the ability of SKF 104078 to be used as a pharmacological tool to identify pre- and post-junctional α_2-adrenoceptors could be limited. Firstly, by its inability to differentiate postjunctional α_1- and α_2-adrenoceptors (Ruffolo *et al.*, 1987), and by the existence of multiple pre- and postjunctional α_2-adrenoceptors showing differing sensitivities to SKF 104078. This may be highlighted by the action of SKF 104078 *in vivo*. In the pithed rat SKF 104078 blocked α_2-mediated pressor responses at low doses, but unlike yohimbine and rauwolscine was limited to a maximum 3 - 4 fold shift in the control response curve. This suggested that there may be 2 populations of postjunctional α_2-adrenoceptors, only one of which was sensitive to SKF 104078. Using a dose of SKF 104078 that produced inhibition of postjunctional pressor responses in the pithed rat, Akers *et al.* (1989) showed no effect on prejunctional inhibition of neurogenic tachycardia induced by BHT 933, but it was effective against xylazine, suggesting multiple prejunctional α_2-adrenoceptors exist with different sensitivity to SKF 104078. However, another study in the rat vas deferens showed that SKF 104078 blocked prejunctional agonist activity of clonidine and but not that of xylazine, UK 14304, and BHT 920 (Ruffolo *et al.*, 1991). The authors concluded that the sympathetic neurones in the rat vas deferens contained a mixed population of prejunctional α_2-adrenoceptors, one of which was sensitive to SKF 104078 and similar to the α_2-adrenoceptors found postjunctionally in the vasculature.

Evidence for two populations of prejunctional α_2-adrenoceptors with differential selectivity for xylazine have also been obtained in other *in vitro* studies in the rat vas deferens, where like SKF 104078,

idazoxan was shown to be completely ineffective against xylazine responses (Harsing & Vizi, 1992). Therefore two subtypes of prejunctional α_2-adrenoceptors may exist in the rat vas deferens, one which is activated by clonidine and inhibited by SKF 104078 and idazoxan, and another which can be activated by xylazine and is insensitive to SKF 104078 and idazoxan. These receptors may be different from those found prejunctionally in the atria, where xylazine responses were inhibited by SKF 104078 (Akers *et al.*, 1989).

The identification of anatomically and pharmacologically distinct α_2-adrenoceptors would be aided by direct radioligand binding studies. Binding studies with postjunctional preparations such as isolated adipocytes and platelets have demonstrated that these adrenoceptors show characteristics of the α_{2A}-subtype. However, the low density of α_2-adrenoceptors on sympathetic neurones within a tissue, and the problems associated with separating pre- and post-junctional tissue has limited the use of this technique in characterising prejunctional adrenoceptors.

1.2.6. Second-messenger systems

Activation of α_2-adrenoceptors in a variety of cell types has been shown to decrease cellular cAMP levels by inhibiting the activity of the membrane bound adenylate cyclase. This has been directly observed in several studies including;- human platelets (Jakobs *et al.*, 1979; Clare *et al.*, 1984), adrenocortical carcinoma cells (Jaishwal & Sharma, 1985), hamster adipocytes (Aktories *et al.*, 1979), rat renal collecting tubules (Edwards *et al.*, 1992), rat pancreatic islets, (Yamazaki *et al.*, 1982), prejunctional α_2-adrenoceptor activation in the rat neocortex (Schoffelmeer & Mulder, 1983), and postjunctional α_2-adrenoceptors in the vasculature of the pithed rat (Boyer et *al.*, 1983). α_2-Adrenoceptors have also been shown to inhibit adenylate cyclase activity in a number of cell lines, such as NG 108-15 cells (Sabol & Nirenberg, 1979; Bylund & Ray-Prenger, 1988) and HT29 cells (Bylund & Ray-Prenger, 1988). The inhibition of adenylate cyclase activity appears to be mediated via a pertussis toxin sensitive G-protein named G_i (Katada & Ui, 1982), and the ability of pertussis toxin to abolish responses to α_2-agonists has been used as evidence for the role of adenylate cyclase in the transduction of these responses. Thus, pertussis toxin has been shown to inhibit α_2-adrenoceptor mediated antilipolytic responses in hamster adipocytes

(Garcia-Sainz, 1980), insulin release from pancreatic islets (Katada & Ui, 1982) and vasoconstriction in the pithed rat (Nichols *et al.*, 1989).

Although the inhibition of adenylate cyclase has been a well documented consequence of α_2-adrenoceptor activation (see above), the importance of this in relation to the final effector response is open to some doubt. For example, in the human platelet some α_2-adrenoceptor agonists could produce an aggregatory response without inhibition of adenylate cyclase, and could in fact block the inhibition of adenylate cyclase by adrenaline (Clare *et al.*, 1984). This suggests that the α_2-mediated aggregatory response may not be completely dependent on a decreased level of cAMP. Indeed for this to be the sole mechanism of action, it would suggest that resting platelets contain a high level of cAMP sufficient for adrenaline to produce an inhibition. It does not detract from the fact that α_2-adrenoceptor agonists could produce an aggregatory response via a decrease in cAMP when cAMP levels are elevated by, for example prostaglandin E_2.

A similar problem has been encountered when interpreting data from the vasoconstrictor responses to α_2-agonists in the vasculature. Thus if an inhibition of adenylate cyclase leads to a contractile response then there must be a resting level of cAMP high enough to produce an effect (Ruffolo *et al.*, 1991). Vasocontrictor responses to α_2-adrenoceptor agonists have also been shown to be accompanied by an influx of extracellular Ca^{2+} (Medgett & Rajanayagam, 1984). The means by which a decreased level of cAMP would increase intracellular Ca^{2+} and thus facilitate a vasoconstrictor response is still unclear, but could be explained by the dual ability of cAMP to increase binding of intracellular Ca^{2+} and hence suppress activity of contractile proteins, and to assist closure of Ca^{2+} channels (McGrath *et al.*, 1989). Thus a decrease in cAMP mediated by α_2-adrenoceptor activation would cause a contraction by increasing intracellular Ca^{2+}.

The role of cAMP and Ca^{2+} in mediating the prejunctional inhibition of neurotransmitter release is however, contradictory, as one would expect an increase in intracellular Ca^{2+} to facilitate release. Indeed, in rat cortical synaptosomes, α_2-adrenoceptor agonists have been shown to reduce intracellular Ca^{2+} (Adamson *et al.*, 1987). In addition, several studies have demonstrated that the presynaptic α_2-adrenoceptor mediated inhibition of noradrenaline release is not sensitive to pertussis toxin (Musgrave *et al.*, 1988; Nichols *et al.*, 1988).

Thus it appears that there is not a uniform mechanism whereby α_2-adrenoceptors mediate their functional responses via cAMP and Ca^{2+}.

There is less evidence suggesting a role of phospholipase C in mediating α_2-adrenergic responses, although it has been shown that in hamster fibroblasts transfected with cloned α_2-adrenoceptors from kidney and platelets, adrenaline can produce a increase in inositol phosphates (Cotecchia *et al.*, 1990). How much an overexpression of the receptor in the host cell led to this response is not clear. Limbird (1984) has suggested that an indirect stimulation of phospholipase C may, via the breakdown of diacylglycerol and phosphorylation to phosphatidic acid, serve as a substrate for phospholipase A_2 to release the aggregatory prostanoids in the human platelet. This action was thought to be through the direct stimulation of a membrane bound Na^+/H^+ antiporter. α_{2A}-Adrenoceptors have also been demonstrated to cause arachidonic acid mobilisation via phospholipase A_2 in transfected hamster ovary cells, which was Ca^{2+} and pertussis toxin sensitive, and blocked by yohimbine (Jones *et al.*, 1991). Transduction of α_2-adrenoceptor mediated events, therefore do not occur via a common pathway, and may involve two or more different second-messenger systems, or may in fact be directly coupled to ion channels. It remains to be elucidated whether different subtypes of α_2-adrenoceptor utilise distinct transduction systems.

1.2.7. Molecular biology of α_2-adrenoceptor subtypes

Recent advances in molecular biology have allowed for the isolation and cloning of genes coding for α_2-adrenoceptor subtypes. The first of these studies was carried out by Koblika *et al.* (1987), who purified and generated oligonucleotide probes to the human platelet α_{2A}-adrenoceptor. When screened against a human genomic library, two of the probes hybridised to a 5.5 kilobase fragment of genomic DNA, localised to chromosome 10, which was subsequently sequenced and found to encode for a protein of 450 amino acids. Analysis of the primary structure revealed seven distinct hydrophobic domains of 20 - 25 amino acids connected by hydrophilic loops of polar and charged residues, and thus demonstrated the characteristics of a G-protein coupled receptor (Savarese & Fraser, 1992). Figure 1.1. shows the primary structure of the human platelet α_{2A}-adrenoceptor. The proposed arrangement within the membrane is such that the seven

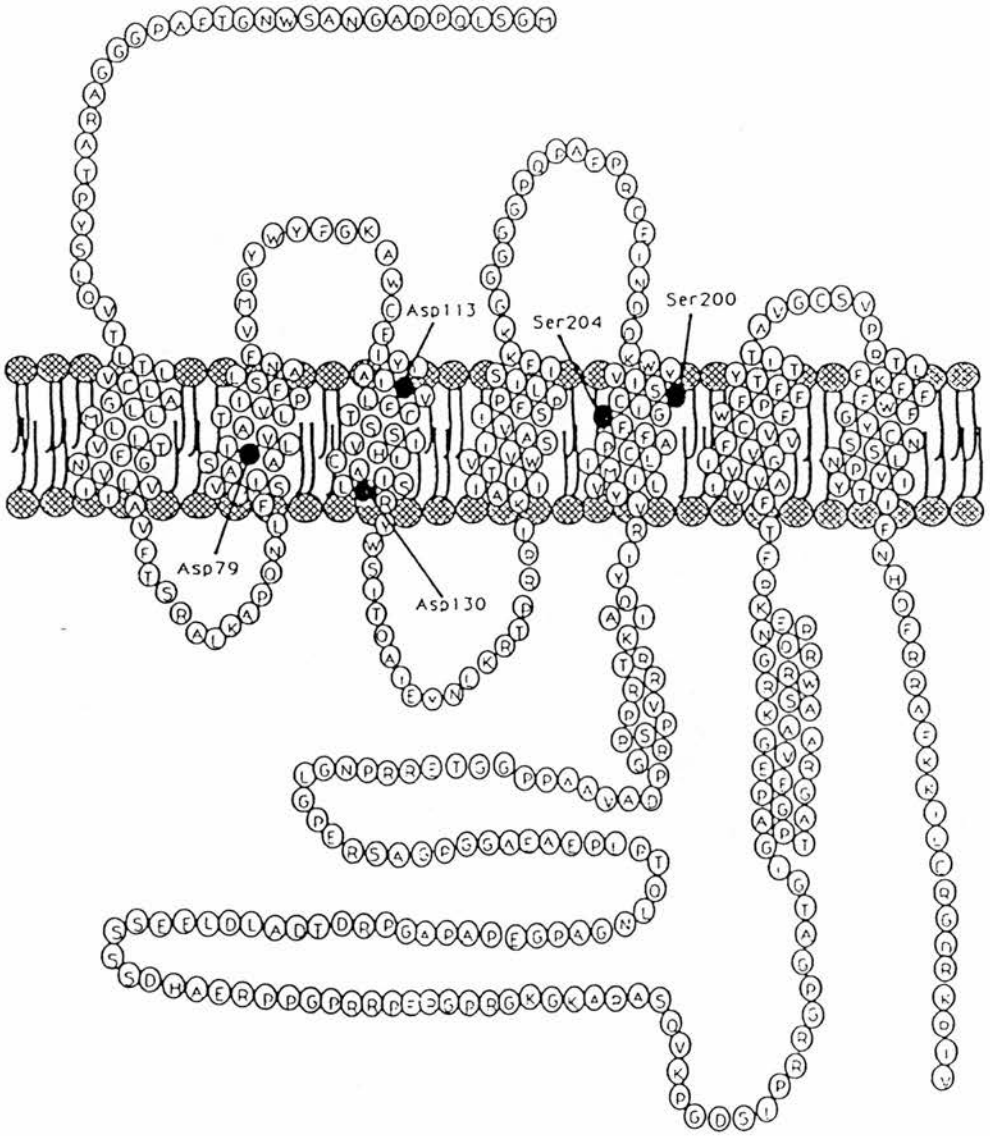


Figure 1.1. Structure of the human platelet α_{2A}-adrenoceptor using the one letter code for amino acid sequence, and the proposed arrangement of the receptor within the membrane. The conserved amino acid residues identified by site-directed mutagenesis are indicated. From Wang *et al.*, (1991).

hydrophobic domains represent the seven membrane-spanning regions similar to that described for rhodopsin, muscarinic receptor subtypes, and the β-adrenoceptors (Savarese & Fraser, 1992). Pst1 restriction enzyme digests of the platelet gene identified 2 additional bands of 1.8 and 5.9 kilobases which were subsequently localised to different human chromosomes, C 4 and C 2, suggesting the existence of 2 more closely related genes.

Since the initial isolation of the platelet α_{2A}-adrenoceptor, several additional clones have been identified from human, pig and rat libraries. The sequences for several of these genes have been analysed, and show some structural differences, particularly in the presence of consensus sites for N-linked glycosylation. The derived gene products have been expressed in host cells. Careful analysis of the binding and functional characteristics of the transfected receptors has allowed for the classification of these receptors into respective α_2-adrenoceptor subtypes. Table 1.3. summarises the nature of the clones so far identified. The observation that the C 2 clone from human kidney, which demonstrates the pharmacology of an α_{2B}-adrenoceptor subtype, would not hybridise with mRNA from neonatal rat lung (the prototypical α_{2B} preparation), whereas the rat kidney α_{2B} clone, RNGα_2, does, suggests species differences in the α_{2B}-adrenoceptor or multiple subtypes. The rat RG 20 clone showed greatest homology with the α_{2D}-adrenoceptor subtype, however an equivalent human clone remains to be identified. Thus, molecular biology has provided additional evidence for the existence of multiple subtypes of α_2-adrenoceptors.

1.3. IMIDAZOLINE BINDING SITES

1.3.1. Discovery

Although it was well established that catecholamines and imidazoline compounds such as clonidine interacted with α_1- and α_2-adrenoceptors to produce their pharmacological effects (see section 1.1.), in 1977 Ruffolo and co-workers (Ruffolo *et al.*, 1977) showed that the interaction of these two classes of compounds with the α_2-adrenoceptor was also dependent on their chemical structure. Desensitisation of the rat vas deferens to oxymetazoline rendered the

Table 1.3. Characteristics of the α_2-adrenoceptor clones so far described.

Clone	source	glycosylated	mRNA	α_2-subtype	human chromosome
C 10	human platelet	yes	widespread	α_{2A}	10
porcine	pig brain	yes	?	α_{2A}	10
RNG α_2	rat kidney	no	neonate lung	α_{2B}	?
5A	human spleen	no	?	α_{2B}	?
C 2	human kidney	no	liver/kidney not neonate lung or brain	α_{2B} like?	2
RG 10	rat kidney	yes	brain	α_{2C}	4
C 4	human kidney	yes	brain, OK cells NG108-15, not neonate lung	α_{2C}	4
GRα_2B	rat kidney	yes	brain	α_{2C}	4
pA2d	rat brain	?	brain	α_{2C}	4
RG 20	rat kidney	yes	brain, kidney, salivary gland	α_{2D}	?

C 10 (Koblika *et al.*, 1987; Lorenz *et al.*, 1990), porcine (Guyer *et al.*, 1990) C 2 (Koblika *et al.*, 1987; Lorenz *et al.*, 1990; Lomasney *et al.*, 1990), C4 (Koblika *et al.*, 1987; Regan *et al.*, 1988; Lorenz *et al.*, 1990;) RNG α_2 (Zeng *et al.*, 1990), 5A (Weinshank *et al.*, 1990), RG 10 and RG 20 (Lanier *et al.*, 1991; Harrison *et al.*, 1991), GRα_2B (Flordellis *et al.*, 1991), pA2d (Voigt *et al.*, 1991).

tissue insensitive to other imidazoline agonists but responses to the catecholamines were unaffected. A different mode of interaction of these two classes of compounds, with α-adrenoceptors was suggested.

The imidazoline α_2-agonist clonidine, has been shown in several studies to produce a centrally-mediated hypotension in cats (Timmermans *et al.*, 1981), rats (Ernsberger *et al.*, 1988) and dogs (Schmitt *et al.*, 1973). Although originally proposed to be mediated by central α_2-adrenoceptors, it was subsequently shown that the site of action of clonidine and the potent α_2-adrenoceptor agonist, α-methyl noradrenaline, were different (Bousquet *et al.*, 1984). Bousquet *et al.* (1984) showed that clonidine and other structurally related drugs which lacked α_2 selectivity, could produce a fall in blood pressure when injected into the medullary area, particularly the nucleus reticularis lateralis (NRL) of the cat, whereas α-methyl noradrenaline was inactive. The existence of imidazoline preferring receptors mediating the hypotensive actions of clonidine was therefore suggested. Later studies in the anaesthetised rat showed that the intracisternal administration of idazoxan (an α_2-adrenoceptor antagonist containing an imidazoline ring), but not yohimbine could block the hypotensive effect of intravenously administered clonidine (Tibiriça *et al.*, 1991). Recent data suggests that other centrally acting antihypertensives such as rilmenidine may act preferentially via imidazoline preferring receptors in the rostral medulla, and this may explain the lack of sedative effects observed with this compound (Fillastre *et al.*, 1988; Gomez *et al.*, 1991) However, other studies have disputed this, suggesting that the effects of rilmenidine may be primarily via spinal or ganglionic structures (Sannajust *et al.*, 1992). Therefore if clonidine and related drugs act at imidazoline preferring sites in the medulla, this will not be their only site of action.

1.3.2. Radioligand binding

Direct evidence for an imidazoline preferring "receptor" came from studies on [^{3}H]-*p*-aminoclonidine binding in membranes prepared from the bovine ventrolateral medulla. It was found that only 70% of the sites labelled were competed for by noradrenaline and other catecholamines, while compounds containing an imidazoline moeity inhibited the remaining noradrenaline insensitive binding (Ernsberger *et al.*, 1987). The imidazoline preferring sites were localised in the

medullary region, as membranes from the frontal cortex were entirely sensitive to noradrenaline. Studies in other species revealed that [3H]-clonidine binding was completely displacable with catecholamines in rat brainstem membranes, while in membranes prepared from the human NRL region, noradrenaline and adrenaline had virtually no effect on [3H]-clonidine binding. This suggests a species difference in the density and distribution of imidazoline preferring "receptors" in brain.

Based on ligand binding studies, the site labelled by [3H]-*p*-aminoclonidine exhibited a rank order of affinity for displacing agents of clonidine (6 nM) > phentolamine (42 nM) >> guanabenz (10000 nM) (Michel & Insel, 1989). Recent data has shown that rilmenidine also has high affinity for these sites (K_i = 6 nM, Gomez *et al.*, 1991). However, it must be stressed that these affinities were calculated based on the relative affinity of these compounds for only 30% of the specifically bound [3H]-*p*-aminoclonidine, and interpretation was complicated by the presence of a large population of α_2-adrenoceptors which [3H]-*p*-aminoclonidine labelled at the concentrations used in the studies. The nature of these sites, therefore, would best be studied either in an homogeneous tissue (human NRL), or in the presence of a selective α_2-adrenoceptor antagonist.

Idazoxan behaves functionally as a potent and selective α_2-adrenoceptor antagonist, and the radiolabelled ligand has been used to label α_2-adrenoceptors in homogenate preparations (see sections 1.2.1. to 1.2.6.). However, recent studies in a variety of tissues such as rabbit kidney (Hamilton *et al.*, 1988), rabbit urethral tissue (Yablonsky *et al.*, 1988; Yablonsky & Dausse, 1989), rabbit adipocytes (Langin & Lafontan, 1989), hamster adipocytes (MacKinnon *et al.*, 1989), guinea pig cerebral cortex (Wikberg & Uhlen, 1990), rat cerebral cortex (Brown *et al.*, 1990a), rat and human kidney (Michel *et al.*, 1989a), and pig kidney (Vigne *et al.*, 1989), have shown that [3H]-idazoxan labelled additional sites that were not competed for by catecholamines and were therefore not adrenoceptors. These non-adrenergic binding sites were competed for with high affinity by several imidazoline compounds such as cirazoline (Wikberg & Uhlen, 1990), and also by guanabenz (Michel *et al.*, 1989a) and amiloride (Yablonsky & Dausse, 1989), and have been termed non-adrenoceptor idazoxan binding sites (NAIBS) or imidazoline-guanidinium receptive sites (IGRS) by some authors (Kilpatrick *et al.*, 1992; Coupry *et al.*, 1989). These sites were shown

to be pharmacologically distinct from those labelled by [3H]-*p*-aminoclonidine in bovine and human brain, as clonidine had low affinity (MacKinnon *et al.*, 1989). Subsequent studies have shown this to be the case in rat, pig, human and rabbit tissues, although rabbit tissues displayed additional differences with regard to a higher affinity for amiloride (Michel & Insel, 1989). Table 1.4. shows the affinity of some imidazoline and non-imidazoline agents in competing for [3H]-*p*-aminoclonidine, [3H]-clonidine and [3H]-idazoxan binding in various tissues.

1.3.3. Functional imidazoline sites

Although much is known about the nature of the imidazoline binding sites, there is little information regarding their function. It appears that central imidazoline sites labelled by [3H]-*p*-aminoclonidine and [3H]-clonidine may be partly involved in the hypotension observed with some imidazoline and oxazoline compounds (see Section 1.3.1.), although the mechanism through which this occurs is uncertain. A number of systems have been shown to be sensitive to clonidine and other imidazoline related compounds which could not be explained by an action at an α_2-adrenoceptor. These include catecholamine release in adrenal chromaffin cells (Regunathan *et al.*, 1991); acid secretion in parietal cells (Houi *et al.*, 1987); electrolyte transport in the rabbit ileum (Dharmsathaphorn *et al.*, 1984) and stimulation of insulin release from rat pancreatic islets (Schulz & Hasselblatt, 1989). A detailed analysis of the structure-activity of the compounds active in these systems would help elucidate the possible involvement of an imidazoline receptor.

Some studies have shown an effect of idazoxan which could not be adequately explained by α_2-adrenoceptor blockade, including the blockade of nonadrenergic-noncholinergic relaxations of the rat anococcygeus muscle (Ramagopal & Leighton,1989), the inhibition of Na^+/H^+ exchange in renal proximal tubule cells (Bidet *et al.*, 1990), the inhibition of noradrenaline release in rabbit aorta and pulmonary artery (Gothert & Molderings, 1991), and the inhibition of prolactin release (Krulich *et al.*, 1989). In addition, idazoxan has been suggested to reduce focal cerebral ischaemic damage by an action at an imidazole receptor (Maiese *et al.*, 1992). Although suggestive of imidazoline receptor mediated events, in most of these studies the concentration of

Table 1.4. Inhibition of [^{3}H]-*p*-aminoclonidine, [^{3}H]-clonidine and [^{3}H]-idazoxan binding in various tissues.

radioligand/tissue	pK_i idazoxan	pK_i clonidine	pK_i amiloride	pK_i guanabenz
[^{3}H]-*p*-aminoclonidine				
bovine brain	n.d.	8.2*a*	<5.0	< 5.0*c*
[^{3}H]-clonidine				
human NRL	7.5*d*	7.0*d*	n.d.	n.d.
[^{3}H]-idazoxan				
rabbit kidney	8.8*e*	6.5*e*	7.4*e*	8.7*e*
rabbit adipocytes	8.7*f*	6.0*f*	7.3*f*	n.d.
rabbit liver mitochondria	8.5*g*	5.1*g*	6.8*g*	8.3*g*
human kidney	8.5*e*	5.6*e*	6.4*e*	8.0*e*
rat brain	8.4*h*	6.3*h*	6.3*h*	8.2*h*
hamster adipocytes	8.7*i*	5.7*i*	n.d.	n.d.
pig kidney	8.8*j*	5.0*j*	n.d.	7.7*j*

*a*Ernsberger *et al.*, 1988, *b*Michel & Insel, 1989, *c*Gomez *et al.*, 1991, *d*Bricca *et al.*, 1989, *e*Lachaud-Pettiti *et al.*, 1991, *f*Langin & Lafontan, 1989, *g*Tesson *et al.*, 1991, *h*Brown *et al.*, 1990a, *i*MacKinnon *et al.*, 1989, *j*Vigne *et al.*, 1989, n.d. not determined.

idazoxan used was higher than may be expected for a selective effect, thus an interaction with serotonergic, α_1-adrenoceptors or indeed a subtype of α_2-adrenoceptor could not be completely ruled out. In addition, the lack of additional data on other high affinity imidazoline compounds, such as cirazoline or guanabenz, could not unequivecably implicate the [^{3}H]-idazoxan labelled imidazoline sites in these systems. The identification of a high affinity imidazoline compound with low affinity for other receptor types would greatly assist our understanding of imidazoline site function.

In a search for an endogenous ligand for the imidazoline site(s), Atlas & Burnstein (1984) isolated and partially purified an extract from bovine brain which displaced [^{3}H]-clonidine from bovine cerebral cortex membranes. This endogenous clonidine displacing substance (CDS) was not a catecholamine but the exact chemical nature of its active ingredient(s) has not yet been established. CDS has been found to bind to α_2-adrenoceptors in human platelets (Diamant *et al.*, 1987) and imidazoline sites in adrenal chromaffin cells (Regunathan *et al.*, 1991) and rat liver cells (Zonnenschein *et al.*, 1990). Preparations of CDS from different laboratories have been shown to increase blood pressure in the cat (Bousquet *et al.*, 1986) or decrease blood pressure in the rat (Meeley *et al.*, 1986) suggesting species differences in the actions of CDS, or, more likely, that different extracts were isolated from the different laboratories. CDS has been shown to contract the rat gastric fundus (Felson *et al.*, 1987), inhibit the twitch response in the rat vas deferens (Diamant & Atlas, 1986) and potentiate ADP-stimulated aggregation of human platelets (Diamant *et al.*, 1987) and in these respects mimicked the actions of clonidine. CDS has been found to be present in human serum, and its levels are increased in patients with pregnancy-induced hypertension suggesting its physiological relevance in humans (Atlas, 1991). The purification and elucidation of the exact chemical structure of the compound(s) will help in understanding its physiological role, and whether it represents the endogenous imidazoline ligand.

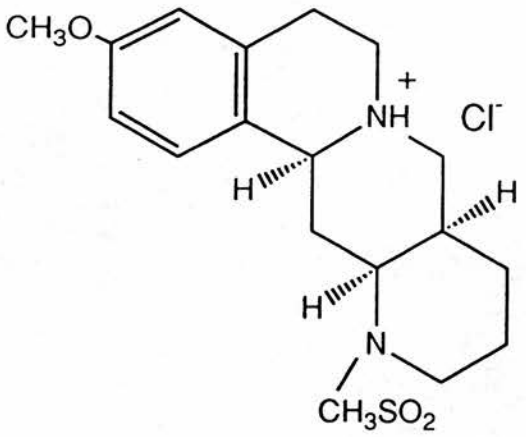
1.4. BACKGROUND TO THESIS

Previous studies from this laboratory (Brown *et al.*, 1990a,b) demonstrated that [^{3}H]-yohimbine labelled two subtypes of α_2-

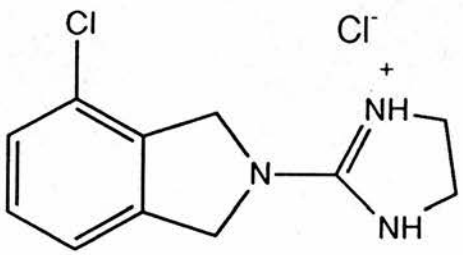
adrenoceptor in rat cortex membranes; one which was sensitive to prazosin (α_{2B}) and another which was insensitive (α_{2A}). In contrast, another α_2-adrenoceptor antagonist ligand, [^{3}H]-idazoxan, labelled only one subtype of α_2-adrenoceptor in rat cerebral cortex, which was insensitive to prazosin. However, it was noted in these studies, that although the site in rat cerebral cortex displaying low affinity for prazosin was similar to the α_{2A}-adrenoceptor on human platelets, a tissue reported to contain an homogeneous population of α_{2A}-adrenoceptors (Cheung *et al.*, 1982; Nahorski *et al.*, 1985), there were a number of differences, particularly in regard to the affinity of [^{3}H]-yohimbine itself, which had 8 fold higher affinity in the human platelet. Therefore, it was suggested that the α_2-adrenoceptor subtypes in rat cerebral cortex may not be consistent with the current classification.

In the mid eighties my colleagues and I at Syntex Edinburgh, and in collaboration with the Institute of Chemistry, Syntex, Palo Alto were involved in an extensive screen of novel compounds synthesised to have α_2-adrenoceptor antagonist properties. From this work, RS-15385-197 (Figure 1.2) a tetracyclic structure based on rauwolscine emerged as having high affinity (pK_i = 9.5 - 10.2) for α_2-adrenoceptors on rat cortex, human platelets, neonatal rat lung and baboon cerebral cortex membranes, and more than 1000 fold selectivity over all other subtypes studied (Table 1.5.). This work, and additional work describing the pharmacology of RS-15385-197 and its modulation of central noradrenergic functioning has been published or has been submitted for publication (Brown *et al.*, 1989,1993; Clark *et al.*, 1989,1990b; MacKinnon *et al.*, 1990,1991a,b; Redfern *et al.*, 1993). RS-15385-197 has since been radiolabelled. The use of a high affinity radiolabelled antagonist with high selectivity for α_2-adrenoceptors will help in reassessing the classification of α_2-adrenoceptor subtypes.

Further analysis of [^{3}H]-idazoxan binding in the rat cerebral cortex revealed that this ligand labelled an additional non-adrenergic site in this tissue, which was defined in the presence of 3 μM yohimbine and when 0.3 μM cirazoline was used to determine non-specific binding (Brown *et al.*, 1990a). Characterisation of the additional site revealed it to be similar to the recently described imidazoline site in rabbit kidney (Hamilton *et al.*, 1988), rabbit adipocytes (Langin & Lafontan, 1989) and rabbit urethral tissue (Yablonsky *et al.*, 1988; Yablonsky & Dausse, 1989), as described in detail in Section 1.3.. Further characterisation of



RS-15385-197



RS-45041-190

The structure of RS-45041-190 is Syntex company confidential information and is the subject of a patent application

Figure 1.2. Structure of RS-15385-197 and RS-45041-190

Table 1.5. Receptor profiles of RS-15385-197 and RS-45041-190.

RS-15385-197 α_2-affinity

rat cerebral cortex	$pK_i = 9.45$
baboon cerebral cortex	$pK_i = 10.12$
human platelets	$pK_i = 9.90$
neonatal rat lung	$pK_i = 9.70$
hamster adipocyte	$pK_i = 8.13$

RS-45041-190 imidazoline affinity

rabbit kidney	$pK_i = 9.37$
hamster adipocytes	$pK_i = 8.50$
rat kidney	$pK_i = 8.66$

				pK_i						
α_1	α_2	β_1	β_2	**5-HT_{1A}**	**5-HT_2**	**D_1**	**D_2**	**M_1**	**M_2**	**CEB**
RS-15385-197										
5.29		5.27	<5.0	6.50	5.10	<5.0	<5.0	<5.0	<5.0	<5.0
RS-45041-190										
<5.0	5.70	<5.0	<5.0	5.03	4.20	<5.0	<5.0	<5.0	<5.0	<5.0

Methods as described in MacKinnon *et al.* (1989) and Brown *et al.* (1993). Imidazoline affinity of RS-15385-197 was assessed in hamster adipocyte membranes (pK_i <4.0).

[3H]-idazoxan binding in hamster adipocyte membranes revealed that 70 - 80% of the sites labelled were to imidazoline-like binding sites (MacKinnon *et al.*, 1989) with only 20 - 30% of the sites inhibited by yohimbine, noradrenaline and RS-15385-197 (MacKinnon *et al.*, 1989,1990). Although classed as imidazoline binding sites it was noted that there were marked differences between these sites and those previously described for [3H]-*p*-aminoclonidine in bovine ventrolateral medulla (Meeley *et al.*, 1986; Ernsberger *et al.*, 1987) and [3H]-clonidine in human brainstem (Bricca *et al.*, 1988; 1989). Possible heterogeneity of the imidazoline binding site was thus suggested (MacKinnon *et al.*, 1989; Brown *et al.*, 1990a).

When a number of novel compounds with an imidazoline structure were estimated for affinity at the imidazoline binding site in hamster adipocyte and rabbit kidney membranes, RS-45041-190 (Figure 1.2.) was identified as having high affinity (pK_i = 8.5 at hamster adipocytes and pK_i = 9.7 at rabbit kidney, unpublished observations, methods as described in MacKinnon *et al.*, 1989 and Stewart *et al.*, 1992). When this compound was studied for affinity at the α_2-adrenoceptor it was found that it had very low affinity for [3H]-yohimbine (pK_i = 5.2) and [3H]-RS-15385-197 (pK_i = 5.7) binding in rat cerebral cortex membranes (unpublished observations, methods as described in Brown *et al.*, 1993, and in this thesis). In addition, Table 1.5. shows that it had very low affinity for all other receptor types studied including α_1, β_1, β_2, 5-HT_{1A}, 5-HT_2, D_1, D_2, M_1, M_2 and the dihydropyridine binding site (CEB) labelled by [3H]-nitrendipine (methods as described in Brown *et al.*, 1993). RS-45041-190 was thus the most selective agent for the imidazoline site so far described. Synthesis of a high affinity radiolabelled agent for the imidazoline site which has low affinity for α_2-adrenoceptors would greatly increase our understanding of the nature of the imidazoline site(s) and whether true heterogeneity exists.

1.5. AIMS OF THIS THESIS

The primary aims of this piece of work is to investigate subtypes of α_2-adrenoceptors using the selective antagonist ligand, [3H]-RS-15385-197, and the agonist ligand [3H]-adrenaline (Chapter three). In addition the autoradiographical distribution of α_2-adrenoceptors in rat

brain will be studied using [3H]-RS-15385-197. Subtypes of imidazoline sites on rat kidney will also be studied using [3H]-idazoxan, [3H]-*p*-aminoclonidine and [3H]-RS-45041-190 (Chapter four). The distribution of imidazoline sites in rat brain will be studied by autoradiography. In addition, the functional consequences of imidazoline binding site and α_2-adrenoceptor subtype activation will be studied (Chapter five). The impact of this work for future studies is considered in Chapter six. Chapter two describes the methods used in this thesis.

CHAPTER TWO

MATERIALS AND METHODS

2.1. GENERALISED RECEPTOR BINDING PROTOCOL

2.1.1 Membrane Preparation

In the majority of studies, tissues from male Sprague Dawley rats (Charles River U.K. 200-250g) were used. The animals were pair housed and fed standard Laboratory chow *ad lib*. On the day of the experiment the animals were brought to the animal preparation area. Rats were sacrificed by a blow to the head and the neck broken. The tissues required were rapidly dissected out onto ice and either used immediately for membrane preparation or stored in plastic capped vials under liquid nitrogen until required. All further procedures were carried out at 4°C unless otherwise indicated. All buffers and solutions were freshly prepared each day. Fresh or thawed-frozen tissue was suspended in the appropriate volume of homogenising buffer and homogenised using a Polytron P10 Tissue Homogeniser (2 x 10 sec bursts at maximum setting). Homogenates were centrifuged in a refrigerated centrifuge (Sorval Superspeed RC 5B with a fixed angle rotor). The speeds and times of centrifugation and the number of washes varied with each individual preparation and will be detailed separately in the following sections. Final membrane pellets (approximate protein concentration 1 to 3 mg/ml) were suspended in a small volume (~3 ml) of assay buffer and stored under liquid nitrogen until required.

2.1.2. Saturation Binding Assay

All buffers and solutions were freshly prepared each day. Binding assays were conducted in 4.9 ml round bottomed polypropylene tubes (Fisons U.K.). The assay volume varied for each receptor / radioligand studied but was generally between 0.5 - 2.0 ml. Assay buffer was added to the tubes first using an Eppendorf automatic pipette fitted with disposable plastic tips which were capable of dispensing repeated volumes of buffer from 10 µl to 1 ml. A series of radioligand dilutions were next prepared to cover at least a concentration range of ~10 fold higher to ~10 fold lower than the K_d value for that receptor. Stock radioligands were purchased from Amersham International plc (Buckingham, UK), Du-Pont (Stevenage, UK) or synthesised by Dr. H. Parnes, Institute of Chemistry, Syntex Palo Alto. Small quantities were removed from the undiluted stock radioligand (usually kept at a

concentration of 1 mCi/ml in ethanol and stored at -20°C) with a Hamilton glass syringe and added to an appropriate volume of buffer to produce the highest concentration of radioligand required for that assay taking into account the dilution factor incurred when added to the particular assay volume. Seven serial dilutions were prepared by sequentially diluting 1 : 2 in assay buffer to cover the range of concentrations required, a new tip was used for each dilution. Generally 8 concentrations of radioligand were used in each saturation experiment for each of 4 total binding tubes and 2 non-specific binding (NSB) tubes. In addition, for each radioligand concentration, 2 total binding tubes were included which were not filtered but from which aliquots (10 - 20 %) were taken for the determination of total radioligand concentration. Radioligand was added to each total, NSB and unfiltered tube for that radioligand concentration using Pipetteman Automatic pipettes fitted with disposable plastic tips, a new tip was used for each concentration of radioligand. The appropriate NSB drug was added to NSB tubes. Membrane samples were removed from storage and thawed before adding to the appropriate volume of assay buffer. The suspended pellets were homogenised briefly in the required volume of assay buffer (for protein concentrations see following sections and Table 2.2.) and aliquots were added to the assay using an Eppendorf automatic pipette. Each assay tube was vortexed and placed in order in racks contained in a water bath at the appropriate temperature for that assay. The samples were left to equilibrate in the water bath before filtration. Table 2.1. illustrates a typical assay protocol.

A Brandel Cell Harvester (Semat, St. Albans, UK) was used to separate bound from free radioligand. This apparatus is capable of filtering 24 samples simultaneously under a constant vacuum pressure of 24 mm Hg. Bound ligand was collected on Whatman GF/B filters which had been pre-wetted with filtration buffer. In general filtered material was washed twice by drawing filtration buffer over the filter for 5 sec. Filters were left to dry thoroughly and placed in plastic scintillation mini-vials to which 4 ml Beckman Ready Safe Scintillation Cocktail was added. The samples were capped and left for at least 8 hours before counting for tritium in a Beckman LS 5000 or 1700 series scintillation counter. Each sample was counted for 3 min and the average radioactivity was expressed as the mean dpm (disintigrations per minute) per sample based on a counting efficiency of 40 - 45 %.

Table 2.1 Assay additions for a typical saturation experiment.

Addition	Total	NSB	unfiltered
Buffer	400 µl	300 µl	400 µl
radioligand	100 µl	100 µl	100 µl
(for each of 8 concentrations)			
NSB drug	-	100 µl	-
membranes	500 µl	500 µl	500 µl
assay volume	1000 µl	1000 µl	1000 µl

Radioligand and NSB drug were prepared at a stock concentration 10 x the required concentration and added to the assay tube. Membranes were homogenised in the appropriate volume of buffer prior to addition to the assay (see Sections 2.1.2. and 2.3.1. to 2.4.3. for individual assay requirements).

2.1.3. Competition Assay

A competition curve comprised 2 total binding tubes 2 NSB tubes and a series of 13 concentrations of competing drug tubes in duplicate. Buffer was added to the assay tubes first. Radioligand was added next at a concentation lower than or equal to the K_d value for that receptor. A series of 13 dilutions of competing drug was constructed by diluting 1 : 10 and 1 : 3.33 from the highest concentration of drug taking into account the dilution factor incurred when aliquots of the dilution were added to the assay volume. When serial 1 : 10 dilutions were made from these 2 dilutions a series of concentrations of decreasing half a log order of magnitude was produced. Dilutions were constructed using a Pipetteman Automatic pipette fitted with disposable plastic tips, a new tip was used for each dilution. The appropriate concentration of competitor was added to the assay tubes in duplicate. Aliquots of membrane were added at the same concentration as for the saturation assay. Samples were equilibrated and filtered as described for the saturation assay (section 2.1.2.).

2.1.4. Kinetics Assay

To determine the kinetics of a binding reaction, association and dissociation experiments were carried out. For an association experiment triplicate total and NSB tubes were incubated for various times before filtration. Plotting specific binding against time yielded an exponential time course from which the association rate (K_{obs}) and time to equilibrium could be calculated (see section 2.9.2.). For a dissociation experiment triplicate total and NSB tubes were incubated to equilibrium (equilibrium time being estimated from association experiments). Excess cold ligand (generally 100 x K_d), or preferably a structurally unrelated competing drug, was added to each assay tube in a small volume (<5 % assay volume), the tubes were vortexed briefly and incubated for various times before filtration. A one way process of dissociation was thus initiated as re-association of the radioligand to the binding site was unlikely due to the large excess of unlabelled competitor present. In most dissociation experiments, for each time point, an additional series of total and NSB tubes received the same volume of buffer. This was included to correct for any dilution error and drift in equilibrium over the dissociation period. Analysis of the exponential time course gave the dissociation rate constant K_2 (see

section 2.9.2.)

2.1.5. Protein determination

Protein content of membrane homogenates was determined using the Pierce BCA Protein Assay Reagent (Pierce, UK) using bovine serum albumin (BSA) as the protein standard. This reagent kit is based on the biuret reaction and allows for the accurate determination of protein concentration in a sample from 20 - 2000 µg protein/ml. A standard curve for BSA was constructed over this range. Aliquots (100 µl) of sample was added to reagent tubes in triplicate (sample was diluted to be within the standard curve as appropriate); the tubes were vortexed for 3 sec and incubated for at least 2 hours at 25°C or 30 min at 37°C before reading for absorbance at 562 nm in a Unicam SP1800 spectrophotometer against reagent blank.

2.2. MEMBRANE PREPARATION

2.2.1. Preparation of rat cerebral cortex membranes

Rat cerebral cortex membranes were prepared as described by Brown *et al.* (1990a) by homogenising freshly dissected or thawed frozen rat cerebral cortices in 25 volumes of ice cold 50 mM Tris HCl, 5 mM EDTA, (pH 8.0). The homogenate was centrifuged at 48000 g for 15 min at 4°C. The pellet was resuspended in the same volume of buffer and washed once more under the conditions described. The pellet was washed twice in assay buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 8.0 at 4°C) by repeated centrifugation at 48000g for 15 min at 4°C. The final pellet was resuspended in assay buffer at an approximate protein level of 3 mg/ml.

2.2.2. Preparation of human platelet membranes

Platelet membranes were prepared essentially as previously described by Cheung *et al.* (1982). Blood was donated by healthy male and female Syntex employees who undergo an annual screen for Hepatitis-B virus. No more than 200 ml blood was collected from any volunteer in any eight week period. Blood was collected into 50 ml blood tubes containing 5 ml of 3.5 % (w/v) trisodium citrate. Disposable gloves, face mask and safety glasses were worn at all times when handling blood or derived products. All homogenisation

procedures were carried out in an externally vented Grade 3 fume hood.

Platelet rich plasma (PRP) was obtained by centrifugation at 200g for 15 min in a MSE Centaur bench centrifuge. The PRP was centrifuged in aliquots of 25 ml at 48000 g for 10 min at 4°C and the resulting pellet resuspended in cold lysing buffer (5 mM Tris HCl, 5 mM EDTA, pH 8.0). The pellet was washed once more in lysing buffer by resuspension and centrifugation followed by a wash in 50 mM Tris HCl, 0.5 mM EDTA pH 8.0 at 4°C. The final pellet was resuspended in assay buffer at a protein concentration of 1.0 mg/ml. Membranes from individual volunteers were stored separately under liquid nitrogen until required for use in the binding assay. For [^{3}H]-adrenaline binding an additional step was included by incubating the membrane suspension in assay buffer for 15 min at 37°C prior to centrifugation to facilitate removal of endogenous agonist and GTP.

2.2.3. Preparation of neonatal rat lung membranes

Neonatal Sprague Dawley rats (< 24 hours old) of mixed sexes were sacrificed by decapitation and the lungs dissected out and frozen under liquid nitrogen until use. Membranes were prepared as described for cerebral cortex (Section 2.2.1.) by homogenising 3 lungs in 25 ml homogenising buffer. Because lung tissue is quite fibrous the homogenate was strained through a layer of muslin to remove non-homogenised material before the first centrifugation step. The final pellet was suspended in 3 ml assay buffer at an approximate protein concentration of 2.0 mg/ml.

2.2.4. Preparation of rat kidney membranes

Kidney membranes were prepared as previously described (MacKinnon *et al.*, 1991a). Kidneys were dissected from male Sprague Dawley rats and each kidney homogenised in 25 ml 50 mM Tris HCl buffer pH 7.4 at 25°C containing 5 mM EDTA. Membranes were prepared as described for rat cortical membranes (section 2.2.1.). The final pellet was suspended in 3 ml assay buffer to a protein concentration of 3 mg/ml.

2.3. BINDING TO α_2-ADRENOCEPTOR SUBTYPES

2.3.1 [3H]-RS-15385-197 binding

[3H]-RS-15385-197 was synthesised to a specific activity of 57 Ci/mmole by Dr. H. Parnes (Institute of Chemistry, Syntex, Palo Alto, U.S.A.) and stored at -20°C at a concentration of 0.75 mCi/ml. Under these storage conditions [3H]-RS-15385-197 retained > 98 % purity (determined by HPLC) throughout the duration of this study. [3H]-RS-15385-197 (0.01 - 2.0 nM) was incubated to equilibrium in 50 mM Tris HCl buffer, pH 7.4 at 25°C containing 0.5 mM EDTA with 50-250 µg membrane protein. Incubations were carried out at 25°C in a final volume of 1 ml for 90 min for rat cerebrocortical membranes and for 60 min for human platelet and neonatal rat lung membranes. Competition experiments were carried out with 0.1 - 0.2 nM [3H]-RS-15385-197 and 13 concentrations of competing drug. Bound ligand was determined as described (Section 2.1.2.). Non-specific binding at each free ligand concentration was determined in the presence of 1 µM phentolamine.

2.3.2. [3H]-adrenaline binding

[3H]-(-)-Adrenaline was purchased from Du-Pont (specific activity 65-75 Ci/mmole). In all cases during this study the (-) isomers of adrenaline and noradrenaline were used. [3H]-Adrenaline (0.2 -25 nM) was incubated with 100 -200 µg neonatal rat lung or human platelet membranes to equilibrium (60 min at 25°C). Competition experiments were carried out with 2 - 3 nM [3H]-adrenaline and 13 concentrations of competing drug. The assay buffer 50 mM Tris HCl pH 7.4 at 25°C contained 0.3 mM ascorbic acid, 5 mM $MgCl_2$, 0.8 mM dithiothreitol and 0.1 mM catechol which mimimised degradation of the radioligand (Sénard *et al.*, 1988). For neonatal rat lung membranes the assay also contained 10 nM propranolol to prevent binding to β-adrenoceptors. Bound ligand was determined as described (Section 2.1.2.). Non-specific binding was carried out in the presence of 10 µM (-)-noradrenaline.

2.4. IMIDAZOLINE SITE BINDING

2.4.1. [3H]-*p*-Aminoclonidine binding to rat kidney membranes

[3H]-*p*-Aminoclonidine (2.0 - 3.0 nM, Du-Pont U.K., 50 - 60

Ci/mmole) was incubated to equilibrium (30 min at 4oC unless otherwise indicated) with 0.8 - 1.0 mg rat kidney membranes in the absence or presence of 13 concentrations of competing drug in a final assay volume of 0.5 ml (50 mM Tris HCl pH 7.4 at 4oC containing 0.1 μM RS-15385-197 to preclude binding to α_2-adrenoceptors). Non-specific binding was determined in the presence of 100 μM clonidine. Bound ligand was determined as described (Section 2.1.2.) followed by 2 x 5 sec washes (unless otherwise indicated) with ice cold assay buffer. In some experiments centrifigation at 15000 g for 90 sec in a Wifug microfuge was used to separate bound ligand. In these experiments the filtrate was tipped out and the resultant pellet washed rapidly and superficially with 0.5 ml ice cold assay buffer and then solubilised in 100 μl formic acid for 30 min. Filters or solubilised pellets were suspended in 4 ml scintillation cocktail and bound ligand estimated by counting in a Beckman 5000CE Scintillation counter. Due to the low affinity of [^{3}H]-p-aminoclonidine for its binding site and to reduce the amount of label required for a saturation assay, equilibrium binding parameters (K_d and B_{max}) were calculated from competition experiments with unlabelled *p*-aminoclonidine (see Section 2.8.).

2.4.2. [^{3}H]-idazoxan binding to rat kidney membranes

Rat kidney membranes (300-500 μg protein) were incubated with 1.0 - 2.0 nM [^{3}H]-idazoxan (Amersham, Bucks, U.K., 40 - 50 Ci/mmole) for 90 min at 25oC (unless otherwise indicated) in the presence of 0.1 μM RS-15385-197 and various concentrations of drugs in a final assay volume of 0.5 ml assay buffer (50 mM Tris HCl; pH 7.4 containing 0.5 mM EDTA). Bound ligand was separated from free as described (Section 2.1.2.) followed by 2 x 5 sec washes with assay buffer at room temperature. Non-specific binding was determined in the presence of 1 μM cirazoline.

2.4.3. [^{3}H]-RS-45041-190 binding to rat kidney membranes

[^{3}H]-RS-45041-190 was synthesised to a specific activity of 28 Ci/mmole by Dr. H. Parnes (Institute of Chemistry, Syntex Palo Alto, U.S.A.), and stored at -20oC at a concentration of 1 mCi/ml. Under these storage conditions [^{3}H]-RS-45041-190 retained > 96 % purity (determined by HPLC) throughout the duration of this study. 0.2 - 25 nM [^{3}H]-RS-45041-190 was incubated with rat kidney membranes (200-

400 µg protein) to equilibrium (60 min at 25°C) in an assay volume of 500 µl 50 mM Tris HCl pH 7.4 at 25°C containing 0.5 mM EDTA. In competition experiments 1.0 - 2.0 nM [^{3}H]-RS-45041-190 was incubated with 13 concentrations of competing drug. Non-specific binding was defined in the presence of 10 µM cirazoline. Bound ligand was determined as described (Section 2.1.2.).

A summary of the receptor binding conditions used in this thesis is given in Table 2.2.

2.5. *IN VITRO* RECEPTOR AUTORADIOGRAPHY

2.5.1. Preparation of slides for autoradiography

Double frosted glass microscope slides were cleaned in running tap water and dried in an oven at 60°C. A subbing solution was prepared by dissolving 10 g gelatin and 0.5 g chromic potassium sulphate in 1 L deionised water. The solution was heat stirred to a temperature of 60 - 70°C and filtered through Whatman 91 filter paper. The solution was cooled to below 40°C and racks containing the cleaned slides dipped in the subbing solution to coat thoroughly. The slides were dried overnight in an oven at 60°C with copper sulphate dessicant. The subbing solution could be used to coat several batches of slides at once but was discarded after use.

2.5.2. Preparation of sections for autoradiography

Male Sprague-Dawley rats (250-350 g) were anaesthetised with 60 mg/kg pentobarbitone i.p. and the thorax opened. A clamp was placed around the descending arteries and the animal was perfused intracardially with 20 mls 0.9% saline by means of a blunted needle inserted into the left ventricle and up into the aorta. A nick was made in the right atria to allow passage of blood and perfusate. The brains were carefully removed and frozen in isopentane at -45°C (temperature was maintained by cooling in a dry ice / acetone mixture). Tissue blocks were frozen onto orientating microtome chucks with Tissue Tek and dipped in Lipshaw Embedding Matrix. 20 µm coronal sections were cut on a Bright cryostat and thaw mounted onto gelatin subbed slides. For a detailed brain receptor map sections were taken in the caudal to rostral direction in a plane related to Bregma as described by

Table 2.2. Summary of binding conditions

ligand (concentration)	buffer	membranes (protein)	NSB	incubation
[^{3}H]-RS-15385-197 0.1 - 0.2 nM	50 mM Tris pH 7.4; EDTA 0.5 mM	rat cortex (150-250 µg)	1 µM phentolamine	25°C 90 min
[^{3}H]-RS-15385-197 0.1 - 0.2 nM	50 mM Tris; pH 7.4 EDTA 0.5 mM	human platelet (50-150 µg)	1 µM phentolamine	25°C 60 min
[^{3}H]-RS-15385-197 0.1 -0.2 nM	50 mM Tris; pH 7.4 EDTA 0.5 mM	rat neonate lung (100-200 µg)	1 µM phentolamine	25°C 60 min
[^{3}H]-adrenaline 2.0 - 3.0 nM	50 mM Tris; pH 7.4 $MgCl_2$ 5 mM dithiothreitol 0.8 mM ascorbate 0.3 mM catechol 0.1 mM	human platelet (100-200 µg)	10 µM noradrenaline	25°C 60 min
[^{3}H]-adrenaline 2.0 - 3.0 nM	50 mM Tris; pH 7.4 $MgCl_2$ 5 mM dithiothreitol 0.8 mM ascorbate 0.3 mM catechol 0.1 mM propranolol 10 nM	rat neonate lung (100-200 µg)	10 µM noradrenaline	25°C 60 min
[^{3}H]-*p*-aminoclonidine 2.0 - 3.0 nM	50 mM Tris; pH 7.4 EDTA 0.5 mM RS-15385-197 0.1 µM	rat kidney (800-1000 µg)	0.1 mM clonidine	4°C 30 min
[^{3}H]-idazoxan 1.0 - 2.0 nM	50 mM Tris; pH 7.4 EDTA 0.5 mM RS-15385-197 0.1 µM	rat kidney (300-500 µg)	1 µM cirazoline	25°C 90 min
[^{3}H]-RS-45041-190 1.0 - 2.0 nM	50 mM Tris; pH 7.4 EDTA 0.5 mM	rat kidney (200-400 µg)	10 µM cirazoline	25°C 60 min

Paxinos & Watson (1982). Six sections were taken (3 sections per slide) for each brain area with 15 sections discarded between areas, in this way the receptor distribution in approximately 50 brain areas could be made from 1 animal. Sections were allowed to dry at room temperature and then stored at -20°C for at least 3 days before use to facilitate adhesion to the slide.

2.5.3. Preparation of labelled sections for autoradiography

The preparation of labelled sections for autoradiography requires; 1) a preincubation step to remove any endogenous ligands which may interfere with the binding of the radioligand 2) an incubation step in an appropriate buffer system with radioligand to maximise radioligand binding; parallel sections are incubated in the presence of non-specific binding drug to define the level of non-specific binding in the brain section 3) a washing stage to remove unbound radioligand whilst retaining a high proportion of bound ligand and a good ratio of specific to non-specific binding 4) a drying stage prior to exposure to radioactive film 5) quantification.

2.5.4. Establishing a labelling protocol

Prior to the generation of autoradiograms, the optimum incubation and washing conditions were established for each radioligand. This was achieved by bringing the sections to room temperature and preincubating in assay buffer for 20 min at room temperature in racks which hold 25 slides. The assay buffer used was the same as that used in membrane binding experiments except that isotonic buffers were used e.g. 170 mM Tris HCl. The slides were then transferred to 25 ml Coplin jars containing 20 ml assay buffer and radioligand. These jars are capable of holding 10 slides back to back in parallel. The incubation time and concentration of radioligand used were the same as that used in membrane binding experiments. Parallel incubations were performed in the presence of non-specific drug. Sections were washed by placing slides individually in metal racks in troughs containing ice-cold assay buffer. The slides were removed at timed intervals, dipped in ice-cold distilled water and the sections wiped immediately from the slide with a circle of filter paper. Filters representing total and non-specific binding for each wash time were placed in scintillation mini-vials and 4 ml scintillation cocktail added.

Samples were counted for radioactivity in a Beckman 5000CE Scintillation counter. From these experiments a graph of wash time against specific dpm bound/section could be constructed and the optimum washtime for further experiments established. The optimum incubation time for each radioligand was established similarly by incubating in assay buffer with radioligand for various times before washing. The wash time used would be that established from the washing experiments. From these experiments the time to reach equilibrium could be established by plotting incubation time against specific dpm bound/section. Using the optimised assay conditions, labelled sections were prepared for the generation of autoradiograms.

2.5.5. Preparation of [^{3}H]-RS-15385-197 labelled sections

Sections were brought to room temperature and preincubated for 20 min at room temperature in 170 mM Tris HCl buffer pH 7.4 containing 0.5 mM EDTA. Incubations were performed in the same buffer containing 0.1 nM [^{3}H]-RS-15385-197 for 60 min, non-specific binding was determined in the presence of 1 μM phentolamine. Labelled sections were washed in ice-cold Tris EDTA buffer for 10 min, dipped in ice cold distilled H_2O and dried by placing on dry ice and blowing with ambient air with a domestic Clairol Coolset hairdryer for several hours. The sections were left overnight at room temperature to dry thoroughly and opposed to [^{3}H]-sensitive Hyperfilm (Amersham) with appropriate standards (^{3}H microscales Amersham) in X-ray cassettes for 12 weeks. [^{3}H]-Microscales are supplied in 2 radioactivity ranges, RPA 505 (0.07 to 6.5 nCi/mg tissue equivalent) and RPA 507 (0.1 to 16 nCi/mg tissue equivalent), each containg 8 activity levels in a polymer matrix. The tissue equivalent activity is calculated as an estimate of the optical density expected in a section of brain tissue (gray and white matter) of an equivalent activity and this data is supplied in an accompanying data sheet. The activity in each brain region can be calculated from a standard curve constructed from the optical density of the standards against activity in moles/mg tissue equivalent (see Section 2.5.8.), or as calculated for [^{3}H]-RS-15385-197 autoradiography in moles/unit area (in this case mm^2).

The films were developed in Agfa X-ray developer (diluted 1 : 5 with tap water) for 4 min at room temperature, rinsed briefly in water and fixed in Agfa X-ray fixer (diluted 1 : 4 with tap water) for 4 min

Films were rinsed for 10 min in running water and dried in a drying cabinet at 30°C.

2.5.6. Preparation of [^{3}H]-idazoxan labelled sections

Sections were brought to room temperature and preincubated for 20 min at room temperature in 170 mM Tris HCl buffer pH 7.4 containing 0.5 mM EDTA, 100 µM phenyl methyl sulphonyl fluoride (PMSF) and 0.1 µM RS-15385-197. Incubations were performed in the same buffer containing 3 nM [^{3}H]-idazoxan for 3 hours, non-specific binding was determined in the presence of 10 µM cirazoline. Labelled sections were washed in ice cold preincubation buffer for 10 min and dipped in ice cold distilled H_2O. The sections were dried and exposed to [^{3}H]-sensitive Hyperfilm for 12 weeks as described (Section 2.5.5.).

2.5.7 Preparation of [^{3}H]-RS-45041-190 labelled sections

Sections were brought to room temperature and preincubated for 20 min at room temperature in 170 mM Tris HCl buffer pH 7.4 containing 0.5 mM EDTA. Incubations were performed in the same buffer containing 3 nM [^{3}H]-RS-45041-190 for 60 min, non-specific binding was determined in the presence of 10 µM cirazoline. Labelled sections were washed in ice cold preincubation buffer for 10 min, dipped in ice cold distilled H_2O and dried. Autoradiograms were generated as described (Section 2.5.5.).

2.5.8. Quantitative Autoradiography

Autoradiograms were analysed using a Quantimet 970 Image Analyser run on a PDP 11 computer with a Video Camera. After background subtraction by a matrix shading corrector facility, the optical density (O.D.) in each of the 8 Amersham microscale standard sections was calculated and stored on disc as ln O.D. versus ln radioactivity in fmoles/mg tissue equivalent. [^{3}H]-RS-15385-197 autoradiography sections were sent to Dr. N. Sharif, Syntex Palo Alto for quantification. In these studies, standard curves were calculated as O.D. vs radioactivity in moles/unit area (mm^2). O.D. measurements of the brain structures were performed by selecting a measuring box of appropriate dimensions which was used to overlay the structure in a total binding section and measure the optical density of that area. Once all the measurements in a particular section was complete the image area

was stored, and after edge enhancement and alignment of the corresponding non-specific section, the specific O.D. of all structures in that section was made automatically by subtraction of the non-specific from the total. Measurements from replicate sections were collated and stored on disc to be later printed to give the mean specific binding for a defined brain area. Prior to calculation of activity in the autoradiograms, a protocol was established to outline the structures to be measured in each section plus the area of the box used to measure each structure. Often more than one measurement would be made of any one structure in a given section, and also measurements of left and right portions of a structure where appropriate. Once the protocol was established it was adhered to for all sections and animals in that study group.

2.5.9. Photography

For diagramatic representation in this thesis, autogadiograms were photographed using a Canon SLR camera with Kodak Ortholith black and white film. All further procedures were carried out under safe-light conditions. Exposed films were developed in Microfen developer for 5 min at 22°C, rinsed with cold water, and fixed in Ilford Hypam fixer for 2 min at 22°C. The developed film was washed for 10 min in running water and dried at 30°C for at least 30 min. Prints were made from the film using Ilford grade 5 black and white printing paper. The prints were developed in Ilford Bromofen developer for 2 min at 22°C, rinsed in cold water, and fixed in Hypam fixer for 2 min at 22°C. The prints were washed in running water for 10 min, and dried at 30°C. Individual autoradiograms were mounted onto white card, and the corresponding legend attached. This was then rephotographed and printed as described above.

2.6. LIPOLYSIS IN HAMSTER ADIPOCYTES

2.6.1. Preparation of Hamster adipocytes

Hamster adipocytes were prepared essentially as previously described (MacKinnon *et al.*, 1989). Male Syrian hamsters (2-3 months old; 100 - 150 g) were killed by gassing with nitrogen. White adipose tissue from the epididymal area, kidneys and subcutaneous layer was removed onto ice and minced finely. Normally 8 - 10 g fat was

obtained per animal. Minced tissue was suspended in Krebs Ringer bicarbonate buffer (3ml/g tissue) of the following composition; 25 mM $NaHCO_3$, 120 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 6 mM glucose, 3.5 % fatty acid free bovine serum albumin and 1 mM $CaCl_2$ (pH 7.4; gassed with 95% O_2 / 5% CO_2) and contained 1 mg/ml collagenase (Sigma Type 1A). The tissue was incubated at 37°C for 20 min, with vigorous shaking by hand every 5 min. The suspension containing the adipocytes was filtered through 2 layers of muslin to remove undigested fat and was left to settle at room temperature for 5 min. During this time the adipocytes floated to the surface of the supension and were separated from the digestion buffer by aspiration of the infranatant. The adipocytes were washed 3 times by suspension in equal volumes of collagenase free buffer.

2.6.2. Measurement of glycerol release

Aliquots (200 µl) of isolated adipocytes were suspended in Krebs-Ringer bicarbonate buffer pH 7.4 containing 100 nM propranolol and various concentrations of agonist and antagonist in a final volume of 500 µl. Lipolysis was initiated by the addition of 100 µM theophylline and 2 µg adenosine deaminase (72 units/mg protein). Batches of adenosine deaminase were made up to 2 mg/ml with deionised water on arrival and then split into 100 µl aliquots and frozen at -20°C. One aliquot of thawed enzyme was suspended in 10 ml of 0.5 mM theophylline in assay buffer and 100 µl of this mixture added to each assay tube. The cells were incubated at 37°C for 20 min with agitation, unless otherwise indicated, and the reaction stopped on ice. Aliquots of infranatant (200 µl) were removed for the determination of glycerol. Glycerol was measured using spectrophotometric kits supplied by Randox (Ireland). The direct colometric procedure uses a quinoneimine chromogen system in the presence of peroxidase and glycerol phosphate oxidase and is linear up to a glycerol concentration of 5 mM. 30 µl of sample or glycerol standard was incubated with 1 ml reagent at 25°C for 10 min and read for absorbance at 520 nm. Agonist responses were expressed as a percentage of the theophylline/adenosine deaminase stimulation.

2.7. MEASUREMENT OF CAMP

2.7.1. Whole platelet preparation

Whole platelets were prepared by the method of Tymkewycz *et al.* (1991). Blood (200 ml) from male and female volunteers, was collected into 50 ml tubes containing 5 ml of 3.5% (w/v) trisodium citrate and centrifuged at 250 g for 15 min (MSE Centaur bench centrifuge) to produce a platelet-rich plasma (PRP). Idomethacin (10 μM) was added to the PRP and whole platelets were sedimented by centrifugation at 450g for 20 min. The pellet was supended in 20 ml 50 mM Tris HCl; 5 mM EDTA pH 7.4 and swirled gently to disperse the platelets. Aliquots (0.5 ml, ~1.5 mg protein) were incubated at 37°C for 1 min with cicaprost (8 nM) to stimulate adenylate cyclase. Where inhibitory agonists were included they were preincubated with the platelets for 2 min prior to the addition of cicaprost. The reaction was stopped with addition of 1 ml ethanol and the precipitate sedimented by centrifugation at 3000g for 15 min. The supernatant was evaporated overnight at 50°C and the residue resuspended in 0.5 ml Tris EDTA buffer for analysis of cAMP (Section 2.7.3.). Standard cAMP samples were extracted in parallel to assess the percentage recovery of cAMP. In this system cAMP recovery was not less than 94%.

