AGONIST REGULATION OF ADRENERGIC RECEPTORS IN THE RABBIT

by

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DECLARATION

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been submitted previously for a higher degree.

This research was carried out in the Department of Materia Medica, University of Glasgow, under the supervision of Dr. C.A. Hamilton and Professor J.L. Reid.

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SUMMARY

Acute elevations of the circulating catecholamines, adrenaline and noradrenaline are observed in physiological situations such as exercise, posture change and stress. Pathological settings such as phaeochromocytoma, heart failure and cirrhosis may be associated with chronic increases in plasma catecholamines. These high catecholamine levels have been associated with decreases in adrenergic receptor responsiveness or desensitisation. A reduction in adrenergic receptor density or down regulation has also been detected under these conditions. However, although the *in vitro* effects of raised concentrations of plasma catecholamines have been widely studied, similar investigations *in vivo* are not so well characterised. In addition, some *in vivo* experimental models (e.g. rat phaeochromocytoma) have produced plasma catecholamine levels vastly exceeding those found in many pathological settings. Therefore, the current investigations principally aimed to evaluate the effects of moderate increases in circulating catecholamines *in vivo* on adrenergic receptor function and number in a rabbit model.

The first series of experiments were designed to validate the methods used and characterise the receptor populations under investigation. To begin with, experiments were carried out to determine if the pro-aggregatory response to adrenaline in rabbit platelets was mediated (like human platelets) by $alpha_2$ adrenoceptors. The ability of the alpha adrenoceptor antagonists, idazoxan, yohimbine and prazosin to inhibit the platelet pro-aggregatory responses to adrenaline was recorded and

IC_{50} values (the concentration of antagonist required to produce fifty percent inhibition of response) were calculated. The $alpha_2$ adrenoceptor antagonists, idazoxan and yohimbine were found to be more potent than the $alpha_1$ adrenoceptor antagonist, prazosin at inhibiting the platelet pro-aggregatory response to adrenaline. Therefore, these results demonstrated that $alpha_2$ adrenoceptors primarily mediate the platelet pro-aggregatory response to adrenaline in the rabbit.

The specificity of [^{3}H] yohimbine binding to $alpha_2$ adrenoceptors in rabbit platelets and kidney was also determined. Displacement assays were performed and the ability of various unlabelled alpha adrenoceptor antagonists to compete for [^{3}H] yohimbine binding sites was examined and expressed as K_I (a measure of affinity) values. Yohimbine was found to have a considerably higher potency than prazosin for displacing [^{3}H] yohimbine binding sites on rabbit platelets. Similarly, in rabbit kidney; prazosin and phentolamine were both less potent than yohimbine at displacing [^{3}H] yohimbine binding. These results were consistent with [^{3}H] yohimbine binding to $alpha_2$ receptors in rabbit platelets and kidney.

Subpopulations of beta adrenoceptors were characterised in the platelets, lymphocytes, heart and lung of the rabbit. The ability of the $beta_1$ (atenolol or metoprolol) and $beta_2$ (ICI 118551) adrenoceptor selective antagonists to displace the beta adrenoceptor selective [^{125}I](-) Iodocyanopindolol (ICYP) from its binding sites in platelets and was assessed. ICI 118551 was found to be more potent than atenolol in displacing [^{125}I](-)

ICYP specific binding in both tissues. These findings were in agreement with previous studies showing that the beta adrenoceptors in rabbit platelets and lymphocytes (like humans) were largely $beta_2$ in type. In a separate study, the $beta_1$ adrenoceptor selective antagonist, metoprolol had a higher affinity than the $beta_2$ selective ICI 118551 at inhibiting $[^{125}]$((-) ICYP specific binding in rabbit heart and lung. These results confirmed that both the heart and the lung of the rabbit harbour a higher proportion of $beta_1$ adrenergic receptors in the rabbit. Interestingly, the proportion of $beta_1$ and $beta_2$ adrenoceptors in rabbit lung were shown to be quite the inverse of most other mammalian species.

The investigations carried out in chapter four examine the effects of short term agonist infusion on platelet and vascular $alpha_2$ adrenoceptor responses in the conscious rabbit. The first agonist to be studied was alpha methylnoradrenaline which is selective for $alpha_2$ adrenoceptors. Vasopressor responses to bolus injections of alpha methylnoradrenaline (3 ug/kg) were measured before and during intravenous infusions of alpha methylnoradrenaline (0.5 umol/kg/hr and 2.5 umol/kg/hr) or the $alpha_1$ adrenoceptor agonist, phenylephrine (1.8 umol/kg/hr). Pressor responses to alpha methylnoradrenaline during alpha methylnoradrenaline infusion were decreased within 2.5 minutes of infusion and in a dose dependent manner. Similar responses during phenylephrine infusion were unchanged. In conjunction with the *in vivo* experiments, *in vitro* platelet pro-aggregatory responses to adrenaline were measured before and at the end of 2.5, 5 and 10 minute alpha methylnoradrenaline (2.5 umol/kg/hr)

and 10 minute phenylephrine (1.8 umol/kg/hr) infusions. Similar responses were also measured at the end of 10 and 30 minutes alpha methylnoradrenaline (0.5 umol/kg/hr) infusion. Attenuations in the maximum aggregatory response (Emax) to adrenaline were dependent upon both, the dose and the duration of infusion. Infusions of phenylephrine had no effect on the aggregatory responses to adrenaline. These results were consistent with a rapid dose dependent desensitisation of platelet and vascular $alpha_2$ adrenoceptors similar to that observed in beta adrenoceptor systems.

The effect of acute infusion (10 minute) of alpha methylnoradrenaline (2.5 umol/kg/hr) on [^{3}H] yohimbine binding to rabbit platelets was also examined and there was no significant alteration in the concentration of $alpha_2$ receptors. Therefore, the acute <u>in vitro</u> reductions in response could not be explained in terms of changes in receptor number.

Short term administration of the endogenous catecholamine adrenaline (1.5 umol/kg/hr) also caused a reduction in vascular pressor responses within 2.5 minutes. These changes were again found to be dose dependent as no alterations in pressor responses were recorded during 0.05umol/kg/hr adrenaline infusion. Platelet pro-aggregatory responses to adrenaline were measured before and after ten minute intravenous infusions of adrenaline (1.5 umol/kg/hr). Reductions in the pro-aggregatory responses were observed in five out of the six animals treated although Emax did not significantly change. Thus, short term administration of adrenaline may also lead to a rapid

desensitisation of $alpha_2$ adrenoceptors.

Acute administration of noradrenaline (0.09 umol/kg/hr) produced no alteration in the pressor responses to alpha methylnoradrenaline (3 ug/kg) even after one hour of infusion. However, an attenuation of response may have been detected if infusions had been prolonged or if higher doses of noradrenaline had been administered.

In conclusion, short term exposure to alpha methylnoradrenaline or adrenaline both resulted in a dose dependent desensitisation of the $alpha_2$ adrenoceptor mediated responses examined, however the time course of desensitisation apparently differed according to the response examined.

Chapters five and six investigate the effects of long term administration of the endogenous agonists, adrenaline and noradrenaline on adrenergic receptor function and number. In the studies described in chapter five, rabbits were treated with intravenous infusions of adrenaline (0.05 umol/kg/hr) for ten days via osmotic minipumps implanted at the femoral vein. Control animals received the vehicle (0.1% ascorbate). Mean arterial blood pressure, heart rate and plasma catecholamines were measured at five intervals during the period of infusion. Eight fold elevations in circulating adrenaline concentrations were achieved within 24 hours of commencing infusion and these levels were sustained after ten days adrenaline infusion. This chronic increase in plasma adrenaline was not accompanied by significant changes in the mean arterial blood pressure or heart rate. Rabbits were killed after ten days and blood was withdrawn for preparation of platelets and lymphocytes. The whole heart,

lung and one kidney were also collected. Platelet aggregation studies were carried out as well as [^{3}H] yohimbine binding studies in platelets and kidney. [^{125}I](-) ICYP binding to platelets, lymphocytes, heart and lung was also quantified at the end of infusion. Ten day adrenaline (0.05 umol/kg/hr) infusions led to a significant reduction in the maximum aggregatory response to adrenaline concomitant with a decrease in alpha$_2$ adrenoceptor number in platelets but no significant fall in kidney alpha$_2$ receptors. There were marked falls in [^{125}I] ICYP binding to heart and lung without any changes in platelets or lymphocytes. The K_D values for either [^{3}H] yohimbine or [^{125}I] ICYP binding did not significantly alter with long term adrenaline treatment. Thus, long term adrenaline induced down regulation of adrenergic receptors in the rabbit was dependent upon the location and subtype of adrenoceptor.

Similar studies were conducted to determine the effects of chronic noradrenaline infusion on the function and density of adrenergic receptors in the rabbit (chapter six). Animals were treated with intravenous noradrenaline (0.09 umol/kg/hr) or ascorbate (0.1%) infusions for ten days via osmotic minipumps implanted at the femoral vein. The mean arterial blood pressure, heart rate and catecholamine levels were monitored before commencing and after 24 hours and ten days infusion. There were five fold increases in circulating plasma noradrenaline levels after 24 hours of noradrenaline infusion, these levels increasing to six fold at the end of ten days infusion. There were no significant alterations in blood pressure while a significant

decrease in the heart rate was evident only after 24 hours infusion. Rabbits were killed after ten days and similar measurements to those described in chapter five, were taken. A significant decrease in the number of $alpha_2$ adrenoceptors in the kidney was observed. This down regulation was in marked contrast to the lack of alteration in platelet $alpha_2$ receptor number and the platelet $alpha_2$ adrenoceptor mediated pro-aggregatory response. Long term exposure to noradrenaline also resulted in significant reductions in the number of beta adrenoceptors in heart and lung with no changes in the density of lymphocyte $beta_2$ receptors. Therefore, a moderate increase in circulating plasma noradrenaline resulted in substantial decreases in adrenoceptor number which were confined to solid tissues.

The observed discrepancies between the results following long term adrenaline and long term noradrenaline infusions could be due to the differing affinities of each agonist for the various receptor subtypes.

The final series of experiments (chapter seven) address the time course of down regulation in $alpha_2$ adrenoceptor and beta adrenoceptor systems. Groups of rabbits were treated with intravenous infusions of adrenaline (0.05 umol/kg/hr) for 12 hours, 24 hours, two, three or ten days using minipumps. Plasma concentrations of adrenaline were raised approximately ten fold after each duration of adrenaline infusion. At the end of infusions, rabbits were killed for removal of blood, heart and lung. [^{3}H] yohimbine binding studies were carried out in the platelets of control and noradrenaline infused animals. The first evidence of a decrement in platelet $alpha_2$ adrenoceptor

number took place after three days exposure to adrenaline with significant decreases obvious by the end of ten days infusion. Beta adrenoceptors were quantified in rabbit heart and lung using [^{125}I](-) ICYP. A trend towards a reduction in heart and lung beta adrenoceptor density was apparent within 12 hours exposure to adrenaline, these reductions becoming significant following ten days of infusion. Therefore, alpha$_2$ adrenergic receptor systems could be more resistant to down regulation in response to chronic adrenaline infusion.

In conclusion, acute adrenoceptor agonist infusion led to an alpha$_2$ adrenoceptor desensitisation without changes in adrenoceptor number while chronic agonist infusion reduced both adrenoceptor function and number. Reductions in both alpha$_2$ and beta adrenoceptor concentration were found to be tissue specific. Agonist selectivity, receptor specificity and the rate of receptor degradation may all be important factors influencing differential changes in adrenergic receptor density in response to chronically elevated catecholamines.

CHAPTER ONE

INTRODUCTION

Chapter One

Introduction

1.1 THE HISTORY OF ADRENOCEPTOR CLASSIFICATION

The catecholamines, adrenaline and noradrenaline play an important role as key modulators of many physiological events, in particular, the regulation of the cardiovascular system. Adrenaline functions as a circulatory hormone released from the chromaffin cells of the adrenal medulla whilst noradrenaline acts primarily as a neurotransmitter released from the postganglionic sympathetic nerves. These catecholamines exert their actions on target organs by binding with high affinity to specific recognition sites; the adrenergic receptors, commonly called adrenoceptors. The receptors act as the initial translators of extracellular messages by relaying signals so that the characteristic cellular response follows.

Dale (1906) first suggested that there may be subtypes of adrenergic receptors at myoneural junctions due to the differential effects of ergot alkaloids on smooth muscle. Since these pioneering studies, considerable advances have been made regarding the classification and function of adrenergic receptors. The work of Ahlquist in 1948 extended Dale's theories by proposing that the different vascular responses to catecholamines were mediated through two broad categories of receptor population which he designated, alpha and beta. Observation of the effects of some sympathomimetic amines on certain animal tissues allowed the functions of these receptors to be determined. The alpha adrenoceptor was shown to be

associated with excitatory functions such as vasoconstriction and stimulation of the uterus whereas, the beta adrenoceptor was associated with inhibitory functions like vasodilation and inhibition of the uterine and bronchial musculature, with one excitatory function, myocardial stimulation.

1.1.1 Beta adrenoceptor subclassification

Further subdivision of beta adrenoceptors was demonstrated by Furchgott (1967) and Lands et al (1967a) by examination of the relative potency of a series of agonists and antagonists in producing specific responses in isolated tissues from the rabbit and the guinea pig. These authors concluded that there were two different types of beta receptors which they termed $beta_1$ and $beta_2$. At $beta_1$ receptors, the efficacy of isoprenaline was greater than adrenaline which approximately equalled noradrenaline. This subtype was responsible for mediating positive inotropic cardiac responses and lipolysis. At $beta_2$ receptors, controlling broncho-dilatation and vasodilatation, isoprenaline was more potent than adrenaline which was substantially more potent than noradrenaline (Table 1.1). Subsequent validation of this subclassification was shown with the development of subtype selective antagonists and the pharmacology of $beta_1$ and $beta_2$ receptors was found to be identical in all mammalian species (Minneman, 1981).

1.1.2 Alpha adrenoceptor subclassification

In contrast to the beta adrenergic receptors, identification of alpha adrenergic receptor subtypes proved to be more difficult due to the complex physiological responses elicited via these

receptors and the slower development of subtype selective drugs.

In 1974, Langer proposed that postsynaptic and presynaptic alpha adrenoceptors could be distinguished from each other by function and agonist selectivity. Presynaptic alpha adrenoceptors were involved in the regulation of transmitter (e.g. noradrenaline) release through a negative feedback mechanism mediated by the transmitter itself (Starke, 1977). Postsynaptic alpha adrenoceptors represented the "classic" postjunctional receptors, stimulation of which produced a pharmacological effect. In rabbit heart (Starke, 1981) and cat spleen (Langer, 1973) the presynaptic alpha adrenoceptors could be differentiated from the postsynaptic ones with respect to the relative activities of various agonists and antagonists. The postsynaptic receptor was classified as an $alpha_1$ adrenoceptor with a relatively high affinity for the antagonist prazosin and the agonist methoxamine. Its functions included mediation of excitatory responses such as vasoconstriction. The presynaptic receptor was termed an $alpha_2$ adrenoceptor and exhibited a high affinity for the agonists, clonidine and alpha methylnoradrenaline and the antagonist, yohimbine (Starke, 1977; Wikberg, 1978).

The existence of $alpha_2$ adrenoceptors outside noradrenergic terminal axons at postsynaptic sites (e.g. smooth muscle and central nervous system) as well as non-synaptic sites (e.g. platelets and adipocytes) imposed limitations on the original anatomical subclassification of Langer (1974). Jauering et al (1978) showed that noradrenaline responses in human digital

arteries, unlike visceral arteries, were resistant to blockade by prazosin, implicating the presence of postjunctional $alpha_2$ receptor. These findings then led to the possibility that both $alpha_1$ and $alpha_2$ receptors mediated smooth muscle contraction and in particular vasoconstriction (Drew and Whiting, 1979). Therefore, alpha receptors are now defined by using pharmacological specificity rather than by location (Berthelson and Pettinger, 1977; Starke and Langer, 1979). A summary of the location and function of alpha adrenergic receptors is given in Table 1.1.

1.1.3 Subdivisions of $alpha_2$ adrenoceptors

Over the last few years, there has been increasing evidence to suggest that the $alpha_2$ adrenoceptors are not homogeneous. Investigations into this possible heterogeneity of $alpha_2$ adrenoceptors have been facilitated by the use of radiolabelled alpha adrenoceptor agonists and antagonists (Section 1.1). In 1981, Bylund conducted a study to compare the number of binding sites for [^{3}H] yohimbine and [^{3}H] clonidine in several tissues and species. He found that by choosing an appropriate tissue and species almost any ratio of [^{3}H] antagonist to [^{3}H] agonist could be obtained indicating that $alpha_2$ adrenoceptor subtypes may be present in some tissues. Similar studies by Dickinson et al (1986) demonstrated that $alpha_2$ receptor sites in human spleen, kidney, colon and platelets differed significantly from those in either rabbit spleen and kidney or in rat brain membranes.

Table 1.1

Classification of adrenergic receptors

Type	Location/Agonist Potency	Function
α-adrenergic	NORADR > ADR >> ISO	Vasoconstriction, excitation of uterine contractions, inhibition of intestinal peristalsis
α_1 adrenergic	Postsynaptic	Vasoconstriction
α_2 adrenergic	ADR > NORADR >> ISO Presynaptic	Negative feedback inhibition of noradrenaline release
	Postsynaptic	Vasoconstriction
	Nonsynaptic	Platelet activation, lipolysis, insulin release etc.
β-adrenergic	ISO > ADR > NORADR	Vasodilatation, inhibition of uterine contraction, myocardial stimulation
β_1 adrenergic	ISO > ADR = NORADR Postsynaptic	Positive inotropic cardiac responses. Fatty acid mobilisation from adipose tissue
β_2 adrenergic	ISO > ADR >> NORADR	Bronchodilatation, vasodepression
	Presynaptic	Positivefeedback stimulation of noradrenaline release
	Postsynaptic	Bronchodilatation, vasodepression, cardiac stimulation
	Nonsynaptic	Platelet inhibition etc.

ADR = Adrenaline
NORADR = Noradrenaline
ISO = Isoprenaline

Within species and within tissue heterogeneity of $alpha_2$ receptors has also been observed. Studies by Hamilton et al (1988) have shown that [^{3}H] idazoxan and [^{3}H] yohimbine bind to different sites in rabbit forebrain and kidney membranes. Differences in radioligand binding between $alpha_2$ adrenoceptors in rabbit, kidney and platelet prompted Nahorski and colleagues (1985) to propose that two putative subtypes of this adrenoceptor, A and B, existed with different properties depending on their locality (Chapter eight). Two subpopulations of the $alpha_2$ adrenoceptor have also been identified within tissues such as rat cerebral cortex and submandibular gland (Bylund, 1985).

The recent advent of quantitative autoradiographic techniques has allowed high resolution anatomical data to be obtained in certain animal tissues (Altar et al, 1984). Boyajian et al (1987) have recently presented evidence of differential autoradiographic distributions of [^{3}H] Rauwolscine and [^{3}H] Idazoxan in the rat brain indicating the presence of more than one $alpha_2$ adrenoceptor subtype within this tissue.

1.1.4 $Alpha_1$ adrenoceptor subclassification

$Alpha_1$ adrenoceptors have been shown to have different pharmacological properties in different tissues. Analysis of the pA_2 (affinity) values obtained for prazosin and yohimbine against $alpha_1$-adrenergic contractile responses in blood vessels from rodents and rabbits suggested the presence of two subtypes of this receptor; one with high affinity for prazosin and yohimbine and one with lower affinity for these two antagonists (Flavahan

and Vanhoutte, 1986). These authors demonstrated the existence of $alpha_1$ adrenoceptor subtypes in the pulmonary artery of the rabbit and dog while, in contrast, Docherty (1987) presented results in favour of a homogeneous population of $alpha_1$ adrenoceptors in rabbit pulmonary artery. Thus, the presence of subdivisions of the $alpha_1$ receptor appears to be equivocal (Hieble et al, 1986). However, a recent study by Han et al (1987) provides evidence to suggest that two separate subtypes (α_{1a} and α_{1b}) of the $alpha_1$ adrenoceptor are present in the smooth muscle of the rat, each subtype having a different function in the control of Ca^{2+} release by neurotransmitters and hormones.

1.1.5 The history of radioligand binding studies

During the last decade, rapid progress has been made in our understanding of receptor mediated events due to the advent of radioactively labelled hormone and drug derivatives with high specific activity. These radioligands have permitted direct identification of adrenergic receptors, quantification and determination of drug-receptor affinities. They provide a powerful tool for exploration of receptor structure and function in normal and pathological situations.

The basic concepts of this technique involves the incubation of a cell membrane preparation or whole cells (containing the receptors) with the radioactive adrenergic agonist or antagonist (radioligand) in the presence and absence of a nonradioactive drug that will bind to all the receptors being studied (e.g. phentolamine and propranolol for alpha and

beta receptors respectively). Specific binding is then defined by subtraction of the radioactivity observed in the presence of this unlabelled compound (nonspecific binding) from that observed in its absence (total binding). Radioligands bind to adrenergic receptors in a saturable manner with specific binding approaching a maximum (Bmax). The concentration of ligand that binds to half this number of receptors is the dissociation constant (K_D). These parameters are derived from the scatchard plot and description of this type of analysis has been given in Section 2.5. Several experiments have to be carried out to prove that tissue bound radioactivity represents receptors.

Thus, Motulsky and Insel (1984) have defined the following criteria that must be satisfied to demonstrate that radioligand binding sites may be equated with adrenergic receptors:-

1. Specific binding should be saturable and proportional to tissue concentration.
2. Binding should be as rapid and reversible as are the physiologic responses to the ligand.
3. Unlabelled drugs should compete for radioligand binding with appropriate relative potencies as predicted from pharmacologic experiments.
4. Alternative methods should yield nearly identical values for the equilibrium dissociation constant.
5. The radioligand should remain unaltered during the experiment.

The first successful radioligand binding studies were reported in 1974 with the use of [^{3}H](-) alprenolol to measure beta adrenergic receptors in turkey erythrocytes (Levitzki et al) while corresponding work in alpha receptors was started in 1976 using [^{3}H] dihydroergocryptine (Williams and Lefkowitz; Greenberg et al). In some of these earlier investigations, the ligands were not highly specific and large amounts would bind to nonreceptor proteins (nonspecific binding). Since then, ligands with higher affinities for the alpha and beta adrenoceptors have been developed. The selective alpha$_2$ antagonist [^{3}H] yohimbine was introduced in the studies of Motulsky et al in 1980. Binding of this ligand to human platelets was shown to be rapid, readily reversible and of high affinity for alpha$_2$ adrenoceptors. Similarly, the beta adrenoceptor selective antagonist [^{125}I] iodocyanopindolol (ICYP) was shown to have advantages over previously employed beta adrenoceptor ligands. [^{125}I] ICYP had lower nonspecific binding, a higher specific activity and required smaller volumes of blood for lymphocyte studies (Brodde et al, 1981a). These ligands have enabled more detailed investigation of the molecular and physiological regulation of the adrenergic receptors and therefore, more information on the underlying mechanisms of disease.

1.2 BIOCHEMICAL MECHANISMS OF SIGNAL TRANSDUCTION

The interaction between an agonist and adrenergic receptor induces changes that lead to a cascade of events which ultimately result in the observed physiologic response. Radioligand binding studies have made possible the exploration of molecular details

and dissection of the biochemical components involved in these steps.

1.2.1 Beta adrenoceptor effector coupling

The fundamental difference between agonist and antagonist drugs is that agonists activate a biological process by virtue of their binding to receptors whereas antagonists that bind to the same receptors cause no such activation. Therefore, there will be differences in the way in which agonists, as opposed to antagonists, activate beta adrenergic receptors.

Both $beta_1$ and $beta_2$ adrenoceptors stimulate the membrane bound enzyme, adenylate cyclase (AC) (Sutherland and Rall, 1960). The first step involves binding of the agonist to receptor (R) which leads to a conformational change in the receptor molecule (R^1) so that the agonist-R^1 complex can bind to the guanine nucleotide protein (G_S) forming a transient agonist-R^1-G ternary complex. Formation of this complex also facilitates the exchange of GTP for GDP which then causes a dissociation of G from agonist-R^1 and allows it to bind to and activate adenylate cyclase (AC) (Figure 1.1).

The activated adenylate cyclase stimulates the conversion of adenosine triphosphate (ATP) to adenosine-3^1, 5^1-monophosphate or cyclic AMP which serves as a "second messenger" for the hormone and activates intracellular protein kinases. These then cause phosphorylation of enzymes that lead to the cellular response such as smooth muscle relaxation.

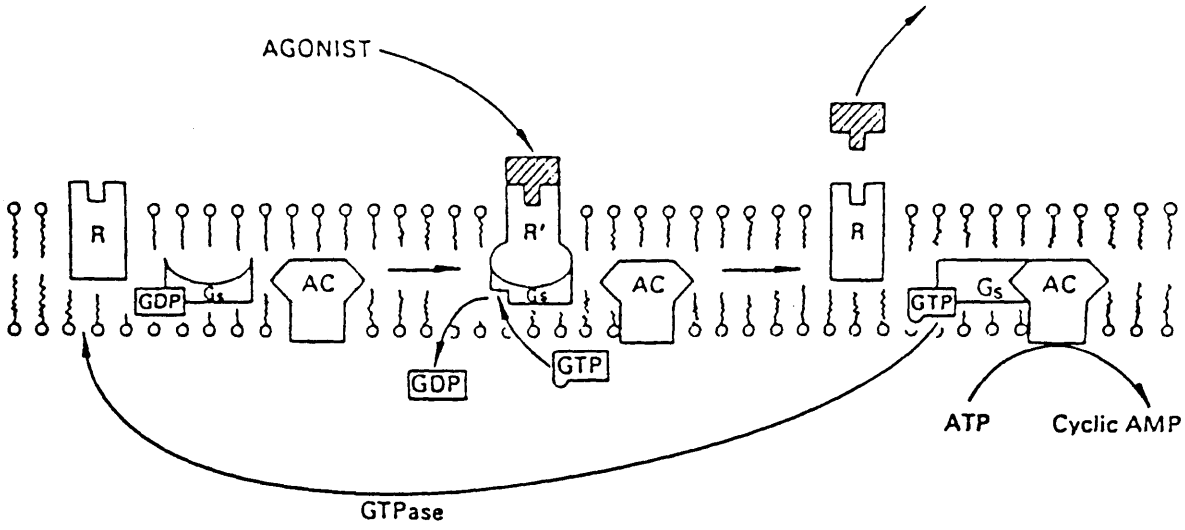


Figure 1.1

Hormonal activation of adenylate cyclase
(adapted from Nahorski and Barnett, 1982)

(1) Agonist binds to receptor R inducing a conformational change, R^1.

(2) The agonist - R^1 complex then binds to the guanine nucleotide protein, G forming the agonist-R^1-G ternary complex which facilitates the exchange of GTP for GDP on G.

(3) G then dissociates from agonist-R^1, and binds to and activates adenylate cyclase (AC).

(4) Dissociation of G converts R^1 into R.

(5) Synthesis of cyclic AMP continues until GTP is hydrolysed by the GTPase "turn off" reaction.

The most extensively studied modulator of binding to adrenergic receptors is the guanine nucleotide guanosine triphosphate (GTP) (Rodbell, 1980; Hoffman and Lefkowitz, 1980). In its absence, agonists discriminate between two classes of receptors termed high affinity and low affinity receptors. In the presence of GTP, however, only low affinity binding can be detected.

In a radioligand binding assay, the ability of agonists to promote the formation of a receptor-G-protein complex corresponds to the ability of the agonist to form a high affinity state of the receptor and is the molecular basis for the "shallow" curves that are characteristic of agonists competing against labelled antagonist ligands. Addition of GTP to the assay results in a shift of the agonist curve to the right and a steepening of the curve. This parallels the ability of guanine nucleotides to dissociate the receptor-G-protein complex resulting in a homogeneous population of receptors with lower affinity for agonists. Thus, the shape of an agonist displacement curve and the change induced by GTP gives a measure of the relative coupling of the receptor to adenylate cyclase (Nahorski and Barnett, 1982; Motulsky and Insel, 1982).

1.2.2 Alpha$_2$ adrenoceptor effector coupling

The coupling mechanisms following activation of alpha$_2$ adrenoceptors are very similar to those described for the beta adrenergic system although the alpha$_2$ receptors are negatively coupled to adenylate cyclase (Fain and Garcia-Sainz, 1980). Less

information is available concerning the hormonal activation of $alpha_2$ receptors, however like beta receptors, they exist in both high and low affinity states (Bylund and U'Prichard, 1983) with antagonists only recognising a homogeneous population of $alpha_2$ receptors (Nahorski et al, 1985). Guanine nucleotides have also been shown to exert important agonist specific regulatory effects (Tsai and Lefkowitz, 1979). Inhibition of adenylate cyclase occurs through activation of a different guanine nucleotide binding protein (Gi) which reduces adenylate cyclase activity (Jakobs et al, 1984). One subunit of this protein is clearly distinct from the units making up the protein mediating adenylate cyclase stimulation (Jakobs et al, 1985). At present there are conflicting opinions regarding the mechanism by which the guanine nucleotide protein causes decreases in adenylate cyclase activity. However decreases in cyclic AMP levels have been shown in platelets, adipocytes, hepatocytes, pancreatic islets, cerebral microvessels and cloned neuroblastoma cells (Jakobs et al, 1983).

1.2.3 $Alpha_1$ adrenoceptor effector coupling

In contrast to $alpha_2$ and beta adrenergic receptors, $alpha_1$ adrenoceptor activation does not alter intracellular levels of cyclic AMP.

Binding of an agonist to the $alpha_1$ receptor activates phospholipase C, an enzyme that hydrolyzes the phosphatidylinositol-4, 5-bisphosphate (PIP_2) to form myo-inositol-1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DG) (Homcy and Graham, 1985). Availability of IP_3 leads to

intracellular Ca^{2+} release which can then in turn activate cellular responses such as actin-myosin coupling or promotion of protein kinase C activation (Michell, 1985). Thus the mobilisation of Ca^{2+} from intracellular vesicles and/or the influx of extracellular Ca^{2+} appears to be linked closely to activation of the $alpha_1$ adrenoceptor.

1.3 REGULATION OF ADRENERGIC RECEPTORS

Adrenergic receptors possess the ability to increase (up regulate) or decrease (down regulate) the synthesis of their own receptor protein in response to drug administration and many physiological and pathological settings.

For the purpose of this thesis regulation of adrenergic receptors that stimulate (beta) and inhibit ($alpha_2$) adenylate cyclase will be examined.

1.3.1 Supersensitivity and Up-regulation

Depletion of catecholamines by destruction of noradrenergic neurons or treatment with adrenergic antagonists may lead to a supersensitivity of tissues to catecholamines and an up-regulation of receptor number (Glaubiger et al, 1978). When nerve terminals were destroyed by treatment with 6-hydroxydopamine a fall in tissue catecholamines associated with an increase in beta adrenoceptors in rat brain has been observed (Sporn et al, 1976). This increase was accompanied by a rise in isoprenaline-stimulated cyclic AMP production. Chronic blockade of beta adrenergic receptors resulting from propranolol administration resulted in an elevation of beta adrenoceptor

number in human lymphocytes (Aarons et al, 1980) and a doubling of beta receptor density in rat heart (Glaubiger and Lefkowitz, 1977). The "propranolol withdrawal syndrome" has been associated with beta adrenergic hypersensitivity, arrhythmias and myocardial ischaemia (Alderman et al, 1974) due to persistent up-regulation of beta receptors. On withdrawal of propranolol excessive numbers of receptors are left exposed to catecholamines accounting for the increased sensitivity (Davies and Lefkowitz, 1981). Another example of up-regulation is in patients with orthostatic hypotension who have low concentrations of circulating catecholamines and enhanced responses to administered catecholamines. In these subjects the number of $alpha_2$ adrenoceptors on platelets and beta adrenoceptors on lymphocytes are several times higher than in normal subjects (Hui and Connolly, 1981; Davies et al, 1981).

1.3.2 Desensitisation

The converse of supersensitivity occurs when there is a decreased sensitivity to circulating catecholamines, a phenomenon known as receptor sub-sensitivity or densensitisation. This blunted responsiveness takes place when the adenylate cyclase system is subjected to persistent agonist stimulation. Desensitisation is thought to represent some sort of homeostatic mechanism whereby tissues may be protected from prolonged agonist exposure (Catt et al, 1979).