2.7.2. Human platelet and neonatal rat lung membranes

Human platelet and neonatal rat lung membranes were prepared as described (Sections 2.2.2 and 2.2.3.), diluted to give an approximate protein concentration of 2 mg/ml, and frozen at -80°C prior to use. Aliquots (50 μl ~ 100 μg protein) of thawed membrane were incubated at 37°C for 15 min (unless otherwise indicated) in 0.5 ml of cAMP generating buffer (50 mM Tris HCl; pH 7.4, 0.5 mM EDTA, 2.5 mM $MgCl_2$, 5 mM creatine phosphate, 50 U/ml creatine phosphokinase (Sigma, type 1), 1 mM IBMX, 0.1 mM GTP, 1 mM ATP, 1 mM ascorbate). Adenylate cyclase activity was stimulated with cicaprost (platelet membranes) or isoprenaline or forskolin (lung membranes) and inhibitory agonists added 15 min prior to the addition of cicaprost. The reaction was stopped by boiling for 5 min and the precipitated proteins sedimented at 10000g for 2 min. Aliquots of supernatant were assayed for cAMP with standards prepared in cAMP generating buffer.

2.7.3. Assay of cAMP

cAMP was assayed using a cAMP binding protein suplied by BDH (BDH, Poole, UK). This assay is based on the competitive binding of [^{3}H]-cAMP (28 Ci/mmole; Amersham, Buckingham, UK) and unlabelled cAMP to a protein kinase isolated from bovine adrenal cortex. It is largely specific for cAMP and binds much less readily to cGMP. The assay procedure was essentially as described in the accompanying data sheet, but the protein was diluted 1.5 ml in 20 ml assay buffer (50 mM Tris HCl, 4 mM EDTA pH 7.4) prior to use.

Duplicate 50 µl aliquots of extracted cAMP and 50 µl [^{3}H]-cAMP (20-25 Ci/mmole, Amersham Bucks UK, diluted 5 µl in 10 ml assay buffer to give 0.025 µCi/assay) were incubated with 100 µl diluted binding protein for 2 hours in an ice bath. A standard curve (0, 1, 2, 4, 8, 16, 30, 100 pmoles cAMP/50 µl) was constructed in duplicate and assayed in parallel. Unbound cAMP was separated by the addition of 100 µl charcoal adsorbent (0.52g charcoal, 0.4g bovine serum albumin in 20 ml ice cold distilled water), vortexed and centrifuged in a Wifug microfuge for 90 sec. Charcoal blank tubes containing [^{3}H]-cAMP in the absence of binding protein were included to assess the efficiency of the charcoal adsorbent. Aliquots (200 µl) of supernatant were suspended in 5 ml Ready Safe Scintillation cocktail. The samples were capped and vortexed thoroughly, and left for at least 8 hours before counting for tritium as described in Section 2.1.2.

After subtraction of the charcoal blank, a standard curve was constructed by plotting C0/Cx against pmoles cAMP, where C0 is the dpm in the absence of unlabelled cAMP and Cx is the dpm in the presence of each concentration of unlabelled cAMP. This plot was essentially linear over the range 1 - 100 pmoles cAMP/50 µl. Linear regression analysis was carried out using the Kaleidagraph programme run on an Apple Macintosh computer. Unknown cAMP samples were calculated from the standard curve using the equation of the line. A typical standard curve is presented in Figure 2.1.

2.8. RAT ANOCOCCYGEUS MUSCLE PREPARATION

Male Sprague-Dawley rats (250 - 300 g) were sacrificed by a blow to the head and cervical dislocation. The abdomen was opened and the bilateral anococcygeus muscles exposed by breaking the pelvic bone

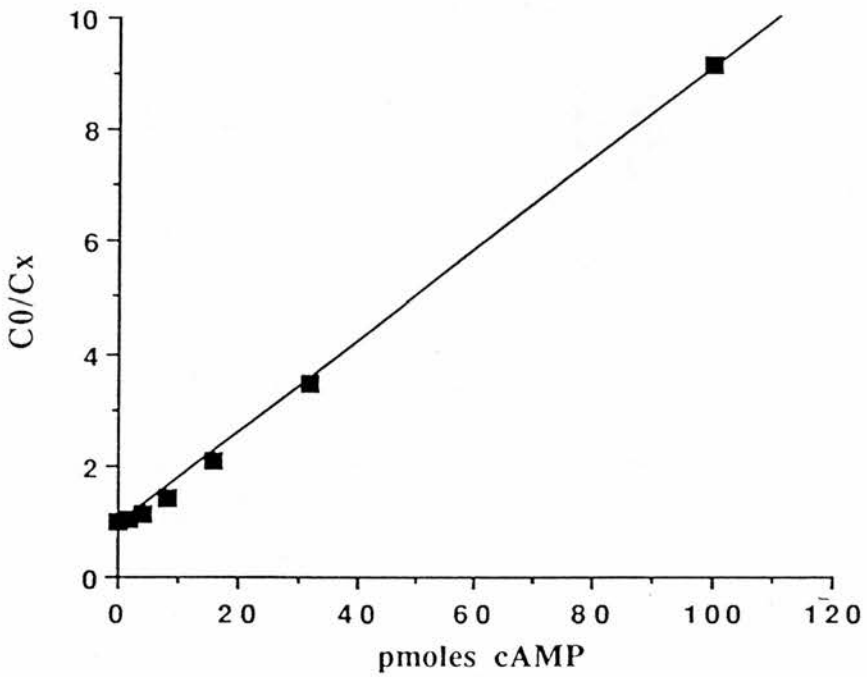


Figure 2.1. An example of a typical cAMP standard curve. The curve was constructed over the range 1 - 100 pmoles cAMP/50 µl, and is essentially linear over this range. Unknown cAMP samples are read from the curve using the equation of the straight line $y = mx + c$. Linear regression analysis was carried out using the Kaleidagraph programme run on an Apple Macintosh computer.

and carefully retracting the descending colon. Ligatures were placed around each muscle at the intestinal and spinal attachments and tied. The muscles were removed with care to avoid stretching of the tissue and placed in Krebs Henselheit buffer (118 mM NaCl, 5.6 mM KCl, 1.19 mM $MgSO_4$, 1.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 2.5 mM $CaCl_2$ gassed with 95% O_2/5% CO_2 pH 7.4 at 37°C). Each tissue was mounted on a stainless steel field-stimulating electrode under 0.5 g tension in a 30 ml organ bath maintained at 37°C. Changes in tension were recorded by Dynamometer UF1 isometric force displacement transducers (25 g range) coupled to a BBC Georz Melrawatt SE 460 chart recorder. Tissues were allowed to equilibrate for 45 min with frequent changes of medium before commencement of the experiment. After equilibration, the tissues were contracted to various agonists. The concentration of agonist was selected so as to produce equal increases in tension in the tissue (approximately 5 g, or 75 % noradrenaline maximum). Upon attainment of the contraction plateau (10 - 15 min) the tissues were field stimulated via a Grass S88 stimulator (square wave, 0.5 msec duration; 50V). Cumulative frequency inhibition response curves (0.025 - 32 Hz) were constructed by increasing the frequency 2 fold sequentially after the attainment of a steady response. After determination of a control response, the stimulator was switched off and the tissues washed frequently for 30 min or until the precontraction baseline was achieved. The antagonist was added, and allowed to equilibrate with the tissue for 60 min before addition of agonist and the construction of a second frequency response.

2.9. DATA ANALYSIS

2.9.1. Equilibrium binding parameters

Equilibrium binding parameters (K_d and B_{max}) were obtained using the iterative, non-linear least-square curve-fitting programme "Ligand" (Munson & Rodbard, 1980) run on an HP 3000 computer. Analysis of the data was carried out after initial graphical display of the data on an IBM PC which enabled the elimination of rouge points. In several cases throughout this thesis saturation data will be presented in the form of Scatchard plots. These plots are for graphical illustration only and are linear transformations, plotting bound ligand against bound/free ligand. K_d and B_{max} values for [^{3}H]-*p*-aminoclonidine

binding were obtained from competition studies with unlabelled *p*-aminoclonidine where $K_d = IC_{50}$ - [ligand] and $B_{max} = Bo \times IC_{50}$ / [ligand] (Deblasi *et al.*, 1989). IC_{50} is the concentration of competing drug producing 50% inhibition of specific binding and Bo is the amount of labelled ligand specifically bound.

Binding isotherms from competition studies were analysed using a non-linear least-square parametric curve-fitting programme run on a BBC model B computer (Michel & Whiting, 1981). This programme was designed for the interpretation of sigmoidal concentration response curves and capable of estimating IC_{50} and curve steepness (Hill slope; nH) and iterative curve-fitting to a single or two site model. The IC_{50} was converted to a pK_i (when Hill slopes were equal to 1) or a pIC_{50} corrected for ligand concentration (when Hill slopes deviated from 1) by the equation (Eq.1) of Cheng & Prussof (1973). Where [L] = the concentration of free ligand and K_d is the dissociation constant for the radioligand.

$$pK_i/pIC_{50} = -\log \frac{IC_{50}}{1 + [L]/K_d} \qquad \text{(Eq.1)}$$

All data were initially analysed assuming a one site model of radioligand binding. The data with Hill slopes of less than unity were then analysed assuming a 2 site model and the results of the fit were statistically compared to those of the one site fit by the differential F value defined by Eq.2:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2} \qquad \text{(Eq.2)}$$

where SS_1 is the sum of squares error for the single site, SS_2 is the sum of squares error for the two site model, df_1 is the degrees of freedom for the single site model and df_2 the degrees of freedom for the two site model (Munson & Rodbard, 1980; Petrash & Bylund, 1986). A two site fit was assumed to be significantly better than a single site fit if the determined F value was significant (95% confidence limits). For the purpose of graphical presentation in this thesis, competition curves were

plotted using the Kaleidagraph programme run on an Apple Macintosh computer.

2.9.2. Kinetic analysis

For kinetic experiments, the pseudo first order rate constant (K_{obs}) was initially calculated from the slope of the plot ln(Be/Be-Bt) versus time where Be is the binding at equilibrium and Bt is the binding at time t. Essentially the same results were obtained with a non-linear least squares fit to a single exponential function (Eq. 3), and this was subsequently used in all determinations of K_{obs}, where Be and k are, respectively, the amount of equilibrium binding and the rate constant (K_{obs}) for the single exponential model. Experiments for which semilogarithmic plots were non-linear, were analysed according to a double exponential fit (Eq. 4).

$$Bt = Be\ (1 - e^{-k}) \qquad \text{(Eq. 3)}$$

$$Bt = Be_f(1 - e^{-kf}) + Be_s(1 - e^{-ks}) \qquad \text{(Eq. 4)}$$

Be_f and Be_s are the amplitudes, and k_f and k_s are the rates (K_{obs}) of the fast and slow binding components in the double exponential model. The fits for a one or two site model were compared using the differential F value (Section 2.8.1. and Eq.2). Non-linear fits were analysed by the Kaleidagraph programme run on an Apple Macintosh computer. The association rate constant K_1 was determined from the equation $K_1 = (K_{obs}-K_2)/[Ligand]$, where K_2 is the dissociation rate constant calculated from the slope of the plot ln(Bt/B0) against time, where B0 represents binding at time 0 and Bt the binding at time t . Again, the same results were obtained with a non-linear least-squares fit to a single or double exponential function (Eq. 5 and 6), and this was used in all determinations of K_2.

$$Bt = B0\ (- e^{-k}) \qquad \text{(Eq. 5)}$$

$$Bt = B0_f(- e^{-kf}) + B0_s(- e^{-ks}) \qquad \text{(Eq. 6)}$$

The equilibrium dissociation constant (K_d) was calculated from the equation $K_d = K_2/K_1$.

2.9.3. Functional data analysis

In functional experiments the dissociation constant for antagonists was calculated using the procedures developed by Arunlukshana & Schild (1959). This procedure permits the determination of the dissociation constant (K_B) while making no assumptions regarding the relationship between fractional occupancy of the receptor and the ultimate response. In each experiment a family of four dose response curves were constructed. One in the absence of antagonist (control), and one in the presence of each of 3 concentrations of antagonist. From these response curves EC_{50} values were determined; where EC_{50} is the concentration of agonist producing 50% of the maximum response. The log of the dose ratio (i.e. the EC_{50} in the presence of antagonist divided by the EC_{50} of the control) minus one was plotted against [log antagonist]. The y intercept determined by least squares linear regression was the pA_2.

2.9.4. Statistical analysis

The majority of figures in this thesis are diplayed as a representative experiment from at least three experiments carried out on preparations from different animals (unless otherwise indicated) and the meaned data and standard error are given in the text or in a table referred to in the text or figure legend. Where only two determinations were made, the meaned data are presented. The statistical tests used in this thesis were the Students t-test and the differential F test test (Section 2.9.2.).

2.10. MATERIALS

2.10.1. Chemicals and drugs

[^{3}H]-RS-15385-197 (57 Ci/mmole) and [^{3}H]-RS-45041-190 (28 Ci/mmole) were synthesised by Dr H. Parnes, Syntex, Palo Alto, US; [^{3}H]-(-)-adrenaline (65-75 Ci/mmole) and [^{3}H]-*p*-aminoclonidine (50 - 60 Ci/mmole) were purchased from Du Pont, Hertfordshire, UK; [^{3}H]-idazoxan (60 - 70 Ci/mmole) and [^{3}H]-cAMP (28 Ci/mmole) were purchased from Amersham, Bucks, UK. Photographic chemicals and films were purchased from H.A.West, Edindurgh, UK. Tissue Tek was purchased from Lab-Tek Products, Illinois, US; Lipshaw Embedding Matrix was purchased from Lipshaw Detroit, US; 5-HT, yohimbine,

clonidine, oxymetazoline, (-)-adrenaline, (-)-noradrenaline, (-)-isoprenaline, Gpp(NH)p, dopamine, naphazoline, GTP, ATP, cAMP, amiloride, glibenclamide, histamine, adenosine deaminase, theophyline, indomethacin, cAMP, IBMX, creatine phosphate and creatinephosphokinase and collagenase were purchased from Sigma; ritanserin, haloperidol, *p*-aminoclonidine and 8-OH-DPAT from Research Biochemicals (Semat); phentolamine from Ciba Geigy; forskolin from Calbiochem; cicaprost from Schering; Rauwolscine from Carl Roth; WY 26703, guanabenz and indoramin from Wyeth; prazosin from Pfizer; MDL 72222 from Merrel Dow; WB 4101 from Ward Bleckinsop; cirazoline from Synthelabo; cimetidine from Smith Klein & Beecham; methysergide from Sandoz; RS-15385-197, RS-45041-190, BRL 44409, SKF 104078, idazoxan, imiloxan and UK 14304 were synthesised by Dr R. Clark, Syntex, Palo Alto, US. All other chemicals were of the highest purity and purchased from Fisons, UK.

CHAPTER THREE

ANTAGONIST AND AGONIST BINDING TO α_2-ADRENOCEPTOR SUBTYPES

3.1. INTRODUCTION

In Chapter one of this thesis the history of α_2-adrenoceptor classification was described, and also how the current classification could be misinterpreted due to the inadequate nature of the [^{3}H]-antagonists used. In addition, it was shown, that characterisation of α_2-adrenoceptors using [^{3}H]-agonist ligands may not be consistent with the current classification. The discovery of the novel compound RS-15385-197 as a potent and selective α_2-adrenoceptor antagonist was described and has been radiolabelled. In this chapter the characterisation of α_2-adrenoceptors on rat cortical membranes with [^{3}H]-RS-15385-197 is described and the autoradiographical distribution in brain sections examined. In addition the characterisation of the α_2-adrenoceptor subtypes on human platelet and neonatal rat lung membranes with [^{3}H]-RS-15385-197 and the α_2-agonist ligand [^{3}H]-(-) adrenaline will be compared.

3.2 [^{3}H]-RS-15385 BINDING TO RAT CEREBRAL CORTEX MEMBRANES

3.2.1. Association and dissociation kinetics

Association of [^{3}H]-RS-15385-197 to rat cerebral cortex membranes was rapid at 25^{o}C with a $t_{1/2}$ = 15 min. (Figure 3.1.). Equilibrium was reached after 90 min and the mean observed initial rate constant (K_{obs}) calculated from 4 separate experiments was 0.045 $\pm$ 0.006 min^{-1}. Dissociation of [^{3}H]-RS-15385-197 initiated by the addition of 1 μM phentolamine followed first order kinetics with $t_{1/2}$ = 53 min (Figure 3.1.). The mean rate constant for dissociation (K_2) was 0.013 $\pm$ 0.001 min.$^{-1}$. The equilibrium dissociation constant (K_d) determined from the equation $K_d = K_2/K_1$ where $K_1 = (K_{obs}-K_2)/$[^{3}H-RS-15385-197] was 0.062 $\pm$ 0.015 nM. Thus initial kinetic analysis revealed that [^{3}H]-RS-15385-197 labelled a single high affinity binding site in rat cortex membranes. In all further experiments with [^{3}H]-RS-15385-197 in rat cortical membranes, incubations were carried out for 90 min at 25^{o}C.

3.2.2. Saturation analysis

Saturation analysis of [^{3}H]-RS-15385-197 binding in rat cerebral

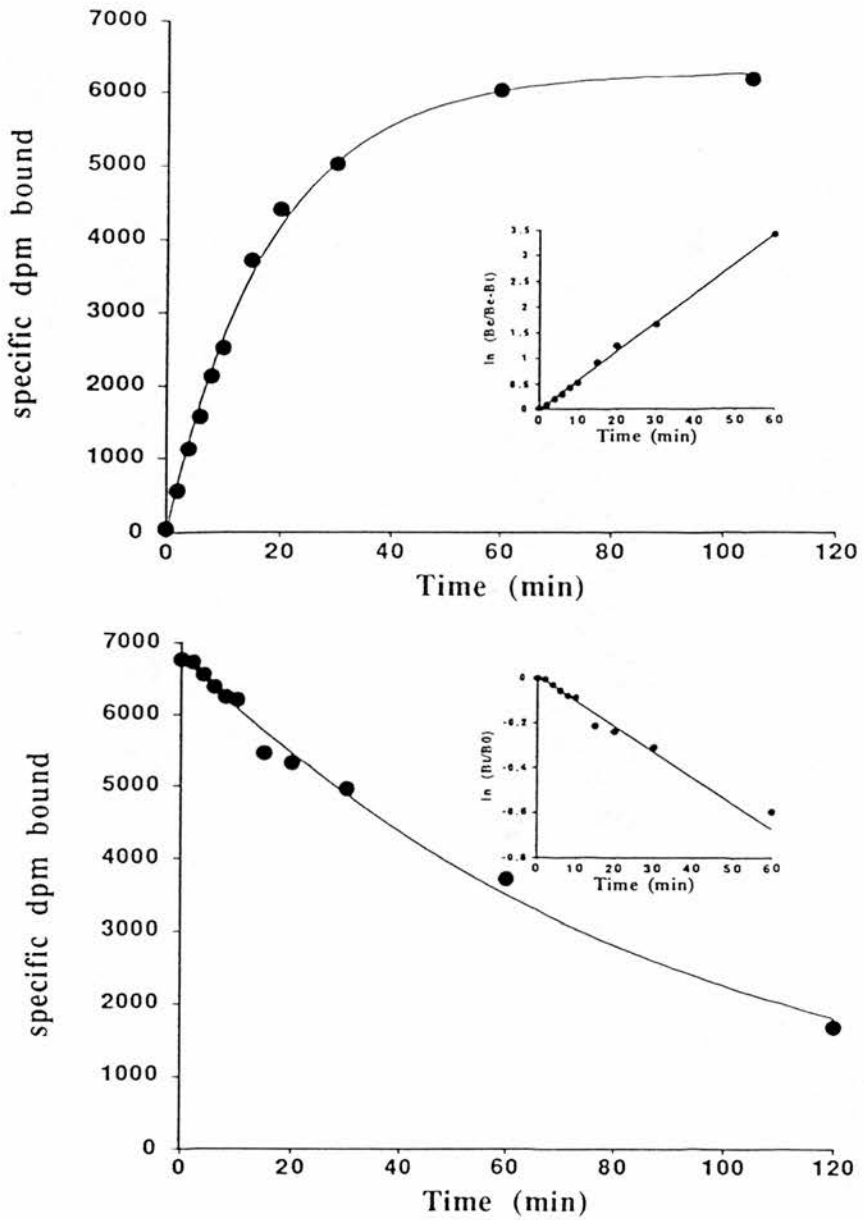


Figure 3.1. Kinetic analysis of 0.2 nM [^{3}H]-RS-15385-197 binding in rat cerebral cortex membranes at 25^{o}C. The top panel shows the association plots and the bottom panel shows the dissociation plots. The insets show the semilogarithmic transformations of the data. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in three other experiments (see text for meaned values). Association was rapid ($t_{1/2}$ = 12.6 min) and the association rate constant (K_{obs}) = 0.055/min. Dissociation, initiated by the addition of 1 μM phentolamine was slow ($t_{1/2}$ = 53 min) had a dissociation rate constant (K_2) = 0.013/min, giving a derived affinity K_d = 0.058 nM.

cortex membranes is shown in Figure 3.2.. The affinity (K_d) = 0.12 ± 0.01 nM and the density of sites (B_{max}) = 275 ± 19 fmoles/mg protein (n = 4). Thus, saturation analysis of [^{3}H]-RS-15385-197 binding revealed that this ligand labelled a single population of high affinity sites in rat cerebral cortex membranes, and that the sites were saturable with increasing concentrations of radioligand. Previous studies from this laboratory have shown that [^{3}H]-yohimbine labelled a population of sites in the same preparation but with a lower affinity and density than that seen with [^{3}H]-RS-15385-197 (K_d = 5.2 nM, B_{max} = 121 fmol/mg protein, Brown *et al.*, 1990a,b). The reasons for this discrepancy will be discussed (Section 3.8.).

3.2.3. Effect of ions and membrane protein on [^{3}H]-RS-15385-197 binding to rat cerebral cortex membranes

The monovalent cation Na^+ had a small facilitatory effect on [^{3}H]-RS-15385-197 binding in rat cortical membranes at concentrations up to 100 mM, but the divalent cations Mg^{2+} and Ca^{2+} inhibited [^{3}H]-RS-15385-197 binding with 58 and 49% inhibition respectively at 100 mM (Figure 3.3.). Further studies with [^{3}H]-RS-15385-197 were carried out in 50 mM Tris HCl buffer containing 0.5 mM EDTA to chelate divalent cations. Specific [^{3}H]-RS-15385-197 binding was dependent on membrane protein (Figure 3.4) and was linear up to 600 µg protein/assay. Subsequent experiments were conducted with 150 - 250 µg membrane protein to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

3.2.4. Effects of temperature on [^{3}H]-RS-15385-197 binding to rat cerebral cortex membranes

Measurements of [^{3}H]-RS-15385-197 dissociation from rat cortical membranes at different temperatures was characterised to investigate what effects the fluid state of the membrane had on its interaction with the receptor. Arrhenius plots of ln K_2 vs 1/T, i.e. the natural logarithm of the dissociation rate (time^{-1}) against temperature (degrees Kelvin^{-1}) were prepared to assess temperature effects. A linear plot would suggest that the binding is dependent on aqueous diffusion, whereas a plot with a "break"at 17°C corresponding to the phase transition temperature of the lipid component of the membrane would suggest that the interaction was aided by diffusion through the

lipid component of the membrane (Berg & Hippel, 1985).

[^{3}H]-RS-15385-197 dissociation from rat cortical α_2-adrenoceptors was characterised by a simple exponential time course with half times varying more than 50 fold over the temperature range 2-30^{o}C (Figure 3.5.). An Arrhenius plot of the dissociation rates was linear over the temperature range studied. Analysis of the slope of the plot provided an activation energy of 94.82 kJ/mole/degree. This suggests that [^{3}H]-RS-15385-197 binding is limited predominately by aqueous diffusion, and that probably 3 or more hydrogen bonds are involved in the interaction of RS-15385-197 with the receptor site.

3.2.5. Pharmacological characterisation of [^{3}H]-RS-15385-197 binding to rat cerebral cortex membranes

In order to determine the nature of the binding site labelled by [^{3}H]-RS-15385-197 in the rat cortex, the affinities of a number of selective α_2-adrenoceptor agonist and antagonist ligands were determined at this site (Table 3.1). The antagonists, yohimbine, rauwolscine, idazoxan and WY 26703 each inhibited [^{3}H]-RS-15385-197 with high affinity and with Hill slopes not significantly different from unity. The mixed α_1/α_2-antagonist phentolamine showed very high affinity for [^{3}H]-RS-15385-197 binding (pKi = 8.96). The α_2-adrenoceptor agonists clonidine and oxymetazoline, and the endogenous catecholamines adrenaline and noradrenaline, displayed high affinity and inhibited [^{3}H]-RS-15385-197 from rat cortical membranes with Hill slopes that were significantly lower than unity, consistent with these agonists displaying high and low affinity for α_2-adrenoceptor states. Thus, the profile of α_2-adrenoceptor agonist and antagonist affinities is consistent with [^{3}H]-RS-15385-197 labelling an α_2-adrenoceptor in rat cortical membranes.

The α_1-adrenoceptor antagonists prazosin and indoramin competed for [^{3}H]-RS-15385-197 binding with low affinity and with Hill slopes close to unity. Dopamine, haloperidol, 5-HT and the mixed 5-HT$_1$ and 5-HT$_2$ ligand methysergide also had low affinity; 8-OH-DPAT a 5-HT$_{1A}$ agonist, showed moderate affinity for [^{3}H]-RS-15385-197 binding. These data suggest that [^{3}H]-RS-15385-197 did not label α_1-adrenoceptors, dopamine or 5-HT receptors and is consistent with the high selectivity of RS-15385-197 for α_2-adrenoceptors previously reported (Clark *et al.*, 1989,1990b; MacKinnon *et al.*, 1990,1992b;

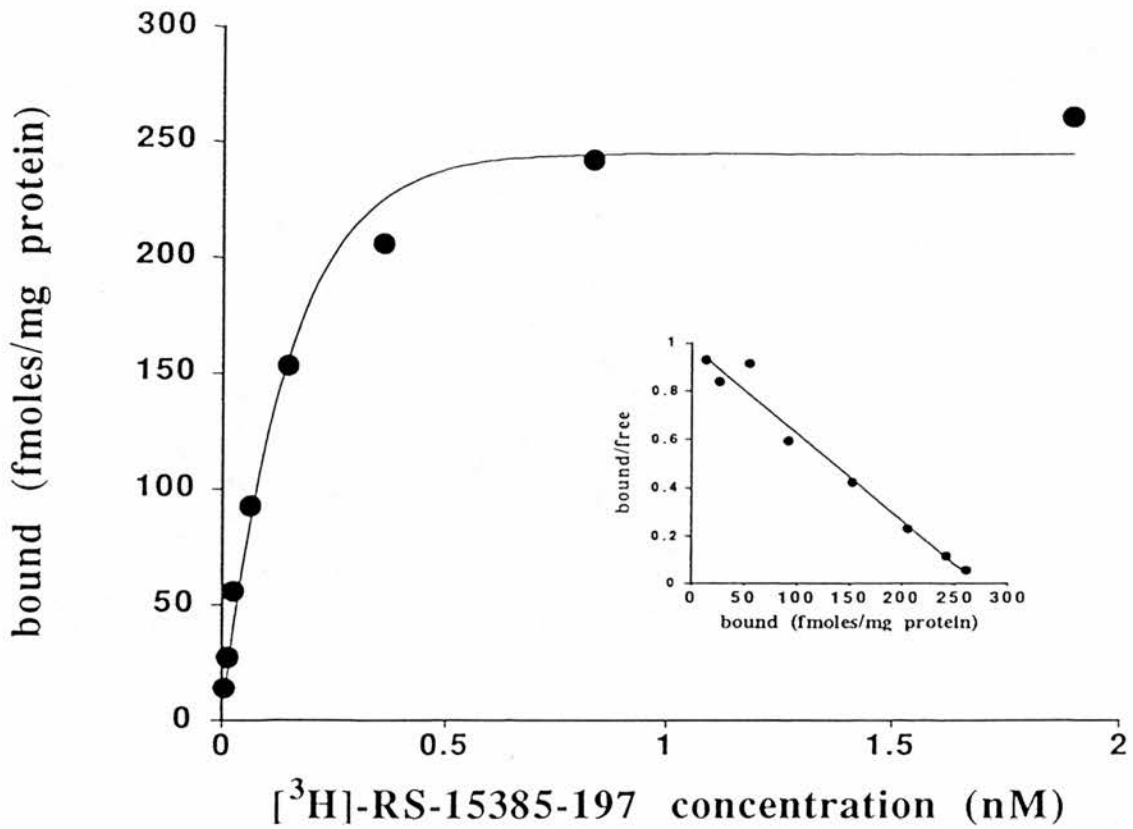


Figure 3.2. Saturation analysis of [3H]-RS-15385-197 binding to rat cerebral cortex membranes. The inset shows the Scatchard transformation of the data. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in three other experiments (see text for meaned data). The equilibrium binding constants K_d and B_{max} = 0.14 nM and 248 fmoles/mg protein.

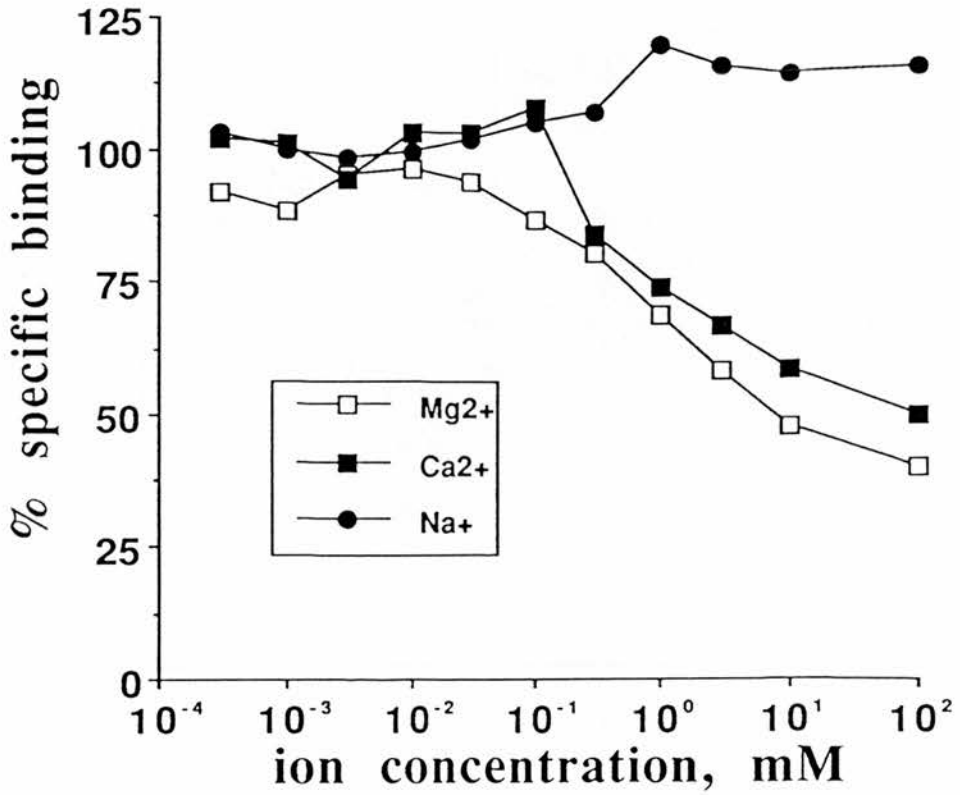


Figure 3.3. Effect of $MgCl_2$, $CaCl_2$ and NaCl on [^{3}H]-RS-15385-197 binding in rat cerebral cortex membranes. Incubations were performed with 0.1 nM [^{3}H]-RS-15385-197 in EDTA-free Tris HCl; pH 7.4. The data represents the mean of 2 experiments on separate preparations each performed in duplicate.

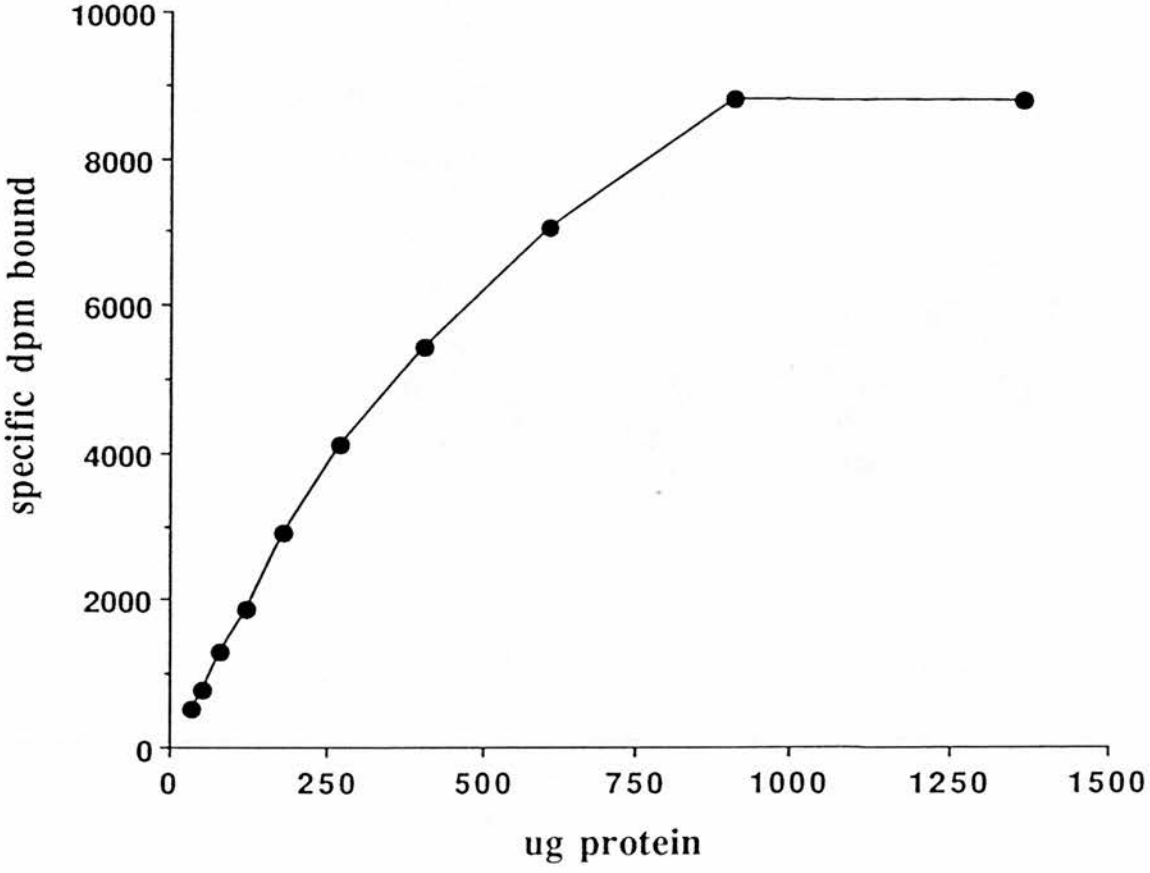


Figure 3.4. Tissue linearity curve for [^{3}H]-RS-15385-197 binding in rat cerebral cortex membranes. Increasing concentrations of membrane protein were incubated with 0.1 nM [^{3}H]-RS-15385-197 as described in Chapter 2. The data represents a single experiment performed in triplicate.

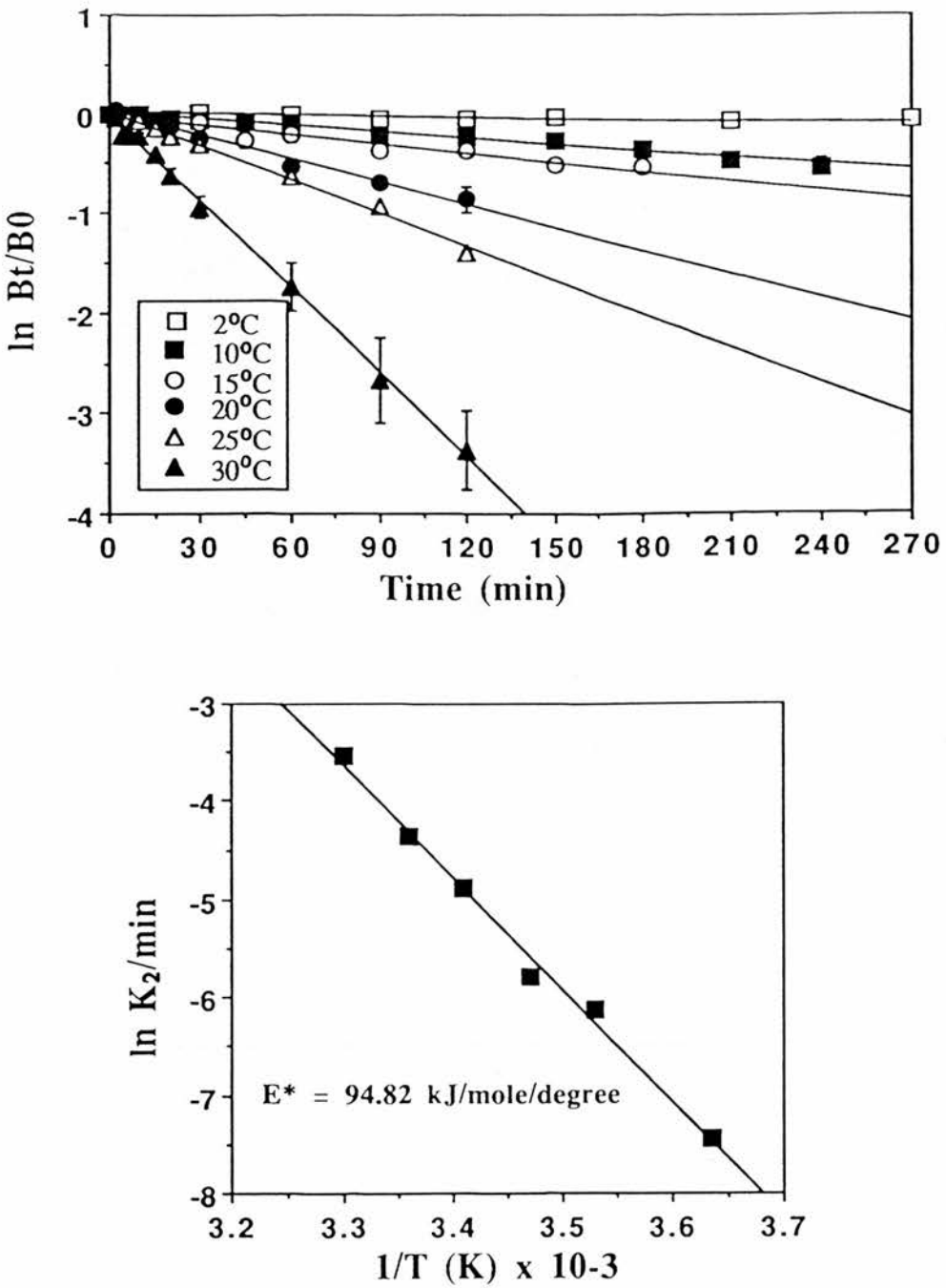


Figure 3.5. Effect of temperature on [^{3}H]-RS-15385-197 dissociation from rat cerebral cortex membranes. The timecourse was followed after the addition of 1 μM phentolamine at the temperatures indicated. The semilogarithmic plots of the mean ± s.e.mean of 3-4 experiments each performed in triplicate are shown. The bottom panel shows an Arrenhius plot of the meaned data. The plot was linear with a calculated activation energy (E*) = 94.82 kJ/mole/degree (see text for details).

Table 3.1. Inhibition of [^{3}H]-RS-15385-197 from rat cerebral cortex membranes.

compound	n	pK_i/pIC_{50}	nH
RS-15385-197	3	9.46 ± 0.04	1.01 ± 0.01
phentolamine	3	8.96 ± 0.20	0.99 ± 0.01
idazoxan	3	8.45 ± 0.23	0.92 ± 0.04
WY 26703	3	8.16 ± 0.17	0.98 ± 0.05
yohimbine	6	7.85 ± 0.08	0.91 ± 0.04
rauwolscine	3	7.68 ± 0.04	0.92 ± 0.04
clonidine	3	7.79 ± 0.22	0.77 ± 0.04
adrenaline	3	7.34 ± 0.15	0.63 ± 0.01
noradrenaline	3	7.01 ± 0.25	0.70 ± 0.06
oxymetazoline	4	7.94 ± 0.15	0.74 ± 0.01
SKF 104078	3	7.36 ± 0.02	0.94 ± 0.05
BRL 44409	3	7.69 ± 0.06	0.90 ± 0.02
imiloxan	3	7.14 ± 0.02	0.95 ± 0.04
WB 4101	3	7.52 ± 0.05	1.04 ± 0.05
prazosin	5	6.17 ± 0.18	1.04 ± 0.05
indoramin	3	5.24 ± 0.10	0.97 ± 0.05
5-HT	3	4.68 ± 0.18	1.15 ± 0.08
methysergide	3	5.94 ± 0.11	0.94 ± 0.07
ritanserin	3	7.38 ± 0.19	0.87 ± 0.01
MDL 72222	3	4.69 ± 0.12	0.96 ± 0.04
8-OH-DPAT	3	6.47 ± 0.08	0.90 ± 0.10
naphazoline	3	7.83 ± 0.15	0.80 ± 0.03
cirazoline	3	6.45 ± 0.04	0.95 ± 0.05
dopamine	3	6.00 ± 0.04	0.76 ± 0.05
haloperidol	3	5.85 ± 0.15	0.96 ± 0.08

Compounds were incubated with 0.1 nM [^{3}H]-RS-15385-197 and rat cortical membranes for 90 min at 25°C as described in Chapter two. The data are expressed as pK_i (nH = 1) or pIC_{50} (nH < 1) and represent the mean ± s.e.mean of n separate determinations on different preparations performed in duplicate.

Brown *et al.*, 1992; Redfern *et al.*, 1992).

To determine the nature of the α_2-adrenoceptor subtype labelled by [^{3}H]-RS-15385-197 in rat cerebral cortex, the affinities of several compounds reported to be selective for the α_{2A}- or α_{2B}-adrenoceptor were determined. Oxymetazoline and BRL 44409; compounds showing selectivity for the α_{2A} subtype (Bylund, 1985; Nahorski *et al.*, 1985; Young *et al.*, 1989), had high affinity. Prazosin and imiloxan have been reported to show selectivity for the α_{2B} subtype (Bylund, 1985; Nahorski *et al.*, 1985; Michel *et al.*, 1989b). As stated previously, prazosin had low affinity (pK_i = 6.17) and imiloxan had moderate affinity, however, the affinities of these compounds are intermediate between their reported affinities for α_{2A} and α_{2B} subtypes (see Section 3.4. and Discussion). SKF 104078, an antagonist with apparent post-junctional α_2-adrenoceptor selectivity (Hieble *et al.*, 1988; Akers *et al.*, 1989; Ruffolo *et al.*, 1991), had moderate affinity for [^{3}H]-RS-15385-197 binding. These results suggest that the α_2-adrenoceptor labelled by [^{3}H]-RS-15385-197 in rat cortical membranes does not fit with the presently defined α_{2A}- or α_{2B}-adrenoceptor classification and, therefore, probably represents an additional subtype.

3.2.6. Effect of GTP on [^{3}H]-RS-15385-197 binding to rat cerebral cortex membranes

As shown in Section 3.2.5. the inhibition curves for the agonists, clonidine, adrenaline, noradrenaline and oxymetazoline were shallow, whilst the displacement curves for the antagonists were closer to unity. Fitting the data to a two site model revealed that the agonists competed for [^{3}H]-RS-15385-197 binding with biphasic inhibition curves consistent with these compounds distinguishing high and low affinity agonist states of the receptor (Table 3.2. Figure 3.6.). The antagonist yohimbine displayed monophasic inhibition curves against [^{3}H]-RS-15385-197, suggesting that yohimbine had equal affinity for both states of the receptor. The percentage of sites in the high affinity state for agonists varied from between 42% to 56%. In the presence of 100 μM GTP, the agonist inhibition curves were shifted to the right and there was a steepening of the Hill slope (Figure 3.6.), such that the data was better fitted to a 1 site model. The inhibition curve to yohimbine, however, was unaffected. This suggests that two populations of sites were labelled by [^{3}H]-RS 15385-197, which had high and low affinity

Table 3.2. Effect of GTP on the inhibition of [^{3}H]-RS-15385-197 from rat cerebral cortex membranes.

competing ligand	Control pIC_{50}	Control nH	+100 μM GTP pIC_{50}	+100 μM GTP nH
adrenaline	7.37±0.09	0.66±0.02	6.32±0.04*	0.87±0.09*
high (%)	7.96±0.11 (42%)			
low (%)	6.37±0.14 (58%)			
noradrenaline	7.08±0.13	0.70±0.03	6.10±0.11*	0.81±0.03*
high (%)	7.21±0.08 (49%)			
low (%)	5.60±0.15 (51%)			
clonidine	7.80±0.10	0.75±0.03	6.95±0.03*	0.93±0.01*
high (%)	8.08±0.19 (56%)			
low (%)	6.94±0.06 (44%)			
oxymetazoline	8.16±0.09	0.71±0.01	7.39±0.06*	0.99±0.04*
high (%)	8.41±0.17 (49%)			
low (%)	7.03±0.08 (51%)			
yohimbine	7.68±0.03	1.00±0.08	7.94±0.19	0.80±0.04

The affinity values were determined from the inhibition of 0.1 nM [^{3}H]-RS-15385-197 to rat cerebral cortex membranes in the absence and presence of 100 μM GTP. The results are expressed as pIC_{50} and Hill slope for a one site fit, and for a two-site fit the pIC_{50} and % contribution of each component is given. Values shown are the mean ± s.e.mean of 3 - 4 experiments on different preparations. *Statistically significant difference ($p < 0.05$) relative to corresponding control value.

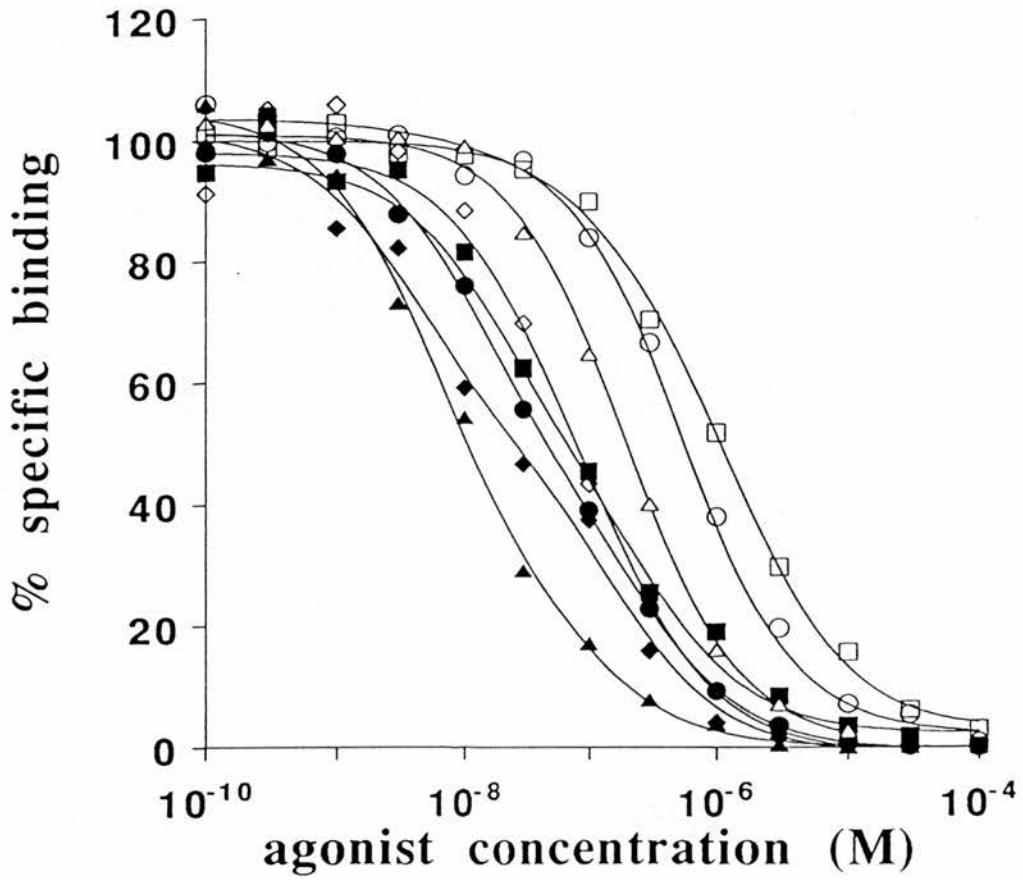


Figure 3.6. Effect of GTP (100 μM) on agonist inhibition curves in rat cerebral cortex membranes. The data represents a typical experiment performed in duplicate. Essentially similar data was obtained in at least two other experiments; ●adrenaline control, pIC_{50} = 8.14 (53%), 6.63 (47%),○adrenaline + GTP pIC_{50} = 6.36, nH = 0.96; ■ noradrenaline control pIC_{50} = 7.69 (59%), 6.12 (41%), □noradrenaline + GTP pIC_{50} = 6.20, nH = 0.85;◆oxymetazoline control pIC_{50} = 8.44 (50%), 6.78 (50%),◇oxymetazoline + GTP pIC_{50} = 7.11, nH = 0.96;▲clonidine control pIC_{50} = 8.29 (77%), 7.08 (23%), △clonidine + GTP pIC_{50} = 6.74, nH = 0.97. Meaned values in Table 3.2..

for agonists, but equal affinity for antagonists. In the presence of GTP the low affinity agonist state predominates; this results in low affinity, monophasic inhibition curves for agonists.

3.3. [^{3}H]-RS-15385-197 AUTORADIOGRAPHY IN RAT BRAIN

To maximise the specific [^{3}H]-RS-15385-197 binding to brain sections, the wash time was varied and the sections wiped from the slide for analysis by liquid scintillation spectrometry. Figure 3.7. shows that after 10 min wash, non-specific binding was reduced to ~10% of total binding. Further washing produced no appreciable decrease in non-specific binding, so in all further experiments a wash time of 10 min was employed.

The autoradiographic distribution of [^{3}H]-RS-15385-197 binding sites was studied in serial sections from rat brain. Plates 3.1 and 3.2. illustrate images of [^{3}H]-RS-15385-197 binding, and the corresponding binding density in each of 62 regions is tabulated in Table 3.3. The distribution of α_2-adrenoceptors identified with [^{3}H]-RS-15385-197 in rat brain sections revealed high density binding in areas receiving noradrenergic innervation. Particularly, the anterior olfactory nucleus, septum, striatum, entorhinal and piriform cortex, hypothalamic, amygdaloid and thalamic nuclei, central gray, locus coeruleus and the nucleus of the solitary tract. In the forebrain a low density of [^{3}H]-RS-15385-197 binding sites was found in the globus pallidus. In the midbrain, the CA-layers of the hippocampus, and in the hindbrain, the molecular layer of the cerebellum, had the lowest level of [^{3}H]-RS-15385-197 binding sites. The white matter of the corpus callosum and internal capsule exhibited the lowest density of α_2-adrenoceptors.

3.4. [^{3}H]-RS-15385 BINDING TO HUMAN PLATELET AND NEONATAL RAT LUNG MEMBRANES

3.4.1. Association and dissociation kinetics

Association of [^{3}H]-RS-15385-197 to human platelet membranes was rapid with a $t_{1/2}$ value of 12 min (Figure 3.8.). Equilibrium was reached within 60 min and the mean observed initial rate constant (K_{obs}) calculated from 3 separate experiments was 0.059 ± 0.010 min^{-1}.

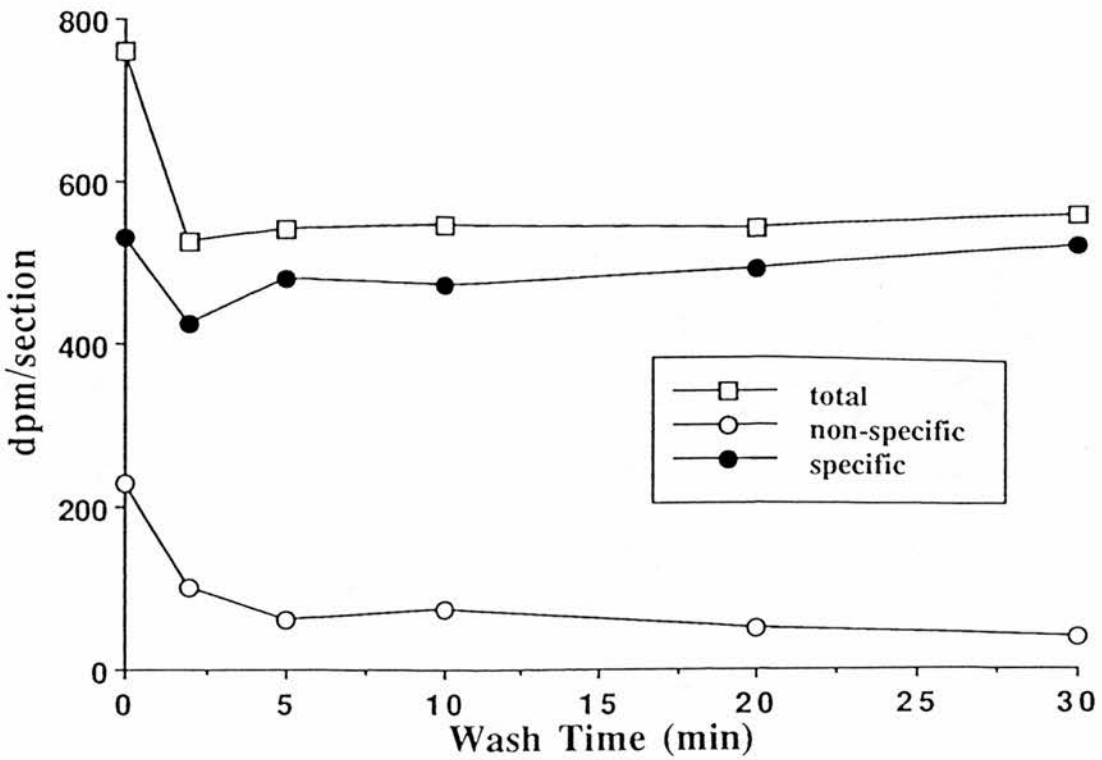


Figure 3.7. Effect of increasing washtime on [^{3}H]-RS-15385-197 dissociation from rat brain sections *in vitro*. The sections were incubated with 0.1 nM [^{3}H]-RS-15385-197 in the presence or absence of 1 μM phentolamine for 60 min as described in chapter two, and washed by immersion in ice-cold assay buffer for the times indicated. The sections were dipped in ice cold distilled water, wiped from the slide and counted for bound [^{3}H]-RS-15385-197 by scintillation spectroscopy. The results represent the means of two experiments on different rat brains each performed in triplicate.

Table 3.3. Autoradiographic localisation of α_2-adrenoceptors in rat brain with [^{3}H]-RS-15385-197.

Region	Specific Binding (amol/mm^2)	Region	Specific Binding (amol/mm^2)
Olfactory System		**Amygdaloid nucleus**	
claustrum	2646 ± 226	basolateral nucleus	4757 ± 411
olfactory tubercle	2804 ± 308	basomedial nucleus	5106 ± 433
anterior olfactory nuc.	3517 ± 249	central nucleus	4491 ± 218
Septal Area		cortical nucleus	5218 ± 497
lat. septum	3701 ± 696	medial nucleus	3344 ± 381
medial septum	3763 ± 978	**Thalamic nuclei**	
Corpus Striatum		dor. med. nucleus	2919 ± 297
nucleus accumbuns	1841 ± 214	ventrolateral nucleus	421 ± 44
striatum, head	1663 ± 130	ventromedial nucleus	3529 ± 592
striatum, body	1212 ± 109	periventricular nucleus	3058 ± 178
bed. nuc. stria term.	3086 ± 282	**Hippocampal Regions**	
globus palidus	399 ± 45	molecular layer	1686 ± 111
ventral palladium	2769 ± 342	pyramidal CA1 layer	496 ± 79
endopiriform nucleus	2781 ± 316	pyramidal CA2 layer	657 ± 67
Cortical Regions		pyramidal CA3 layer	565 ± 99
frontal cortex	1565 ± 220	**Mid-Hindbrain**	
angular insular cortex	3030 ± 318	dor. lat. geniculate body	2962 ± 288
entorhinal cortex	3578 ± 677	dor. med. geniculate body	1139 ± 219
piriform cortex	3319 ± 278	superior colliculus	2813 ± 511
cerebral cortex l. 1	2348 ± 195	inferior colliculus	1923 ± 203
cerebral cortex l. 2-3	1861 ± 126	central gray	2612 ± 304
cerebral cortex l. 4	2164 ± 161	subs. nigra, compacta	2309 ± 480
cerebral cortex l. 5-6	1121 ± 97	ventral tegmental area	2296 ± 668
Hypothalamic Regions		dorsal tegmental area	1574 ± 122
Stria terminalis	2304 ± 387	**Pons/Medulla Regions**	
medial preoptic area	2095 ± 257	locus coeruleus	2961 ± 293
supraoptic nucleus	2089 ± 446	nuc. solitary tract	4118 ± 341
dorsomedial	3319 ± 277	area postrema	2294 ± 597
ventomedial	2615 ± 508	nuc. spinal trig. nerve	1601 ± 209
ventrolateral	2277 ± 104	med. vestibular nucleus	1511 ± 319
mammilliary nucleus	3216 ± 791	facial nucleus	950 ± 86
arcuate nucleus	2240 ± 366	lateral reticular area	1050±220
White Matter		cerebellum, mol layer	442 ± 234
internal capsule	190 ± 56	cerebellum, granular layer	1287 ± 234
corpus callosum	154 ± 42		

Frozen sections (20 μm) were labelled with 0.1 nM [^{3}H]-RS-15385-197 as described in Chapter two. Quantimet analysis was carried out by Dr. N. Sharif, Institute of Chemistry, Syntex Palo Alto. Density is expressed as specific binding in amol/mm^2 and represents the mean ± s.e.mean for 3-4 rats.

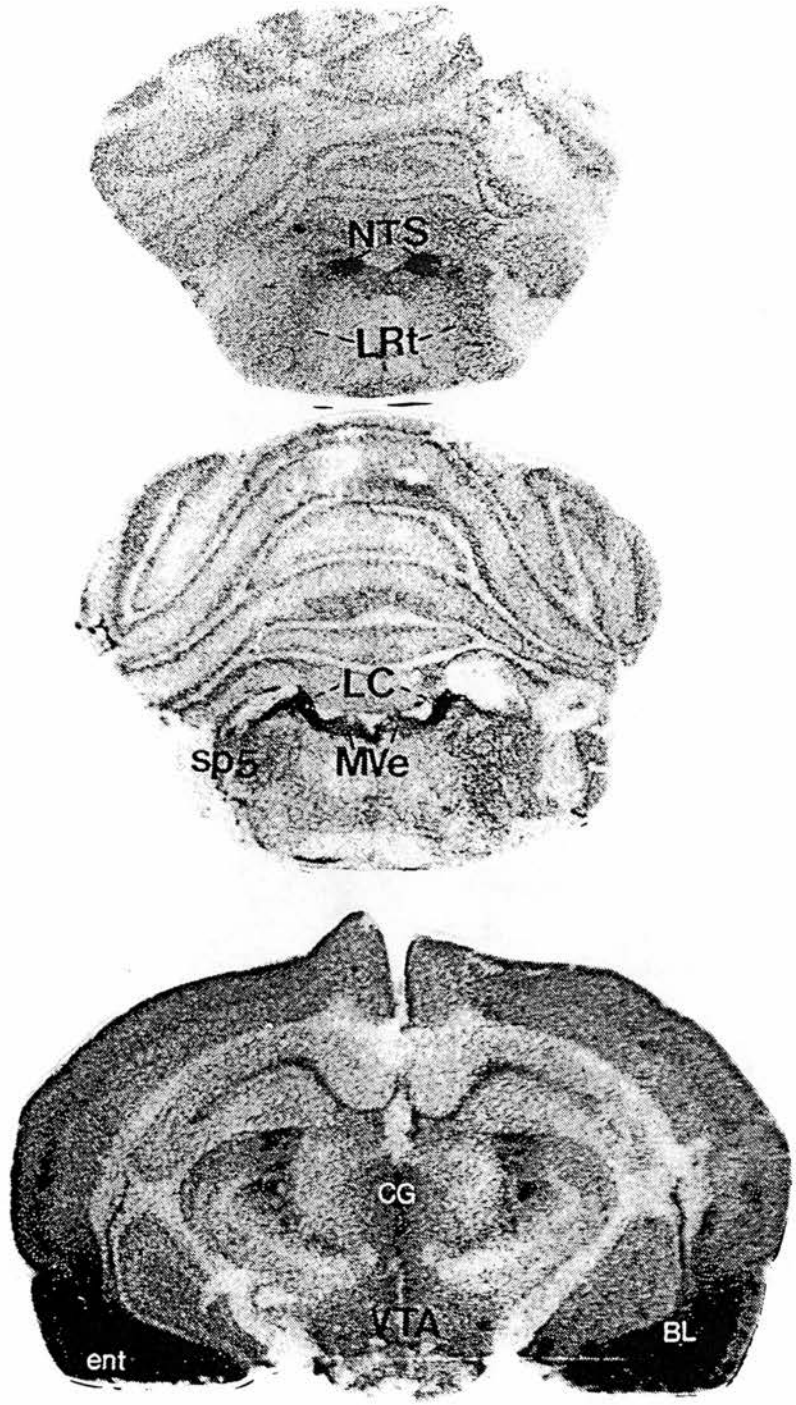


Plate 3.1. [^{3}H]-RS-15385-197 binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. BL (basolateral amygdaloid nucleus), CG (central gray), ent (entorhinal cortex), LC (locus coeruleus), LRt (lateral reticular area), MVe (medial vestibular nucleus), NTS (nucleus of the solitary tract), sp5 (spinal trigeminal nerve), VTA (ventral tegmental area).

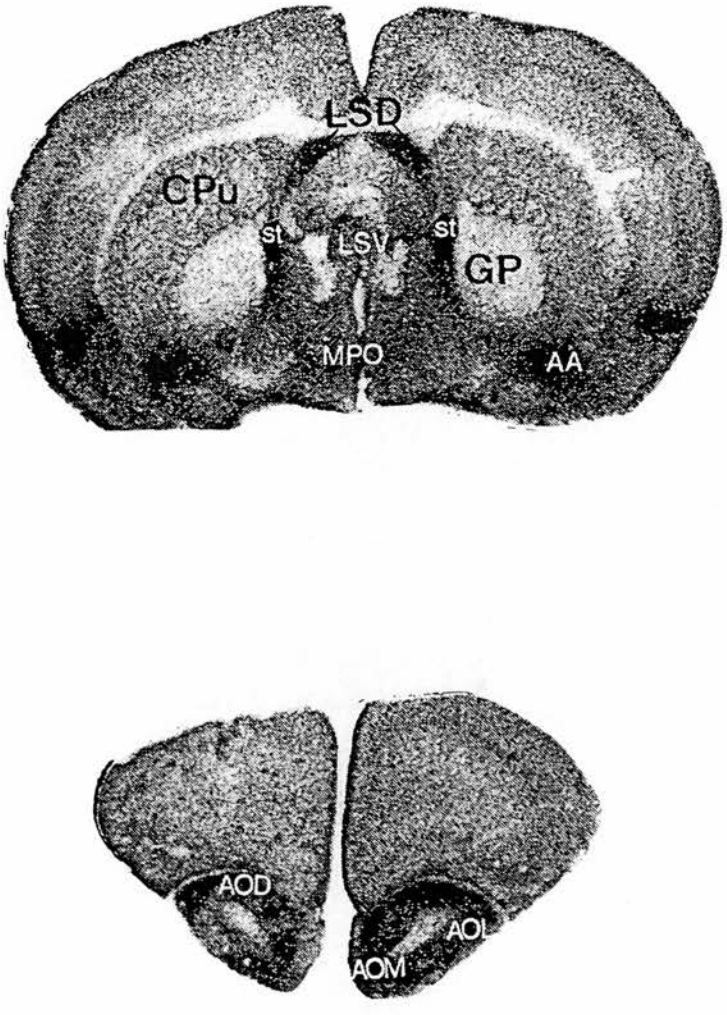


Plate 3.2. [^{3}H]-RS-15385-197 binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. AA (anterior amygdaloid area), AOD (anterior olfactory nucleus, dorsal), AOL (anterior olfactory nucleus, lateral), AOM (anterior olfactory nucleus, medial), CPu (caudate putamen), GP (globus pallidus), LSD (lateral septal nucleus, dorsal), LSV (lateral septal nucleus, ventral), MPO (medial preoptic area), st (stria terminalis).

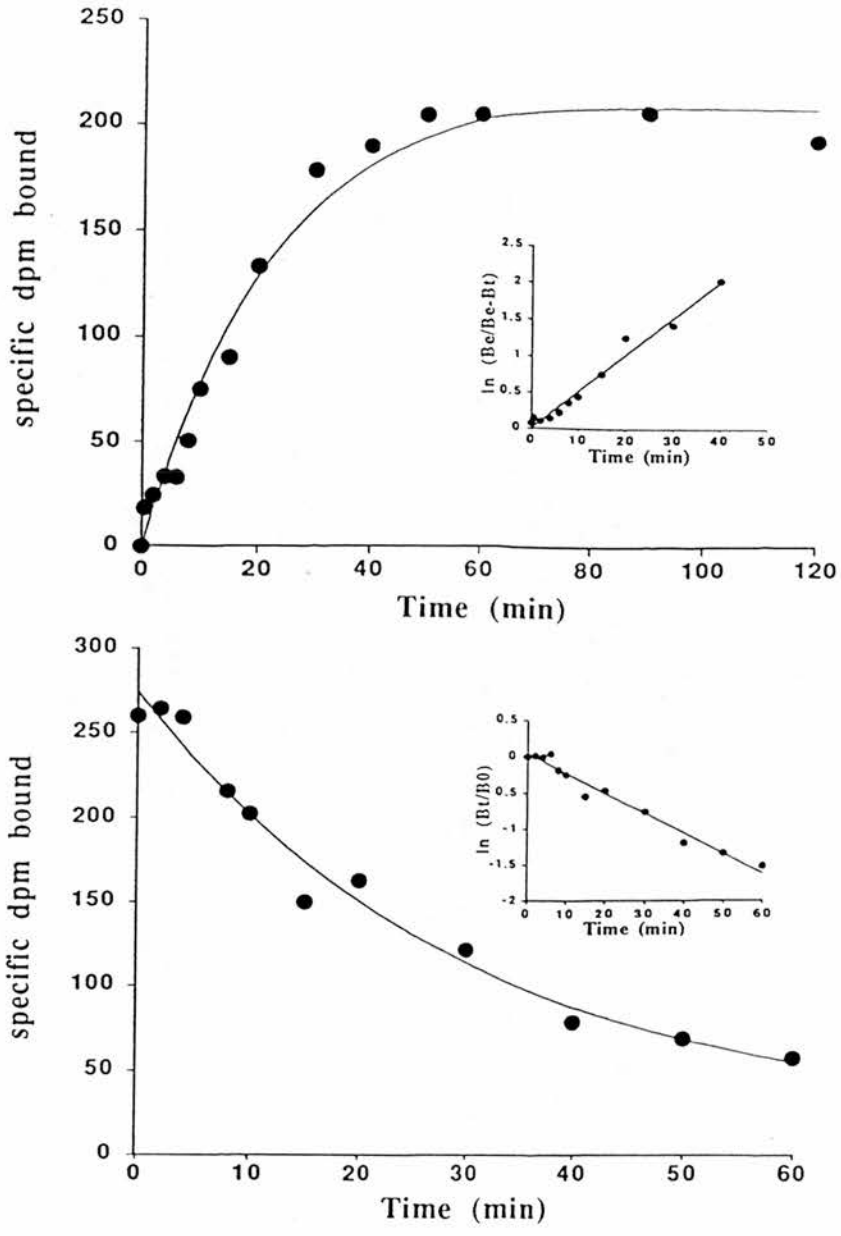


Figure 3.8. Kinetic analysis of 0.05 nM [^{3}H]-RS-15385-197 binding to human platelet membranes at 25°C. The insets show the semilogarithmic transformations of the data. The data represent a single experiment performed in triplicate. Essentially similar data was obtained in at least two other experiments on different preparations (see text for meaned values). Association (top panel) was rapid ($t_{1/2}$ = 17.7 min) and the association rate constant (K_{obs}) = 0.0391/min. Dissociation (bottom panel), initiated by the addition of 1 μM phentolamine had a dissociation rate constant (K_2) = 0.0279/min ($t_{1/2}$ = 24.8 min), giving a derived affinity K_d = 0.125 nM.

Dissociation of [^{3}H]-RS-15385-197 from the human platelet initiated by the addition of 1 μM phentolamine followed first order kinetics with a $t_{1/2}$ = 19 min (Figure 3.8.). The mean rate constant for dissociation (K_2) was 0.0357 ± 0.005 min.$^{-1}$. The equilibrium dissociation constant (K_d) determined from the equation $K_d = K_2/K_1$ where $K_1 = (K_{obs} - K_2)/[^3$H-RS-15385-197] was 0.113 ± 0.021 nM. In all further experiments with [^{3}H]-RS-15385-197 in human platelets, incubations were carried out for 60 min at 25°C.

In neonatal rat lung membranes, the $t_{1/2}$ for association was 8 min and the binding reaction reached equilibrium within 42 min (Figure 3.9.). The association rate constant K_{obs} calculated from 3 experiments was 0.082 ± 0.012 min^{-1}. Dissociation of [^{3}H]-RS-15385-197 from neonatal rat lung membranes initiated by 1 μM phentolamine had a $t_{1/2}$ = 44 min and K_2 = 0.0158 ± 0.001 min^{-1}.(Figure 3.9.). The kinetically derived K_d = 0.056 ± 0.007 nM.

3.4.2. Saturation analysis

Saturation analysis of [^{3}H]-RS-15385-197 binding revealed a single high affinity binding site in human platelet membranes (Figure 3.10.) with an affinity (K_d) of 0.13 ± 0.01 nM and B_{max} = 111.6 ± 7.4 fmoles/mg protein (n=7). In neonatal rat lung membranes (Figure 3.11.) K_d = 0.081 ± 0.01 nM, B_{max} = 174 ± 20 fmoles/mg protein (n=4).

3.4.3. Pharmacological characterisation of [^{3}H]-RS-15385-197 binding to human platelet and neonatal rat lung membranes

In order to determine the nature of the binding sites labelled by [^{3}H]-RS-15385-197 in subtype selective tissues, the affinity constants (pK_i) of several compounds against [^{3}H]-RS-15385-197 binding in the human platelet (α_{2A}) and neonatal rat lung (α_{2B}) were determined (Table 3.4). Rauwolscine and yohimbine were equipotent in the human platelet, but rauwolscine was more potent than yohimbine in the neonatal rat lung. Adrenaline was more potent than noradrenaline in the platelet, although the rank order of potency was reversed in the neonatal rat lung. Prazosin and imiloxan, compounds reported to show selectivity for the α_{2B} subtype (Michel *et al.*, 1989b), displayed high affinity in the neonatal lung but only weak affinity in the human platelet, whereas the selective α_{2A} compounds oxymetazoline and BRL

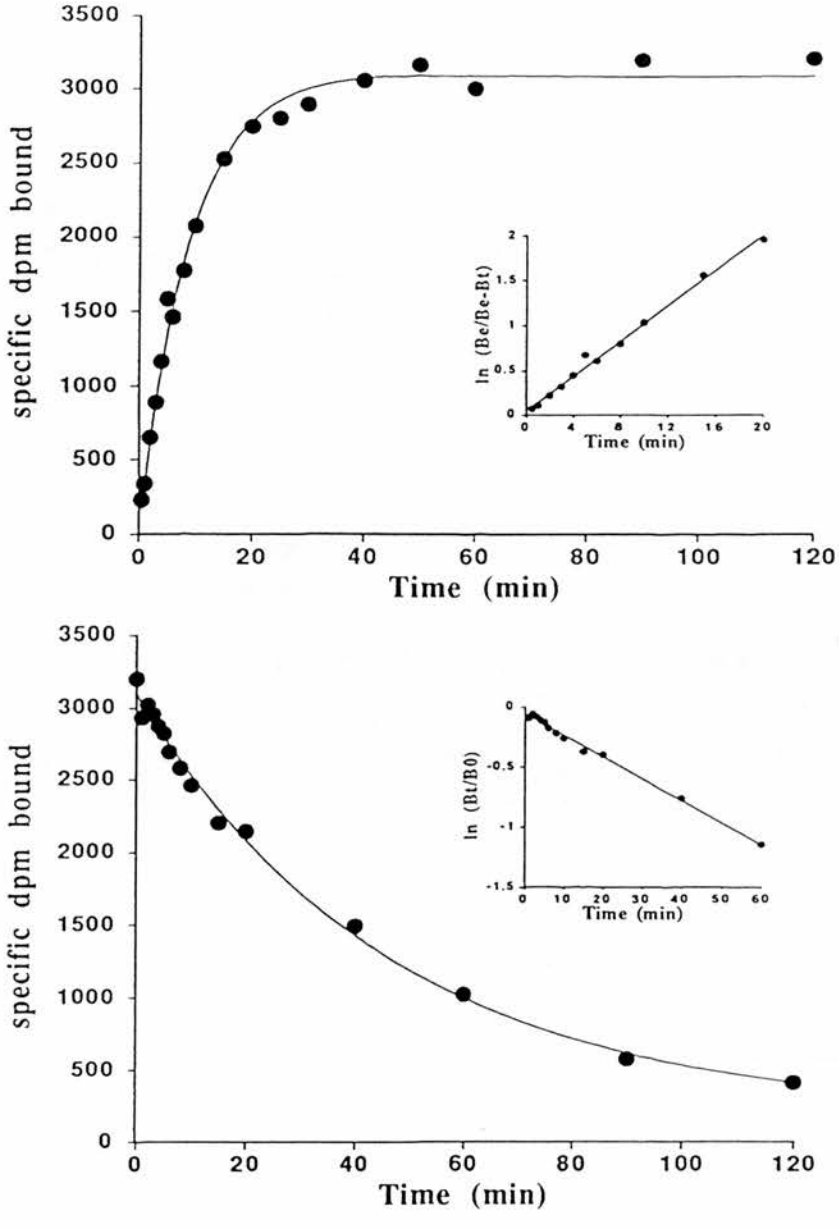


Figure 3.9. Kinetic analysis of 0.2 nM [^{3}H]-RS-15385-197 binding in neonatal rat lung membranes at 25^{o}C. The insets show the semilogarithmic transformations of the data. The data represent a single experiment performed in triplicate. Essentially similar data was obtained in at least two other experiments on different preparations (see text for meaned values). Association (top panel) was rapid ($t_{1/2}$ = 7.1 min) and the association rate constant (K_{obs}) = 0.097/min. Dissociation (bottom panel), initiated by the addition of 1 μM phentolamine had a dissociation rate constant (K_2) = 0.0181/min. ($t_{1/2}$ = 38.3 min), giving a derived affinity K_d = 0.05 nM.

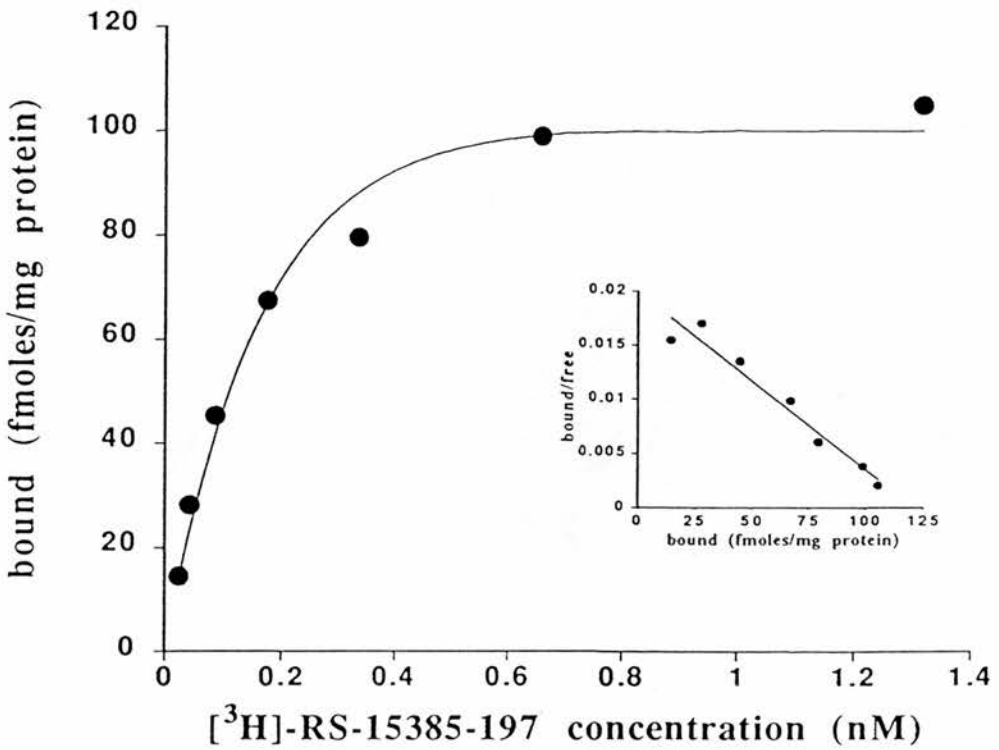


Figure 3.10. Saturation analysis of [^{3}H]-RS-15385-197 binding in human platelet membranes. The inset shows the Scatchard transformation of the data. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in four other experiments on different preparations (see text for meaned values). The equilibrium binding constants K_d and B_{max} = 0.12 nM and 112 fmoles/mg protein respectively.

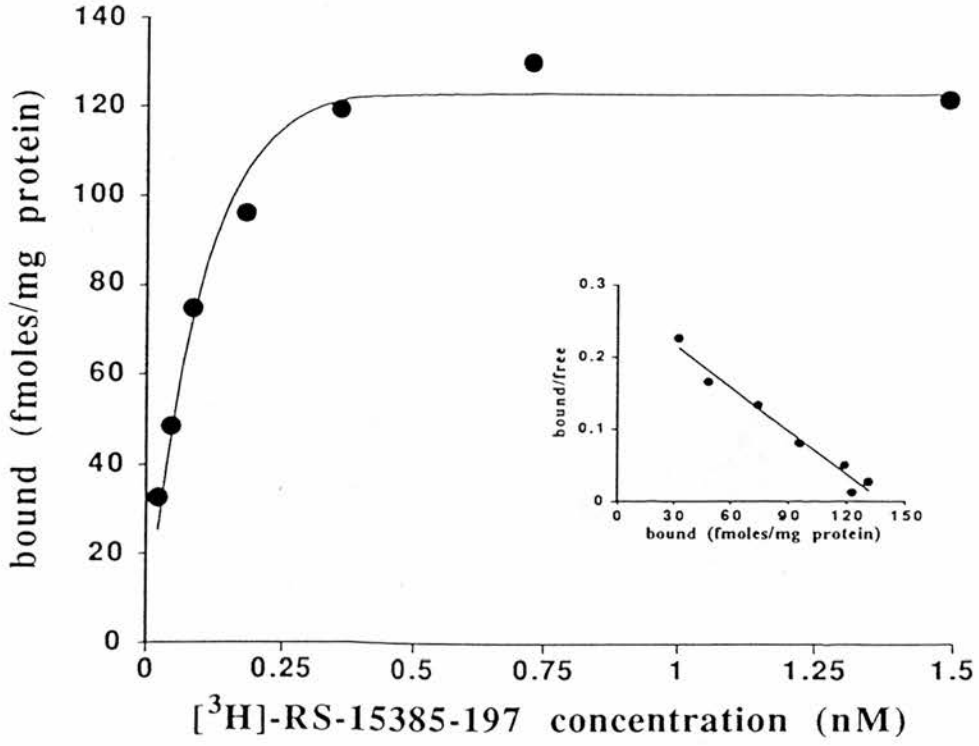


Figure 3.11. Saturation analysis of [^{3}H]-RS-15385-197 binding in neonatal rat lung membranes. The inset shows the Scatchard transformation of the data. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in three other experiments on different preparations (see text for meaned values) The equilibrium binding constants K_d and B_{max} = 0.07 nM and 157 fmoles/mg protein.

Table 3.4. Inhibition of [^{3}H]-15385-197 from human platelet and neonatal rat lung membranes.

	human platelet			rat neonatal lung		
	n	pK_i/pIC_{50}	nH	n	pK_i/pIC_{50}	nH
RS-15385-197	4	9.51±0.10	0.99±0.01	3	9.58±0.31	1.01±0.02
yohimbine	4	8.98±0.10	0.89±0.04	3	8.16±0.08	0.99±0.01
rauwolscine	4	8.91±0.12	1.04±0.09	3	8.62±0.07	0.85±0.02
phentolamine	3	8.70±0.22	1.25±0.35	3	8.11±0.08	1.01±0.08
idazoxan	4	8.22±0.03	1.01±0.04	3	7.46±0.08	0.99±0.01
adrenaline	6	7.55±0.11	0.81±0.07	5	7.08±0.04	0.75±0.05
noradrenaline	4	6.93±0.04	0.75±0.01	3	7.25±0.03	0.68±0.04
BHT 920	3	7.17±0.03	1.05±0.06	3	6.89±0.04	0.83±0.02
clonidine	3	7.47±0.06	1.01±0.04	3	7.30±0.01	1.05±0.04
UK 14304	3	7.87±0.04	0.97±0.13	3	7.04±0.10	0.79±0.05
prazosin	4	6.13±0.08	0.94±0.15	4	7.49±0.12	1.04±0.09
imiloxan	3	6.30±0.04	1.30±0.06	3	7.36±0.02	1.03±0.04
oxymetazoline	4	8.49±0.01	0.90±0.06	3	6.91±0.04	0.73±0.07
BRL 44409	3	7.77±0.05	0.98±0.08	3	6.38±0.05	1.07±0.07

The affinity values were obtained from the inhibition of 0.1 nM [^{3}H]-RS-15385-197 from human platelet and neonatal rat lung membranes as described in Chapter two. The values represent the pK_i (nH = 1) or pIC_{50} (nH < 1). Each value represents the mean ± s.e.mean of n determinations on different preparations.

44409 (Young *et al.*, 1989) showed higher affinity in the human platelet. These results suggest that [^{3}H]-RS-15385-197 labelled α_{2A} and α_{2B}-adrenoceptor subtypes in human platelet and rat neonatal lung membranes with high affinity, but was itself not able to distinguish between subtypes.

3.4.4. Effect of $MgCl_2$, Gpp(NH)p and prazosin on [^{3}H]-RS-15385-197 binding to neonatal rat lung and human platelet membranes

The effects of 100 μM Gpp(NH)p, a stable GTP analogue, and 5 mM $MgCl_2$ are shown in Table 3.5. As in the rat cortex (Section 3.2.6.), the inhibition curves for the agonist adrenaline in the human platelet and the neonatal rat lung was shallow and could be fitted to a 2 site model, consistent with adrenaline distinguishing high and low affinity agonist states of the receptor. In the presence of 5 mM $MgCl_2$ the curves became more markedly biphasic, this was manifest by an increased affinity for the higher affinity component of agonist binding, without an apparent change in the % of sites in the high affinity agonist state. Antagonist inhibition curves were unaffected by $MgCl_2$ in both tissues. In the presence of 100 μM Gpp(NH)p, there was a decrease in affinity and a steepening of the Hill slope for the agonists in the neonatal rat lung such that they gave monophasic inhibition curves. Gpp(NH)p had no significant effect on the concentration response curves to yohimbine or prazosin in either tissue. In the human platelet, although Gpp(NH)p had no significant effect on control inhibition curves to adrenaline, there was a significant reduction in affinity when compared to the inhibition in the presence of $MgCl_2$. Therefore, as in the rat cortex, the α_2-adrenoceptor labelled by [^{3}H]-RS-15385-197 in the human platelet and the neonatal rat lung showed high and low affinity states for agonists. The proportion of receptors in the high affinity state could be modified by the GTP analogue Gpp(NH)p. The effect of the guanyl nucleotide in the human platelet was less marked and only evident when compared to agonist responses in the presence of $MgCl_2$.

Given that prazosin has historically been the drug of choice to define α_{2B}-adrenoceptors, it was decided to investigate the mode of interaction of prazosin with the α_{2B} receptor on neonatal rat lung membranes, its effects on [^{3}H]-RS-15385-197 saturation curves and dissociation kinetics were examined. At 30 nM prazosin (a

Table 3.5. Effect of Gpp(NH)p and $MgCl_2$ on the inhibition of [^{3}H]-RS-15385-197 from neonatal rat lung and human platelet membranes.

	1 site fit		2 site fit			
	pIC_{50}	nH	pIC_{50}	(%)	pIC_{50}	(%)
neonate lung						
adrenaline						
control	7.12±0.09	0.64±0.04	7.36±0.20	(46±8)	6.07±0.16	(54±8)
$MgCl_2$	7.23±0.03	0.56±0.01	8.25±0.08	(35±4)	6.41±0.09	(65±4)
Gpp(NH)p	6.60±0.09*	0.79±0.06*				
yohimbine						
control	8.21±0.05	0.94±0.02				
$MgCl_2$	8.02±0.15	0.96±0.01				
Gpp(NH)p	8.01±0.08	1.01±0.02				
prazosin						
control	7.39±0.11	0.96±0.02				
$MgCl_2$	7.26±0.20	0.96±0.08				
Gpp(NH)p	7.24±0.13	1.04±0.01				
platelet						
adrenaline						
control	7.36±0.01	0.80±0.05	7.70±0.37	(71±12)	6.03±0.43	(29±12)
$MgCl_2$	8.00±0.06*	0.58±0.04	8.09±0.02	(65±2)	6.08±0.12	(35±2)
Gpp(NH)p	7.34±0.21$	0.73±0.05				
yohimbine						
control	8.98±0.10	0.89±0.04				
$MgCl_2$	8.72±0.16	0.95±0.07				
Gpp(NH)p	8.52±0.29	0.97±0.06				
prazosin						
control	6.19±0.08	1.02±0.04				
$MgCl_2$	5.76±0.06*	0.82±0.15				
Gpp(NH)p	5.87±0.12	0.83±0.08				

The affinity values were determined from the inhibition of specific binding of 0.1 nM [^{3}H]-RS-15385-197 in the absence and presence of 5 mM $MgCl_2$ or 100 µM Gpp(NH)p as shown. The results are expressed as pIC_{50} and Hill slope (nH) for a one site fit, and pIC_{50} and % contribution for a 2 site fit where appropriate. Values shown are the mean ± s.e.mean of 3 experiments. *Statistically significant difference ($p < 0.05$) relative to corresponding control; $ relative to $MgCl_2$

concentration around the pK_i for prazosin) there was a decrease in affinity of [3H]-RS-15385-197 binding with no change in B_{max} (Figure 3.12.); control K_d = 0.12 ± 0.02 nM, B_{max} = 178 ± 11 fmoles/mg protein; 30 nM prazosin K_d = 1.14 ± 0.23, B_{max} = 172 ± 7 fmoles/mg protein (n=3, $p<0.01$ for an effect on K_d). This suggests a competitive type of interaction. In dissociation experiments (Figure 3.13.), a 10 fold higher concentration of prazosin than the K_d (300 nM) had no effect on phentolamine induced dissociation (control K_2 = 0.0158 min^{-1}, prazosin K_2 = 0.0148 min^{-1}, n=3). This further supports a competitive interaction of prazosin for α_{2B} binding sites in neonatal rat lung membranes.

A correlation of affinities for the sites labelled with [3H]-RS-15385-197 in different tissues is shown in Figures 3.14.a-c. There was a poor correlation between affinity estimates obtained in the rat cerebral cortex and neonatal rat lung (r = 0.296, Figure 3.14.a.), and in the neonatal rat lung and human platelet (r = 0.384, Figure 3.14.b.). The correlation between the human platelet and the rat cerebral cortex was better (r = 0.667, Figure 3.14.c.), but yohimbine and rauwolscine had greater than 10 fold selectivity for the human platelet, whereas imiloxan was 7 fold selective for the rat cerebral cortex. This suggests that the α_2-adrenoceptors in the rat cortex, human platelet and neonatal rat lung probably represent 3 distinct subtypes of the α_2-adrenoceptor.

3.5. [3H]-ADRENALINE BINDING

[3H]-Adrenaline binding in human platelet and rat neonatal lung membranes was carried out in a 50 mM Tris HCl buffer; pH 7.4 containing 5 mM $MgCl_2$, 0.3 mM ascorbate, 0.1 mM catechol and 0.8 mM dithiothreitol. This buffer system was shown by Sénard *et al.* (1988) to sufficiently protect (-)adrenaline from degradation at 25°C for at least 2 hours. Monoamine oxidase inhibitors pargyline and iproniazid were not included as they were shown to directly inhibit [3H]-adrenaline binding to HT29 cell membranes. HT29 cells are a human carcinoma cell line containing an homogeneous population of α_{2A}-adrenoceptors (Bylund *et al.*, 1988). Garcia-Sevilla & Fuster (1986) showed previously that [3H]-adrenaline preferentially labelled α_2-adrenoceptors on human platelet and that propranolol had very low affinity (pK_i = 10 μM), however Figure 3.15. shows that in neonatal rat

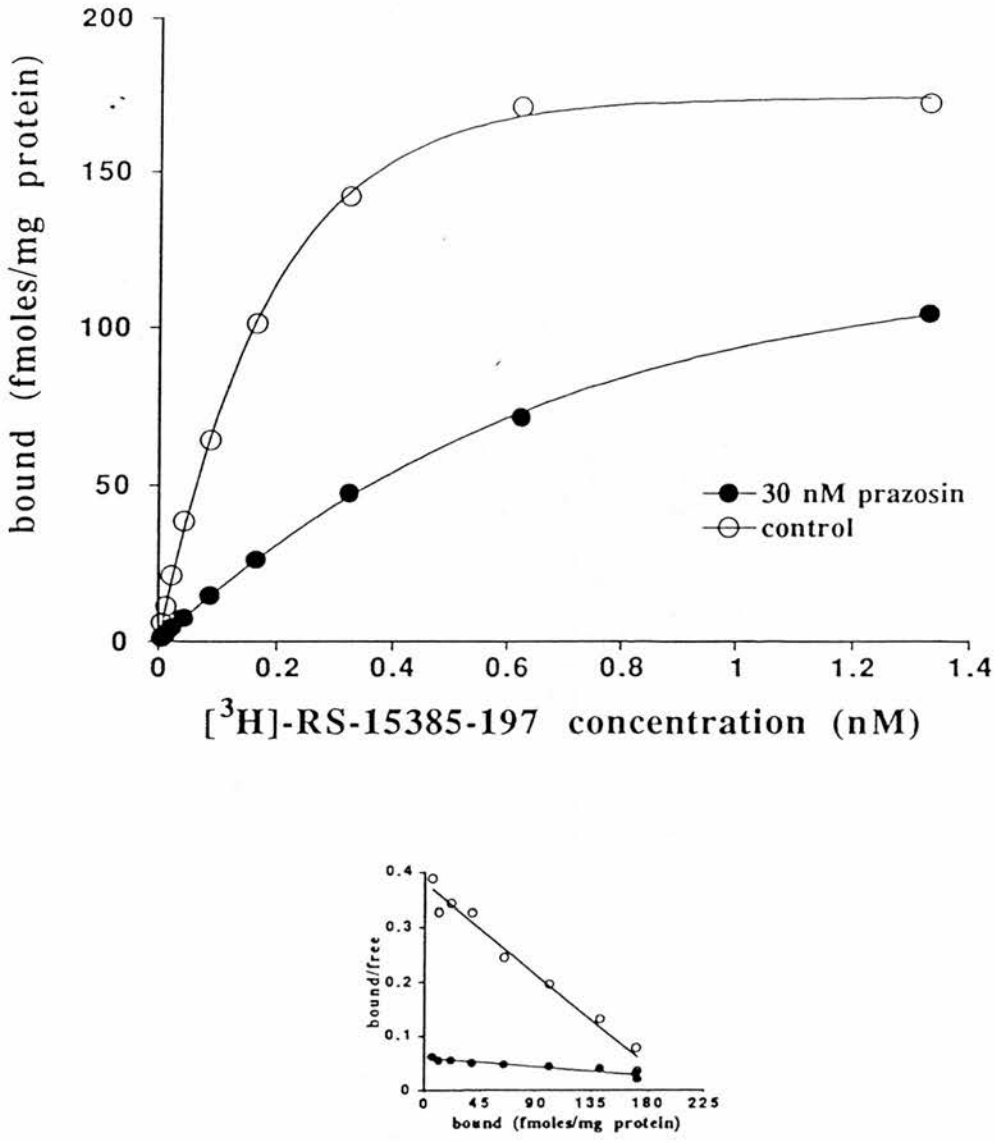


Figure 3.12. Effect of 30 nM prazosin on [3H]-RS-15385-197 saturation curves in neonatal rat lung membranes. The results represent a single experiment performed in triplicate. Essentially similar data was obtained in at least two other experiments on different preparations (see text for meaned values). The bottom panel shows the Scatchard transformation of the data. Control K_d = 0.14 nM, B_{max} = 203.9 fmoles/mg protein; prazosin K_d = 0.76 nM, B_{max} = 163.1 fmoles/mg protein.

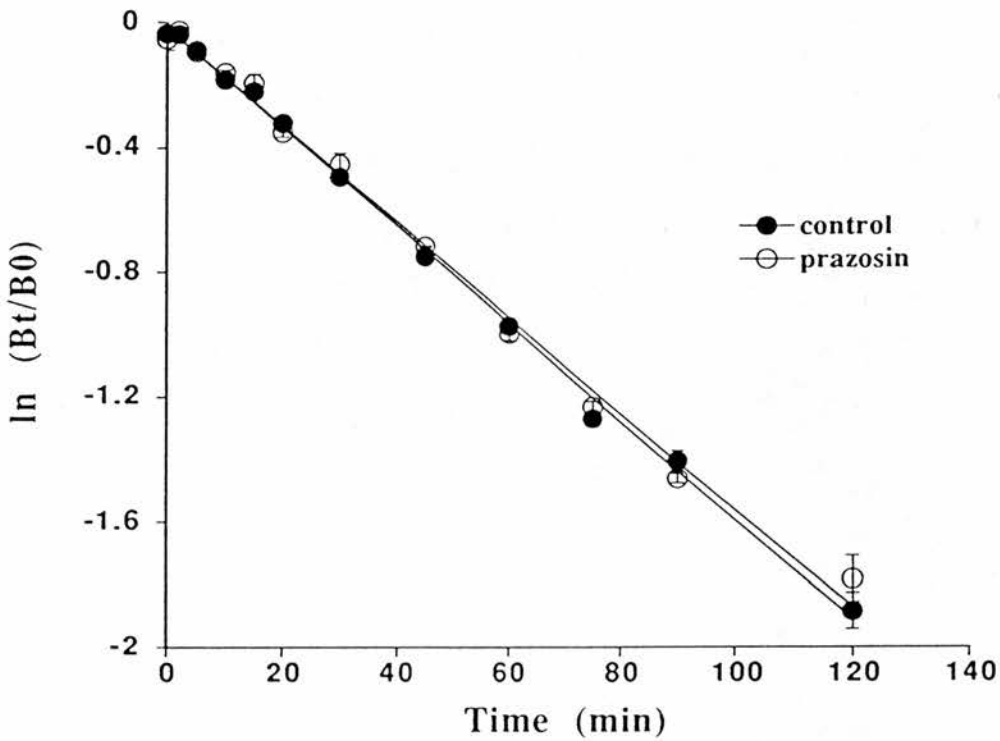


Figure 3.13. Effect of 300 nM prazosin on [^{3}H]-RS-15385-197 dissociation from rat neonatal lung membranes. The timecourse of dissociation was followed after the addition of 1 μM phentolamine (control) or 1 μM phentolamine plus 300 nM prazosin (prazosin). The results represent the mean ± s.e.mean of 3 experiments performed in triplicate. Calculated values given in text.

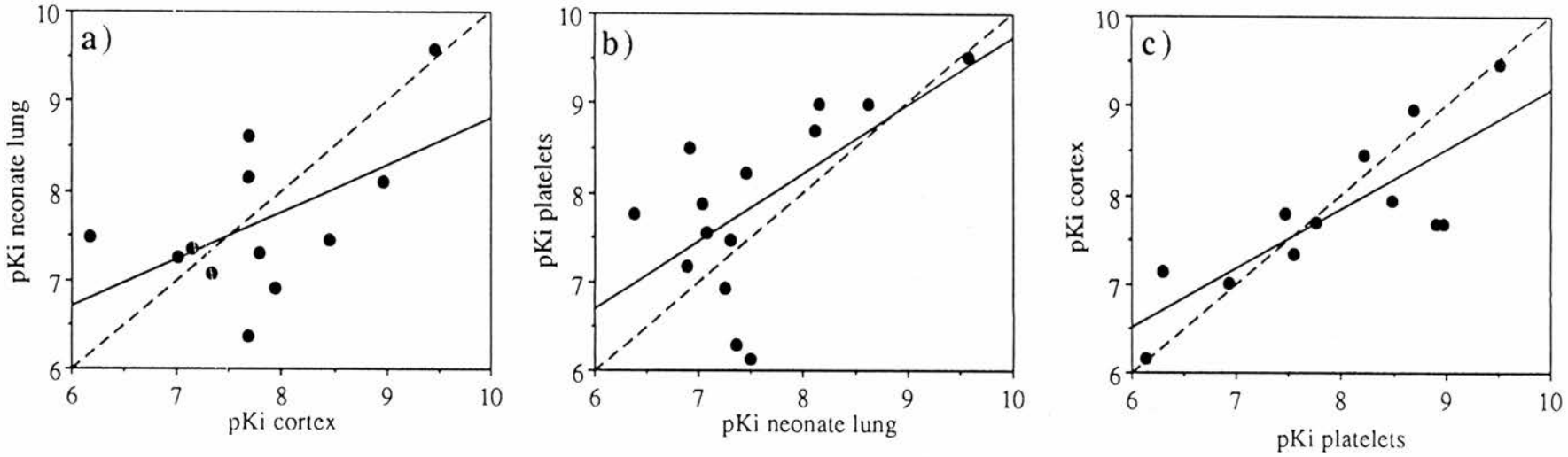


Figure 3.14. Correlations of binding affinity (pK_i) of several compounds for [^{3}H]-RS-15385-197 binding in rat cerebral cortex, human platelet and neonatal rat lung membranes. The data is taken from Tables 3.1. and 3.4. The solid line represents the line of best fit and the dotted line represents the line of identity. a) rat cerebral cortex vs neonatal rat lung (slope = 0.53, r = 0.296); b) rat neonatal lung vs human platelet (slope = 0.76, r = 0.384); c) human platelet vs rat cerebral cortex (slope = 0.67, r = 0.667).

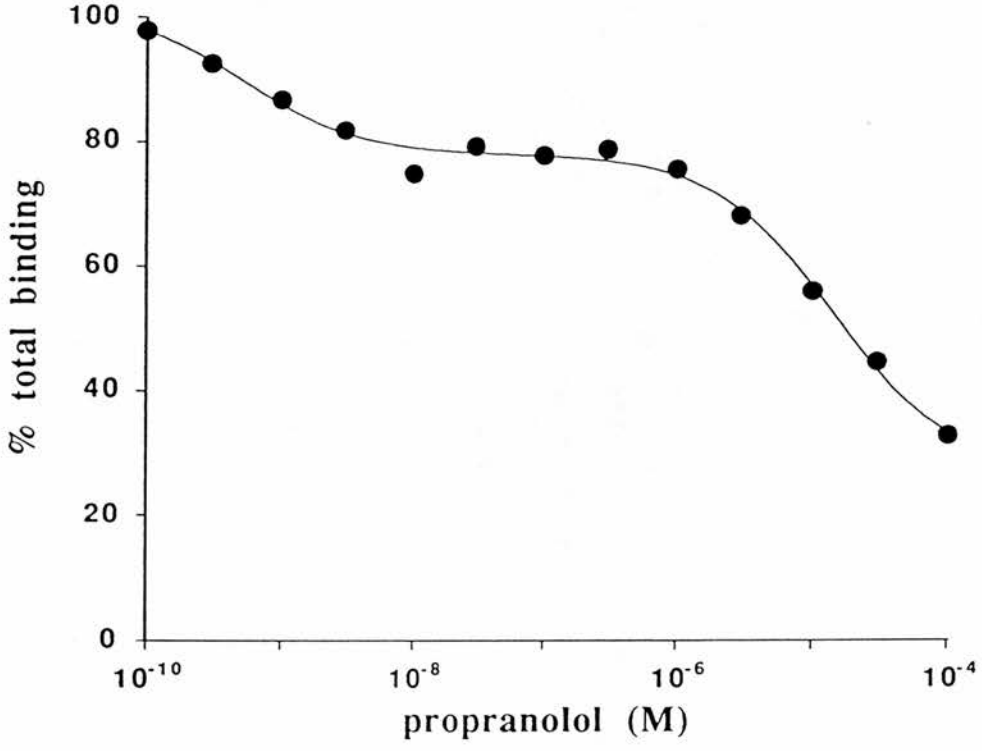


Figure 3.15. Inhibition of [^{3}H]-adrenaline binding to neonatal rat lung membranes by propranolol. The data represents the mean of 2 experiments performed in different preparations each performed in duplicate. The inhibition curve was better fitted to a two-site model and showed high affinity ($pK_i = 9.30$) for 24% of the binding.

lung membranes propranolol displaced 24 % of the [3H]-adrenaline binding with high affinity (pK_i = 9.30). Subsequent experiments in neonatal rat lung membranes were carried out in the presence of 10 nM propranolol to prevent binding to β-adrenoceptors.

3.5.1. Kinetic and saturation analysis of [3H]-adrenaline binding to human platelet and neonatal rat lung membranes

Kinetic analysis revealed that [3H]-adrenaline binding to neonatal rat lung membranes was rapid and fully reversible on the addition of 10 μM noradrenaline (Figure 3.16.) with K_{obs} = 0.0324 ± 0.003 min^{-1}, and K_2 = 0.021 ± 0.004 min^{-1}, giving a kinetically derived K_d = 5.03 ± 2.99 nM (n=3). In the human platelet (Figure 3.17.) equilibrium was reached after 60 min with K_{obs} for association = 0.215 min^{-1}, and K_2 = 0.171 min^{-1}. Figure 3.17. shows that the data were possibly better described by a 2 site model but due to the limited availability of platelets, kinetic experiments were performed only once. Saturation analysis (Figure 3.18.) revealed that [3H]-adrenaline labelled a single high affinity site in the neonatal rat lung (K_d = 3.04 ± 0.44 nM, B_{max} = 77.7 ± 11 fmoles/mg protein, n=7) and human platelet (K_d = 2.62 ± 0.56 nM, B_{max} = 95.1 ± 17 fmoles/mg protein, n=3). Therefore, the percentage of sites labelled by the agonist, [3H]-adrenaline compared to that labelled by the antagonist, [3H]-RS-15385-197, was 44% in the neonatal rat lung and 85% in the human platelet.

3.5.2. Pharmacological characterisation of [3H]-adrenaline binding to human platelet and neonatal rat lung membranes

To determine the nature of the site labelled by [3H]-adrenaline in subtype specific tissues, the affinity of a number of competing agonist and antagonist compounds were studied in human platelet and neonatal rat lung membranes (Table 3.6.). pK_i Determinations in platelet membranes were very similar to those determinations in rat neonatal lung membranes. The agonists adrenaline, noradrenaline, oxymetazoline and BHT 920 showed high affinity for [3H]-adrenaline binding in both tissues. The α_2-antagonists yohimbine, rauwolscine, idazoxan and phentolamine displayed moderate to high affinity in both tissues, whereas prazosin had very low affinity. In Figure 3.19. a correlation between affinity estimates obtained in the two preparations are presented. There was a good correlation between affinities obtained

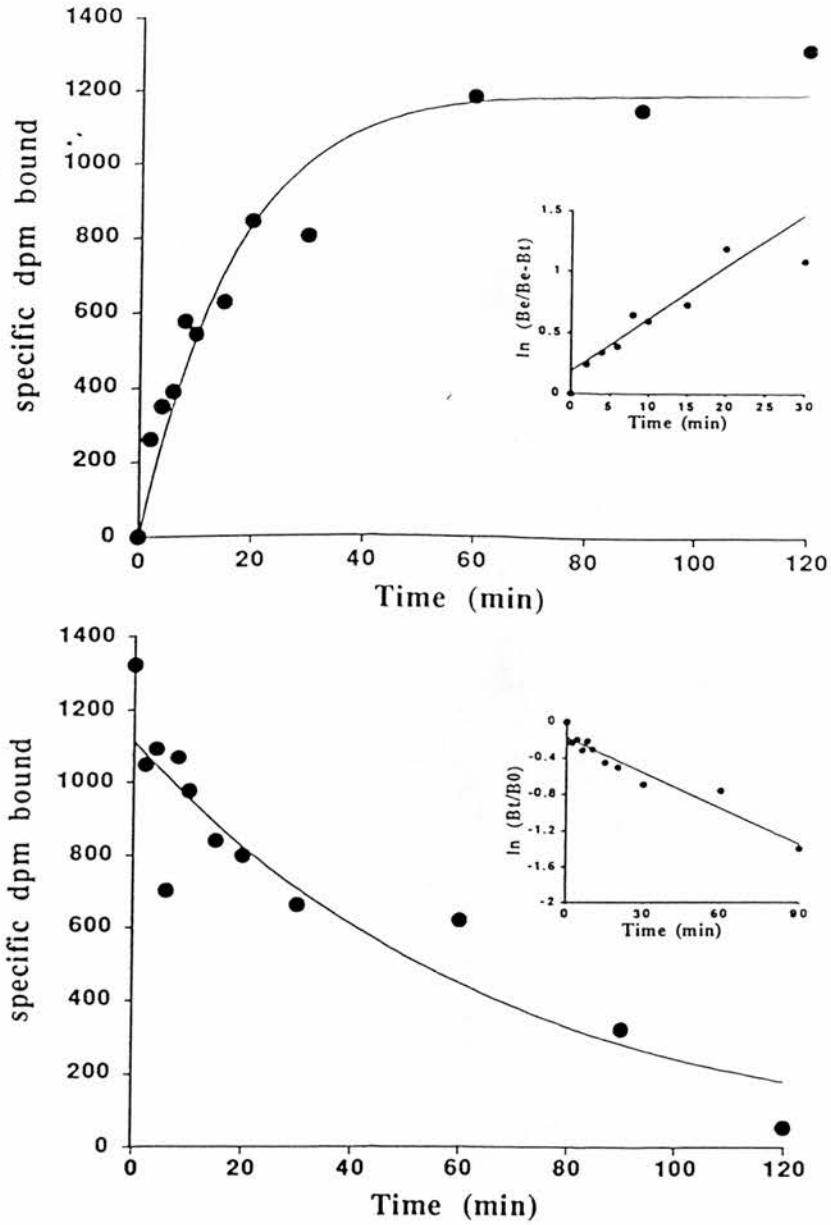


Figure 3.16. Kinetic analysis of 3 nM [^{3}H]-adrenaline binding to neonatal rat lung membranes at 25^{o}C. The insets show the semilogarithmic transformations of the data. The data represent a single experiment performed in triplicate. Essentially similar data was obtained in at least two other experiments on different preparations (see text for meaned values). Association (top panel) was rapid ($t_{1/2}$ = 11.8 min) and the association rate constant (K_{obs}) = 0.0585/min. Dissociation (bottom panel), initiated by the addition of 10 μM noradrenaline had a dissociation rate constant (K_2) = 0.0251/min. ($t_{1/2}$ = 27.6 min), giving a derived affinity K_d = 2.25 nM.

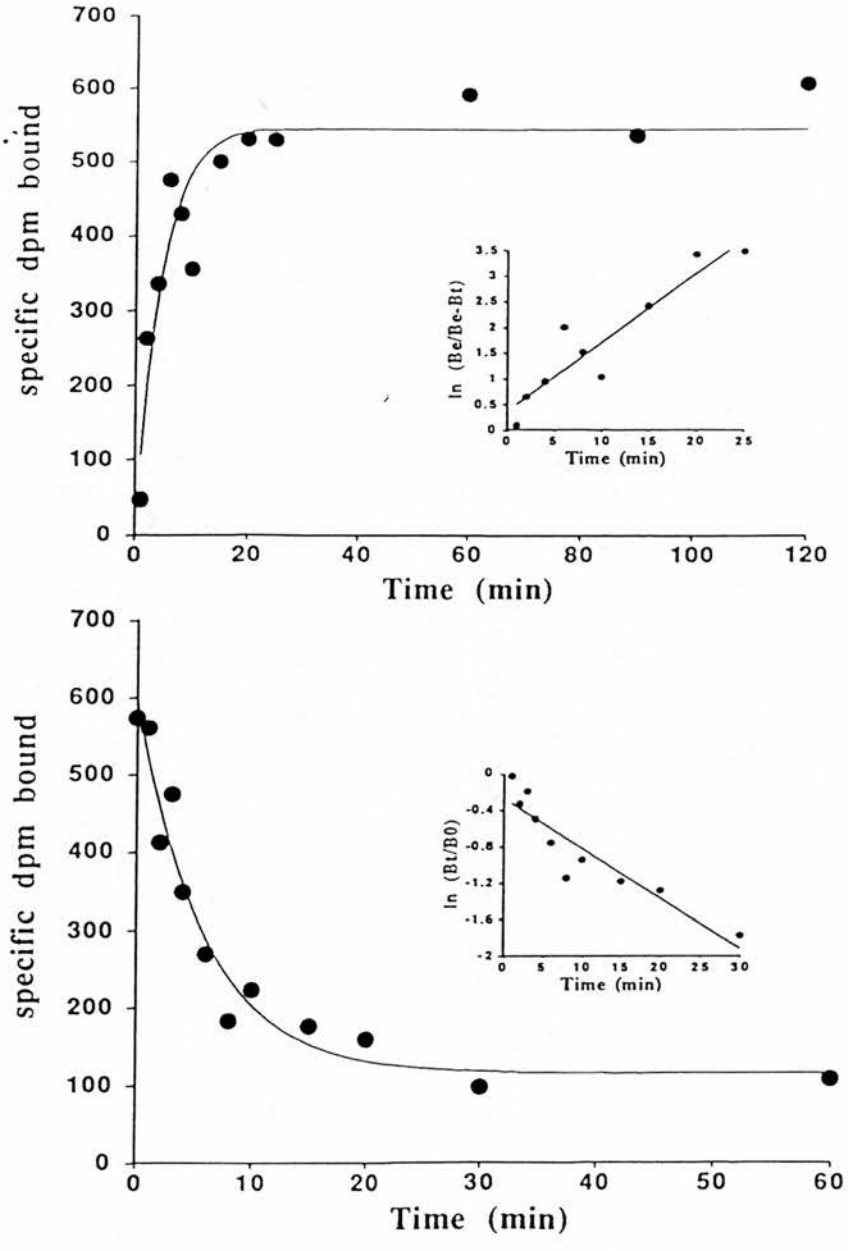


Figure 3.17. Kinetic analysis of 1 nM [^{3}H]-adrenaline binding to human platelet membranes. The insets show the semilogarithmic transformations of the data. The data represent a single experiment performed in triplicate. Association (top panel) was rapid ($t_{1/2}$ = 3.22 min) and the association rate constant (K_{obs}) = 0.2152/min. Dissociation (bottom panel), initiated by the addition of 10 μM noradrenaline had a dissociation rate constant (K_2) = 0.171/min ($t_{1/2}$ = 4.05 min), giving a derived affinity K_d = 3.87 nM.

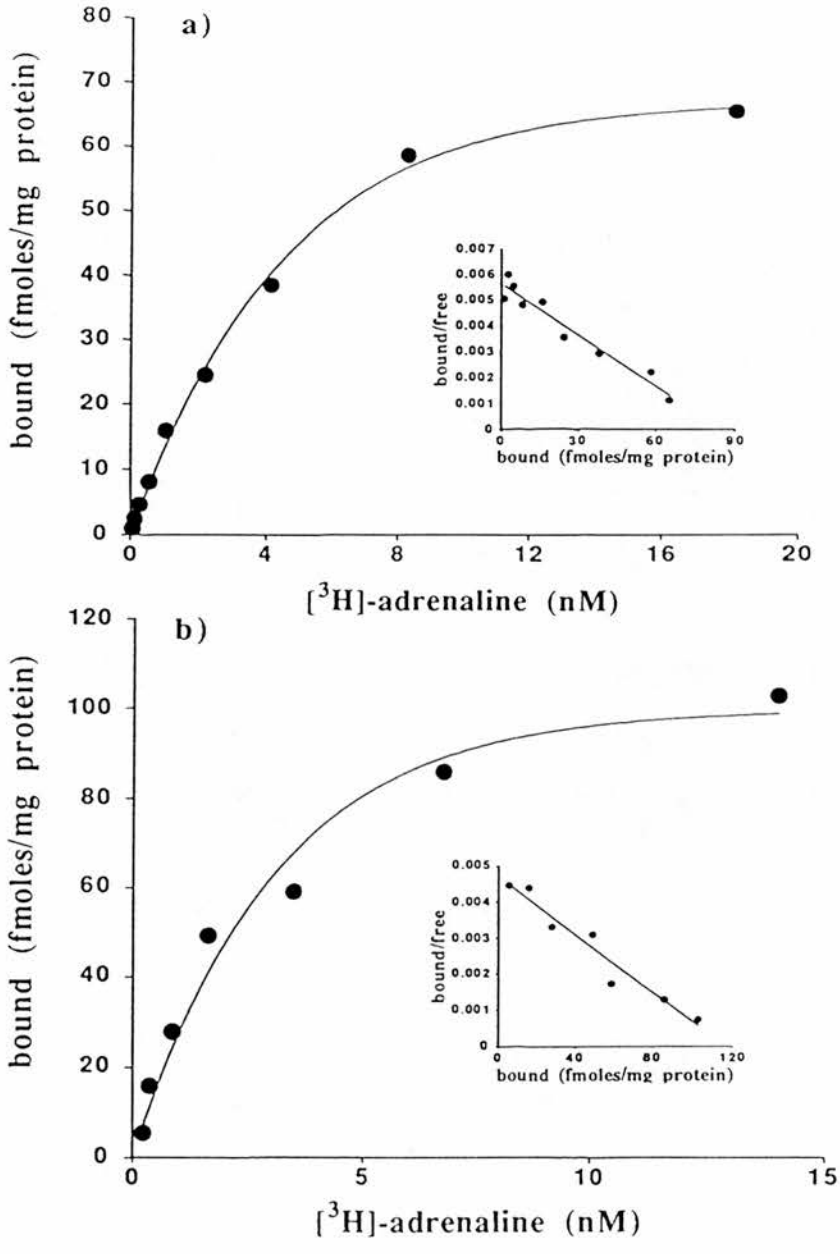


Figure 3.18. Saturation analysis of [3H]-adrenaline binding to neonatal rat lung and human platelet membranes. The data represents a typical experiment performed in triplicate. Essentially similar data was obtained in at least two other experiments on different preparations (see text for meaned values). The equilibrium binding parameters K_d and B_{max} were 2.75 nM and 82 fmoles/mg protein respectively in the neonatal rat lung (a) and 2.68 nM and 112 fmoles/mg protein respectively in the human platelet (b).

Table 3.6. Affinity values for a number of competing agonists and antagonists for [^{3}H]-adrenaline binding in human platelet and neonatal rat lung membranes.

	neonatal rat lung			human platelets		
	n	pK_i/pIC_{50}	nH	n	pK_i/pIC_{50}	nH
yohimbine	5	7.37±0.20	0.98±0.20	3	8.20±0.28	0.78±0.11
rauwolscine	3	7.89±0.12	0.80±0.07	2	8.36	0.79
phentolamine	3	7.84±0.11	0.93±0.07	2	7.99	1.20
idazoxan	3	7.58±0.06	0.98±0.02	2	7.68	0.89
imiloxan	3	5.61±0.12	0.81±0.10	3	5.68±0.14	0.81±0.09
WB 4101	3	6.78±0.01	0.89±0.11		nd	nd
prazosin	4	5.68±0.16	0.99±0.01	3	5.12±0.14	1.09±0.09
noradrenaline	4	8.42±0.14	0.97±0.03	3	8.15±0.32	1.18±0.31
adrenaline	4	8.41±0.16	1.00±0.01	3	8.56±0.15	0.89±0.19
UK 14304	3	7.83±0.17	0.82±0.13	2	8.21	0.94
clonidine	3	7.45±0.23	0.79±0.11	2	7.92	0.88
BHT 920	3	7.97±0.11	0.88±0.09	2	7.79	0.79
oxymetazoline	3	8.22±0.09	0.90±0.10	3	8.33±0.13	0.78±0.17
BRL 44409	3	6.67±0.24	1.06±0.06	3	7.19±0.19	0.77±0.07

The affinities are determined from competition experiments with [^{3}H]-adrenaline as described in Chapter two. The values represent the pK_i (nH = 1) or pIC_{50} (nH < 1) of the mean (n=2) or the mean ± s.e.mean of n determinations from different preparations performed in duplicate, (nd = not determined).

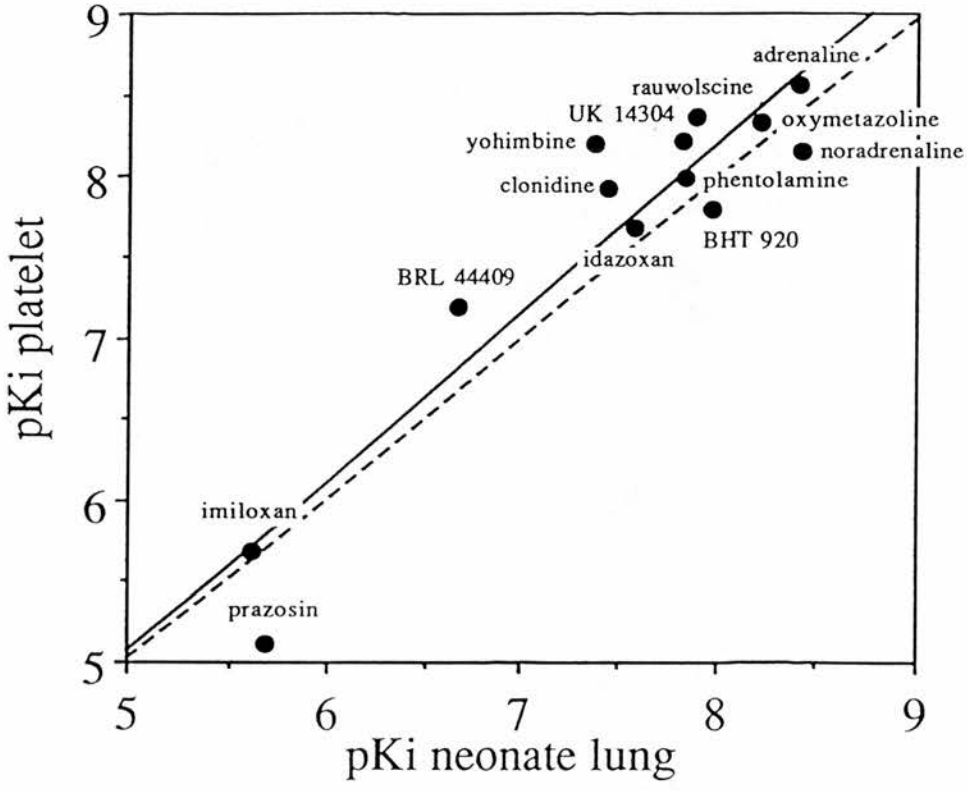


Figure 3.19. Correlation of binding affinity (pK_i) of several compounds for [^{3}H]-adrenaline binding in human platelet and neonatal rat lung membranes. The data is taken from Table 3.6. The solid line represents the line of best fit and the dotted line represents the line of identity. The data is best described by the equation $y = 1.04x + 0.11$; $r = 0.85$.

in human platelet and neonatal rat lung membranes (r=0.90). Therefore, unlike [3H]-RS-15385-197, which distinguished 2 subtypes with distinct pharmacology, the profile of the agonist state of the receptor was essentially the same in both tissues.

3.6. COMPARISON OF [3H]-RS-15385-197 AND [3H]-ADRENALINE BINDING

In the previous sections, the α_2-antagonist and the α_2-agonist radioligands, [3H]-RS-15385-197 and [3H]-adrenaline were characterised in the putative subtype specific tissues, the human platelet (α_{2A}) and the neonatal rat lung (α_{2B}). Studies with the [3H]-antagonist revealed marked differences in the nature of the α_2-receptor in the two tissues with regard to compound selectivity, e.g. prazosin was more potent in the neonatal lung whereas oxymetazoline was more potent in the human platelet. However, a comparison of affinities with the [3H]-agonist revealed broadly similar affinities in the two tissues. In this section the results of these studies will be compared. In Figures 3.20. a-d. correlations between affinity estimates obtained from the two radioligands in the two preparations are presented. As can be seen from Figure 3.20.a. there was a poor correlation (r=0.003) between [3H]-RS-15385-197 and [3H]-adrenaline binding in the neonatal rat lung, and between the two radioligands in the human platelet (Figure 3.20.b. r=0.526). The correlation in the human platelet was improved considerably when agonist affinities were removed from the analysis (r=0.99), and although there was an improvement in the neonatal rat lung correlation (r=0.31) there was still an obvious heterogeneity within the antagonists alone. This suggests that in the human platelet, [3H]-RS-15385-197 and [3H]-adrenaline label largely the same population of sites, and this is further substantiated by the similar number of sites labelled by the two ligands. In the neonatal rat lung, however, [3H]-RS-15385-197 recognises additional, low affinity, agonist sites which have higher affinity for antagonists.

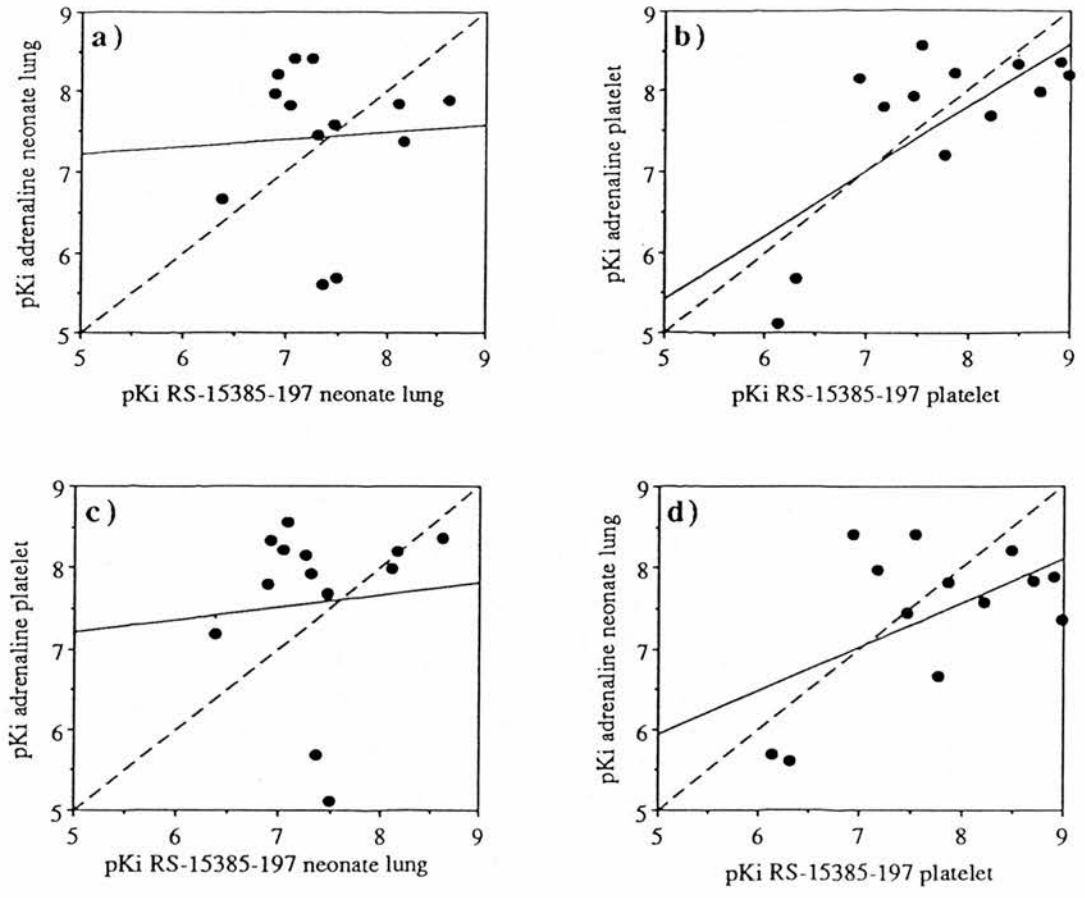


Figure 3.20. Correlations of binding affinity (pK_i) for several coumpounds for [^{3}H]-RS-15385-197 and [^{3}H]-adrenaline binding in neonatal rat lung and human platelet membranes. The solid line represents the line of best fit and the dotted line represents the line of identity in each case. (a) [^{3}H]-RS-15385-197 and [^{3}H]-adrenaline binding in neonatal rat lung (slope = 0.09, r = 0.003); (b) [^{3}H]-RS-15385-197 and [^{3}H]-adrenaline binding in human platelet (slope = 0.79, r = 0.526); (c) [^{3}H]-adrenaline binding in platelets and [^{3}H]-RS-15385-197 binding in neonatal rat lung (slope = 0.15, r = 0.008); (d) [^{3}H]-adrenaline binding in neonatal rat lung and [^{3}H]-RS-15385-197 binding in platelets (slope = 0.55, r = 0.302).

3.7. EFFECT OF BUFFER CONSTITUENTS AND NA+ ON [3H]-AGONIST AND [3H]-ANTAGONIST BINDING TO NEONATAL RAT LUNG MEMBRANES

To determine whether buffer constituents were responsible for the differences seen between the two radioligands, prazosin inhibition curves to [3H]-RS-15385-197 were performed in the absence of EDTA and contained $MgCl_2$ (5 mM), ascorbate (0.3 mM), catechol (0.1 mM) and dithiothreitol (0.8 mM) as used in the [3H]-adrenaline binding assay (Table 3.7.). Inhibition curves to [3H]-adrenaline under [3H]-RS-15385-197 binding conditions were not determined as degradation of radioligand and the absence of $MgCl_2$ from the buffer would make interpretation of the results difficult. Under conditions used for [3H]-adrenaline binding, the specific binding of 0.1 nM [3H]-RS-15385-197 was reduced by 30%. This was most likely due to the effect of $MgCl_2$, as Figure 3.3. showed that $MgCl_2$ inhibited [3H]-RS-15385-197 binding by 35% at 5 mM. However, in competition experiments prazosin retained high affinity for [3H]-RS-15385-197 in the neonatal rat lung. This suggests that differences in assay buffer cannot be responsible for the difference seen in the affinity of prazosin for agonist and antagonist binding.

Previous studies by Limbird *et al.*, (1982) have shown that Na+ increases the affinity for antagonist binding to α_2-adrenoceptors whilst agonist binding is decreased. In the present study, saturation experiments showed that 100 mM NaCl had no effect on the affinity, but increased the density of sites labelled with [3H]-RS-15385-197 in neonatal rat lung membranes by 52% (control, K_d = 0.081 ± 0.010 nM, B_{max} = 174 ± 20 fmoles/mg; NaCl, K_d = 0.092 ± 0.005 nM, B_{max} = 265.4 ± 3.2 fmoles/mg; $p < 0.05$ for an effect on B_{max}, n=3). NaCl (100 mM) also increased the density of sites labelled by [3H]-adrenaline in neonatal rat lung membranes, but there was a consequent decrease in affinity (control, K_d = 3.04 ± 0.44 nM, B_{max} = 77.7 ± 11 fmoles/mg; NaCl, K_d = 11.06 ± 1.73 nM, B_{max} = 208.7 ± 37.5 fmoles/mg; $p < 0.05$ for an effect on K_d and B_{max}, n=3). In the human platelet, 100 mM NaCl had no significant effect on [3H]-RS-15385-197 binding (control, K_d = 0.118 ± 0.012 nM, B_{max} = 99.2 ± 11.7 fmoles/mg; NaCl, K_d = 0.157 ± 0.011 nM, B_{max} = 92.78 ± 10.8 fmoles/mg; n = 3). In the presence of 100 mM NaCl, no specific [3H]-adrenaline binding was

Table 3.7. Effect of buffer composition on the affinity of prazosin for [3H]-RS-15385-197 and [3H]-adrenaline binding in the neonatal rat lung.

	[3H]-RS-15385-197	[3H]-adrenaline
	prazosin	**prazosin**
	pK_i	pK_i
	nH	nH
Tris EDTA (control)	7.20 ± 0.08 (3) 0.99 ± 0.06	nd
[3H]-adrenaline buffer system *	7.01 ± 0.03 (3) 1.01 ± 0.03	5.79 ± 0.06 (4) 0.97 ± 0.19

Membranes were incubated with radioligand as described in Chapter two. The number of determinations performed in duplicate is indicated in brackets. * Tris-EDTA-free buffer containing 5 mM $MgCl_2$, 0.8 mM dithiothreitol, 0.1 mM catechol, 0.3 mM ascorbate and 10 nM propranolol. The results represent the mean ± s.e.mean.

observed in human platelet membranes.

Table 3.8. shows the effect of 100 mM NaCl on the affinity of several competing drugs for [^{3}H]-adrenaline and [^{3}H]-RS-15385-197 binding to neonatal rat lung membranes. There was no significant effect on the affinity of prazosin for [^{3}H]-RS-15385-197 binding in the neonatal rat lung, however, imiloxan affinity was increased 13 fold. The affinity of the catecholamine agonists adrenaline and noradrenaline for [^{3}H]-RS-15385-197 binding was significantly decreased, whereas oxymetazoline affinity was much less affected and there was no significant change in the affinity of UK 14304. The affinity of prazosin for [^{3}H]-adrenaline binding was significantly increased ($p < 0.05$) from 5.68 to 6.22 in the presence of 10 mM NaCl and to 6.70 in the presence of 100 mM NaCl. Similarly, the affinity of imiloxan was increased from 5.61 to 6.69 in the presence of 100 mM NaCl. Conversely, the affinity of adrenaline and noradrenaline was decreased in the presence of NaCl, whereas there was no change in the affinity of oxymetazoline or UK 14304.

In platelet membranes (Table 3.9.) 100 mM NaCl had no effect on the affinity of prazosin for [^{3}H]-RS-15385-197 binding but the affinity of imiloxan was significantly increased. NaCl (100 mM) significantly decreased the affinity of the catecholamines adrenaline and noradrenaline. There was a small decrease in the affinity of UK 14304, whereas oxymetazoline affinity was unaffected. Competition experiments with [^{3}H]-adrenaline in the presence of NaCl in platelets were difficult to characterise as the specific binding was very low under these conditions.