Hormone induced desensitisation may be divided into two categories referred to as homologous (agonist specific) and heterologous (agonist non-specific). The latter process refers

to a decreased responsiveness to the desensitising hormone as well as to other hormones and in some instances non-hormonal stimulators such as guanine nucleotides and fluoride ion. Su and colleagues (1976) were among the first to investigate heterologous desensitisation using clonal astrocytoma cells. Prolonged incubation with either beta adrenergic agonists or prostaglandins diminished the subsequent capacity of both hormones to elevate intracellular cyclic AMP levels. In this form of desensitisation receptor function is shown to be regulated by phosphorylation in the absence of receptor sequestration or down regulation (Sibley and Lefkowitz, 1985).

Homologous desensitisation leads to an attenuated response only to the desensitising hormone. The first stage of this type of desensitisation involves a rapid uncoupling of the receptors within the plasma membrane which alters the conformational state of the receptor so that it is unable to activate adenylate cyclase (Homburger et al, 1980). This process has been shown to take place within minutes (Staehelin and Simons, 1982). In addition to uncoupling from the G protein, the receptors are sequestered or "internalised" away from the cell surface into endocytotic vesicles which are devoid of adrenylate cyclase activity (Stadel et al, 1983). At this stage these redistributed receptors have their binding site facing inwards and can still be detected by conventional antagonist radioligand binding techniques which label total receptor populations. On removal of agonist the process may be rapidly reversed with receptors reappearing on the cell surface (Sibley and Lefkowitz, 1985). A model describing these events is shown in Figure 1.2.

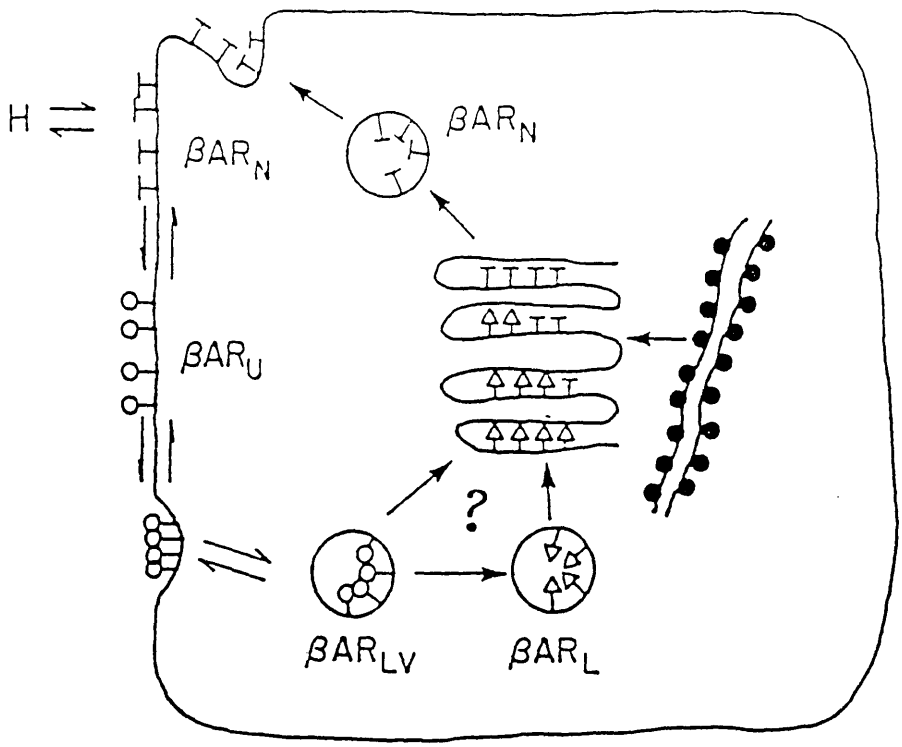


Figure 1.2

A hypothetical model for catecholamine induced homologous desensitisation
(adapted from Hertel et al, 1983)

The model depicts the binding of agonists (H) to native receptors (BAR_N) leading to rapid uncoupling of receptors (BAR_U), endocytosis to form vesicles containing receptors (BAR_{LV}), conversion of receptors to a form undetectable in ligand binding assays (BAR_L) and recycling of the modified BAR or new synthesis of BAR.

The first evidence of catecholamine induced internalisation of adrenergic receptors came from the in vitro studies of Chuang and Costa (1979) when they incubated frog erythrocytes with isoprenaline. A loss of beta adrenergic receptors from the plasma membrane fraction was associated with an increase in binding activity in the cell cytosol. This work was extended to demonstrate that the erythrocyte beta receptors were internalised subsequent to receptor clustering mediated by transglutaminase (Chuang et al, 1980). The development of a hydrophilic beta antagonist radioligand [^{3}H]CGP-12177 allowed specific measurement of cell surface beta receptors to be made (Staehelin et al, 1983). This ligand is membrane impermeant unlike the other more lipophilic antagonist radioligands [^{125}I] ICYP and [^{3}H] DHA which assess the total receptor concentration. Recent studies using this ligand have presented evidence that agonist modified receptors are not "internalised" into endocytotic vesicles but are "redistributed" in the plasma membrane with a proportion of the cell surface receptors (dependent on the dose and duration of agonist exposure) losing the ability to bind [^{3}H] CGP (Mahan et al, 1985). Another group of workers have demonstrated that the first molecular event to take place in the desensitisation process is phosphorylation of the agonist occupied receptor by a specific beta adrenoceptor kinase (Sibley et al, 1986). This phosphorylation results in the functional uncoupling of the receptors and triggers their sequestration from the cell surface. Dephosphorylation is thought to restore receptor function and recycle the receptors back to the cell surface.

Rapid desensitisation of $alpha_2$ adrenergic receptors has been much less extensively studied partly due to the lack of development of suitable hydrophilic radioligands. It appears that this phenomenon is not a prominent feature of many alpha adrenergic responses however rat parotid cells have been shown to lose their alpha adrenergic mediated potassium secretory response after 2-4 minutes of *in vitro* exposure to adrenaline (Strittmatter et al, 1977).

1.4 Possible modes of homologous desensitisation

Regulation of an adrenergic effect may involve changes in receptor affinity or number, altered coupling of receptor to adenylate cyclase or other effector mechanisms or alterations at more distal steps.

1.4.1 Changes in affinity

Subtle changes in receptor function may be mediated via changes in the ratio of high and low affinity states of the receptor (Harden et al, 1979). Assessment of changes in affinity state may provide information on receptor "status" rather than measurement of the total receptor population. Moreover, receptor concentration may be unaltered while the proportion of high affinity agonist sites of the receptor (closely reflecting the coupling to adenylate cyclase) is significantly changed (Davies et al, 1981). Alterations in affinity state during agonist induced desensitisation have been observed in both beta and $alpha_2$ adrenergic systems (Limbird et al, 1980; Michel et al, 1981).

Adrenergic receptor affinity may also be modulated by cations. It has been observed that a divalent cation such as magnesium is necessary for formation of the high affinity agonist-beta receptor complex (Tsai and Lefkowitz (1979)). In the absence of magnesium, heterogeneity of agonist binding is not apparent and even agonist curves are steep, shifted to the right and not affected by guanine nucleotides (Hoffman and Lefkowitz, 1980). In contrast, monovalent cations such as sodium have been reported to decrease hormone and guanine nucleotide-induced stimulation of adenylate cyclase (Jakobs et al, 1984). Magnesium and sodium ions have been shown to have opposite effects in other beta adrenergic systems such as the beta adrenoceptor affinity for agonists in the membranes of guinea pig lung and S49 lymphoma cells. Magnesium ions were found to increase the affinity for agonists and were also found to modulate the decrease in affinity caused by sodium ions (Minuth and Jakobs, 1986).

Agonist binding to $alpha_2$ adrenoceptors is also shown to be modulated by ions. Sodium ions have been demonstrated to mediate an agonist specific decrease in $alpha_2$ receptor binding affinity in rabbit platelets (Greenberg et al, 1978). In another study displacement curves of agonists in competition with [^{3}H] Dihydroergocryptine DHE were shifted to the right by sodium ions and other univalent cations (Tsai and Lefkowitz, 1978). The site of action of sodium ions in regulating alpha and beta adrenoceptor binding could be a subunit of the G protein (Northup et al, 1983) or, alternatively, the receptors themselves (Minuth and Jakobs, 1986).

1.4.2 Changes in the efficiency of coupling to biologic effectors

Alterations in the components making up the adenylate cyclase system or transmembrane signalling processes can affect the coupling of hormone to receptor and it has become increasingly clear that adrenoceptors may alter their coupling independently of any change in total receptor density (Su et al, 1980; Iyengar et al, 1981). Desensitisation of cyclic AMP mediated cellular responses could occur through a reduction in the rate of cyclic AMP synthesis, enhancement of cyclic AMP degradation or egress of cyclic AMP from the cell. The major regulating factor appears to be modulation of the rate of receptor stimulated cyclic AMP, however cyclic AMP levels are also regulated by degradation of the nucleotide catalysed by phosphodiesterase. Increases in phosphodiesterase activity have been reported following chronic exposure of several cell types to catecholamines (Bourne et al, 1973; Browning et al, 1976). Closer examination of beta adrenergic receptor coupling mechanisms has been made possible due to the availability of a toxin produced by the cholera bacillus. Cholera toxin covalently modifies the G_S protein and transducin by adding an adenosine diphosphate (ADP) ribose to the G protein. The ADP ribosylation of G_S inhibits its GTPase activity and prolongs the life of the GTP-G_S complex, causing persistent activation of adenylate cyclse with continual production of cyclic AMP (Berridge, 1985). Similarly, the pertussis toxin has proved a useful inhibitor of the $alpha_2$ adrenoceptor transduction process (Ui et al, 1984). Pre-treatment of tissue with this bacterial toxin is known to

block the receptor mediated suppression of adenylate cyclase. Furthermore, it is believed that the ability of this toxin to inactivate these systems relates to its ability to cause ADP-vibosylation of a specific sub-unit of the guanine nucleotide binding protein G_I (Nahorski et al, 1985).

1.4.3 Down regulation

Homologous desensitisation may also be associated with an actual disappearance or proteolytic degradation of receptor protein, known as down regulation. This process usually occurs after prolonged periods (hours or days) of agonist stimulation. At this stage, receptors cannot be readily recovered, in contrast to the earlier uncoupling phase or desensitisation. Resensitisation or recovery of receptor number may be a slow and gradual process (Scarpace et al, 1985). Dramatic reductions in adrenergic receptor concentration may have implications in diseased situations where supraphysiological levels of catecholamines are often found. A typical example is the repeated administration of beta adrenoceptor agonist drugs to asthmatic patients which has been shown to result in a progressive decrease in the observed therapeutic response due to a down regulation of beta adrenoceptors. Attempts to compensate for this loss of drug efficacy by administering gradually larger doses may increase morbidity and mortality (Connolly and Greenacre, 1976). Further examples of beta receptor down regulation are given in Section 1.6.

1.5 Receptor life cycles

The rate of metabolism or turnover of receptors by the cells on which they are situated could affect the rate of down regulation. Steady state levels of receptor expression in target cells are dependent on several intricate cellular processes which result in receptor appearance and disappearance from the cell surface. Recovery or recycling of receptor protein represents a slow process which could result in a period of impaired regulation of circulatory control. New receptor synthesis involves processing by the golgi apparatus and movement through the cell's cytoplasm with ultimate insertion or externalisation of receptors from intracellular pools to the membrane surface.

1.5.1 Measurement of receptor appearance and disappearance

Antagonist studies

Studies on the metabolism of alpha adrenergic receptors involve the use of protein synthesis inhibitors which have been shown to delay return of the maximum number of binding sites to control levels (Hamilton et al, 1983). An example is the selective $alpha_1$ adrenoceptor antagonist, phenoxybenzamine. This non-competitive antagonist irreversibly blocks the receptor by alkylation, thereby allowing the rate of new receptor synthesis to be measured. Studies of this kind have shown that adrenergic receptors in the central nervous system turn over more slowly than those in peripheral tissues (Hamilton et al 1985). Beta receptor turnover has also been investigated using irreversible and slowly dissociating beta adrenoceptor antagonists such as

alprenolol derivatives (Baker and Pitha, 1982). These compounds have been useful probes to help gain more insight into the rate of beta adrenoceptor synthesis after irreversible antagonism (Fraser and Venter, 1980) and the coupling efficiency between beta adrenoceptors and adenylate cyclase or physiologic responses (Tolkovsky and Levitzki, 1978; Venter, 1979). Cellular metabolism of $alpha_2$ adrenergic receptors has been difficult to study using irreversible antagonists due to their limited specificity.

Agonist studies

Another approach involves computer analysis of the kinetics of receptor loss during agonist infusion and of reappearance of receptors upon removal of the agonist (Snavely et al, 1984). This method involves application of a steady state model that allows estimation of the rate constants for receptor appearance and disappearance. Snavely and colleagues (1984) gave continuous infusions of isoprenaline to rats via osmotic minipumps and determined rates of receptor disappearance and reappearance after removal of agonist. Rat renal cortical membranes contain seventy percent $beta_1$ and thirty percent $beta_2$ receptors. A similar time course of down regulation was observed for both beta adrenoceptor subtypes although $beta_1$ receptor recovery was slower. These *in vivo* studies revealed that the basal turnover of beta adrenergic receptors is slow while there is an accelerated rate of beta receptor reappearance after agonist induced down regulation. This model may be widely used in both *in vitro* and *in vivo* receptor systems and facilitates an understanding of how

important changes in the rates of receptor loss and recovery may be in relation to changes in receptor number that are evoked by various stimuli. Thus, the described techniques for agonist and antagonist studies may help to further elucidate the molecular mechanisms controlling the cellular metabolism of adrenergic receptors.

1.6 Beta adrenoceptor down-regulation

1.6.1 In vitro studies

Early investigations of agonist induced beta adrenoceptor down regulation were carried out using amphibian and avian erythrocytes. Chronic exposure (24 hours) of frog erythrocytes to isoprenaline resulted in fifty percent reductions in both the total numbers of receptors and the isoprenaline stimulated adenylate cyclase activity (Mukherjee et al, 1975; Mickey et al, 1975). These studies suggested that loss of erythrocyte beta receptors was the result of the formation of a slowly reversible complex between the catecholamine and receptor. Down regulation of beta receptors was also demonstrated in lymphocytes from asthmatic patients after prolonged incubation with either isoprenaline or terbutaline in vitro (Galant et al, 1978; Tashkin et al, 1982). These studies also induced a two step process of adenylate cyclase desensitisation accompanied by a slower down regulation. These findings differ from those found in response to acute infusions of isoprenaline which have been associated with a rapid loss of adenylate cyclase activity without a reduction in beta receptor number (Krall et al, 1980). The majority of in vitro studies on receptor down regulation used

high concentrations of added catecholamines which resulted in grossly unphysiological conditions and were therefore difficult to relate to the intact organism. Unresolved issues with *in vitro* experiments were whether down regulation occurred *in vivo* in physiological or pathological settings and whether all receptors of a given type were down regulated in a similar manner and to a similar extent.

1.6.2 *In vivo* studies

Adrenergic agonist therapy in asthmatic patients has also been shown to down regulate beta adrenergic receptors *in vivo*. Aarons *et al* (1983) showed that asthmatic or healthy subjects treated with terbutaline or ephedrine for eight days had fewer lymphocyte beta receptors compared to those found before treatment.

The frog erythrocyte model has also been investigated with respect to *in vivo* treatment with isoprenaline. Mickey et al (1975) showed that changes *in vitro* paralleled those *in vivo* resulting in a fifty percent decline in the isoprenaline stimulated adenylate cyclase activity associated with a fall in beta adrenoceptor number. Isoprenaline has also been administered *in vivo* in rats by continuous infusion (4-7 days) from osmotic minipumps. The subsequent *in vitro* responsiveness to the same agonist was decreased and correlated with a fifty percent loss in the number of beta adrenoceptor sites (Chang et al, 1982; Kenakin and Ferris, 1983). Thus, reductions in responsiveness corresponded with reduced beta receptor density in both *in vitro* and *in vivo* studies however the time courses for

receptor degradation may differ. A longer time course of down regulation may occur in vivo due to a lower concentration of the agonist in proximity to the receptor with infusion into an intact animal compared to the concentrations that can be achieved in cell culture (Snavely et al, 1985).

Several in vivo studies of beta adrenoceptor down regulation have presented evidence in support of differential regulation of the $beta_1$ and $beta_2$ adrenoceptor subtypes. Drug selectivity may be one possible explanation for this subtype specific down regulation. Long term infusions (fourteen days) of various catecholamines via minipumps into rats resulted in subtype selective down regulation of the beta adrenoceptor subtypes in renal cortical membranes (Snavely et al, 1985). In this study, adrenaline infusion produced a seventy five percent decrease in $beta_2$ receptor density as might be expected, considering its higher affinity for the $beta_2$ adrenoceptor (Lands et al, 1967b). Likewise, noradrenaline infusion led to a more marked down regulation of $beta_1$ receptors and isoprenaline infusion down regulated both $beta_1$ and $beta_2$ adrenoceptors to a similar extent, as would be predicted by their relative affinities for the beta adrenoceptor subtypes (Minneman et al,1981).

However, a recent study by Cohen and Schenck (1987) revealed a selective down regulation of vascular $beta_1$ adrenergic receptors following prolonged isoprenaline infusion in rats. These authors proposed that vascular $beta_1$ receptors are considerably more susceptible to down regulation than are $beta_2$ adrenergic receptors. Further discussion of the independent

regulation of beta adrenoceptor subtypes is given in chapters five and six.

1.7 Alpha$_2$ adrenoceptor down regulation

There is less information available regarding the mechanisms of down regulation of alpha$_2$ adrenergic receptors. Blood platelets have been widely adopted as a model of alpha$_2$ adrenoceptor systems in other organs and tissues due to their accessibility and ease of sampling. Cooper et al (1978) demonstrated a reduction in [^{3}H] DHE dihydroergocryptine (DHE) binding to human platelets comparable with a fall in adrenaline induced aggregation after prolonged incubation with adrenaline *in vitro*. However a later study by Karliner and colleagues (1982) reached opposite conclusions with [^{3}H] yohimbine binding to platelets following *in vitro* incubations with catecholamines. They observed a "pseudo" down regulation and two fold increase in K_D resulting from retained agonist. These conflicting findings could be due to the use of different ligands to measure these alpha$_2$ receptor populations. Motulsky and Insel (1982) suggested that [^{3}H] DHE may bind to more sites than [^{3}H] yohimbine in platelets however, later studies by Pfeifer and others (1984) showed the number of alpha$_2$ adrenoceptor sites to be compatible for the two ligands.

This controversy of opinion also featured in the *in vivo* agonist regulation of alpha$_2$ adrenoceptors. Treatment of hypertensive patients with alpha$_2$ adrenergic agonists for several days to weeks have demonstrated either no change (Motulsky et al, 1983; Boon et al, 1983) or a thirty percent decrease in platelet

alpha$_2$ receptor number (Brodde et al, 1982).

Platelet alpha$_2$ adrenoceptors have also been quantified in vivo in experimental animals. Administration of clonidine for four days to dogs resulted in no modification in the total numbers of [^{3}H] yohimbine binding sites (Villeneuve, 1985b). Down regulation of adipocyte alpha$_2$ adrenergic receptors also did not occur when hamsters were treated with clonidine or adrenaline (Pecquery et al, 1984; Villeneuve, 1985a).

Thus, alpha$_2$ adrenergic receptors appear to be less susceptible than beta adrenergic receptors to down regulation. Although protein degradation may play a limited role in the agonist regulation of alpha$_2$ receptors, it is possible that changes distal to the receptor may predominate, such as changes in affinity state or cyclic AMP levels.

1.8 Physiological situations associated with acute elevations of catecholamines

Elevated concentrations of the major catecholamines, adrenaline and noradrenaline have been reported in physiological settings such as stress, physical exercise and postural changes. Alterations in the central nervous system activity accompanying stress may also lead to changes in the density of beta adrenergic receptors. For example, high blood levels of catecholamines during stress may lead to a decrease in the density of beta receptors located on the peripheral side of the cerebral vasculature (Minneman et al, 1981).

Dynamic exercise is known to rapidly and vigorously increase plasma noradrenaline and adrenaline levels (Christensen and

Galbo, 1983). These high circulating catecholamines have been associated with acute changes in lymphocyte beta adrenoceptor number and responsiveness (Brodde et al, 1984). De Blasi and others (1986) have shown that moderate exercise leads to increased isoprenaline stimulated cyclic AMP accumulation without any changes in lymphocyte beta receptor density. These authors also reported increases in beta adrenoceptor responses following upright posture for three hours however these observations contrasted with earlier findings of Sowers et al (1983) who found that acute postural changes and circadian fluctuations in plasma catecholamines in the physiological range did not change beta adrenoceptor numbers in human lymphocytes.

1.9 Pathological conditions associated with chronic elevations of plasma catecholamines

1.9.1 Phaeochromocytoma

Phaeochromocytoma is a catecholamine secreting tumor of the adrenal medulla which produces plasma concentrations of adrenaline and noradrenaline vastly exceeding those found in normal situations. This excessive production of catecholamines, particularly noradrenaline, is usually associated with hypertension due to alpha adrenoceptor stimulation mediating vasoconstriction and beta adrenoceptor stimulation causing cardiac arrhythmias. However, these elevations in blood pressure are sometimes lower than might be anticipated (Bravo et al, 1979) and may be due to reduced responsiveness of adrenergic receptors (Lefkowitz, 1982).

In man, phaeochromocytomata have been associated with

reductions in lymphocyte beta adrenoceptor density (Greenacre and Connolly, 1978; Valet et al, 1987) while extensive studies with transplantable tumors in rats have demonstrated down regulation of renal, cardiac and adipocyte beta adrenoceptors (Snavely et al, 1982; Tsujimoto et al, 1984). Selective changes in $beta_1$ and $beta_2$ adrenoceptor density have been observed in many of the animal studies of this diseased situation and these have been discussed in chapters five, six and eight. Changes in $alpha_2$ adrenergic receptors have also been reported in association with the chronically elevated catecholamines in phaeochromocytoma. A fifty percent decrease in human platelet $alpha_2$ adrenoceptors was observed by certain groups (Davies et al, 1981; Brodde and Bock, 1984) while others have shown a lack of change in human platelet $alpha_2$ receptor number in this condition (Jones et al, 1985a; Valet et al, 1987). Possible resistance of $alpha_2$ adrenoceptors to down regulation is discussed in chapter six.

1.9.2 Heart failure

Beta adrenergic and alpha adrenergic receptors are present in the myocardial cells (Ahlquist, 1948; Benfey, 1982) and serve as transducers linking hormone mediated chemical signals to the mechanical event of augmented cardiac contractility. Congestive heart failure may be characterised by attenuated myocardial inotropy associated with decreased cardiac beta adrenoceptors and responsiveness to beta adrenoceptor agonists (Thomas and Marks, 1978). It has been suggested that systemic changes such as elevated circulating noradrenaline levels may contribute to the down regulation of cardiac and lymphocyte beta receptors during

heart failure (Colucci et al, 1981).

These findings were supported by Brodde and co-workers (1986) while quantifying beta adrenoceptors in explanted hearts from patients with end stage heart failure. This group demonstrated a selective down regulation of cardiac $beta_1$ receptors and postulated that these results were due to raised plasma noradrenaline concentrations.

In experimental animals with heart failure, myocardial adrenergic receptors were shown to be increased (Karliner et al, 1980; Vatner et al, 1985) or unchanged (Ho et al, 1980). However, recent studies by Fan et al (1987) reported reduced numbers of beta adrenoceptors in the failing right ventricles of dogs. Whether these decreases in beta receptor density are an example of down regulation in response to high circulating catecholamines remains to be determined.

1.9.3 Cirrhosis

Plasma noradrenaline levels are raised in patients with cirrhosis (Burghard et al, 1982; Henriksen et al, 1984). The characteristic haemodynamic disturbance associated with this disease is that of a hyperdynamic circulation with diminished peripheral vascular resistance. It is possible, therefore, that there is an impaired haemodynamic response to noradrenaline which in turn could be in part to desensitisation of adrenergic receptors. There is a scarcity of information on changes in adrenoceptor function and number during this condition, however there have been two conflicting reports concerning alterations in lymphocyte $beta_2$ adrenoceptor density. Gerber and colleagues

(1986) proposed that an observed reduction in lymphocyte $beta_2$ receptors in patients with severe ascites could be interpreted as down regulation while MacGilchrist (1988) found an absence of alteration in lymphocyte $beta_2$ adrenoceptors or platelet $alpha_2$ receptors in similar studies. Therefore, it remains debatable whether changes in adrenoceptor number contribute to the decreased vascular resistance observed in cirrhosis.

1.10 Scope of the thesis

The studies described in this thesis were designed to investigate the effects of acute and chronic agonist treatment on adrenoceptor function and number in a rabbit model. Several studies have focused on the *in vitro* effects of raised concentrations of plasma catecholamines on adrenergic receptor responsiveness and density. However, similar investigations *in vivo* have been less widely reported. When *in vivo* studies have been undertaken, very large increases in plasma catecholamines were achieved. In animal models of phaeochromocytoma, plasma noradrenaline levels were often found to be highly elevated (50-200 fold). Thus, the principal objectives of the present studies were to examine changes in adrenoceptor function and number in response to modest rises (10 fold) in circulating plasma catecholamines in a rabbit model.

A range of tissues were used throughout the course of the following studies. The circulating blood elements (platelets and lymphocytes) were taken due to the ease of sampling and to make comparisons with human studies. Tissues (heart, lung and kidney) were also used for comparisons between tissues and blood elements

and extrapolation to tissues in human studies. In addition to looking at the number of binding sites in platelets, the pro-aggregatory response to adrenaline was examined so that changes in receptor number could be related to changes in function. In other studies, pressor responses to alpha adrenoceptor agonists were examined to give a measure of postsynaptic alpha adrenoceptor function.

To begin with, it was necessary to define the adrenoceptor populations within the tissues used. The first studies (chapter three) aimed to provide evidence that the pro-aggregatory response of rabbit platelets to adrenaline is mediated by $alpha_2$ adrenoceptors. Displacement binding assays were also carried out to characterise the adrenoceptor subpopulations in the various tissues used.

In chapter four the effects of acute infusions of three different adrenoceptor agonists (alpha methylnoradrenaline, adrenaline and noradrenaline) on $alpha_2$ adrenoceptor responses were assessed. Changes in vascular pressor responses to alpha adrenoceptor agonists and changes in platelet aggregation were used to determine the time courses of vascular and platelet $alpha_2$ adrenoceptor desensitisation. If changes in response were observed further investigation of these responses was then required to examine if alterations were dependent on dose and/or duration of infusion.

The purpose of the investigations undertaken in chapter five was to evaluate the effects of long term (10 day) intravenous minipump infusions of the endogenous agonist, adrenaline on adrenergic receptor function (measured by platelet aggregation)

and number (measured by radioligand binding). These studies particularly focused on whether down regulation was confined to certain tissues or subtypes of adrenoceptor.

In a separate study, ten day intravenous minipump infusions of the other main endogenous agonist, noradrenaline were given to groups of rabbits (chapter six) and changes in the function and concentration of adrenoceptors observed as described for the studies in chapter five. Tissue and subtype variations in susceptibility to down regulation were again examined.

The main objective of the final set of studies was to ascertain the time course of down regulation in $alpha_2$ and beta adrenoceptor systems in response to long term administration of adrenaline. Differences in the rates of $alpha_2$ and beta adrenoceptor down regulation were observed with particular emphasis on estimation of the critical time for receptor degradation in each system.

The final chapter aims to make a comparison of the findings for acute and chronic adrenaline and noradrenaline treatments together with proposals for future work.

SUMMARY OF AIMS

CHAPTER THREE:

To define the methods used in this thesis.

Firstly, to provide evidence that the pro-aggregatory response of rabbit platelets is mediated through activation of alpha$_2$ adrenoceptors. Secondly, to provide evidence that [^{3}H] yohimbine binding corresponds to alpha$_2$ adrenoceptor sites in rabbit platelet and kidney. Thirdly, to characterise the beta adrenoceptor subpopulations in rabbit platelets, lymphocytes, heart and lung.

CHAPTER FOUR:

To examine the effects of agonist infusions on platelet and vascular alpha$_2$ adrenoceptors.

CHAPTER FIVE:

Examination of the effects of chronic (10 day) adrenaline infusions on the function and number of adrenergic receptors.

CHAPTER SIX:

An examination of the effects of chronic (10 day) infusions of noradrenaline on adrenergic receptor function and number.

CHAPTER SEVEN:

An investigation to determine the time course of alpha$_2$ and beta adrenoceptor down regulation in response to adrenaline infusion.

CHAPTER EIGHT:

A general discussion/comparison of the effects of acute and chronic administration of the agonists studied on adrenoceptor function and number.

Proposals for future studies.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

Chapter Two

General Materials and Methods

The animal studies described in this thesis were carried out using male New Zealand White rabbits weighing 2 - 3 kg. Groups of six or more animals were used for all experiments.

2.1 Measurement of blood pressure and heart rate

A catheter was inserted into the central artery of the ear under local anaesthesia (2% lignocaine) for direct monitoring of mean arterial pressure (mmHg) via a Statham P231D transducer connected to a Grass polygraph recorder. The heart rate (beats/min.) was counted directly from the pressure trace. All animals were allowed to rest unrestrained for one hour prior to readings being taken.

2.2 Plasma catecholamines

Arterial blood samples (5 mls) were removed into lithium heparin tubes and plasma harvested after immediate centrifugation at 1700 g for five minutes at 4°C. Samples were stored at -70°C and assayed by a sensitive radioenzymatic assay (COMT)(Peuler and Johnson, 1977) with inter-assay coefficients of variation for adrenaline and noradrenaline of 13% and 10%.

2.3 Preparation of blood elements and tissues for adrenoceptor studies

2.3.1 Platelet preparation for aggregation studies

Whole blood was anticoagulated with sodium citrate (3.8% w/v, 1 volume to 9 volumes of blood). Platelet-rich plasma

(PRP) was prepared by centrifugation of blood at 150 g for 15 minutes at 20°C. An aliquot (10 mls) of PRP was aspirated into a polyethylene tube which was kept tightly capped at room temperature to minimise the air-platelet interface. Further centrifugaion of whole blood at 1700 g produced platelet poor plasma (PPP) which was used as a reference sample. Platelet aggregation was performed as described in Method 2.4.

2.3.2 Preparation of platelets for receptor binding studies

An aliquot (20 mls) of PRP was aspirated free from the red cells and centrifuged at 1700 g for 15 minutes at 4°C to produce a platelet pellet. This pellet was suspended in 0.1% EDTA, 150 mM NaCl Buffer, pH 7.4 to give a platelet concentration of 100×10^9/litre for $alpha_2$ adrenoceptor binding and 200×10^9/litre for beta adrenoceptor binding as determined by Coulter counting. If necessary, platelets were washed with the resuspension buffer to remove any retained agonist.

2.3.3 Preparation of lymphocyte membranes

After collection of PRP, the remaining red cells were used to isolate lymphocytes according to the method of Boyum (1968). The red cells were diluted with Hanks balanced salt solution and carefully layered onto a Ficoll/Hypaque solution (6%/10%) and the samples centrifuged at 400 g for 40 minutes at 20°C. The lymphocyte band was harvested by aspiration and a broken cell lysate prepared by a modification of the method of Aarons and Molinoff, (1982). The lymphocyte band was resuspended in 30 mM NaCl and centrifuged at 180 g for ten minutes at 4°C to remove remaining red blood cells by hypotonic lysis. The pellet was

resuspended in ice cold distilled water and centrifuged at 50,000 g for 45 minutes at 4°C. The final pellet was resuspended in 2 ml of ice cold assay buffer (150 mM NaCl with 12.5 mM $MgCl_2$, 1.5 mM EDTA buffered with 50 mM Tris HCl, pH 7.4) and stored at -70°C until assayed.

2.3.4 Preparation of kidney, heart and lung membranes

Tissues were chopped up finely in ice cold 0.32 M sucrose (40 mls), then homogenised using a Brinkman Polytron at setting 6 and centrifuged at 400 g for 15 minutes at 4°C. The pellets consisting of fibrous tissues, red cell and other high density debris were discarded and the supernatant recentrifuged twice at 50,000 g for 15 minutes at 4°C. The resulting membranes were washed in cold tris HCl 50 mM buffer both times and were finally resuspended in 50 mM Tris HCl buffer pH 7.5 and assayed at a wet weight concentration of 25 mg/ml (kidney) or stored at -70°C (heart and lung).