NaCl therefore produced complex interactions with the α_2-adrenoceptor labelled by [^{3}H]-agonists and [^{3}H]-antagonists. In the neonatal rat lung, NaCl reduced agonist affinity whilst increasing the total number of receptors labelled by both the agonist and antagonist. The profile of the agonist binding site in the presence of NaCl was such that the affinities of prazosin and imiloxan, were increased, whilst those for the catecholamines were decreased. The affinity of the imidazolines agonists, oxymetazoline and UK 14304 were much less affected by the presence of NaCl. Possible mechanisms whereby NaCl exerts these effects will be addressed in Section 3.8.

Table 3.8. The effect of 100 mM NaCl on agonist and antagonist binding to α_2-adrenoceptors on neonatal rat lung membranes.

		[^{3}H]-adrenaline		[^{3}H]-RS-15385-197	
		control	NaCl 100 mM	control	NaCl 100 mM
adrenaline	pIC_{50}	8.41±0.16	7.78±0.20*	7.05±0.04	6.25±0.05*
	nH	1.00±0.01	0.92±0.07	0.82±0.02	1.01±0.03
noradrenaline	pIC_{50}	8.42±0.14	8.00±0.13*	7.25±0.03	6.19±0.06*
	nH	0.97±0.03	0.82±0.10	0.68±0.04	0.94±0.13
oxymetazoline	pIC_{50}	8.22±0.09	8.34±0.25	6.91±0.04	6.28±0.04*
	nH	0.90±0.10	0.75±0.02	0.73±0.07	1.06±0.04
UK 14304	pIC_{50}	7.83±0.17	7.82±0.10	7.04±0.10	6.81±0.07
	nH	0.82±0.13	0.87±0.13	0.79±0.05	0.92±0.04
prazosin	pIC_{50}	5.68±0.16	6.70±0.23*	7.49±0.12	7.66±0.02
	nH	0.99±0.01	0.98±0.04	1.04±0.12	1.18±0.09
imiloxan	pIC_{50}	5.61±0.12	6.69±0.27*	6.30±0.04	7.40±0.22*
	nH	0.81±0.10	0.91±0.12	1.03±0.04	0.98±0.13

The results represent the mean ± s.e.mean for at least 3 determinations performed in duplicate.

* $p < 0.05$ compared to corresponding control.

Table 3.9. The effect of 100 mM NaCl on agonist and antagonist inhibition of [^{3}H]-RS-15385-197 binding to human platelet membranes.

		[^{3}H]-RS-15385-197	
		control	NaCl 100 mM
adrenaline	pIC_{50}	7.36±0.01	6.48±0.15*
	nH	0.80±0.05	0.65±0.04
noradrenaline	pIC_{50}	6.93±0.04	5.70±0.05*
	nH	0.76±0.07	0.85±0.05
oxymetazoline	pIC_{50}	8.49±0.01	8.57±0.08
	nH	0.90±0.06	0.81±0.12
UK 14304	pIC_{50}	7.87±0.04	7.44±0.06*
	nH	0.97±0.13	0.82±0.17
prazosin	pIC_{50}	6.13±0.08	6.13±0.08
	nH	0.94±0.06	1.24±0.14
imiloxan	pIC_{50}	6.30±0.04	7.00±0.22*
	nH	1.30±0.16	0.67±0.12

The results represent the mean ± s.e.mean for at least 3 determinations performed in duplicate.
* $p < 0.05$ compared to corresponding control.

3.8. DISCUSSION

3.8.1. [^{3}H]-RS-15385-197 binding

In this chapter the interaction of a novel α_2-antagonist ligand [^{3}H]-RS-15385-197 has been determined in three different membrane preparations and in autoradiography studies in rat brain. The binding of [^{3}H]-RS-15385-197 in all tissues was reversible, saturable and of high affinity, and had advantages over other α_2-antagonist ligands in several respects. Firstly, it displayed very low levels of non-specific binding (< 5%) at concentrations around the K_d, and was highly selective over other receptor types. The finding that the α_2-adrenoceptor antagonists, yohimbine, rauwolscine, idazoxan, WY 26703 and phentolamine, and the α_2-adrenoceptor agonists, clonidine, noradrenaline and adrenaline displayed high affinity towards this site whereas a variety of ligands with affinity for a number of other receptor types displayed low affinity, confirms our previous studies showing RS-15385-197 to be a highly potent and selective α_2-adrenoceptor antagonist (Brown *et al.*, 1989,1992; Clark *et al.*, 1989,1990b; Redfern *et al.*, 1992).

Saturation studies revealed [^{3}H]-RS-15385-197 (K_d = 0.12 nM) to have higher affinity than [^{3}H]-yohimbine (K_d = 5.3 nM, Brown *et al.*, 1990a) and [^{3}H]-idazoxan (K_d = 4.1 nM, Brown *et al.*, 1990b) at α_2-adrenoceptors in rat cerebral cortex. The density of α_2-adrenoceptors labelled by [^{3}H]-RS-15385-197 (275 fmoles/mg protein) was significantly greater than the number identified with [^{3}H]-yohimbine and [^{3}H]-idazoxan in the same preparation (B_{max} = 121 and 87 fmoles/mg protein for [^{3}H]-yohimbine and [^{3}H]-idazoxan respectively; Brown *et al.*, 1990a,b).

The higher density of α_2-adrenoceptors labelled by [^{3}H]-RS-15385-197, relative to the number of sites identified by [^{3}H]-yohimbine, is unlikely to be caused by [^{3}H]-RS-15385-197 labelling another receptor, as the compound has > 800 fold selectivity over any other receptor subtype studied (Clark *et al.*, 1989,1990b and Table 1.5). Furthermore, all of the α_2-adrenoceptor antagonists fully inhibited the binding of [^{3}H]-RS-15385-197 to rat cerebral cortex with Hill slopes not significantly different from unity, confirming that [^{3}H]-RS-15385-197 is binding only to α_2-adrenoceptors. Thus, if the difference in B_{max} values obtained with [^{3}H]-RS-15385-197 and [^{3}H]-yohimbine is not due to additional binding sites for [^{3}H]-RS-15385-197, then [^{3}H]-yohimbine

must not label the total population of α_2-adrenoceptors in rat cerebral cortex. In rabbit adipocyte membranes, [^{3}H]-yohimbine failed to bind to α_2-adrenoceptors (Langin & Lafontan, 1989), whereas [^{3}H]-UK 14304 labelled the high affinity state and [^{3}H]-RX821002 labelled the whole population of α_2-adrenoceptors (B_{max} = 289 fmoles/mg protein, Langin *et al.*, 1990). The inability of [^{3}H]-yohimbine to label α_2-adrenoceptors in these studies was explained by the relatively low affinity and the high level of non-specific binding. The present study showed that yohimbine had only moderate affinity for [^{3}H]-RS-15385-197 binding in rat cortical membranes (K_i = 14 nM), and previous studies in the same preparation (Brown *et al.*, 1990a,b) showed [^{3}H]-yohimbine to have moderate affinity (K_d = 5.3 nM). The lower affinity of [^{3}H]-yohimbine may suggest that a faster dissociation rate with this ligand could result in dissociation from a proportion of α_2-adrenoceptors during the filtration process.

[^{3}H]-Idazoxan binding has been studied in the rat cortex (Boyajian & Leslie, 1987; Boyajian *et al.*, 1987; Brown *et al.*, 1990a). In the former study [^{3}H]-idazoxan was proposed to label two populations of α_2-adrenoceptor, one of which was recognised by [^{3}H]-rauwolscine, however the latter study showed that an additional site labelled by [^{3}H]-idazoxan was due to binding to a non-adrenergic imidazoline binding site which had a distinct pharmacology. When [^{3}H]-idazoxan binding was analysed using 3 μM phentolamine to define non-specific binding (thus isolating the α_2-component) the density of α_2-adrenoceptors labelled with [^{3}H]-idazoxan was less than that labelled with [^{3}H]-yohimbine. Therefore, in these studies, [^{3}H]-yohimbine was proposed to label two α_2-adrenoceptors (Brown *et al.*, 1990a,b) α_{2A} and α_{2B}, the latter subtype being defined as having high affinity for prazosin.

The number of binding sites identified with [^{3}H]-yohimbine is dependent upon the concentration range chosen for the experiment. Saturation studies carried out over a wider [^{3}H]-yohimbine concentration range (0.2 - 80 nM) have demonstrated binding to a second site in rat cortex (Michel & Whiting, 1984). Conditions for [^{3}H]-yohimbine binding to rat cortex have to be carefully defined in order to exclude binding to this second component which, if unresolved, would increase the density of binding sites. [^{3}H]-rauwolscine, a stereoisomer of yohimbine, also binds in a biphasic manner to rat cortical membranes at high concentrations (Diop *et al.*, 1983; Broadhurst & Wyllie, 1986).

Recent studies have shown that [3H]-rauwolscine labels 5-HT_{1A} receptors in human cortex (Convents *et al.*, 1989) and a spiperone-sensitive site that most likely represents a 5-HT_{1A} receptor in rat cerebral cortex (Broadhurst *et al.*, 1988). Rauwolscine and yohimbine also have high affinity for the $5HT_{1D}$ binding site identified in non-rodent brain (Heuring & Peroutka, 1987). In autoradiography studies, [3H]-rauwolscine was shown to bind to areas receiving primarily dopaminergic innervation and corresponded closely to [3H]-spiroperidol binding distribution (Boyajian *et al.*, 1987). However the same authors demonstrated that selective D_1 and D_2 compounds failed to inhibit [3H]-rauwolscine binding in an homogenate preparation, suggesting that the binding was not to dopaminergic receptors (Boyajian & Leslie, 1987). [3H]-Rauwolscine binding to dopaminergic areas (caudate-putamen, nucleus accumbens and hippocampus) may represent binding to a 5-HT-receptor subtype. Indeed, previous studies from this laboratory have shown that like prazosin, some 5-HT agents, 8-OH-DPAT, RU 24969 and methysergide, show high affinity for ~40% of specifically bound [3H]-yohimbine in rat cortical membranes (Brown *et al.*, 1990b).

Because of the inability of [3H]-yohimbine to adequately label the entire population of α_2-adrenoceptors in a variety of preparations (see above), and because of the non-adrenergic interactions of both [3H]-yohimbine and [3H]-idazoxan, [3H]-RS-15385-197 is proposed to be a superior ligand for the characterisation of α_2-adrenoceptor subtypes.

3.8.2. Autoradiographical distribution

The distribution of α_2-adrenoceptors was characterised with [3H]-RS-15385-197. The highest density binding was observed over areas reported to receive dense noradrenergic innervation (Loughin & Fallon, 1985), namely the anterior olfactory nuclei, amygdala, entorhinal cortex, locus coeruleus and the nucleus of the solitary tract.

Previous studies with the agonists [3H]-*p*-aminoclonidine (Unnerstall *et al.*, 1984) and [3H]-clonidine (Young & Kuhar, 1981) and with the antagonist [3H]-idazoxan (Bruning *et al.*, 1987; Boyajian *et al.*, 1987) have demonstrated a similar distribution to that shown with [3H]-RS-15385-197 in the present study. The presence of α_2-adrenoceptors in these areas corresponded closely to the neuronal populations receiving innervations from the noradrenergic and adrenergic cell groups (amygdaloid nucleus, entorhinal cortex, anterior olfactory

nucleus, paraventricular and periventricular hypothalamic nucleus and bed nucleus of the stria terminalis) and to those regions where the cell bodies of adrenergic and noradrenergic neurons are found (locus coeruleus, nucleus of the solitary tract), and suggest that α_2-adrenoceptors in rat brain are located both pre- and post-synaptically. A diagram of the noradrenergic projections from the locus coeruleus is given in plate 3.3.

Some anomalies between this present and previous studies include the high density binding observed with [^{3}H]-idazoxan over parts of the hypothalamus and the subfornical organ and area postrema (Bruning *et al.*, 1987). Binding in these areas was not displaceable with yohimbine and was therefore unlikely to represent binding to α_2-adrenoceptors. In the following chapter, evidence will be presented suggesting that binding in these areas is to imidazoline binding sites and the nature of these sites will be discussed.

The distibution of α_2-adrenoceptors has also been studied with [^{3}H]-rauwolscine in rat brain (Boyajian *et al.*, 1987). Unlike the disribution observed with [^{3}H]-idazoxan (Bruning *et al.*, 1987; Boyajian *et al.*, 1987), [^{3}H]-*p*-aminoclonidine (Unnerstall *et al.*, 1984), [^{3}H]-clonidine (Young & Kuhar, 1981) and [^{3}H]-RS-15385-197 (this study), [^{3}H]-rauwolscine binding sites were observed over areas which receive primarily dopaminergic innervation, namely the nucleus caudate-putamen, nucleus accumbens, olfactory tubercle, islands of Calleja, hippocampus, parasubiculum, basolateral amygdaloid nucleus and substantia nigra (Boyajian *et al.*, 1987), and corresponded closely to [^{3}H]-spiroperidol binding distributions. However, very low density binding was observed over areas receiving noradrenergic innervation and labelled by the other α_2-radioligands, particularly in the hypothalamus and pons medullary area. It must be stressed, however, that the density of sites labelled by [^{3}H]-rauwolscine were consistently lower than, or equal to, those determined from parallel incubations with [^{3}H]-idazoxan, even in the highest density areas. From these studies the authors concluded that [^{3}H]-rauwolscine labelled a subset of sites labelled by [^{3}H]-idazoxan.

[^{3}H]-Rauwolscine has, until now, been the highest affinity α_2-adrenoceptor antagonist available. It is therefore surprising, that although [^{3}H]-rauwolscine and [^{3}H]-idazoxan were used at concentrations around their respective K_d values, [^{3}H]-rauwolscine did

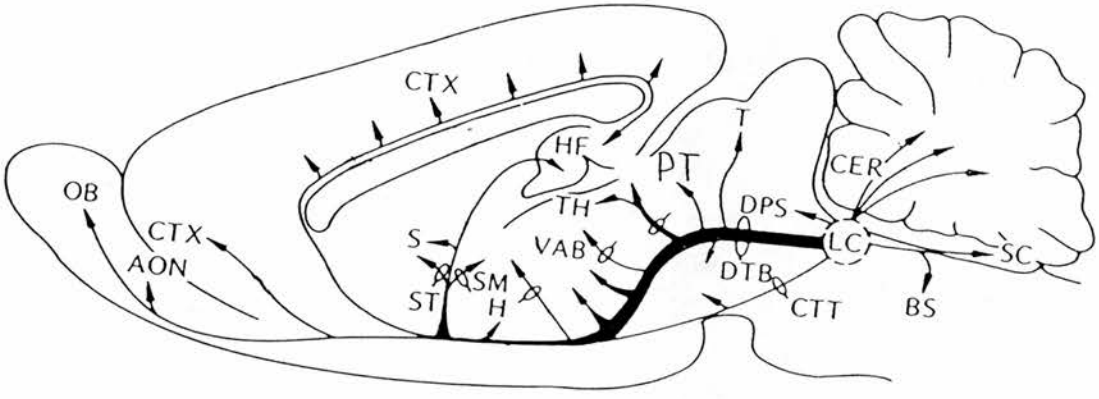


Plate 3.3. Diagram of the projections of the locus coeruleus viewed in the sagittal plane. AON, anterior olfactory nucleus; BS, brainstem nuclei; CER, cerebellum; CTT, central tegmental tract; CTX, cerebral cortex; DPS, dorsal periventricular system, DTB, dorsal catecholamine bundle; H, hypothalamus; HF, hippocampal formation; LC, locus coeruleus; OB, olfactory bulb; PT, pretectal area; S, septal area; SC, spinal chord; ST, stria terminalis; T, tectum; TH, thalamus; VAB, ventral amygdaloid bundle system.

not label sites which all other selective α_2-adrenoceptor agonists and antagonists label. An alternative explanation for the discrepancy between the two radioligands could be that the wash times chosen for the autoradiography experiments resulted in the removal of unequal amounts of specifically bound ligand. The washtime chosen for [^{3}H]-idazoxan (2 min at 0^{o}C) with a $t_{1/2}$ of 19 min would have resulted in a loss of less than 5% bound ligand at 0.3 nM, whereas a washtime for [^{3}H]-rauwolscine (30 min. at 0^{o}C) with a $t_{1/2}$ of 105 min would have resulted in a loss of more than 20% bound ligand at 0.6 nM. Thus, it is possible that the [^{3}H]-rauwolscine bound to those regions labelled by [^{3}H]-idazoxan was lost during the washout period.

3.8.3. α_2-adrenoceptor subtypes

The pharmacological basis for suggesting that heterogeneity exists within α_2-adrenoceptors is primarily based upon the relative affinities of prazosin or oxymetazoline to inhibit [^{3}H]-yohimbine and [^{3}H]-rauwolscine binding to sites in a variety of tissues (Bylund, 1985; Cheung *et al.*, 1982; Latifpour *et al.*, 1982) and not upon differences in receptor densities. Prazosin is a more potent inhibitor of [^{3}H]-yohimbine and [^{3}H]-rauwolscine binding to α_2-adrenoceptors in neonatal rat lung (Latifpour *et al.*, 1982) and rat cerebral cortex (Cheung *et al.*, 1982) than from α_2-adrenoceptors present on human platelets (Cheung *et al.*, 1982). In contrast, oxymetazoline displays higher affinity towards α_2-adrenoceptors on human platelets than at the receptors on neonatal rat lung.

There are no highly selective antagonists for α_2-adrenoceptor subtypes, although a number of α_2-adrenoceptor antagonists have been reported in recent years, including imiloxan (Michel & Whiting, 1981), idazoxan (Doxey *et al.*, 1983b), RX 821002 (Stillings *et al.*, 1985), WY 26703 (Lattimer *et al.*, 1984), L-654,284 (Pettibourne *et al.*, 1986), L-657,743 (Pettibourne *et al.*, 1987) and CH-38083 (Vizi *et al.*, 1986). These antagonists have the advantages over yohimbine and rauwolscine of either being more potent and/or selective relative to the other adrenoceptor subtypes. Amongst the new antagonists, idazoxan, RX 821002, L-657,743 and L-654,284 have been radiolabelled. [^{3}H]-RX 821002, an imidazoline analogue of idazoxan is described as a new radioligand for the identification of α_{2A}-adrenoceptors (Langin *et al.*, 1989), but has also been reported recently to label 2 classes of α_{2B}-

adrenoceptor in rat kidney (Uhlen &Wikberg, 1991). The binding of the non-imidazoline α_2-adrenoceptor radioligands [^{3}H]-L-654,284 (Randall *et al.*, 1988) and [^{3}H]-L-657,743 (Pettibourne *et al.*, 1988), to rat cerebral cortex have also been characterized. Prazosin displaced each radioligand with low affinity and with Hill slopes that did not deviate from unity, a finding consistent with binding to the α_{2A}-adrenoceptor subtype. Recently, BRL 41992, structurally related to mianserin, has been described as a potent and selective ligand for α_{2B}-adrenoceptors in rat neonatal lung (Young *et al.*, 1989). The synthesis of [^{3}H]-BRL 41992 and its confirmation as a selective, high affinity radioligand for the α_{2B}-adrenoceptor subtype would help resolve the precise nature of the α_{2B}-adrenoceptor subtype.

This study showed that [^{3}H]-RS-15385-197 labels both the α_{2A} adrenoceptor subtype in the human platelet and the α_{2B} adrenoceptor subtype in the neonatal rat lung, as defined by relative affinities of prazosin and oxymetazoline. However, the lower affinity for prazosin in the rat cerebral cortex is consistent, using current classification, with [^{3}H]-RS-15385-197 labelling an α_{2A} subtype in this tissue. However, it is unlike the α_{2A} site in the human platelet which has very high affinity for yohimbine and rauwolscine and low affinity for prazosin (Cheung *et al.*, 1982; Brown *et al.*, 1990b) and in this study a relatively poor correlation (r=0.67, Figure 3.14.c.) was acheived when affinity values from 11 compounds were compared in the two tissues. It is also unlike the α_{2A} site described for the rabbit spleen (Michel *et al.*, 1989b, Table 1.2), which showed 10 fold lower affinity for prazosin (pK_i = 5.33) and 50 fold lower affinity for imiloxan (pK_i = 5.52, Michel *et al.*, 1989b).

Michel *et al.* (1989b) have characterised a site in the rat submaxillary gland which showed intermediate affinity for prazosin, moderate affinity for imiloxan, and only moderate affinity for yohimbine and rauwolscine, and in this respect shows some similarities to the site in the rat cerebral cortex labelled by [^{3}H]-RS-15385-197. In Figure 3.21. a correlation between affinity estimates obtained from 10 compounds against [^{3}H]-rauwolscine in the submaxillary gland, and [^{3}H]-RS-15385-197 binding in the rat cortex is presented and shows the best degree of homology (r=0.80). Recently, Simonneaux *et al.* (1991) showed the rat submaxilliary site to be similar to a site in the bovine pineal gland and has tentatively termed this site α_{2D}. In addition, a gene isolated from a rat gene library (clone RG20), when expressed in COS-

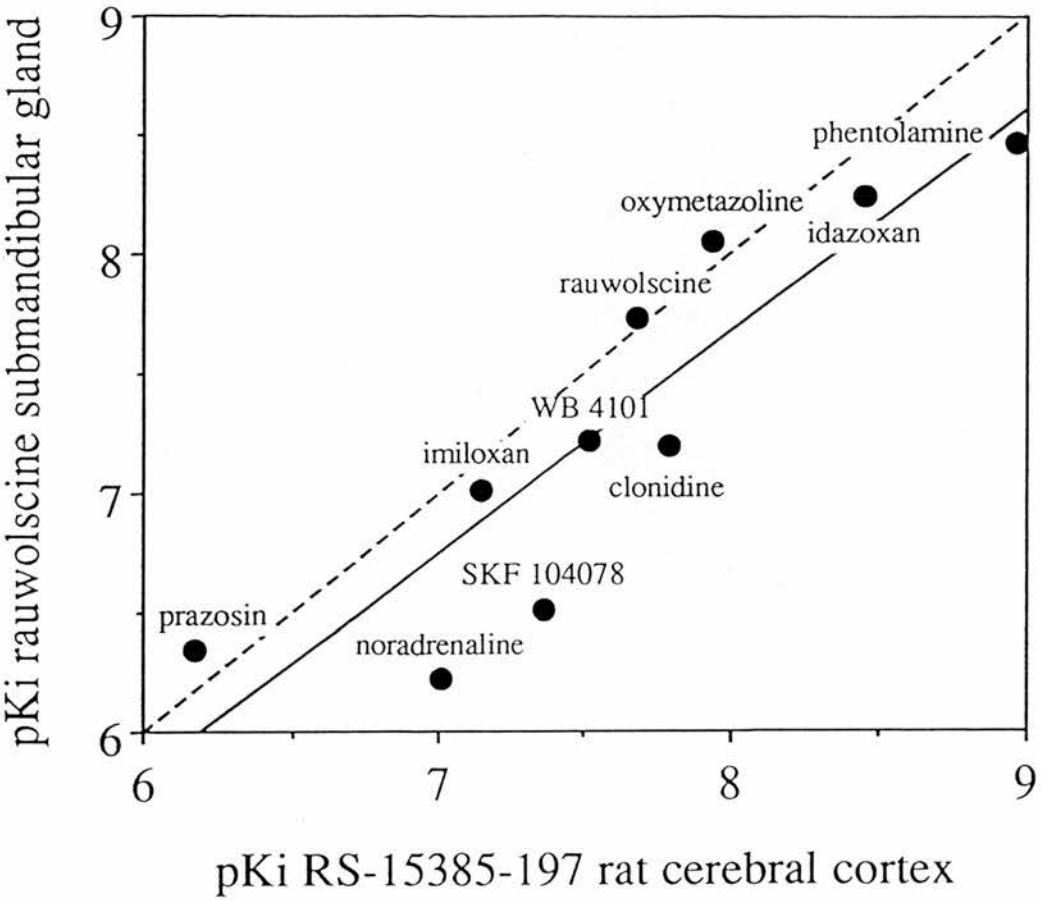


Figure 3.21. Correlation of binding affinity (pK_i) of several compounds for [^{3}H]-RS-15385-197 binding rat cerebral cortex membranes and [^{3}H]-rauwolscine binding to rat submaxillary gland. The solid line represents the line of best fit and the dotted line represents the line of identity. The data is best described by the equation $y = 0.928x + 0.24$, $r = 0.80$. Data for the submaxillary gland is taken from Michel *et al.* (1989).

1 cells, exhibited binding properties similar to the α_{2D} subtype (Lanier *et al.*, 1991), and to the cerebral cortex binding site described in this thesis.

In the present study, therefore, a paradox is evident, as α_{2A} and α_{2B} subtypes have been reported to exist in the rat cerebral cortex as labelled by [^{3}H]-yohimbine and as defined by a prazosin-sensitive component (Nahorski *et al.*, 1985; Bylund, 1985, Brown *et al.*, 1990a,b) although other studies have failed to demonstrate this (Dickinson *et al.*, 1986; Boyajian *et al.*, 1987). In addition, a single class of adrenoceptor showing, until now, the greatest similarity with the α_{2A}-adrenoceptor subtype, has been labelled with [^{3}H]-idazoxan (Brown *et al.*, 1990a), [^{3}H]-L-654-284 (Randall *et al.*, 1988) and [^{3}H]-L-657-743 (Pettibourne *et al.*, 1988) in rat cerebral cortex membranes. On reflection, it now seems more likely that the subtype labelled by these agents is of the α_{2D} rather than the α_{2A} subtype.

The nature of the prazosin-sensitive component of [^{3}H]-yohimbine binding in the rat cerebral cortex is therefore not clear. If indeed it represented binding to an α_{2B}-adrenoceptor subtype as suggested (Nahorski *et al.*, 1985; Bylund, 1985; Brown *et al.*, 1990a,b) then [^{3}H]-RS-15385-197 would be expected to label it with high affinity, as [^{3}H]-RS-15385-197 had equal affinity for α_{2A} and α_{2B}-adrenoceptor subtypes in human platelet and rat neonatal lung. However, in the rat cortex, inhibition curves to prazosin were monophasic and of low affinity, suggesting that the α_{2B}-adrenoceptor was not labelled. Since the density of sites labelled by [^{3}H]-yohimbine in an identical membrane preparation was only 44% of those labelled by [^{3}H]-RS-15385-197, and given that only 40% of those sites were sensitive to prazosin (Brown *et al.*, 1990a,b), then it could be calculated that only 18% of sites labelled by [^{3}H]-RS-15385-197 would be prazosin-sensitive. It is possible therefore, that due to the small percentage of sites and the relatively low selectivity of prazosin (20-100 fold, Nahorski *et al.*, 1985; Brown *et al.*, 1990a,b) resolution was limiting in identifying a high affinity prazosin-sensitive component with [^{3}H]-RS-15385-197.

A recent study with [^{3}H]-MK 912 has suggested that the rat cortex contains two populations of α_2-adrenoceptors; one designated α_{2A} (296 fmoles/mg protein) and a smaller population designated α_{2C} (33 fmoles/mg protein) for which prazosin showed 16 fold selectivity. Although classed as α_{2A}-adrenoceptors, the former population was of a

similar density to those described in the present study, and showed only moderate affinity for yohimbine and rauwolscine (K_d = 49.8 and 34.5 nM respectively) and was therefore more similar to the α_{2D}-adrenoceptor described here. The latter population, indeed showed characterisitcs similar to the α_{2C}-adrenoceptor and could represent the prazosin-sensitive component of [^{3}H]-yohimbine binding previousely designated α_{2B} (Nahorski *et al.*, 1985; Bylund, 1985; Brown *et al.*, 1990a,b). Both [^{3}H]-yohimbine and [^{3}H]-MK 912 show high selectivity for the α_{2C} adrenoceptor (Murphy & Bylund, 1988; Uhlen *et al.*, 1992), which could explain why a prazosin-sensitive component was evident in rat cerebral cortex with these ligands and not others.

The identification of a selective, high affinity, radioligand for the α_{2B}-adrenoceptor subtype would help resolve the precise nature of this receptor, and whether or not it exists in the rat cerebral cortex. However, based on the available evidence, I would now suggest that [^{3}H]-RS-15385-197 and other α_2-radioligands label a site in the rat cerebral cortex of the α_{2D} subclass, and that this forms the major population of α_2-adrenoceptors in the rat cortex.

3.8.4. Agonist versus antagonist binding

The ability of the agonist [^{3}H]-adrenaline to label α_{2A}- and α_{2B}-adrenoceptors was assessed in human platelet and neonatal rat lung membranes, and compared with [^{3}H]-RS-15385-197 binding. Marked differences were noted in the nature of the sites labelled by the antagonist and the agonist. [^{3}H]-Adrenaline labelled 85% and 44% of the sites labelled by [^{3}H]-RS-15385-197 in the human platelet and the neonatal rat lung respectively. Unlike [^{3}H]-RS-15385-197 binding, however, the pharmacology of the agonist labelled site in both tissues was essentially similar (r=0.85). Prazosin had low affinity and oxymetazoline had high affinity for the agonist labelled site in both tissues suggesting that an adrenoceptor characteristic of the α_{2B}-subtype could not be labelled with [^{3}H]-adrenaline. Kahn *et al.* (1982) have demonstrated on NG 108-15 cells, a cell line reputed to express an homogeneous population of α_{2B}-adrenoceptors (Bylund *et al.*, 1988), that binding sites labelled by the agonists [^{3}H]-*p*-aminoclonidine and [^{3}H]-adrenaline exhibited low micromolar affinity for prazosin, but that sites labelled by [^{3}H]-yohimbine possessed 10-100 fold higher affinity for antagonists (prazosin affinity 16 nM) and 20 fold lower affinity for

agonists. In these respects the data resembles data from the present study in the neonatal rat lung.

In another study, [^{3}H]-clonidine and [^{3}H]-UK 14304 failed to distinguish α_{2A}- and α_{2B}-adrenoceptor subtypes on HT29 and NG 108-15 cells ($r > 0.9$, Gleason & Hieble, 1991), although it was suggested that closer examination of the slope of the plots revealed that prazosin and ARC 239 retained some selectivity for the α_{2B}-adrenoceptor subtype labelled by the agonists (slope < 0.6). Correlations in the present study revealed slope values closer to unity for displacement of agonist binding to human platelet and neonatal rat lung membranes and no selectivity of prazosin and imiloxan for the α_{2B}-adrenoceptor was evident. Therefore, no marked differences were observed in the binding characteristics of the activated state of the α_{2A} and α_{2B}-adrenoceptor subtypes in the human platelet and neonatal rat lung.

Unlike antagonists, agonist binding is influenced by guanine nucleotides which results in a complex interaction of the receptor with the agonist and a guanine nucleotide binding protein. In the present study it was demonstrated that GTP shifted adrenaline inhibition curves to [^{3}H]-RS-15385-197 to the right in rat cerebral cortex, and that Gpp(NH)p, a stable GTP analogue had a similar effect in neonatal rat lung, and to a lesser extent in the human platelet. The affinity of the antagonists yohimbine and prazosin, however, were unaltered. The ability of Gpp(NH)p to decrease agonist affinity in the human platelet, was only evident when compared to agonist inhibition in the presence of Mg^{2+}. In the absence of Mg^{2+} and Gpp(NH)p, agonist inhibition curves were less markedly biphasic and in fact clonidine and UK 14304 were monophasic. The differential effects of Mg^{2+} in the human platelet compared to other tissues has been previousely reported (Bylund & U'Prichard, 1983). In human platelet membranes Mg^{2+} has been reported to produce a leftward shift of adrenaline competition curves at [^{3}H]-yohimbine labelled sites (U'Prichard *et al.*, 1983), but not to cortical membranes (U'Prichard & Snyder, 1980) or NG 108-15 cell membranes (Bylund & U'Prichard, 1983). In addition, the ability of GTP and Gpp(NH)p to inhibit [^{3}H]-adrenaline binding in platelets was enhanced in the presence of $MgCl_2$ (U'Prichard & Snyder, 1980). These data indicate that in the human platelet, Mg^{2+} enhances binding to the high affinity agonist state of the α_2-adrenoceptor, and suggests differences in the effects of guanyl nucleotides on agonist interactions

with the receptor.

Although antagonists are historically reported to have equal affinity for agonist receptor states, variable affinity for α_2-adrenoceptor states by some antagonists has been reported (Perry & U'Prichard, 1983). In human platelets, [^{3}H]-yohimbine has been reported as having some selectivity for the lower affinity agonist state of the receptor (Daiguji *et al.*, 1981). In the current study, yohimbine and prazosin exhibited monophasic inhibition curves for [^{3}H]-RS-15385-197 binding which were unaltered by the presence of Gpp(NH)p or GTP or $MgCl_2$ in all tissues, although the affinity of prazosin was decreased slightly in the presence of $MgCl_2$ in the human platelet.

The fact that prazosin had >100 fold lower affinity for the high affinity agonist state of the α_{2B}-adrenoceptor, as delineated by [^{3}H]-adrenaline binding, cannot explain why this compound exhibited monophasic inhibition curves for [^{3}H]-RS-15385-197, if it is assumed that the latter compound labels both high and low affinity states of the receptor. It cannot be explained by a non-competitive interaction of prazosin, as prazosin does not influence the dissociation rate of, or the number of sites labelled by [^{3}H]-RS-15385-197. Alternatively, [^{3}H]-adrenaline may label an additional high affinity state of the α_{2B}-adrenoceptor, which has low affinity for some antagonists, particularly prazosin and imiloxan, and may not be labelled by [^{3}H]-RS-15385-197. Multiple high affinity agonist states of the α_2-adrenoceptor on the human platelet have been previously suggested using [^{3}H]-adrenaline (U'Prichard *et al.*, 1983) and [^{3}H]-UK-14,304 (Neubig *et al.*, 1988). It is possible, therefore, that the same is true for the α_{2B}-adrenoceptor, and could be elucidated by a detailed kinetic analysis of [^{3}H]-adrenaline binding in the neonatal rat lung. The interaction of prazosin and RS-15385-197 with the various states of the α_2-adrenoceptor must therefore be more complicated than previously thought, and the difference in the ability of selective antagonists to inhibit [^{3}H]-antagonist and not [^{3}H]-agonist binding suggests additional differences in the interaction of these radioligands with the α_2-adrenoceptor subtypes.

3.8.5. Effects of Na^+

Another factor shown to differentially modulate agonist and antagonist binding to α_2-adrenoceptors is Na^+ (Limbird *et al.*, 1982). The reciprocal effects of Na^+ on agonist versus antagonist interactions

appear to be a property of all receptor populations linked to inhibition of adenylate cyclase activity (Jakobs, 1979). For the α_2-adrenoceptor at least, the effect of Na^+ is to decrease agonist potency for [^{3}H]-antagonist binding and to increase receptor affinity for antagonists. In the present study, the effects of Na^+ on α_2-adrenoceptor subtypes were studied in more depth by examining changes in affinity of a variety of compounds for agonist and antagonist labelled sites in the absence or presence of 100 mM NaCl. Previous studies have looked only at agonist and antagonist inhibition of [^{3}H]-yohimbine binding which, for the reasons stated in the previous section, may not adequately explain changes observed in agonist and antagonist binding at α_2-adrenoceptor subtypes.

The present data showed that complex interactions of agonist and antagonist binding occurred in the presence of NaCl in tissues containing the α_{2A} and α_{2B}-adrenoceptor subtypes. The effects were unlikely to be due to changes in osmolality, as previous studies have shown that the regulatory effects of NaCl on the porcine α_{2A}-adrenoceptor and the NG 108-15 cell α_{2B}-adrenoceptor were not produced by equimolar concentrations of N-methyl-D-glucamine-Cl (Guyer *et al.*, 1990; Wilson *et al.*, 1991a). They are also not likely to be due to Cl^- ions as LiCl and KCl were much less effective (Nunnari *et al.*, 1987; Guyer *et al.*, 1990; Wilson *et al.*, 1991a).

100 mM NaCl decreased the affinity of adrenaline by 4 fold in the neonatal rat lung and by 9 fold in the platelet. In the human platelet, the decreased affinity was so great that no detectable binding was observed with [^{3}H]-adrenaline. No change in the density of binding was seen for [^{3}H]-RS-15385-197 in the platelet, however, in the neonatal rat lung, an increase in the density of both [^{3}H]-RS-15385-197 and [^{3}H]-adrenaline was observed, suggesting that Na^+ brought about changes in the conformation of the receptor so that more sites in the low affinity agonist state was seen by both ligands.

The allosteric effects of Na^+ on the cloned porcine α_{2A}-adrenoceptor showed that this cation increased the affinity of [^{3}H]-yohimbine binding (Guyer *et al.*, 1990). The interaction was non-competitive in that there was an increase in the dissociation rate of [^{3}H]-yohimbine. Although an increase in dissociation rate would not be consistent with an increase in affinity, a previous study indicated that this is offset by a 3 fold increase in the association rate, resulting in a net increase in affinity (Limbird *et al.*, 1982). Another study on the

solubilised α_{2B}-adrenoceptor from NG 108-15 cells showed, that although Na^+ decreased adrenaline affinity for [^{3}H]-yohimbine binding it did not facilitate [^{3}H]-yohimbine dissociation, (Wilson *et al.*, 1991a). These studies would therefore support the results from the present study, that Na^+ modulates agonist and antagonist interactions with the α_{2A}- and α_{2B}-adrenoceptors via different mechanisms.

In the neonatal rat lung, competition experiments with [^{3}H]-adrenaline in the presence of Na^+, showed that the affinity of imiloxan and prazosin was increased more than 10 fold whilst adrenaline and noradrenaline affinity was decreased by a similar magnitude. The decreased agonist affinity was highlighted in that adrenaline and noradrenaline inhibition of [^{3}H]-RS-15385-197 binding was decreased by Na^+ and the Hill slopes were closer to unity, suggesting a predominance of a low affinity agonist state. Similar changes in the affinity of noradrenaline and adrenaline were observed in the human platelet. The affinities of the imidazoline agonists, oxymetazoline and UK 14304, however, were unchanged by Na^+ when inhibiting [^{3}H]-adrenaline binding, and there was only a small decrease in the affinity of oxymetazoline for [^{3}H]-RS-15385-197 binding. These data suggest that the interaction of imidazoline and catecholamine agonists with the α_2-receptor may be different, and support an earlier hypothesis by Ruffolo *et al.* (1977).

A recent study has identified a specific aspartate residue (asp79) on the porcine α_{2A}-adrenoceptor to which Na^+ binds and exerts its allosteric effects (Horstman *et al.*, 1990). By mutating this residue to an asparagine residue, the mutatant receptor resembled the wild-type receptor in the presence of Na^+, but, further allosteric effects of Na^+ were prevented. An aspartate residue reported to lie in a topographically identical position (asp63) has been detected in the derived amino acid sequence from a rat cDNA encoding an α_{2B}-adrenoceptor (Zeng *et al.*, 1990). Thus, it appears that Na^+ plays a crucial role in the regulation of α_{2A}-and α_{2B}-adrenoceptor function via an interaction with a specific amino acid residue.

3.9. CONCLUSIONS

In conclusion, this study has demonstrated that the new α_2-adrenoceptor antagonist ligand [^{3}H]-RS-15385-197, labelled α_{2A}- and

α_{2B}-adrenoceptor subtypes in human platelet and neonatal rat lung membranes, and an additional subtype, similar to the α_{2D}-adrenoceptor, in rat cerebral cortex. However, [^{3}H]-adrenaline binding to the α_{2A}- and α_{2B}-adrenoceptor subtypes revealed that agonist binding to these receptors was essentially the same. The study emphasised, that to accurately characterise receptor subtypes in ligand binding studies, the receptor should be studied with both an agonist and an antagonist ligand. The functional consequences of α_{2A}- and α_{2B}-adrenoceptor activation will be addressed in Chapter five of this thesis.

The allosteric effect of Na^+ on the α_{2B}-adrenoceptor was to increase the density of sites in a low affinity (catecholamine insensitive) agonist state, whilst increasing the affinity of antagonists. Although qualitatively similar effects were observed at the α_{2A}-adrenoceptor there was no increase in the density of agonist sites labelled. Competition experiments in the presence of 10 and 100 mM NaCl revealed, that UK 14304 and oxymetazoline inhibition curves were less sensitive to Na^+, suggesting differences in the interaction of the catecholamines and the imidazolines with the receptor. The impact of this work for future studies on α_2-adrenoceptors is considered in Chapter six of this thesis.

CHAPTER FOUR

CHARACTERISATION OF IMIDAZOLINE BINDING SITES

4.1. INTRODUCTION

In Chapter one of this thesis the discovery of imidazoline binding sites distinct from the α_2-adrenoceptor was described, and how structures with an imidazoline moeity were found to interact with these sites with differing degrees of affinity. To date, the classification of these sites is defined with [^{3}H]-idazoxan binding in peripheral tissues and with [^{3}H]-clonidine or [^{3}H]-*p*-aminoclonidine in the brain. In this chapter, the pharmacology of [^{3}H]-idazoxan and [^{3}H]-*p*-aminoclonidine binding sites is compared in the same tissue; the rat kidney, to determine whether imidazoline site heterogeneity exists within a tissue. In addition a novel, selective, imidazoline ligand [^{3}H]-RS-45041-190 will be described. The binding characteristics, distribution in rat brain, and its contribution to the understanding of imidazoline site pharmacology will be discussed.

4.2. [^{3}H]-IDAZOXAN BINDING TO RAT KIDNEY

4.2.1. [^{3}H]-idazoxan binding to α_2-adrenoceptors

Initial studies with [^{3}H]-idazoxan were performed in the absence of α_2-antagonist to assess the contribution of α_2-adrenoceptor binding in the rat kidney preparation. Inhibition of total [^{3}H]-idazoxan binding by noradrenaline, RS-15385-197 and idazoxan are shown in Figure 4.1.. RS-15385-197 displaced < 10% [^{3}H]-idazoxan binding at 0.1 mM, whereas noradrenaline displaced 40-50% with low affinity (pIC_{50} = 4.86 ± 0.30, n=3) and unlabelled idazoxan displaced 80% of the total binding with high affinity (pIC_{50} = 8.15, nH = 0.89, n=2). Due to the low affinity of noradrenaline and RS-15385-197, it was concluded that [^{3}H]-idazoxan did not label α_2-adrenoceptors in rat kidney at 1 nM. However, subsequent experiments with [^{3}H]-idazoxan were carried out in the presence of 0.1 μM RS-15385-197 to exclude possible binding to α_2-adrenoceptors at higher ligand concentrations and tissue variations.

4.2.2. Kinetics of [^{3}H]-idazoxan binding to imidazoline sites

The interaction of [^{3}H]-idazoxan with the imidazoline binding site on rat kidney membranes was rapid and reversible (Figure 4.2.). Equilibrium was attained within 90 min with K_{obs} = 0.0727 ± 0.0065/min (n=5). In 3 out of 5 experiments however, the association

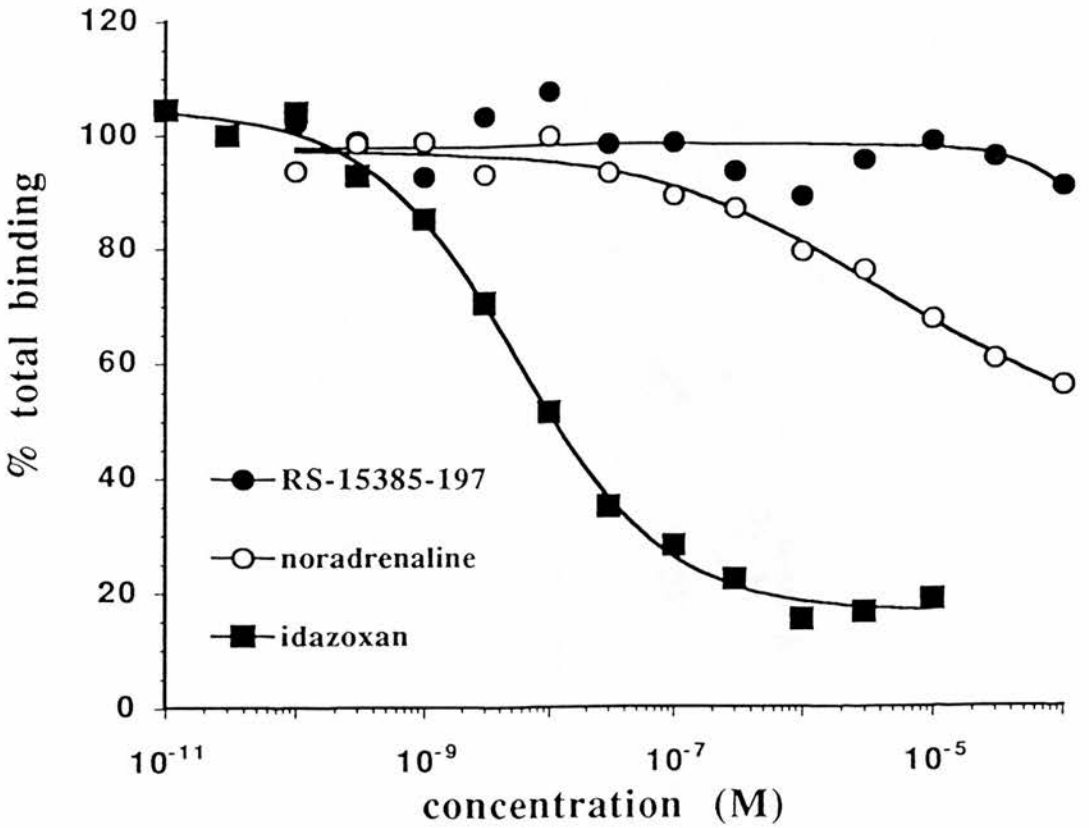


Figure 4.1. Inhibition of [^{3}H]-idazoxan binding in rat kidney membranes. The results represent a typical experiment performed in duplicate. Essentially similar data was obtained in one (idazoxan) or two (RS-15385-197 and noradrenaline) other experiments on different preparations (see text for meaned values). Idazoxan displaced 80% of the total binding (pIC_{50} = 8.22), whereas noradrenaline displaced 46% (pIC_{50} = 5.34) and RS-15385-197 displaced <10% of the binding at 0.1 mM.

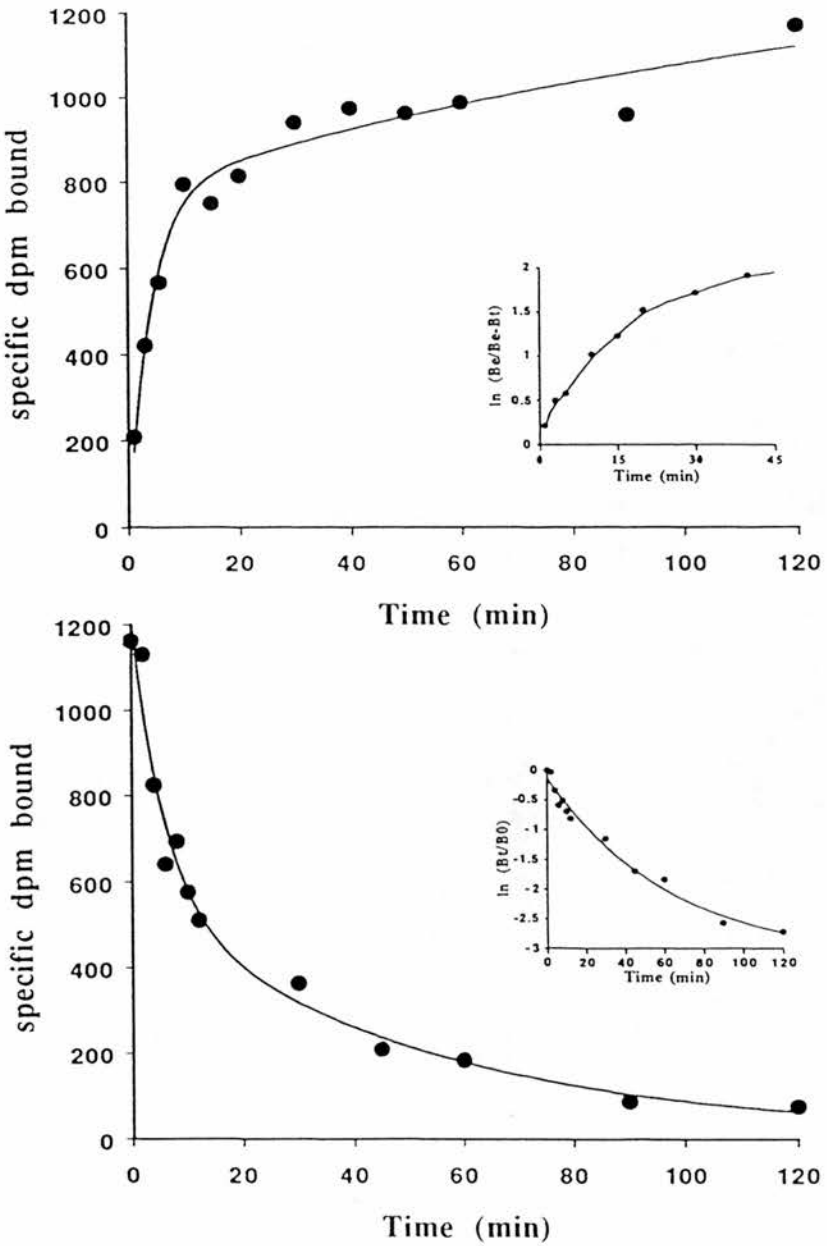


Figure 4.2. Kinetic analysis of [^{3}H]-idazoxan binding to rat kidney membranes. The insets show the semilogarithmic plots of the data. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in two other experiments on different preparations (see text for meaned values). Two association rate constants (top panel) were calculated; K_{obs1} = 0.307/min and K_{obs2} = 0.0299/min. On the addition of 1 μM cirazoline, [^{3}H]-idazoxan dissociated with two rate constants K_{21} = 0.1701/min and K_{22} = 0.0197/min (bottom panel). Calculated K_d values for the fast and slow components were 2.23 and 3.46 nM respectively.

log plots were better fitted to 2 site model with $t_{1/2}$ for the fast and slow associating components = 1.59 and 30.1 min respectively (% contribution = 62 and 38 respectively). On the addition of 1 μM cirazoline, [3H]-idazoxan binding was fully reversed but showed fast and slow dissociation components, with 65% of the specific [3H]-idazoxan binding dissociated within the first 15 min (K_2 = 0.5176 ± 0.1823/min, $t_{1/2}$ = 1.3 min). The remaining binding dissociated more slowly (K_2 = 0.0234 ± 0.007/min, $t_{1/2}$ = 30 min). In experiments showing biphasic association plots K_d values were calculated based on the percentage of ligand associating and dissociating rapidly and yielded affinity constants for the fast and slow dissociation components of 4.38 ± 1.10 and 2.24 ± 1.23 nM respectively (n=3).

4.2.3. Affinity and density of imidazoline sites labelled by [3H]-idazoxan

In saturation studies (Figure 4.3.), [3H]-idazoxan labelled a single site with high affinity in the presence of 0.1 μM RS-15385-197 (K_d = 3.78 ± 0.28 nM, B_{max} = 45.94 ± 11.4 fmoles/mg, n = 4). The slope of the associated Hill plot (nH) = 0.95 ± 0.06. At the K_d concentration, non-specific [3H]-idazoxan binding represented 25-35% of the total binding. The inability of saturation analysis to detect two binding sites (as derived from kinetic experiments) is probably due to the fact that [3H]-idazoxan had similar affinity for these sites (K_d = 4.38 and 2.24 nM). It is unlikely that saturation analysis would differentiate two binding sites in this case.

4.2.4. Pharmacology of [3H]-idazoxan binding

Table 4.1. shows affinity values (pK_i) for a variety of agents for the imidazoline binding site labelled by [3H]-idazoxan. Noradrenaline and RS-15385-197 had low affinity, emphasising the non-adrenergic nature of the interaction. Clonidine and *p*-aminoclonidine showed low affinity (pK_i = 5.64 and 5.05) and the imidazolines phentolamine and cimetidine also had low affinity (pK_i = 4.48 and 4.31). However, some imidazoline structures, idazoxan and the α_1-adrenoceptor agonist cirazoline, showed high affinity (pK_i = 7.95 and 8.48 respectively). High affinity was not restricted to compounds containing an imidazoline ring as guanabenz, which has a guanido structure, had high affinity (pK_i = 7.26). The K+ sparing diuretic, amiloride, which shows high

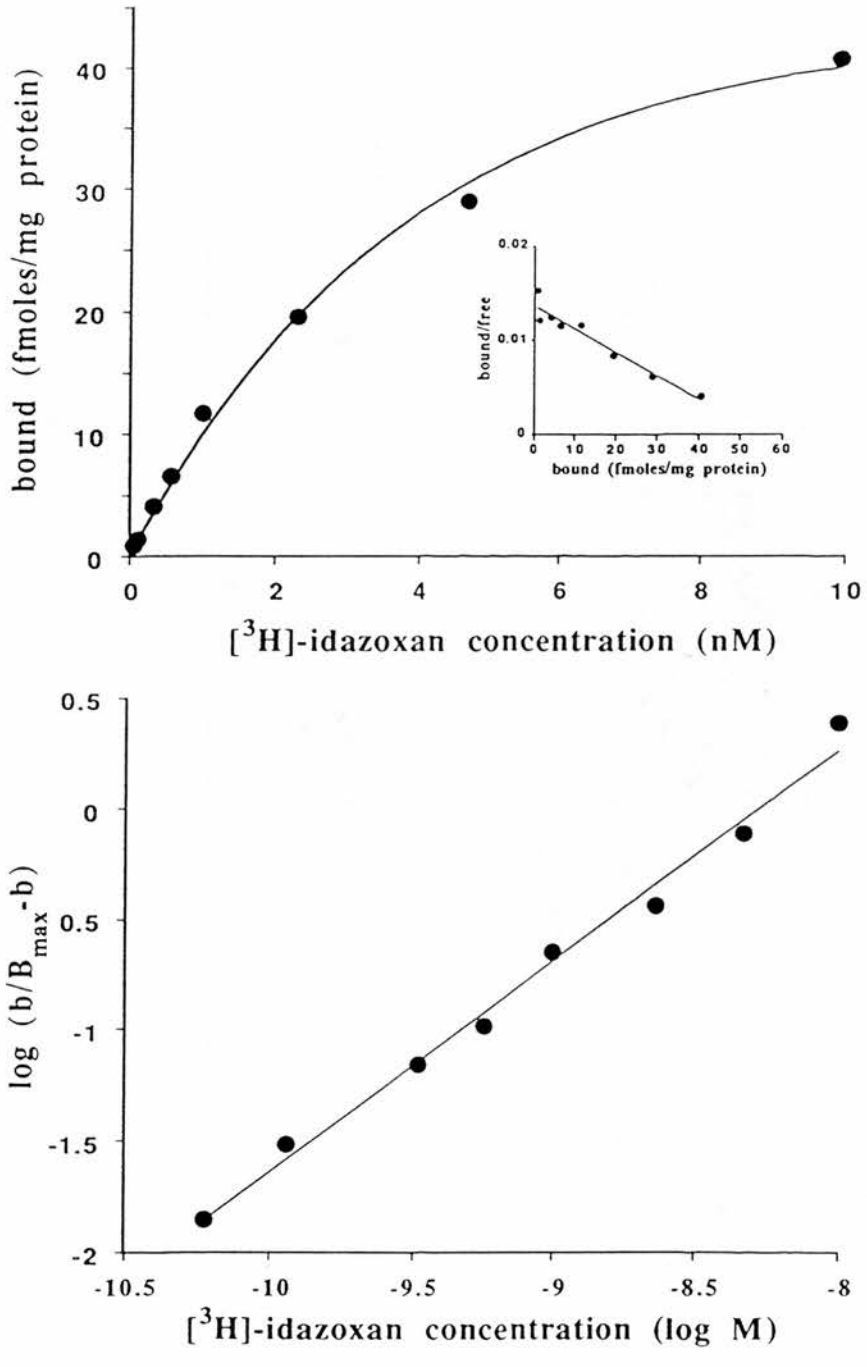


Figure 4.3. Saturation analysis of [3H]-idazoxan binding to rat kidney membranes. The inset shows the Scatchard plot of the data and the associated Hill plot is shown in the bottom panel. The results represent a single experiment performed in triplicate. Essentially similar data was obtained in three other experiments on different preparations (see text for meaned values). The affinity (K_d) = 3.35 nM and the B_{max} = 57.2 fmoles/mg protein. The Hill coefficient (nH) = 0.953.

Table 4.1. [^{3}H]-Idazoxan binding to imidazoline sites on rat kidney membranes.

compound	n	pK_i	nH
RS-45041-190	3	8.66 ± 0.09	0.92 ± 0.08
cirazoline	3	8.48 ± 0.10	0.96 ± 0.04
idazoxan	4	8.06 ± 0.16	1.00 ± 0.01
guanabenz	3	7.26 ± 0.07	0.90 ± 0.10
amiloride	3	6.36 ± 0.06	0.92 ± 0.08
clonidine	5	5.64 ± 0.16	0.92 ± 0.08
p-aminoclonidine	6	5.05±0.16	1.02 ± 0.10
noradrenaline	4	4.53 ± 0.20	-
phentolamine	3	4.48 ± 0.07	-
cimetidine	3	4.31 ± 0.19	-
RS-15385-197	3	4.26 ± 0.21	-
GTP	3	3.48 ± 0.07	-

Compounds were incubated with 1 nM [^{3}H]-idazoxan and 0.1 μM RS-15385-197 for 90 min at 25°C as described in Chapter two. Values represent the mean ± s.e.mean. Each assay was performed in duplicate and the number of determinations on separate preparations is indicated by n.

affinity for [^{3}H]-idazoxan binding in rabbit tissues (see chapter one and Michel *et al.*, 1989a), had only moderate affinity (pKi = 6.36). The most potent compound was RS-45041-190 (pK_i = 8.66), a novel imidazoline agent which has low affinity (pKi < 5) for a wide range of other receptor types including α_2- and α_1-adrenoceptors (see Table 1.5). GTP inhibited [^{3}H]-idazoxan binding (pK_i = 3.48), however, in saturation experiments (Figure 4.4.) 300 μM GTP had no significant on the K_d or B_{max} (control; K_d = 3.78 ± 0.28 nM, B_{max} = 45.94 ± 8.6 fmoles/mg protein, GTP; K_d = 4.05 ± 0.70 nM, B_{max} = 41.7 ± 2.9 fmoles/mg, n=3).

Figure 4.5. shows inhibition curves for the α_1-adrenoceptor agonist compounds naphazoline and cirazoline for [^{3}H]-idazoxan binding to imidazoline sites in the rat kidney. The inhibition curve for cirazoline was monophasic and of high affinity (pK_i = 8.48 ± 0.10, n=3), however, the inhibition curve for naphazoline was better fitted to a 2 site model and gave affinity values for the high and low affinity components of 8.65 ± 0.18 and 6.88 ± 0.21 respectively (n=4), with the high affinity component comprising 68 ± 6% of the specifically bound [^{3}H]-idazoxan. In the rat kidney therefore, these studies suggest that [^{3}H]-idazoxan labels two populations of imidazoline sites with similar affinity in the presence of RS-15385-197 to exclude binding to α_2-adrenoceptors, and these sites could be differentiated with naphazoline.

4.3. [^{3}H]-*p*-AMINOCLONIDINE BINDING IN RAT KIDNEY

4.3.1. [^{3}H]-*p*-Aminoclonidine binding to α_2-adrenoceptors

Initial studies with [^{3}H]-*p*-aminoclonidine were performed at 25°C. In competition experiments (Figure 4.6.) RS-15385-197 and noradrenaline showed high affinity for 50-60% of the total [^{3}H]-*p*-aminoclonidine binding (pIC_{50} = 8.81 ± 0.09, n=5 and 7.23 ± 0.48, n=3 respectively), whereas clonidine displaced 70-80% (pIC_{50} = 7.64, n=2). These initial studies suggested that ~60% of the [^{3}H]-*p*-aminoclonidine binding was to α_2-adrenoceptors (high affinity for noradrenaline and RS-15385-197) and the remaining binding was insensitive to noradrenaline but displaceable with clonidine (non-adrenergic).

In the following work, to study the non-adrenergic component of

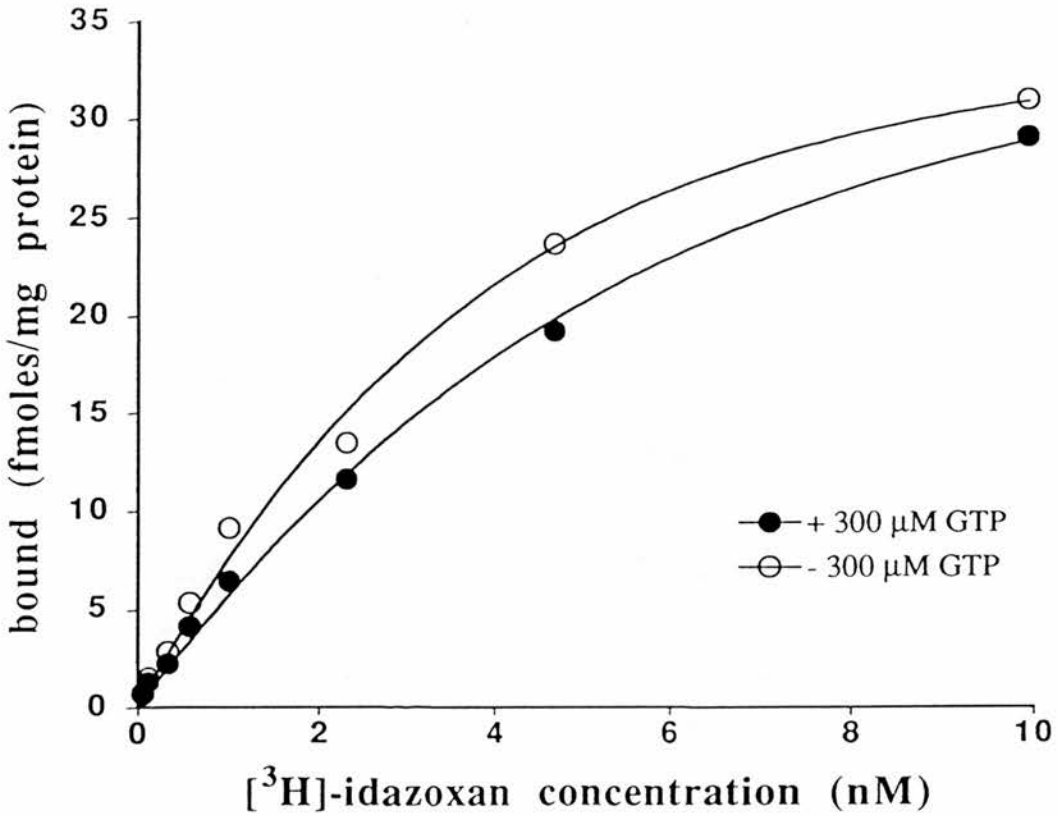


Figure 4.4. Effect of 300 μM GTP on [3H]-idazoxan binding in rat kidney. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in two other experiments on different preparations (see text for meaned values). Control, K_d = 3.22 nM, B_{max} = 40.01 fmoles/mg protein; GTP K_d = 3.54 nM, B_{max} = 36.1 fmoles/mg protein.

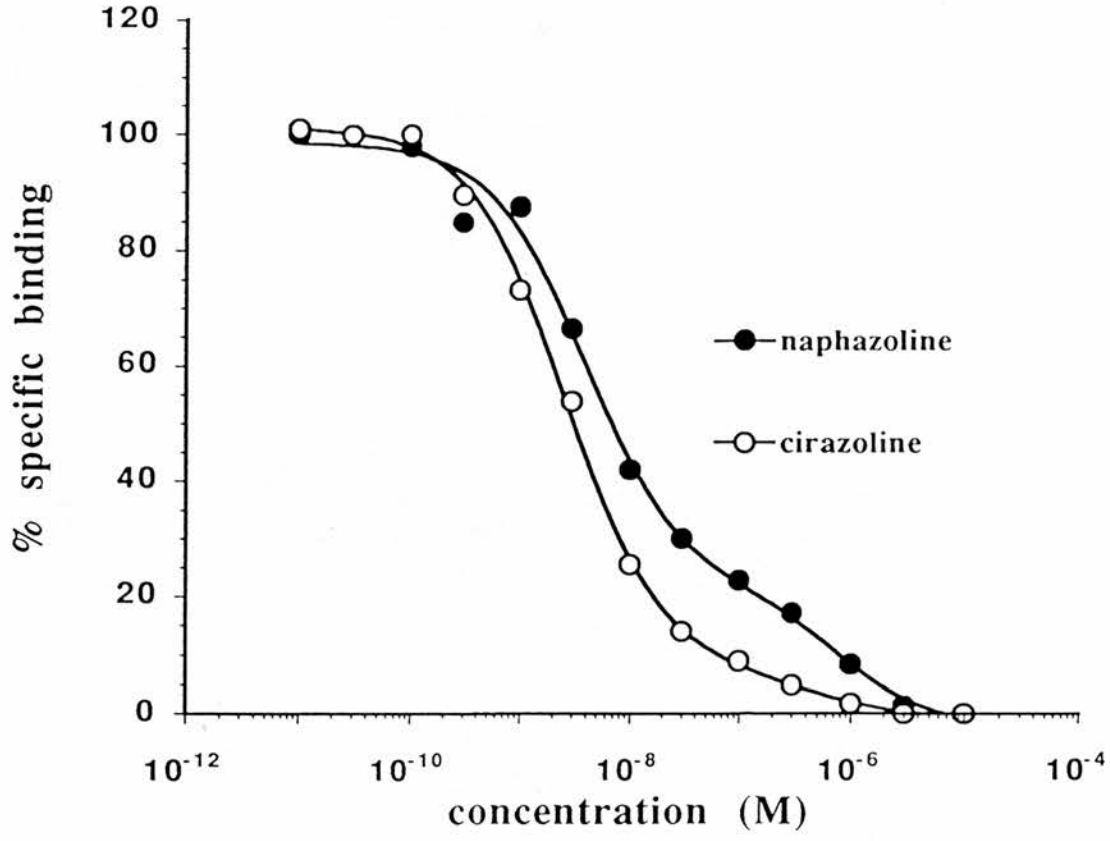


Figure 4.5. Inhibition of 1 nM [^{3}H]-idazoxan binding in rat kidney by cirazoline and naphazoline. The data represents a single experiment performed in duplicate. Essentially similar data was obtained in at least two other experiments on different preparations (see text for meaned values). One and two site fits were compared for the data. The cirazoline curve was better fitted to a 1 site model $pIC_{50} = 8.52$, whereas the naphazoline curve was better fitted to a two site model with pIC_{50} values for the high and low affinity components = 8.32 (75%) and 6.18 (25%).