2.4 Measurement of platelet aggregation

Platelet aggregation was quantified by the standard turbidometric method of Born (1962) measuring the change in light transmission through the sample with time in a Payton dual-channel aggregometer. This instrument was set so that PRP gave 10% and PPP 90% light transmittance and performed at 37°C with a stir speed of 800 rpm.

Rabbit platelets fail to aggregate to adrenaline alone (Section 3.1.1) therefore the potentiation of the pro-aggregatory response to 1 uM adenosine diphosphate (ADP) was measured

according to the method of Grant and Scrutton (1980). Before additions of agonist were made, saline (50 ul) was added to the PRP (450 ul) in one channel and L-propranolol, 1 uM (50 ul) added into the other channel to eliminate any inhibitory beta adrenoceptor effects (Kerry and Scrutton, 1983).

In vitro additions (50 ul) were made of (-) adrenaline bitartrate (0.1 nM - 100 uM) followed by addition (50 ul) of adenosine diphosphate (1 uM) thirty seconds later. These aggregating agents and their dilutions were prepared from stock solutions (stored at -70°C) and dissolved in 0.9% saline with 1 mM ascorbic acid. The maximum change in optical density (max. O.D) was recorded in terms of light transmittance units per minute. The sigmoidal dose response relationship was fitted to a generalised model of the Hill equation by non linear least squares fitting procedure (Figure 2.1). Parameter values were obtained for Emax, the maximum rate of primary aggregation (cm) and C_{50}, the concentration of adrenaline required to produce 50% maximum aggregation (uM) and γ the slope of the dose response relationship:-

$$\text{Effect} = \frac{\text{Emax} \cdot (C/C_{50})\,\gamma}{1 + (C/C_{50})\,\gamma}$$

2.5 Alpha$_2$ adrenoceptor binding assay on whole platelets and kidney membranes

Aliquots of tissue suspension (0.8 ml) were incubated for 24 minutes at 25°C with eight concentrations of [^{3}H] yohimbine (90 Ci/mmol) (Jones et al, 1986) 1.2 - 25 nM in triplicate.

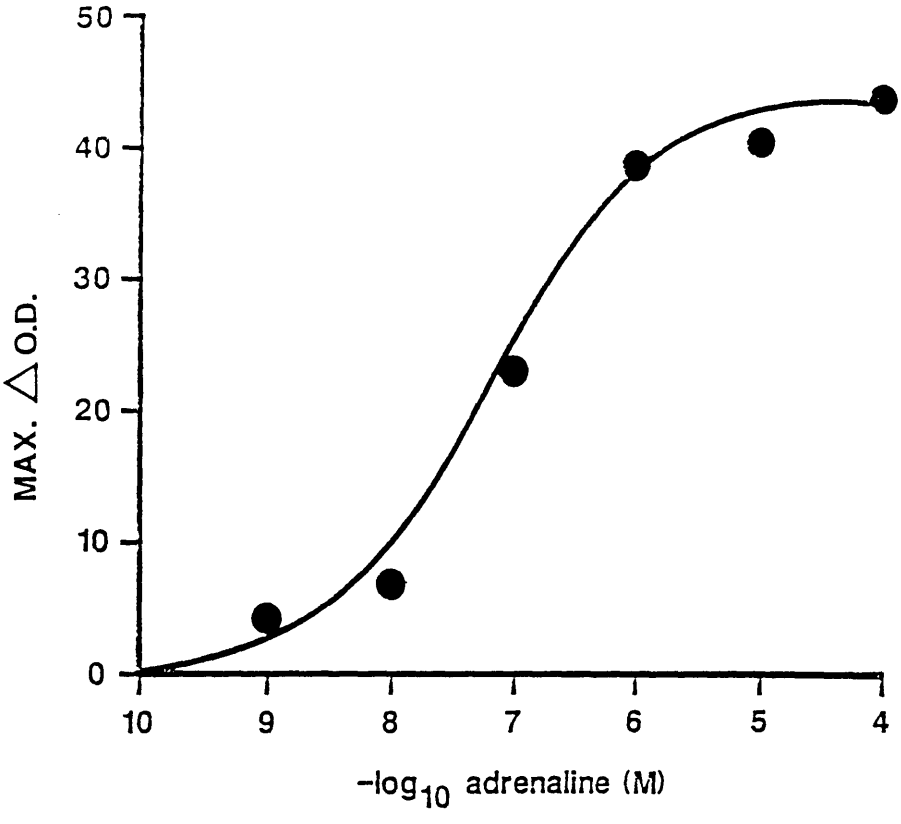


Figure 2.1

A typical dose response curve showing the pro-aggregatory response of rabbit platelets to adrenaline (10^{-4}-10^{-10}M).

The parameters derived from the Hill equation were:-

Emax (cm) = 44 ± 5

C_{50}(uM) = 0.3 ± 0.2

γ = 1.1 ± 0.6

The results shown here are for one rabbit.

Specific binding was defined as total minus non specific binding and was saturable over the range of concentrations used. The non specific binding was determined in the presence of 10 uM phentolamine.

Incubations were terminated by filtration with 20 ml of ice cold tris HCl (50 mM, pH 7.5) through Whatman GFC (platelets) or GFB (kidney) filters using a millipore multiport filtration apparatus (Alexander et al, 1978). The filters were dried overnight at room temperature and the bound radioactivity was determined by liquid scintillation counting at an efficiency of 36%. Saturation binding isotherms were analysed by plotting free/bound radioactivity vs free using least squares fitting to obtain values for the antagonist dissociation (K_D, nM) and the maximum number of binding sites (Bmax, fmoles/10^9 platelets or fmoles/mg protein). Specific binding of [^{3}H] yohimbine equilibrated by ten minutes and was stable for 40 minutes (Figure 2.2). The protein concentration was assessed calorimetrically using the method of Lowry (1951).

The intra assay and inter assay coefficients of variation for platelet [^{3}H] yohimbine binding (Bmax) were 4.4% and 14.8% respectively.

For analysis of ligand binding data in this thesis, Hane plots of Free/Bound vs Free were used in preference to plots of bound/free vs bound (Scatchard, 1949). Both plots linearise the binding isotherm but in Hane plots the variable with the greatest error (bound ligand) only contributes to the vertical coordinate. In the Scatchard plot the bound concentration affects the

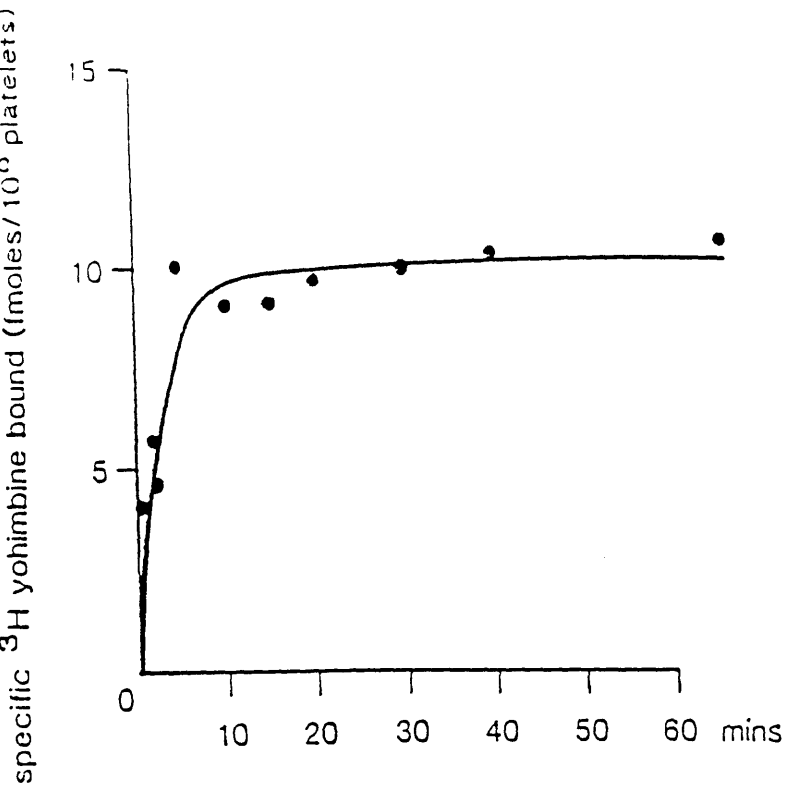


Figure 2.2

The time course for equilibration of specific [^{3}H] yohimbine binding.

Results were expressed as the mean for two experiments in duplicate.

vertical and the horizontal axes (Hamilton et al, 1984). Both plots are shown for comparison with the methods for deriving the parameters, Bmax and K_D from the regression analysis in Figure 2.3. The binding data from one sample of rabbit platelets is illustrated in Figure 2.4.

2.6 Beta adrenoceptor binding assay on whole platelets, lymphocyte, heart and lung membranes

Intact platelets were resuspended in 0.1% EDTA, 150 mM NaCl buffer, pH 7.4 and assayed on the day of preparation (Section 2.3.3) while lymphocyte heart and lung membranes were thawed and recentrifuged at 50,000 g for 15 minutes at 4°C in ice cold incubation buffer. Aliquots (100 ul) of lymphocytes, lung (15 - 30 ug protein), heart (30 - 50 ug) and platelets (200,000/ul) were incubated with eight concentrations of [^{125}I](-) Iodocyanopindolol (ICYP) (Amersham, U.K. 2200 Ci/mMol) 10 - 150 pM according to the method of Brodde et al (1982). Incubations were for 60 minutes at 25°C and terminated by addition of 10 ml of incubation buffer and vacuum filtration over Whatman GFB (lymphocytes, heart and lung) and GFC (platelets) glass fibre filters. The radioactivity of the wet filters was determined in a gamma counter (Berthold model LB2100) at an efficiency of 80%.

Non specific binding of [^{125}I] ICYP was defined as radioactivity bound to membranes which was not displaced by 1 uM L-Propranolol. Specific binding was defined as total radioactivity minus non specific binding and equilibrated by 60 minutes (Figure 2.5). The dissociation constant K_D (pM) and maximum number of binding sites Bmax (fmoles/mg protein,

ANALYSIS OF RADIOLIGAND BINDING DATA

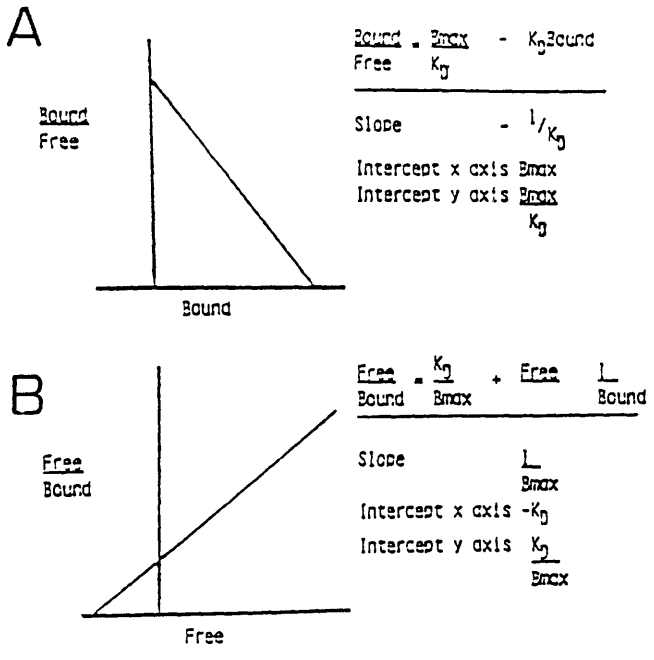


Figure 2.3

The classical scatchard analysis (A) and the Hane plot (B).

Bmax = The maximum number of binding sites or receptors

K_D = The equilibrium dissociation constant or the affinity of the ligand for the receptor

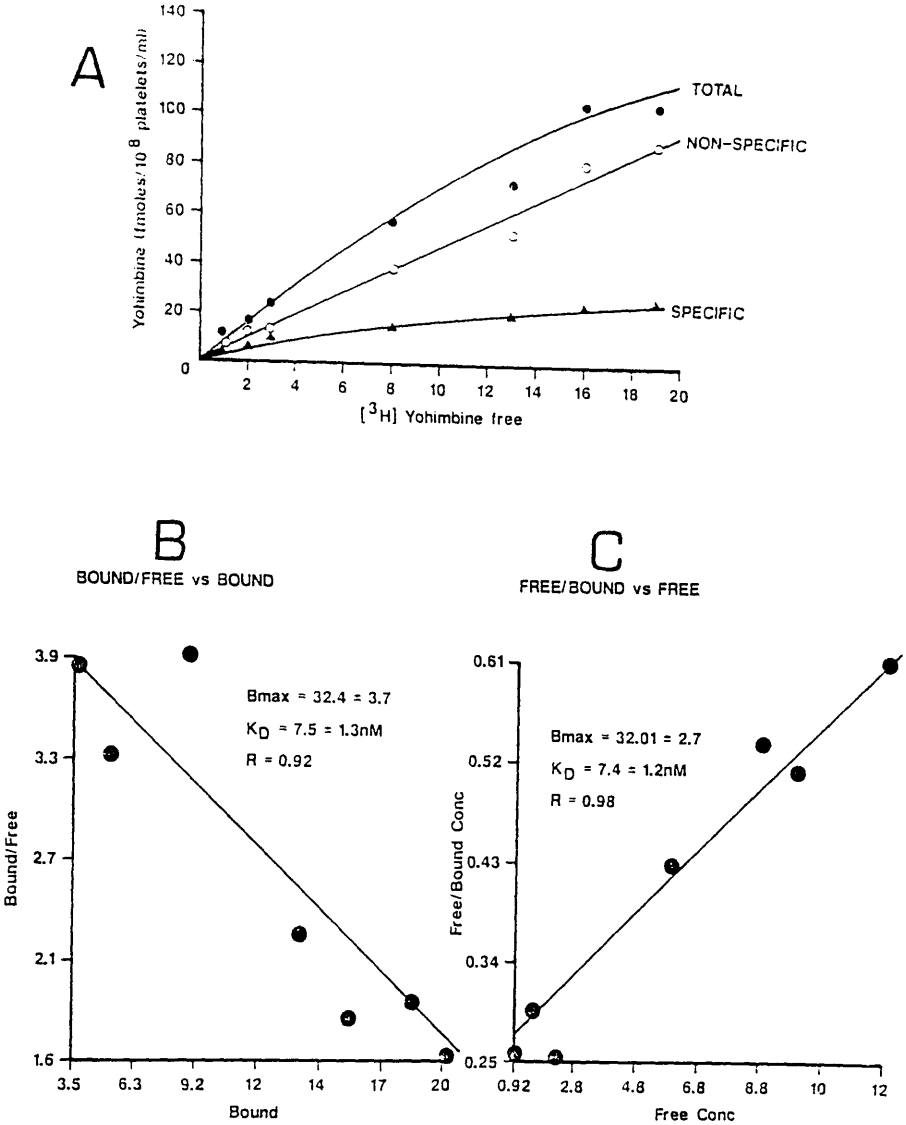


Figure 2.4

Scatchard analysis of [^{3}H] yohimbine binding to rabbit platelets.

A = The saturation binding isotherm
B = The classical scatchard plot
C = The Hane plot

Results shown here are for one rabbit.

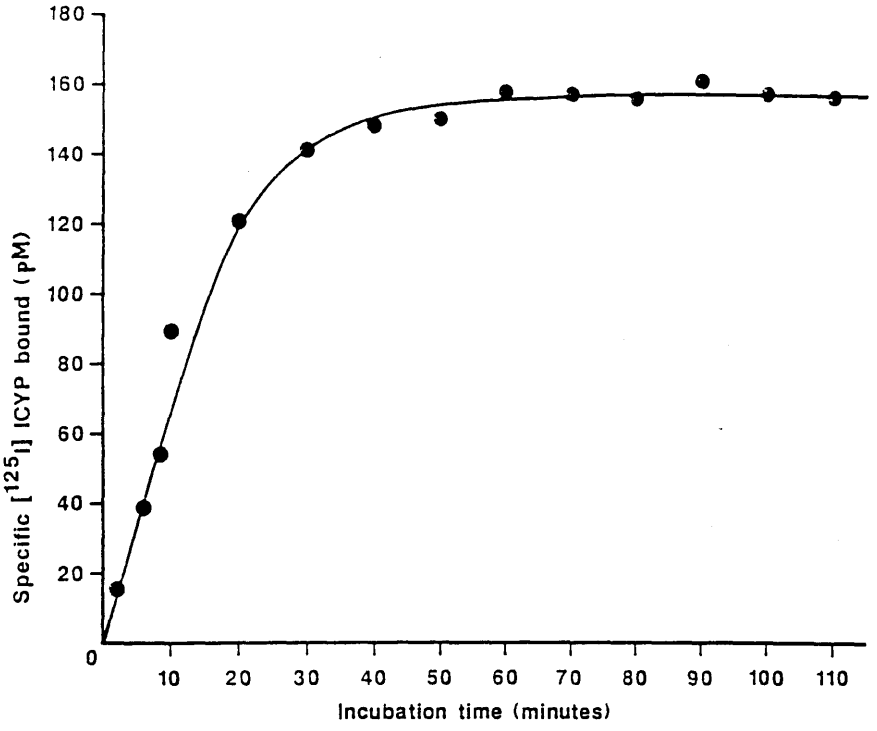


Figure 2.5

The time course for equilibration of specific [^{125}I] Iodocyanopindolol binding to heart membranes.

Results were expressed as the mean for two experiments in duplicate.

fmoles/10^9 platelets) were calculated from plots of free/bound vs free ICYP by least squares fitting of the regression analysis (Figure 2.6). Protein concentration was determined by the method of Lowry (1951).

2.7 Materials

This section covers the materials used in all the studies carried out in this thesis. New Zealand White rabbits were obtained from Cheshire rabbit farms in Tarporley. The radioactively labelled antagonists, [O-methyl-^{3}H] yohimbine, [^{3}H] - Dihydroalprenolol and [^{125}I] Iodocyanopindolol were supplied by Amersham International U.K. Ficoll/Hypaque solution (for lymphocyte isolation) was supplied by Pharmacia, Uppsala in Sweden and Hanks buffer from Gibco, Scotland. Alzet osmotic minipumps were obtained from Scientific Marketing, London. The following drugs were all purchased from Sigma : (-) adrenaline bitartrate, (+ noradrenaline HCL, phenylephrine HCL, isoprenaline HCL, alpha-methyl noradrenaline HCL, and adenosine 5'-diphosphate disodium salt. Phentolamine mersylate was obtained from Ciba-Geigy laboratories, Horsham, West Sussex, l-propranolol and metoprolol from ICI, Macclesfield, Cheshire. Most drugs were prepared in Tris HCl buffer (50 mM) pH 7.5 apart from catechol containing compounds which were dissolved in ascorbic acid (114 uM) to reduce oxidation of the catechol moiety.

2.8 Statistics

The majority of experiments were analysed using the Wilcoxon-Mann Whitney non-parametric test for unpaired data. This test is employed when the data do not conform to a normal

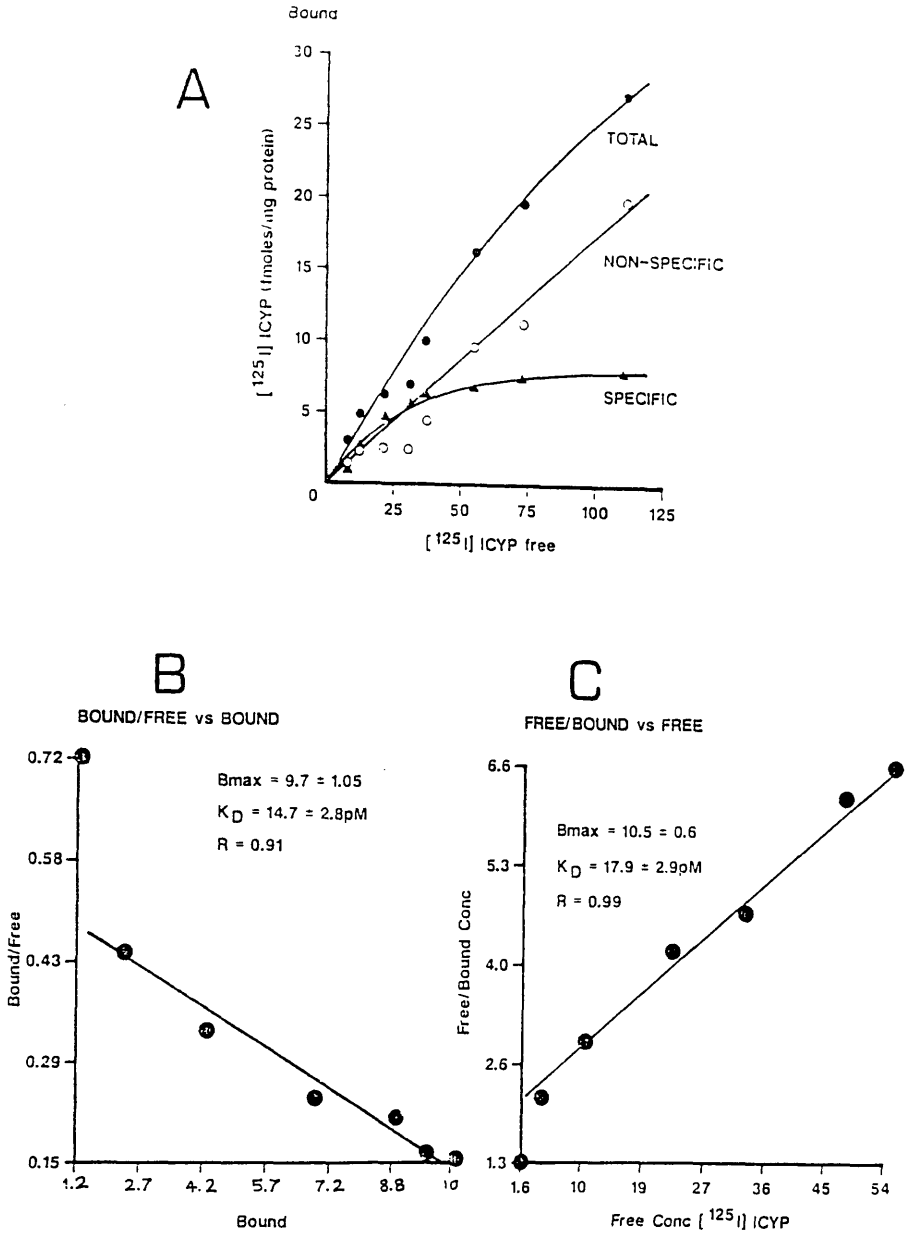


Figure 2.6

Scatchard analysis of [^{125}I](-) Iodocyanopindolol binding to rabbit heart membranes.

A = The saturation binding isotherm
B = The classical scatchard plot
C = The Hane plot

Results shown here are for one rabbit.

distribution.

Groups of six or more animals were used for all studies and results were expressed as the mean $\pm$ standard deviation. Alternative statistical tests have been carried out in chapter seven and are described therein.

CHAPTER THREE

SPECIFICITY OF PLATELET AGGREGATION AND RADIOLIGAND BINDING

Chapter Three

Specificity of Platelet Aggregation and Radioligand Binding

3.1 Evidence that rabbit platelet aggregation is mediated by alpha$_2$ adrenoceptors

3.1.1 Introduction

Marked species variations exist in the response of blood platelets to catecholamines (Grant and Scutton, 1980). Human platelets aggregate and secrete their contents when stimulated by adrenaline (O'Brien, 1963) while platelets from most other mammalian species fail to aggregate on exposure to adrenaline even in the presence of other excitatory agonists e.g. rat platelets (Yu and Latour, 1977). Dog and rabbit platelets however, exhibit a pro-aggregatory response to a sub-maximal concentration of an excitatory agonist such as adenosine 5' diphosphate (ADP), this response being potentiated by prior addition of adrenaline (Meyers et al, 1983; Drummond, 1976) (Figure 3.1).

Previous investigations using selective alpha adrenoceptor agonists and antagonists have demonstrated that the aggregatory response of human platelets, and the pro-aggregatory response of rabbit platelets to adrenaline are both mediated by alpha$_2$ adrenoceptors (Hsu et al, 1979; Grant and Scrutton, 1980). Beta adrenoceptors; mediating inhibition of platelet function by adrenaline are also present on human, rabbit and rat platelets (Mills and Smith, 1971; Yu and Latour, 1977). The current study aims to provide further evidence to confirm that rabbit platelet aggregation is mediated by alpha$_2$ adrenoceptors.

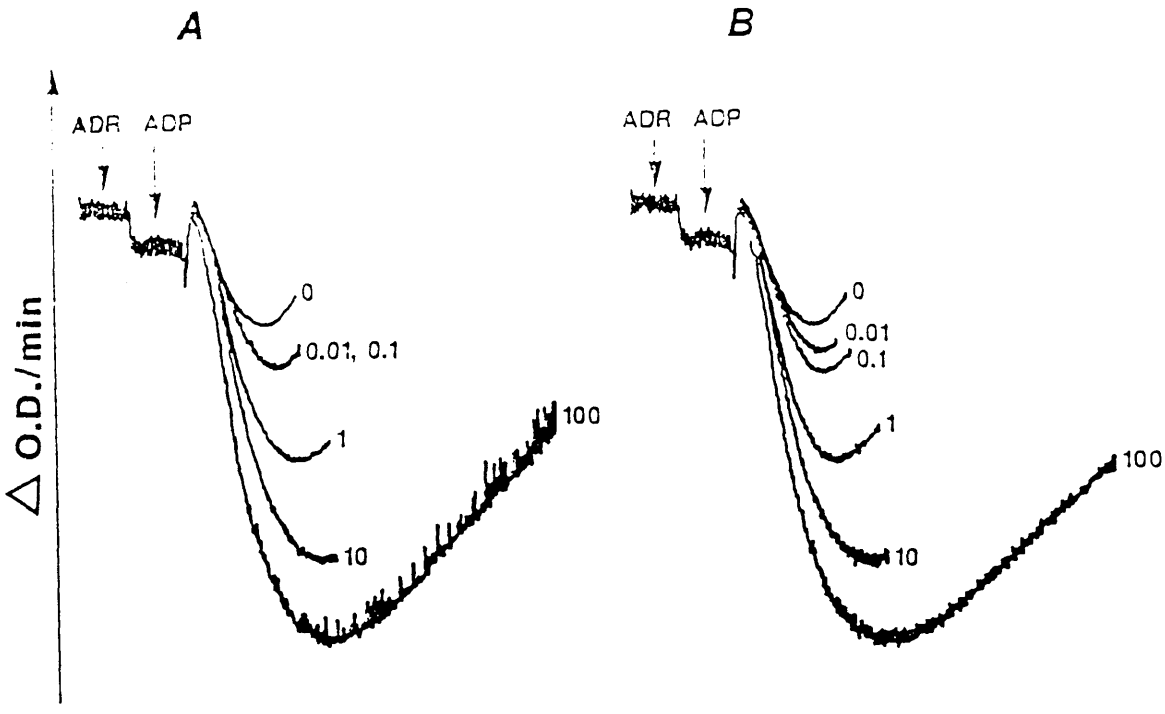


Figure 3.1

Tracings showing the potentiation by adrenaline (0.01 - 100 uM) of the pro-aggregatory response of rabbit platelets to ADP.

The maximum change in optical density was recorded in the presence and absence of propranolol (1 uM).

A	=	Absence of propranolol
B	=	Presence of propranolol
ADR	=	Adrenaline
ADP	=	Adenosine diphosphate

3.1.2 Methods

A group of four rabbits were used in these experiments. Platelets were prepared for aggregation studies as previously described (Section 2.3.1 and 2.4). Platelet rich plasma (450 ul) was added to the cuvette followed by 50 ul of propranolol to eliminate beta adrenoceptor effects. Then additions (50 ul) of the alpha adrenoceptor antagonists, idazoxan (0.1 - 10 uM), yohimbine (0.5 - 100 uM) and prazosin (100 and 500 uM) were made thirty seconds before the agonist, adrenaline (7.6 uM) was added (50 ul). Finally, additions (50 ul) of ADP (1 uM) were made and inhibition of the platelet pro-aggregatory responses to adrenaline was then recorded for each alpha adrenoceptor antagonist. The concentration of antagonist required to produce fifty percent inhibition or IC_{50} was then calculated.

The non-parametric Wilcoxon test was employed for analysis in this chapter (Section 2.7). All results are expressed as means $\pm$ standard deviation.

3.1.3 Results

A highly significant difference in IC_{50} (uM) was demonstrated for the $alpha_1$ antagonist, prazosin (> 750, $p < 0.001$), in comparison with the values for the $alpha_2$ antagonists, yohimbine (1.2 ± 1.7) and idazoxan (1.1 ± 1.4) which were not significantly different from each other.

3.1.4 Discussion

These results demonstrated that the $alpha_2$ antagonists, idazoxan (RX 781094) and yohimbine were more potent than the $alpha_1$ antagonist prazosin at inhibiting the platelet aggregatory

response to adrenaline in the rabbit. These findings were consistent with those of Grant and Scrutton (1980) who showed that the aggregatory responses to adrenaline and UK-14304 in rabbit platelets were blocked by yohimbine but not by prazosin or indoramin. Thus, the present data agree with previous findings that the pro-aggregatory response of rabbit platelets to adrenaline is mediated primarily by alpha$_2$ adrenoceptors.

3.2 Specificity of [^{3}H] Yohimbine binding to alpha$_2$ adrenoceptors in platelets and kidney

3.2.1 Introduction

Historically, the two antagonists most commonly used to discriminate between alpha adrenergic receptor subtypes have been the alpha$_2$ adrenoceptor antagonist, yohimbine and the alpha$_1$ adrenoceptor antagonist, prazosin. Yohimbine is approximately one hundred times more potent at human platelet alpha$_2$ adrenoceptors than prazosin (Hoffman, 1979a).

Extensive studies have reported [^{3}H] yohimbine binding to be saturable of high affinity for alpha$_2$ adrenoceptors with low non-specific binding (Langer, 1974; Bennett, 1978; Daiguji et al, 1981). The potency of non-radioactive compounds in competition with [^{3}H] yohimbine binding to intact platelets has been shown to be stereoselective as expected for alpha$_2$ adrenergic receptors with rank order - yohimbine > dihydroergocryptine > phentolamine > prazosin > propranolol (Motulsky et al, 1980).

To confirm the specificity of [^{3}H] yohimbine binding to alpha$_2$ adrenoceptors in rabbit platelets and kidney, displacement

assays were performed and the ability of various unlabelled antagonists to displace [^{3}H] yohimbine binding was examined.

3.2.2 Methods

Whole platelets and kidney membranes were prepared as described in methods 2.3.1, 2.3.2 and 2.3.4 (n = 3). Specific [^{3}H] yohimbine binding was then measured in the absence and presence of increasing concentrations (8 - 10) of alpha adrenoceptor antagonist drugs. The displacement curves were then fitted using non-linear least squares fitting to find the concentration of cold drug which reduced binding by 50% (IC_{50}) and the slope or Hill coefficient of the curve. These parameters was used to determine the K_I (nM) (a measure of affinity) according to the equation of Cheng and Prusoff (1973):

$$K_I = \frac{IC_{50}}{\frac{S}{K_D} + 1}$$

Where S is the concentration of [^{3}H] yohimbine in the assay (6.25 nM) and K_D is the equilibrium dissociation constant for [^{3}H] yohimbine binding obtained from saturation experiments.

3.2.3 Results

Specific binding of [^{3}H] yohimbine to rabbit platelets was consistent with binding to $alpha_2$ adrenoceptor sites since the K_I (nM) for the $alpha_1$ antagonist prazosin (4375 $\pm$ 1105) was substantially greater than the K_I for the selective $alpha_2$ antagonist, yohimbine (5.9 $\pm$ 0.7). Similarly, for rabbit kidney, the K_I for prazosin was considerably greater than the K_I for the

alpha$_2$ antagonists, yohimbine (13 ± 2) or phentolamine (106 ± 13). The displacement curves are illustrated in Figures 3.2 and 3.3 and the summary of results in Table 3.1.

Table 3.1

K_I (nM) values for α -adrenoceptor antagonists competing with [^{3}H] yohimbine

	Antagonist		
Tissue	Yohimbine	Phentolamine	Prazosin
Platelet	5.9 ± 0.7	-	4375 ± 1105
Kidney	13 ± 2	106 ± 13	< 5000

Results are expressed as mean ± S.D. for a group of three rabbits.

3.2.4 Discussion

The present results show that the alpha$_2$ antagonist, yohimbine was highly potent at displacing [^{3}H] yohimbine from its binding sites and confirm that [^{3}H) yohimbine is binding to alpha$_2$ adrenoceptors in rabbit platelets and kidney. The values were consistent with previous reports on the selectivity of [^{3}H] yohimbine binding (Motulsky et al, 1980; Daiguji et al, 1981).

3.3 Specificity of beta adrenoceptor binding

3.3.1 Introduction

Beta$_1$ and beta$_2$ adrenoceptors are not only organ-specifically distributed but both subtypes may co-exist in a single organ (Daly and Levy, 1979). Beta adrenoceptor antagonist

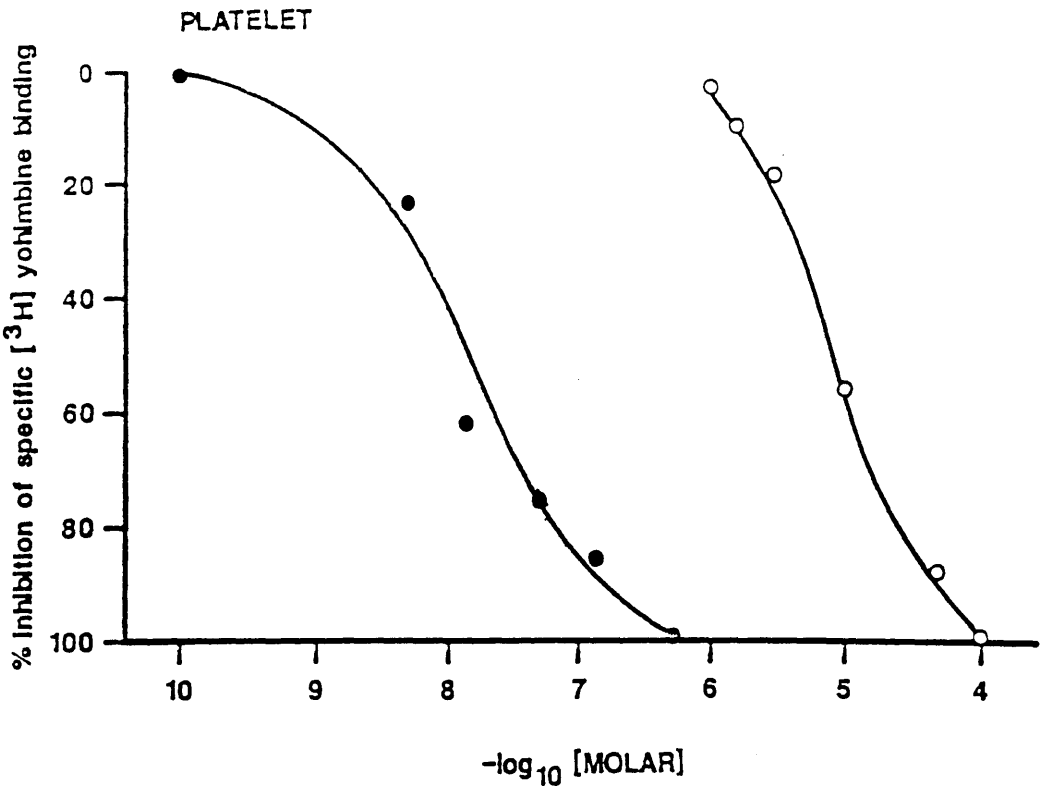


Figure 3.2

Displacement curves for alpha adrenergic antagonists competing for [^{3}H] yohimbine binding to rabbit platelets.

● Yohimbine

○ Prazosin

Results are the mean for three rabbits.

K_I (nM) values are shown in Table 3.1.

The Hill coefficients did not differ significantly from one.

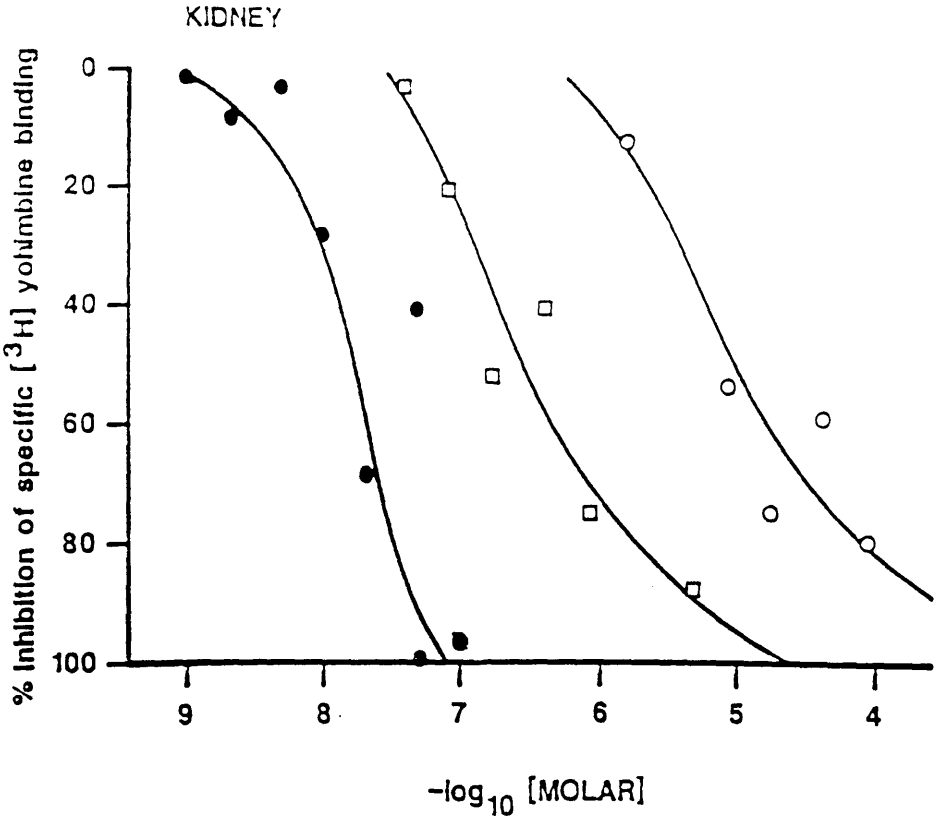


Figure 3.3

Displacement curves for alpha adrenergic antagonists competing with [^{3}H] Yohimbine binding to rabbit kidney.

● Yohimbine

□ Phentolamine

○ Prazosin

Results were expressed as the mean for three rabbits.

K_I (nM) values are shown in Table 3.1.

The Hill coefficients did not differ significantly from one.

drugs with different affinities for $beta_1$ and $beta_2$ adrenoceptors have been used to characterise the subtypes within tissues. The $beta_1$ selective antagonists, metoprolol, atenolol and practolol and the $beta_2$ selective antagonist, ICI 118551 have frequently been used to define beta adrenoceptor sub-populations (Jones et al, 1986; Brodde et al, 1983).

In the rabbit, platelets and lymphocytes have been shown to contain exclusively $beta_2$ adrenoceptors (Jones et al, 1985b; Hamilton et al, 1986) while those in the heart were predominantly $beta_1$ in type (Brodde et al, 1982). In contrast to most other species, the beta adrenoceptors in rabbit lung also contain a majority of $beta_1$ receptors (Rugg et al, 1978; Brodde et al, 1983).

This study aimed to substantiate these findings by carrying out displacement studies to characterise the sub-populations of beta adrenoceptors in the platelets, lymphocytes, heart and lung of the rabbit.

3.3.2 Methods

Platelets, lymphocytes, heart and lung tissues were prepared according to Methods 2.3.1, 2.3.2, 2.3.3 and 2.3.4 (n = 3). The beta adrenoceptor ligand [^{125}I] Iodocyanopindolol was then used to measure specific binding in the absence and presence of 10 - 15 doses of the $beta_2$ adrenoceptor antagonist, ICI 118551 and the $beta_1$ adrenoceptor antagonists, atenolol and metoprolol. Displacement curves were constructed and K_I (nM) values calculated where applicable as described in Section 3.2.2.

3.3.3 Results

Platelet and Lymphocyte

These studies suggest that rabbit platelets contain exclusively $beta_2$ adrenoceptors indicated by the low K_I value for the $beta_2$ adrenoceptor selective ICI 118551 (151 $\pm$ 67 nM) and the higher value the $beta_1$ selective atenolol (5843 $\pm$ 633 nM). Rabbit lymphocytes were also shown to contain largely $beta_2$ adrenoceptors, K_I values were 0.98 $\pm$ 0.8 nM for ICI 118551 and 5707 $\pm$ 769 nM for atenolol. The results are illustrated as displacement curves in Figures 3.4 and 3.5.

3.3.4 Heart and Lung

In the rabbit heart and lung calculation of K_I values for metoprolol and ICI 118551 was not valid due to the shallow displacement curves for the inhibition of [^{125}I] ICYP binding by metoprolol and ICI 118551 indicating the presence of more than one subtype of beta adrenoceptor in these tissues. However, there were rightward shifts in the displacement curves for inhibition of [^{125}I] ICYP binding by ICI 118551 compared to the inhibition curves for metoprolol in the heart (Figure 3.6). In this tissue, IC_{50} values were 2×10^{-11}M for metoprolol compared to 8×10^{-7}M for ICI 118551. In the rabbit lung, corresponding IC_{50} values were 4×10^{-7}M for metoprolol in comparison to 1×10^{-6}M for ICI 118551 (Figure 3.7). Therefore these results demonstrated that the $beta_1$ adrenoceptor selective antagonist metoprolol was more potent than the $beta_2$ selective antagonist, ICI 118551 in displacing [^{125}I] ICYP from beta adrenoceptor binding sites in rabbit heart and lung.

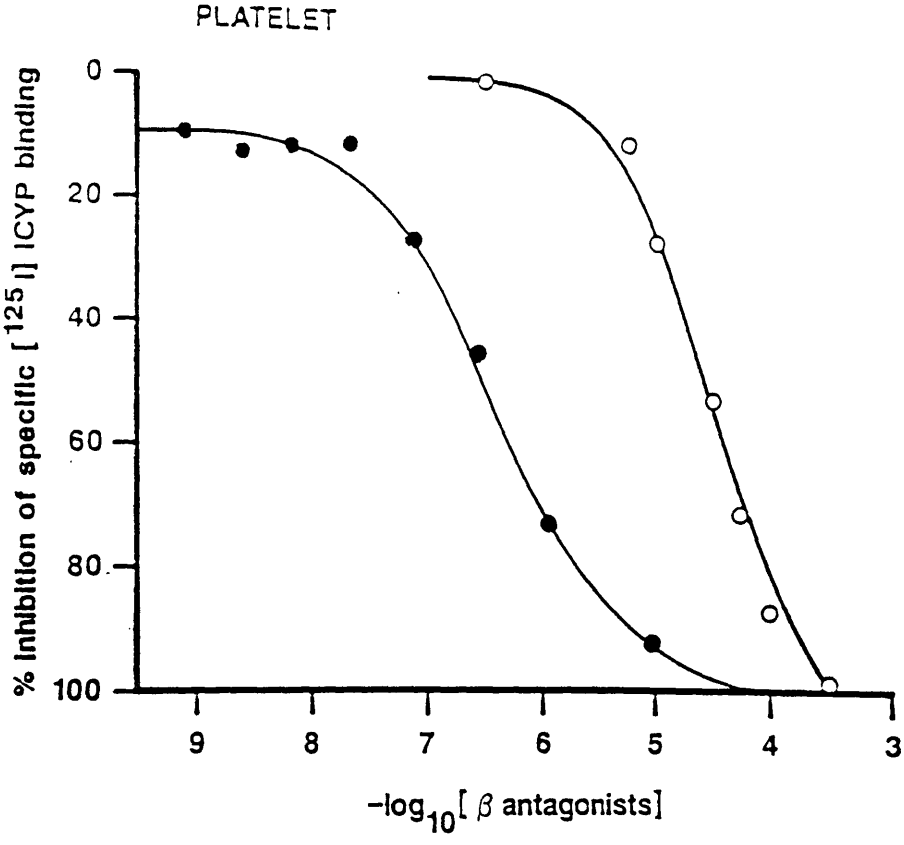


Figure 3.4

Displacement curves for beta adrenergic antagonists competing with [^{125}I] ICYP binding to rabbit platelets.

● ICI 118551

○ Atenolol

Results are expressed as the mean for three rabbits.

K_I values are shown in Table 3.2.

The Hill coefficients did not differ significantly from one.

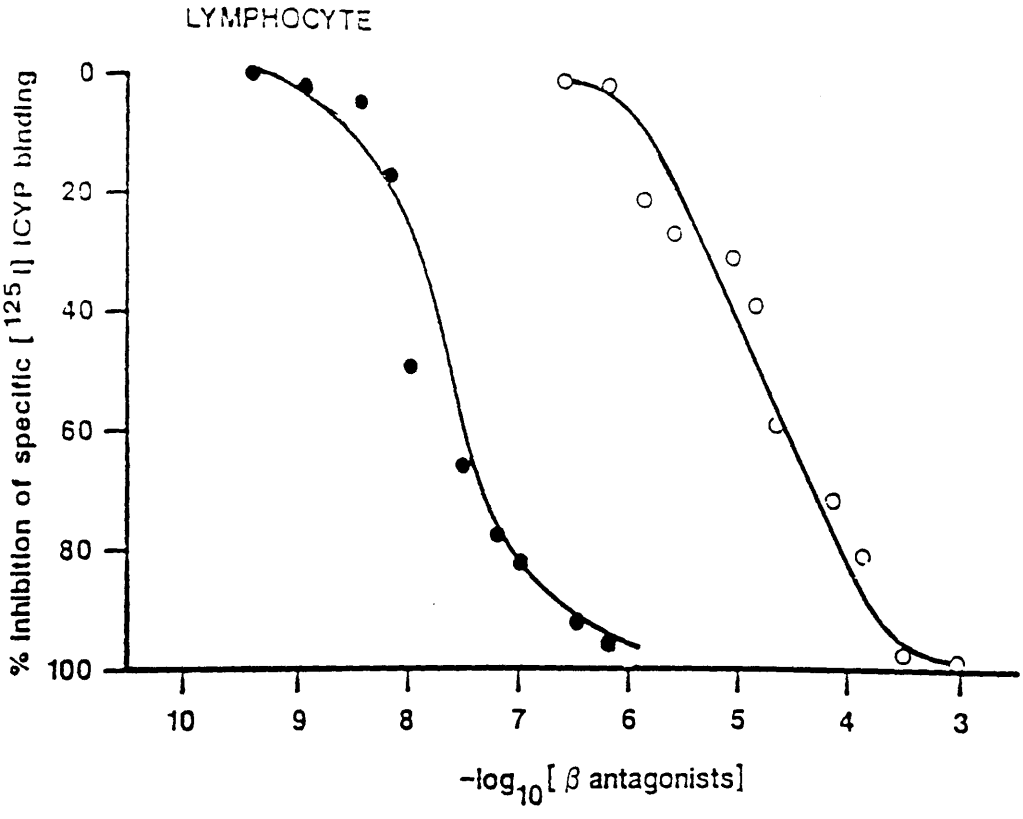


Figure 3.5

Displacement curves for beta adrenergic antagonists competing with [^{125}I] ICYP binding to rabbit lymphocyte membranes.

● ICI 118551

○ Atenolol

Results are expressed as the mean for three rabbits.

K_I values are shown in Table 3.2.

The Hill coefficients did not differ significantly from one.

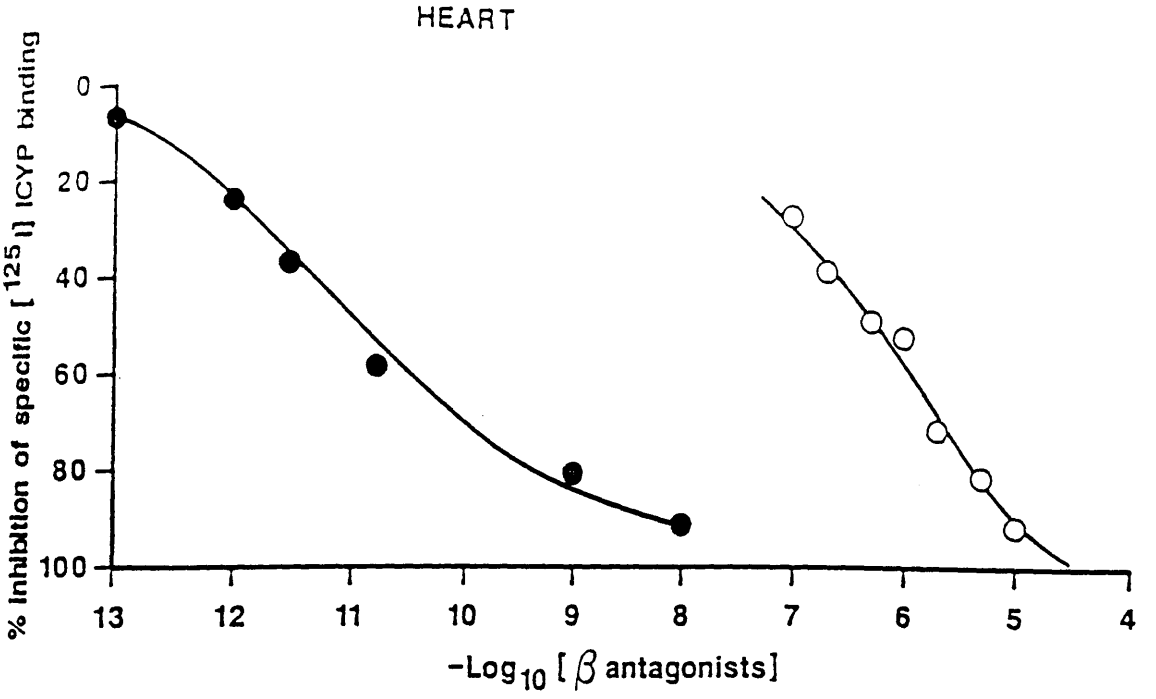


Figure 3.6

Displacement curves for beta adrenergic antagonists competing with [^{125}I] ICYP binding to rabbit heart membranes.

● Metoprolol

○ ICI 118551

Results are expressed as the mean for three rabbits.

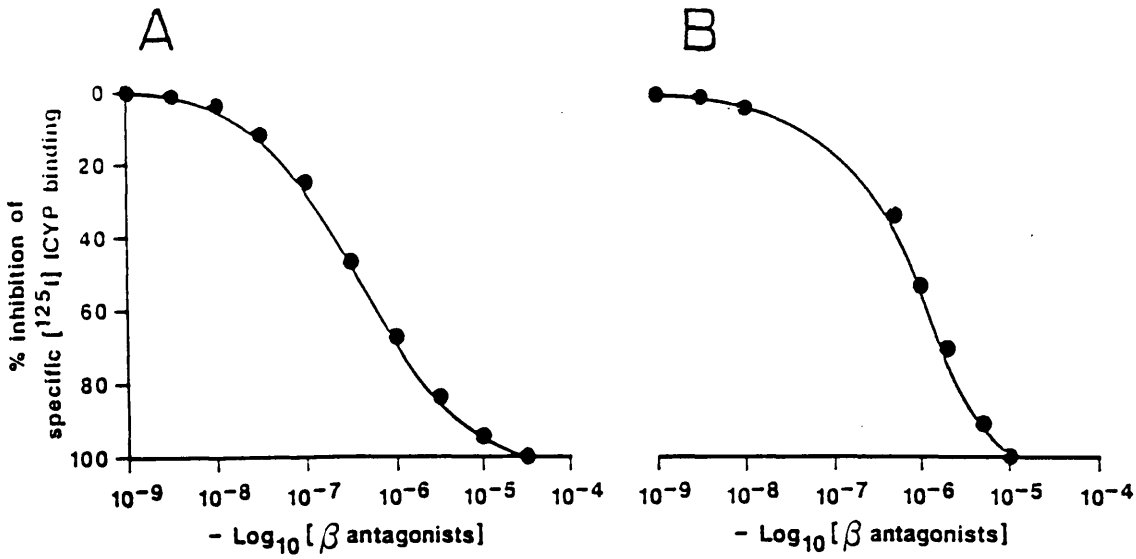


Figure 3.7

Displacement curves for beta adrenergic antagonists competing with [^{125}I] ICYP binding to rabbit lung membranes.

A Metoprolol

B ICI 118551

Each point indicates the mean for three rabbits.

3.3.5 Discussion

The present findings were consistent with others (Hamilton et al, 1986a; Jones et al, 1985b) showing that rabbit platelets and lymphocytes harbour a homogeneous population of $beta_2$ adrenoceptors.

The data for rabbit heart suggests a predominance of the $beta_1$ adrenoceptor in this tissue which would be consistent with previous investigations (Brodde et al, 1981b; Jones et al, 1985b). These earlier studies also revealed that the highest number of $beta_2$ receptors were localised in the right atrium while the ventricles contained nearly exclusively $beta_1$ adrenoceptors. Beta adrenoceptors in the human heart are also shown to be largely $beta_1$ in type (Brodde et al, 1986).

The higher potency of metoprolol for the beta adrenoceptors in rabbit lung suggests that there may be a higher proportion of $beta_1$ adrenoceptors within this tissue. Although, Hoftsee analysis was not employed in the present studies to determine the proportions of $beta_1$ and $beta_2$ adrenoceptor sub-types in rabbit lung, previous studies have confirmed a preponderance of $beta_1$ receptors in this tissue. Indeed, Rugg et al (1978) demonstrated that rabbit lung contained approximately sixty percent $beta_1$ adrenoceptors and forty percent $beta_2$ receptors. Further evidence was provided by Brodde et al (1983) who found a ratio of 80% $beta_1$: 20% $beta_2$ receptors in lung membranes of the rabbit.

Interestingly, these relative proportions are quite the inverse of most other mammalian species including humans (Engel et al, 1981) which show a predominance of $beta_2$ adrenoceptor in the lung.

CHAPTER FOUR

CHANGES IN ADRENOCEPTOR FUNCTION AND NUMBER DURING ACUTE AGONIST INFUSION

Chapter Four

Changes in Adrenoceptor Function and number during Acute Agonist Infusion

4.1 Introduction

Agonist induced attenuation of physiological responsiveness or desensitisation may or may not be accompanied by a decrement in adrenoceptor number. Long term agonist stimulation has been associated with decreased responsiveness concurrent with a decrease in adrenergic receptor density (Mickey et al, 1975; Scarpace and Abrass, 1982) while short term manipulations of plasma agonist concentrations are shown to desensitise adrenergic responses without degradation of receptor protein (Harden et al, 1979; Strasser et al, 1985).

Acute elevations of circulating plasma catecholamines occur in physiological settings such as posture change, exercise and stress (Section 1.8). Alterations in beta adrenoceptor response without changes in total receptor density have been recorded in these situations (Sowers et al, 1983; De Blasi et al, 1986). Also, acute isoprenaline infusions in man led to a fall in isoprenaline dependent cyclic AMP production in lymphocytes at forty and sixty minutes of infusion without significant changes in receptor number (Krall et al, 1980). This early desensitisation of beta adrenoceptors was shown to involve a rapid uncoupling of the beta receptor from adenylate cyclase which took place within 2-3 minutes (Staehelin and Simons, 1982; Hertel et al, 1983). Although there have been many reports concerning the acute desensitisation of beta adrenoceptors

(linked to stimulation of adenylate cyclase) fewer studies have focused on the short term agonist regulation of alpha$_2$ adrenoceptors (linked to inhibition of adenylate cyclase). A study by Jones et al (1986) revealed that acute agonists infusions (60-120 minutes) in man could be correlated with an attenuation of the platelet aggregatory response to adrenaline and a reduction in the ability of adrenaline to reduce cyclic AMP levels. These workers also found a lack of change in the number of [^{3}H] yohimbine binding sites in platelets. This agonist induced attenuation of platelet aggregatory responses to adrenaline has been observed by several investigators (O'Brien, 1964; Cooper et al, 1978; Hollister et al, 1983) although the mechanisms remain unknown.

The following studies address the short term agonist regulation of vascular and platelet alpha$_2$ adrenoceptors in the rabbit. Alpha$_2$ receptors are located postsynaptically as well as presynaptically on vascular smooth muscle (Drew and Whiting, 1979; Hamilton and Reid, 1980). Changes in blood pressure during alpha$_2$ adrenoceptor agonist infusion are mediated through these receptors (Hamilton and Reid, 1980). Alpha$_2$ adrenergic receptors are also present on the cell surface of platelets and function to mediate the aggregatory response to adrenaline (Grant and Scrutton, 1979). Vascular and platelet aggregatory responses have been used to investigate the effects of three different agonists on adrenergic receptor function. The first agonist to be studied was alpha methylnoradrenaline which is selective for alpha$_2$ adrenoceptors and possesses very weak alpha$_1$ and beta adrenoceptor activity. This agonist was chosen in preference to

clonidine which is a partial agonist at the $alpha_2$ receptor and acts on central $alpha_2$ receptors as well as peripheral $alpha_1$ and $alpha_2$ adrenoceptors. The second series of experiments examines the effects of acute infusion of the endogenous non-selective adrenoceptor agonist, adrenaline and compares adrenoceptor responses with those resulting from acute alpha methylnoradrenaline infusion. The third agonist tested was noradrenaline using the same dose as that used in the long term infusions (Chapters five and six). A summary of the experimental protocol is shown in Figure 4.1.

4.2 Alpha methylnoradrenaline infusions

In vivo studies - Methods

The following experiments used groups of 6-10 rabbits. One arterial and two venous catheters were inserted into vessels of the ears under local anaesthesia (2% lignocaine). The mean arterial blood pressure (mmHg) and heart rate (beats/min) were monitored via an arterial line as described in Section 2.1. One venous catheter was used for administration of drugs to permit continuous infusion using a Perfusor infusion pump, while bolus injections were given by the second catheter. Rabbits were left unrestrained for one hour prior to all recordings.

4.2.1 Specificity of vascular responses to αmethyl noradrenaline and phenylephrine during alpha adrenoceptor agonist infusion

In the conscious rabbit, phenylephrine and alpha methylnoradrenaline show $alpha_1$ and $alpha_2$ adrenoceptor

AGONIST INFUSION

MEASUREMENT	α METHYLNORADRENALINE 0.5 μmol/kg/hr	α METHYLNORADRENALINE 2.5 μmol/kg/hr	ADRENALINE 0.05 μmol/kg/hr	ADRENALINE 1.5 μmol/kg/hr	NORADRENALINE 0.09 μmol/kg/hr
VASCULAR PRESSOR RESPONSES TO ALPHA ADRENOCEPTOR AGONISTS	✓	✓	✓	✓	✓
PLATELET PRO--AGGREGATORY RESPONSES TO ADRENALINE	✓	✓	—	✓	—
[^{3}H] YOHIMBINE BINDING TO PLATELETS	—	✓	—	—	—

Figure 4.1

Experimental protocol for the acute agonist infusions

selectivity respectively (Hannah et al, 1984).

In order to examine the effects of alpha methylnoradrenaline and phenylephrine infusions on $alpha_1$ and $alpha_2$ adrenoceptors, responses to bolus doses of phenylephrine and alpha methylnoradrenaline were examined before and during infusion of the drugs.

The bolus dose of phenylephrine (10 ug/kg) or alpha methylnoradrenaline (3 ug/kg) was selected after pilot experiments designed to find the dose that evoked a rise in the mean arterial pressure of 25-30 mmHg. Three consistent measurements of the acute pressor response were obtained and then phenylephrine (1.8 umol/kg/hr) or alpha methylnoradrenaline (2.5 umol/kg/hr) was infused through the other venous catheter. Ten minutes into this infusion, a further intravenous bolus dose of phenylephrine or alpha methylnoradrenaline was administered and the rise in mean arterial pressure recorded. Four sets of rabbits were studied with six in each group as outlined below:-

1. Alpha methylnoradrenaline infusion + alpha methylnoradrenaline bolus injection.
2. Alpha methylnoradrenaline infusion + phenylephrine bolus injection.
3. Phenylephrine infusion + alpha methylnoradrenaline bolus injection.
4. Phenylephrine infusion + phenylephrine bolus injection.

Values for mean arterial pressure and heart rate were obtained at all times examined.

In a separate group of animals, changes in blood pressure

and heart rate were monitored before commencing and after five minutes infusion of each of the alpha$_2$ agonists.

4.2.2 Time and dose effects

Alpha methylnoradrenaline, 0.5 umol/kg/hr or 2.5 umol/kg/hr was infused intravenously into groups of rabbits and the pressor responses to further intravenous doses of alpha methylnoradrenaline (3 ug/kg) examined before commencing and after 2.5, 5 and 10 minutes of infusion.

4.2.3 In vitro studies - Platelet aggregation

In conjunction with the in vivo studies, experiments were carried out to examine the effects of alpha methylnoradrenaline infusion on the platelet pro-aggregatory responses to adrenaline. Groups of rabbits (6-8) received intravenous infusions of alpha methylnoradrenaline (0.5 umol/kg/hr or 2.5 umol/kg/hr) via a Perfussor infusion pump and blood (10 mls) was withdrawn from an arterial line before infusion and after 2.5, 5 or 10 minutes infusion with 2.5 umol/kg/hr alpha methylnoradrenaline or after ten or 30 minutes infusion with 0.5 umol/kg/hr. Another set of rabbits were given ten minute phenylephrine (1.8 umol/kg/hr) infusions and 10 mls of blood removed at the end of infusion.

Platelets were prepared and aggregation experiments performed according to Methods 2.3.1 and 2.4. Additions (50 ul) of propranolol (1 uM) were made to all cuvettes to eliminate any beta adrenoceptor effects. Hill plot analysis was also carried out to obtain estimates for the parameters Emax (the maximum change in optical density, cm) and C_{50} (the concentration of adrenaline required to produce 50% response).

4.2.4 Measurement of plasma alpha methyladrenaline levels

Blood samples (2.5 mls) were withdrawn into ice cold heparanised tubes and plasma harvested following centrifugation (1700 g) at 4°C for five minutes. This plasma was then stored at -70°C until measurement of alpha methylnoradrenaline levels was made by high pressure liquid chromatography (H.P.L.C.) (Howes et al, 1985).

Plasma alpha methylnoradrenaline levels were measured by reverse phase H.P.L.C. The method was modified from that of Jenner et al (1981). Plasma (1 ml) was mixed for 30 minutes with 1 M Tris (1 ml) containing 2% EDTA. 20 mg of alumina and 4 ng dihydroxylbenzylamine as the internal standard. The alumina was washed with 10 mls of water and alpha methylnoradrenaline eluted with 130 ul of 0.2 M perchloric acid. The sample (120 ul) was injected onto a 25 cm x 4.5 mm column lane packed with 5 micron octadecylsilane. The mobile phase consisted of 70 mM potassium dihydrogen phosphate, 3 mM octanesulphonate and 0.4 mM EDTA pH 2.2. The flow rate was 1 ml/min and the working potential +0.7 V. The retention time for alpha methylnoradrenaline was 17.5 minutes.

4.2.5 [^{3}H] Yohimbine binding to platelets

Arterial blood samples (15 mls) were withdrawn from six rabbits before and at the end of ten minutes intravenous infusion of alpha methylnoradrenaline. Intact platelets were prepared (Section 2.3.1) and [^{3}H] yohimbine binding to rabbit platelets was assessed as previously described in Sections 2.3.2 and 2.5.

Statistical analysis was performed using the non-parametric Wilcoxon test (Section 2.8) and all results expressed as means ± standard deviation.

4.2.6 Responses to alpha methylnoradrenaline during acute adrenaline infusion

Rabbits were prepared for in vivo studies as described in Section 4.2 (n = 8). Pressor responses to alpha methylnoradrenaline (3 ug/kg) were recorded before and at the end of 2.5 and ten minutes of a ten minute adrenaline (1.5 umol/kg/hr) infusion.

Vascular responses were also measured using a lower infusion rate of adrenaline. Pressor responses to bolus injections of alpha methylnoradrenaline (3 ug/kg) were recorded at the end of 2.5, 10, 30 and 60 minutes of a 2-3 hour intravenous infusion of adrenaline (0.05 umol/kg/hr) Control animals received 2-3 hour infusions of the vehicle, 0.1% ascorbate and pressor responses to alpha methylnoradrenaline were recorded as described for the treated animals. All rabbits were allowed to recover to baseline blood pressure and heart rate between bolus injections. Arterial blood samples (2.5 mls) were withdrawn at the end of ten minutes adrenaline (1.5 umol/kg/hr) infusion and forty-five mintues adrenaline (0.05 umol/kg/hr). Plasma adrenaline concentrations were then assessed according to Method 2.2. Blood pressure and heart rate changes throughout the agonist infusions were also examined and a comparison made with long term agonist infusions at the same dose.