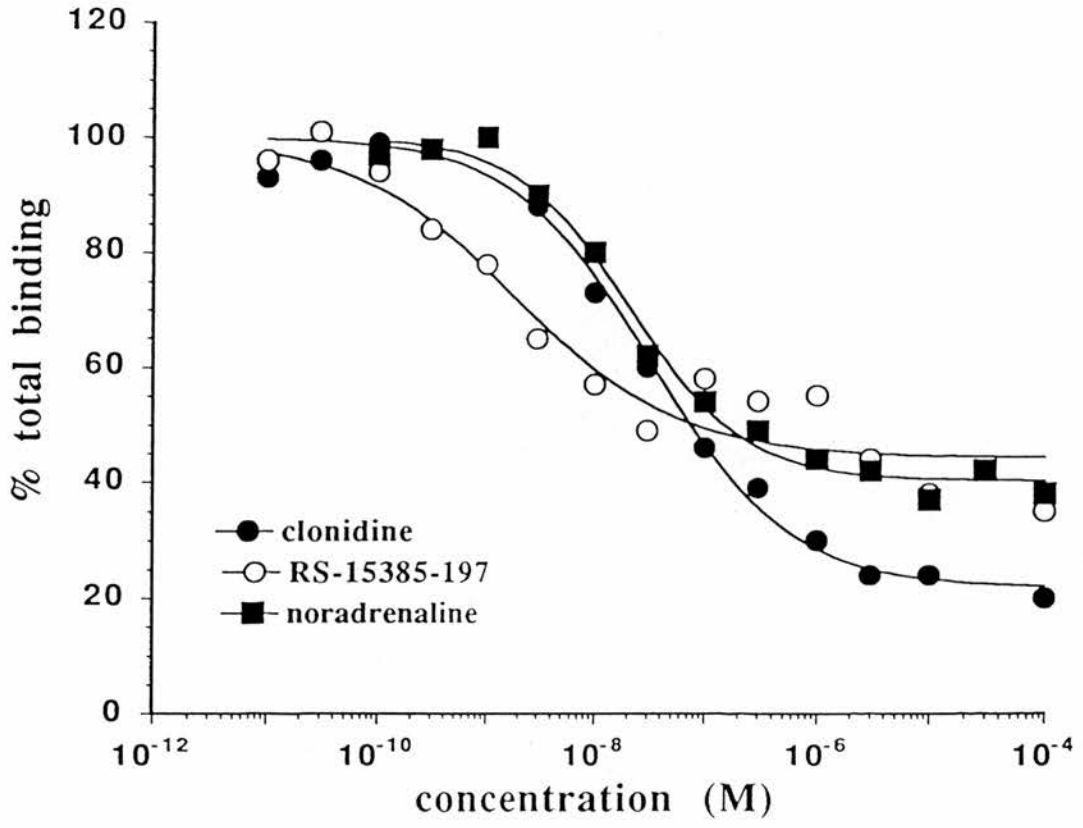


Figure 4.6. Inhibition of 3 nM [^{3}H]-*p*-aminoclonidine binding from rat kidney membranes. Incubations were performed 25°C. The data represents a single experiment performed in duplicate. Essentially similar data was obtained in at least one other experiment on a different preparation (see text for meaned values). RS-15385-197 displaced 56% and noradrenaline 60% with affinity values pIC_{50} = 8.73 and 7.68 respectively. Clonidine displaced 78% with pIC_{50} = 7.49.

the binding in isolation, and to make the data comparable with the [3H]-idazoxan studies, experiments with [3H]-*p*-aminoclonidine were performed in the presence of 0.1 μM RS-15385-197 to prevent binding to α_2-adrenoceptors.

4.3.2. Kinetics of [3H]-*p*-aminoclonidine binding to imidazoline sites

Initial studies with [3H]-*p*-aminoclonidine showed the radioligand to be fully associated within 1 min at 25oC and fully dissociated on the addition of 100 μM clonidine within 90 seconds. Experiments with [3H]-*p*-aminoclonidine were subsequently performed at 4oC to slow the reaction rate, in an attempt to establish the kinetically derived affinity of [3H]-*p*-aminoclonidine, and to minimise dissociation of the ligand during filtration. Figure 4.7. shows that even at 4oC the kinetics for [3H]-*p*-aminoclonidine were rapid with equilibrium being attained within 10 min. On the addition of 0.1 mM clonidine, [3H]-*p*-aminoclonidine binding was rapidly reversed and fully dissociated within 10 min. Due to the rapid rate of association and dissociation it was not possible to determine reliable estimations of association and dissociation rate constants for [3H]-*p*-aminoclonidine in consecutive experiments.

4.3.3. Affinity and density of sites labelled with [3H]-*p*-aminoclonidine

To determine the saturability of [3H]-*p*-aminoclonidine binding, K_d and B_{max} values were calculated from competition experiments with unlabelled *p*-aminoclonidine. This was found to be necessary as the low affinity of the ligand required concentrations of [3H]-*p*-aminoclonidine greater than 100 nM to approach saturation. K_d and B_{max} values from competition experiments with unlabelled *p*-aminoclonidine were calculated from a knowledge of the IC_{50} and free ligand concentration, and based on the assumption that labelled and unlabelled compound bind to the same site with equal affinity (Deblasi *et al.*, 1990). Figure 4.8. shows that [3H]-*p*-aminoclonidine binding was monophasic and of low affinity (K_d = 127.6 ± 19.7 nM) and labelled more than 20 times the number of sites than [3H]-idazoxan (B_{max} = 978 ± 172 fmoles/mg protein, n=8; compared to 45.94 ± 11.4 fmoles/mg protein for [3H]-idazoxan). The percentage of non-specific binding was greater than that for [3H]-idazoxan and represented 40-50% of the total [3H]-*p*-

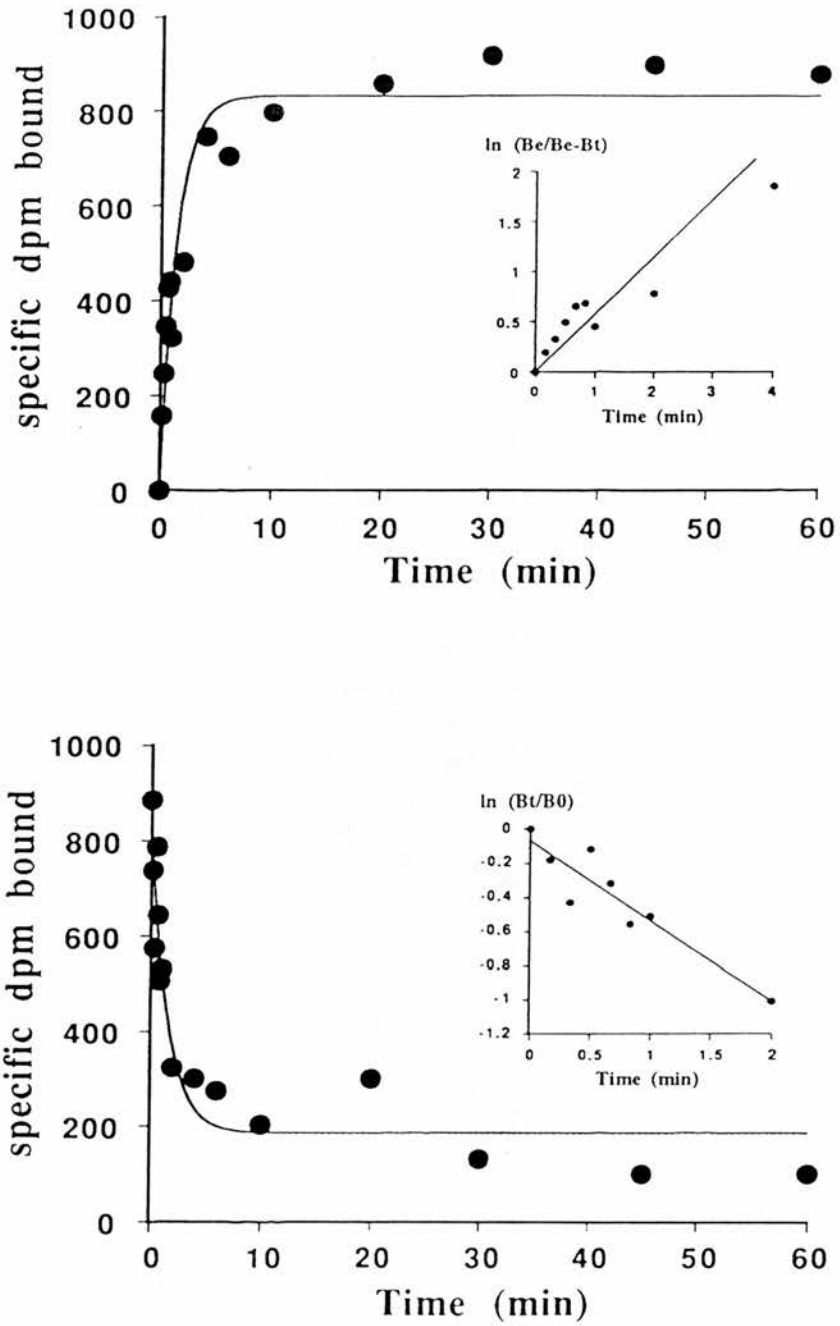


Figure 4.7. Kinetic analysis of [^{3}H]-*p*-aminoclonidine binding in rat kidney membranes at 4^{o}C. The insets show the semilogarithmic plots of the data. The data represents a single experiment performed in triplicate. The association rate (top panel) was rapid, and the ligand fully associated after 10 min (K_{obs} = 0.707/min). The dissociation rate (bottom panel) was also rapid, with 66% of the bound ligand dissociated within the first 2 min (K_2 = 0.681/min), giving a derived affinity K_d = 81.2 nM. Accurate determination of the rates in consecutive experiments was not possible due to the rapidity of the reaction.

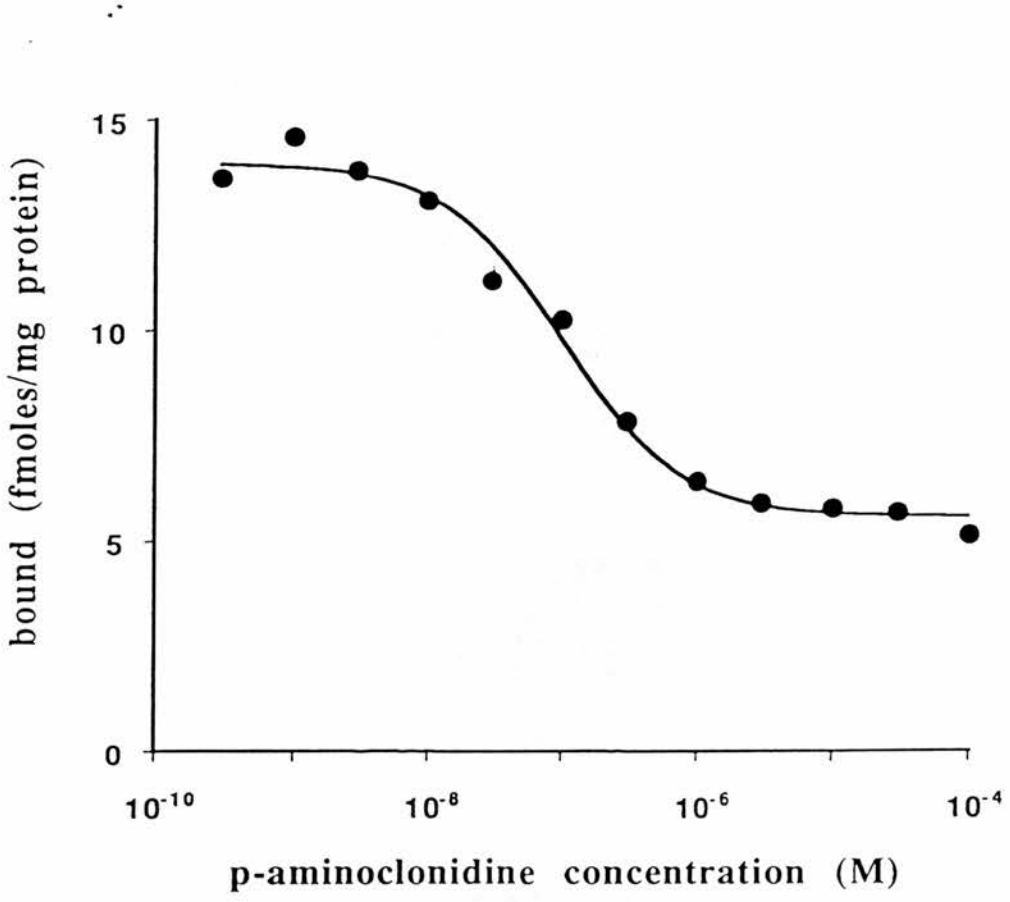


Figure 4.8. Saturation analysis of [^{3}H]-*p*-aminoclonidine binding to rat kidney membranes. Experiments were performed with 3 nM [^{3}H]-*p*-aminoclonidine and increasing concentrations of unlabelled *p*-aminoclonidine. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in seven other experiments on different preparations (see text for meaned values). K_d = 127 nM, B_{max} = 1020 fmoles/mg protein; nH = 1.02.

aminoclonidine binding.

Due to the low affinity of [^{3}H]-*p*-aminoclonidine, it was surprising that specific binding could be demonstrated using filtration to separate bound from free ligand, as a high percentage of the specifically bound ligand would be expected to dissociate during the separation process. Figure 4.7. showed that 25-40 % of the specifically bound [^{3}H]-*p*-aminoclonidine dissociated rapidly between 0 and 30 sec after the addition of 100 μM clonidine. It was possible that a large percentage of bound ligand would be lost during the washing stage of the filtration process. This problem was addressed in two ways. Firstly, using filtration to separate bound ligand, [^{3}H]-*p*-aminoclonidine binding was determined at equilibrium with different wash times with ice cold assay buffer. Secondly, bound [^{3}H]-*p*-aminoclonidine was separated by centrifugation at 15000g for 90 sec. Figure 4.9. shows that between a 2 and 10 sec wash, no significant decrease in specific binding was observed (24.2 ± 3.1 fmoles/mg at 2 sec, 22.7 ± 1.7 fmoles/mg at 10 sec) suggesting minimal dissociation occurred at these wash times. Thereafter, [^{3}H]-*p*-aminoclonidine dissociated with time with less than 40% of the initial binding remaining after 2 min. The washtime of 10 sec used in previous experiments with [^{3}H]-*p*-aminoclonidine was therefore maintained in subsequent experiments, as this was shown to minimise ligand dissociation whilst ensuring removal of unbound ligand.

When centrifugation was used to separate bound radioligand, the affinity and density of sites labelled by [^{3}H]-*p*-aminoclonidine was not significantly different from the filtration assays (K_d = 104.2 ± 21.7 nM, B_{max} = 1480 ± 310 fmoles/mg protein, n=3). These data suggested that, the percentage of [^{3}H]-*p*-aminoclonidine binding lost during filtration and centrifugation experiments was comparable as similar binding parameters were gained using both protocols. It was decided that for ease of sample preparation and throughput, filtration could be justifiably employed to characterise the pharmacology of the binding site. However, for compounds critical to the classification of imidazoline sites, centrifugation experiments were carried out in parallel.

4.3.4. Pharmacology of [^{3}H]-*p*-aminoclonidine binding sites

Table 4.2. shows affinity values (pK_i) for a variety of agents for

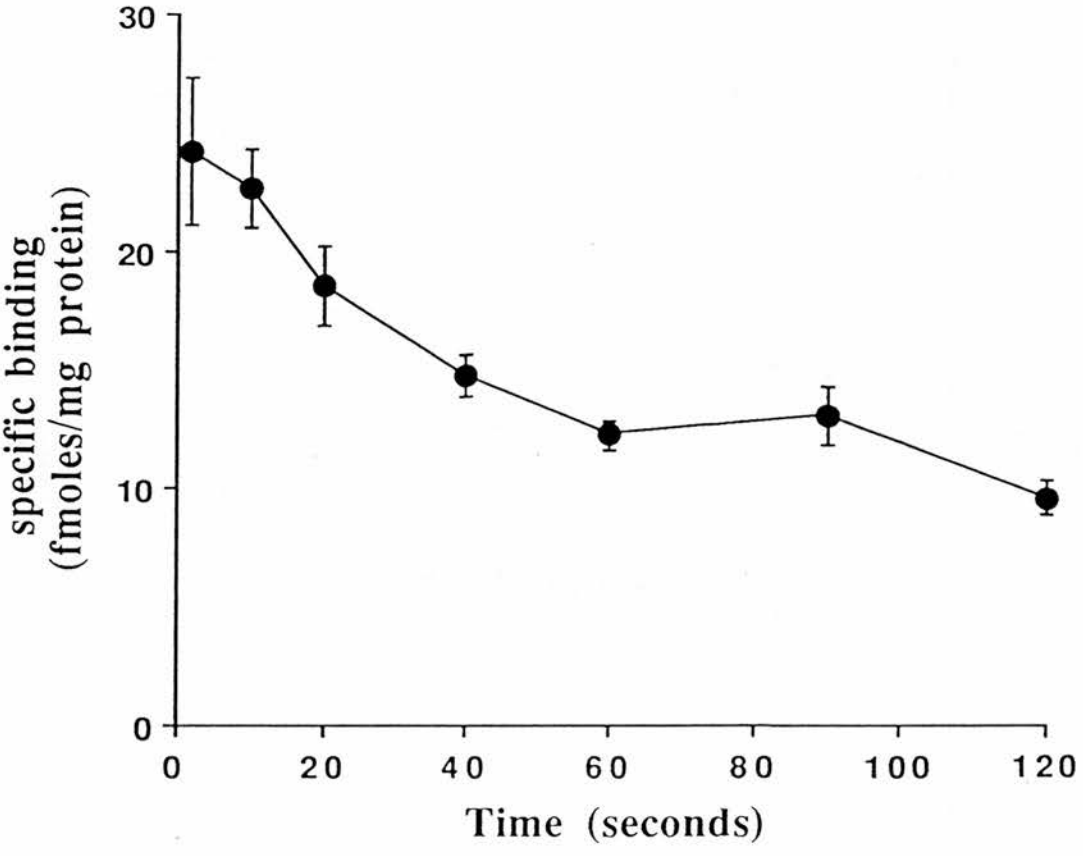


Figure 4.9. Effect of washing [^{3}H]-*p*-aminoclonidine bound to rat kidney membranes on glass fibre filters. Membranes were incubated with 3 nM [^{3}H]-*p*-aminoclonidine as described in Chapter two and filtered over Whatman GF-B filters followed by various wash times with ice-cold assay buffer. The data represents the mean ± s.e.mean of three experiments on different membrane preparations each performed in triplicate.

Table 4.2. [^{3}H]-*p*-Aminoclonidine binding to imidazoline sites on rat kidney membranes.

	[^{3}H]-*p*-aminoclonidine				
	filtration			centrifugation	
	pK_i/pIC_{50}	nH		pK_i	nH
guanabenz	7.81 ± 0.06	1.25 ± 0.11	(3)	7.29±0.37	1.05 ± 0.44 (3)
RS-45041-190	7.18 ± 0.09	0.92 ± 0.10	(3)	-	-
clonidine	6.88 ± 0.07	0.80 ± 0.06	(3)	-	-
p-aminoclonidine	6.98 ± 0.09	0.87 ± 0.07	(6)	6.87 ± 0.21	1.02 ± 0.12 (3)
idazoxan	5.34 ± 0.04	0.77 ± 0.11	(3)	5.34 ± 0.25	1.05 ± 0.05 (3)
phentolamine	5.10 ± 0.23	1.01 ± 0.01	(3)	-	-
cimetidine	4.99 ± 0.09	-	(3)	-	-
RS-15385-197	4.85 ± 0.13	-	(3)	-	-
amiloride	< 4	-	(3)	-	-
noradrenaline	< 4	-	(3)	-	-
GTP	< 3	-	(3)	-	-

Compounds were incubated with 3 nM [^{3}H]-*p*-aminoclonidine and 0.1 µM RS-15385-197 for 30 min at 4°C as described in Chapter two. The values represent the pK_i (nH = 1) or pIC_{50} (nH < 1). Values were obtained from filtration or centrifugation experiments as indicated and represent the mean ± s.e.mean. Each assay was performed in duplicate and the number of separate determinations on different membrane preparations is shown in brackets.

the imidazoline binding site labelled by [^{3}H]-*p*-aminoclonidine. Noradrenaline and RS-15385-197 had low affinity (pKi < 5) emphasising the non-adrenergic nature of the interaction. Clonidine and *p*-aminoclonidine showed moderately high affinity (pK_i = 6.88 and 6.98 respectively), whereas idazoxan and amiloride had very low affinity (pK_i = 5.34 and < 4.00 respectively). Guanabenz and RS-45041-190 showed moderately high affinity (pK_i = 7.26 and 7.18 respectively), while the imidazolines phentolamine and cimetidine had low affinity (pK_i = 5.10 and 4.99). To ensure that the pharmacology of the [^{3}H]-*p*-aminoclonidine binding site was the same irrespective of the method employed to separate bound ligand, the affinity of guanabenz, idazoxan and *p*-aminoclonidine were estimated for [^{3}H]-*p*-aminoclonidine binding using centrifugation to separate bound ligand. The affinity of these compounds was not significantly different from filtration affinity estimates (pK_i = 7.29, 5.34 and 6.87 respectively). GTP did not inhibit [^{3}H]-*p*-aminoclonidine binding up to a concentration of 1 mM. The observation that GTP inhibited [^{3}H]-idazoxan (Table 4.1.) but not [^{3}H]-*p*-aminoclonidine binding in competition experiments, suggested that [^{3}H]-idazoxan may label an agonist GTP-sensitive state of the imidazoline "receptor", while [^{3}H]-*p*-aminoclonidine may label both affinity states (i.e. an antagonist), in the same way that [^{3}H]-RS-15385-197 and [^{3}H]-adrenaline label affinity states of the α_2-adrenoceptor (see Chapter three). Experiments were carried out, therefore, to determine the effect of GTP on idazoxan inhibition curves to [^{3}H]-*p*-aminoclonidine (Figure 4.10.). Inhibition by idazoxan was conducted in the presence of 0.1 and 1 mM GTP (concentrations which inhibit [^{3}H]-idazoxan binding by approximately 25 and 60%) and no effect was seen on the affinity or the Hill slope of the idazoxan displacement curve (control: pIC_{50} = 5.39, nH = 0.83; 0.1 mM GTP pIC_{50} = 5.43, nH = 0.74; 1 mM GTP: pIC_{50} = 5.44, nH = 0.86; n=2).

These results show that imidazoline sites labelled by [^{3}H]-*p*-aminoclonidine are pharmacologically distinct from those labelled by [^{3}H]-idazoxan. It is unlikely that those sites labelled by [^{3}H]-idazoxan represent a GTP-sensitive agonist state of the imidazoline receptor labelled by [^{3}H]-*p*-aminoclonidine.

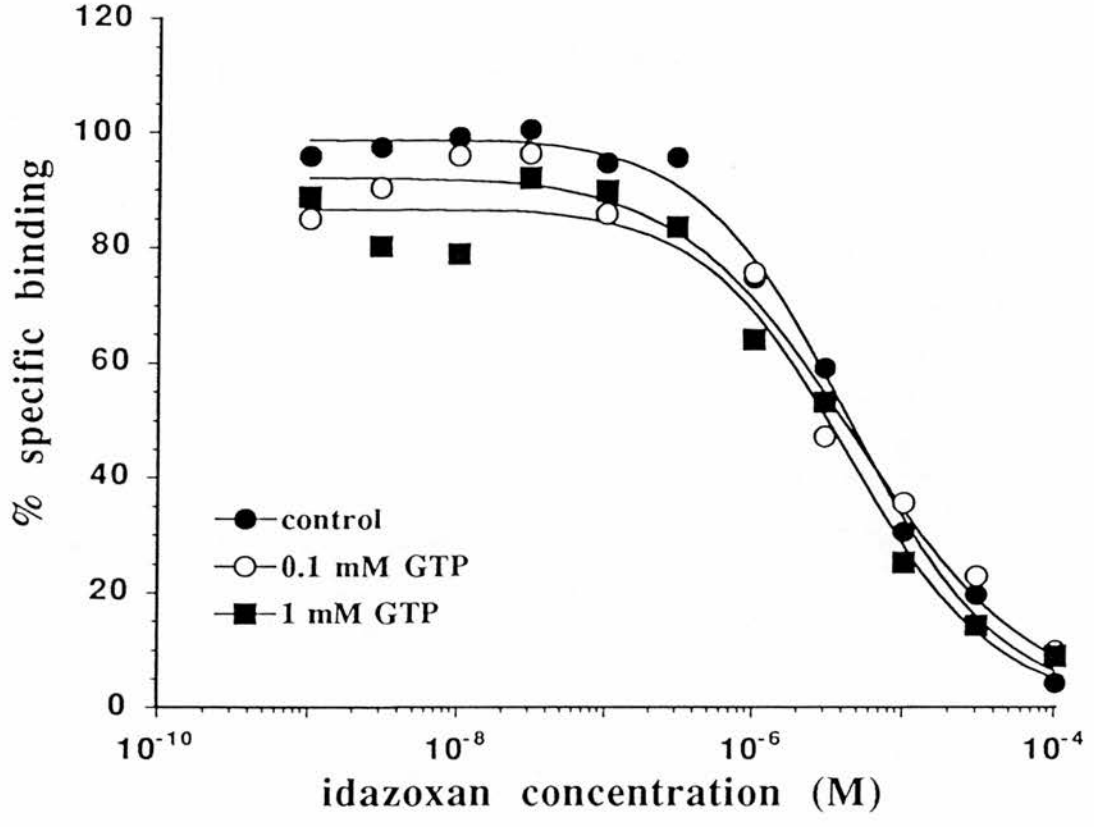


Figure 4.10. Effect of GTP on idazoxan inhibition of [^{3}H]-*p*-aminoclonidine binding to rat kidney membranes. Incubations were performed in the presence of 0.1 μM RS-15385-197 as described in Chapter two. The results represent one of two experiments on different preparations performed in duplicate. Control pIC_{50} = 5.39, 0.1 mM GTP pIC_{50} = 5.43, 1 mM GTP pIC_{50} = 5.44.

4.4. [3H]-RS-45041-190 BINDING TO RAT KIDNEY

4.4.1. Kinetics of [3H]-RS-45041-190 binding to rat kidney

[3H]-RS-45041-190 binding to rat kidney membranes was rapid and reversible (Figure 4.11.). Equilibrium was reached within 60 min, but the semilogarithmic transformations of the association plots were distinctly non-linear. Consequently the time course of [3H]-RS-45041-190 binding was fitted to two exponential phases of binding. The rate constant K_{obs} for the fast phase of binding was 1.395 ± 0.518/min with $t_{1/2}$ = 35 sec and represented 53 ± 6% of the total bound ligand at equilibrium. The remaining binding associated slowly 0.055 ± 0.035/min with $t_{1/2}$ = 12.6 min (n=4 in each case). After the addition of 10 μM cirazoline, [3H]-RS-45041-190 exhibited a biphasic dissociation plot with 57 ± 6% dissociating rapidly ($K_2 = 0.401 \pm 0.118$/min, $t_{1/2}$ = 1.7 min). The remaining binding dissociated slowly ($K_2 = 0.037 \pm 0.102$/min, $t_{1/2}$ = 19 min). K_d values were calculated based on the percentage of rapidly associating and dissociating components, thus, the amount of rapidly associating ligand was more similar to the amount of rapidly dissociating ligand (53% and 57% respectively). The calculation of K_d values based on the slowly associating, rapidly dissociating, components was inappropriate in some cases where K_2 was greater than K_{obs} therefore giving K_1 a negative value. Taking these criteria into account, K_d values of 0.573 ± 0.208 nM for the fast phase and 0.886 ± 0.154 nM for the slow phase were estimated. These results indicated that [3H]-RS-45041-190 labelled 2 sites or 2 affinity states of an imidazoline site on rat kidney.

4.4.2. Affinity and density of sites labelled with [3H]-RS-45041-190

[3H]-RS-45041-190 binding was of high affinity and saturable in the rat kidney (Figure 4.12.). The data was better fitted to a one site model ($K_d = 2.71 \pm 0.59$ nM, $B_{max} = 223.1 \pm 18.4$ fmoles/mg protein, n=6). At the K_d concentration the percentage of binding defined with 10 μM cirazoline was 70-75% of the total [3H]-RS-45041-190 bound. The failure of saturation analysis to define 2 sites for [3H]-RS-45041-190, was probably due to the very similar affinity shown for the two sites in kinetic experiments (see previous section).

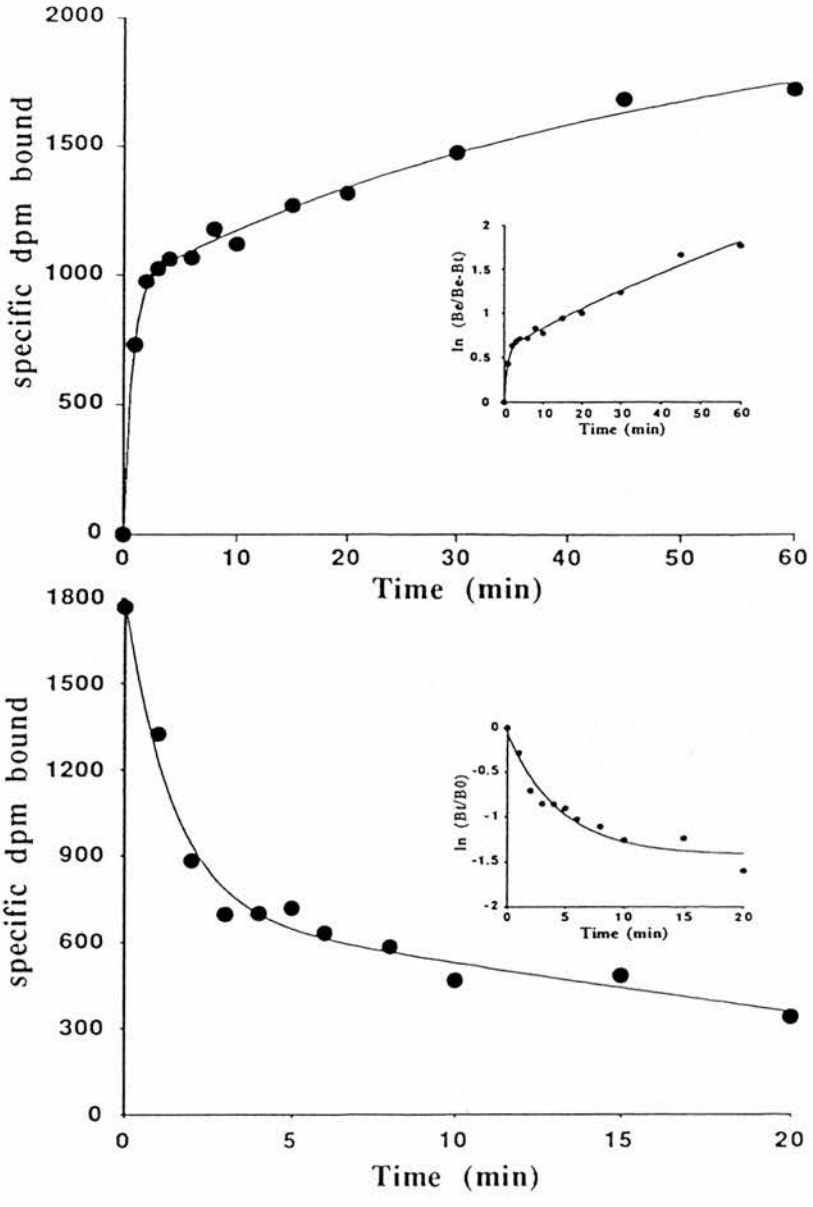


Figure 4.11. Association and dissociation of [^{3}H]-RS-45041-190 binding to rat kidney membranes. The insets show the semilogarithmic plots of the data. The data represents a single experiment performed in triplicate. Essentially similar data was obtained in three other experiments on different preparations (see text for meaned values). Two association rate constants (top panel) were calculated; K_{obs1} = 1.376, K_{obs2} = 0.0346/min. On the addition of 10 μM cirazoline, [^{3}H]-RS-45041-190 dissociated with two rate constants; K_{21} = 0.714, K_{22} = 0.0204/min. Calculated K_d values for the fast and slow components were 1.18 and 1.57 nM respectively.

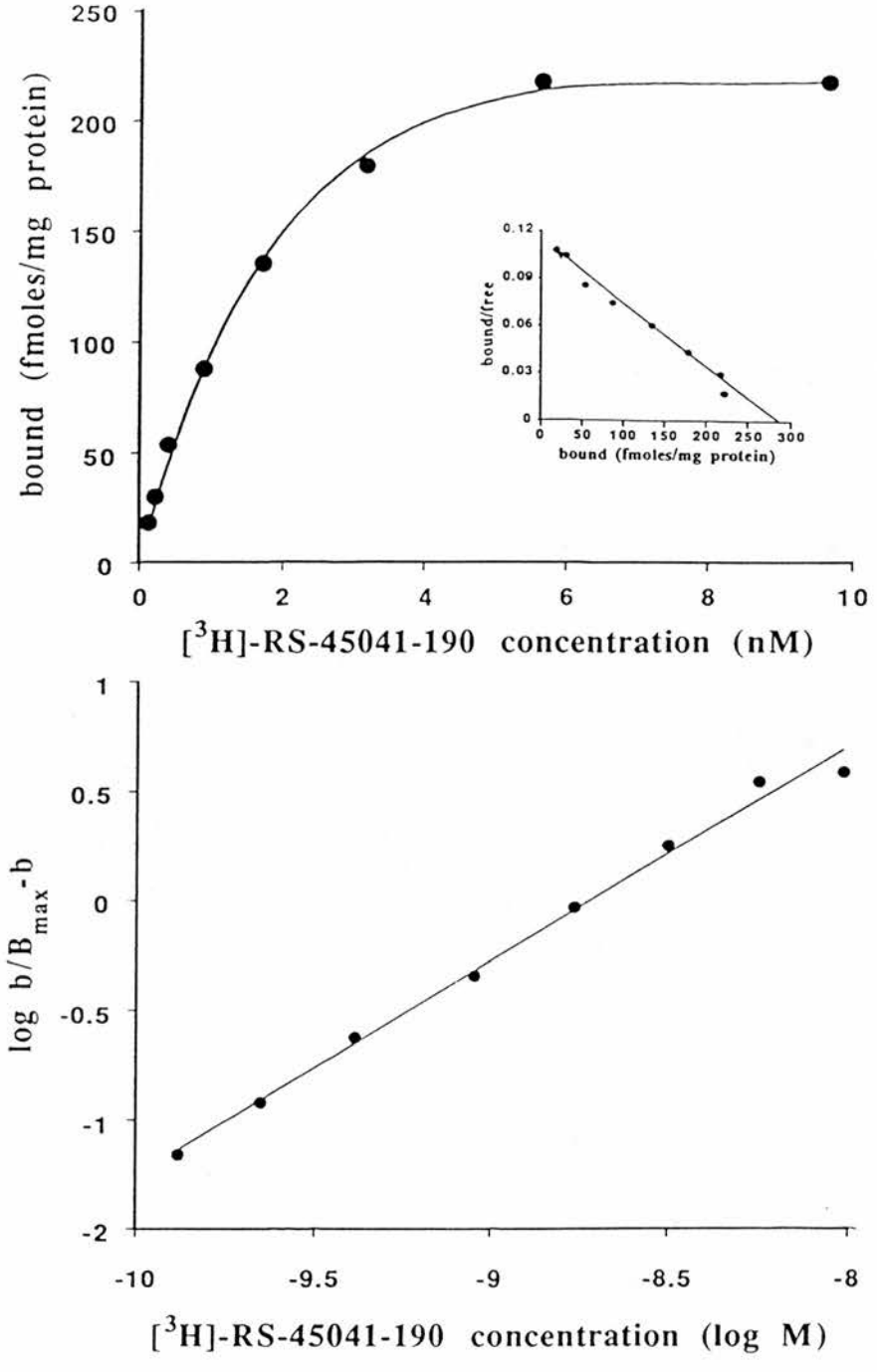


Figure 4.12. Saturation analysis of [^{3}H]-RS-45041-190 binding to rat kidney membranes. The data represents a typical experiment performed in triplicate. Essentially similar data was obtained in five other experiments on different preparations (see text for meaned values). The inset shows the Scatchard plot of the data and the associated Hill plot is shown in the bottom panel. The affinity (K_d) = 1.91 nM and the B_{max} = 280.1 fmoles/mg protein. The Hill coefficient (nH) = 0.979.

4.4.3. Pharmacology of [^{3}H]-RS-45041-190 binding sites in rat kidney

In competition experiments (Table 4.3.), adrenaline, RS-15385-197, dopamine, histamine and prazosin had very low affinity for [^{3}H]-RS-45041-190 ($pK_i < 5.1$). Inhibition curves with unlabelled RS-45041-190 showed high affinity (pK_i = 8.39) and a Hill slope of 0.75, indicating that more that one site was being labelled with [^{3}H]-RS-45041-190, however statistical analysis of the fits revealed that the data was not significantly better fitted to a two site model. The imidazoline compounds idazoxan and cirazoline showed high affinity, but displaced [^{3}H]-RS-45041-190 binding with high and low affinity components (pIC_{50} = 7.85 and 5.80 for idazoxan and 8.16 and 6.11 for cirazoline). Naphazoline displaced [^{3}H]-RS-45041-190 binding with moderate affinity and a shallow Hill slope (pIC_{50} = 6.61 ± 0.11, nH = 0.62 ± 0.03) although this was not significantly better fitted to two sites. Guanabenz showed high affinity for [^{3}H]-RS-45041-190 binding and *p*-aminoclonidine showed very low affinity. These data suggest that 60 - 70% of the sites labelled with [^{3}H]-RS-45041-190 were more similar to the site described for [^{3}H]-idazoxan in rat kidney (see Section 4.2.4.) and other rat tissues (MacKinnon *et al.*, 1991; Michel & Insel, 1989) than to the [^{3}H]-*p*-aminoclonidine binding site in rat kidney (see section Section 4.3.) or bovine brainstem (Ernsberger *et al.*, 1987). Glibenclamide, and phentolamine, compounds which have been shown to have K+ channel blocking activity and hypoglycaemic action (Schulz & Hasselblatt, 1989; Dunne, 1991), had low affinity for [^{3}H]-RS-45041-190 binding. Saturation experiments showed that [^{3}H]-RS-45041-190, labelled more sites than [^{3}H]-idazoxan ([^{3}H]-RS-45041-190 B_{max} = 223, [^{3}H]-idazoxan B_{max} = 46 fmoles/mg protein). These data suggest that [^{3}H]-RS-45041-190 labelled an imidazoline site with characteristics similar to that described for [^{3}H]-idazoxan, but also an additional site which is at present undefined.

4.4.4. Effect of ions and Gpp(NH)p on [^{3}H]-RS-45041-190 binding

In order to determine whether the [^{3}H]-RS-45041-190 binding site(s) couple to a G-protein, the regulation of binding by Gpp(NH)p was determined. Gpp(NH)p did not compete for [^{3}H]-RS-45041-190 binding at concentrations up to 1 mM (Table 4.3.). However, for G-

Table 4.3. Inhibition of [^{3}H]-RS-45041-190 binding to rat kidney membranes.

compound	n	pK_i/pIC_{50}	nH	pIC_{50} (60-70%)	pIC_{50} (30-40%)
RS-45041-190	3	8.39 ± 0.04	0.75 ± 0.02		
idazoxan	3			7.85 ± 0.03	5.80 ± 0.12
cirazoline	3			8.16 ± 0.05	6.11 ± 0.17
guanabenz	3	7.23 ± 0.07	1.23 ± 0.10		
amiloride	3	6.91 ± 0.36	0.72 ± 0.03		
naphazoline	4	6.61 ± 0.11	0.62 ± 0.02		
p-aminoclonidine	3	4.89 ± 0.15	0.77 ± 0.08		
phentolamine	3	4.09 ± 0.05	1.04 ± 0.02		
glibenclamide	3	3.91 ± 0.04	1.48 ± 0.16		
adrenaline	3	3.72 ± 0.05	1.02 ± 0.06		
RS-15385-197	3	<4.00	-		
dopamine	2	5.08	0.72		
histamine	2	4.13	-		
prazosin	2	4.20	-		
Gpp(NH)p	3	<3.00	-		

Competing ligand was incubated with 1 nM [^{3}H]-RS-45041-190 for 60 min at 25°C as described in Chapter two. The results are expressed as pK_i (when nH = 1) or pIC_{50} (nH < 1) and represent the mean ± s.e.mean of n determinations on separate preparations each performed in duplicate. For compounds displaying biphasic displacement curves, the pIC_{50} and % contribution of each component is given.

protein coupled receptors, guanylnucleotides change the affinity state of the receptor for its agonist to a low affinity state. One potential agonist is idazoxan, as in competition experiments idazoxan gave rise to a biphasic inhibition curve; and, as shown in Section 4.2.4., [^{3}H]-idazoxan was sensitive to GTP, therefore inhibition curves to idazoxan were carried out in the presence and absence of 100 μM Gpp(NH)p. Figure 4.13. shows that the affinity and the % of sites defined with idazoxan were unaltered by the presence of 100 μM Gpp(NH)p (control: pIC_{50} = 7.74 ± 0.03 (69%) and 5.75 ± 0.18 (21%); 100 μM Gpp(NH)p: pIC_{50} = 7.93 ± 0.11 (68%) and 5.56 ± 0.31 (22%), n=3). These data suggest that either idazoxan is not an agonist or that the binding sites are not coupled to a G-protein.

Figure 4.14. shows the effects of monovalent and divalent cations on [^{3}H]-RS-45041-190 binding. Monovalent ions were more potent in inhibiting binding; KCl inhibited specific binding by 80% at 500 mM. The rank order of potency was $K^+ > Na^+ = Ca^{2+} = Mg^{2+}$. [^{3}H]-RS-45041-190 binding therefore shows a similar ion dependancy to [^{3}H]-idazoxan (Coupry *et al.*, 1989; Zonnenschein *et al.*, 1990).

4.5. AUTORADIOGRAPHICAL DISTRIBUTION OF IMIDAZOLINE BINDING SITES IN RAT BRAIN

4.5.1. Distribution of [^{3}H]-idazoxan labelled imidazoline binding sites in rat brain

The distribution of imidazoline binding sites labelled by [^{3}H]-idazoxan was studied in rat brain by *in vitro* autoradiography, in the presence of 0.1 μM RS-15385-197 to prevent binding to α_2-adrenoceptors. Table 4.4. shows the density of imidazoline sites measured in 6 areas of rat brain. The optical densities observed over other areas of the brain were not significantly higher than the film background. Imidazoline sites were highly localised in the circumventricular organs; the area postrema and the subfornical organ (plates 4.1. and 4.3.). High density binding was observed in specific hypothalamic nuclei namely the interpeduncular (plate 4.1.), lateral mammilary and arcuate nuclei (plate 4.2.). Imidazoline sites labelled by [^{3}H]-idazoxan were therefore localised over discrete areas of rat brain.

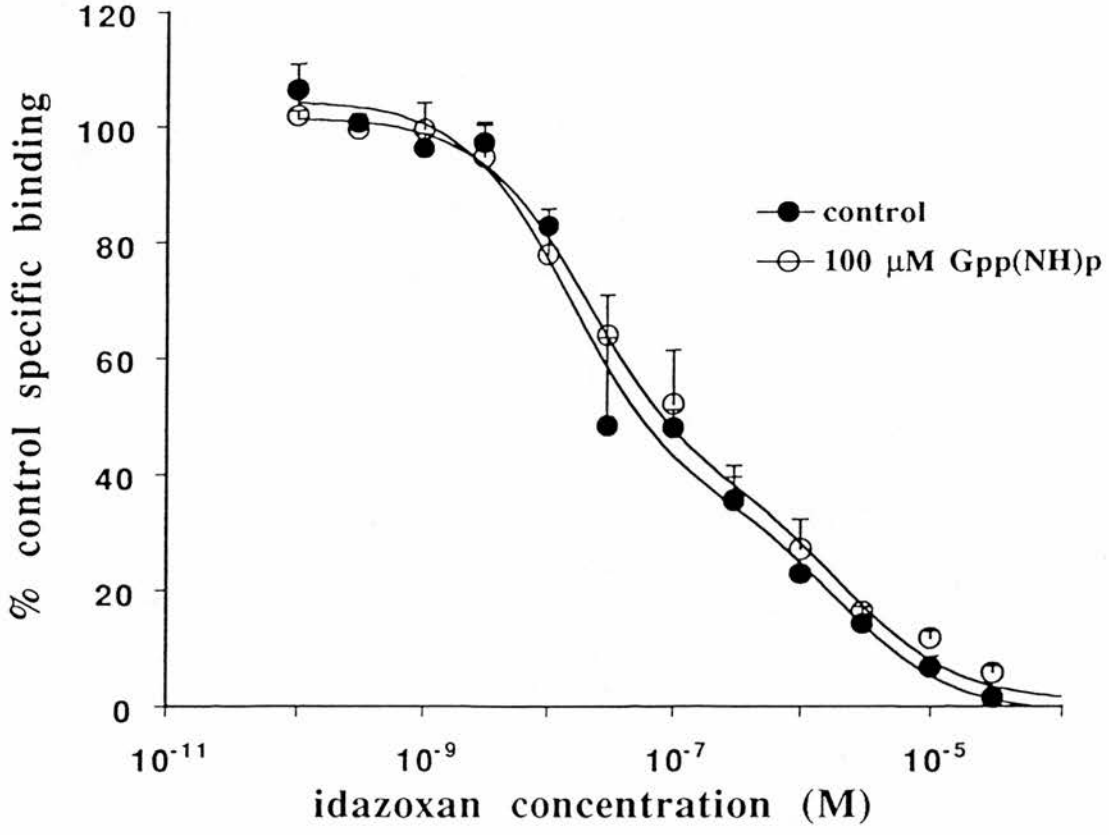


Figure 4.13. Effect of 100 μM Gpp(NH)p on idazoxan inhibition of [^{3}H]-RS-45041-190 binding. The data represents the mean ± s.e.mean of three experiments on different preparations each performed in duplicate. The data was better fitted to a two site model in the presence and absence of Gpp(NH)p. Affinity values as given in the text.

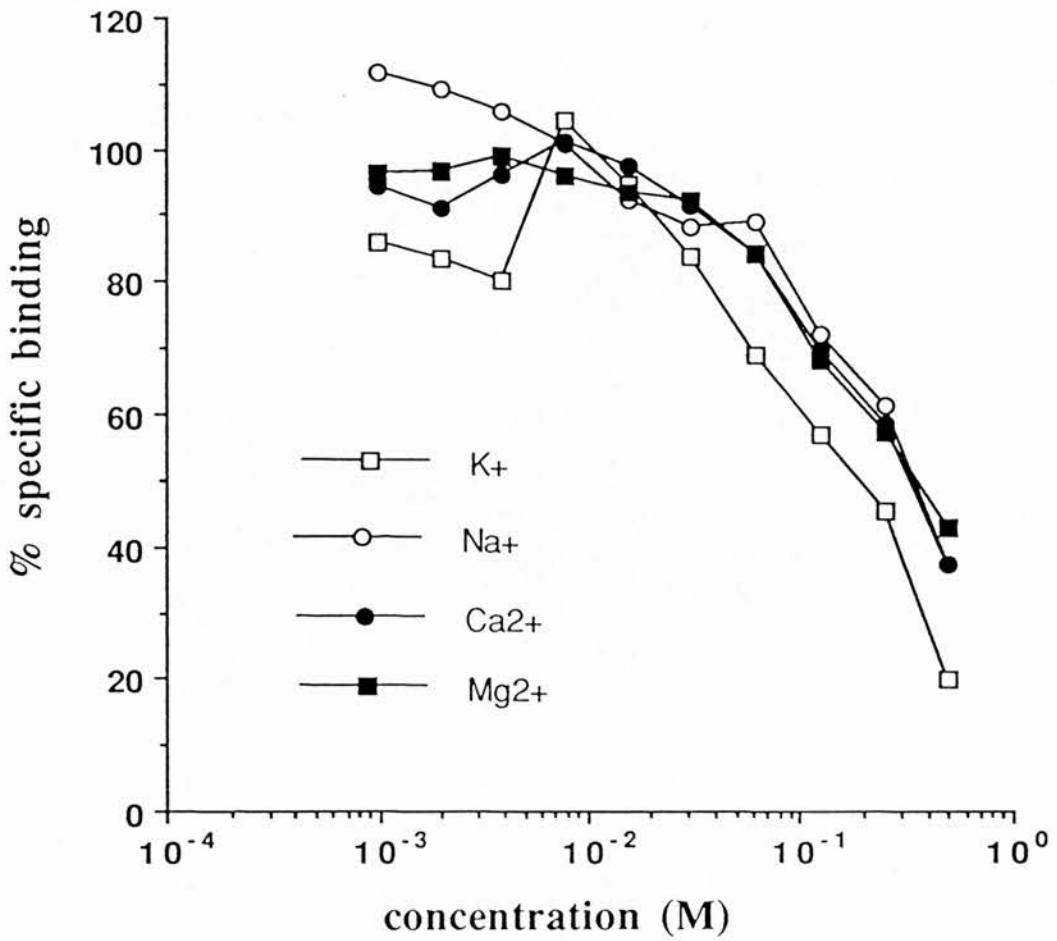


Figure 4.14. Effect of cations on [^{3}H]-RS-45041-190 binding in rat kidney membranes. The chloride salt was used in all cases. The data represents the mean of two experiments on different preparations each performed in duplicate. Affinity values as given in the text.

Table 4.4. Distribution of imidazoline binding sites labelled by [3H]-idazoxan in rat brain.

structure	specific binding fmoles/mg tissue
subfornical organ	151.2 ± 31.3
arcuate nucleus	84.3 ± 17.4
interpeduncular nucleus	71.5 ± 3.6
area postrema	64.9 ± 16
lateral mammillary nucleus	42.8 ± 19.9

Sections were labelled with 1 nM [3H]-idazoxan in the presence of 0.1 μM RS-15385-197 as described in Chapter two. Quantification of image density in fmoles/mg tissue was carried out using a Quantimet 970 image analyser. The results represent the mean ± s.e.mean of multiple measurements made from 4-5 animals.

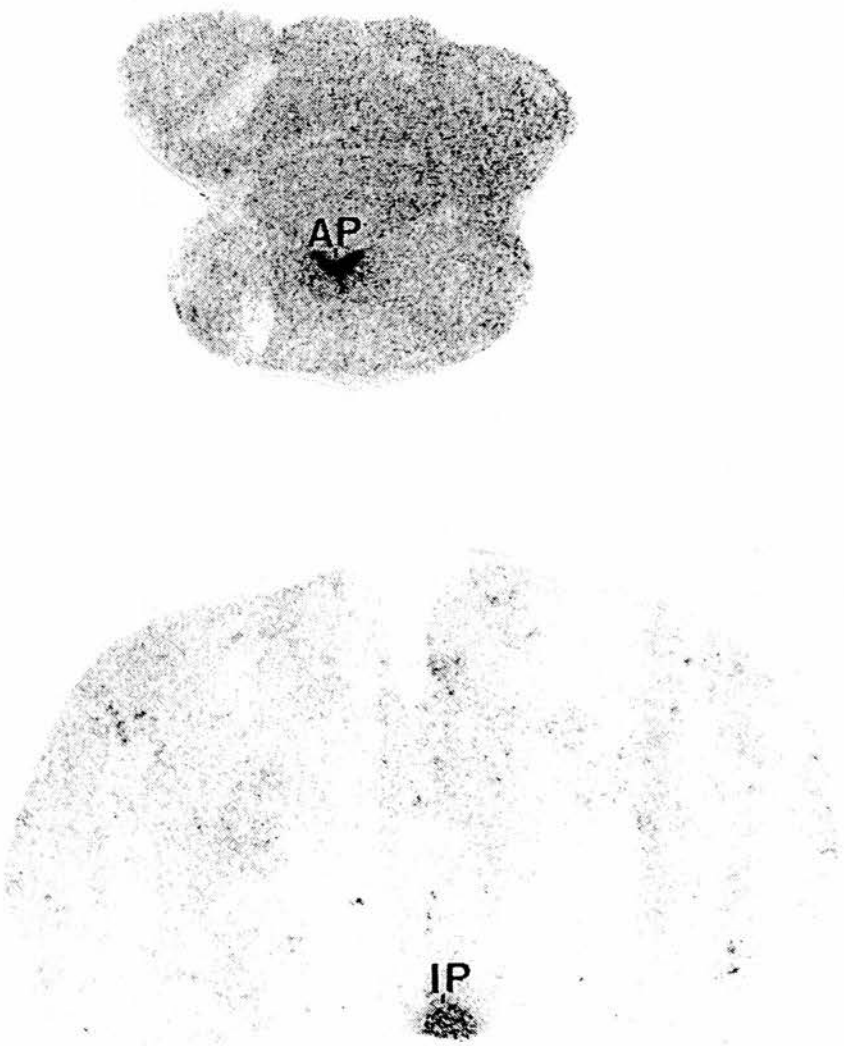


Plate 4.1. [^{3}H]-idazoxan binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. AP (area postrema), IP (interpeduncular nucleus).

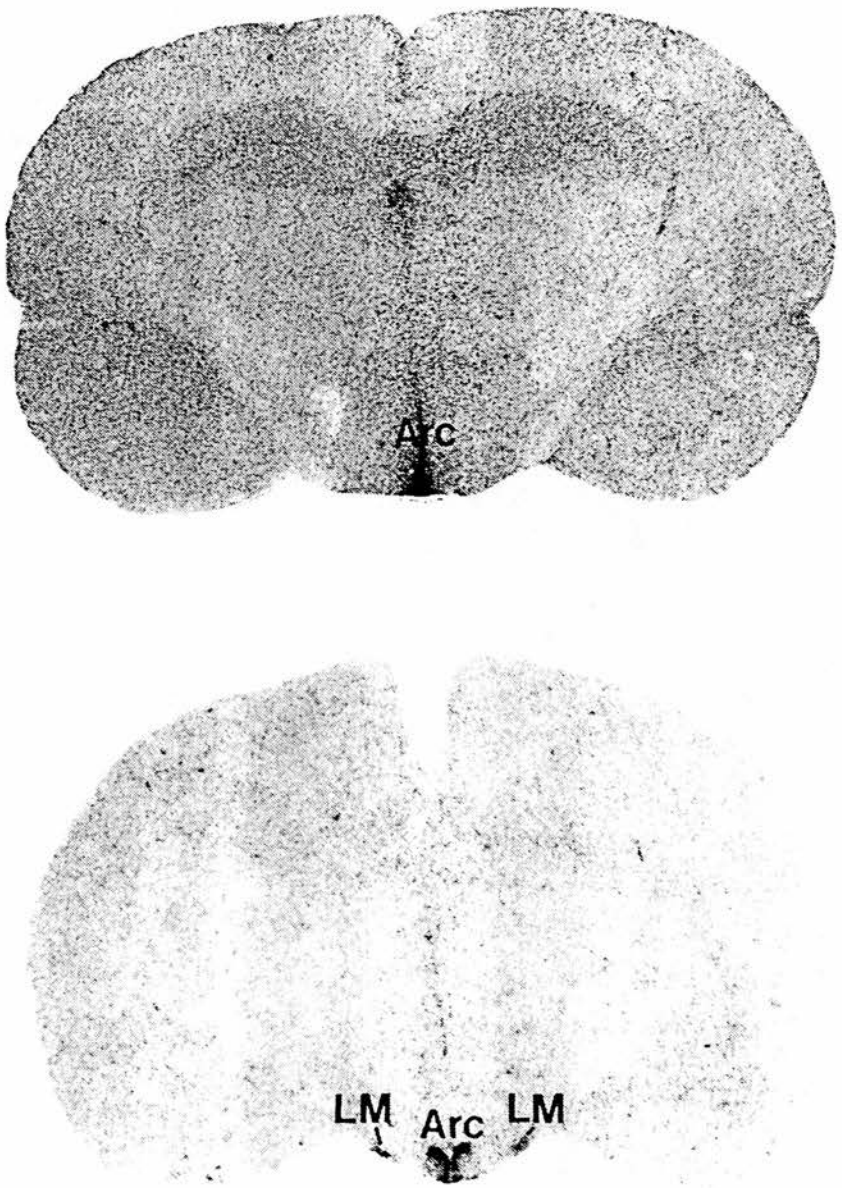


Plate 4.2. [3H]-idazoxan binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. Arc (arcuate nucleus), LM (lateral mammillary nucleus).

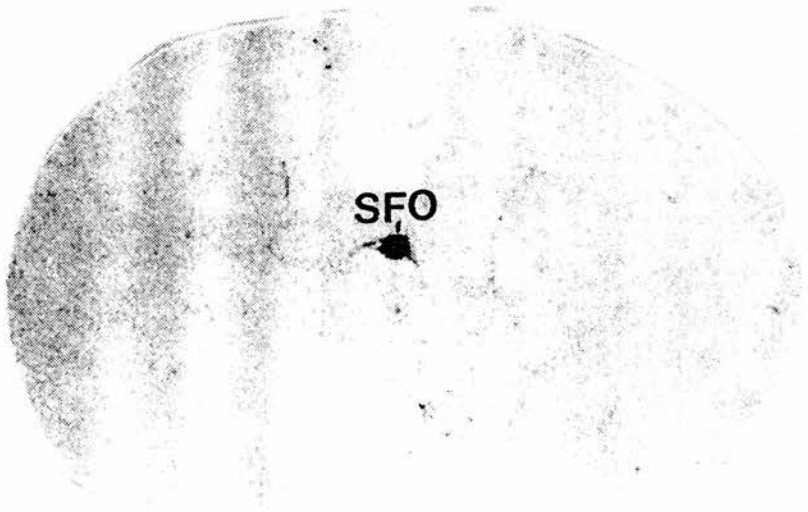


Plate 4.3. [^{3}H]-idazoxan binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. SFO (subfornical organ).

4.5.2. Biochemical characterisation of [3H]-RS-45041-190 binding sites in rat brain

Initial characterisation of [3H]-RS-45041-190 binding was carried out on coronal sections through the hypothalamic area (Figure 4.15.). Equilibrium was reached after 60 min and a washtime of 10 min was chosen to remove unbound ligand. Given that RS-45041-190 has very low affinity for α_2-adrenoceptors in rat cerebral cortex membranes (pK_i = 5.37; unpublished observations), and RS-15385-197 had very low affinity for [3H]-RS-45041-190 binding in rat kidney (pKi < 4.0, Section 4.4.3.), [3H]-RS-45041-190 was considered as being highly selective for imidazoline sites. RS-15385-197 was therefore, not included in these experiments as [3H]-RS-45041-190 would not be expected to label α_2-adrenoceptors in rat brain.

4.5.3. Distribution of [3H]-RS-45041-190 binding sites in rat brain

[3H]-RS-45041-190 binding sites were highly localised in discrete regions of rat brain. Table 4.5. shows the density of binding sites measured in several regions of rat brain. Images of total binding sections are presented in plates 4.4. - 4.6. The distribution of binding sites was similar to that seen with [3H]-idazoxan (Section 4.5.1.) in as much as the highest levels were observed over the area postrema (plate 4.4), the interpeduncular nucleus (plate 4.5), the arcuate and lateral mammillary nuclei, and the subfornical organ (plate 4.6.). However, additional sites were observed over the locus coeruleus, the medial habenular nucleus, the dorsomedial hypothalamic nucleus, the inferior olive and the nucleus of the solitary tract. Very low levels (<30 fmoles/mg tissue) were observed over all other areas.

4.6. DISCUSSION

4.6.1. A comparison of [3H]-idazoxan and [3H]-*p*-aminoclonidine binding

The present study indicated that [3H]-idazoxan and [3H]-*p*-aminoclonidine labelled non-adrenergic binding sites rat kidney membranes. The choice of drug to define non-specific binding was based on the concentration of drug required to maximally inhibit the binding in the presence of RS-15385-197 (1 μM cirazoline for [3H]-

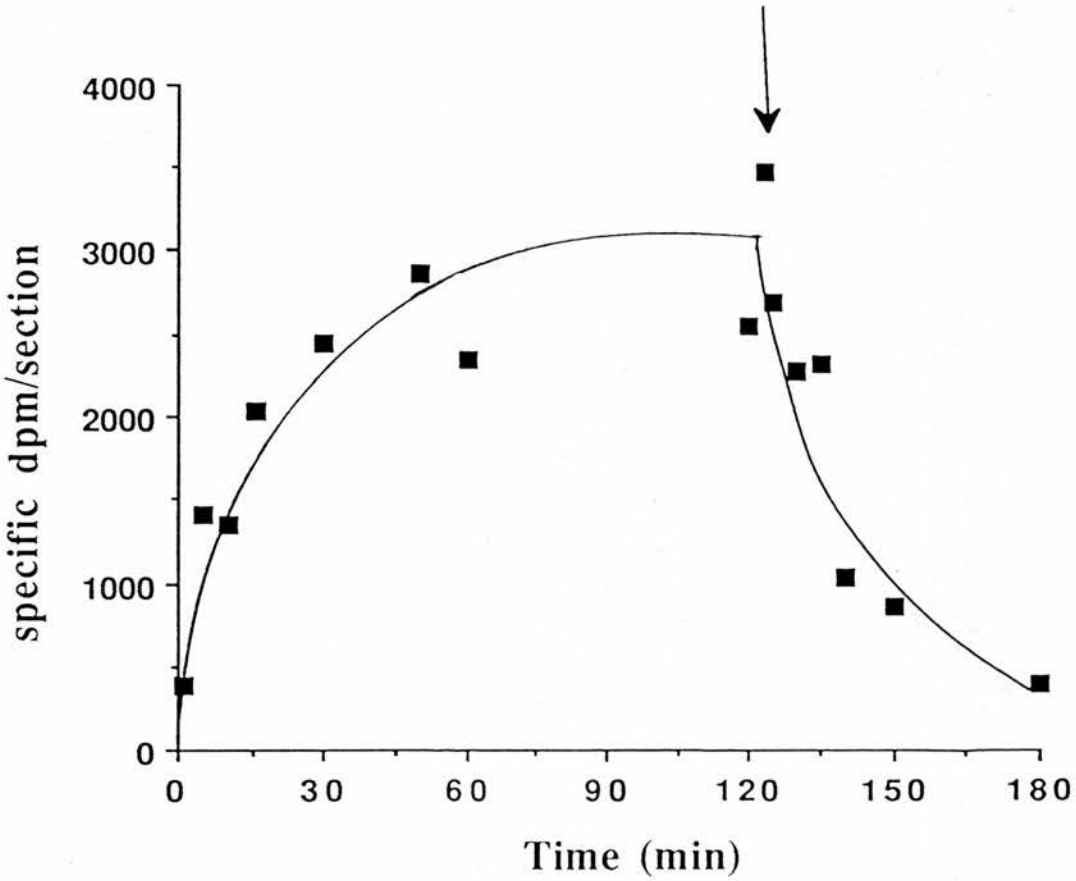


Figure 4.15. Association and dissociation kinetics of [^{3}H]-RS-45041-190 binding to coronal sections of rat brain. Following incubation and washing, the sections were wiped from the slide and counted for radioactivity as described in Chapter two. The data represents a typical experiment performed in triplicate. Dissociation was initiated by the addition of 10 μM cirazoline (indicated by the arrow).