Platelet aggregation

Eight rabbits were treated with ten minute adrenaline (1.5 umol/kg/hr) infusions and arterial blood samples (12 mls) removed before and at the end of each infusion. Platelet rich plasma was then prepared according to Section 4.2.3 and pro-aggregatory responses to adrenaline measured in the presence of propranolol (1 uM).

4.2.7 Responses to alpha methylnoradrenaline during noradrenaline infusions

Animals were prepared for infusions as described in section 4.2. Pressor responses to bolus injections of alpha methylnoradrenaline (3 ug/kg) were recorded following 2.5, 10, 30 and 60 minutes of a 2-3 hour intravenous infusion of noradrenaline (0.09 umol/kg/hr). Similar pressor responses were measured in control animals which received intravenous ascorbate (0.1%) for 2-3 hours. Blood was removed for measurement of plasma noradrenaline levels as described in method 2.2.

4.3 Results

4.3.1 Alpha methylnoradrenaline infusions

Effect of infusion of phenylephrine and α methylnoradrenaline on the mean arterial pressure and heart rate

There were similar significant blood pressure rises of approximately 25 mmHg after the five minute infusions of phenylephrine (1.8 umol/kg/hr) and alpha methylnoradrenaline

(2.5 umol/kg/hr). No significant alterations in the heart rate were detected after five minute infusions of either agonist (Table 4.1). The lower dose of alpha methylnoradrenaline caused a rise of 8 ± 4 mmHg in the mean arterial pressure.

Table 4.1

Haemodynamic changes after five minute infusions of phenylephrine and alpha methylnoradrenaline

	Phenylephrine (1.8 umol/kg/hr)		α-Methylnoradrenaline (2.5 umol/kg/hr)	
	Baseline	5 min	Baseline	5 min
Mean arterial pressure (mmHg)	85 ± 11	106* ± 10.8	75 ± 10	*100 ± 13
Heart rate (beats/min)	215 ± 31	155 ± 37	218 ± 28	189 ± 54

Results are expressed as mean ± S.D. for six rabbits.

* $P < 0.01$

Effect of alpha methylnoradrenaline infusions on the pressor responses to alpha adrenoceptor agonists

A significant ($P < 0.01$) attenuation of the pressor response to alpha methylnoradrenaline during an alpha methylnoradrenaline infusion was observed when compared to pre-infusion values.

However, there were no changes in the phenylephrine responses after ten minutes' administration of alpha methylnoradrenaline, nor were there any changes in the alpha methylnoradrenaline responses, before and during phenylephrine infusion despite the rise (25 mmHg) in blood pressure

(Table 4.2).

4.3.2 Time and dose effects of alpha methylnoradrenaline infusion

The pressor responses evoked by alpha methylnoradrenaline (2.5, 5 and 10 minute) during alpha methylnoradrenaline infusion (2.5 umol/kg/hr) were all significantly reduced ($P < 0.001$) in comparison with baseline values. The maximum attenuation of 52% was achieved with 2.5 minutes of infusion (27 $\pm$ 2.8, baseline compared to 12.6 $\pm$ 1.9, treated). The baseline pressor responses did not differ significantly between groups (23.7 $\pm$ 12.6 compared to 28.6 $\pm$ 4.9 compared to 28.6 $\pm$ 4.9 mmHg for 2.5, 5 and 10 minutes respectively) and were therefore combined for clarity (Figure 4.2).

These decreases in vascular responsiveness were dose related as responses to alpha methylnoradrenaline (0.5 umol/kg/hr) were also significantly reduced ($P < 0.01$) although to a lesser extent (28%). There were no alterations in alpha methylnoradrenaline responses during phenylephrine infusions (Figure 4.2).

4.3.3 Plasma alpha methylnoradrenaline levels

Circulating plasma concentrations of alpha methylnoradrenaline (2.5 umol/kg/hr) reached steady state levels by 2.5 minutes of alpha methylnoradrenaline infusion (395 $\pm$ 74 nM). Similarly, plasma concentrations of alpha methylnoradrenaline (0.5 umol/kg/hr) attained steady state levels by five minutes of infusion (118 $\pm$ 40) (Table 4.3).

Table 4.2

Pressor responses to IV bolus doses of alpha adrenoceptor agonists during alpha adrenoceptor agonist infusion

Infusion	Phenylephrine pressor response (mmHg)		α methylnoradrenaline pressor response (mmHg)	
	Pre-infusion	During infusion	Pre-infusion	During infusion
α methylnoradrenaline 2.5 umol/kg/hr	28 ± 4	29 ± 6	30 ± 5	17 ± 2*
Phenylephrine 1.8 umol/kg/hr	29 ± 5	31 ± 5	28 ± 5	31 ± 1

Results are expressed as mean ± S.D. for groups of 6 rabbits.

The second pressor response was measured after 10 minutes infusion.

* $P < 0.01$ when compared to pre-infusion response

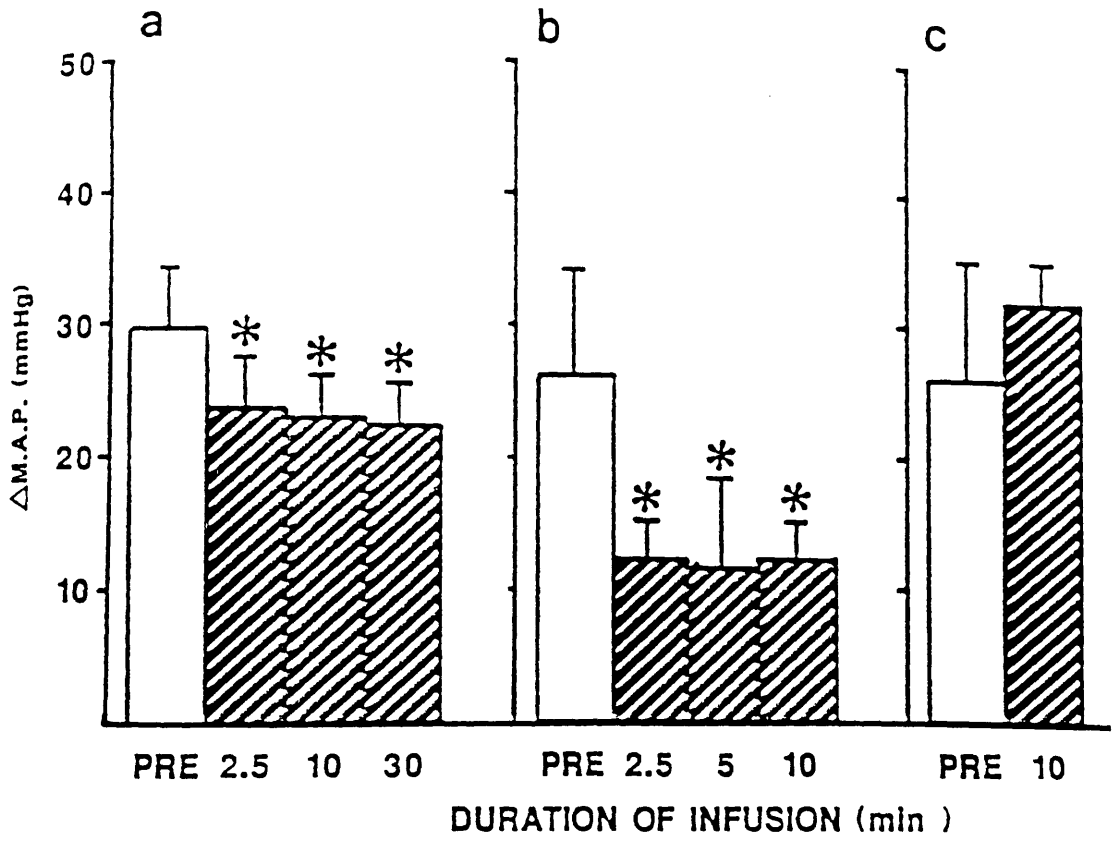


Figure 4.2

Pressor responses to alpha methylnoradrenaline during alpha adrenoceptor agonist infusions

a. Alpha methylnoradrenaline infusion, 0.5 umol/kg/hr.

b. Alpha methylnoradrenaline infusion, 2.5 umol/kg/hr.

c. Phenylephrine infusion, 1.8 umol/kg/hr.

□ Response before infusion

▨ Response during infusion

* $P < 0.01$

Results were expressed as the mean ± standard deviation for groups of six animals.

Table 4.3

Plasma alpha methylnoradrenaline levels during alpha methylnoradrenaline infusion

Time (min)	Plasma alpha methylnoradrenaline concentrations (nM)	
	0.5 umol/kg/hr	2.5 umol/kg/hr
2.5	96 ± 9	395 ± 74
5	118 ± 40	374 ± 73
10	125 ± 34	322 ± 76
15	-	391 ± 94
30	122 ± 24	-

Results are expressed as the mean ± standard deviation for groups of five rabbits.

- = not measured.

4.3.4 The effect of alpha methylnoradrenaline infusions on platelet aggregatory responses to adrenaline

The maximum aggregatory response (Emax, cm) to adrenaline was significantly reduced ($P < 0.001$) after five and ten minute infusions of alpha methylnoradrenaline (2.5 umol/kg/hr). C_{50} (the dose of adrenaline required to produce a half maximal response) values (nM) were found to be significantly increased ($P < 0.001$) following 2.5, 5 and 10 minute alpha methylnoradrenaline infusion. These results are shown in Table 4.4 and Figure 4.3. The lower infusion rate of alpha methylnoradrenaline (0.5 umol/kg/hr) only produced a significant

Table 4.4

The effects of short term alpha adrenoceptor agonist infusion on platelet aggregation

Agonist	Time of Infusion (min)	Emax (△O.D., cm)		C_{50} (nM)		ADP response (△O.D., cm)	
		Pre	During	Pre	During	Pre	During
α Methyl-noradrenaline (2.5 um/kg/hr)	2.5	43 ± 14	38 ± 19	11 ± 7	58 ± 28*	20 ± 18	23 ± 22
	5	55 ± 22	32 ± 11*	59 ± 38	419 ± 427*	28 ± 20	30 ± 21
	10	50 ± 15	32 ± 8*	20 ± 19	94 ± 96*	31 ± 49	46 ± 49
α Methyl-noradrenaline 0.5 um/kg/hr)	10	44 ± 14	46 ± 24	15 ± 15	12 ± 8	20 ± 20	28 ± 20
	30	57 ± 2	47 ± 3*	16 ± 3	22 ± 9	26 ± 15	28 ± 22
Phenylephrine (1.8 um/kg/hr)	10	49 ± 19	43 ± 16	55 ± 56	57 ± 16	28 ± 21	49 ± 11

* $P < 0.001$

Results are expressed as the mean ± standard deviation for groups of six rabbits

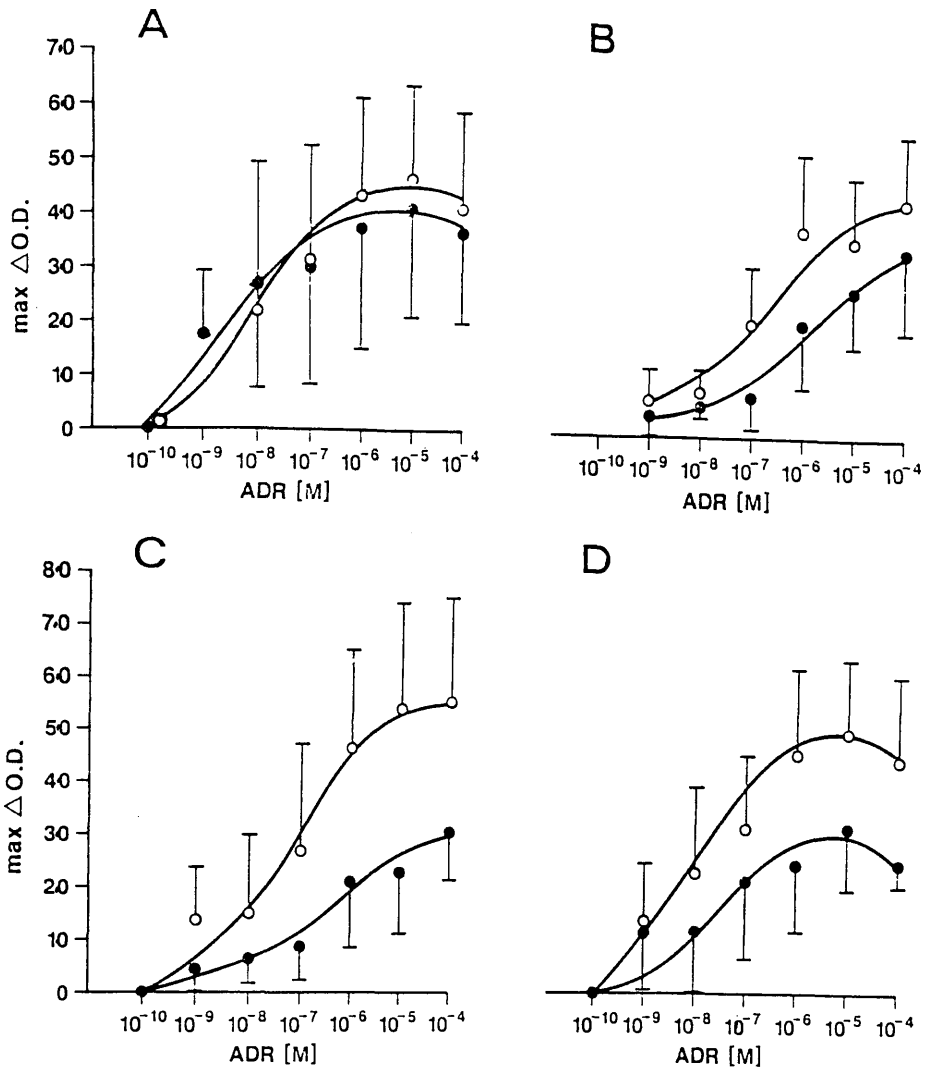


Figure 4.3

Platelet pro-aggregatory responses to adrenaline following acute alpha adrenoceptor agonist infusions

A 10 min. phenylephrine infusion, 1.8 umol/kg/hr

B 2.5 min. α methylnoradrenaline infusion, 2.5 umol/kg/hr

C 5 min. α methylnoradrenaline infusion, 2.5 umol/kg/hr

D 10 min. α methylnoradenaline infusion, 2.5 umol/kg/hr

○ Response before infusion

● Response during infusion

Results were expressed as the mean ± standard deviation for groups of six animals.

($P < 0.01$) decrease in Emax after 30 minutes' infusion (Figure 4.4, Table 4.4). Ten minute infusions of phenylephrine had no effect on aggregatory responses to adrenaline and the responses to ADP alone were not significantly changed before and after any of the infusions The slope of each dose response curve did not differ significantly from 1.0.

4.3.5 [^{3}H] Yohimbine binding to platelets

Acute (ten minute) intravenous administration of alpha methylnoradrenaline (2.5 umol/kg/hr) failed to alter [^{3}H] yohimbine binding to rabbit platelets. Values were 20.6 $\pm$ 2.4 before infusion compared to 20. 2 $\pm$ 1.8 fmoles/10^9 platelets, after infusion. There was also no evidence of a change in the K_D (8.1 $\pm$ 3. 2 before compared to 7.3 $\pm$ 1.6 nM after infusion). These results are illustrated in Figure 4.5.

4.3.6 Alpha methylnoradrenaline responses during acute adrenaline infusions

Plasma adrenaline levels were raised approximately thirty fold aften ten minutes adrenaline (1.5 umol/kg/hr) infusion (1.1 $\pm$ 0.3 nM in controls compared to 30.6 $\pm$ 9.1 nM in treated animals). These high circulating levels of adrenaline were associated with a significant reductions ($P < 0.01$) in the 2.5 minute pressor response to alpha methylnoradrenaline (3 ug/kg) from 32.1 $\pm$ 2 mmHg in ascorbate infused animals compared to 12 $\pm$ 8 mmHg in the adrenaline infused animals. Ten minute adrenaline infusions at the same dose resulted in no further attenuation of pressor responses to alpha methylnoradrenaline

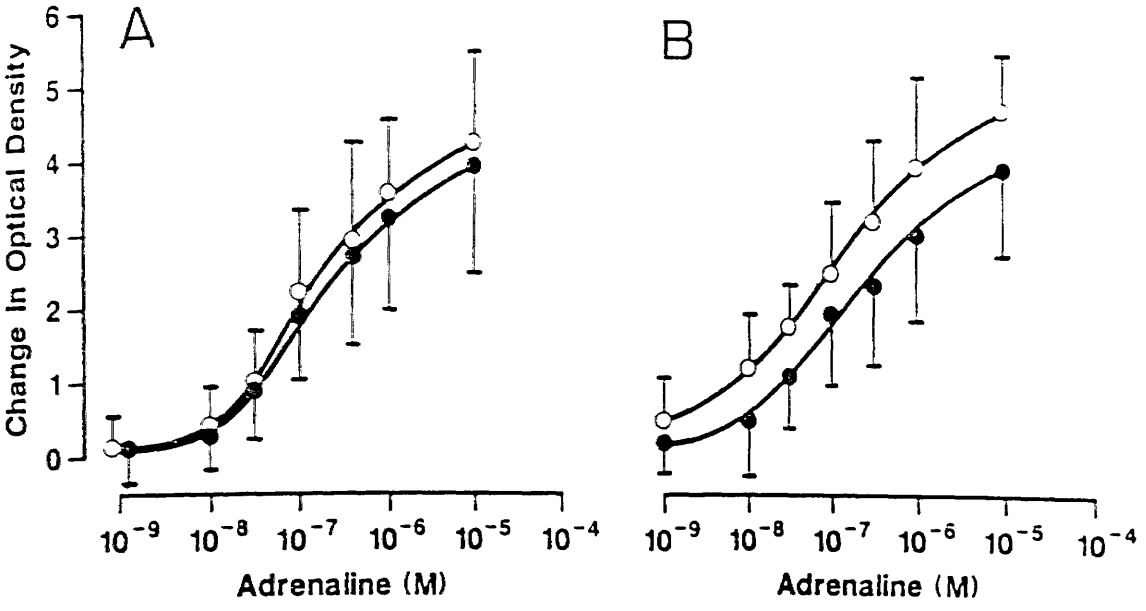


Figure 4.4

Platelet pro-aggregatory responses to adrenaline following acute alpha methylnoradrenaline infusions

A 10 min. α methylnoradrenaline infusion (0.5 umol/kg/hr)

B 30 min. α methylnoradrenaline infusion (0.5 umol/kg/hr)

○ Response before infusion

● Response during infusion

Results were expressed as the mean ± standard deviation for groups of six animals.

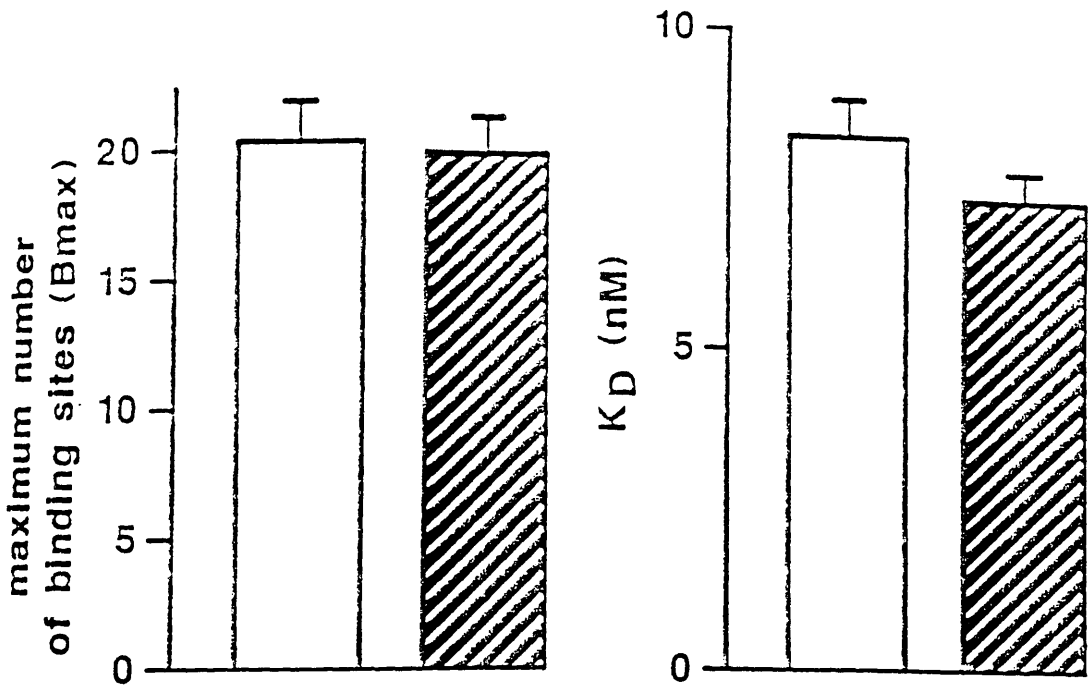


Figure 4.5

[^{3}H] Yohimbine binding to whole platelets at the end of a ten minute infusion of alpha methylnoradrenaline

□ Before infusion

▨ At the end of infusion

Arterial blood samples (15 mls) were removed after ten minutes of alpha methylnoradrenaline (2.5 umol/kg/hr) infusion.

The results shown are expressed as mean ± standard deviation for six animals.

(34 ± 3 in ascorbate treated animals compared to 20 ± 6 mmHg in the adrenaline treated animals) (Figure 4.6). The lower dose of adrenaline (0.05 umol/kg/hr) produced approximately eight fold elevations in plasma adrenaline concentration (1.6 ± 0.2 nM before compared to 12.7 ± 2.1 nM after 45 minutes infusion). There were no significant alterations in the pressor responses to alpha methylnoradrenaline (3 ug/kg) at any of the times examined during these infusions (Figure 4.6).

Platelet aggregation

There was a reduction in the aggregatory responses to adrenaline in five out of six of the animals infused with adrenaline, 1.5 umol/kg/hr, however the maximum aggregatory response (Emax, cm) to adrenaline was not significantly attenuated (44 ± 16 before infusion compared to 28 ± 5 at the end of infusion) (Figure 4.7). Similarly, there were no significant changes in C_{50} (uM) (0.4 ± 0.3 before infusion in comparison to 1.2 ± 1.7 post infusion) or the primary ADP responses (32 ± 21 before infusion in comparison to 29 ± 10 post infusion).

4.3.7 Responses to alpha methylnoradrenaline during noradrenaline infusions

Plasma noradrenaline concentrations were 2.1 ± 1.1 nM before infusion and 18 ± 8.2 nM after 45 minutes of noradrenaline (0.09 umol/kg/hr) infusion.

Vascular pressor responses (2.5, 10, 30 and 60 minute) to alpha methylnoradrenaline (3 ug/kg) during administration of a low dose of noradrenaline (0.09 umol/kg/hr) did not differ

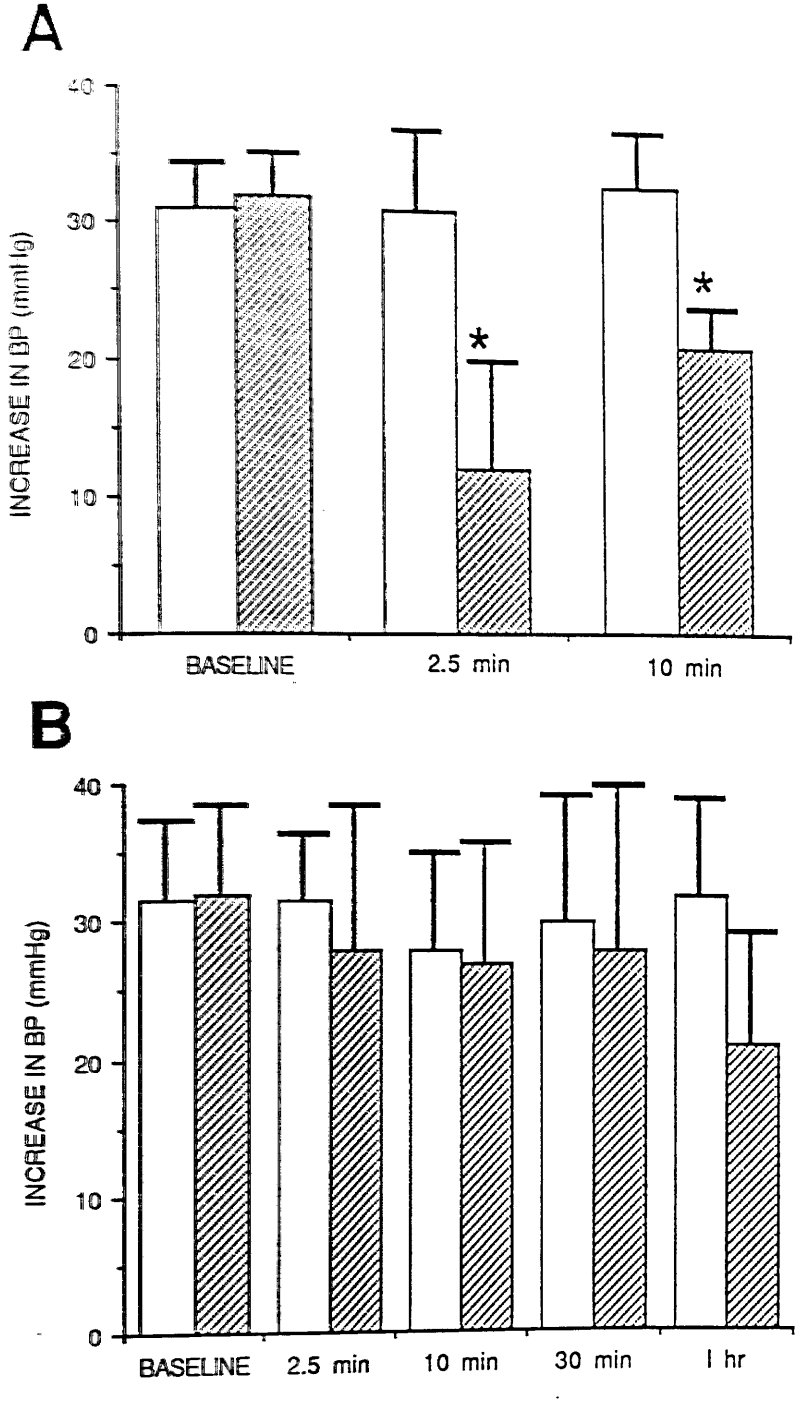


Figure 4.6

Vascular pressor responses to alpha methylnoradrenaline during acute ascorbate and adrenaline infusions

A Adrenaline infusion, 1.5 umol/kg/hr ($P < 0.01$)

B Adrenaline infusion, 0.05 umol/kg/hr

□ Response during ascorbate infusion

▨ Response during adrenaline infusion

Results were expressed as the mean ± standard deviation for groups of six rabbits.

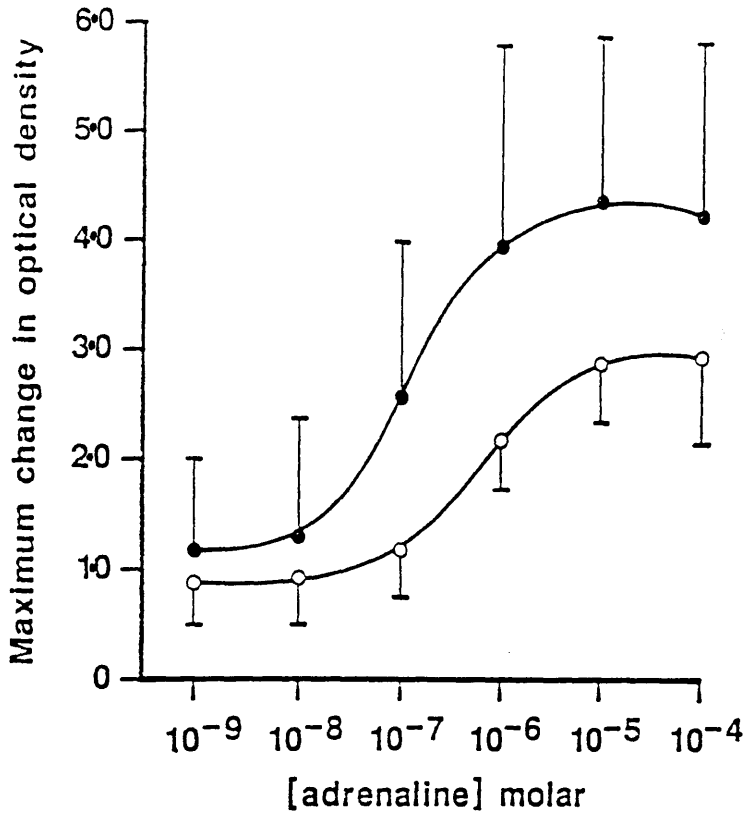


Figure 4.7

Platelet pro-aggregatory responses to adrenaline at the end of ten minutes adrenaline infusion

● Response before infusion

○ Response at the end of infusion

The rate of adrenaline infusion was 1.5 umol/kg/hr. Results were expressed as the mean ± standard deviation for groups of 6 rabbits.

significantly from responses during ascorbate (0.1%) infusions (Figure 4.8). Baseline pressor responses between control and treated animals were not significantly different for each group.

4.3.8 Changes in blood pressure and heart rate during 0.05 umol/kg/hr adrenaline and 0.09 umol/kg/hr noradrenaline infusions

Infusions of adrenaline (0.05 umol/kg/hr) and noradrenaline (0.09 umol/kg/hr) both elicited an increase in blood pressure of approximately 10 mmHg within 2.5 - 3 minutes. During both adrenaline and noradrenaline infusion approximately 50% of the rabbits recovered back to normal baseline blood pressures within one hour while the remainder maintained the initial rise of 10 mmHg. During noradrenaline infusion, the elevation in blood pressure was accompanied by a reduction in heart rate of approximately 30-40 beats/min.

4.4 Discussion

Short term treatment with alpha methylnoradrenaline in the rabbit caused rapid in vivo and in vitro attenuations of $alpha_2$ adrenoceptor mediated responses. These changes were dependent on both the dose and duration of alpha methylnoradrenaline infusion.

Phenylephrine and alpha methylnoradrenaline infusions led to similar rises in the mean arterial pressure although there were no reductions in the pressor response to alpha methylnoradrenaline during phenylephrine infusion. These results suggest that the observed attenuation in vascular response was specific for $alpha_2$ adrenoceptor mediated responses and not a consequence of either a rise in blood pressure or a compensatory

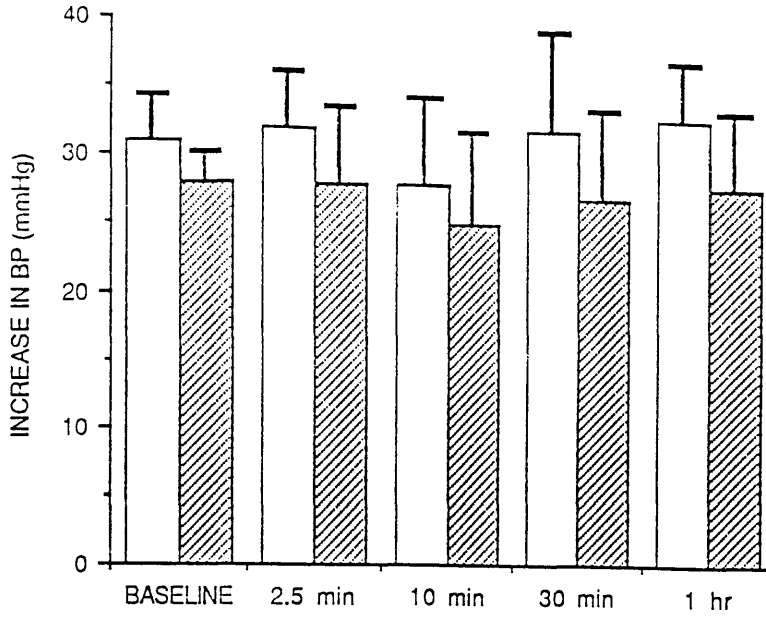


Figure 4.8

Vascular pressor responses to alpha methylnoradrenaline during noradrenaline infusion

□ Response during ascorbate infusion

▨ Response during noradrenaline infusion

The rate of noradrenaline infusion was 0.09 umol/kg/hr.

Results were expressed as the mean ± standard deviation for groups of six rabbits.

baroreflex mechanism. In the in vivo studies, administration of alpha methylnoradrenaline produced a rapid (2.5 minute) desensitisation of postsynaptic alpha$_2$ adrenoceptor mediated responses which was dose related. These findings were of a similar time scale to those observed during the early phase of homologous desensitisation in beta adrenoceptor systems (Staehelin and Simons, 1982; Toews and Perkins, 1984). In these studies, the beta adrenoceptors appeared to be functional although sequestered away from guanine nucleotide proteins and adenylate cyclase (Strasser et al, 1985).

Decreases in the alpha$_2$ adrenoceptor mediated platelet aggregatory responses to adrenaline were also observed and found to be dependent on both the dose and the duration of infusion. Rightward shifts in the dose response relationships and reductions in Emax were detected following five and ten minute infusions of alpha methylnoradrenaline (2.5 umol/kg/hr) but only after thirty minute infusions of alpha methylnoradrenaline (0.5 umol/kg/hr). In contrast to the in vivo data, there were no significant changes in the aggregatory responses to adrenaline after the 2.5 minute alpha methylnoradrenaline infusions (2.5 umol/kg/hr). However, other investigators have shown that desensitisation of platelet aggregatory responses occurs only half maximally within 3-5 minutes with maximum attenuation being reached by 20 minutes (Motulsky et al, 1986). This discrepancy with the present in vivo data could be due to some reversal of desensitisation occurring during the preparation of blood samples for in vitro platelet aggregation studies. Another possible factor is that the vascular alpha$_2$ receptors could be more

susceptible to desensitisation. The observed decreases in aggregatory responses could not be attributed to fluctuations in circulating plasma alpha methylnoradrenaline levels considering that steady state concentrations were achieved within 2.5 minutes, nor could they be due to retention of agonist (Karliner et al, 1982) since there were no significant alterations in the primary responses to ADP.

No changes in specific [^{3}H] yohimbine binding were found after ten minute infusions of alpha methylnoradrenaline therefore reductions in total receptor number were unlikely to be an explanation for the acute in vitro reductions in response. Desensitisation of human platelet alpha$_2$ adrenoceptors without changes in platelet alpha$_2$ receptor density has been observed by Jones and colleagues (1985a) during short term agonist treatment.

Reductions in the pressor response to alpha methylnoradrenaline (2.5 and 10 minutes) during adrenaline (1.5 umol/kg/hr) infusion were of the same magnitude as those evoked during alpha methylnoradrenaline (2.5 umol/kg/hr) infusion. This attenuation of response was also found to be dose dependent demonstrated by the lack of change in blood pressure responses during administration of the lower dose of adrenaline (0.05 umol/kg/hr). However, a reduction in response was observed in sixty-six percent of these animals after one hour of adrenaline (0.05 umol/kg/hr) infusion and it is possible that a significant attenuation of responses would have been observed if the infusions had been continued for longer. These experiments suggest that acute exposure to adrenaline may also be causing

dose and time dependent desensitisation of alpha$_2$ adrenergic receptors. Pressor responses to alpha methylnoradrenaline during administration of noradrenaline (0.09 umol/kg/hr) were unchanged. This may be related to the relatively low dose infused and to the duration of infusion. It would have been interesting to have prolonged the infusion times for noradrenaline as well as adrenaline.

The initial increase in blood pressure noted during 2-3 hour infusions of the lower infusion rate of adrenaline and noradrenaline was likely to be caused by vasoconstriction due to stimulation of postsynaptic alpha$_1$ and alpha$_2$ adrenoceptors. Two hours into infusion the blood pressure had returned to pretreatment baseline values in fifty percent of the rabbits. In the animals treated chronically with adrenaline (0.05 umol/kg/hr) or noradrenaline (0.09 umol/kg/hr), no increase in blood pressure was observed after twenty-four hours of infusion (Sections 5.3.2 and 6.3.2). It is possible that desensitisation of vascular alpha adrenoceptors occurred during the first twenty-four hours of infusion in these animals. This could be caused by either uncoupling from second messengers or a degradation of receptor protein. The bradycardia observed during exposure to noradrenaline could be due to a baroreflex mediated mechanism compensating for the elevation in blood pressure. A reduction in heart rate has also been observed after twenty four hours' infusion of noradrenaline (0.09 umol/kg/hr).

Clearly, further experiments are warranted to determine the molecular mechanisms involved in the acute desensitisation of $alpha_2$ adrenergic receptors.

CHAPTER FIVE

CHANGES IN ADRENERGIC RECEPTOR FUNCTION AND NUMBER FOLLOWING LONG TERM ADRENALINE INFUSION

Chapter Five

Changes in Adrenergic Receptor Function and Number Following Long Term Adrenaline Infusion

5.1 Introduction

Repeated administration of adrenergic agonist drugs to patients may be associated with a progressive decrease in the observed therapeutic response due to down regulation of adrenergic receptors (Section 1.4.3). Attempts to compensate for this loss of drug efficacy by administering gradually larger doses may increase morbidity and mortality (Connolly and Greenacre, 1976).

There is a relative paucity of information concerning the long term effects of the endogenous agonist, adrenaline on adrenoceptor function and number. In one study Tsujimoto and Hoffman (1984) treated rats with adrenaline for seven days resulting in 70 fold elevations in plasma adrenaline and marked reductions in heart and lung beta adrenoceptors. In a similar investigation, elevated adrenaline levels in rats caused down regulation of beta adrenergic receptors in renal cortical membranes (Snavely et al, 1985) however the decrease in beta adrenoceptors was selective for the $beta_2$ subtype of the receptor.

There have been many conflicting reports concerning the relationship between chronic agonist administration and $alpha_2$ adrenoceptor regulation (Section 1.7). Some workers have reported a decrease in platelet $alpha_2$-adrenoceptors on chronic treatment with clonidine (Brodde et al, 1982) while others have

failed to find any alteration in platelet alpha$_2$ adrenoceptor number during treatment of hypertensive patients with another alpha$_2$ adrenoceptor agaonist guanabenz (Motulsky et al, 1983). There were also no changes in platelet alpha$_2$ receptor density observed following long term adrenaline infusion either in humans (Roberts et al, 1986; Pfeifer et al, 1984) or in dogs (Villeneuve et al, 1985a). Thus, further examination of alpha$_2$ adrenoceptor regulation in response to chronic agonist treatment is warranted.

In the present studies, the effects of chronic adrenaline infusion on adrenoceptor function and number was examined. Alterations in alpha$_2$ adrenoceptor response were assessed by platelet aggregation studies and changes in alpha$_2$ receptor number in platelet and kidney, by radioligand binding. Tissues containing both homogenous populations (platelets and lymphocytes) and mixed populations (heart and lung) of beta receptors were also used to evaluate the effects of long term adrenaline infusion *in vivo* on beta adrenergic receptor number in the rabbit.

Adrenaline was administered using osmotic minipumps which infuse the given drug at a constant rate over the duration of treatment. This technique avoids the use of intermittent drug infusions which may be short lived and fail to detect slowly developing changes in adrenoceptor concentration (Chang et al, 1982).

5.2 Methods

Ten day infusions of adrenaline (0.05 umol/kg/hr) were given to groups of rabbits (n = 10) via ALZET^{tm} osmotic minipumps type

2002 (Figure 5.1). Control animals (n = 10) received the vehicle (0.1% acorbate) by the same method.

5.2.1 The dose of adrenaline

In the treated rabbits, the objective was to raise plasma adrenaline levels approximately ten fold. The dose was determined by clearance calculations (Fitzgerald et al, 1979).

$$\text{Clearance} = \frac{\text{Infusion rate}}{\text{Steady state - basal}}$$

The clearance of catecholamines in rabbits was shown to be 5 l/kg/hr (Hamilton and Reid, 1983). In the basal state, the concentration of adrenaline in rabbit plasma is approximately 1.0 nM (Deighton et al, 1986). The rate of infusion for a 2.5 kg rabbit was 15.2 ug/kg/hr or 0.05 umol/kg/hr. Therefore steady state levels of adrenaline were expected to be approximately 10 nM over the period of infusion.

5.2.2 Blood pressure, heart rate and catecholamines

Basal monitoring of the mean arterial pressure (mmHg) and heart rate (beats/min) was undertaken (Section 2.1) in conjunction with removal of blood (5 mls) for simultaneous measurement of adrenaline and noradrenaline concentrations (Section 2.2). These recordings were then repeated at 1, 4, 7 and 10 days of adrenaline or ascorbate infusion.

5.2.3 Femoral vein cannulation

A venous catheter was inserted into the peripheral ear vein under local anaesthesia (2% lignocaine). Animals were then

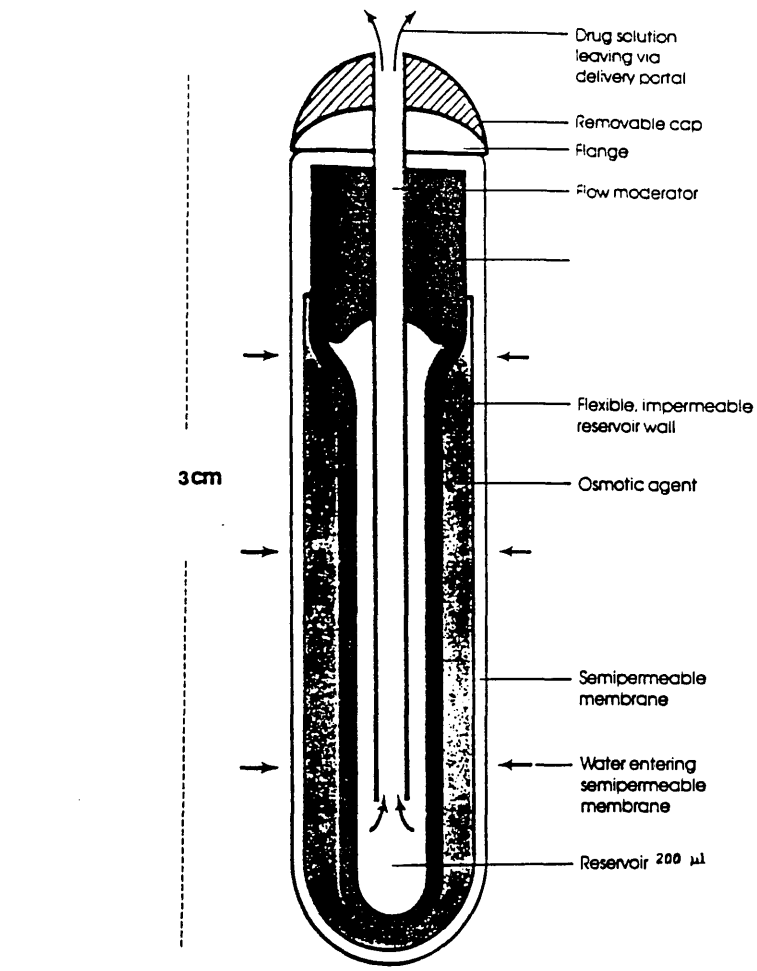


Figure 5.1

A cross section of the osmotic minipump

Thin tubing (1 mm diameter) was tied securely to the delivery portal for intravenous cannulation.

The pumps were used to deliver adrenaline (0.05 umol/kg/hr) or ascorbate (0.1%) at a constant rate of 0.5 ul/hr for 10 days.

anaesthetised with sodium pentobarbitone (60 mg/kg) and shaved at the upper, inner aspect of the thigh to allow a small incision to be made. The femoral vein was isolated and cannulated towards the heart with the distal end of the cannula attached securely to the osmotic minipump which was embedded in the muscle. The wound was then closed and animals left to recover from the anaesthetic. The minipump was left in place for the ensuing ten days to infuse either the drug or the vehicle at a constant rate of 0.5 ul/hr.

5.2.4 Preparation of blood elements and tissues for adrenoceptor studies

On day eleven of infusion, rabbits were killed via an intravenous overdose of sodium pentobarbitone (60 mg/kg) and blood (60 - 80 mls) was withdrawn immediately by cardiac puncture together with collection of whole heart, lung and one kidney. Intact platelets were prepared for aggregation studies and [^{3}H] yohimbine binding as described in Sections 2.3.1, 2.3.2, 2.4 and 2.5. Kidney membranes were also prepared for [^{3}H] yohimbine binding. In addition, lymphocyte, heart and lung membranes were prepared and [^{125}I) ICYP binding carried out according to Methods 2.3.3, 2.3.4 and 2.6.

5.2.5 Statistics

The non-parametric Wilcoxon test was employed for unpaired data (Section 2.8). All results are quoted as means $\pm$ standard deviation. In all experiments groups of six or more animals were studied.

5.3 Results

5.3.1 Catecholamine levels

Plasma adrenaline levels, before starting the infusion were 1.4 + 1.5 nM with approximately eight fold elevations at 24 hrs. of adrenaline treatment (9.2 ± 3.2 nM). These raised levels were sustained through to day 10 (10.3 ± 5.7 nM) with there being no significant alterations in the concentrations of noradrenaline during adrenaline infusion (Figure 5.2). There were also no changes in adrenaline levels during infusions of the vehicle, 1.3 ± 0.8, basal compared to 1.8 ± 1.6 after five days and 0.8 ± 0.6 nM after ten days of ascorbate infusion.

5.3.2 Blood pressure and heart rate

The mean arterial blood pressure and heart rate did not alter significantly from 84 ± 5.2 (mmHg) and 210 ± 31.2 (beats/min) before compared to 80.9 ± 9.4 and 227 ± 27.2 respectively on day 10 of adrenaline infusion (n = 10). The profile for control animals was similar; values were 77.2 ± 9.7 mmHg and 226 ± 16.4 beats/min before to 76.1 ± 8.5 mmHg and 215.7 ± 9.7 beats/min on day 10 of ascorbate infusion. These results and those obtained for intermediate recordings are shown in Figure 5.3.

5.3.3 Platelet aggregation

The effects of long term adrenaline treatment (10 days) on the platelet pro-aggregatory responses to adrenaline are illustrated in Figure 5.4 (n = 8). There were parallel rightward shifts in the dose response curves with significant decreases in

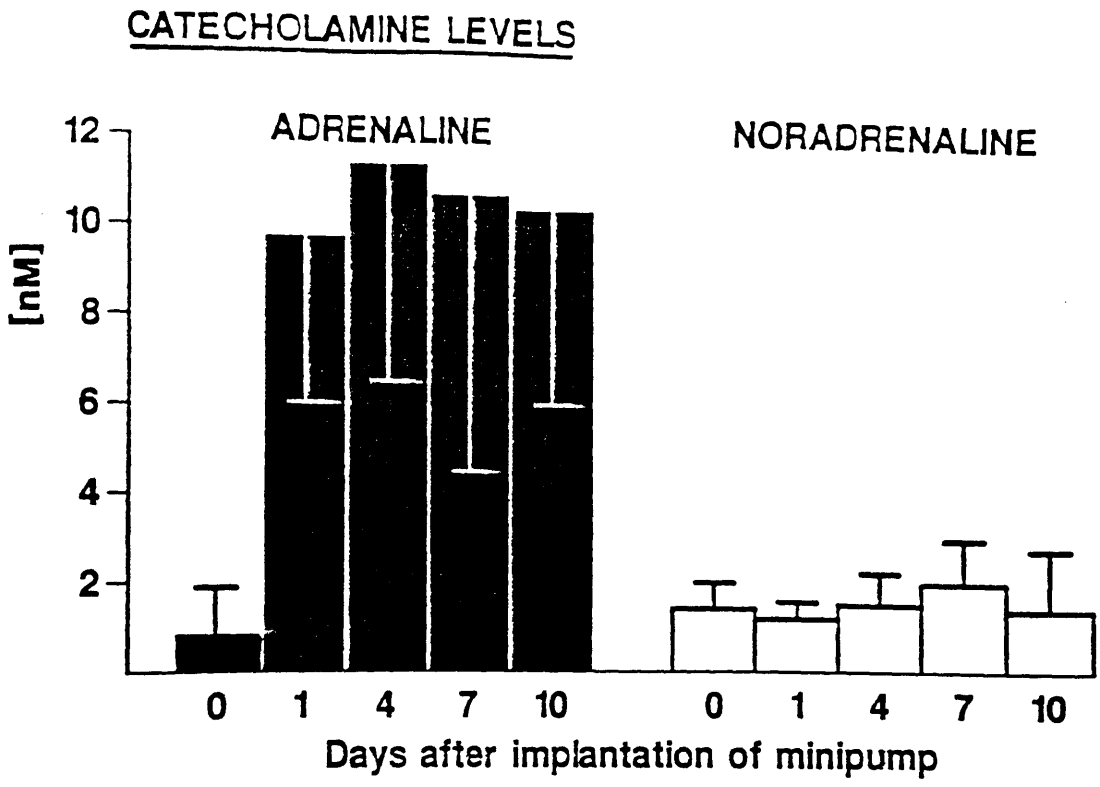


Figure 5.2

The effects of a 10 day adrenaline infusion (0.05 umoles/kg/hr) on catecholamine levels (nM).

Blood samples (5 mls) were removed before and 1, 4, 7 and 10 days into infusion.

Results were expressed as the mean ± standard deviation for groups of ten animals.

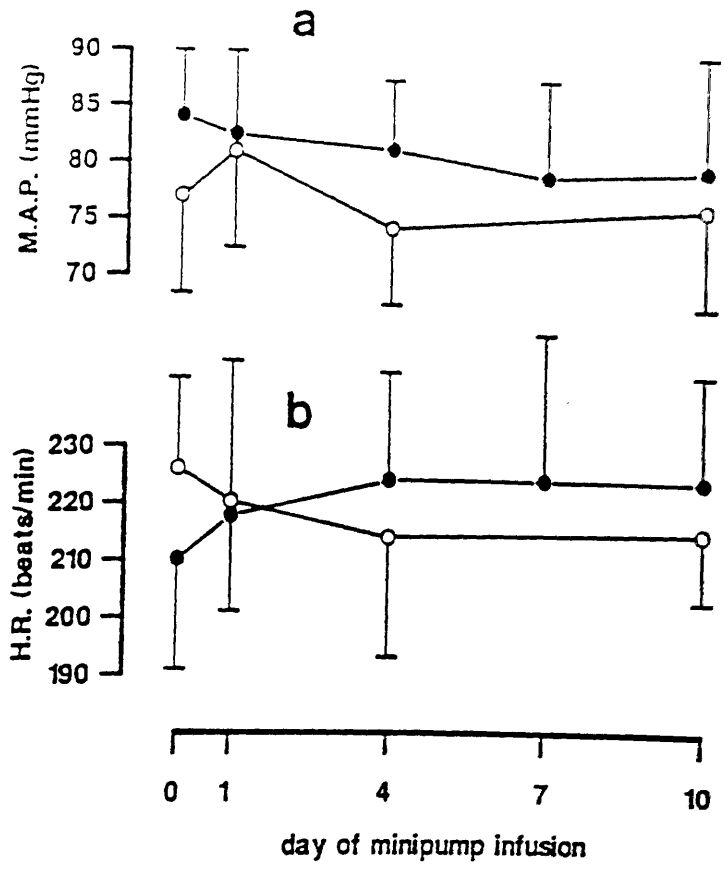


Figure 5.3

Effects of a 10 day adrenaline infusion on blood pressure (A) (mmHg) and heart rate (B) (beats/min). Recordings were made before and after 1, 4, 7 and 10 days of infusion.

○ Control

● Adrenaline treated

Results were expressed as the mean ± standard deviation for groups of nine rabbits.

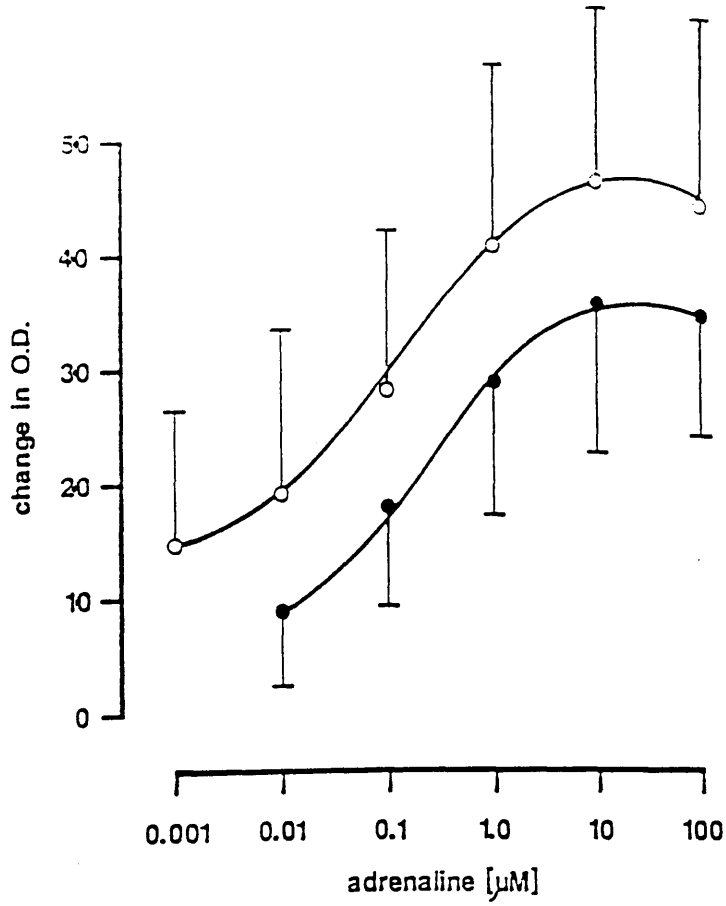


Figure 5.4

The effects of in vitro additions of adrenaline (0.001 - 100 uM) on the ADP induced platelet aggregation following a 10 day adrenaline infusion.

○ Control

● Adrenaline treated

Results were expressed as the mean ± standard deviation for eight rabbits.

Emax (cm) from 50 ± 3 before infusion to 36 ± 2 post infusion ($p < 0.001$). A slight but not significant fall was observed in the C_{50} (uM) from 2.1 ± 3.7 to 0.9 ± 0.4 after adrenaline infusion. The aggregatory responses to ADP alone did not significantly change from 34 ± 22 (control) to 35 ± 10 (post infusion).

5.3.4 Alpha$_2$ adrenoceptor number in platelets and kidney

Chronic adrenaline administration caused a significant reduction in [^{3}H] yohimbine binding to rabbit platelets (23 ± 4 before compared to 10 ± 1 fmoles/10^9 platelets after infusion, $p < 0.001$) without significant alterations in kidney membranes (99 ± 33 before compared to 77 ± 27 fmoles/mg protein after infusion). The K_D (nM) did not change significantly for either tissue as shown, together with the changes in Bmax, in Figure 5.5.

5.3.5 Beta adrenoceptor density in platelet, lymphocyte, heart and lung

Specific [^{125}I] Iodocyanopindolol (ICYP) binding to platelets did not alter significantly from control (0.7 ± 0.4) compared to treated animals (0.5 ± 0.2). Similarly, [^{125}I] ICYP binding to lymphocytes resulted in no changes between control (5 ± 2) and ten day adrenaline treated animals (6 ± 3) (Figure 5.6).

In contrast, a significant and marked reduction of approximately 50% was revealed in [^{125}I] ICYP binding sites in rabbit heart and lung following chronic adrenaline infusion. Bmax (fmoles/mg protein) in heart from control animals was 18 ± 5

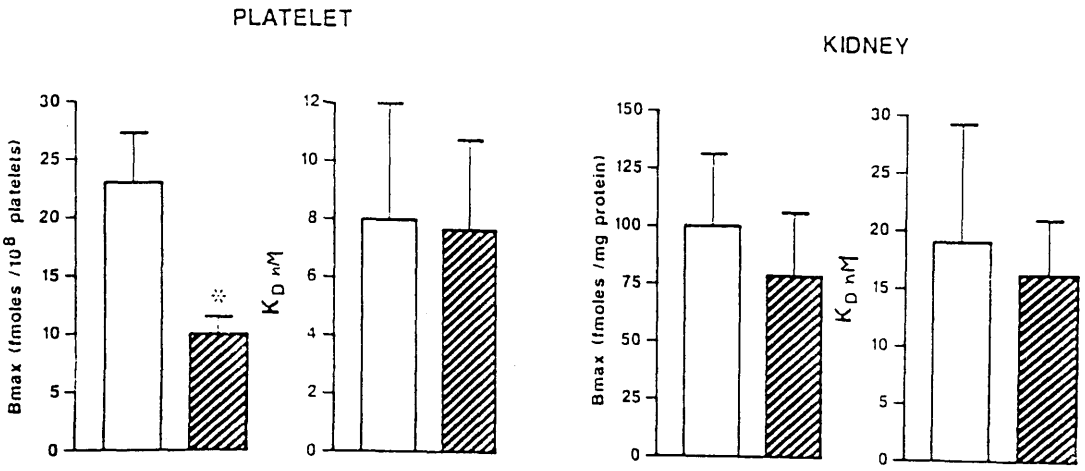


Figure 5.5

Changes in alpha adrenoceptor number (Bmax) and the equilibrium dissociation constant, K_D (nM) in intact platelets and kidney membranes following a 10 day infusion of adrenaline.

□ Control

▨ Adrenaline Treated

* $P < 0.001$

$Alpha_2$ adrenoceptor binding sites were quantified using [^{3}H] yohimbine.

Results were expressed as the mean ± standard deviation for six rabbits.

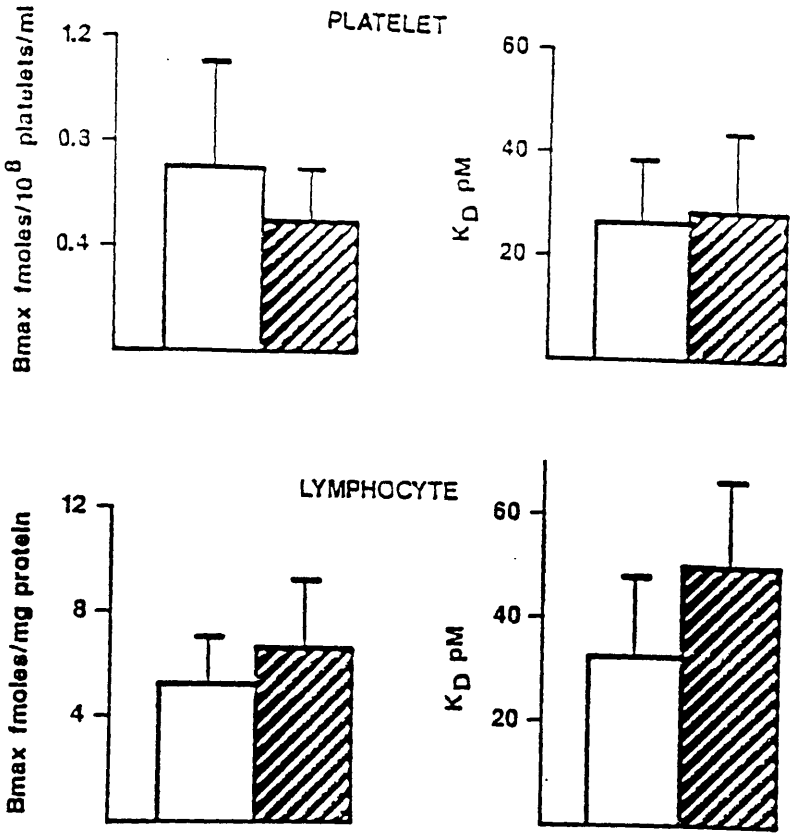


Figure 5.6

Changes in beta adrenoceptor density (Bmax) and equilibrium dissociation constant, K_D (pM) in intact platelets and lymphocyte membranes following a 10 day adrenaline infusion.

□ Control

▨ Adrenaline Treated

Beta adrenoceptor binding sites were quantified using [^{125}I] ICYP.

Results were expressed as the mean ± standard deviation for six rabbits.

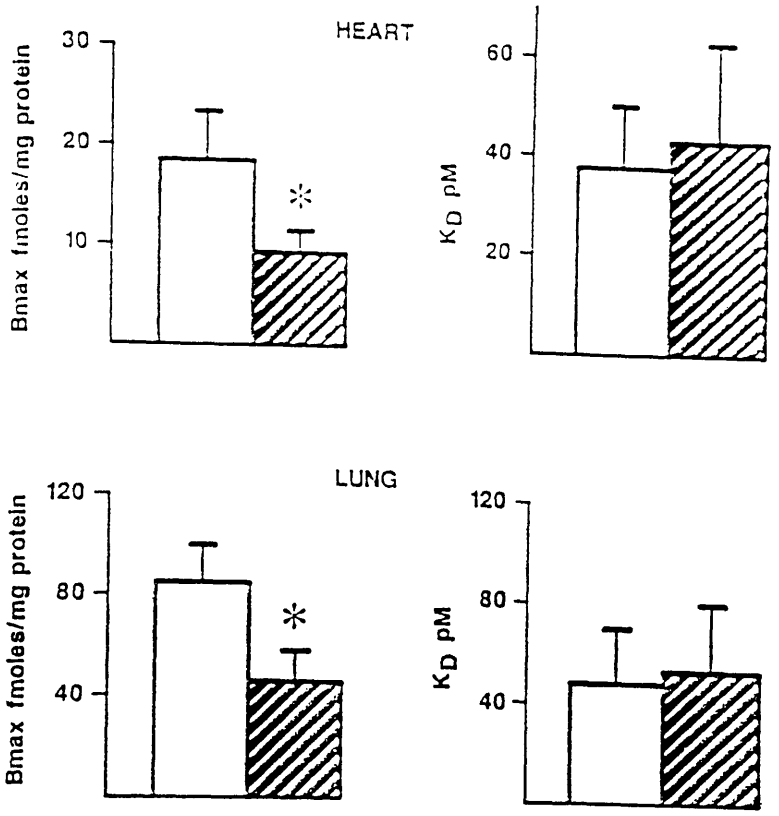


Figure 5.7

Changes in beta adrenoceptor density (Bmax) and equilibrium binding constant K_D (pM) in heart and lung membranes following a 10 day infusion of adrenaline.

□ Control

▨ Treated

* $P < 0.001$

Beta adrenoceptor binding sites were quantified [^{125}I] ICYP. Results were expressed as the mean ± standard deviation for six rabbits.

compared to 9 ± 3 in treated rabbits ($p < 0.001$). Bmax from control lungs was 84 ± 13 with significant falls to 46 ± 10 fmoles/mg protein in treated animals ($p < 0.001$). The K_D (pM) was not significantly different between control and treated animals for all four tissues (Figures 5.6 and 5.7).

5.4 Discussion

Moderate increases in circulating plasma adrenaline for ten days led to an attenuation of the aggregatory response of rabbit platelets to adrenaline. These $alpha_2$ adrenoceptor mediated reductions in response were accompanied by a fall in the density of platelet $alpha_2$ adrenoceptors. The present findings were consistent with other studies which demonstrated desensitisation of $alpha_2$ receptors with concomitant decreases in receptor number (Cooper et al, 1978; Brodde, 1983). In contrast there was a lack of alteration in the number of kidney $alpha_2$ adrenoceptors. Snavely et al, (1983) also recorded an absence of down regulation of renal cortex $alpha_2$ receptors in rats with phaeochromocytoma. These authors postulated that differences in susceptibility to down regulation may occur between tissues. Moreover, in the current studies, intravenous infusion would achieve high and sustained levels of circulating adrenaline in proximity to platelets, while adrenaline levels at kidney receptor sites would not be so high. Further discussion of these results is given in chapter eight where the effects of chronic administration of adrenaline and noradrenaline are compared.

In this study a tissue specific reduction in beta adrenergic receptor concentration was also demonstrated following prolonged

exposure to adrenaline. Significant falls in beta adrenoceptor density were observed in heart and lung membranes without comparable reductions in $beta_2$ receptors in platelets or lymphocytes. These conflicting results for beta adrenoceptor changes were unlikely to be due to differences in tissue drug levels as the platelets and lymphocytes would have been exposed to the highest concentrations of the drug. Secondly, the down regulation was unlikely to be an artifact due to retained agonist (Karliner et al, 1982) considering that the binding data for heart and lung show no increase in the K_D after infusion. The extensive washing in hypotonic Tris HCl buffer should remove retained catecholamines (Cheung et al, 1984). Lastly, these findings cannot be interpreted on the basis of adrenaline's differing affinities at the $beta_1$ and $beta_2$ subtypes of the receptor as if this was the case, a significant decrease in lymphocyte $beta_2$ receptor number would have been expected (Aarons et al, 1983). Tissue selective changes in beta adrenoceptor number were also observed when adrenaline was infused into rats for seven days (Tsujimoto and Hoffman, 1984). No alterations in the beta adrenoceptor number in mesenteric artery were found in contrast to the striking down regulation in lung (both largely of the $beta_2$ subtype). In another animal model where there were high levels of circulating catecholamines, Torda and colleagues (1981) reported a decrease in beta adrenergic receptor density in rat spleen, but not in lung after immobilisation stress. The beta adrenoceptors in rabbit heart and lung are eighty percent $beta_1$ in type while those in the platelets and lymphocytes are almost exclusively $beta_2$ in type (Section 3.3.3). Thus, it is

possible that the present results could reflect a $beta_1$ adrenoceptor selective down regulation, however, the proportions of $beta_1$ and $beta_2$ adrenoceptors within the same tissue were not determined in these studies.