Table 4.5. Distribution of imidazoline binding sites labelled by [^{3}H]-RS-45041-190 in rat brain.

region	specific binding fmoles/mg tissue	region	specific binding fmoles/mg tissue
olfactory system		**cortical regions**	
Ant. olfactory nuc.lat.	97.13±10.64	entorhinal cortex	85.08±8.16
septal area		occipital cortex	62.56±5.55
subfornical organ	195.7±12.26	temporal crtex	62.65±2.93
lateral ventricles	103.04±6.40	parietal cortex	38.31±1.96
corpus striatum		cingulate cortex	43.30±1.60
caudate putamen	52.07±4.63	cort/amygdaloid	
globus pallidus	11.14±1.39	zone	124.48±16.42
nuc. accumbens	101.30±9.63	orbital cortex	88.46±17.73
thalamic regions		**pons**	
paraventricular	125.92±3.09	locus coruleus	184.48±15.63
medial habenular nuc.	159.20±9.44	olivocochlear bundle	110.89±25.53
fascilus		ventrospinal cer. tract	86.35±5.81
retroflexus	88.74±15.02	pontine nuclei	35.70±4.79
hypothalamic regions		dorsal tegmental nuc.	76.91±3.40
dorsomedial	237.60±29.72	dorsal raphe	135.12±20.58
arcuate nuc.	247.32±10.17	**medulla**	
lateral mammilary nuc.	214.43±11.24	gracile nucleus	56.17±12.92
mammilary nuc. med.	152.88±81.4	hypoglossal nuc.	71.25±8.25
mammilary peduncle	112.85±16.2	nuc. spin. tract trig.	9.65±1.16
hippocampal regions		area postrema	215.58±25.83
dentate gyrus	62.67±5.72	lateral reticular nuc.	40.69±4.07
subiculum	97.83±16.45	dor. motor nuc. vagus	149.98±6.79
CA1	105.40±8.70	pyramidal tract	66.3±10.46
CA2	97.58±7.99	nuc. sol. tract med.	100.57±5.65
CA3	89.23±11.02	inferior olive	76.35±3.09
mesencephalon		paramedian lobule	35.41±4.33
central gray	93.43±4.07	**cerebellar regions**	
interpeduncular nuc.	217.41±21.73	C1	57.93±5.74
substantia nigra	32.92±0.78	C9	24.1±3.68
		Copulla of the pyramis	32.99±5.3

Sections were labelled with 8 nM [^{3}H]-RS-45041-190 as described in Chapter two. Quantification of image density in fmoles/mg tissue was carried out using a Quantimet 970 image analyser. The results represent the mean ± s.e.mean of multiple measurements made from 3-4 animals.

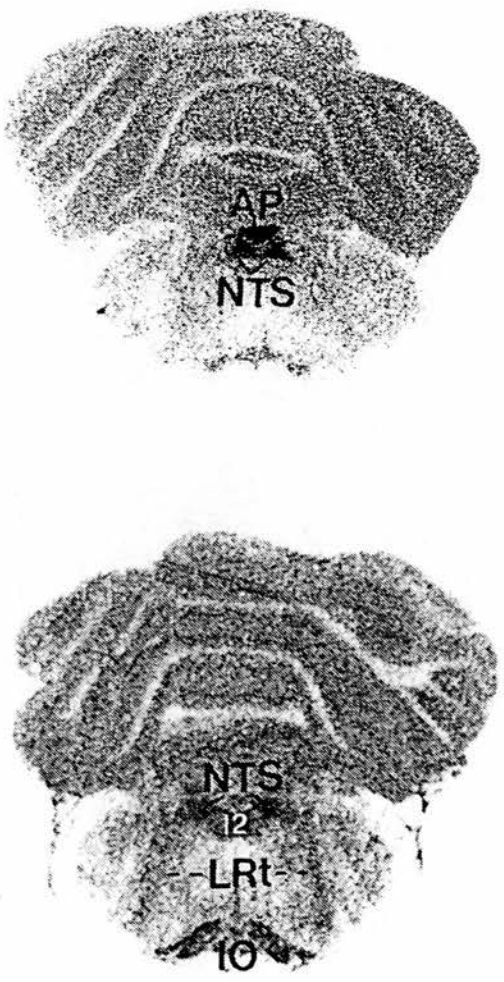


Plate 4.4. [^{3}H]-RS-45041-190 binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. AP (area postrema), IO (inferior olive), LRt (lateral reticular area), NTS (nucleus of the solitary tract), 12 (hypoglossal nucleus).

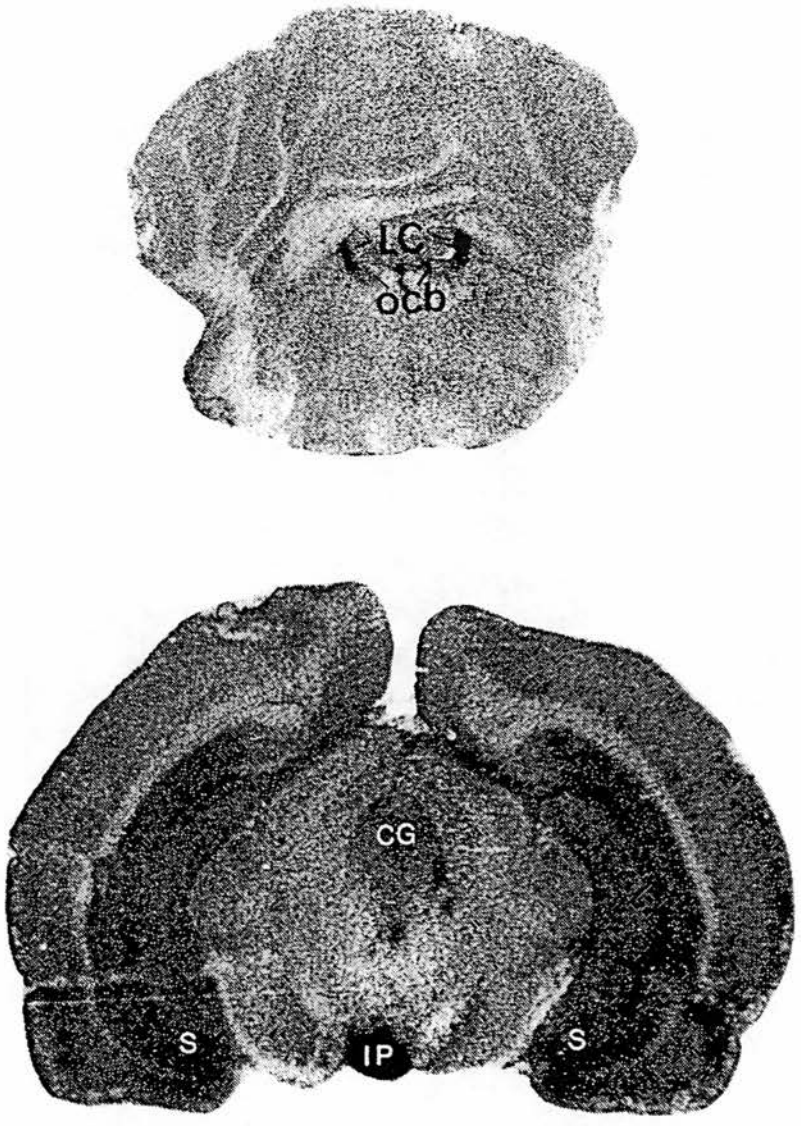


Plate 4.5. [^{3}H]-RS-45041-190 binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. CG (central gray), IP (interpeduncular nucleus), LC (locus coeruleus), ocb (olivocochlear bundle), S (subiculum).

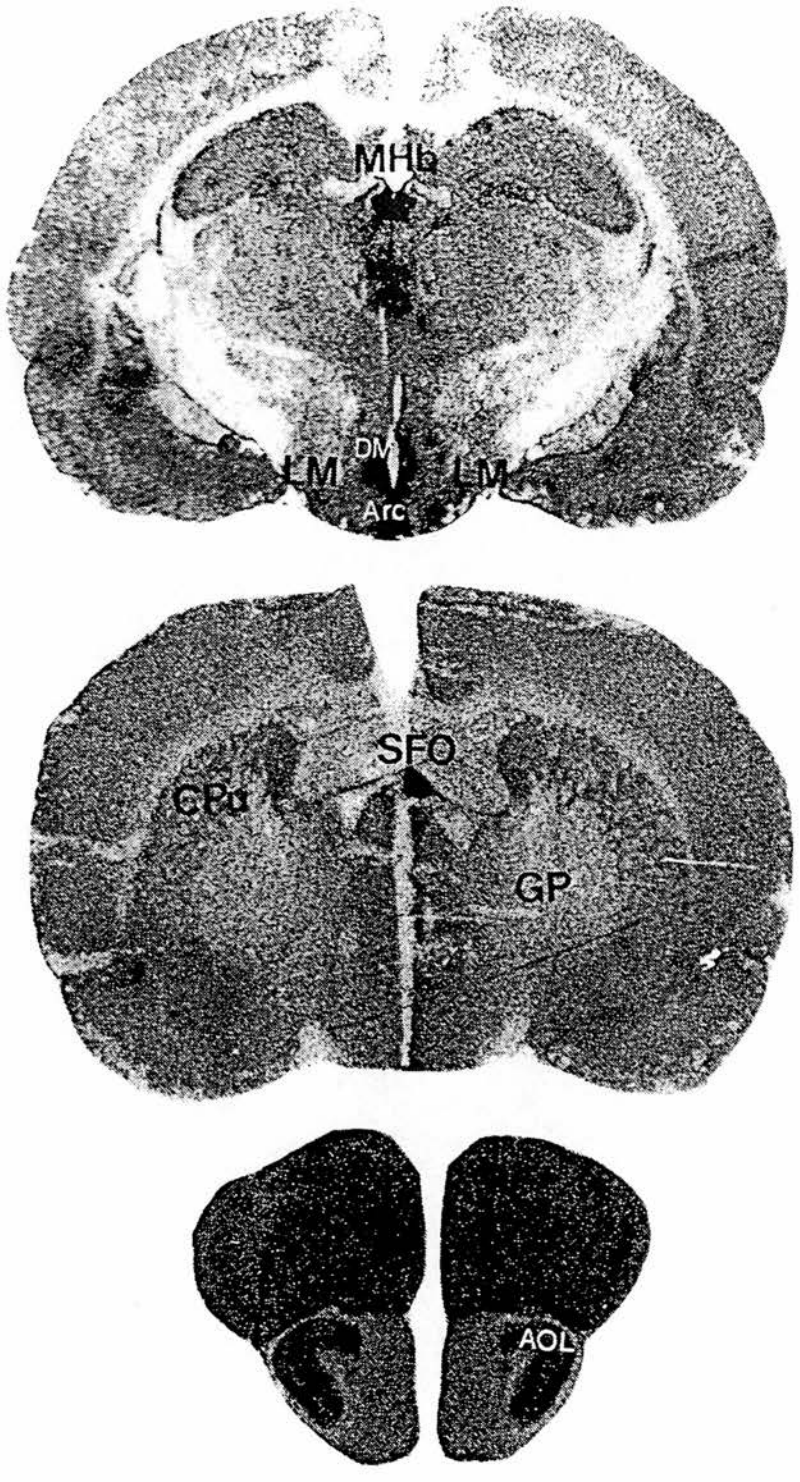


Plate 4.6. [^{3}H]-RS-45041-190 binding in rat brain. The images show representative total binding sections through the rat brain. Non-specific binding in consecutive sections was not significantly greater than background. Arc (arcuate nucleus), AOL (anterior olfactory nucleus, lateral), CPu (caudate putamen), DM (dorsomedial hypothalamic nucleus), GP (globus pallidus, LM (lateral mammillary nucleus), MHb (medial habenular nucleus), SFO (subfornical organ).

idazoxan binding, 100 μM clonidine for [^{3}H]-*p*-aminoclonidine binding). The sites labelled by each ligand had low affinity for the catecholamine noradrenaline and low affinity for the selective α_2-adrenoceptor antagonist RS-15385-197 and were therefore not adrenoceptors. Both ligands labelled a population of sites that showed marked differences in nature. Firstly, [^{3}H]-*p*-aminoclonidine labelled more than 20 times the number of sites than [^{3}H]-idazoxan but with relatively low affinity. Because of the low affinity of [^{3}H]-*p*-aminoclonidine for its binding site, the binding parameters (K_d and B_{max}) were analysed from competition experiments with unlabelled *p*-aminoclonidine. The data showed that *p*-aminoclonidine labelled a single population of low affinity sites (K_d = 128 nM, B_{max} = 978 fmoles/mg protein).

It was surprising that specific [^{3}H]-*p*-aminoclonidine binding using filtration to separate bound from free radioligand, as this is normally only suitable for ligands having nanomolar affinity for their receptor site. Ligands with lower affinity would be expected to dissociate from the receptor site during the washing stage of the separation. However, the experiments were repeated using separation by centrifugation, and no significant difference was found in the affinity of the site compared with that determined by filtration. The pharmacological profile of the site was also similar, as the affinities of *p*-aminoclonidine, idazoxan and guanabenz, compounds critical to the classification, were identical using both separation methods. This lead to the conclusion that the same site was being labelled under the different protocols. By using ice cold washing buffer to minimise ligand dissociation it was shown that a significant proportion of binding remained after a separation time of up to 10 sec. Conducting the separation at 4^{o}C, must slow the dissociation rate sufficiently to allow for the determination of reproducible binding data with [^{3}H]-*p*-aminoclonidine using a filtration protocol. Filtration has been successfully used to label glutamate receptor subtypes and the neurotoxin receptor site associated with Na+ channels using radioligands of moderate affinity (Catterall *et al.*, 1981; Honore *et al.*, 1989).

In competition experiments, the rank order of potencies of a number of compounds for imidazoline sites labelled by the two ligands was markedly different. Figure 4.16 shows that the affinities correlated poorly (r = 0.249, nH = 0.427). In particular, idazoxan had very low

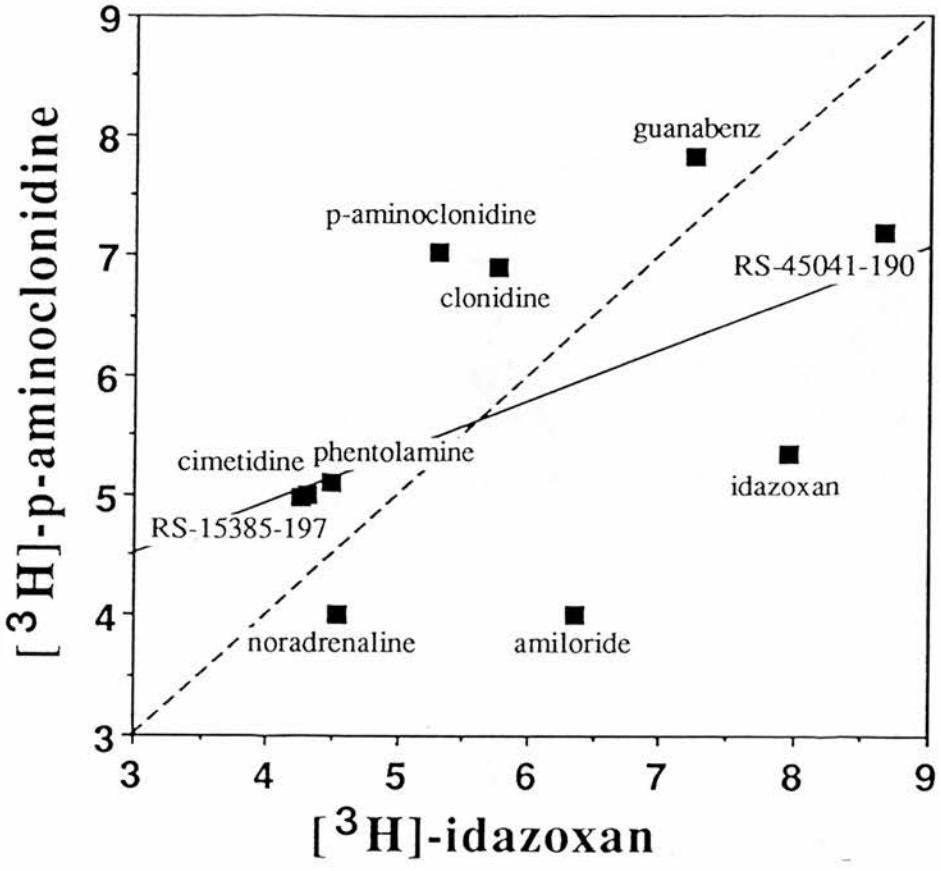


Figure 4.16. Correlation of binding affinities (pK_i) of 9 compounds for [3H]-idazoxan and [3H]-*p*-aminoclonidine binding to rat kidney membranes. The dotted line represents the line of identity and the solid line represents the line of best fit. The data is best described by the equation $y = 0.427x + 3.22$, $r = 0.249$.

affinity for [3H]-*p*-aminoclonidine binding (pK_i = 5.34). Other studies with idazoxan have shown differences in affinities for [3H]-*p*-aminoclonidine binding, ranging from 220 nM in the rat renal cortex (Ernsberger *et al.*, 1990) to 33 nM for [3H]-clonidine binding in the human brainstem (Bricca *et al.*, 1988). The low affinity for idazoxan in the present study may be due to the removal of idazoxan's high affinity for α_2-adrenoceptors with RS-15385-197.

In competition experiments with [3H]-idazoxan, *p*-aminoclonidine and clonidine had low affinity. This is consistent with a number of studies in pig (Vigne *et al.*, 1989), hamster (Mackinnon *et al.*, 1989), human and rat kidney (Michel *et al.*, 1989), whereas clonidine has somewhat higher affinity in rabbit tissues (Yablonsky *et al.*, 1988; Langin & Lafontan, 1989). Guanabenz showed moderately high affinity for [3H]-idazoxan binding which is consistent with other studies (Vigne *et al.*, 1989), but also had high affinity for [3H]-*p*-aminoclonidine binding. Studies with [3H]-*p*-aminoclonidine in the bovine ventrolateral medulla (Ernsberger *et al.*, 1988) show guanabenz to have very low affinity.

Studies by Bricca *et al.* (1989) have shown that [3H]-clonidine labels imidazoline-like binding sites with high affinity in bovine and human brainstem membranes, and that the human nucleus reticularis lateralis provides an homogeneous population of imidazoline binding sites. However, using the same protocol, imidazoline binding sites could not be labelled with [3H]-clonidine in rat brainstem membranes (Bricca *et al.*, 1989). Imidazoline sites have also been labelled with [3H]-*p*-aminoclonidine (Piletz *et al.*, 1991) and [125I]-*p*-iodoclonidine (Piletz *et al.*, 1992) in human platelets. In these studies clonidine and *p*-aminoclonidine had high affinity and idazoxan had low affinity. The site on the human platelet, therefore showed similarities to the site identified in bovine brainstem (Ernsberger *et al.*, 1987; Michel & Insel, 1989). The lack of high affinity [3H]-clonidine and [3H]-*p*-aminoclonidine binding in rat tissues may represent a species difference in the imidazoline binding site.

The finding in the present study that [3H]-*p*-aminoclonidine had low affinity for imidazoline binding sites in rat kidney is, however, not consistent with studies by Ernsberger *et al.* (1990), who demonstrated high affinity for this ligand in rat renal cortex membranes. These studies showed that imidazoline compounds would displace 100% of the

specific binding, while the catecholamines would only displace ~68%. Imidazoline affinity was calculated as the relative affinity for 32% of the specifically bound [^{3}H]-*p*-aminoclonidine defined with 10 μM phentolamine. It is considered that 10 μM phentolamine would not be a sufficiently high enough concentration to displace all imidazoline-like binding with either [^{3}H]-*p*-aminoclonidine or [^{3}H]-idazoxan, as in the present study, phentolamine had an affinity of 7.9 and 33 μM for these two ligands respectively. There are several differences between this study and that of Ernsberger *et al.* (1990) in that the present study used whole kidney homogenate, whereas renal cortex was used by Ernsberger *et al.* (1990). This, however, is unlikely to explain the differences seen, as one would still expect to label the high affinity cortical binding in a whole kidney preparation. The assay conditions were similar in both studies except that the incubation conditions in the present work were set to specifically label imidazoline sites, i.e. performed in the presence of RS-15385-197 to block α_2-adrenoceptors. Under the conditions employed by Ernsberger *et al.* (1990); long incubation time at 25°C and non-specific binding defined with 10 μM phentolamine, one would expect [^{3}H]-*p*-aminoclonidine to label a significant population of α_2-adrenoceptors and would then perhaps not label the whole population of imidazoline sites. True imidazoline affinity is likely to be masked when the radioligand used is also labelling α_2-adrenoceptors.

The imidazoline compound naphazoline showed high affinity for the [^{3}H]-idazoxan binding site on rat kidney, but exhibited a displacement curve which had a Hill slope of less than unity. Naphazoline has been shown to exhibit a shallow displacement curve for [^{3}H]-idazoxan binding to imidazoline sites in rat brain (Brown *et al.*, 1990), rat liver (Zonnenschein *et al.*, 1990), rabbit kidney (Stewart *et al.*, 1992) and hamster adipocyte (Mackinnon *et al.*, 1989). In the present study the displacement curve for naphazoline could be better resolved into a two site model with affinities for the high and low affinity components (pIC_{50}) of 8.65 and 6.88 respectively. This could suggest that naphazoline is defining two non-interacting subtypes of imidazoline binding site for [^{3}H]-idazoxan, or that naphazoline is defining two affinity states of the same receptor. There is some evidence to suggest that imidazoline sites labelled by [^{3}H]-idazoxan may be heterogeneous. Michel & Insel (1989) suggested that imidazoline

sites in rabbit tissues may show differences to those from rat, human and pig tissues based on the affinity of guanabenz, amiloride, phentolamine and clonidine, all of which have much higher affinity in the rabbit. However, the rank order of potency of these compounds was the same in all species (guanabenz > amiloride > clonidine $\geq$ phentolamine) and this has been shown to hold in a number of other studies in rabbit (Yablonsky & Dausse, 1989) and human (Lachaud-Pettiti *et al.*, 1991) tissues. The rabbit binding site may then represent a species homologue of the same imidazoline site.

In some studies heterogeneous binding sites for [^{3}H]-idazoxan have been demonstrated in the same species. Wikberg *et al.* (1991) demonstrated that [^{3}H]-idazoxan labelled two classes of imidazoline site in guinea pig ileum and cerebral cortex membranes which showed different affinities for clonidine, and different stereoselectivity for medetomidine isomers, and named these subtypes I^A and I^B. Further studies are required to elucidate whether naphazoline has selectivity for either of these subtypes.

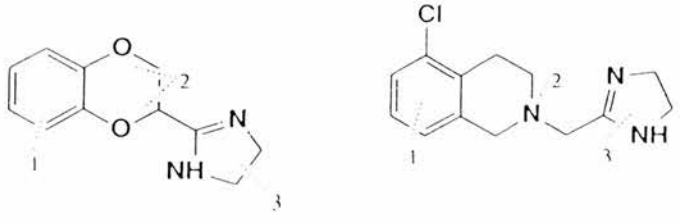
Previous studies have shown that the stable GTP analogue Gpp(NH)p had no effect on the inhibition curves of UK 14304, cirazoline, guanabenz or clonidine at idazoxan binding sites in rat liver membranes (Zonnenschein *et al.*, 1990), however other studies suggested that GTP decreased the number of imidazoline sites labelled by [^{3}H]-idazoxan in the bovine brain (Hussain *et al.*, 1991). In this study GTP inhibited [^{3}H]-idazoxan binding but not [^{3}H]-*p*-aminoclonidine or [^{3}H]-RS-45041-190 binding. From competition studies with [^{3}H]-idazoxan, 300 μM GTP inhibited binding by 15-20%, but had no significant effect in saturation experiments. This is possibly explained by Gpp(NH)p having a selective effect over lower concentrations of [^{3}H]-idazoxan such as that used in competition experiments. When studied in saturation experiments a relative lack of effect at higher concentrations may, in the analysis, mask effects at lower concentrations, particularly when the inhibition is relatively small.

Inhibition by guanyl nucleotides is a phenomenon normally associated with agonist binding, and therefore suggests that idazoxan may be an agonist at the imidazoline binding site. This, coupled with the finding that [^{3}H]-idazoxan labelled a fraction of the number of sites labelled by [^{3}H]-*p*-aminoclonidine, may suggest that the latter ligand

labels the whole population of sites, a subset of which has high affinity for agonists. Studies by Yakabu *et al.* (1990) showed that chronic infusions of idazoxan produced a down-regulation in the number of sites labelled by [3H]-idazoxan but not those labelled by [3H]-yohimbine in the rabbit kidney. Down-regulation is consistent with chronic agonist treatment, further evidence that idazoxan may be an agonist or a partial agonist at the imidazoline site and that this site represents a functional receptor. Unlabelled idazoxan had low affinity for [3H]-*p*-aminoclonidine binding and exhibited a shallow displacement curve. In the presence of GTP however, the inhibition curve to idazoxan was unaffected. Idazoxan also exhibitied a shallow displacement curve to [3H]-RS-45041-190 but again this was unaltered in the presence of Gpp(NH)p. These studies suggest that either idazoxan is not an agonist or that the imidazoline receptor is not linked to a G-protein. Further studies are required to elucidate the effect of GTP on imidazoline sites, and functional studies are neccessary to elucidate the possible agonist effects of idazoxan.

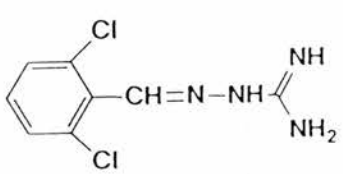
4.6.2. [3H]-RS-45041-190; a selective imidazoline ligand

In the mid-eighties, following extensive structure-activity studies with analogues of idazoxan, it was suggested that three major binding sites exist for this type of compound at the α_2-adrenoceptor (Stillings *et al.*, 1985). These were a planar hydrophobic area that interacts with the benzene ring, a site which binds one or both of the benzodioxan oxygens and an imidazoline binding region. Subsequently, myself and colleagues showed, with a series of substituted 2-(tetrahydroisoquinolin-2-yl methyl) and 2-(isoindolin-2-yl methyl) imidazolines, that α_2-adrenoceptor antagonist potency could be maintained with the proviso that an additional methylene spacer was introduced between the planar and imidazoline rings and that the nitrogen of the isoquinoline or isoindoline group bound to the benzodioxan site (Clark *et al.*, 1990).

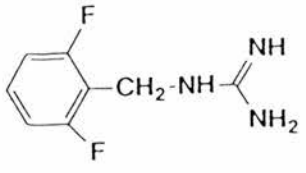


structure of idazoxan and a 2-(tetrahydroisoquinolin-2-ylmethyl) imidazoline compound (5j from Clark *et al.* 1990) showing the proposed points of interaction with the α_2-adrenoceptor

Zonnenschein *et al.* (1990) looked at a series of substituted imidazoline and guanido structures and suggested that shortening the link between the aromatic group and the imidazoline/guanido group, although decreasing α_2-adrenoceptor affinity, increased affinity for imidazoline sites labelled by [^{3}H]-idazoxan in rat liver (e.g. guanabenz and RBII 170(d)). In this case the guanido group presumably occupied the imidazoline binding region of the α_2-adrenoceptor. In addition, substitution of halogen groups on the aromatic moeity facilitated binding to the imidazoline binding site whilst α_2-adrenoceptor affinity was unaffected.



guanabenz
K_i imidazoline = 33.8 nM
K_i α_2 ~50 - 100 nM



RB II 170 (d)
K_i imidazoline = 28.5 nM
K_i α_2 = 3000 nM

Thus, it appears that shortening the link between the planar and imidazoline ring and halogen substitution on the former ring would facilitate imidazoline site vs α_2-adrenoceptor selectivity. RS-45041-190 (Figure 1.2.) is a 5-chloroisoindoline imidazoline compound with a single link bond between the indoline and imidazoline groups and showed high affinity for the imidazoline binding site labelled by [^{3}H]-idazoxan on rat kidney (pK_i = 8.66), rabbit kidney (pK_i = 9.37) and hamster adipocyte (pKi = 8.50) but very low affinity for the α_2-adrenoceptor on rat cortex (pK_i = 5.70). It therefore appears that there are very different structural requirements for an interaction at imidazoline sites and α_2-adrenoceptors.

This study showed that [^{3}H]-idazoxan and [^{3}H]-*p*-aminoclonidine label different subsets of imidazoline binding sites in the rat kidney, and the results emphasised that the lack of selectivity of these ligands necessitates the inclusion of an α_2-antagonist to isolate the imidazoline site. [^{3}H]-RS-45041-190 was shown to be a selective imidazoline ligand which displayed high affinity for an imidazoline site on rat kidney membranes. Competition studies showed noradrenaline and RS-15385-197 to have negligible affinity suggesting that this ligand does not label

α_2-adrenoceptors. Kinetic analysis showed that [^{3}H]-RS-45041-190 labelled two sites in rat kidney (K_d = 0.57 and 0.89 nM), however, in saturation studies and in competition experiments with unlabelled RS-45041-190 the data was better fitted to a one-site model. This could be explained by the lack of resolution in the latter two analyses given that the affinities calculated from kinetic experiments differed by less than two fold. Inhibition curves to idazoxan and cirazoline confirmed that [^{3}H]-RS-45041-190 labelled two sites as they showed >100 fold selectivity for 60-70% of the binding.

The characterisation of [^{3}H]-RS-45041-190 binding showed that 60-70% of the sites labelled were similar to the sites labelled by [^{3}H]-idazoxan. Figure 4.17. shows a very good correlation (r = 0.968, nH = 1.067) between [^{3}H]-RS-45041-190 and [^{3}H]-idazoxan binding in the rat kidney, whereas a poorer correlation was observed against [^{3}H]-*p*-aminoclonidine (r = 0.205, nH = 0.586).

[^{3}H]-RS-45041-190 binding was inhibited by monovalent and divalent cations with a rank order of potency $K^+ > Na^+ = Ca^{2+} = Mg^{2+}$. Studies with [^{3}H]-idazoxan in basolateral membranes from rabbit renal proximal tubules (Coupry *et al.*, 1989) and rat liver (Zonnenschein *et al.*, 1990) showed a similar sensitivity to ions. The interaction of K^+ was allosteric in as much as both the affinity and the dissociation rate of [^{3}H]-idazoxan were increased in the presence of 75 mM K^+ (Coupry *et al.*, 1989). This, coupled with the finding that the K^+ channel blocker 4-aminopyridine inhibited [^{3}H]-idazoxan binding at an effective K^+ channel blocking concentration, led the authors to conclude that imidazoline sites may be linked to K^+ gating (Zonnenschein *et al.*, 1990). However, more recent studies in rat liver membranes showed that a series of K^+ channel openers were devoid of affinity at imidazoline sites with the exception of pinacidil (Ibbotson & Watson, 1992). Furthermore, several potent imidazoline ligands were inactive in displacing [^{3}H]-glibenclamide from rat brain homogenates. In the present study [^{3}H]-RS-45041-190 showed a similar ion sensitivity to [^{3}H]-idazoxan, but glibenclamide, phentolamine and clonidine, agents which increase insulin secretion and inhibit ATP-dependent K^+ channels (Plant & Henquin, 1990; Cook & Quast, 1990; Plant *et al.*, 1991), had low affinity for [^{3}H]-RS-45041-190 binding, confirming the conclusions of Ibbotson & Watson (1992) that the imidazoline site is unlikely to be associated with an ATP-dependent K^+ channel.

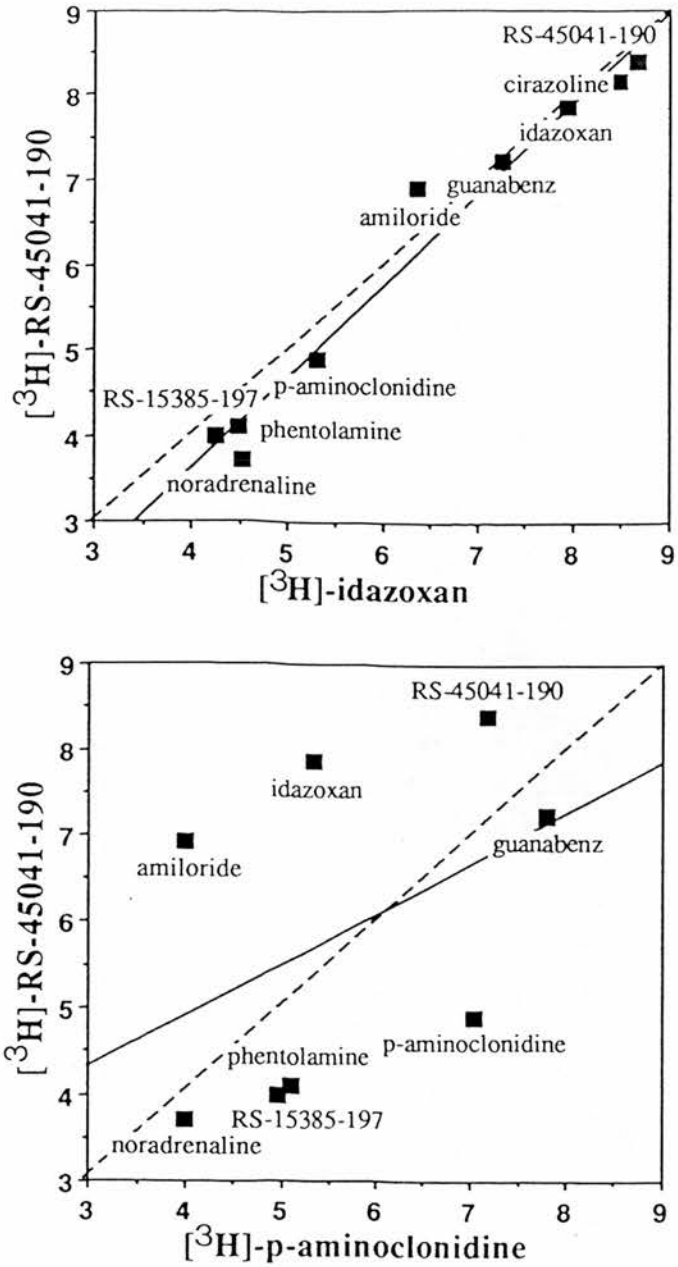


Figure 4.17. Correlation of binding affinities (pK_i) of several compounds for [3H]-idazoxan (top panel) and [3H]-*p*-aminoclonidine binding (bottom panel) versus [3H]-RS-45041-190 binding to rat kidney membranes. The dotted line represents the line of identity and the solid line represents the line of best fit. For [3H]-idazoxan the data is best described by the equation y = 1.067x + 0.655, r = 0.968, and for [3H]-*p*-aminoclonidine y = 0.586x + 2.56, r = 0.205.

The possibility that [3H]-RS-45041-190 was labelling both states of a G protein-linked receptor exhibiting high and low affinity states for agonists was addressed in experiments with the stable GTP analogue Gpp(NH)p. Idazoxan exhibited a biphasic inhibition curve against [3H]-RS-45041-190 and for that reason and those outlined in Section 4.6.1., represented a likely candidate for an agonist at the imidazoline site. However 100 μM Gpp(NH)p had no effect on the affinity or the percentage of sites defined with idazoxan suggesting that either idazoxan is not an agonist or that [3H]-RS-45041-190 binding sites are not associated with a G-protein.

4.6.3. Distribution of imidazoline binding sites in rat brain

The distribution of imidazoline sites was studied in rat brain sections with [3H]-idazoxan and [3H]-RS-45041-190 and showed several similarities. Both ligands labelled discrete regions of the hypothalamus, namely the interpeduncular nucleus, lateral mammilary nucleus, and arcuate nucleus and were also similar to other studies with [3H]-idazoxan (Mallard *et al.*, 1992). The arcuate nucleus is associated with pituitary function and a large number of hormones and peptides are found in this and other imidazoline rich areas. A recent study from this laboratory (MacKinnon *et al.*, 1992b) investigated the affinity of many of these compounds for the [3H]-idazoxan labelled imidazoline binding site on rabbit renal cortex. Unfortunately none were potential candidates for the endogenous ligand; ACTH and LH showed moderate affinity (pK_i = 5.86 and 5.80, respectively). All other compounds showed $pK_i < 5$; LHRH, GHRH, angiotensin I & II, progesterone, testosterone, beta estradiol, endothelin ET-1, ADH, FMRF-NH_2, prolactin, dynorphin, neuropeptide Y, somatastatin, acetylcholine, GABA, substance P, TRH, met-enkephalin, met-enkephalinamide, ouabain, oxytocin, VIP, alpha-MSH, gamma-MSH, neurotensin, galanin, gastrin, renin and beta endorphin. Thus, none of these ligands are likely to have endogenous activity at this site.

High density binding was also observed in the circumventricular organs the area-postrema and the subfornical organ. These organs lack an intact blood-brain barrier and are thought to be the sites associated with angiotensin II mediated changes in blood volume and salt and water intake (Phillips, 1987; Allen *et al.*, 1988). The distribution of angiotensin II receptors in rat brain displays some close parallels with

imidazoline site distribution in as much as high density binding is observed over the circumventricular areas and the ventricular areas, (Mendelsohn *et al.*, 1984). However, as stated above, neither angiotensin I or II have appreciable affinity for imidazoline sites labelled with [^{3}H]-idazoxan, and it is therefore unlikely that imidazoline sites are associated with angiotensin II receptors in these areas.

In studies carried out recently by Mallard *et al.* (1992), it was demonstrated that high density binding was also observed over the pineal gland in rat brain. The rat pineal gland is small, and is located at the dorsal surface of the brain protected by a bony case. Unless particular attention is paid to its removal the gland will remain in the skull during removal of the brain. This would most likely explain why imidazoline binding to the pineal gland was not demonstrated in the present study with either [^{3}H]-idazoxan or [^{3}H]-RS-45041-190.

A recent study by Ruggiero *et al.* (1992) has demonstrated that imidazoline binding sites labelled by [^{3}H]-idazoxan are highly localised in mitochondrial membranes prepared from glial cells, particularly astrocytes in the CNS. Using antibodies raised to the solubilised binding site from rabbit kidney, imidazoline sites were localised using immunocytohistochemistry in rat brain sections. These studies showed that the distribution closely parallelled the distribution of imidazoline sites labelled by [^{3}H]-idazoxan and [^{3}H]-RS-45041-190 in the present autoradiography studies, and also showed several similarities to the distribution of the peripheral-type benzodiazepine sites (ω_3) labelled by [^{3}H]-PK-11195 in rat brain (Benavides *et al.*, 1983). This suggests that central localisation of imidazoline binding sites may be primarily to non-neuronal structures. However, it is unlikely that the benzodiazepine site and the imidazoline site are one and the same as Tesson *et al.* (1991) showed that PK 11195 had low affinity for [^{3}H]-idazoxan labelled imidazoline sites in rabbit and human liver mitochondrial membranes.

In addition to labelling those regions also labelled by [^{3}H]-idazoxan, [^{3}H]-RS-45041-190 labelled other discrete nuclei in rat brain. Binding was observed over the locus coeruleus and nucleus tractus solitaris, the dorsal raphe and the dorsomedial hypothalamic nucleus. The exact nature of the binding site in these areas, and its functions, remain to be elucidated.

The lateral medullary region has been proposed to be the major site for the hypotensive action of imidazolines, particularly the nucleus

reticularis lateralis (NRL) in the cat (Bousquet *et al.*, 1984,1987), and the C1 area of the rostral ventrolateral medulla in the rat (Meeley *et al.*, 1986; Granata & Reis, 1987; Ernsberger *et al.*, 1988). Autoradiography studies with [^{3}H]-*p*-aminoclonidine (Unnerstal *et al.*, 1984) have localised binding in the rat medullary area to the nucleus tractus solitaris (NTS) and the raphe pallidus with lower diffuse areas of binding extending from the lateral NTS toward the ventrolateral medulla (lateral reticular area), and on more rostral sections extended to the ventrolateral surface of the medulla (C1 area). In these respects the distribution of [^{3}H]-*p*-aminoclonidine binding paralleled very closely the distribution of α_2-adrenoceptors identified with [^{3}H]-RS-15385-197, and probably represented binding to α_2-adrenoceptors (see plate 3.1.).

Another study identified [^{3}H]-*p*-aminoclonidine binding in the rostral ventrolateral medulla and the NTS which was sensitive to imidazole-4-acetic acid, suggesting that imidazoline sites were present in these areas (Arango *et al.*, 1986). In studies where imidazolines have been shown to exert a hypotensive action when injected into the medulla of the rat (Meeley *et al.*, 1986; Ernsberger *et al.*, 1988) the injection site corresponded to the lateral reticular area, where diffuse [^{3}H]-*p*-aminoclonidine binding was described by Unnerstall *et al.*, (1984) and [^{3}H]-RS-15385-197 (for illustration see plate 3.1.). Injections into areas 1 mm medial, dorsal or dorsolateral to this area (Meeley *et al.*, 1986), or into the inferior olive or the nucleus raphe magnus (Ernsberger *et al.*, 1988), however, produced no fall in blood pressure. Therefore, the area sensitive to imidazolines was very restricted.

In this thesis it was demonstrated that imidazoline "specific" binding (as defined by [^{3}H]-idazoxan in the presence of α_2-blockade and by [^{3}H]-RS-45041-190) was confined to highly specific areas in the medulla. Low levels of [^{3}H]-idazoxan and only moderate levels [^{3}H]-RS-45041-190 binding were observed over the lateral reticular area of the medulla (plate 4.1. and 4.4., and Table 4.5.). [^{3}H]-RS-15385-197 binding to α_2-adrenoceptors was also present in this region (plate 3.1. and Table 3.3.).

Given that autoradiography studies with different radioligands cannot be directly quantitatively compared, due to the different affinity of the ligands, and the concentrations used. Given also, that it is uncertain what density of receptors would be necessary to produce a functional effect. It remains unclear, whether an action at a binding site

with the characteristics of that described for [3H]-idazoxan and [3H]-RS-45041-190 or an action at α_2-adrenoceptors or central [3H]-*p*-aminoclonidine binding sites, would be essential to elicit to an hypotensive response.

In rat kidney membranes [3H]-RS-45041-190 labelled a secondary component which had low affinity for idazoxan and cirazoline and represented 30-40% of the specifically bound [3H]-RS-45041-190 at 1 - 2 nM. It is unlikely that these sites were similar to those labelled by [3H]-*p*-aminoclonidine for two reasons. The total number of sites labelled by [3H]-RS-45041-190 amounted to less than 20% of that labelled by [3H]-*p*-aminoclonidine, and unlabelled *p*-aminoclonidine had very low affinity for [3H]-RS-45041-190 binding. It is also not due to binding to α_2-adrenoceptors as RS-15385-197 and noradrenaline had very low affinity for [3H]-RS-45041-190 binding. Furtheremore, RS-45041-190 had very low affinity for [3H]-RS-15385-197 binding in rat cerebral cortex membranes. Other studies from this laboratory have also shown that RS-45041-190 has very low affinity (pKi < 5.0) for α_1, β_1, β_2, 5-HT_{1A}, 5-HT_2, D_1, D_2, H_1 receptors and dihydropyridine binding sites (Table 1.5.), ruling out an action at these classes of receptors. The additional component is also unlikely to represent an imidazoline site similar to that described in human and bovine medullary membranes (Ernsberger *et al.*, 1986; Bousquet *et al.*, 1984; Bricca *et al.*, 1989b) as *p*-aminoclonidine and cirazoline had relatively low affinity. The nature of this component is therefore uncertain. Autoradiography studies revealed that [3H]-RS-45041-190 labelled several areas that were not recognised by [3H]-idazoxan, including the inferior olive, NTS and locus coeruleus. It is feasable therefore that these areas contain the additional as yet undefined site identified by [3H]-RS-45041-190 in rat kidney membranes.

4.6.4. Cellular localisation

Several lines of evidence suggest that [3H]-idazoxan labelled imidazoline binding sites are enriched in the mitochondria prepared from human and rabbit liver(Tesson *et al.*, 1991) and rabbit cerebral cortex (Tesson & Parini, 1991). In the rabbit liver, the sites are particularly localised to the outer mitochondrial membrane (Tesson *et al.*, 1991). An enrichment in the mitochondria has also been suggested in chromaffin cells (Reis *et al.*, 1992) and glial cells (Ruggiero *et*

al.,1992). The identification of these sites on the mitochondrial membrane could help in the definition of a function. Other studies, however, have suggested that on human placental trophoblasts, [^{3}H]-idazoxan labelled imidazoline sites are present mainly on the cell surface (Diamant *et al.*, 1992). In the present study a relatively crude preparation of rat kidney membranes was studied which would be expected to contain both plasma membrane and mitochondrial membrane fractions. The subcellular localisation and the consequent function of the imidazoline binding sites in these membrane compartments requires further study.

4.7. CONCLUSIONS

In this chapter, imidazoline binding sites were studied in the same preparation of rat kidney membranes with three different radioligands. Three binding sites with different pharmacology were identified: 1) a moderate affinity [^{3}H]-*p*-aminoclonidine binding site, which had low affinity for idazoxan and moderate affinity for clonidine and guanabenz; 2) a site showing high affinity for idazoxan, guanabenz and cirazoline and labelled by [^{3}H]-idazoxan and [^{3}H]-RS-45041-190; 3) a site labelled by [^{3}H]-RS-45041-190 which had low affinity for *p*-aminoclonidine, idazoxan and cirazoline. In autoradiography studies, [^{3}H]-idazoxan binding was restricted to the circumventricular organs and some areas of the hypothalamus. These areas were also labelled by [^{3}H]-RS-45041-190, and probably represent areas containing high densities of the second type of imidazoline site. Additional sites were recognised by [^{3}H]-RS-45041-190 in the locus coerulues, the NTS and the inferior olive, and may represent areas containing imidazoline sites of the third type described above.

Further characterisation of these binding sites, and the identification of the second messenger systems involved in the transduction of a functional response, is necessary to elucidate whether these sites may be classed as functional receptors. Aspects of imidazoline "receptor" function will be addressed in more detail in the following chapter.

CHAPTER FIVE

FUNCTIONAL CONSEQUENCES OF IMIDAZOLINE AND α_2-ADRENOCEPTOR SUBTYPE ACTIVATION

5.1. INTRODUCTION

In Chapters three and four of this thesis, evidence was presented for the existence of different subtypes of α_2-adrenoceptor and imidazoline binding site. In Chapter one, the current knowledge regarding the function of these subtypes was discussed, and was highlighted by the lack of functional backup supporting the existence of different subtypes. In this chapter, the function of these sites will be addressed.

Several studies have demonstrated that α_2-adrenoceptors are negatively linked to adenylate cyclase in a number of preparations (see Chapter one for references). To determine whether this applies to both the α_{2A}- and α_{2B}-adrenoceptor subtypes, the ability of α_2-adrenoceptor agonists to inhibit cAMP accumulation in human platelet (α_{2A}) and neonatal rat lung (α_{2B}) will be examined.

The function of the imidazoline sites will be addressed in two systems:

Firstly, previous data from this laboratory has demonstrated the presence of imidazoline binding sites on hamster adipocyte membranes (MacKinnon *et al.*, 1989), although a physiological function for these sites was not demonstrated. The imidazoline binding site was not a minor component of the [^{3}H]-idazoxan binding; its density was approximately four times that of the α_2-adrenoceptor. In view of the finding that the imidazoline agonist UK 14304 inhibits lipolysis by 80% in rat adipocytes (Rebourcet *et al.*, 1988), and has high affinity for imidazoline binding sites on hamster adipocytes (pK_i = 7.52, MacKinnon *et al.*, 1989), it is important to establish if UK 14304 is acting via the α_2-adrenoceptor or the imidazoline binding site. Therefore, the functional consequences of imidazoline site activation will be examined in this system by assessing the ability of selective imidazoline (RS-45041-190 and cirazoline), selective α_2 (RS-15385-197, yohimbine and phentolamine) and non-selective (idazoxan) agents to influence the UK 14304-induced inhibition of glycerol release in hamster adipocytes.

Secondly, Ramagopal & Leighton (1989) demonstrated that idazoxan could abolish the field stimulation-induced, frequency-dependent relaxations in the rat anococcygeus muscle, when the tone of the muscle was raised with imidazoline agonists such as clonidine, UK

14304 and oxymetazoline but not when the tone was raised with noradrenaline or phenylephrine. The effect of idazoxan was unlikely to be due to blockade of α_2-adrenoceptors as phentolamine was ineffective in this regard. Instead, it was suggested that idazoxan may be acting via an imidazoline preferring receptor or a novel α_2-adrenoceptor subtype. To determine whether imidazoline sites are responsible for this effect, the effect of idazoxan and the selective imidazoline agent RS-45041-190 will be compared in this system.

5.2. MEASUREMENT OF cAMP ACCUMULATION IN HUMAN PLATELETS (α_{2A}) AND NEONATAL RAT LUNG (α_{2B})

5.2.1. cAMP accumulation in whole platelets

A whole cell platelet preparation was used initially as a system for measuring α_{2A}-adrenoceptor-induced inhibition of adenylate cyclase, as this has been shown by several studies to produce a greater maximal response to α_2-adrenoceptor agonists (Bylund & U'Prichard, 1983). Due to the very low levels of cAMP in resting platelets (11.4 ± 2.69 pmoles cAMP/min/mg protein, n=4), an inhibitory action was measured when the levels of cAMP were raised by a stimulatory agonist. In this study the prostacyclin mimetic, cicaprost, was used to stimulate cAMP. Cicaprost (8 nM) raised cAMP levels in whole platelets by 4 fold to 48.64 ± 6.85 pmoles cAMP/min/mg protein (n=4).

In Figure 5.1. the effects of the α_2-adrenoceptor agonists UK 14304, clonidine and oxymetazoline on the inhibition of cicaprost-stimulated cAMP accumulation in whole platelets is presented. Oxymetazoline was the most potent (IC_{50} = 2.54 ± 1.06 nM, n=3) but only produced a maximum response (I max) of 47%. UK 14304 produced a greater maximum response than oxymetazoline (IC_{50} = 6.35 ± 1.88 nM, I max = 81%, n=4) or clonidine (IC_{50} = 10.45 ± 4.45 nM, I max = 70%, n=3). The ability of the α_2-adrenoceptor agonists to produce an inhibition of cAMP accumulation, confirms several studies demonstrating a functional α_2-adrenoceptor linked to the inhibition of adenylate cyclase activity in the human platelet (Jakobs *et al.*, 1978; Jakobs, 1979; Clare *et al.*, 1984).

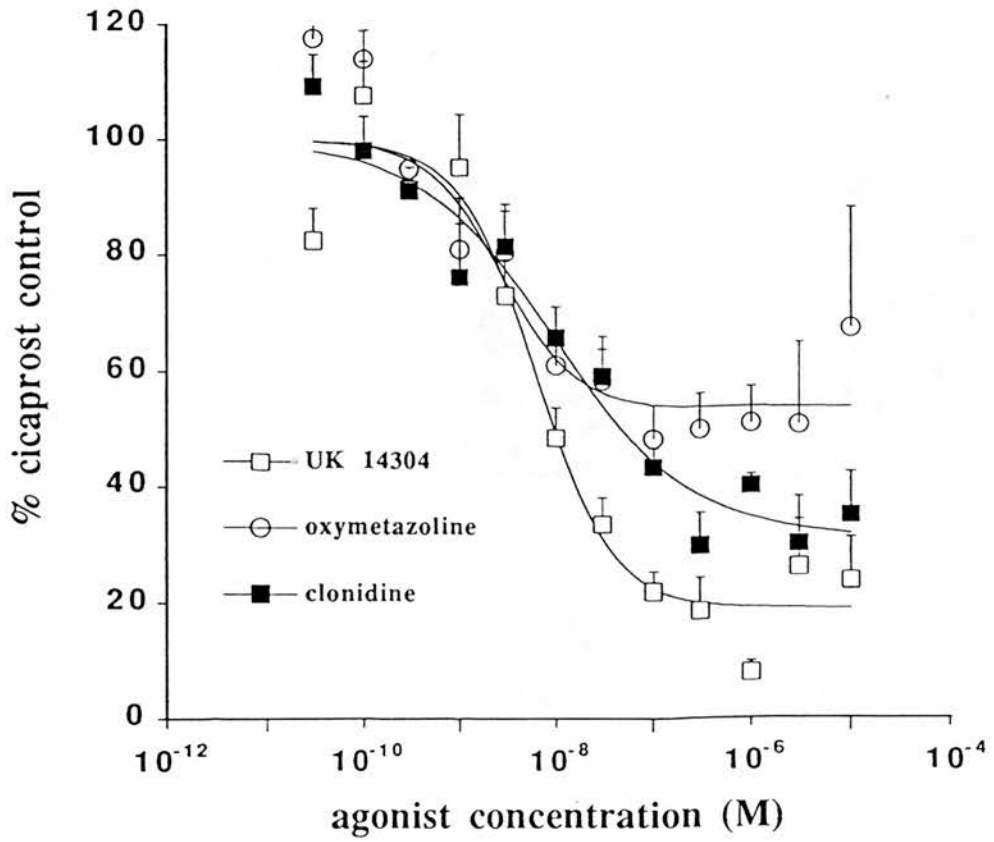


Figure 5.1. Inhibition of cAMP accumulation in human platelets. Agonists were preincubated with whole human platelets for 1 min and incubated in the presence of 8 nM cicaprost for 2 min as described in Chapter two. The results represent the mean ± s.e.mean of 3 experiments (UK 14304, n=4) performed in duplicate. See text for mean values.

5.2.2. cAMP accumulation in platelet membranes

Because an assessment of the α_2-adrenoceptor mediated inhibition of cAMP accumulation in the neonatal rat lung would necessitate the use of a membrane preparation, the platelet studies were repeated in a cell-free, membrane preparation, to make the studies more directly comparable. In both cases, membrane preparations identical to those used in the binding studies were used (see Section 2.2.2., 2.2.3. and 2.7.2.). A buffer containing $MgCl_2$, creatine phosphate, creatine phosphokinase, IBMX, GTP, and ATP was used to ensure adequate levels of the ATP substrate (Golf *et al.*, 1984).

Figure 5.2. shows the effect of incubation time on the accumulation of cAMP in human platelet membranes. Basal, forskolin- and cicaprost-stimulated cAMP levels were linear over the time period 0 - 30 min. In all further experiments an incubation time of 15 min was chosen. Figure 5.3. shows the effect of cicaprost (0.08 - 80 nM) on cAMP production in human platelet membranes. Cicaprost gave a dose dependent increase in cAMP accumulation with an EC_{50} = 3.7 nM. For studies with inhibitory agonists, a concentration of cicaprost which gave 75% of the maximum response i.e. 8 nM, and gave 10 - 13 fold stimulation of cAMP over basal levels (basal, 16.1 ± 3.8 pmoles/min/mg protein; cicaprost 216.5 ± 33.4 pmoles/min/mg protein, n=6) was used to stimulate cAMP.

In Figure 5.4. the effect of UK 14304, clonidine and oxymetazoline on cicaprost-stimulated cAMP accumulation in human platelet membranes is presented. UK 14304 inhibited cAMP accumulation with a maximum inhibition of 40% (IC_{50} = 7.05 ± 1.21 nM, n=3). Clonidine and oxymetazoline were less efficacious, giving maximal inhibition of only 26 and 23% respectively (clonidine IC_{50} = 13.9 ± 5.3 nM, n=3; oxymetazoline IC_{50} = 19.8 ± 8.86 nM, n=3). Therefore, the maximal effectiveness of these agonists was reduced in the membrane preparation as compared to the whole platelet preparation. This suggests that an efficiently coupled system is necessary to observe a maximum α_2-adrenoceptor-induced inhibition of adenylate cyclase.

5.2.3. cAMP accumulation in neonatal rat lung membranes

A time course of cAMP production in neonatal rat lung membranes is presented in Figures 5.5. and 5.6.. Figure 5.5. shows that

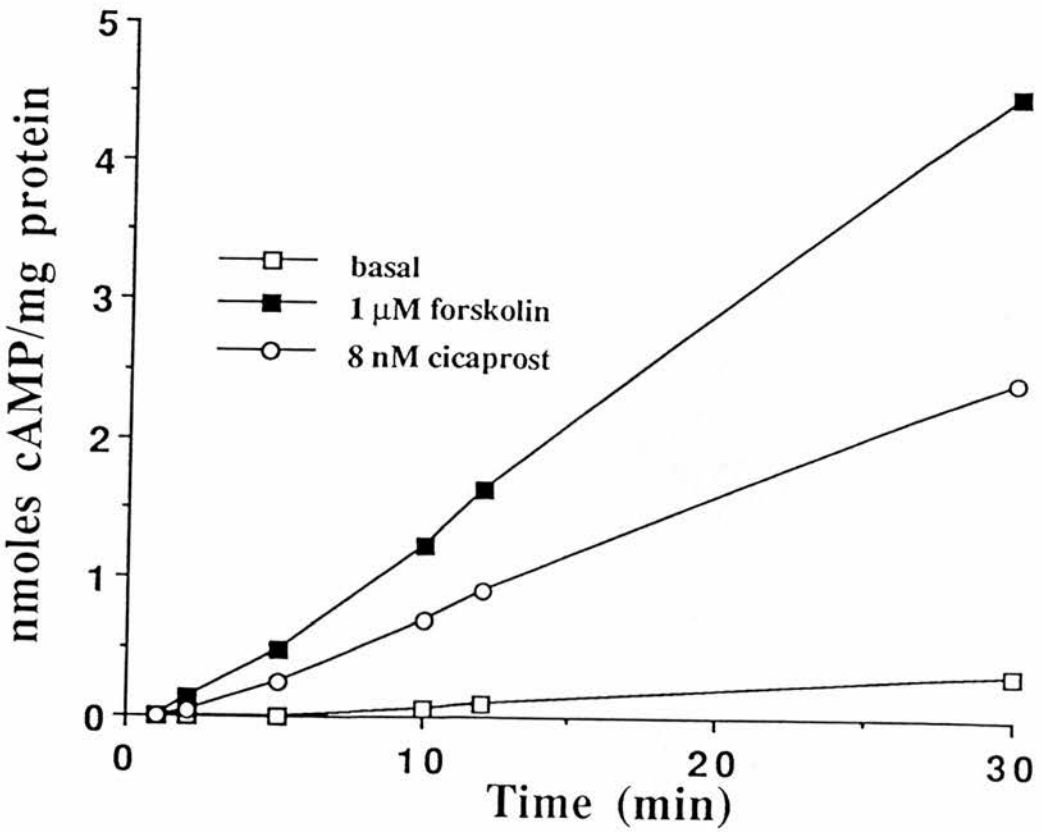


Figure 5.2. Time course of cAMP accumulation in human platelet membranes. Experiments were performed in the absence (control) or presence of 1 μM forskolin or 8 nM cicaprost at 37°C. The results represent the mean of two experiments on different platelet preparations performed in duplicate.

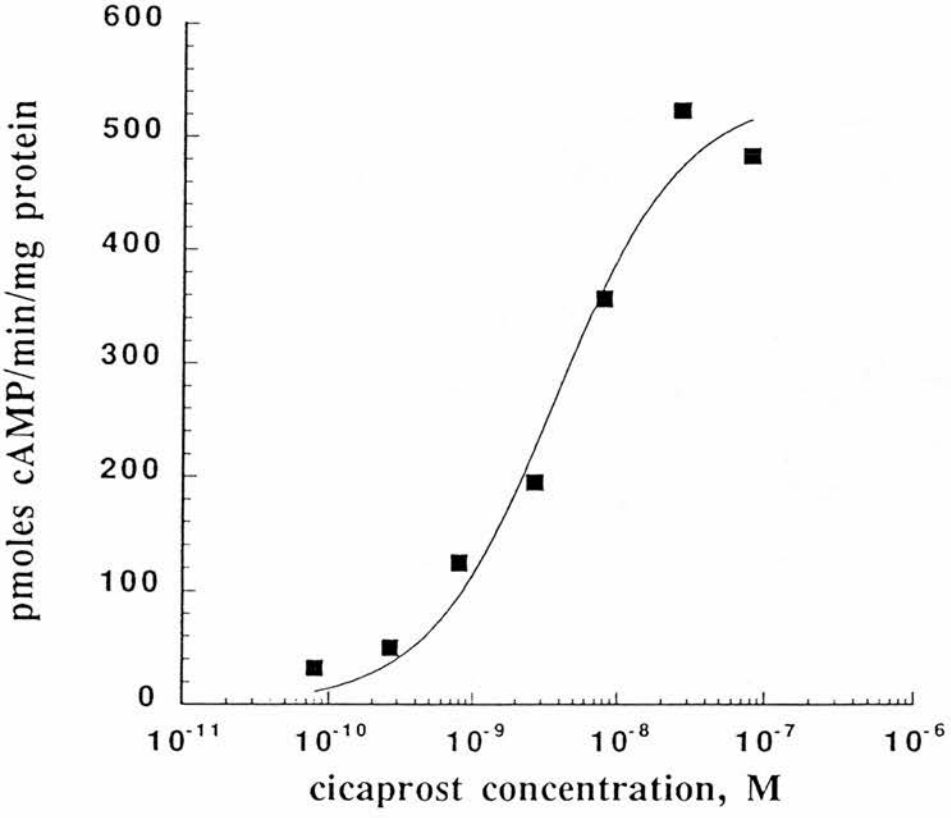


Figure 5.3. Effect of cicaprost on cAMP accumulation in human platelet membranes. Platelet membranes were incubated with increasing concentrations of cicaprost for 1 min at 37°C as described in Chapter two. The results represent the mean of two experiments on different platelet preparations performed in duplicate. Cicaprost EC_{50} = 3.7 nM.

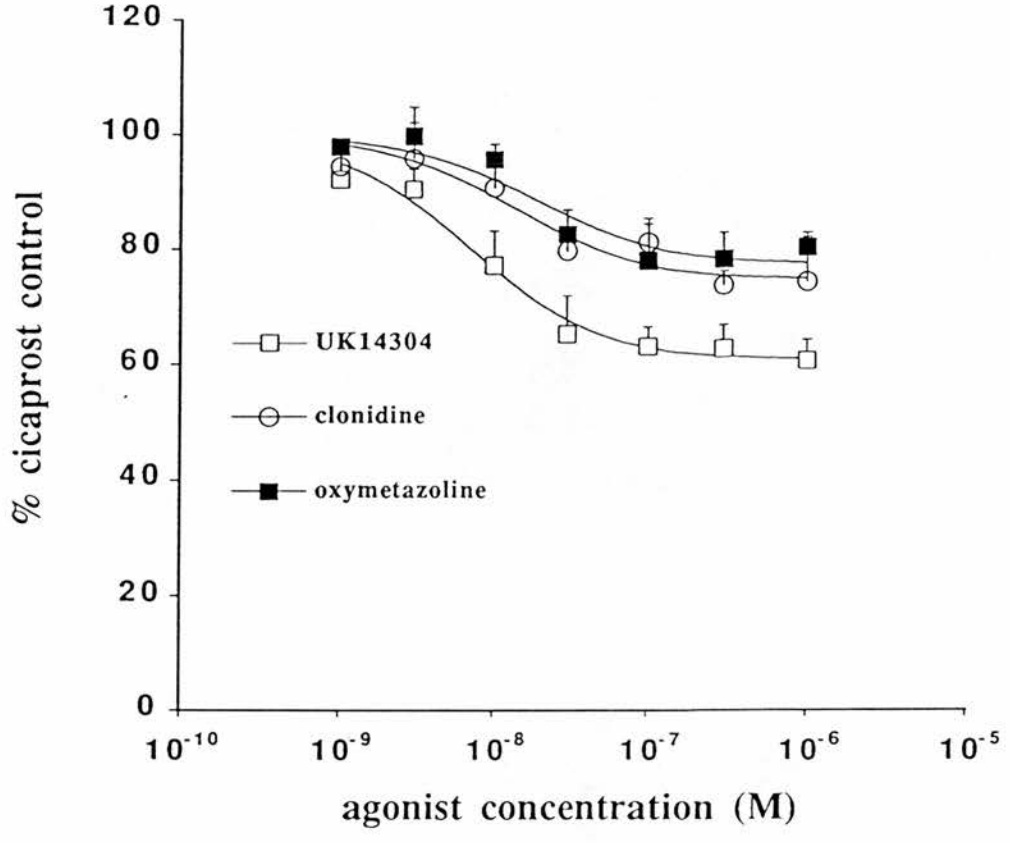


Figure 5.4. Effect of UK 14304, clonidine and oxymetazoline on cicaprost-stimulated cAMP accumulation in human platelet membranes. Platelet membranes were incubated with increasing concentrations of agonist in the presence of 8 nM cicaprost as described in Chapter two. The results represent the mean ± s.e.mean of 3 experiments performed in duplicate. See text for mean values.

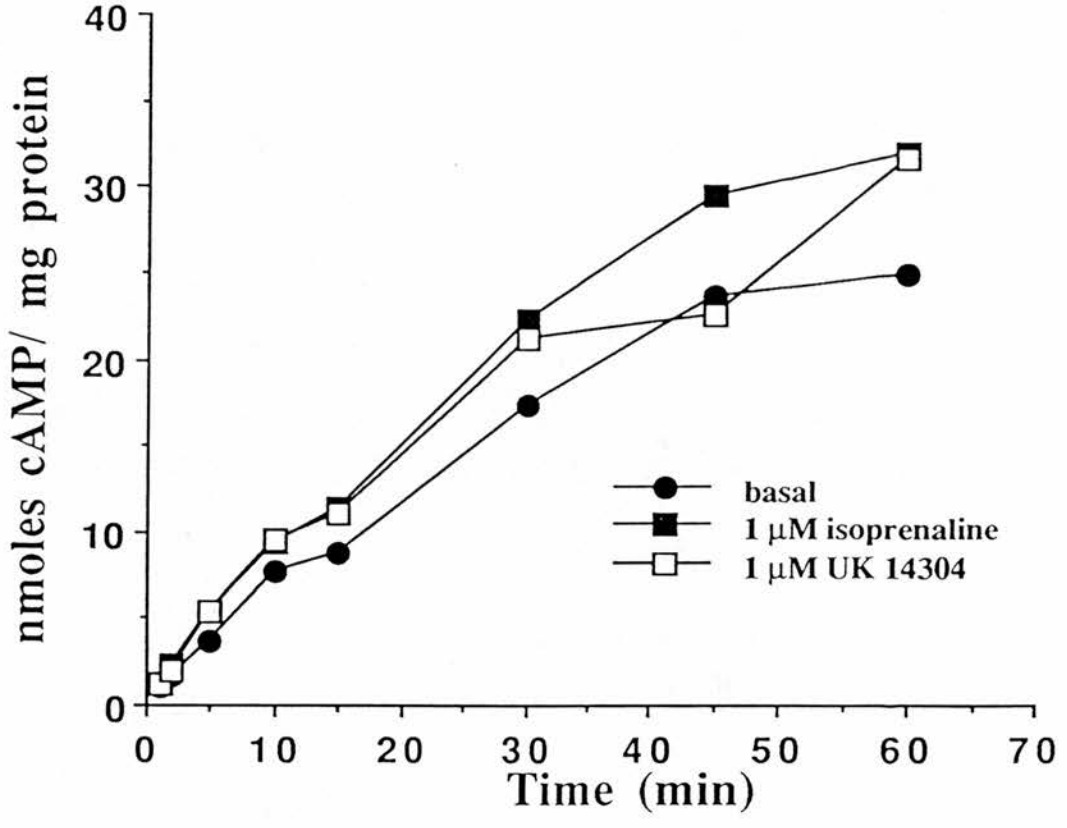


Figure 5.5. Time course of cAMP accumulation in neonatal rat lung membranes at 37°C. Aliquots of membrane preparation were incubated with 1 μM isoprenaline or 1 μM isoprenaline in the presence of 1 μM UK 14304 and compared to the rate of cAMP accumulation in the absence of drugs (basal). The results represent the mean of two experiments performed on separate preparations in duplicate.