Increased levels of circulating adrenaline have been reported to cause elevations in blood pressure in the rat (Majewski et al, 1981), both by acting at post synaptic $alpha_1$ and $alpha_2$ adrenoceptors and at putative presynaptic $beta_2$ adrenoceptors to enhance noradrenaline release. Although adrenaline also acts at presynaptic $alpha_2$ adrenoceptors to inhibit noradrenaline release which would also tend to lower blood pressure. In this study, there were no significant changes in the mean arterial pressure and heart rate during adrenaline infusion and plasma noradrenaline was unchanged. The earliest measurements of blood pressure and heart rate were made 24 hrs after commencing infusion and it is possible that desensitisation and down regulation of alpha adrenoceptors had occurred during this time.

Thus, in conclusion, chronic adrenaline infusion in rabbits resulted in a reduction in $alpha_2$ adrenoceptor mediated platelet aggregation together with tissue selective decreases in both $alpha_2$ and beta adrenergic receptor number. Extrapolations of binding data to other tissues containing similar adrenoceptor populations should be exercised with caution due to the wide differences in susceptibility to down regulation. Furthermore agonist efficacy and differential accessibility of receptors to agonists could both influence down regulation.

CHAPTER SIX

CHANGES IN ADRENERGIC RECEPTOR FUNCTION AND NUMBER FOLLOWING LONG TERM NORADRENALINE INFUSION

Chapter Six

Changes in Adrenergic Receptor Function and Number Following Long Term Noradrenaline Infusion

6.1 Introduction

Changes in adrenoceptor response and number following chronic infusions of adrenaline have been assessed in chapter five. However, raised circulating plasma concentrations of the other main endogenous agonist, noradrenaline may be more common.

Studies using *in vitro* techniques have documented alterations in adrenergic responses subsequent to noradrenaline treatment. Twenty-four hour exposure of guinea pig vas deferens to noradrenaline elicited a long term *in vitro* desensitisation without any changes in alpha adrenoceptor number (Takeyasu et al, 1982). Alterations in adrenoceptor concentration have been recorded in *in vivo* diseased situations such as phaeochromocytoma and heart failure (Sections 1.9.1 and 1.9.2) when plasma noradrenaline levels were high and greater than that of adrenaline (Hermann and Mornex, 1964). Several of these investigations revealed a down regulation of $alpha_2$ and beta adrenoceptors and this down regulation was found to be selective for certain tissues and/or subtypes of adrenoceptor. Brodde and colleagues (1986) observed a selective loss of $beta_1$ adrenoceptors in explanted hearts from patients with end stage heart failure. Similarly, a preferential decrease (64%) in $beta_1$ adrenoceptors without changes in the $beta_2$ receptor population was demonstrated in the renal membranes of rats harbouring

phaeochromocytoma (Snavely et al, 1982).

There still remains a certain amount of controversy surrounding alterations in $alpha_2$ adrenoceptor density in response to raised plasma noradrenaline and further investigation is required. Some workers report a down regulation of platelet $alpha_2$ receptors in human phaeochromocytoma (Davies et al, 1981; Brodde and Bock, 1984) while, more recently, others have failed to show any differences in human platelet $alpha_2$ receptors (Valet et al, 1987) or rat mesenteric artery (Tsujimoto et al, 1987) in the same diseased condition.

Plasma levels of noradrenaline were 50-70 fold greater than basal values in most experimental models and in some studies plasma noradrenaline levels as high as 200 times normal were reported (Tsujimoto et al, 1987). It would, therefore, be interesting to explore the effects of moderate increases in circulating noradrenaline (10-20 fold) on adrenergic responses.

The principal objectives of the present study were to evaluate the effects of ten day noradrenaline infusions on $alpha_2$ adrenergic receptor function, $alpha_2$ adrenoceptor and beta adrenoceptor number in certain tissues of the rabbit. Osmotic minipumps were used to achieve moderate intravenous elevations of noradrenaline. A comparison of the results obtained following chronic adrenaline and noradrenaline administration are discussed in chapter eight.

6.2 Methods

A group of twelve rabbits were given long term (10 day) intravenous noradrenaline infusions (0.09 umol/kg/hr) using

minipumps implanted at the femoral vein (Section 5.2.2). Control animals received minipumps containing the vehicle (0.1% ascorbate) using the same mode of delivery.

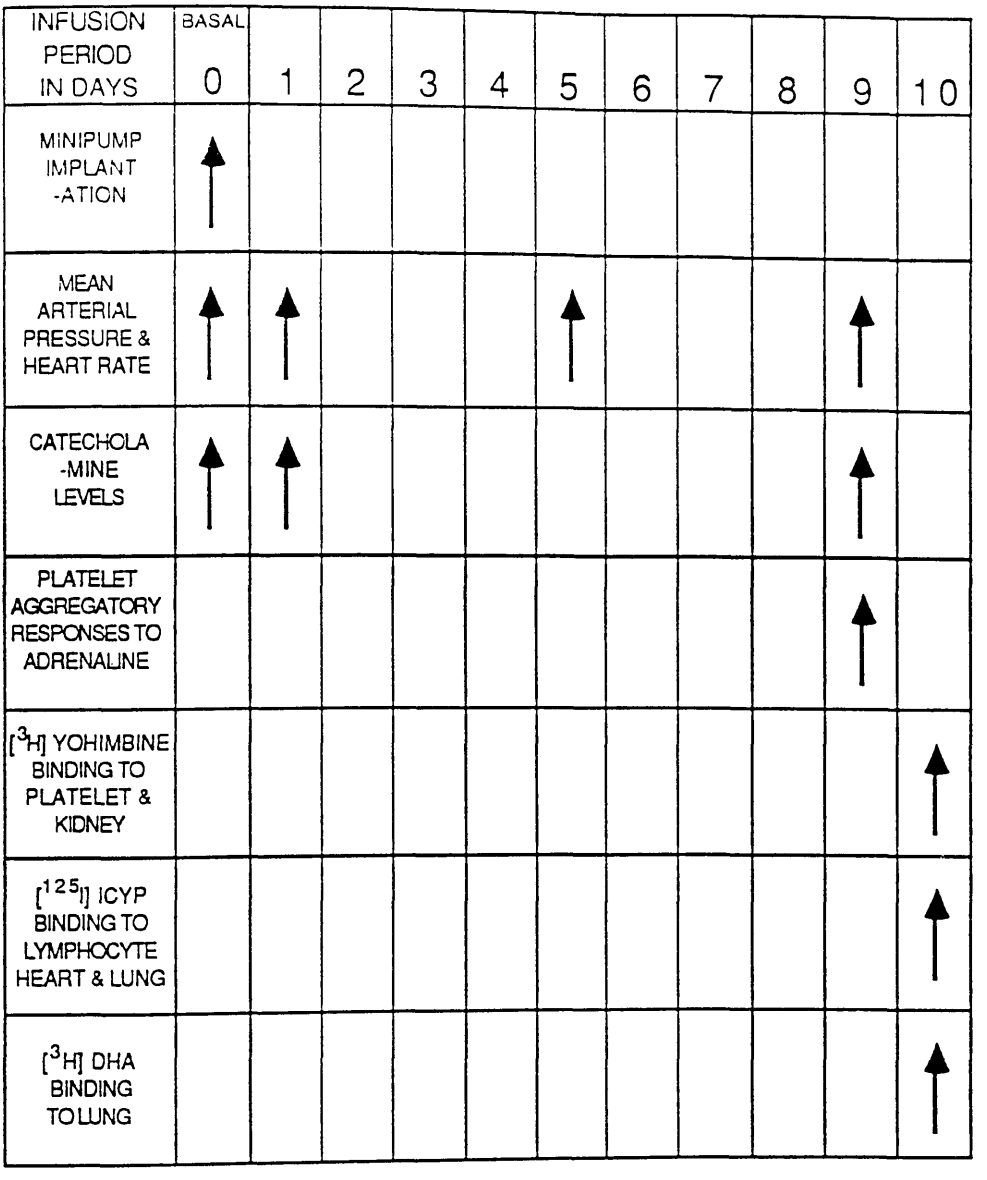
6.2.1 Dose of noradrenaline

The aim, as in the previous study, was to cause a tenfold increase in circulating levels of the catecholamine under investigation. The dose was estimated using clearance calculations as described in Section 5.2.1. Rabbit basal plasma noradrenaline is approximately 2 nM (Brown et al, 1987) therefore to achieve approximately 20 nM plasma concentrations, a dose of 46 ug/hr would be administered. For a 2.5 kg rabbit the rate of infusion is 18.5 ug/kg/hr or 0.09 umol/kg/hr.

6.2.2 Measurements taken during the infusion

The experimental design was similar to that described for the long term adrenaline infusions (Section 5.2) however the protocol has been summarised in Figure 6.1.

The mean arterial pressure (mmHg) and heart rate (beats/min) were monitored at specific intervals in conjunction with measurement of plasma catecholamines. Rabbits were killed on day ten with an intravenous overdose of sodium pentobarbitone (60 mg/kg). Whole blood (80 mls) was withdrawn immediately from the heart followed by removal of whole heart, lung and kidney. Platelets and lymphocytes were prepared for binding studies as described in Sections 2.3.1, 2.3.2 and 2.3.3. Heart, lung and kidney were also prepared for ligand binding according to the methodology in Section 2.3.4.



A group of 12 rabbits administered Noradrenaline (0.09 µmoles/kg/hr) or ascorbate (0.1%) for 10 days.

Figure 6.1

Experimental protocol for the long term noradrenaline infusions

Platelet aggregatory responses to adrenaline were quantified for groups of eight rabbits after ten day ascorbate or noradrenaline infusions (Method 2.4). These experiments were accompanied by measurement of [^{3}H] yohimbine binding to platelets and kidney (Section 2.5) together with [^{125}I] Iodocyanopindolol (ICYP) binding to lymphocytes, heart and lung all in groups of six rabbits (Section 2.6). [^{3}H] Dihydroalprenolol (DHA) (0.15 - 7.2 nM) binding to lung membranes was carried out for a group of four rabbits, these assays following the techniques described for [^{3}H] yohimbine binding to the kidney.

Statistical analysis was performed using the Wilcoxon test (Section 2.8).

6.2.3 Statistics

Results were analysed using the non-parametric Wilcoxon test (Section 2.7) for unpaired data. All numbers are quoted as means $\pm$ standard deviation with six or more animals in each group.

6.3 Results

6.3.1 Catecholamine concentrations

Circulating plasma noradrenaline levels in the treated animals (n = 12) were 3.1 $\pm$ 2.07 nM (basal) rising to 14.7 $\pm$ 10.7 nM after 24 hours infusion and 17.0 $\pm$ 5.6 nM after ten days noradrenaline treatment. There were no significant alterations in adrenaline concentrations during the noradrenaline infusions and no changes in catecholamine levels throughout the ascorbate infusion (Figure 6.2).

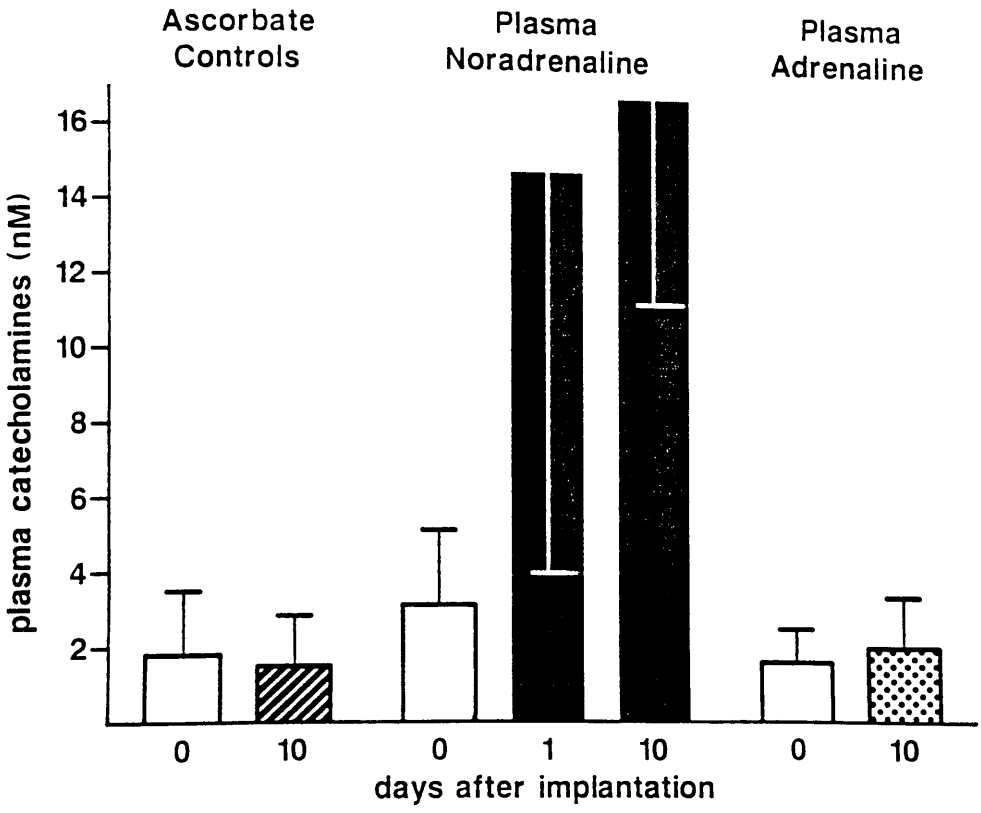


Figure 6.2

Plasma noradrenaline concentrations (nM) during noradrenaline or ascorbate infusion. Adrenaline levels during noradrenaline infusion are also shown.

BASAL

NORADRENALINE TREATED

ASCORBATE TREATED

ADRENALINE

The rate of noradrenaline infusion was 0.09 umol/kg/hr.

(Results are expressed as the mean ± standard deviation for a group of 12 rabbits).

6.3.2 Blood pressure and heart rate responses

Chronic noradrenaline administration in this model resulted in no significant differences in the mean arterial pressure (Figure 6.3). There were also no changes in the heart rate after ten days noradrenaline although a significant decrease was observed after 24 hours (233 $\pm$ 27, basal compared to 202 $\pm$ 21, treated, $P < 0.01$).

6.3.3 Platelet aggregation responses

The effects of chronic intravenous noradrenaline infusion on the ADP induced aggregation to adrenaline (in the presence and absence of propranolol) are illustrated as dose response curves in Figure 6.4. No significant changes in Emax (cm) were observed between the control and the treated animals, nor were there any differences in C_{50} (nM) as shown in Table 6.1. There were also no significant alterations in the responses to ADP (Figure 6.5).

6.3.4 [^{3}H] Yohimbine binding to platelet and kidney

After ten days noradrenaline infusion a significant reduction in [^{3}H] yohimbine binding to kidney membranes was observed (93.4 $\pm$ 38, control compared to 30.4 $\pm$ 15.7, treated, fmoles/mg protein, $P < 0.01$) without comparable falls in the platelet (22.7 $\pm$ 4, control compared to 27.0 $\pm$ 6.2, treated, fmoles/10^9 platelets, $n = 6$). The K_D did not significantly change for either tissue (Figure 6.6).

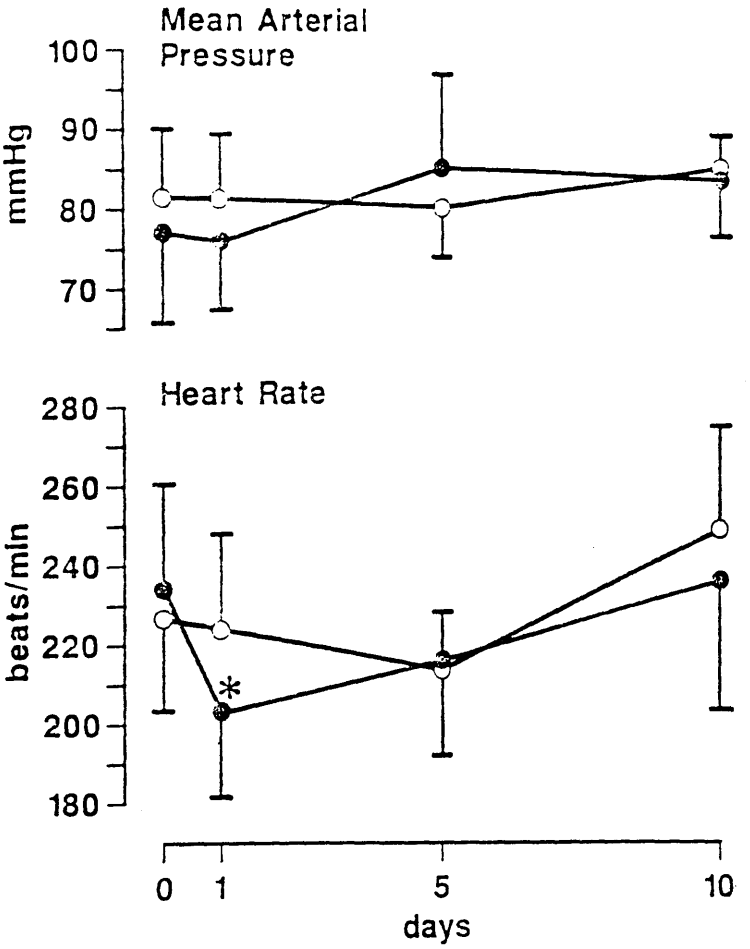


Figure 6.3

A comparison of the effects of ten days ascorbate and ten days noradrenaline infusion on the mean arterial pressure (mmHg) and heart rate (beats/min.)

○ ASCORBATE INFUSION

● NORADRENALINE INFUSION

* $P < 0.01$

The rate of noradreanline infusion was 0.09 umol/kg/hr.

Results are expressed as the mean ± standard deviation for a group of 12 rabbits.

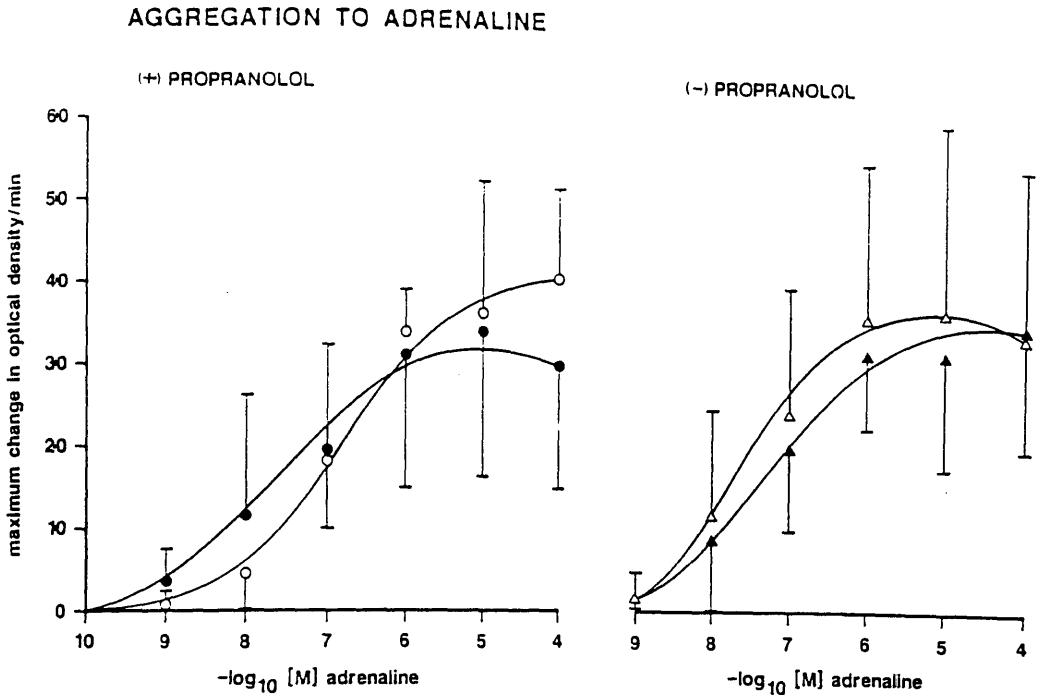


Figure 6.4

The effects of adrenaline (10^{-4}-10^{-10}M) on the pro-aggregatory responses of rabbit platelets from control and ten day noradrenaline treated animals

○ CONTROL) (+) PROPRANOLOL
● TREATED)

△ CONTROL) (-) PROPRANOLOL
▲ TREATED)

Results are expressed as the mean ± standard deviation for a group of eight animals.

Noradrenaline was infused at 0.09 umol/kg/hr.

Table 6.1

Hill plot analysis of the platelet aggregatory responses to adrenaline in control and noradrenaline treated animals

	With Propranolol			Without Propranolol		
	Emax	C_{50}	γ	Emax	C_{50}	γ
Control	33.2 ± 15	0.07 ± 0.08	1.4 ± 1.3	36.3 ± 22	0.03 ± 0.06	1.1 ± 0.3
Noradrenaline Treated	46.1 ± 12.4	0.03 ± 0.03	0.7 ± 0.2	37.6 ±14.1	0.01 ± 0.3	0.9 ± 0.4

Emax = The maximum change in optical density (mm)

C_{50} (nM) = The concentration of adrenaline which produced 50% of the maximum response

γ = The slope of the dose response relationship.

The rate of noradrenaline infusion was 0.09 umol/kg/hr for 10 days.

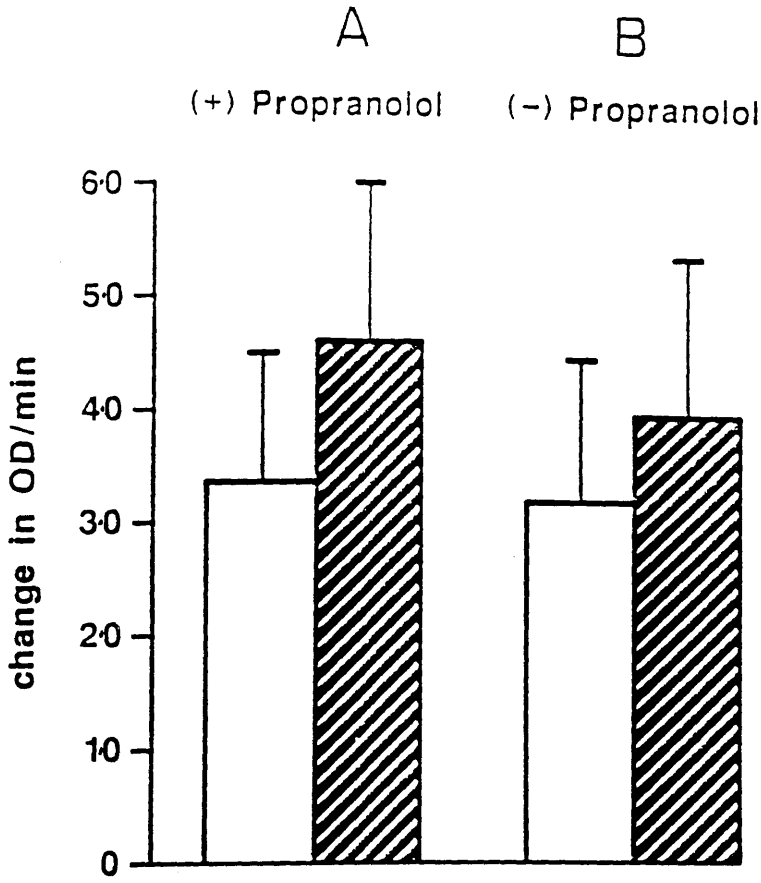


Figure 6.5

Platelet aggregatory responses to ADP in control and noradrenaline infused rabbits

□ CONTROL

▨ NORADRENALINE TREATED

Noradrenaline was infused at 0.09 umol/kg/hr.

(Results are expressed as the mean ± standard deviation for eight rabbits).

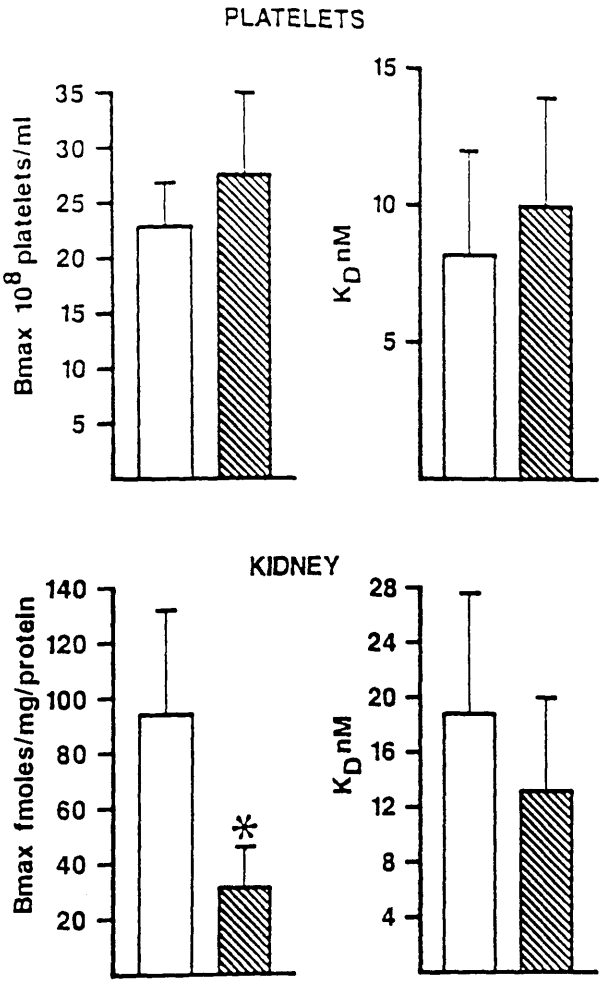


Figure 6.6

Changes in alpha$_2$ adrenoceptor density (Bmax) and the dissociation constant (K_D) in whole platelets and kidney membranes from control and ten day noradrenaline treated rabbits.

□ CONTROL

▨ NORADRENALINE TREATED

* $P < 0.01$

Noradrenaline was infused at 0.09 umol/kg/hr.

(Specific binding was measured using [^{3}H] Yohimbine and results expressed as mean ± standard deviation for six animals).

6.3.5 [^{125}I] Iodocyanopindolol binding to lymphocytes, heart and lung

There were no significant changes in the maximum number of binding sites (Bmax) in lymphocyte membranes (5.1 ± 1.7 control compared to 3.6 ± 0.4, treated fmoles/mg protein, n = 5) following chronic noradrenaline exposure in the rabbit.

These findings were in contrast to the highly significant fall in specific binding (73%) found in heart membranes (16.1 ± 4, control compared to 4.3 ± 1.8, treated, $P < 0.001$, fmoles/mg protein, n = 8). Similarly, significant decreases resulted in specific binding (47%) to lung membranes (75.2 ± 18.3, control and 40.0 ± 18.8, treated, fmoles/mg protein, n = 9, $P < 0.01$). The K_D (pM) was also reduced significantly for heart and lung tissues (Figure 6.7). Using another beta adrenoceptor specific ligand, [^{3}H] dihydroalprenolol, a significant loss of lung, beta receptors (66%) resulted with no alteration in the K_D which was 1.7 ± 0.7 in controls compared to 1.9 ± 0.4 nM in noradrenaline treated animals. **B_{max} values were 286±123 in controls compared to 96±10 in the treated animals (fmoles/mg protein).**

6.4 Discussion

Prolonged administration of the endogenous agonist, noradrenaline in rabbits, resulted in tissue specific decreases in both alpha$_2$ and beta adrenergic receptor number. The absence of an alteration in the alpha$_2$ adrenoceptor concentration in platelets was consistent with there being no alteration in function as measured by platelet aggregation. These results were comparable with studies showing the alpha$_2$ receptor to be more

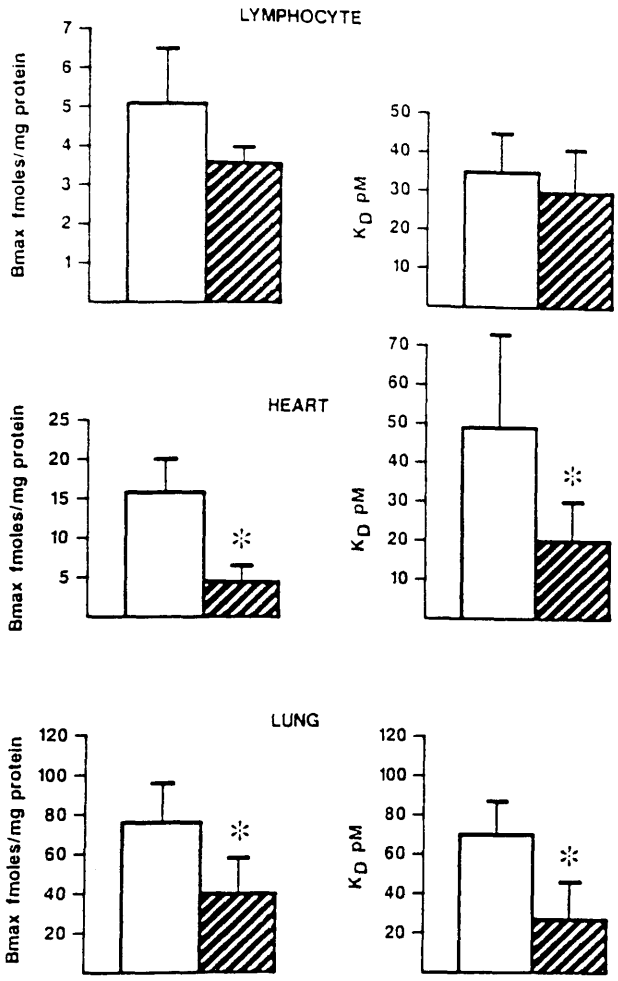


Figure 6.7

Changes in beta adrenoceptor density (Bmax) and dissociation constant (K_D) in lymphocyte, heart and lung membranes from control and noradrenaline treated animals

□ CONTROL

▨ NORADRENALINE TREATED

* $P < 0.001$

Noradrenaline was infused at 0.09 umol/kg/hr.

Specific binding was assessed using [^{125}I] ICYP and results expressed as mean ± standard deviation for a group of six rabbits.

resistant to down regulation in the face of high circulating noradrenaline (Snavely et al, 1982; Jones et al, 1985a). There were, however, marked reductions in the density of kidney alpha$_2$ receptors in this study. These findings were in contrast to those of Snavely et al (1985) who observed a fall in alpha$_1$ receptors without concomitant changes in alpha$_2$ adrenoceptors in rat renal cortex during similar increments in plasma noradrenaline. Possible reasons for these tissue selective decreases in alpha$_2$ receptor number are discussed in chapter eight.

Profound reductions in beta adrenoceptor number were observed for heart and lung, both of which contain largely beta$_1$ adrenoceptors in the rabbit (Section 3.3.1). This down regulation was in accordance with other studies in which raised plasma noradrenaline levels caused a fall in beta$_1$ adrenoceptor density (Tsujimoto et al, 1984; Brodde et al, 1986). Contrasting results were obtained for the lymphocyte showing an absence of change in the number of beta$_2$ adrenoceptors following noradrenaline infusion. These findings could be explained by considering that noradrenaline may possess a lower affinity for the beta$_2$ than the beta$_1$ subtype of the receptor. It has been postulated that the beta$_2$ receptors are extrajunctional receptors particularly sensitive to the hormone, adrenaline, whereas the beta$_1$ receptors are postsynaptic junctional receptors particularly sensitive to neurotransmitter noradrenaline released from the sympathetic nerve endings (Ariens and Simonis, 1983). The beta$_2$ adrenoceptor could be less susceptible to down regulation as described previously in section 5.4. Beta$_2$

adrenoceptor resistance to down regulation has been shown in situations other than phaeochromocytoma and heart failure when noradrenaline levels have also been high. In one such study conducted by Minneman et al (1979), repeated treatment with the tricyclic antidepressant desmethylimipramine to adult rats for ten days caused a forty percent decrease in the density of $beta_1$ adrenergic receptors in the cerebral cortex without concurrent falls in the density of $beta_2$ receptors. Thus there may be independent regulation of the $beta_1$ and $beta_2$ subtypes of the beta adrenergic receptor.