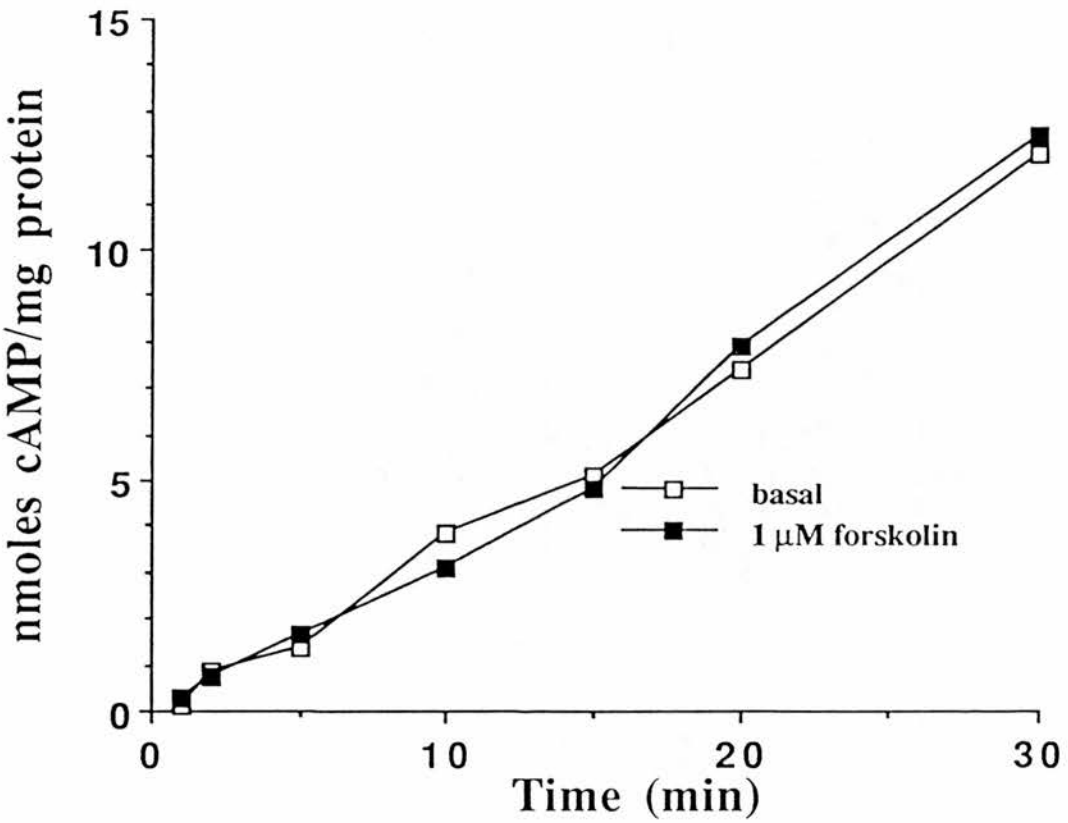


Figure 5.6. Effect of 1 μM forskolin on the rate of cAMP accumulation in neonatal rat lung membranes at 37°C. Aliquots of membrane preparation were incubated in the absence (basal) or presence of 1 μM forskolin as described in Chapter two. The results represent the mean of two experiments performed on separate preparations in duplicate.

the basal rate of cAMP production was linear over the first 30 min and was 30 fold higher that that observed in human platelet membranes (489.9 ± 87.6 pmoles cAMP/min/mg protein, n=6). The rate of cAMP production was not significantly altered by the β-adrenoceptor agonists isoprenaline (1 μM) in the presence or absence of UK 14304 (1 μM). Similarly, as shown in Figure 5.6., 1 μM forskolin had no effect on the basal rate of cAMP production.

5.2.4. Adenylate cyclase activity in neonatal rat lung membranes

Due to the inability of forskolin and isoprenaline to stimulate cAMP levels significantly in neonatal rat lung membranes, the activity of the adenylate cyclase enzyme in this preparation was determined directly by Dr. Graeme Milligan, University of Glasgow, using the method of (Salomon *et al.*, 1974). This method measures the conversion of [^{32}P]-ATP substrate to [^{32}P]-cAMP. A two column system is then employed to separate the labelled [^{3}H]-cAMP. Table 5.1 shows the basal activity of the enzyme and the effect of 10 μM forskolin, 10 μM isoprenaline and 10 μM UK 14304 in the presence of forskolin. Although 10 μM forskolin produced just over a 3 fold increase in adenylate cyclase activity, this stimulation was a lot less than that observed in other systems (13 fold stimulation in rat adipocytes, (Garcia-Sainz & Martinez, 1989); 10 fold stimulation in NG 108-15 cells, (Bylund & Ray-Prenger, (1989)). Similarly, isoprenaline increased activity by less than 2 fold. UK 14304 at a concentration of 10 μM, a maximally effective concentration in the human platelet (Section 5.2.1.), failed to attenuate forskolin- or isoprenaline-induced adenylate cyclase activity in the neonatal rat lung preparation. Therefore, the α_{2B}-adrenoceptor in neonatal rat lung membranes does not couple efficiently to adenylate cyclase to facilitate an inhibition of this enzyme.

5.3. LIPOLYSIS IN HAMSTER ADIPOCYTES

Lipolysis stimulated by theophylline and adenosine deaminase was linear up to 30 min at 37°C in adipocytes isolated from hamster fat pads (Figure 5.7.). All subsequent experiments were carried out at an incubation time of 20 min. The stimulated release of glycerol was

Table 5.1. Adenylate cyclase activity in neonatal rat lung membranes.

additions	adenylate cyclase activity pmol/min/mg protein
basal	129.9 ± 8.5
10 μM forskolin	426.3 ± 9.3
10 μM isoprenaline	235.5 ± 8.5
10 μM forskolin + 10 μM UK 14304	393.0 ± 10.8

Adenylate cyclase activity was measured in neonatal rat lung membranes by Dr. Graeme Milligan, University of Glasgow, using the method of Salomon *et al.* (1974). The results represent the mean ± s.e.mean of four different preparations performed in triplicate.

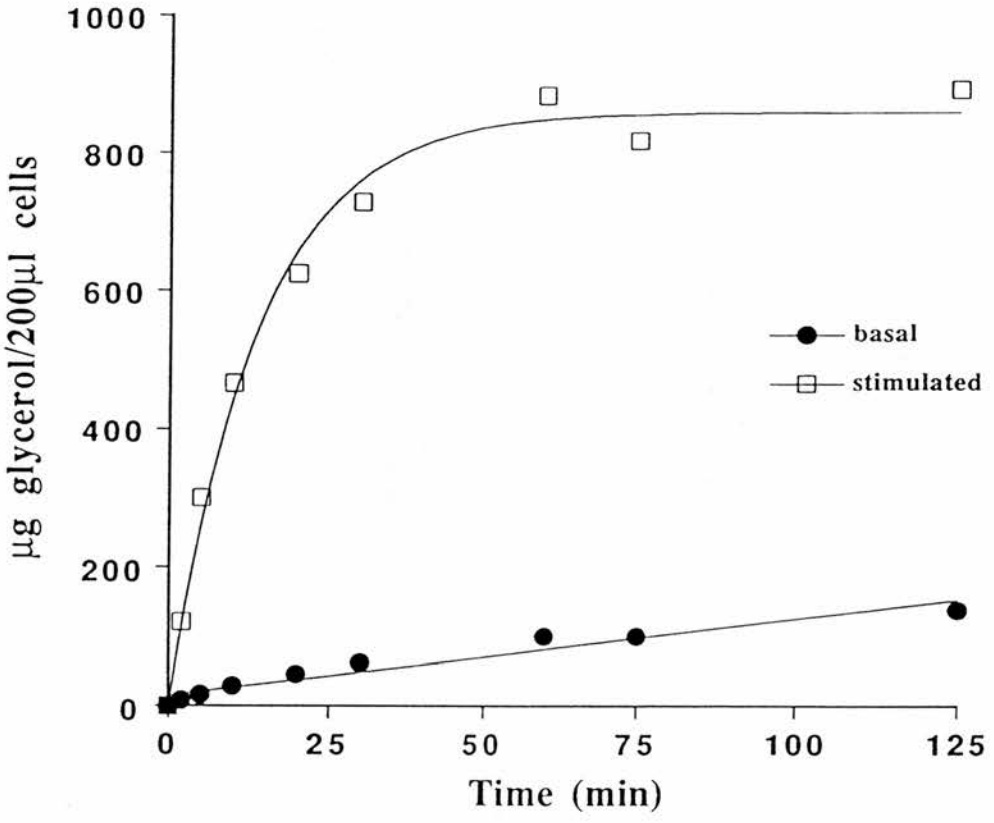


Figure 5.7. Time course of glycerol release from hamster adipocytes at 37°C. Adipocytes were incubated in the absence (basal) or presence of 100 µM theophylline and 0.15U adenosine deaminase (stimulated). Glycerol released into the medium was determined as described in Chapter two. The results represent the mean of two experiments performed on separate preparations in triplicate.

inhibited in a concentration dependent manner by α-adrenergic agonists (Figure 5.8.). UK 14304 and guanabenz appeared to act as full agonists and reversed the stimulated release of glycerol by 80-85%, whereas clonidine and naphazoline acted as a partial agonists, inhibiting release by 40-45%; cirazoline, however, had little effect giving 19% inhibition at 0.1 mM (not shown). The rank order of potency was naphazoline (pIC_{50} = 8.00 ± 0.17, I max = 46%) > guanabenz (pIC_{50} = 7.37 ± 0.07, I max = 84%) > UK 14304 (pIC_{50} = 7.34 ± 0.17, I max = 82%) > clonidine (pIC_{50} = 6.92 ± 0.05, I max = 43%), n=4. RS-15385-197, phentolamine, yohimbine, and RS-45041-190 had no direct effect on basal or stimulated levels of glycerol release at concentrations up to 0.1 mM.

The effects of α_2-antagonists and the imidazoline compounds RS-45041-190 and cirazoline were studied on UK 14304 induced inhibition of glycerol release (Table 5.2.). The effects of idazoxan and RS-15385-197 are also shown in Figure 5.9.. Each antagonist was examined at least three concentrations over the range 10 nM - 10 μM and produced parallel shifts to the right of the concentration response curve for UK 14304 without producing a reduction in maximum response. The pA_2 values and the slope of the Schild plots are summarised in Table 5.2. along with affinity values for [^{3}H]-idazoxan binding to α_2-adrenoceptors and imidazoline binding sites in hamster adipocyte membranes (taken from MacKinnon *et al.*, 1989). The slope of the Schild plot for phentolamine and RS-15385-197 was not significantly different from unity with pA_2 = 7.87 and 7.44 respectively. In contrast, yohimbine, idazoxan and cirazoline gave rise to slopes of less than unity and pA_2 = 7.19, 7.36 and 5.95 respectively. RS-45041-190 was ineffective at concentations up to 0.1 mM. RS-45041-190 was also ineffective in reversing naphazoline induced inhibition of glycerol release (Figure 5.10.). These data indicate that phentolamine and RS-15385-197 antagonised competitively the antilipolytic effects of UK 14304, whereas a more complex interaction occured with idazoxan, yohimbine and cirazoline. Reversal of the antilipolytic effects of UK 14304 occurred with a rank order of potency phentolamine > RS-15385-197 = idazoxan > yohimbine > cirazoline, consistent with an action at an α_2-adrenoceptor.

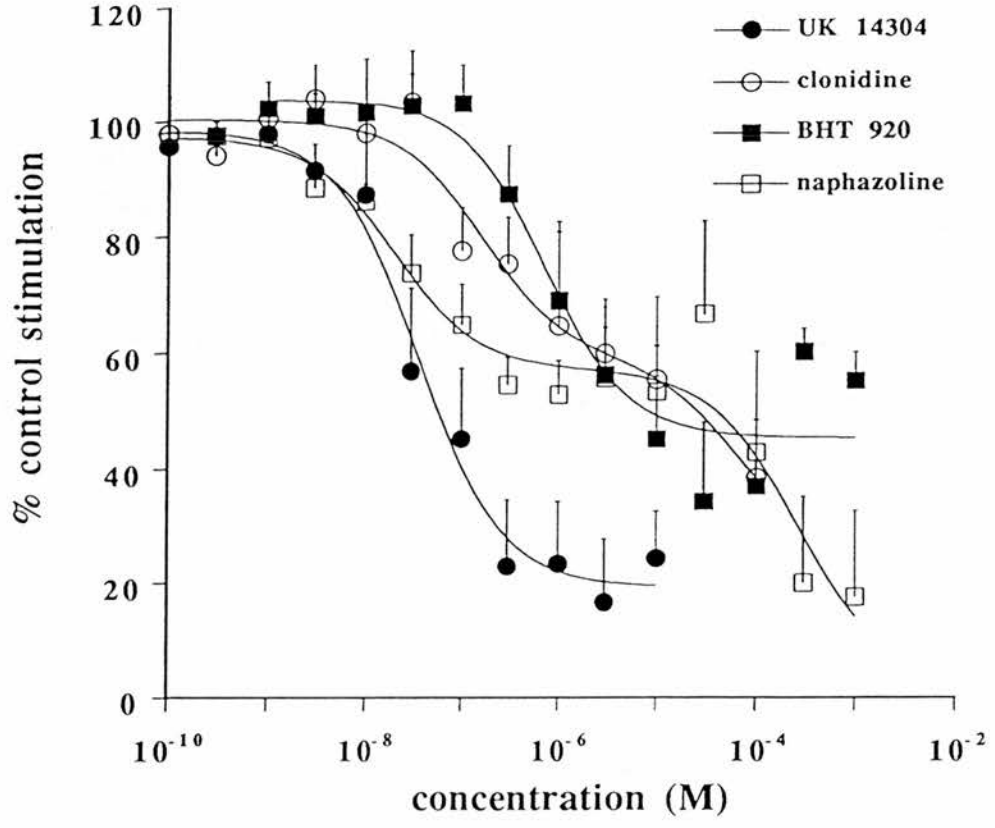


Figure 5.8. Inhibition of theophylline- and adenosine deaminase-stimulated glycerol release from hamster adipocytes. Increasing concentrations of agonist were incubated with 100 μM theophylline and 0.15U adenosine deaminase for 20 min at 37°C as described in Chapter two. The results represent the mean ± s.e.mean of at least 3 separate determinations each performed in duplicate. See text for mean values.

Table 5.2. Reversal of UK 14304 mediated inhibition of glycerol release in hamster adipocytes.

	[3H]-idazoxan binding			glycerol release	
	n	α_2	imidazoline	n	pA_2
RS-15385-197	3	8.38	< 4.00	3	7.44 ± 0.12
idazoxan	4	8.34 ± 0.16		3	7.36 ± 0.32
phentolamine	3	7.92 ± 0.14	5.21 ± 0.02	3	7.87 ± 0.31
yohimbine	3	7.28 ± 0.02	4.26 ± 0.18	3	7.26 ± 0.17
cirazoline	3	6.63 ± 0.09	9.02 ± 0.13	3	5.95 ± 0.31
RS-45041-190	3	4.91 ± 0.22	8.13 ± 0.11	3	<4.00

The data represents the mean ± s.e.mean of n experiments performed in duplicate. Imidazoline and α_2-adrenoceptor affinity were measured in binding experiments with [3H]-idazoxan in hamster adipocyte membranes (taken from MacKinnon *et al.*, 1989).

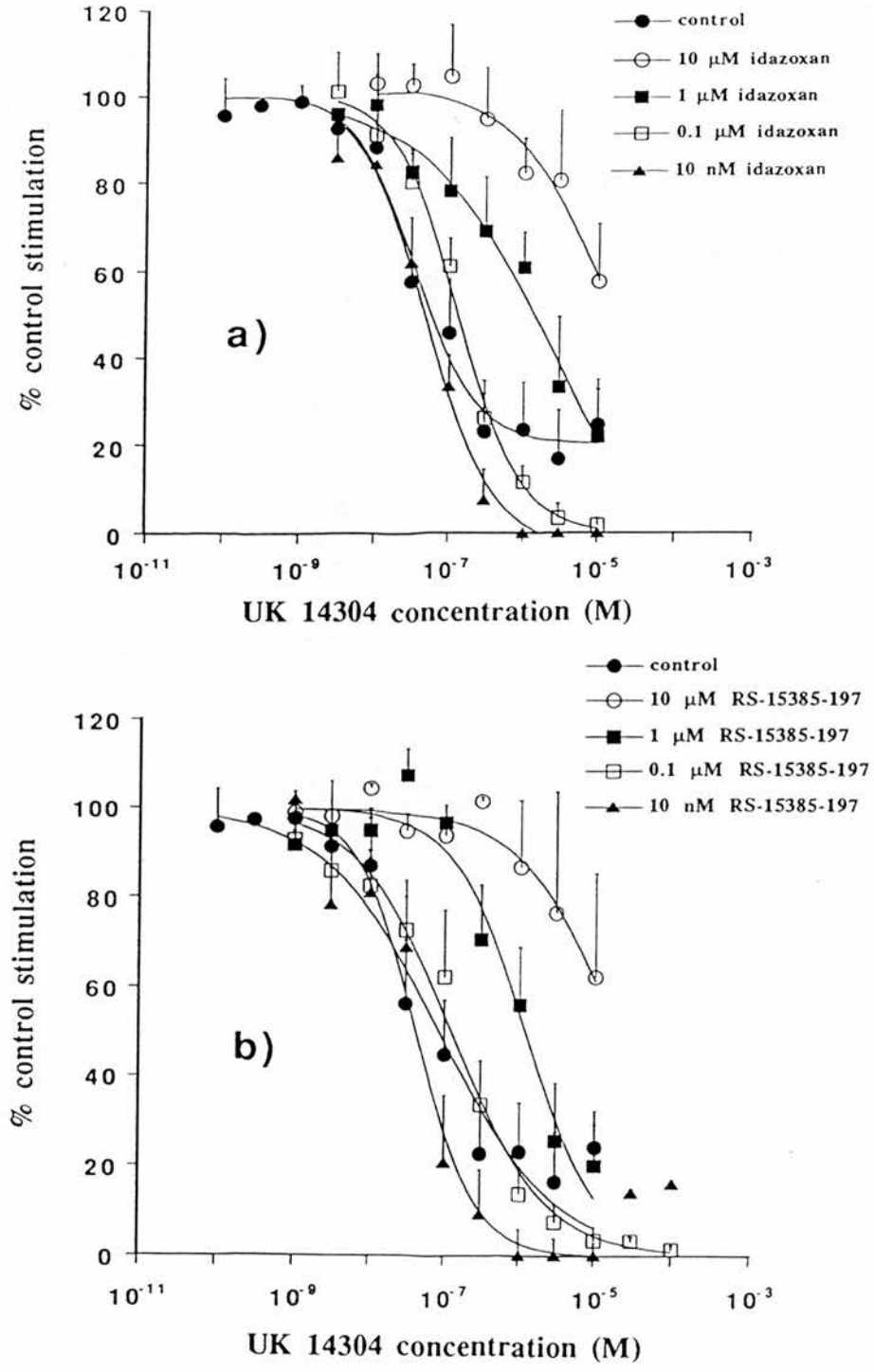


Figure 5.9. Effect of idazoxan and RS-15385-197 on UK 14304-induced inhibition of glycerol release from hamster adipocytes. UK 14304 was incubated in the presence of 100 μM theophylline, 0.15U adenosine deaminase and various concentrations of idazoxan (a) or RS-15385-197 (b) as described in Chapter two. The results represent the mean ± s.e.mean of 3 experiments performed in duplicate. See Table 5.2. for mean values.

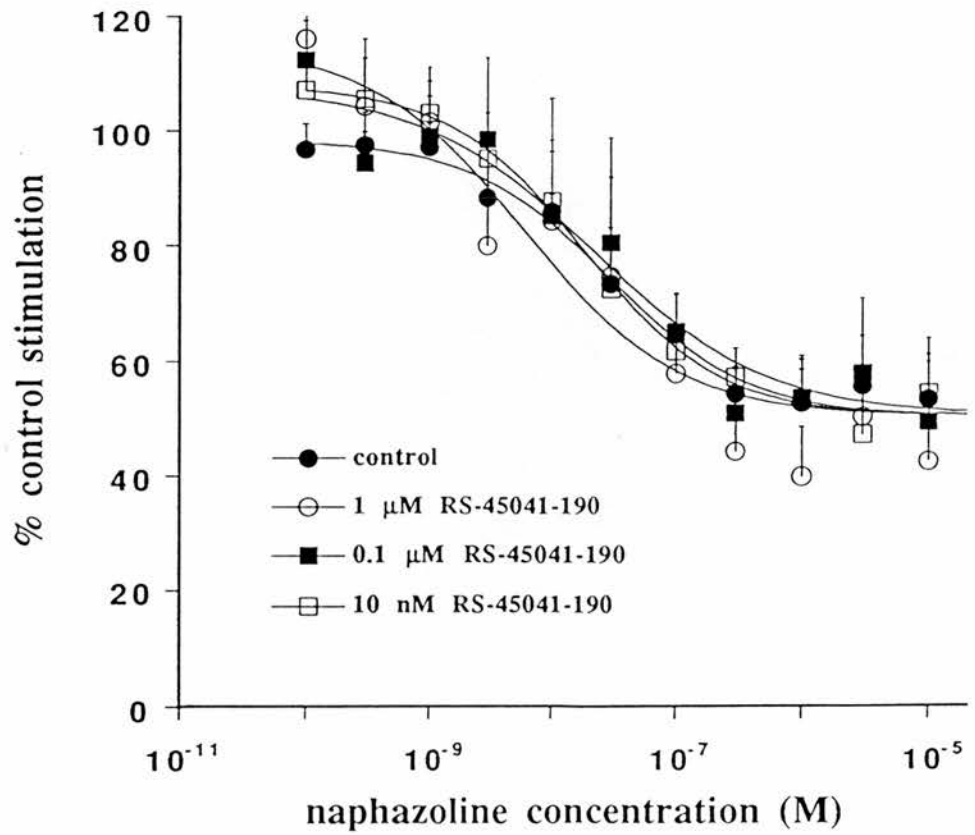


Figure 5.10. Effect of RS-45041-190 on naphazoline-induced inhibition of glycerol release from hamster adipocytes. Naphazoline was incubated in the presence of 100 μM theophylline, 0.15U adenosine deaminase and various concentrations RS-45041-190 as described in Chapter two. The results represent the mean ± s.e.mean of 3 experiments performed in duplicate.

5.4. RAT ANOCOCCYGEUS MUSCLE

To study the effect of field stimulation in response to α-adrenoceptor agonists in the rat anococcygeus muscle, a concentration of agonist which produced equal amounts of tension in the tissue was chosen. In Figure 5.11. a concentration effect curve to noradrenaline is presented. Noradrenaline was added cummulatively; i.e. the next concentration was added when a plateau was reached with the preceeding concentration, without washing between additions. Figure 5.11. shows that noradrenaline produced contractions in the rat anococcygeus muscle with an EC_{50} = 140 ± 17 nM (n=8) and a maximal increase in tension of 6.53 ± 0.36g. Thus a concentration of agonist producing approximately 5.0g tension (75% noradrenaline maximum) in the tissue was chosen for all subsequent experiments.

A representative trace showing the effect of field stimulation on tissues precontracted to clonidine and phenylephrine is presented in Figure 5.12. Clonidine (1 μM) produced a contracture in the anococcygeus muscle of 5.02 ± 0.21g. The same concentration of phenylephrine produced a mean contracture of 5.21 ± 0.31g. When the tissue was contracted with clonidine, field stimulation induced biphasic frequency-dependent changes in tension; i.e., lower frequencies produced relaxations, whereas, higher frequencies produced contractions. Frequencies higher than 8 Hz always produced frequency-dependent contractions. In contrast, when the tissue was contracted with phenylephrine, field stimulation produced only frequency-dependent contractions.

To assess whether antagonist effects could be measured in the same tissue following an initial control response, the reproducability of the clonidine response was established. In these experiments the response to field stimulation was assessed in an initial response curve; the stimulator was switched off, and the tissue washed every 5 min until precontraction baseline activity was observed (30 min). The tissue was then left for 60 min before receiving a second concentration of clonidine and a second frequency response curve. Figure 5.13. shows that the second frequency response was shifted to the left of the first, although the variability of the response this suggested this was not significantly different. It was therefore decided, that antagonists could be assessed in the same tissue following the determination of a control response.

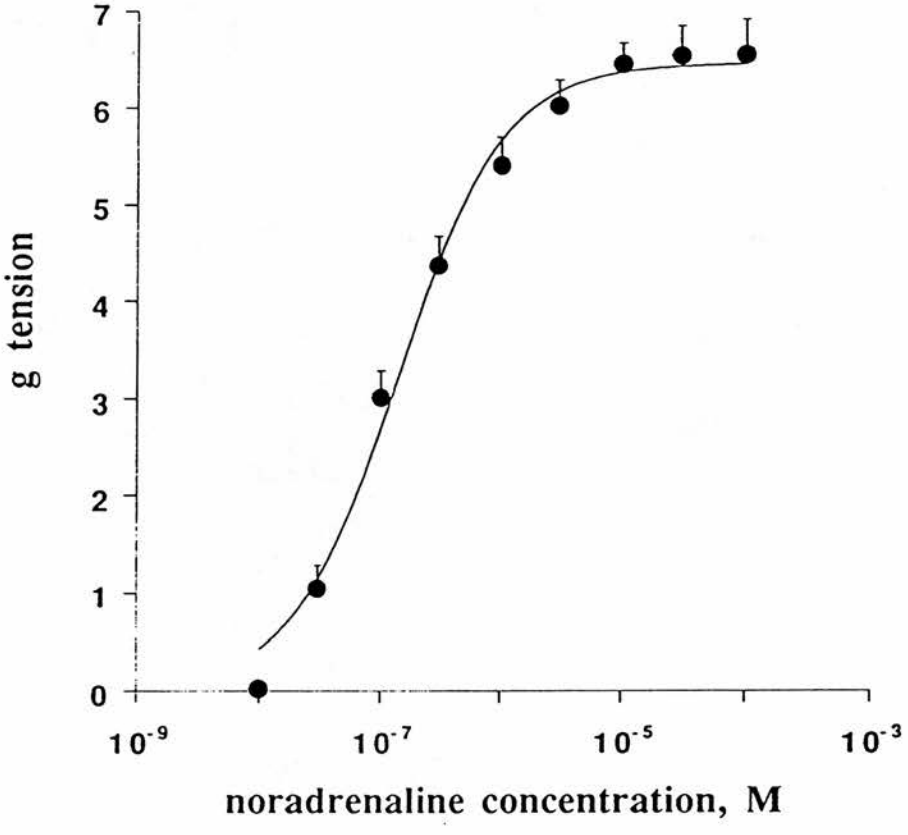


Figure 5.11. Concentration effect curve of noradrenaline on the rat annococcygeus muscle. The results represent the mean ± s.e.mean of 8 experiments. See text for mean values.

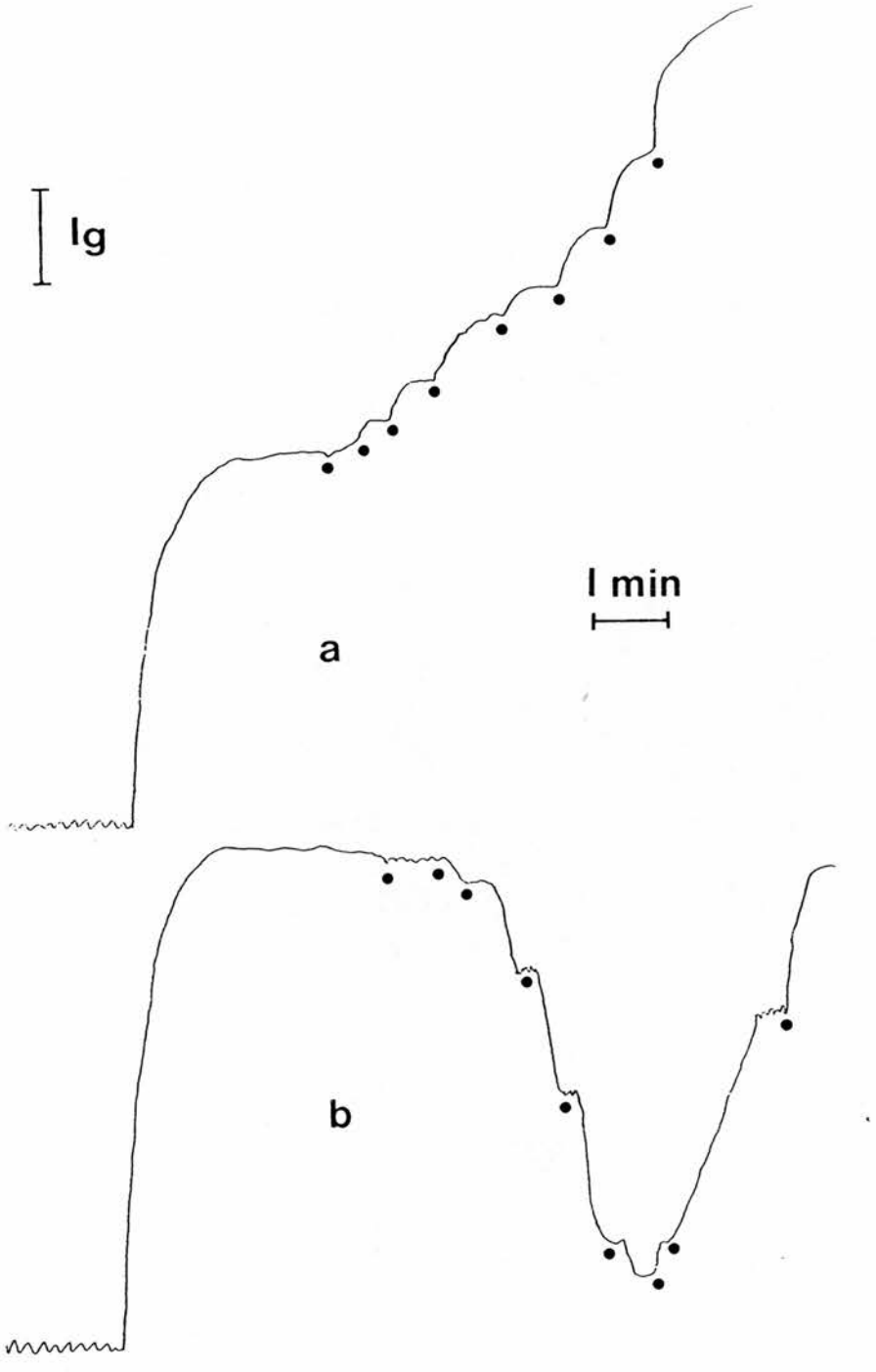


Figure 5.12. A typical trace showing the effect of field stimulation after administration of 1 μM phenylephrine (a) or 1 μM clonidine (b) in the rat annococcygeus muscle. Field stimulation was initiated at a frequency of 0.25Hz and was doubled after attainment of a plateau (idicated by the dots). In (a), field stimulation after phenylephrine produced contractions at all frequencies, whereas after clonidine (b) low frequencies produced relaxations followed by contractions at higher frequencies.

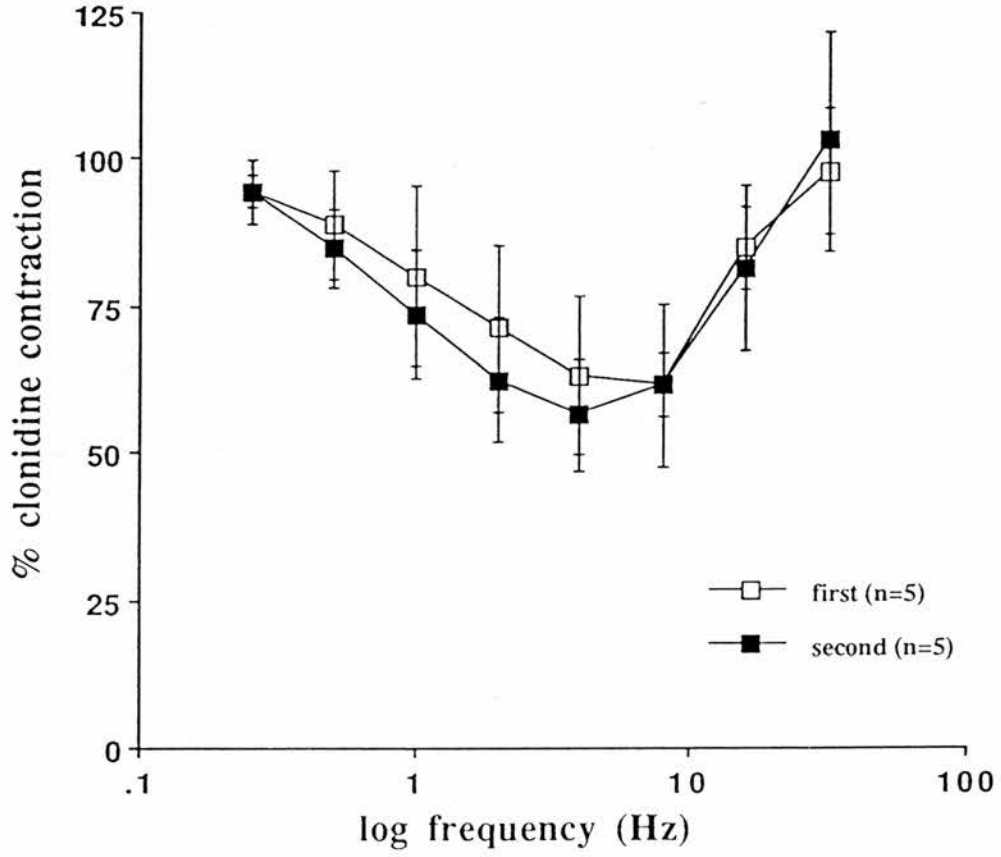


Figure 5.13. Reproducability of frequency response curves induced on clonidine contractions. Frequency response curves were initiated after the administration of clonidine (1 μM). After construction of the first response curve the tissues were washed and allowed to relax back to precontraction baseline. A second response curve was constructed after 60 min. The results represent the mean ± s.e.mean of n experiments. See text for details.

The effect of idazoxan (1 μM) and RS-45041-190 (1 μM) is shown in Figure 5.14.. In the presence of these antagonists, the contraction produced by 1 μM clonidine was 5.71 ± 0.25 and 4.79 ± 0.77g respectively. Neither compound blocked the frequency-dependent relaxations. Indeed, it appeared as though idazoxan in fact potentiated the inhibitory response to low frequencies. However, when the variability of the second response is taken into account (Figure 5.13.), the effect of idazoxan is not significant. Taken together these results suggest that imidazoline sites of the type recognised by RS-45041-190 are probably not involved in the frequency-dependent relaxations observed in the rat anococcygeus muscle.

5.5. DISCUSSION

5.5.1. Inhibition of cAMP accumulation

This study attempted to characterise the functional activity of the α_{2A}- and α_{2B}-adrenoceptor subtypes, by determining the inhibition of cAMP induced by the selective α_2-adrenoceptor agonists UK 14304, oxymetazoline and clonidine in human platelets and neonatal rat lung. In whole platelets, UK 14304 exhibited the greatest efficacy and decreased cAMP stimulated by cicaprost with a IC_{50} of 6.4 nM. The rank order of potency for inhibition of cAMP accumulation was oxymetazoline > UK 14304 > clonidine, and was therefore in good agreement with the affinity of these compounds for α_2-adrenoceptors in binding experiments. A similar potency was observed with UK 14304 in a platelet membrane preparation IC_{50} = 7.1 nM but the maximal inhibition observed was much lower than that observed in the whole cell (40% compared to 81%). The efficacy of oxymetazoline and clonidine was also reduced in the platelet preparation. These studies therefore agree with that of Lenox *et al.* (1980) which indicated that clonidine only exhibits efficacy in a more efficiently coupled system.

In this study, measurement of cAMP accumulation was conducted in the absence of Na^+ ions, as Chapter three demonstrated that agonist potency was greatly reduced in binding experiments in the presence of this cation. However, several studies have suggested that Na^+ ions are essential for the inhibition of adenylate cyclase in a number of cell free systems (for a review see Jakobs, 1979). Studies in whole platelets have shown that the intracellular concentration of Na^+ in platelets suspended

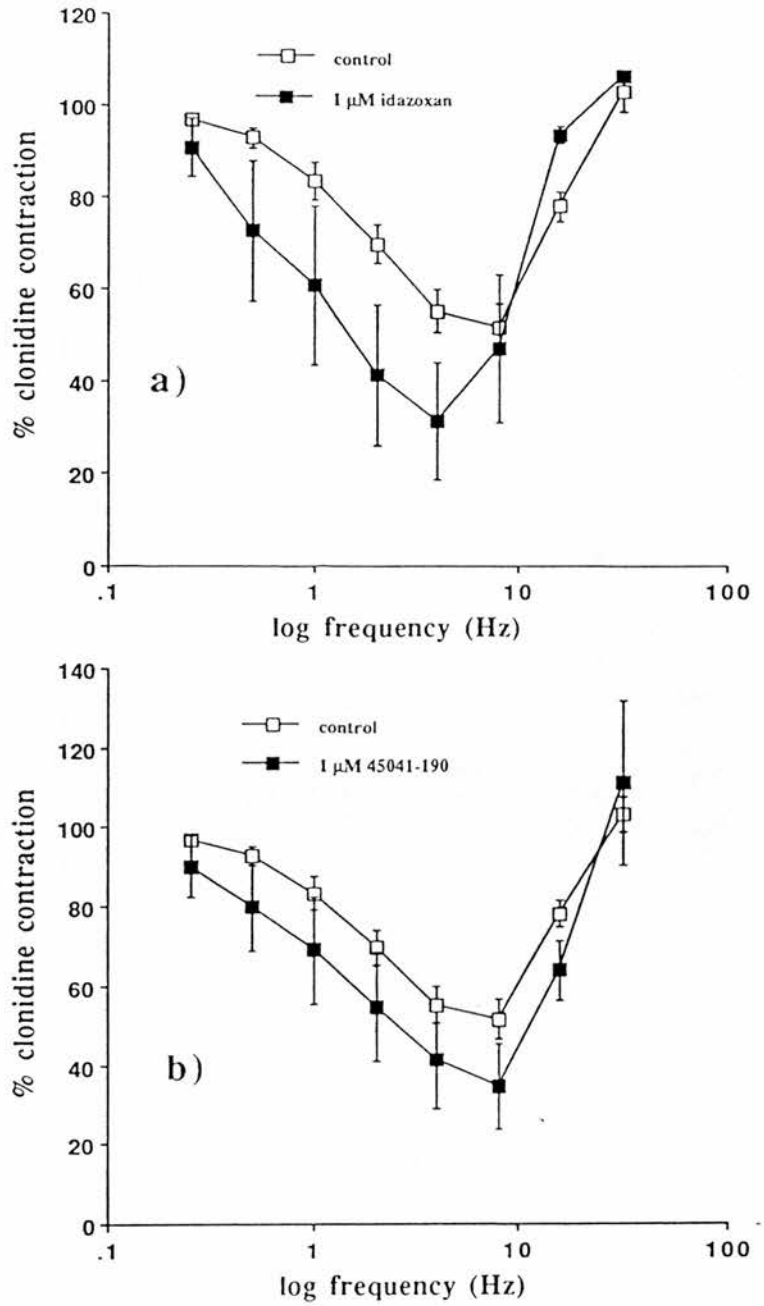


Figure 5.14. Effect of idazoxan (a) and RS-45041-190 (b) on field stimulation induced on clonidine contractions. Idazoxan (1 μM) or RS-45041-190 (1 μM) were added after the construction of a control frequency response, and incubated with the tissue for 60 min before the construction of a second response. The results represent the mean ± s.e.mean of 4 separate experiments in the presence of antagonist.

in a Na+ free medium, as used in the present study, is around 14 mM (Motulsky & Insel, 1983) and that this is sufficient to regulate adenylate cyclase activity (Limbird *et al.*, 1984). Therefore, it is possible that the absence of Na+ in the platelet membrane studies, could account for the reduced efficacy of the agonists compared to the whole cell, and may also explain the complete lack of efficacy in the neonatal lung. Adenylate cyclase activity in the neonatal rat lung was therefore measured directly in the presence of 100 mM Na+ using the method of Salomon *et al.* (1974). However, using this protocol, no inhibition of 10 μM forskolin-stimulated adenylate cyclase activity was observed with 10 μM UK 14304. It appears, therefore, that the α_{2B}-adrenoceptor in the neonatal rat lung does not couple efficiently to adenylate cyclase.

As outlined in Chapter one, there are very few examples of a functional system showing the characteristics of an α_{2B}-adrenoceptor subtype. One study in intact NG 108-15 and HT29 cells; cell lines proposed by Bylund *et al.* (1988) to contain an homogeneous population of α_{2B}- and α_{2A}-adrenoceptors respectively, showed that UK 14304-induced inhibition of VIP-stimulated (HT29) and forskolin-stimulated (NG 108-15) adenylate cyclase was reversed by antagonists with K_b values similar to the K_i values in binding experiments with [^{3}H]-yohimbine (Bylund & Ray-Prenger, 1989). The authors concluded that the functional studies supported the existence of α_{2A}- and α_{2B}-adrenoceptor subtypes. What was not addressed in these studies, was that the potency of UK 14304 was approximately 100 fold higher in the HT29 cell, and whether this correlated with the pK_i of UK 14304 in binding experiments.

The characterisation of the NG 108-15 cell α_2-adrenoceptor as α_{2B} has recently been questioned, as a probe constructed from the α_2-adrenoceptor gene on human chromosome 4 (α_2-C4), which shows the pharmacology of the α_{2C}-adrenoceptor subtype, hybridised with mRNA present in NG 108-15 cells but not neonatal rat lung (Lorenz *et al.*, 1990). This suggests that the α_2-adrenoceptor mediating an inhibition of adenylate cyclase in NG 108-15 cells (Bylund & Ray-Prenger,1989) could be the α_{2C}-adrenoceptor. It therefore remains to be confirmed whether α_{2B}-adrenoceptors couple functionally to adenylate cyclase. Results from this study suggest that they do not. Early studies by Latifpour & Bylund (1982) suggested that the density of α_2-adrenoceptors in the neonatal rat lung decreases rapidly with age;

decreasing by 60% in the first week, and to undetectable levels from 5 weeks. The physiological role of the α_{2B}-adrenoceptor on neonatal rat lung therefore, remains to be determined. Future studies are required to elucidate whether or not an alternative second messenger system is utilised by this α_2-adrenoceptor subtype.

5.5.2. Lipolysis in hamster adipocytes

This present study extends previous observations from this laboratory that [^{3}H]-idazoxan labels an α_2-adrenoceptor and an imidazoline binding site in hamster adipocytes (MacKinnon *et al.*, 1989). Thus, the hamster adipocyte was considered to be an ideal model to explore the functional consequences of imidazoline site activation. Adipocytes are the storage sites for triglycerides in white and brown fat tissue. Considerable evidence indicates that catecholamines alter the rate of free fatty acid and glycerol production through two distinct receptors: stimulation through beta-adrenoceptors and inhibition through α_2-adrenoceptors. This dual effect of catecholamines operates in hamster (Giudicelli *et al.*, 1977b; Garcia-Sainz *et al*, 1980), rabbit (Lafontan, 1981), dog (Berlan *et al.*, 1982) and human (Lafontan & Berlan, 1980; Burns *et al.*, 1981) adipocytes.

The low level of lipolysis in isolated fat cells makes it necessary to first stimulate the system if an antilipolytic effect of α_2-adrenergic agonists is to be observed. A number of stimulants have been used including adrenaline and noradrenaline (Pecquery *et al.*, 1984), isoprenaline (Berlan & Lafontan, 1982), methyl isobutylxanthine (Tan & Curtis-Prior, 1984), theophylline (Lafontan & Berlan, 1980), theophylline and adenosine deaminase (Lafontan *et al.*, 1983) and forskolin (Burns *et al.*, 1982). In this study, lipolysis was stimulated by theophylline together with adenosine deaminase. The mechanisms through which theophylline and adenosine deaminase stimulate lipolysis most likely involve an elevation of cAMP through the inhibition of cAMP dependent phosphodiesterase (theophylline) and the removal, by adenosine deaminase, of the inhibitory effects of adenosine released into the medium. Clonidine has been shown to inhibit stimulated lipolysis from adipocytes isolated from human and hamster fat tissue with a profile consistent with an interaction with α_2-adrenoceptors (Lafontan & Berlan, 1980; Tan & Curtis-Prior, 1984). In a study in isolated rat adipocytes, the antilipolytic action of clonidine was compared with UK

14304, a potent and more selective α_2-adrenoceptor agonist (Cambridge, 1981). Clonidine at concentrations up to 100 μM had only a small antilipolytic effect whereas UK 14304 at 1 μM inhibited lipolysis by approximately 80% (Rebourcet *et al*, 1988). The findings of the present study that clonidine only partially reversed lipolysis in hamster adipocytes, while UK 14304 reversed lipolysis by 80% at the maximally active concentration, provides additional evidence for the greater antilipolytic effects of UK 14304 over clonidine.

The α_2-adrenoceptor nature of the antilipolytic effects of clonidine in a number of functional studies are based solely on the rank order of affinity of idazoxan > yohimbine > prazosin. The concentrations of the α_2-adrenoceptor antagonists required to reverse the antilipolytic effects of clonidine are generally in the micromolar range whereas their affinity at the α_2-adrenoceptor is in the nanomolar range. Although UK 14304 is more selective than clonidine for α_2-adrenoceptors, it also displays high affinity for the imidazoline site on hamster adipocyte membranes (MacKinnon *et al.*, 1989; Saulnier-Blache *et al.*, 1989). Thus it was important to establish if the antilipolytic effects of clonidine and UK 14304 were mediated through the α_2-adrenoceptor or the imidazoline binding site.

Competition studies on the displacement of [^{3}H]-idazoxan from imidazoline binding sites and α_2-adrenoceptors suggest that idazoxan can be considered as a non-selective ligand, having high affinity for both receptors (MacKinnon *et al.*, 1989). Yohimbine, phentolamine and RS-15385-197 show selectivity towards the α_2-adrenoceptor, and cirazoline and RS-45041-190 are selective for imidazoline sites (see Chapters three and four of this thesis). The finding from functional studies, that the antilipolytic effects of UK 14304 were reversed with a rank order of potency of phentolamine > RS-15385-197 = idazoxan > phentolamine > yohimbine > RS-45041-190 agrees with the rank order of affinity for the displacement of [^{3}H]-idazoxan from α_2-adrenoceptors, and suggests that the antilipolytic effects of these agents are mediated via an α_2-adrenoceptor and not an imidazoline receptor.

The subtype of α_2-adrenoceptor involved in the antilipolytic effect is not certain. In hamster adipocyte membranes [^{3}H]-idazoxan labelled a population of α_2-adrenoceptors with a rank order of affinity RS-15385-197 = idazoxan > phentolamine > yohimbine = rauwolscine > prazosin (MacKinnon *et al.*, 1989, 1990). Studies with [^{3}H]-RX 821002,

a ligand reported to be α_{2A} selective from studies in the HT29 cell line (Langin *et al.*, 1989) showed that it had low affinity for imidazoline sites and labelled a site in the hamster adipocyte which resembled α_{2A} in that it had low affinity for prazosin and higher affinity for oxymetazoline, but was unlike the α_{2A} in that it had low affinity for yohimbine (Saulnier-Blanche *et al.*, 1989). The low affinity for yohimbine reported in these studies and others (Pecquery *et al.*, 1984) is probably responsible for the inability of [^{3}H]-yohimbine to label the entire population of α_2-adrenoceptors in fat cell membranes. It also casts doubt on the α_{2A} classification of the adrenoceptor mediating antilipolytic effects.

In Chapter three of this thesis the existence of an α_{2D}-adrenoceptor subtype was discussed and has been reported to exist on rat submaxillary gland (Michel *et al.*, 1989b), bovine pineal gland (Simonneaux *et al.*, 1991) and from this thesis, in rat cerebral cortex. This site is characterised as having high affinity for phentolamine, and low affinity for yohimbine, rauwolscine and prazosin, and in this respect may better describe the α_2-adrenoceptor mediating lipolysis in the adipocyte. However, Michel *et al.* (1989b, 1990) described an α_2-adrenoceptor labelled with [^{3}H]-rauwolscine on rabbit spleen which they termed α_{2A} but which had lower than expected affinity for rauwolscine, and may thus represent a species variant of the α_{2A}-adrenoceptor.

An important finding is that the affinity of RS-15385-197 for the α_2-adrenoceptor on hamster adipocytes labelled by [^{3}H]-idazoxan (pK_i = 8.34, MacKinnon *et al.*, 1990) and in functional studies of glycerol release (pA_2 = 7.44, this study) is more than 30 times lower than that reported for any other α_2-adrenoceptor subtype (MacKinnon *et al.*, 1991a; 1992b and this thesis) and lends credence to the possibility that the adipocyte receptor mediating antilipolytic effects in hamster adipocytes is via a species variant of the α_{2A}-adrenoceptor and not an imidazoline site.

5.5.3. Rat anococcygeus muscle

There are several pieces of evidence to suggest that functional imidazoline receptors exist in smooth muscle. Ruffolo *et al.* (1977) was the first to show that structurally dissimilar α-adrenoceptor agonists (imidazolines and catecholamines) failed to cross-desensitise responses in the rat vas deferens. This suggested that the two types of agonists act

at different receptors or different parts of the receptor. However, this has recently been disputed (Rice *et al.*, 1991), and an alternative explanation based on agonist intrinsic efficacy has been proposed. Another study in the rat vas deferens showed that the imidazoline, naphazoline, but not noradrenaline produced spontaneous phasic activity in this normally quiescent tissue after washout (Grana *et al.*, 1991). The contractile activity could be blocked by cromakalim suggesting that naphazoline could block K+ channels. Presynaptic imidazoline receptors have also been suggested to modulate noradrenaline release in a number of vascular preparations (Göthert & Molderings, 1991; Göthert *et al.*, 1991).

In the present study the effect of idazoxan and RS-45041-190 was studied in the rat anococcygeus muscle. This muscle originally described by Gillespie (1972), responds to agents of several pharmacological classes, and exhibits a relaxation response to field stimulation when the tone of the tissue is raised with guanethidine (Gillespie, 1972,1980). However, Ramagopal & Leighton (1989), demonstrated that the relaxation response observed was dependent on the agent used to initially raise the tone in the tissue. Using a series of α-adrenoceptor agonists, they demonstrated that the relaxation response was observed when clonidine, UK 14304 and oxymetazoline but not noradrenaline or phenylephrine were used to raise the tone of the tissue. Results from the present study showed that relaxation responses were observed at low frequencies with clonidine but not phenylephrine, and were thus in good agreement with the studies of Ramagopal & Leighton (1989). However, in contrast to these studies, the present study did not demonstrate that the relaxation response was blocked by idazoxan, indeed, it appeared as though idazoxan in fact potentiated the response to field stimulation. Moreover, RS-45041-190 (1 μM) had no significant effect on the relaxation response. Thus, results from this study would suggest that imidazoline "receptors" of the idazoxan type, are not involved in the relaxation response to field stimulation in the rat anococcygeus muscle. It remains to be shown whether a different subclass of imidazoline "receptor" may be involved in the response.

It is not clear at present why these two studies gave conflicting results with idazoxan. One reason could be the type of electrode used; steel electrodes were used in the present study whilst platinum electrodes were used in the studies of Ramagopal & Leighton.

However, given that that the control responses were very similar in both studies suggests that the stimulation received by each tissue was essentially the same. Another reason could be the length of time the tissue was allowed to recover following the initial contraction. In the present study the tissue was washed for 30 min until precontraction baseline was acheived and idazoxan or RS-45041-190 were incubated for 60 min prior to the second response. In the studies of Ramagopal & Leighton (1989), this length of time was not stated. It is possible therefore, that the time allowed for recovery, and the exposure time of the antagonist, could be crucial in demonstrating an antagonist effect of idazoxan.

Recently, the transmitter mediating the inhibitory response in the rat anococcygeus muscle has been identified as nitric oxide based on the ability of the competitive inhibitors of L-arginine, N^G-monomethyl L-arginine (L-NMMA, Gillespie *et al.*, 1989) and L-N^G-nitro-arginine (L-NOARG, Gibson *et al.*, 1989) to inhibit the response. Ramagopal & Leighton (1990), in an additional study, suggested that the effects of idazoxan seen in their earlier experiments (Ramagopal & Leighton, 1989) could be via the generation of nitric oxide. Therefore, it remains to be elucidated, whether imidazoline "receptors" can modulate nitric oxide metabolism in this or other tissues.

5.6. CONCLUSIONS

In the present chapter, the functional characteristics of the α_{2A}- and the α_{2B}-adrenoceptor subtypes, and the imidazoline binding sites were addressed. The α_2-adrenoceptor subtypes were assessed by their ability to inhibit adenylate cyclase activity in tissue preparations containing the α_{2A} and α_{2B} subtypes. The studies showed that the α_{2A}-adrenoceptor subtype on human platelets, could inhibit adenylate cyclase activity in whole cells and membranes, although the efficacy of the agonists was improved in the whole cell preparation. In contrast, in neonatal rat lung membranes, a preparation shown to contain the α_{2B}-adrenoceptor subtype in binding studies, failed to show an inhibitory response to a supramaximal concentration of UK 14304. The α_{2B}-adrenoceptor subtype in this preparation was therefore not efficiently coupled to adenylate cyclase.

The imidazoline site on hamster adipocyte membranes was studied

in a functional assay of glycerol release. The ability of several compounds to reverse UK 14304-induced glycerol release was consistent with these agents affinity for α_2-adrenoceptors, suggesting that the imidazoline site was not involved in the antilipolytic effect of UK 14304. In the rat anococcygeus muscle, the frequency-dependent, field stimulation-induced relaxations were not affected by idazoxan and RS-45041-190, suggesting that imidazoline sites are probably not involved in this response. The impact of this work for future studies on α_2-adrenoceptors and imidazoline binding sites is considered in Chapter six of this thesis.

CHAPTER SIX

DISCUSSION AND CONCLUSIONS

6.1. α_2-ADRENOCEPTOR SUBTYPES

This thesis has described the interaction of the novel antagonist ligand, [^{3}H]-RS-15385-197, and the agonist ligand, [^{3}H]-adrenaline, with α_2-adrenoceptor subtypes on human platelets, neonatal rat lung and rat cerebral cortex, in an attempt to classify the subtypes present on these tissues. Binding data with the antagonist described three pharmacologically distinct binding sites, which were characterised as α_{2A} (human platelet), α_{2B} (neonatal rat lung) and α_{2D} (rat cerebral cortex). So far, gene cloning has identified 5 distinct subtypes (see Table 1.3 and references therein). Although classified as distinct receptor subtypes, not all of these subtypes have been shown to exist in more than one species. Thus, a gene coding for the α_{2A}-adrenoceptor has been demonstrated in human and pig tissues, but an equivalent rat gene has so far not been identified. Conversely, although a gene has been isolated from human and rat tissues, which shows α_{2B}-adrenoceptor pharmacology, the human kidney gene did not hybridise with mRNA present in the neonatal rat lung, the prototypical α_{2B} tissue, or brain. The human kidney gene should therefore be termed α_{2B}-like, and may represent a species variant of the α_{2B}-adrenoceptor. The α_{2C}-adrenoceptor, however, has been isolated from rat and human kidney and rat brain and shows a high degree of homology between species. This leaves the α_{2D}-adrenoceptor subtype, for which a gene has been identified in rat kidney. So far, this gene has not been identified in any other species other than the rat and it could be tempting to suggest that this gene may represent the rat homologue of the α_{2A}-adrenoceptor for which there is as yet no known gene.

Limberger *et al.* (1992) suggested that the prejunctional α_2-adrenoceptors in the rat submaxillary gland and the rat vas deferens, previously designated α_{2A} could be better described as α_{2D}. Further supporting the view that the α_{2D}-adrenoceptor may represent a rat α_{2A}-adrenoceptor. However, the α_{2D}-adrenoceptor was first characterised in bovine tissue suggesting that this subtype is not unique to the rat. Duda *et al.* (1990) demonstrated that an α_2-adrenoceptor cDNA clone from a rat brain library hybridised with four different sized mRNAs in rat brain and three in both heart and adrenal gland, suggesting that the range of α_2-adrenoceptor diversity may be larger than originally thought. Elucidation of the structure and pharmacological

characteristics of these gene products, and the identification of more selective compounds, would help in the classification of α_2-adrenoceptor subtypes.

Chapter three of this thesis demonstrated that the identification of different α_2-adrenoceptor subtypes, depends on whether a radiolabelled agonist or antagonist is used to label the receptor. Thus, normally selective antagonists could not discriminate between the subtypes in the "activated" state. I would like to propose a model of agonist and antagonist interactions with the α_2-adrenoceptor subtypes, which is based on the receptor existing in three states with regard to agonist and Gi.

Neubig *et al.* (1988) demonstrated by a series of elegant kinetic studies of [^{3}H]-yohimbine and [^{3}H]-UK 14304 binding in the human platelet that approximately one third of the receptors were precoupled to Gi and bound agonist with high affinity to form a tightly coupled ternary complex (precoupled). Another third had low affinity for agonist and were unable to couple to Gi (free) while the remainder bound agonist with high affinity and coupled with Gi following an agonist induced conformational change and diffusional translocation in the membrane (couplable). If this model is correct then it could be assumed that [^{3}H]-agonists would label the precoupled and couplable receptors, while [^{3}H]-antagonists (which don't discriminate between states) would label the precoupled and free receptors. Antagonists, however, could not label the couplable receptors if it is assumed that antagonists, having no efficacy, can not induce the conformational change required for diffusional-dependent G-protein coupling. This latter aspect is highlighted by the linear Arrhenius plots observed with [^{3}H]-RS-15385-197 binding in the rat cortex. Previous studies in the human platelet have shown linear Arrhenius plots with [^{3}H]-yohimbine, but non-linear plots with [^{3}H]-UK 14304, suggesting that the agonist required protein diffusion in the membrane presumably for coupling to Gi (Gantzoz & Neubig, 1988).

This model therefore suggests firstly that, not all states of the receptor are labelled by antagonists and secondly, that the couplable state is labelled only by agonists. The possibility does exist however that antagonists may label the couplable state but only in the presence of agonist, thus explaining the shallow displacement curves observed with agonist inhibition of antagonist binding.

The model can be used to explain some of the data in the present study: 1) The idea that agonist labelled sites may not meerly a subset of those labelled by the antagonist could explain the vastly different affinities of prazosin for agonist and antagonist binding in the neonatal rat lung. Prazosin may not influence agonist-induced G-protein-coupling but may instead have high affinity for the free receptor. 2) The percentage of sites existing in the three states may be different in the human platelet and the neonatal lung. If as suggested by Neubig *et al.* (1988) the proportion of precoupled, couplable and free receptors on the human platelet are 1:1:1 then the density of sites labelled by [^{3}H]-adrenaline (precoupled and couplable) would be approximately equal to the density of sites labelled by [^{3}H]-RS-15385-197 (precoupled and free). The present study showed that [^{3}H]-adrenaline labelled 85% of those sites labelled by [^{3}H]-RS-15385-197 which would be consistent with this model. However, in the neonatal rat lung the number of agonist labelled sites represented only 44% suggesting that a large proportion of these receptors may be uncoupled and show higher affinity for antagonists. This may also explain the inability of the α_{2B}-adrenoceptor to inhibit adenylate cyclase activity in the neonatal rat lung. In this tissue, agonists may not be able to induce the formation of a ternary complex due either to the inability of the α_{2B}-adrenoceptor to couple, or, to the lack of the required G-proteins.

The finding that agonist affinities in the 2 tissues correlated well whilst antagonist affinities correlated poorly, suggest that the differences in the α_{2A} and α_{2B}-adrenoceptor reside in the characteristics of the free receptor. Antagonists such as prazosin and imiloxan may have higher affinity for the free α_{2B}-adrenoceptor. The relationship of this selectivity to functional antagonism of the α_{2B}-adrenoceptor is however unclear.

Based on these findings I would like to suggest that [^{3}H]-antagonist binding may overestimate the postulated potency of antagonists in functional experiments and lead to a dubious estimation of receptor subtype selectivity. The combined use of [^{3}H]-antagonist and [^{3}H]-agonist binding data may better describe the physiological nature of the receptor. This may also be applicable to receptor systems other than the α_2-adrenoceptor.

The differential effects of Na^+ ions on [^{3}H]-agonist and [^{3}H]-antagonist binding to the α_{2A}- and α_{2B}-adrenoceptor subtypes suggests

that the concentration of Na+ arround the vicinity of the receptor may affect its physiological characteristics. In addition, the concentration of Na+ may determine whether α_{2A}- or α_{2B}-adrenoceptor pharmacology is displayed functionally. Given that the residue reputed to be responsible for Na+ regulation of ligand binding faces intracellulary (Hoffman *et al.*, 1990), the intracellular Na+ concentration may be important. For instance different characteristics may be shown in depolarised cells. As described in Chapter one, α_2-adrenoceptors have been shown to influence Na+/H+ exchange in the human platelet and in NG 108-15 cells (Limbird, 1984; Isom *et al.*, 1987). The consequent increase in intracellular Na+ following α_2-adrenoceptor activation could alter receptor sensitivity to agonists and antagonists. Given that prazosin showed high affinity for the α_{2B}-adrenoceptor labelled by the agonist only in the presence of Na+, a high antagonist potency for this compound in functional experiments may only be observed when intracellular Na+ levels are high.

Functional studies of α_2-adrenoceptor mediated inhibition of adenylate cyclase showed that the platelet α_2-adrenoceptor coupled more efficiently to adenylate cyclase than the neonatal rat lung α_2-adrenoceptor. The possibility exists that α_{2B}-adrenoceptor in the neonatal rat lung prefers an alternative second messenger pathway. Chapter one described that other second messenger pathways have been reputed to be utilised by α_2-adrenoceptors, but that the subtype of α_2-adrenoceptor involved has not been elucidated. Jones *et al.* (1990) demonstrated that that the stably transfected α_{2A}-adrenoceptor gene could activate three distict signal transduction systems in hamster ovary cells; inhibition of adenylate cyclase, stimulation of phospholipase A_2 and potentiation of cAMP production, the latter being insensitive to pertussis toxin. Federman *et al.* (1992) suggested a more complex scheme, and showed that the interaction of a transfected α_{2A}-adrenoceptor with adenylate cyclase was inhibitory or facilitatory, depending on the cell type in which it was transfected. They suggested that the effect observed depended on the subtype of adenylate cyclase present, and the ability of the $\beta\gamma$ subunit of Gi to stimulate cyclase. Presumably, a similar scenario could be possible with other α_2-adrenoceptor subtypes. This indicates that the ability of a receptor to produce a functional response may not be limited purely by the structure of the receptor itself, but also by the nature of the other

components of the transduction pathway present in the cell.

Therefore, ligand binding studies and molecular biology have identified unique subtypes of α_2-adrenoceptors in a variety of tissues and species. The manner in which these subtypes produce their functional effects depends upon a number of physiological variables; the nature of which is begining to be understood.

6.2. IMIDAZOLINE BINDING SITES

Chapter four of this thesis demonstrated that the α_2-adrenoceptor antagonist [^{3}H]-idazoxan, the α_2-adrenoceptor agonist [^{3}H]-*p*-aminoclonidine and the novel imidazoline compound [^{3}H]-RS-45041-190 labelled distinct populations of imidazoline binding site in rat kidney. These sites were termed imidazoline binding sites as the compounds themselves contain an imidazoline ring, and the binding sites labelled by these compounds recognised other imidazoline containing structures. However, I would hesitiate to suggest that these sites meerly represent a chemical recognition site as compounds of other chemical classes also display affinity, e.g. guanabenz.

The binding sites identified by these three radioligands in themselves showed marked differences. Thus, [^{3}H]-RS-45041-190 labelled two populations, a subset of which was labelled by idazoxan. The characteristics of the of site labelled by [^{3}H]-*p*-aminoclonidine did not resemble previously reported data with this compound. However, previous data showing high affinity [^{3}H]-*p*-aminoclonidine binding was carried out in higher species, and suggests that the [^{3}H]-*p*-aminoclonidine binding site described in the present study may represent a species variant. A recent study by Wikberg *et al.* (1992) showing [^{3}H]-*p*-aminoclonidine to label a low affinity site (K_d = 213 nM) in guinea-pig kidney would be consistent with this arguement. At present it is unclear why [^{3}H]-*p*-aminoclonidine should label a site in rat kidney given its low affinity, however, separation by centrifugation and filtration gave essentially similar results, suggesting that the characterisation is valid.

A recent symposium on imidazoline receptors (Paris June 29 -30, 1992; Proceedings published in Fundam. Clin. Pharmacol. volume 6, Suppl. 1), in an attempt to simplify nomenclature, suggested that imidazoline sites labelled with high affinity by clonidine and *p*-

aminoclonidine, and which are sensitive to rilmenidine and insensitive to guanabenz, should be termed I_1; and that sites labelled by [3H]-idazoxan which are sensitive to guanabenz and insensitive to clonidine, should be termed I_2.

In the present study, the binding sites labelled by [3H]-idazoxan and the majority of those labelled by [3H]-RS-45041-190, showed the characteristics of the I_2 binding site. However, none of the sites identified in the kidney, showed the characteristcis of the I_1 binding site. The [3H]-*p*-aminoclonidine binding site described in the present study, may represent a species homologue of the I_1 binding site.

To classify these binding sites as receptors they must be shown to elicit a functional response, and the functional pharmacology of the binding site should relate to the response. Using this criteria, the I_1 subtype was suggested, at the above symposium, to be involved in the hypotensive effect observed with some imidazoline compounds when administered directly into the ventrolateral medulla. However, no conclusive functional effect of the I_2 subtype demonstrated. Unfortunately, using glycerol release in the hamster adipocyte and inhibition of field stimulation-induced relaxation in the rat anococcygeus muscle, no functional I_2 imidazoline effect could be observed in the present study. Autoradiography studies showed that the I_2 subtype exhibited an unique distribution in rat brain. A recent study in human brain showed that [3H]-idazoxan labelled sites were more widespread and localised particularly over the basal ganglia (De Vos *et al.*, 1991). The anatomical and intracellular localisation of these sites to the mitochondria, and the purification and characterisation of the endogenous ligand, may provide some clues as to the functional role of these binding sites.

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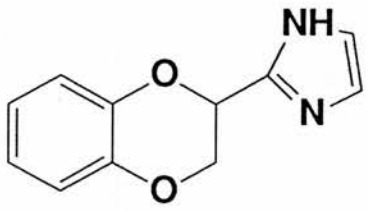
appendix 1



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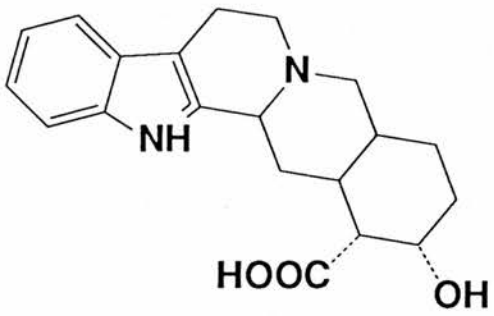

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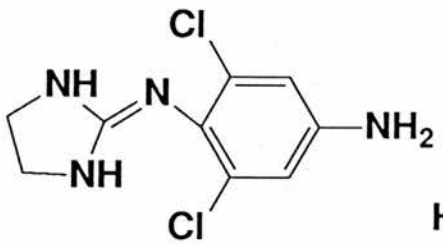

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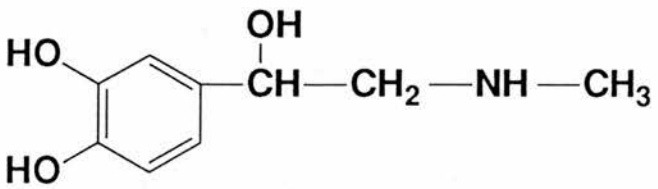

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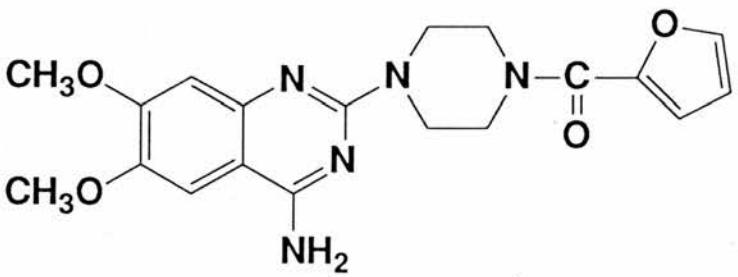

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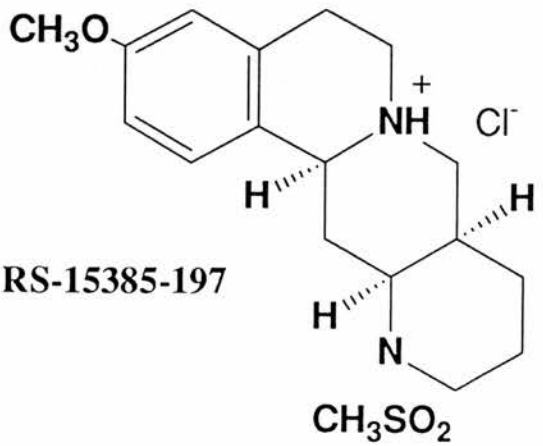

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[^{3}H]p-Aminoclonidine and [^{3}H]idazoxan label different populations of imidazoline sites on rat kidney

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In the presence of RS-15385-197 to preclude binding to α_2-adrenoceptors, [^{3}H]p-aminoclonidine labelled a low affinity high capacity site, ($K_d = 127.6 \pm 19.7$ nM, B_{max} 978 ± 172 fmol/mg protein) whereas [^{3}H]idazoxan labelled a high affinity low capacity site ($K_d = 1.66 \pm 0.28$ nM, B_{max} 45.3 ± 11.4 fmol/mg protein). Clonidine and p-aminoclonidine showed moderate affinity for the site labelled by [^{3}H]p-aminoclonidine, but low affinity for the site labelled by [^{3}H]idazoxan, whereas idazoxan showed high affinity for [^{3}H]idazoxan and low affinity for [^{3}H]p-aminoclonidine binding. Naphazoline inhibited [^{3}H]idazoxan in a biphasic manner suggesting that [^{3}H]idazoxan may label an heterogeneous population of imidazoline sites. GTP inhibited [^{3}H]idazoxan but not [^{3}H]p-aminoclonidine binding. These results suggest that [^{3}H]idazoxan labelled imidazoline I_2 binding sites, whereas [^{3}H]p-aminoclonidine labelled a novel subtype which showed marked differences to the imidazoline I_1 binding site reported in bovine and human brainstem.

[^{3}H]p-Aminoclonidine; [^{3}H]Idazoxan; Imidazoline binding sites; RS-15385-197; Kidney (rat)

1. Introduction

The imidazoline radioligands [^{3}H]p-aminoclonidine and [^{3}H]idazoxan, in addition to labelling α_2-adrenoceptors, also label populations of non-adrenoceptor binding sites which show high affinity for some imidazoline structures. Imidazoline binding sites labelled with [^{3}H]p-aminoclonidine (I_1 sites) in bovine ventrolateral medulla (Ernsberger et al., 1987) and with [^{3}H]clonidine in human brainstem (Bricca et al., 1989) show high affinity for clonidine and oxymetazoline and low affinity for the catecholamines noradrenaline and adrenaline. The imidazoline binding sites labelled with [^{3}H]idazoxan (I_2 sites) in a variety of rabbit tissues show moderate affinity for clonidine and low affinity for catecholamines and yohimbine (Yablonsky et al., 1988; Hamilton et al., 1988), but show high affinity for the guanido compound guanabenz and the diuretic amiloride (Coupry et al., 1989; Yablonsky and Dausse, 1989). In rat and pig kidney [^{3}H]idazoxan labels a site that has low affinity for clonidine and amiloride and moderate affinity for guanabenz (Michel et al., 1989; Vigne et al., 1989) which is similar to the site reported on the hamster adipocyte (MacKinnon et al., 1989). This evidence led Michel and Insel (1989) to propose that imidazoline binding sites may form two or three distinct subgroups, depending on the species and the radioligand used.

In rat kidney membranes, imidazoline binding sites have been identified with [^{3}H]p-aminoclonidine (Ernsberger et al., 1990) and [^{3}H]idazoxan (Michel et al., 1989). Although some differences in the pharmacology of the sites were evident, particularly the affinity of p-aminoclonidine, which was very low against [^{3}H]idazoxan, interpretation was complicated by the presence of a large population of α_2-adrenoceptors which both ligands labelled at the concentrations used in the studies. RS-15385-197 is a potent (pK_i for α_2-adrenoceptors in a variety of tissues 9.3–10.1, Clark et al., 1990) and selective (> 1000-fold selective over α_1-adrenoceptors, Clark et al., 1990) α_2-adrenoceptor antagonist which has been shown to label all subtypes of α_2-adrenoceptor so far described (MacKinnon et al., 1992), but has very low affinity for imidazoline sites in hamster adipocyte membranes (MacKinnon et al., 1990) and would thus be an ideal compound to selectively inhibit binding to α_2-adrenoceptors. It was the purpose of this study to directly compare imidazoline binding sites labelled by [^{3}H]idazoxan and [^{3}H]p-aminocloni-

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dine in the rat kidney in the presence of RS-15385-197 to preclude binding to α_2-adrenoceptors, therefore allowing the characterisation of imidazoline binding site(s) as a single binding component. Some aspects of this work have been published in abstract form (MacKinnon et al., 1991).

2. Materials and methods

2.1. Preparation of rat kidney membranes

Male Sprague-Dawley rats, 200–250 g, were killed by cervical dislocation. Kidneys were homogenised in 25 volumes (w/v) 50 mM Tris HCl; 5 mM EDTA, pH 8.0 at 4°C with a polytron PT 10 tissue disruptor. The homogenate was centrifuged at $48\,000 \times g$ for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in the original volume of homogenisation buffer and recentrifuged. The pellet was washed twice by centrifugation in 50 mM Tris HCl; 0.5 mM EDTA, pH 8.0 at 4°C, and the final pellet resuspended in 3 ml 50 mM Tris HCl; pH 8.0 at 4°C and stored under liquid N_2 until used in the binding assay.

2.2. [^{3}H]p-Aminoclonidine binding

[^{3}H]p-Aminoclonidine, 3–4 nM, was incubated to equilibrium (30 min at 4°C) with 0.8–1.0 mg rat kidney membranes in the absence or presence of various drugs in a final assay volume of 0.5 ml (50 mM Tris HCl pH 7.4 at 4°C containing 0.1 μM RS-15385-197 to preclude binding to α_2-adrenoceptors). Non-specific binding was determined in the presence of 100 μM clonidine. Bound ligand was separated from free by filtration over Whatman GF/B filters in a Brandel Cell Harvester followed by 2×5 s washes (unless otherwise indicated) with ice cold assay buffer. In some experiments centrifugation at $15\,000 \times g$ for 90 s was used to separate bound ligand. In these experiments the resultant pellet was washed once with 0.5 ml ice cold assay buffer and solubilised in 100 μl 0.19 M formic acid for 30 min. Filters or solubilised pellets were suspended in 4 ml scintillation cocktail and bound ligand estimated by counting in a Beckman 5000CE Scintillation counter. Protein was determined using Pierce BCA protein assay reagent and bovine serum albumin as the protein standard.

2.3. [^{3}H]Idazoxan binding

Rat kidney membranes (300–500 μg) were incubated with 1 nM [^{3}H]idazoxan for 90 min at 25°C in the presence of 0.1 μM RS-15385-197 and various concentrations of drugs in a final assay volume of 0.5 ml assay buffer (50 mM Tris HCl; 0.5 mM EDTA, pH 7.4). Bound ligand was separated from free by filtration followed by 2×5 s washes with assay buffer at room temperature. Non-specific binding was determined in the presence of 1 μM cirazoline.

2.4. Data analysis

Binding isotherms from competition studies were analysed by a non-linear least squares curve fitting programme capable of fitting to a one- or two-site model. The IC_{50} (concentration of drug displacing 50% specific binding) was converted to the inhibitory constant (K_i) by the equation of Cheng and Prusoff (1973) where $K_i = IC_{50}/([\text{ligand}]/K_d + 1)$. All data were initially analysed assuming a one site model of radioligand binding. The data with Hill slopes of less than unity were then analysed assuming a two-site model and the results of the fit were statistically compared to those of the one site fit by the differential F value defined by Eq. 1.

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2} \qquad \text{(Eq. 1)}$$

where SS_1 is the sum of squares error for the single site, SS_2 is the sum of squares error for the two-site model, df_1 is the degrees of freedom for the single-site model and df_2 the degrees of freedom for the two-site model (Munson and Rodbard, 1980). A two-site fit was assumed to be significantly better than a single-site fit if the determined F value was significant (95% confidence limits). Equilibrium binding parameters (K_d and B_{max}) for [^{3}H]idazoxan binding were obtained by the iterative non-linear least squares curve fitting programme ligand (Munson and Rodbard, 1980). K_d and B_{max} values for [^{3}H]p-aminoclonidine binding were obtained from competition studies with unlabelled p-aminoclonidine where $K_d = IC_{50} - [\text{ligand}]$ and $B_{max} = BoIC_{50}/[\text{ligand}]$ (Deblasi et al., 1989).

For kinetic experiments, the pseudo first order rate constant (K_{obs}) was calculated from the slope of the plot ln(Be/Be–Bt) versus time where Be is the binding at equilibrium and Bt is the binding at time t. Essentially the same results were obtained with a non-linear least squares fit to a single exponential function (Eq. 2), Experiments for which semilogarithmic plots were non-linear, were analysed according to a double exponential fit (Eq. 3).

$$Bt = Be(1 - e^{-kt}) \qquad \text{(Eq. 2)}$$

$$Bt = Be_f(1 - e^{-kf^t}) + Be_s(1 - e^{-ks^t}) \qquad \text{(Eq. 3)}$$

Be and k are, respectively, the amount of equilibrium binding and the rate constant (K_{obs}) for the single exponential model. Be_f and Be_s are the amplitudes, and k_f and k_s are the rates of the fast and slow binding components in the double exponential model. The fits for a one- or two-site model were compared using the

differential F value. Linear and non-linear fits were analysed by the Kaleidagraph programme run on an Apple Macintosh computer. The association rate constant K_1 was determined from the equation $K_1 = (K_{obs} - K_2)/[ligand]$, where K_2 is the dissociation rate constant calculated from the slope of the plot ln(Bt/B0) against time, where B0 represents binding at time 0 and Bt the binding at time t. The equilibrium dissociation constant (K_d) was calculated from the equation $K_d = K_2/K_1$.

2.5. Chemicals and drugs

[^{3}H]Idazoxan (40 Ci/mmol) was purchased from Amersham International plc U.K. and [^{3}H]p-aminoclonidine (56 Ci/mmol) was purchased from Dupont N.E.N. Division U.K. Drugs were obtained from the following sources; noradrenaline, naphazoline and clonidine from Sigma; p-aminoclonidine from Research Biochemicals Incorporated; cirazoline from Synthelabo; phentolamine from Ciba Geigy; cimetidine from SmithKlein & Beecham; guanabenz from Wyeth; RS-15385-197 ((8aR,12aS,13aS)-5,8,8a,9,10,11,12,12a, 13,13a-decahydro-3-methoxy-12-(methylsulfonyl)-6H-isoquino[2,1-g][1,6]-naphthyridine) and idazoxan were synthesised by Dr. R. Clark, Syntex Palo Alto. All other chemicals were of the highest purity commercially available.

3. Results

3.1. Specificity of [^{3}H]idazoxan and [^{3}H]p-aminoclonidine binding

Initial experiments were carried out in the absence of RS-15385-197 to establish the experimental conditions required to isolate the imidazoline component. In competition experiments (fig. 1A) RS-15385-197 did not displace total [^{3}H]idazoxan binding at concentrations up to 0.1 mM, whereas noradrenaline displaced 40% with low affinity ($pIC_{50} = 4.86 \pm 0.30$, $n = 3$) and unlabelled idazoxan displaced 80% of the total binding with high affinity ($pIC_{50} = 8.15$, $n = 2$). RS-15385-197 and noradrenaline showed high affinity for 50–60% of the total [^{3}H]p-aminoclonidine binding at 25°C ($pIC_{50} = 8.81 \pm 0.09$, $n = 5$ and 7.23 ± 0.48, $n = 3$ respectively), whereas clonidine displaced 70–80% ($pIC_{50} = 7.64$, $n = 2$, fig. 1B). Although [^{3}H]idazoxan did not appear to label α_2-adrenoceptors in competition experiments at 1 nM, subsequent experiments with both [^{3}H]idazoxan and [^{3}H]p-aminoclonidine were carried out in the presence of 0.1 μM RS-15385-197 to exclude possible binding to α_2-adrenoceptors at higher ligand concentrations.

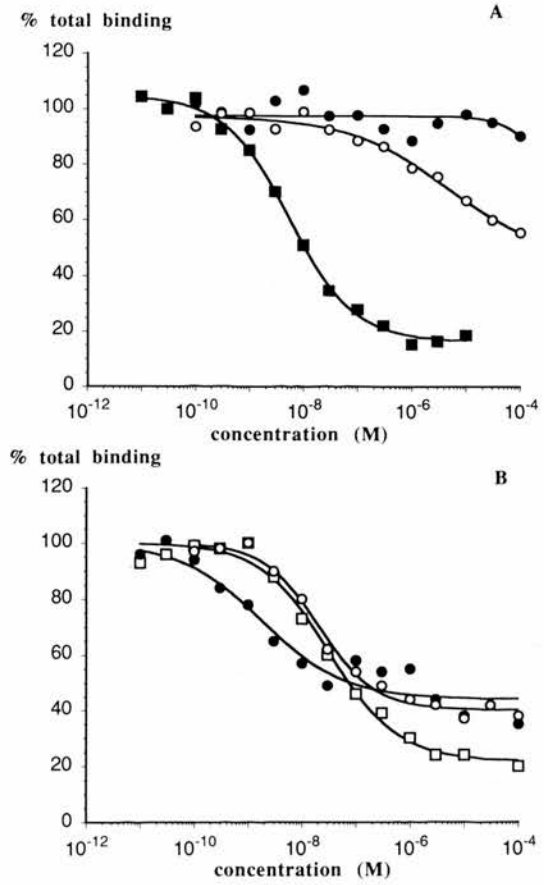
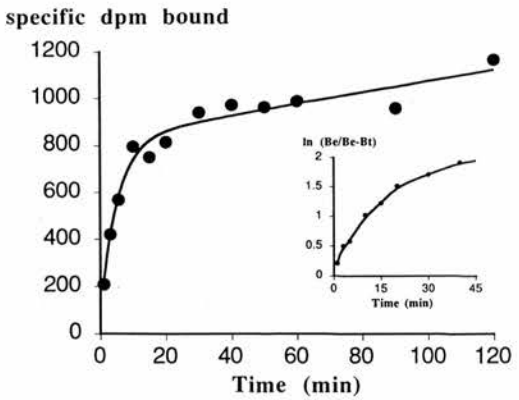


Fig. 1. Inhibition of 1 nM [^{3}H]idazoxan binding (A) and 3 nM [^{3}H]p-aminoclonidine (B) binding to rat kidney membranes by RS-15385-197 (●), noradrenaline (○), idazoxan (■) and clonidine (□). Incubations were performed at 25°C as described in Methods. The data represents a typical experiment performed in duplicate.

3.2. Kinetics of [^{3}H]idazoxan and [^{3}H]p-aminoclonidine binding to imidazoline sites

The interaction of [^{3}H]idazoxan with the imidazoline binding site on rat kidney membranes was rapid and reversible (fig. 2). Equilibrium was attained after 90 min with $K_{obs} = 0.0727 \pm 0.0065$/min ($n = 5$). In three out of five experiments however, the association log plots were better fitted to two-site model with $t_{1/2}$ for the fast and slow associating components = 1.59 and 30.1 min respectively. On the addition of 1 μM cirazoline, [^{3}H]idazoxan binding was fully reversed but showed fast and slow dissociation components, with 65% of the specific [^{3}H]idazoxan binding dissociated within the first 15 min ($K_2 = 0.5176 \pm 0.1823$/min, $t_{1/2} = 1.3$ min). The remaining binding dissociated more slowly ($K_2 = 0.0234 \pm 0.007$/min, $t_{1/2} = 30$ min). In experiments showing biphasic association plots K_d values were calculated based on the percentage of ligand associating and dissociating rapidly and yielded affinity constants for the fast and slow dissociation components of 4.38 ± 1.10 and 2.24 ± 1.23 nM ($n = 3$).

Initial studies with [^{3}H]p-aminoclonidine showed the radioligand to be fully associated after 1 min at 25°C and fully dissociated on the addition of 100 μM clonidine within the first 90 s. Experiments with [^{3}H]p-aminoclonidine were subsequently performed at 4°C to slow the reaction rate. Figure 3 shows that even at 4°C the kinetics for [^{3}H]p-aminoclonidine were rapid with equilibrium being attained after 10 min. On the addition of 0.1 mM clonidine, [^{3}H]p-aminoclonidine binding was rapidly reversed and fully dissociated after 10 min. The rapidity of the interaction did not allow for the determination of reliable rate constants in consecutive experiments, but the data was consistent with [^{3}H]p-aminoclonidine labelling a low affinity site in rat kidney.



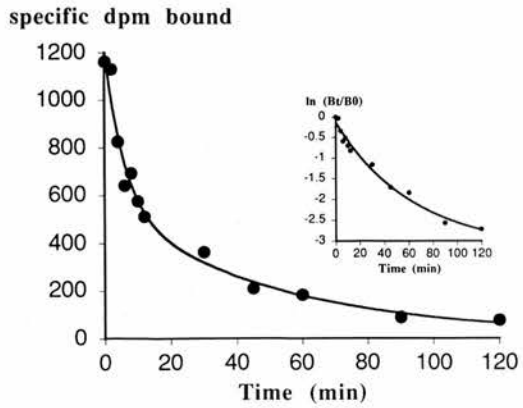
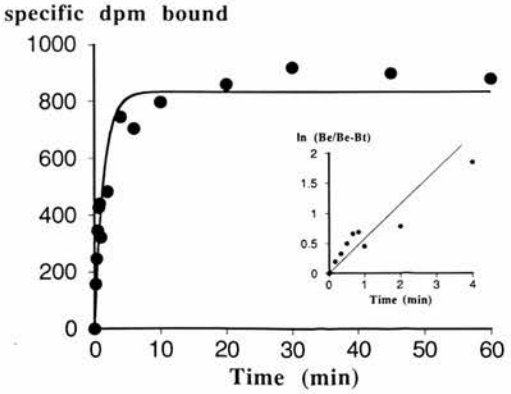


Fig. 2. Kinetic analysis of [^{3}H]idazoxan binding to rat kidney membranes at 25°C. The insets show the semilogarithmic plots of the data. The data represents a single experiment performed in triplicate. Two association rate constants (top panel) were calculated; $K_{obs1} = 0.307$/min and $K_{obs2} = 0.0299$/min. On the addition of 1 μM cirazoline, [^{3}H]idazoxan dissociated with two rate constants $K_{21} = 0.1701$/min and $K_{22} = 0.0197$/min (bottom panel). Calculated K_d values for the fast and slow components were 2.23 and 3.46 nM respectively.



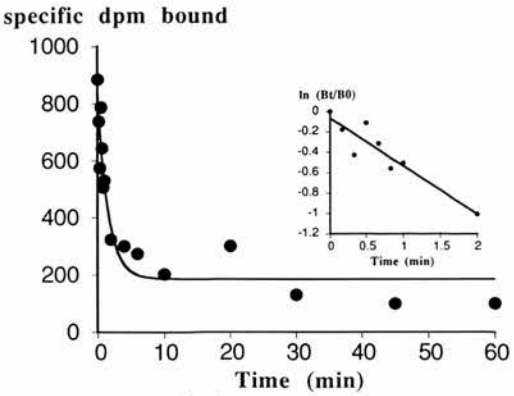


Fig. 3. Kinetic analysis of [^{3}H]p-aminoclonidine binding in rat kidney membranes at 4°C. The insets show the semilogarithmic plots of the data. The data represents a single experiment performed in triplicate. The association rate (top panel) was rapid, and the ligand fully associated after 10 min ($K_{obs} = 0.707$/min). The dissociation rate (bottom panel) was also rapid, with 66% of the bound ligand dissociated within the first 2 min ($K_2 = 0.681$/min), giving a derived affinity $K_d = 81.2$ nM. Accurate determination of the rates in consecutive experiments was not possible due to the rapidity of the reaction.

3.3. Number and affinity of [^{3}H]idazoxan and [^{3}H]p-aminoclonidine binding sites

In saturation studies (fig. 4) [^{3}H]idazoxan labelled a single site with high affinity in the presence of RS-15385-197 ($K_d = 1.66 \pm 0.28$ nM, $B_{max} = 45.33 \pm 11.4$ fmol/mg protein, $n = 8$). At the K_d concentration, non-specific [^{3}H]idazoxan binding represented 20–30% of the total binding. The site labelled with [^{3}H]p-aminoclonidine was monophasic and of low affinity ($K_d = 127.6 \pm 19.7$ nM, $n = 12$) but [^{3}H]p-aminoclonidine labelled more than 20 times the number of sites than [^{3}H]idazoxan ($B_{max} = 978 \pm 172$ fmol/mg protein). The percentage of non-specific binding with this ligand was greater than that for [^{3}H]idazoxan and represented 40–50% of the total [^{3}H]p-aminoclonidine binding at 3 nM. Because of the low affinity of [^{3}H]p-aminoclonidine, we were surprised that we were able to show specific binding using filtration to separate bound from free ligand as a high percentage of the

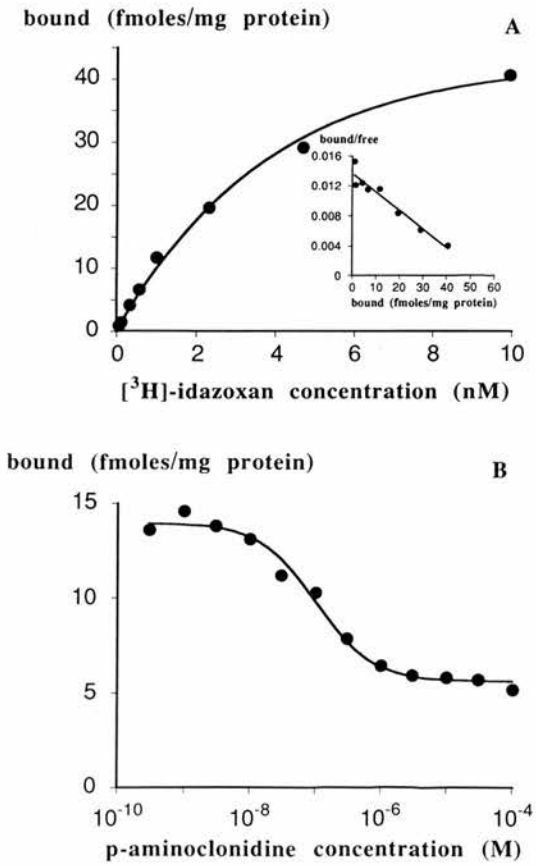


Fig. 4. Saturation analysis of [^{3}H]idazoxan (A) and [^{3}H]p-aminoclonidine (B) binding in rat kidney. The results represent a typical experiment performed in triplicate. K_d and B_{max} values were calculated as described in Methods. (A) $K_d = 1.88$ nM, $B_{max} = 54.7$ fmol/mg. (B) $K_d = 100$ nM, $B_{max} = 775.4$ fmol/mg protein.

specifically bound ligand would be expected to dissociate during the separation process, however, when centrifugation was used to separate bound radioligand, the affinity and density of sites labelled by [^{3}H]p-aminoclonidine were not significantly different from the filtration assays ($K_d = 104.2 \pm 21.7$ nM, $B_{max} = 1480 \pm 310$ fmol/mg protein, n = 4). Given that the method of separation yielded essentially similar results, the characterisation of [^{3}H]p-aminoclonidine binding sites was continued using filtration to make our study more comparable with other work (Ernsberger et al., 1990).

3.4. Pharmacology of [^{3}H]idazoxan and [^{3}H]p-aminoclonidine binding sites

Table 1 shows affinity values (pK_i) for a variety of agents for the imidazoline binding site labelled by [^{3}H]p-aminoclonidine and [^{3}H]idazoxan. Clonidine and p-aminoclonidine showed moderately high affinity for the site labelled by [^{3}H]p-aminoclonidine ($pK_i = 6.88$ and 6.98 respectively) but only low affinity for the site labelled by [^{3}H]idazoxan ($pK_i = 5.64$ and 5.05). Idazoxan and amiloride had low affinity for the site labelled with [^{3}H]p-aminoclonidine ($pK_i = 5.34$ and < 4 respectively) but high affinity for [^{3}H]idazoxan binding ($pK_i = 7.95$ and 6.36 respectively). Noradrenaline and RS-15385-197 had low affinity for both ligands emphasising the non-adrenergic nature of the interaction. Guanabenz showed high affinity for both radioligands ($pK_i = 7.81$ and 7.26), while the imidazolines phentolamine and cimetidine had low affinity (pK_i for [^{3}H]p-aminoclonidine binding 5.10 and 4.99 and for [^{3}H] idazoxan binding 4.48 and 4.31). To ensure that the pharmacology of the [^{3}H]p-aminoclonidine binding site was the same irrespective of the method employed to separate bound ligand, the affinity of guanabenz, idazoxan and p-aminoclonidine were estimated for [^{3}H]p-aminoclonidine binding using centrifugation to separate bound ligand. The affinity of these compounds

TABLE 1

Affinity values (pK_i) for [^{3}H]p-aminoclonidine and [^{3}H]idazoxan binding in the rat kidney in the presence of 0.1 μM RS-15385-197. Assays were performed as described in Methods. For [^{3}H]p-aminoclonidine binding values were obtained from filtration or centrifugation experiments as indicated. Values represent the means ± S.E.M. Each assay was performed in duplicate and the number of determinations is shown in parentheses.

	[^{3}H]p-Aminoclonidine				[^{3}H]Idazoxan	
	Filtration		Centrifugation		pK_i	nH
	pK_i	nH	pK_i	nH		
Clonidine	6.88 ± 0.07	0.80 ± 0.06 (3)	–	–	5.64 ± 0.16	0.92 ± 0.08 (5)
p-Aminoclonidine	6.98 ± 0.09	0.87 ± 0.07 (6)	6.87 ± 0.21	1.02 ± 0.12 (3)	5.05 ± 0.16	1.02 ± 0.10 (6)
Idazoxan	5.34 ± 0.04	0.77 ± 0.11 (3)	5.34 ± 0.25	1.05 ± 0.05 (3)	7.95 ± 0.17	0.99 ± 0.01 (4)
Guanabenz	7.81 ± 0.06	1.25 ± 0.11 (3)	7.29 ± 0.37	1.05 ± 0.04 (3)	7.26 ± 0.07	0.90 ± 0.10 (3)
Phentolamine	5.10 ± 0.23	1.01 ± 0.01 (3)	–	–	4.48 ± 0.07	– (3)
Cimetidine	4.99 ± 0.09	– (3)	–	–	4.31 ± 0.19	– (3)
RS-15385-197	4.85 ± 0.13	– (3)	–	–	4.26 ± 0.21	– (3)
Amiloride	< 4	– (3)	–	–	6.36 ± 0.06	– (3)
Noradrenaline	< 4	– (3)	–	–	4.53 ± 0.20	– (4)
GTP	≪ 3	– (3)	–	–	3.48 ± 0.07	– (3)

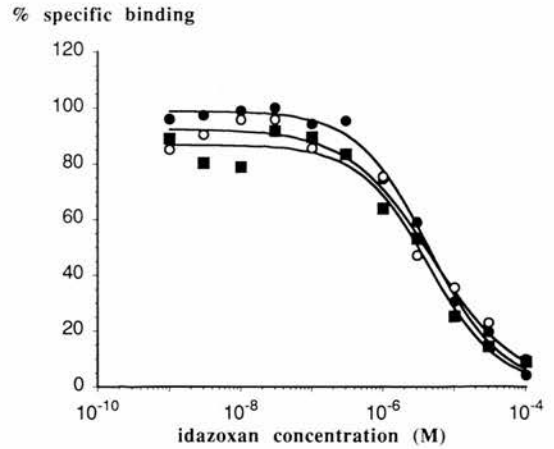


Fig. 5. Inhibition of [^{3}H]p-aminoclonidine binding by idazoxan in the absence and presence of GTP. Assays were performed as described in Methods. The figure represents a single experiment performed in duplicate. Specific binding is calculated as a percentage of binding in the absence of GTP. Control (•) $pIC_{50} = 5.39$, $nH = 0.83$; 0.1 mM GTP (○) $pIC_{50} = 5.43$, $nH = 0.74$; 1 mM GTP (■) $pIC_{50} = 5.44$, $nH = 0.86$.

was not significantly different from filtration affinity estimates.

3.5. Effect of guanyl nucleotides and naphazoline

GTP inhibited [^{3}H]idazoxan binding ($pK_i = 3.48$; 60% inhibition at 1 mM) but did not inhibit [^{3}H]p-aminoclonidine binding up to a concentration of 1 mM. When inhibition of [^{3}H]p-aminoclonidine binding by idazoxan was conducted in the presence of 0.1 and 1 mM GTP (concentrations which inhibit [^{3}H]idazoxan binding by approximately 25 and 60%) no effect was seen on the affinity or the Hill slope of the idazoxan displacement curve (control $pIC_{50} = 5.39$, $nH = 0.83$; 0.1 mM GTP $pIC_{50} = 5.43$, $nH = 0.74$; 1 mM GTP $pIC_{50} = 5.44$, $nH = 0.86$, fig. 5).

Figure 6 shows the inhibition of [^{3}H]idazoxan binding by naphazoline and cirazoline in the presence of RS-15385-197. The inhibition curve for cirazoline was monophasic and of high affinity ($pK_i = 8.48 \pm 0.10$, $n = 4$) however, the inhibition curve for naphazoline was better fitted to a two-site model and gave affinity values for the high and low affinity components of 8.65 ± 0.18 and 6.88 ± 0.21 respectively ($n = 4$), with the high affinity component comprising 50–60% specific binding.

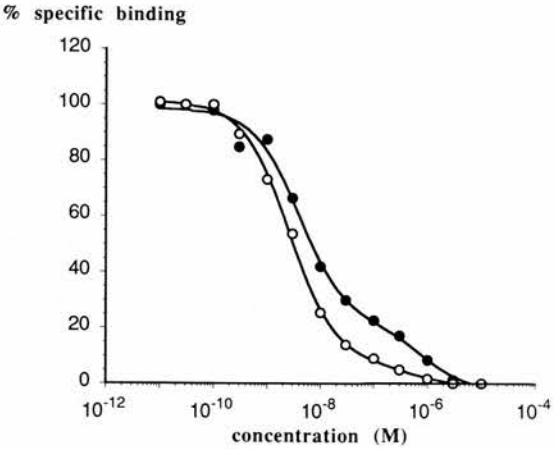


Fig 6. Inhibition of 1 nM [^{3}H]idazoxan binding in rat kidney by naphazoline (•) and cirazoline (○) in the presence of 0.1 μM RS-15385-197. Assays were performed as described in Methods. The data represents a single experiment performed in duplicate. The inhibition curve for cirazoline was monophasic and gave a pK_i of 8.43, however the inhibition curve for naphazoline was better fitted to a two-site model ($P < 0.05$), and gave pIC_{50} values for the high (60%) and low (40%) affinity components of 8.73 and 6.89 respectively.

4. Discussion

This study indicated that [^{3}H]idazoxan and [^{3}H]p-aminoclonidine labelled imidazoline binding sites in rat kidney membranes. Previous studies on imidazoline binding sites in this tissue have been hindered by the presence of a large population of α_2-adrenoceptors, so it was the purpose of this study to isolate the imidazoline site(s) by performing the experiments in the presence of RS-15385-197. Under these conditions, [^{3}H]p-aminoclonidine and [^{3}H]idazoxan labelled populations of sites that showed marked differences in nature. Firstly, [^{3}H]p-aminoclonidine labelled more than 20 times the number of sites than [^{3}H]idazoxan but with relatively low affinity. We were surprised to show specific [^{3}H]p-aminoclonidine binding using filtration to separate bound from free radioligand, as this is normally only suitable for ligands having nanomolar affinity for their receptor site. Ligands with lower affinity would be expected to dissociate from the receptor site during the washing stage of the separation. However, we repeated the experiments using separation by centrifugation and found no significant difference in the affinity of the site determined by filtration. The pharmacological profile of the site was also similar as the affinities of p-aminoclonidine, idazoxan and guanabenz, compounds critical to the classification, were identical using both separation methods. This lead us to believe that the same site was being labelled under the different protocols. Conducting the separation at 4°C must slow the dissociation rate sufficiently to allow for the determination of reproducible binding data with [^{3}H]p-aminoclonidine using a filtration protocol. Filtration has been successfully used to label glutamate receptor subtypes and the neurotoxin receptor site associated with Na^+ channels using radioligands of low affinity (Honore et al., 1989; Catterall et al., 1981).

In competition experiments, idazoxan had low affinity for [^{3}H]p-aminoclonidine binding ($K_i = 4571$ nM). Other studies with idazoxan have shown differences in affinities for [^{3}H]p-aminoclonidine binding, ranging from 220 nM in the rat renal cortex (Ernsberger et al., 1990) to 33 nM for [^{3}H]clonidine binding in the human brainstem (Bricca et al., 1988). The low affinity for idazoxan in this study may be due to the removal of idazoxan's high affinity for α_2-adrenoceptors with RS-15385-197. In competition experiments with [^{3}H] idazoxan, p-aminoclonidine and clonidine had low affinity. This is similar to the imidazoline I_2 binding site in pig (Vigne et al., 1989), hamster (MacKinnon et al., 1989), and human kidney (Michel et al., 1989), whereas clonidine has somewhat higher affinity in rabbit tissues (Yablonsky et al., 1988; Langin and Lafontan, 1989). The imidazoline site labelled by [^{3}H] idazoxan in the rat kidney is therefore consistent with an imidazoline I_2 subclass. The affinity of the diuretic amiloride has been used as a means of subclassifying imidazoline sites (Michel et al., 1989). In this study, amiloride had very low affinity ($pK_i < 4$) for [^{3}H]p-aminoclonidine binding, but had higher affinity for [^{3}H]idazoxan binding ($K_i = 436$ nM). The affinity of amiloride for [^{3}H]idazoxan binding was lower than that reported in rabbit tissues ($K_i = 30$–48 nM) but higher than that reported in human and pig tissues ($K_i > 1100$ nM), see Michel et al., 1989 for review) but was similar to that reported in rat brain (Brown et al., 1990). The affinity of clonidine and amiloride, therefore, shows a large variation in affinity between species.

Studies by Bricca et al. (1989) have shown that [^{3}H]clonidine labels imidazoline I_1 binding sites in bovine and human brainstem membranes with high affinity, and that the human nucleus reticularis lateralis provides an homogeneous population of imidazoline I_1 sites. However, using the same protocol, they could not label high affinity I_1 sites with [^{3}H]clonidine in rat brainstem membranes. The lack of high affinity [^{3}H]clonidine and [^{3}H]p-aminoclonidine binding in rat tissues may represent a species difference. A recent study by Wikberg et al. (1992) showed that [^{3}H]p-aminoclonidine labelled a low affinity site in guinea pig kidney ($K_d = 213$ nM) with a similar pharmacology to that shown in the present study. Imidazoline (I_1) binding in brainstem tissues from higher mammals may therefore represent a different subset of imidazoline sites.

The finding that [^{3}H]p-aminoclonidine has low affinity for imidazoline binding sites in rat kidney is not consistent with studies by Ernsberger et al. (1990) who demonstrated high affinity for this ligand in rat renal cortex membranes. These studies showed that imidazoline compounds would displace 100% of the specific binding while the catecholamines would only displace ~ 68%. Imidazoline affinity was calculated as the relative affinity for 32% of the specifically bound [^{3}H]p-aminoclonidine defined with 10 μM phentolamine. We consider that 10 μM phentolamine would not be a sufficiently high enough concentration to displace all imidazoline-like binding with either [^{3}H]p-aminoclonidine or [^{3}H]idazoxan. There are several differences between our studies and those of Ernsberger. In our studies whole kidney homogenate rather than renal cortex was used, however, this is unlikely to explain the differences seen as one would still expect to label the high affinity cortical binding in a whole kidney preparation. The assay conditions were similar in both studies except that the incubation conditions in our studies were set to specifically label imidazoline sites, i.e. performed in the presence of RS-15385-197 to block α_2-adrenoceptors. Under the conditions employed by Ernsberger (long incubation time at 25°C and non-specific binding defined with 10 μM phentolamine) one would expect [^{3}H]p-aminoclonidine to label a significant population of α_2-adrenoceptors. True imidazoline affinity is likely to be masked when the radioligand used is also labelling a large population of α_2-adrenoceptors.

The imidazoline compound naphazoline showed high affinity for the [^{3}H]idazoxan binding site on rat kidney but exhibited a displacement curve which had a Hill slope of less than unity. Naphazoline has been shown to exhibit a shallow displacement curve for [^{3}H]idazoxan binding to I_2 sites in rat brain (Brown et al., 1990), rat liver (Zonnenschein et al., 1990) and hamster adipocyte (MacKinnon et al., 1989). In the present study the displacement curve for naphazoline could be better resolved into a two-site model with affinities for the high and low affinity components (pIC_{50}) of 8.65 and 6.88 respectively. In addition, kinetic analysis of [^{3}H]idazoxan binding revealed that two binding components of similar affinity were labelled by this ligand. Studies in guinea pig tissues suggest that [^{3}H]idazoxan may label two classes of imidazoline site which show different affinities for clonidine (Wikberg et al., 1991). These subtypes have been termed IA and IB.

Inhibition by guanyl nucleotides is a phenomenon normally associated with agonist binding. In this study we showed that GTP inhibited [^{3}H]idazoxan but not [^{3}H]p-aminoclonidine binding. Previous studies have shown that GTP decreases the number of imidazoline binding sites labelled by [^{3}H]idazoxan in the bovine brain (Hussain et al., 1991), and therefore suggests that idazoxan may be an agonist at a G protein-linked imidazoline receptor. This coupled with the finding that [^{3}H]idazoxan labelled a fraction of the number of sites labelled by [^{3}H]p-aminoclonidine lead us speculate whether the latter ligand was labelling the whole population of sites, a subset of which had high affinity for the agonist idazoxan. However, when idazoxan inhibition curves were carried out in the presence of GTP,

there was no effect on the affinity or the Hill slope for idazoxan. Further studies are required to elucidate the effect of GTP on imidazoline sites, and functional studies are neccessary to elucidate the possible agonist effects of idazoxan.

[^{3}H]p-Aminoclonidine binding to I_1 sites in the ventrolateral medulla have been claimed to be responsible for the hypotensive actions seen by some imidazoline compounds when injected into this region, and an endogenous substance extracted from bovine brain ('clonidine displacing substance' or CDS) has been shown to have high affinity for these sites (Ernsberger et al., 1987), and for I_2 sites in rabbit kidney (Coupry et al., 1989). In this study we show that [^{3}H]p-aminoclonidine labelled a site which was unlike the I_1 site in bovine brainstem, and may not be a functional receptor on the basis of low affinity, high capacity and rapid kinetics. I_2 sites labelled by [^{3}H]idazoxan have been suggested to be involved in Na^+ transport in renal proximal tubule cells (Bidet et al., 1990), and on noradrenaline release in rabbit pulmonary artery and aorta (Göthert and Molderings, 1991). Further characterisation of these effects with selective agonists/antagonists is necessary to elucidate whether these sites may be classed as receptors.

It is clear from this study that [^{3}H]idazoxan and [^{3}H]p-aminoclonidine label different subsets of imidazoline binding site in the rat kidney and the results emphasise the necessity to perform studies on imidazoline sites in the presence of an α_2-antagonist until more selective imidazoline ligands become available. Further studies are required to substantiate the possibility that non-adrenoceptor [^{3}H]idazoxan binding may form an heterogeneous population of sites

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Non-α_2-adrenoceptor idazoxan binding sites; a new target for drug development

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Non α_2-adrenoceptor idazoxan-binding sites: hamster adipocytes a functional model for these sites

The introduction of selective agonists/antagonists has permitted the pharmacological characterization of α_2-adrenoceptors. Clonidine, *p*-aminoclonidine, and UK 14304 are considered selective α_2-adrenoceptor agonists and yohimbine, rauwolscine and idazoxan are high-affinity α_2-adrenoceptor antagonists. For review see [1]. The availability of these compounds as high-specific-activity radioligands combined with the technique of ligand binding has contributed greatly to our understanding of α_2-adrenoceptors.

Ligand-binding studies indicate that clonidine, *p*-aminoclonidine, UK 14304 and idazoxan, in addition to binding to α_2-adrenoceptors, also interact with a non-adrenoceptor binding site in a wide variety of tissues. Considerable interest exists at present in defining a functional role for these non-adrenoceptor idazoxan-binding sites (NAIBS).

Ligand-binding studies

Saturation experiments

A number of studies have demonstrated that [^{3}H]idazoxan binds to a second site in a wide range of tissues, with an affinity comparable with that determined at α_2-adrenoceptors. The number of binding sites defined in rabbit kidney with [^{3}H]idazoxan is approximately 3–4 times greater than that identified with [^{3}H]yohimbine [2]. A similar difference in the density of binding sites is observed in rabbit basolateral membranes if [^{3}H]yohimbine is replaced by [^{3}H]rauwolscine; [^{3}H]idazoxan labels 3–5 times more sites than [^{3}H]rauwolscine [3]. This variability in the density of binding sites and its dependence on the radioligand employed has been reported in rabbit urethral tissue [4, 5], rat liver cells [6], hamster adipocytes [7] and rat and human kidneys [8, 9].

Saturation studies demonstrate that [^{3}H]*p*-aminoclonidine labels two distinct populations of binding sites in the ventrolateral medulla of bovine brain, an α_2-adrenoceptor and a non-adrenoceptor site [10]. In addition to [^{3}H]*p*-aminoclonidine, [^{3}H]clonidine labels a non-adrenoceptor site in human brainstem [11]. Evidence, presented later, suggests that the non-adrenoceptor sites labelled by [^{3}H]*p*-aminoclonidine and [^{3}H]clonidine may be different from the sites labelled by [^{3}H]idazoxan.

Competition experiments

The phenylethanolamines, adrenaline and noradrenaline either display low affinity or fail to interact, at concentrations up to 10^{-4}, with NAIBS in rabbit kidney [2, 3], adipocytes [12], urethral tissue [4, 5], hamster and human adipocytes [7, 13], rat liver cells [6] and brain [14]. Standard non-imidazoline α_2-adrenoceptor antagonists such as yohimbine and rauwolscine have low affinity towards NAIBS, whereas compounds with an imidazoline/

Abbreviation used: NAIBS, non-adrenoceptor idazoxan-binding sites.

guanidinium structure, such as cirazoline, guanabenz and naphazoline bind with high affinity. An affinity rank order of cirazoline (K_i 2.6 nM) > guanabenz (K_i 6.2 nM) > naphazoline (K_i 46.7 nM) > clonidine (K_i 537 nM) is reported for NAIBS in rat brain [14]. A similar affinity rank order exists for NAIBS in human fat cells [13] whereas in rat liver cells the order is cirazoline (K_i 2.21 nM) > naphazoline (K_i 8.99 nM) > guanabenz (K_i 33.8 nM) > clonidine (K_i 15066.9 nM) [6].

The imidazoline α_2-adrenoceptor agonist, UK 14304 [15] has a relatively high affinity at NAIBS in human fat cells (K_i 53.4 nM) [16] and rat liver cells (K_i 42.0 nM) [6], modest affinity at NAIBS in human kidney (K_i 241 nM) [8], but low affinity at the site in rabbit colon epithelial cells (K_i 1049 nM) [17]. Hill slopes of less than unity have been reported for UK 14304 at NAIBS in hamster fat cells, rat liver cells and rat colon epithelial cells.

NAIBS are distinct from α_2-adrenoceptors

Phenyoxybenzamine and benextramine are irreversible antagonists at a number of receptors, including α_2-adrenoceptors. Receptor inactivation experiments provide evidence that NAIBS and α_2-adrenoceptors have distinct binding domains. Pretreatment of human adipocytes with phenoxybenzamine reduces the density of α_2-adrenoceptors without altering the number of NAIBS [16]. In the same tissue, benextramine acts as a reversible antagonist at NAIBS whereas irreversible antagonism occurs at the α_2-adrenoceptor [16].

NAIBS and α_2-adrenoceptors display differing sensitivity towards mono- and divalent ions. The affinity of [^{3}H]yohimbine at α_2-adrenoceptors is increased by monovalent ions with a rank order potency of $Na^+ > Li^+ \gg K^+$ [18]. Binding at NAIBS in human kidney basolateral membranes is inhibited in a concentration-dependent manner by K^+ [8]. Maximum inhibition of binding (70%) occurs at 150 mM with an IC_{50} (concentration producing 50% inhibition) of 70 mM. The divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} have no effect in human kidney NAIBS at concentrations up to 75 mM. The NAIBS in human fat cells are also sensitive to monovalent ions. Specific [^{3}H]idazoxan binding to these sites is reduced by 37% for K^+, 27% for Li^+ and 13% for Na^+ [16]. In contrast with the NAIBS, binding of the selective α_2-adrenoceptor antagonist [^{3}H]RX 821002 [19] is unaffected by K^+, Li^+ and Na^+ [16].

Differential centrifugation experiments demonstrate that α_2-adrenoceptors of human kidney are predominantly enriched in fractions containing the basolateral membranes from proximal tubules [8]. A similar finding is reported for rabbit [20] and rat kidney [21]. In contrast with α_2-adrenoceptors, NAIBS appear to exist in both basolateral and intracellular membranes [18], indicating that NAIBS and α_2-adrenoceptors may have different subcellular distributions.

Solubilization and partial purification of NAIBS and α_2-adrenoceptors from rabbit kidney has been reported [22]. Separation of solubilized NAIBS and α_2-adrenoceptors is achieved using heparin–agarose chromatography. Under these conditions, α_2-adrenoceptors are retained by the chromatography medium, whereas the NAIBS appear in the fall-through fraction. The failure of NAIBS to be retained by the heparin–agarose matrix, under conditions in which α_2-adrenoceptors are readily absorbed [23, 24], provides evidence that these two entities are distinct.

Soluble NAIBS from rabbit kidney are trypsin-sensitive indicating that they are proteins and not simply a lipid membrane component [22]. In solubilization experiments, NAIBS are not dissociated from rabbit-kidney-membrane preparations by high salt concentrations, suggesting that they are intrinsically bound.

A unique distribution of NAIBS in rat brain has been reported [25]. They are located in discrete areas, associated with brain nuclei, such as the arcuate nucleus, the interpenduncular nucleus and area postrema. The physiological role of the NAIBS in these nuclei has still to be defined.

Heterogeneity within NAIBS

Affinity values derived from ligand-binding studies suggest the existence of more than one non-adrenoceptor binding site. Three distinct binding sites are suggested to exist: (1) [^{3}H]*p*-aminoclonidine binding sites distinct from NAIBS, (2) NAIBS in various rabbit tissues and (3) NAIBS in non-rabbit tissue [26]. The non-adrenoceptor sites identified with [^{3}H]*p*-aminoclonidine are pharmacologically different from NAIBS (Table 1). Clonidine displaces [^{3}H]*p*-aminoclonidine binding to bovine and rat brainstem with nanomolar affinity, whereas guanabenz has low (710000 mM) affinity [27]. In contrast, NAIBS, irrespective of whether they are located in rabbit or non-rabbit tissue, display high affinity (mM) towards guanabenz but low affinity (μM) towards clonidine and *p*-aminoclonidine [14, 26].

The argument for multiple NAIBS is primarily based upon differing affinities reported in ligand-binding studies for the K^+-sparing diuretic,

Table 1

Drug affinities at NAIBS and *p*-aminoclonidine binding sites

All values are K_i (nM) except for bovine brain which are IC_{50} (nM) values.

Binding site	Cirazoline	Clonidine	Nephazoline	Amiloride	Guanabenz	UK 14304
[^{3}H]*p*-aminoclonidine binding site						
Bovine brain		6[a]	5.24[a]	> 10,000[a]	> 10,000[a]	n.d.
NAIBS (rabbit)						
Kidney	0.8[b]	170[b]	n.d.	30[c]	1.8[c]	120[b]
Urethra	2.93[c]	1265[e]	n.d.	26[e]	4.4[e]	297[d]
Colon epithelial cells	8.5[f]	n.d.	15.3[f]	n.d.	n.d.	1049[f]
Adipocytes	n.d.	975[g]	9.2[g]	48[g]	n.d.	948[g]
NAIBS (non-rabbit tissue)						
Human kidney	0.64[h]	2575[h]	n.d.	369[h]	9.6[h]	2.41[h]
Human adipocytes	8.5[i]	19054[i]	68.2[i]	n.d.	n.d.	53.4[i]
Rat brain	2.57[j]	537[j]	46.7[j]	562.3	6.2[j]	n.d.
Rat liver cells	2.21[k]	15067[k]	8.89[k]	4808[k]	33.8[k]	42.0[k]
Hamster adipocytes	0.95[l]	1950[l]	12.6[l]	n.d.	n.d.	30.2[l]

Key to references: [a]Ernsberger, P. *et al.* (1988) J. Hypertens. 6 (Suppl 4), S554-S557; [b]Langin, D. *et al.* (1990) Mol. Pharmacol. 188, 261-272; [c]Coupry, I. *et al.* (1989) FASEB J. 3, A1187; [d]Yablonsky, F. *et al.* (1988) Eur. J. Pharmocol. 164, 167-170; [e]Yablonsky, F. & Dausse, J. P. (1989) Eur. J. Pharmocol. 164, 167-170; [f]Senard, J. M. *et al.* (1990) Eur. J. Pharmacol. 191, 59-68; [g]Langin, D. & Lafontan, M. (1989) Eur. J. Pharmacol. 159, 199-203; [h]Lachaud-Pettiti, V. *et al.* (1991) Eur. J. Pharmacol. 206, 23-31; [i]Langin, D. *et al.* (1990) Eur. J. Pharmacol.-Mol. Pharmacol. Section. 188, 261-272; [j]Brown, C. M. *et al.* (1990) Br. J. Pharmacol. 99, 803-809; [k]Zonnenschein, R. *et al.* (1990) Eur. J. Pharmacol. 190, 203-215; MacKinnon, A. C. *et al.* (1989) Br. J. Pharmacol. 98, 1143-1150.

amiloride at NAIBS in rabbit and non-rabbit tissue (Table 1). Amiloride displays high affinity at NAIBS in rabbit urethra (K_i 26 nM), kidney (K_i 30 nM) and adipocytes (K_i 48 nM), modest affinity towards human kidney (K_i 369 nM) and rat brain (K_i 562 nM) [8, 14, 28], and low affinity in rat liver cells (K_i 4808 nM) and rat kidney (K_i 7760 nM) [6, 9]. The widely different affinity values reported for amiloride at NAIBS in rabbit and non-rabbit tissue may simply reflect a species difference among NAIBS and not an example of heterogeneity within this class of sites. Confirmation that heterogeneity exists within NAIBS will require identification of a rabbit tissue displaying low affinity towards amiloride or conversely, a non-rabbit tissue displaying nM affinity towards this drug.

Physiological function of NAIBS

The non-adrenoceptor binding sites labelled by [^{3}H]*p*-aminoclonidine are implicated in the central hypotensive effects of clonidine-like substances [29]. An endogenous substance termed clonidine displacing substances (CDS) has been partially purified and shown to interact with brainstem [^{3}H]*p*-aminoclonidine-binding sites and produce hypotensive effects when injected into the rostral ventrolateral medulla of the rat through a mechanism independent of α-adrenoceptors [30]. It has also been suggested that these sites may be a subtype of the histamine H_2-receptor [31].

In contrast with the non-adrenoceptor sites labelled by [^{3}H]*p*-aminoclonidine, less is known of the physiological relevance of NAIBS. Cirazoline and idazoxan inhibit $^{22}N^+$ uptake into isolated rabbit renal proximal tubule cells by a mechanism independent of the α_2-adrenoceptor [32]. It has been suggested that this effect could be mediated through inhibition of the Na^+/H^+ antiporter in these cells [32]. NAIBS may also be involved in regulating insulin release [33] and inhibition of K^+-channel opening in vascular smooth muscle [34]. Recently, it has been demonstrated that NAIBS in human and rabbit liver are mainly located in the outer mitochondrial membrane and not with the plasma membrane [35]. Cirazolone, idazoxan or guanabenz have no effect on respiratory control indicating that NAIBS are not involved in the regulation of respiratory activity and oxidative phosphorylation. Furthermore, PK 11195, a selective ligand for the mitochondrial peripheral-type benzodiazepine receptor [36] has low affinity for mitochondrial NAIBS, indicating that these two sites are

different. Additional studies are required to confirm that NAIBS present in non-liver tissue are also located on the outer mitochondrial membrane. The identification of selective agonists/antagonists for NAIBS will allow the physiological/biochemical function of these mitochondrial sites to be established.

NAIBS in adipocytes

The adipocyte may be a simple model in which to examine α_2-adrenoceptor function and the possible effects of interactions at NAIBS. Human adipocyte α_2-adrenoceptors are labelled with high affinity by [^{3}H]clonidine and [^{3}H]yohimbine [37, 38]. Hamster adipocytes are labelled with high affinity by [^{3}H]clonidine whereas binding with [^{3}H]yohimbine has proved difficult [39]. α_2-Adrenoceptors have been shown to co-exist with NAIBS in human [13], rabbit [12] and hamster adipocytes [7].

Catecholamine-induced lipolysis is regulated through stimulatory β-adrenoceptors and inhibitory α-adrenoceptors in adipocytes of white-fat tissue from man [40], dog [41] and hamster [42]. Functional studies indicate that α_1- and α_2-adrenoceptors exist in human [34] and hamster fat cells [43, 44]. The anti-lipolytic effects of α-adrenoceptor agonists are mediated through the α_2-adrenoceptor in hamster [45] and man [46].

We have reported previously the existence of α_2-adrenoceptors and NAIBS in hamster adipocytes [7]. We have extended our initial observations and report that from ligand-binding studies, an affinity rank order of idazoxan (pK_i 8.34) > phentolamine (pK_i 7.92) > UK 14304 (pK_i 7.52) > yohimbine (pK_i 7.28) > cirazoline (pK_i 6.63) exists for α_2-adrenoceptors in hamster adipocytes (Table 2). In contrast, the affinity rank order for hamster NAIBS of cirazoline (pK_i 9.02) > yohimbine (pK_i 4.26) is different to that defined from α_2-adrenoceptors. The derived phentolamine/cirazoline affinity ratio of 1:19 at α_2-adrenoceptors and 0.58 at NAIBS is sufficiently different to potentially act as a predictor of the relative contribution that each site makes to an anti-lipolytic effect produced in functional studies.

UK 14304 acts as a full agonist (relative to clonidine), promoting a strong anti-lipolytic effect in rabbit [13] and hamster adipocytes [47]. The anti-lipolytic effect of UK 14304 in functional studies on isolated hamster adipocytes is inhibited with an affinity rank order of phentolamine (pA_2 7.87) > idazoxan (pA_2 7.36) > yohimbine (pA_2 7.26) > cirazoline (pA_2 5.95). The derived phentolamine/cirazoline affinity ratio of 1.32 is indicative of an α_2-adrenoceptor-mediated effect. Thus, although UK 14304 is a non-selective high-affinity

Table 2

Affinity of idazoxan, yohimbine, phentolamine and cirazoline at hamster adipocytes

The isolation of hamster adipoctye membranes and binding studies were carried out as described previously [7]. In functional studies, intact adipocytes (approximately 10000–20000 cells) were suspended in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 3.5% (w/v) fatty-acid-deficient BSA, 2 mM-$CaCl_2$ and 10 μM-propranolol in a final volume of 0.5 ml. Adipocytes were preincubated with increasing concentrations of UK 14304 for 20 min at 37°C before being stimulated by the addition of 100 μM-theophylline and 2 μg of adenosine deaminase (72 units/mg of protein). Incubations were carried out for 20 min. at 37°C. Test drugs were added 20 min. before stimulation by UK 14304. Glycerol in the infranatant was measured spectrophotometrically. The UK 14304 concentration–response curves were repeated in the presence of at least three concentrations of the drug and each value represents the mean ± S.E.M. of five separate experiments.

	[^{3}H]Idazoxan binding		Lipolysis
	α_2	NAIBS	pA_2
Idazoxan	8.34 ± 0.16		7.36 ± 0.32
Yohimbine	7.28 ± 0.02	4.26 ± 0.18	7.26 ± 0.17*
Phentolamine	7.92 ± 0.14	5.21 ± 0.02	7.87 ± 0.31
UK 14304	7.52 ± 0.22*		
Cirazoline	6.63 ± 0.09	9.02 ± 0.13	5.95 ± 0.31

* denotes a slope significantly less than unity, $P < 0.01$.

ligand at α_2-adrenoceptors and NAIBS and displays Hill slopes of less than unity at both sites, the anti-lipolytic effects of UK 14304 are mediated through the α_2-adrenoceptor. The failure of NAIBS to modify the anti-lipolytic effects of UK 14304 suggest that either UK 14304 is not an agonist at NAIBS or that these sites are not involved in α_2-adrenoceptor regulation of lipolysis. However, if it is confirmed that hamster adipocyte NAIBS are located within the mitochondria, the results obtained with UK 14304 may simply reflect ease of access to α_2-adrenoceptors relative to NAIBS. An indication of intracellular accumulation of UK 14304 in isolated adipocytes is required.

Initial data suggest that NAIBS are not involved in the regulation of lipolysis. However, the adipocyte may still represent a relatively simple model for exploring the possible relationship between α_2-adrenoceptors and NAIBS and provide the means of assigning a physiological function to this particular binding site.

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Inter-species variants of the 5-HT$_3$ receptor

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The 5-HT$_3$ receptor

The 5-HT$_3$ receptor is located on neurones in the peripheral and central nervous systems where it mediates some of the excitatory effects of 5-HT (see [1] for review). While the 5-HT$_3$ receptor has not yet been cloned, biochemical and electrophysiological evidence support the view that this receptor belongs to the multi-subunit ligand-gated ion channel family of receptors, a family to which the acetylcholine, nicotinic and GABA$_A$ receptors belong. This contrasts with the other receptors for 5-HT which are all thought to belong to the single-subunit G-protein-linked class of receptors (see [2]).

Electrophysiological analysis of 5-HT$_3$-receptor-mediated events reveals that it is directly linked to a monovalent cation channel [3]. Stimulation of the receptor results in a fast depolarization which rapidly desensitizes, often leaving a small maintained response (see [4]). The 5-HT$_3$ receptor has been solubilized from several sources [5–8]; the molecular mass of the solubilized receptor complex has been estimated to be 250–600 kDa using gel filtration techniques [6, 9]. Affinity chromatography using covalently attached 5-HT$_3$-receptor antagonists has allowed purification of the receptor, and PAGE of the purified receptor reveals either two bands of molecular masses 38 and 54 kDa (NCB20 cells, [9]) or a single band of 55 kDa (N1E 115 cells, [8]). The disparity between the molecular masses of the receptor as determined by gel filtration and PAGE support the view that the 5-HT$_3$ receptor exists as a multimeric complex which can be dissociated into its monomer units.

The 5-HT$_3$ receptor has been classified as such on the basis of its pharmacological profile assessed using agonist and antagonist compounds. A limited number of selective agonists for the 5-HT$_3$ receptor have been identified. The most frequently used compound is 2-methyl-5-HT which was first identified by Richardson *et al.* [10]. This compound is nearly as potent as 5-HT at 5-HT$_3$ receptors, but has little activity at other 5-HT receptors. Phenylbiguanide has a similar profile [11, 12]. The most potent agonist available is *meta*chlorophenylbiguanide (*m*CPBG; [13, 14]). This compound is significantly more potent than 5-HT at 5-HT$_3$ receptors, with minimal effect on other 5-HT receptors.

Recent major advances in 5-HT$_3$-receptor research stem from the availability of highly selective and potent antagonists. Some of the first to be identified were MDL 72222 [15], ICS 205-930 [10]; ondansetron (GR38032F; [16]) and granisetron (BRL43694; [17]), but there are now more than 20 such compounds. As a group, these compounds are peculiar in as much as they are highly selective for the 5-HT$_3$ receptor – each shows at least 1000-fold selectivity for the 5-HT$_3$ receptor over the other neurotransmitter receptors, upon which they have been tested (note that ICS 205-930 has weak affinity for the 5-HT$_4$ receptor) – and possess high affinity. This means that commonly observed effects with these compounds at reasonable doses/concentrations can be confidently ascribed to antagonism at 5-HT$_3$ receptors.

These selective antagonists are not only good pharmacological tools for the study of 5-HT$_3$ receptors but are also useful clinically in the treatment of emesis resulting from cancer therapy. Data from animal behavioural studies and preliminary clinical trials indicate that they may also be useful in the treatment of various psychiatric disorders [18, 19].

Heterogeneity of the 5-HT$_3$ receptor

In the absence of sequence data on the 5-HT$_3$ receptor, the best means of identification of receptor subtypes remains the classical pharmacological

Abbreviation used: *m*CPBG, *meta*chlorophenylbiguanide.