No alterations in the K_D values for [^{125}I] ICYP binding were observed in lymphocyte membranes at the end of ten days noradrenaline infusion however, significant reductions were demonstrated in heart and lung. No changes in affinity were noted when the infusions were repeated using another beta adrenoceptor selective ligand, [^{3}H] DHA, yet there was a similar significant loss of beta adrenoceptors sites. Therefore, the current findings could be attributed to an artifact of the ligand, [^{125}I] ICYP related to its very high affinity for the beta adrenoceptor. Tsuji et al (1987) also observed a significant decrease in the K_D with this ligand and differences in K_D have also been encountered while using other iodinated ligands (Hedberg and Mattson, 1981). Reasons for these discrepancies remain to be fully resolved.

Chronic noradrenaline infusion did not significantly alter the blood pressure at each of the recorded intervals. Infusions of this agonist would, however, be expected to produce a rise in

blood pressure by acting via $alpha_1$ and $alpha_2$ postsynaptic adrenoceptors causing vasoconstriction (Reid and Hamilton, 1980). Increases in blood pressure have been recorded by Tsujimoto et al (1984) in rat phaeochromocytoma, however they achieved forty fold elevations in plasma noradrenaline, considerably higher than the present study. Earlier investigations (chapter four) examined acute administration of this agonist (2-3 hr infusion) and there was an initial rise in blood pressure which then returned to pre-infusion levels after approximately two hours of infusion in fifty percent of the animals. These observations suggested a rapid desensitisation of adrenoceptors. This diminished responsiveness, followed by down regulation would explain the lack of changes in blood pressure, although measurement of adrenoceptor changes in blood vessels would have helped to clarify the present findings. A significant fall in the heart rate was observed at twenty-four hours. The reason for this is unknown but it could be an adaptive mechanism resulting from an initial rise in blood pressure during noradrenaline infusion.

Therefore, in summary, these studies are in support of others which suggest that noradrenaline induced down regulation of $beta_1$ and $beta_2$ adrenoceptors does not always run in parallel.

Furthermore, platelet $alpha_2$ adrenoceptors appear to be more resistant to down regulation than kidney $alpha_2$ receptors in response to noradrenaline infusion.

CHAPTER SEVEN

THE TIME COURSE OF ALPHA$_2$ AND BETA ADRENOCEPTOR DOWN REGULATION

Chapter Seven

The Time Course of Alpha$_2$ and Beta Adrenoceptor Down Regulation

7.1 Introduction

A variety of drugs, diseases and physiologic states are associated with either an up regulation or a down regulation of adrenergic receptors and there is now an extensive supply of literature available to describe these changes. However there has been less mechanistic information documenting the time courses of adrenoceptor degradation and subsequent resynthesis under these conditions. Adrenoceptor down regulation has been implicated in certain clinical situations, for instance, the beneficial actions of some antidepressant drugs may be partly due to a progressive desensitisation of beta adrenergic receptors (Dye et al, 1983). Also, the degree of heart failure has been associated with a progressive decrease in beta adrenoceptor number (Fowler et al, 1986). The factors responsible for the rate at which these receptors are lost and the mechanism through which the extent of receptor loss is controlled are unknown. Su et al (1980) have suggested that the rate of receptor loss may be dependent on the extent of the initial uncoupling reaction while Pittman et al (1984) observed that the rate at which adrenoceptors were lost was correlated with the efficacies of the agonists used for desensitisation.

Agonist promoted down regulation of receptors has provided a useful tool for examination of changes in receptor expression with time (Mahan et al, 1987). The t 1/2 for β receptor down regulation was

four hours during *in vitro* incubation of cardiac myocytes with high concentrations of isoprenaline (1 umol/litre) (Karliner et al, 1986). However the rates of *in vivo* adrenoceptor down regulation tend to be much slower as the receptors are less accessible to agonists. Indeed a half life of twelve hours has been recorded for renal cortical beta receptor down regulation *in vivo* in the rat (Snavely et al, 1984) (Section 1.4.3). These authors suggest that agonist induced up and down regulation of adrenoceptors may be more relevant to *in vivo* receptor regulation and function than the irreversible antagonists which have frequently been used to examine receptor turnover.

The studies so far described in this thesis have assessed changes in receptor number in response to ten minute and ten day agonist infusions resulting in either no change or approximately fifty percent decreases in receptor number respectively. The current investigation aims to examine the course of *in vivo* down regulation with time by measuring the degree of receptor reduction after different durations of adrenaline infusion in the rabbit. Particular interest lay with examination of any differences in $alpha_2$ and beta adrenergic receptor systems as well as the critical time at which receptor degradation began to take place. Quantification of platelet $alpha_2$ adrenoceptors together with heart and lung beta adrenoceptors was carried out beginning with ten day administration of adrenaline and progressively decreasing infusion time down to twelve hours. Adrenaline was administered using osmotic minipumps at all infusion times.

7.2 Methods

Groups of rabbits (n = 6) were treated with adrenaline (0.05 umol/kg/hr) for 12 hours, 24 hours, 2, 3 or 10 days via osmotic minipumps implanted at the femoral vein. The control animals for each group (n = 4-8) received the vehicle, 0.1% ascorbate. The minipumps were primed at 37^{o}C to ensure that infusion commenced as soon as they were implanted for the early time points. Arterial blood samples (5 mls) were removed before and at the end of each adrenaline infusion for measurement of plasma catecholamine levels (Section 2.2). Recordings of the mean arterial blood pressure (mmHg) and heart rate (beats/min.) were obtained before and at the end of 24 hour and ten day infusions (Section 2.1). When infusions were completed, rabbits were killed with an intravenous overdose of sodium pentobarbitone (60 mg/kg) and blood (80 mls) was rapidly withdrawn by cardiac puncture together with collection of the whole heart and lung. Platelets were prepared and [^{3}H] yohimbine binding performed as described in Sections 2.3.1., 2.3.2., 2.4 and 2.5. Heart and lung membranes were prepared for [^{125}I] ICYP binding according to Methods 2.3.4 and 2.6.

Statistical analysis was carried out using a two way analysis of variance (ANOVA). This test examined the individual effects of treatment and time as well as an interaction of the two. In all three cases, Bonferoni multiple comparisons were calculated to see where the differences lay.

7.3 Results

7.3.1 Haemodynamic measurements

Plasma concentrations of adrenaline were raised approximately tenfold after each group of timed adrenaline infusions (Figure 7.1). The plasma adrenaline levels at the end of 12 hours adrenaline infusion may have been higher due to anaesthetic effects. There were no significant changes in the mean arterial blood pressure (mmHg) or heart rate (beats/min.) at the times recorded as previously illustrated in Figure 5.3.

7.3.2 Radioligand binding

[^{125}I] ICYP binding to heart and lung

A significant decrease in [^{125}I] ICYP binding to heart was apparent after ten days adrenaline infusion (18 $\pm$ 4 in controls compared to 9 $\pm$ 3 in treated animals, $P < 0.001$) (Figure 7.2). There were also significant falls in [^{125}I] ICYP binding to lung following both two and ten day infusions of adrenaline (83 $\pm$ 14 in controls compared to 53 $\pm$ 9 after two days adrenaline infusion and 86 $\pm$ 14 compared to 46 $\pm$ 10 after ten days adrenaline infusion) (Figure 7.3). Although similar reductions in specific binding were apparently reached at other intermediate times between 24 hrs and ten days for both tissues, the results failed to achieve significance at the 5% level. However, large differences in the 95% confidence intervals were observed as illustrated in Figures 7.4. The K_D (pM) values did not significantly alter between control and treated animals at all infusion times (Table 7.1).

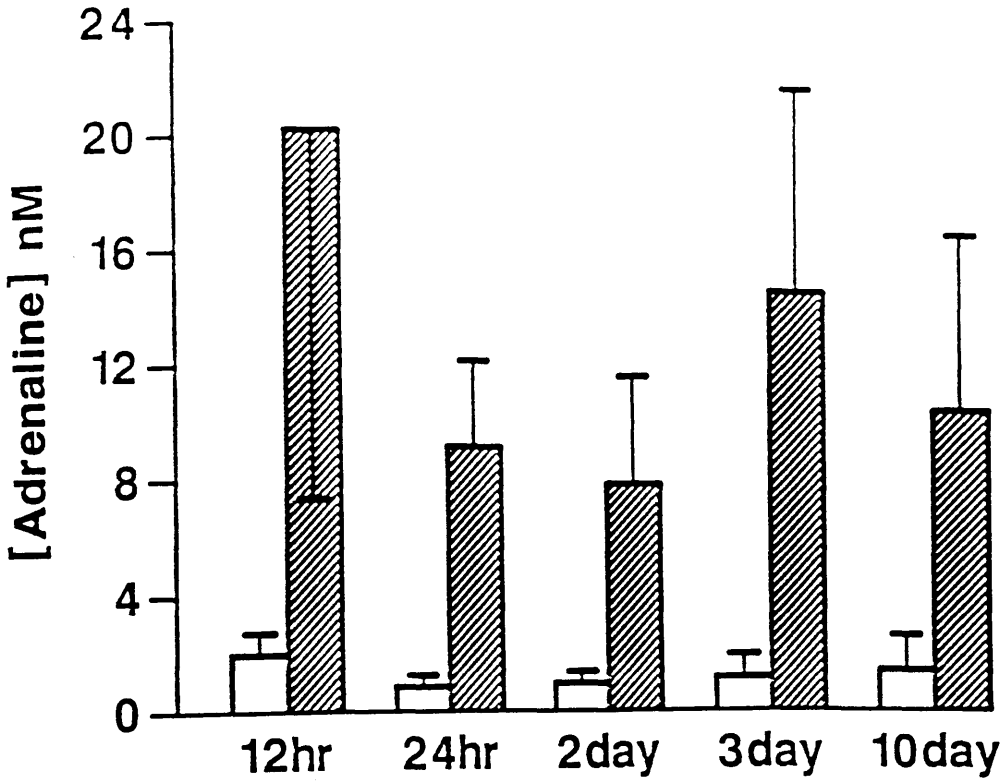


Figure 7.1

Plasma adrenaline concentrations following different durations of adrenaline infusion.

□ Control animals

▨ Adrenaline treated animals

Adrenaline was infused at 0.05 umol/kg/hr.

Control groups were comprised of between four and eight animals and treated groups, between five and seven animals.

Results were expressed as the mean ± standard deviation.

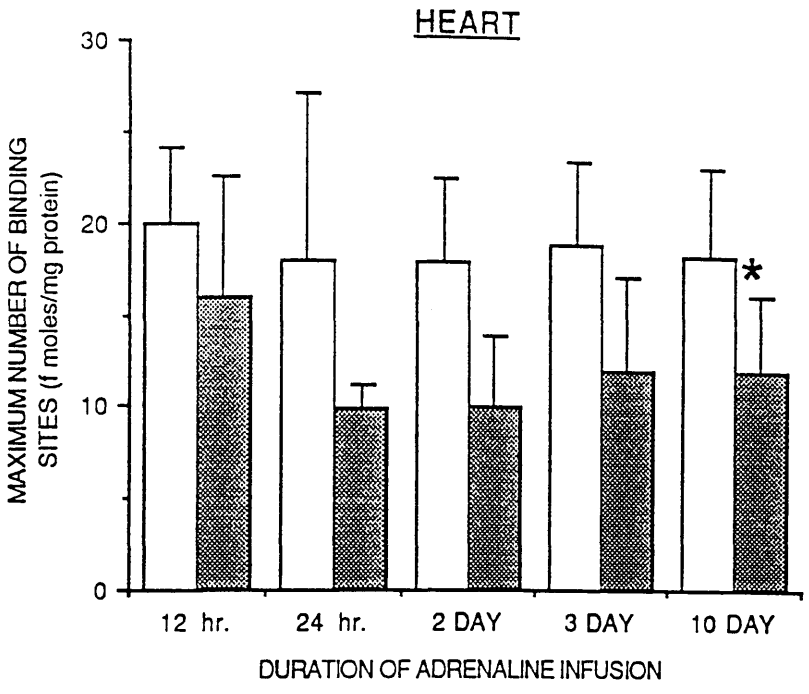


Figure 7.2

Changes in heart beta adrenoceptor number following different durations of adrenaline infusion

* $P < 0.05$

□ Control animals

■ Treated animals

Adrenaline was infused at 0.05 umol/kg/hr.

Results are expressed as the mean ± standard deviation for between four and eight controls and between five and seven treated rabbits.

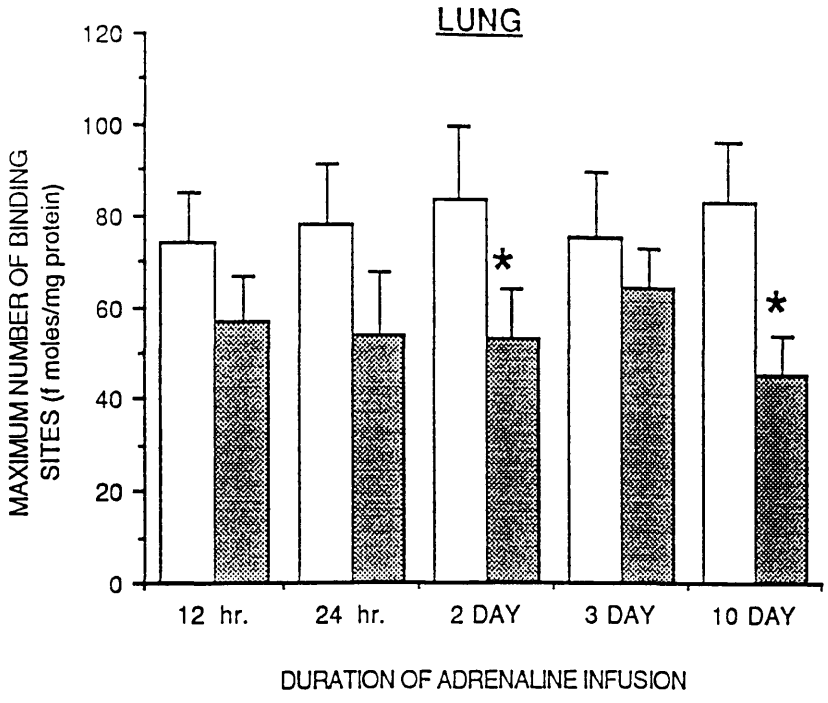


Figure 7.3

Changes in lung beta adrenoceptor number following different durations of adrenaline infusion

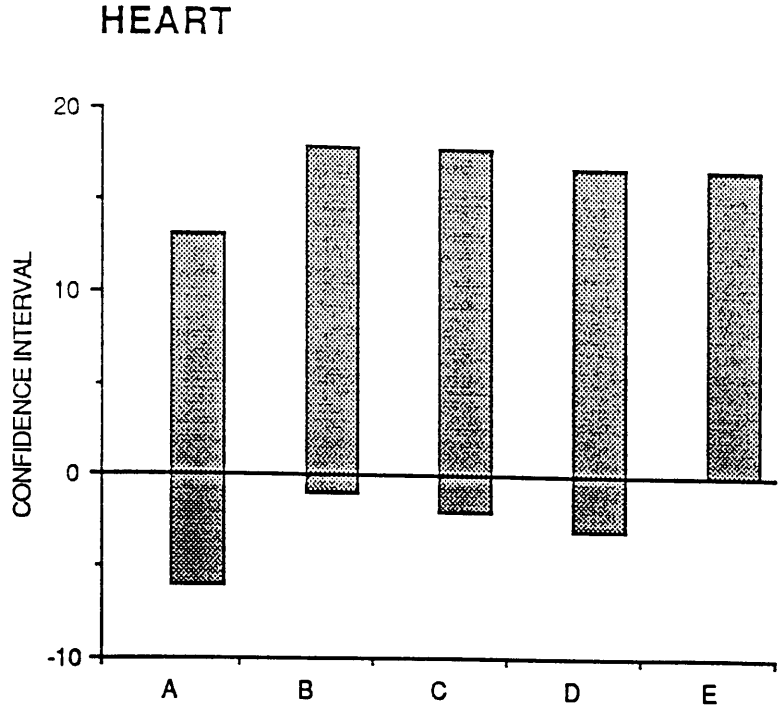
* $P < 0.05$

□ Control animals

■ Treated animals

Adrenaline was infused at 0.05 umol/kg/hr.

Results were expressed as the mean ± standard deviation for between four and eight controls and between five and seven treated animals.



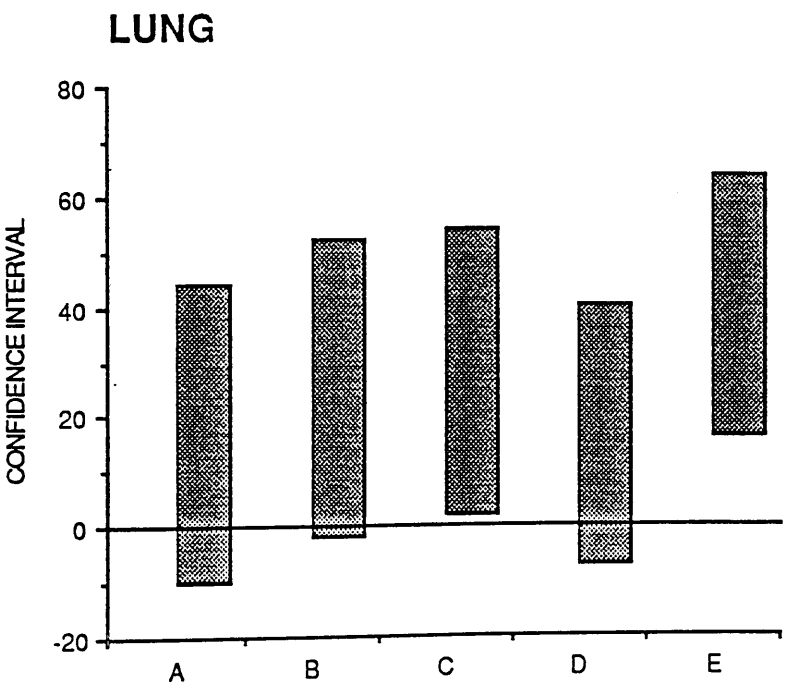


Figure 7.4

The 95% confidence intervals obtained from statistical analysis of [^{125}I] ICYP specific binding to heart and lung membranes

A 12 hour adrenaline infusion

B 24 hour " "

C 2 day " "

D 3 day " "

E 10 day " "

Results were analysed using a two way analysis of variance.

[^{3}H] Yohimbine binding to platelets

[^{3}H] Yohimbine specific binding was significantly reduced by ten days adrenaline infusion (22 $\pm$ 5 in controls compared to 10 $\pm$ 1). ($P < 0.01$) (Figure 7.5). The two way analysis of variance yielded a significant difference between control and adrenaline treated groups over time with no significant alterations detected between the platelet control groups. The first suggestion of a reduction in platelet alpha$_2$ adrenoceptor number occurred after three days of adrenaline infusion. Large differences in the confidence intervals at this time indicated a trend towards significance (Figure 7.6). There were no significant changes in the K_D for any of the tissues studied (Table 7.1). The reductions in alpha$_2$ and beta adrenoceptor density with time were finally expressed as a percentage of the control values as shown in Table 7.2.

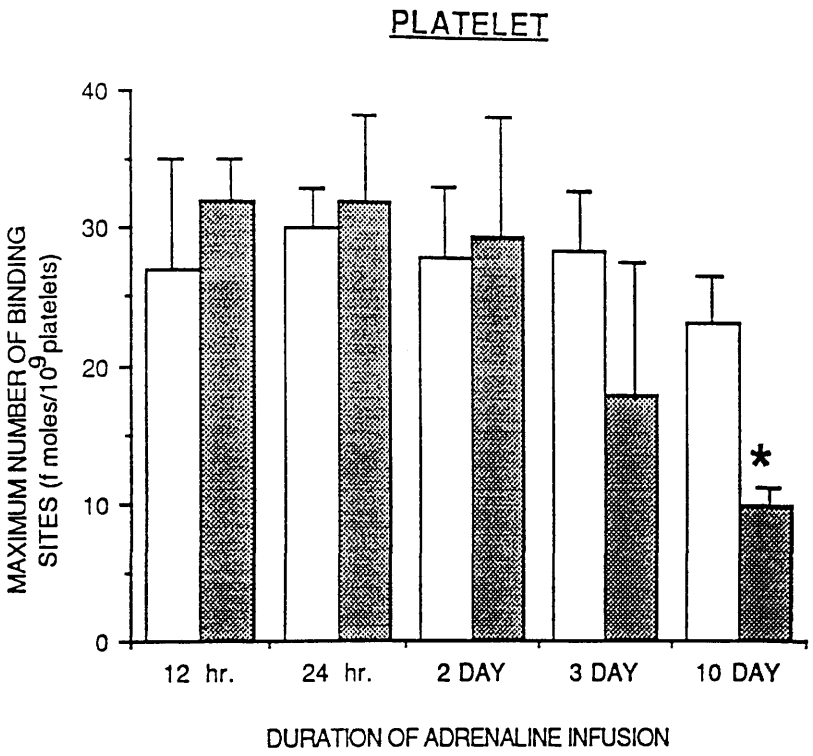


Figure 7.5

Changes in platelet alpha$_2$ adrenoceptor number following different durations of adrenaline infusion

* P < 0.01

□ Control animals

■ Treated animals

Adrenaline was infused at 0.05 umol/kg/hr.

Results were expressed as the mean ± standard deviation for between four and eight controls and between five and seven treated animals.

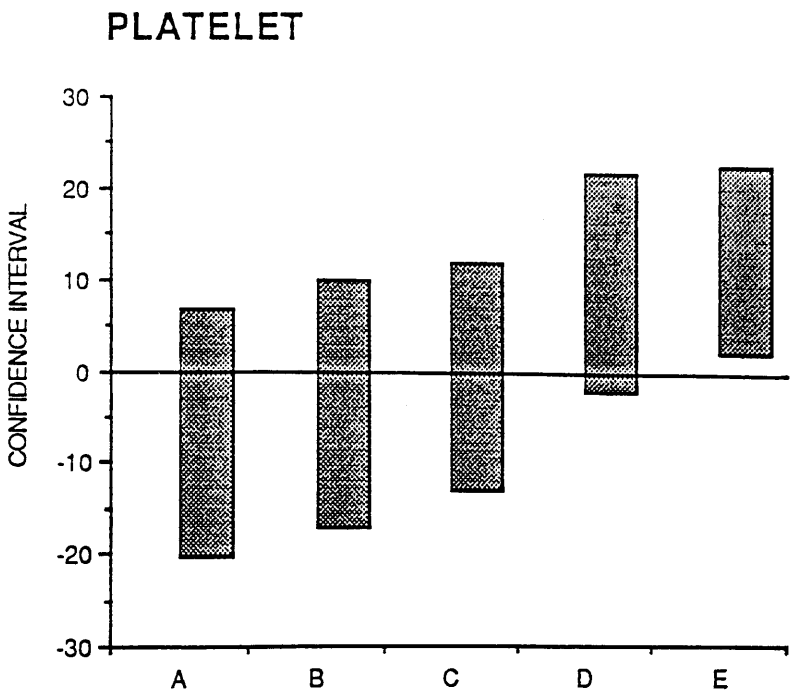


Figure 7.6

The 95% confidence intervals obtained from statistical analysis of [^{3}H] yohimbine binding to platelets

A	12 hour	adrenaline infusion
B	24 hour	" "
C	2 day	" "
D	3 day	" "
E	10 day	" "

Results were analysed using a two way analysis of variance.

Table 7.1

Changes in affinity (K_D) during adrenaline infusion

Tissue	Control	12 hr	Control	24 hr	Control	2 day	Control	3 day	Control	10 day
Heart (pM)	36 ± 12	26 ± 14	39 ± 11	42 ± 15	42 ± 7	37 ± 14	34 ± 9	23 ± 5	38 ± 12	44 ± 19
Lung (pM)	29 ± 10	22 ± 9	32 ± 13	29 ± 7	40 ± 13	40 ± 17	34 ± 10	22 ± 8	58 ± 5	44 ± 15
Platelets (nM)	13 ± 7	11 ± 3	8 ± 2	12 ± 7	15 ± 4	10 ± 3	10 ± 2	13 ± 6	10 ± 3	6 ± 2

Groups of between four and eight animals were used for control experiments and between five and seven animals for each adrenaline treatment.

Adrenaline was fused at 0.05 umol/kg/hr.

Results are expressed as the mean ± S.D.

Table 7.2

Reductions in adrenoceptor number (%) with different durations of adrenaline infusion

	12 hr	24 hr	2 day	3 day	10 day
Platelet alpha$_2$ adrenoceptors	0	0	0	36	57
Heart beta adrenoceptors	19	44	44	37	50
Lung beta adrenoceptors	23	32	36	14	45

The treated animals were comprised of groups of six while controls numbered between four and eight. Each value was expressed as a percentage of the control group.

7.4 Discussion

The present studies suggest that there may be differences in the rates of alpha$_2$ and beta adrenoceptor down regulation during *in vivo* agonist exposure. There was a twenty percent diminution of beta adrenergic receptors in both heart and lung within twelve hours of adrenaline treatment whereas a decrease in platelet alpha$_2$ adrenoceptor density was not detected until the end of three days' exposure to adrenaline. These studies suggest that disappearance of beta adrenoceptors began to take place earlier than twelve hours of adrenaline infusion thus further investigations to examine the effects of shorter adrenaline infusions would be useful. Similarly, the alpha$_2$ adrenoceptor changes suggest that the critical time of receptor disappearance

may be estimated by carrying out adrenaline infusions for times between two and three days. These findings are consistent with previously reported time courses for in vivo agonist promoted adrenoceptor down regulation. A reduction in [^{125}I] ICYP binding to renal cortex was observed within twelve hours of a three day isoprenaline infusion in rats (Snavely et al, 1984). However a much slower rate of down regulation was recorded by Brodde and co-workers (1982) during the treatment of hypertensive patients with clonidine for seven days. A reduced concentration of platelet alpha$_2$ adrenoceptors was revealed within three days of clonidine infusion in these studies. Differences in the time course of alpha$_2$ and beta adrenoceptors in these studies may have been related to differences in the doses together with differences in the agonist efficacy and intrinsic activity. More conclusive evidence of a differential down regulation of alpha and beta adrenoceptors could be achieved with assessment of agonist induced alterations in alpha$_2$ and beta adrenergic receptors within the same tissue. It has been suggested that in some tissues, the end organ response may depend on the ratio of alpha$_2$/beta adrenoceptors (Hamilton et al, 1986a). If down regulation of the two subtypes of adrenoceptor occurred at different rates, the ratio of alpha$_2$/beta adrenoceptors would change which in turn would modify tissue responses. In such tissues, responses would depend on both the extent and rate of desensitisation of alpha$_2$ and beta adrenoceptors.

In summary, the current findings strongly suggest that $alpha_2$ adrenergic receptor systems are more resistant to down regulation in the face of high circulating plasma adrenaline.

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE CONSIDERATIONS

Chapter Eight

General Discussion, Conclusions and Future Considerations

In the rabbit, short and long term intravenous infusion of alpha adrenoceptor agonists revealed reductions in $alpha_2$ adrenoceptor function, however only long term administration of agonist led to decreases in the density of $alpha_2$ adrenergic receptors. These findings were in accordance with those obtained during acute and chronic agonist treatment in man (Brodde et al, 1982; Davies et al, 1982; Jones et al, 1985a).

Acute administration of alpha methylnoradrenaline led to a rapid dose and time dependent densensitisation of platelet and vascular $alpha_2$ adrenergic receptors in the rabbit. Short term infusions of the endogenous agonist, adrenaline (1.5 umol/kg/hr) also caused a similar rapid reduction in platelet and vascular $alpha_2$ adrenoceptor mediated responses. The lower rate of adrenaline (0.05 umol/kg/hr) infusion failed to produce an attenuation of the vasopressor responses which were measured up to one hour into infusion. It is possible that the time course of desensitisation could be slower during these relatively low dose infusions and with continuation of infusion times, a decrease in vascular responsiveness could be seen.

One other group have reported a rapid (2-4 minute) time and concentration dependent desensitisation of alpha adrenoceptors during incubation of rat parotid cells with adrenaline (Strittmatter et al, 1977). It is postulated that this early loss of responsiveness may represent an "internalisation" of receptors similar to that observed in beta adrenoceptor systems.

Toews and Perkins (1984) measured the competition by beta adrenergic agonists and antagonists for [^{125}I](-) pindolol binding sites on intact cells (human astrocytoma and rat glioma) using short term binding assays. The change in the ligand binding properties of the receptor was found to be rapid (t 1/2 - 1-2 min), reversible and dose dependent.

The development of a hydrophilic alpha$_2$ adrenoceptor ligand analogous to the beta adrenoceptor antagonist [^{3}H] CGP-12177 would allow measurement of cell surface alpha$_2$ receptors to be made. Other techniques such as differential centrifugation have been used to examine the mechanisms of beta adrenoceptor desensitisation and might also be applied to studies of alpha$_2$ adrenoceptor desensitisation (Sibley et al, 1986). However, the location of the sequestered beta adrenergic receptors in desensitised cells is not yet known with certainty. Evidence has accumulated that the sequestered beta receptors can be recovered in small vesicular membrane particles and it is not yet clear whether these light membrane particles actually represent endocytotic vesicles or whether they might be a sequestered domain of the plasma membrane (Mahan et al, 1985; Sibley et al, 1986). Future studies may involve more detailed exploration of the mechanisms involved in alpha$_2$ adrenoceptor desensitisation.

When agonist infusions were extended to ten days, decreases in adrenergic receptor number were observed which were tissue specific. Chronic adrenaline infusions led to a decrease in the density of alpha$_2$ receptors in platelets without concomitant reductions in the kidney. These findings were in contrast to those found during long term noradrenaline infusion when a

significant decrement in kidney alpha$_2$ adrenoceptor number was observed, without comparable changes in the alpha$_2$ receptors in platelets (Table 8.1). A possible explanation of these opposing results for the two agonists could be the existence of subpopulations of the alpha$_2$ adrenoceptor, each agonist possessing a preferential affinity for one type. A report by Nahorski and colleagues (1985) outlines differences between species in the rank order of potency of antagonists determined by displacement binding experiments. These workers proposed that two separate subtypes (A and B) of the alpha$_2$ adrenoceptor could be discriminated, rabbit platelets containing predominantly the A type while rat and rabbit kidney were found to be largely B in type. A later study by the same group (Cheung et al, 1986) revealed that adrenaline had a higher affinity than noradrenaline for the A subtype in human platelets (with similar K_I values to rabbit platelets) and noradrenaline had a higher affinity than adrenaline for the B subtype in rat and rabbit kidney. Thus, the possible existence of discrete subgroups of alpha$_2$ receptors in the platelet and kidney may help to explain these results. Indeed, over the last few years, evidence indicating pharmacologic heterogeneity of alpha$_2$ adrenoceptors has mounted (section 1.1.3). Further investigations using both pharmacologic and autoradiographical techniques will help to achieve a clearer definition of alpha$_2$ adrenoceptor subgroups.

Table 8.1

Comparison of changes in $alpha_2$ adrenoceptor number following chronic adrenaline and noradrenaline infusions

	CONTROL (Bmax)	ADRENALINE (Bmax)	CONTROL (Bmax)	NORADRENALINE (Bmax)
PLATELET	23 ± 4	*10 ± 1	22 ± 4	27 ± 6
KIDNEY	99 ± 33	77 ± 27	93 ± 38	*30 ± 16

* $P < 0.01$

Bmax was expressed as fmoles/10^9 platelets or fmoles/mg protein and each group represents the mean ± standard deviation for six or more animals.

Desensitisation and down regulation in response to agonist stimulation does not appear to be a ubiquitous feature of $alpha_2$ adrenoceptor systems (Insel and Motulsky, 1987). Indeed, within the present studies, long term exposure to noradrenaline failed to alter either the aggregatory response to adrenaline or the concentration of platelet $alpha_2$ receptors. However, chronic administration of adrenaline was associated with a decrease in $alpha_2$ adrenoceptor response and number. Thus, adrenaline may be more important in the regulation of platelet $alpha_2$ adrenoceptor number. Other drugs and hormones have been reported to cause tissue specific changes in $alpha_2$ adrenoceptor number. Oestrogen treatment in rabbits increases the number of $alpha_2$ adrenoceptors in the uterus and bladder (Elliot et al, 1980; Roberts et al, 1977) but decreases that in the platelet and causes no change or a relatively small decrease in number in brain, spleen and kidney (Mishra et al, 1985; Roberts et al, 1979) while treatment with the antidepressant, amitriptyline has been reported to attenuate responses to intracisternal clonidine and reduce the density of $alpha_2$ receptors in the high affinity state in rabbit hindbrain without causing concomitant changes in platelet $alpha_2$ adrenoceptor number or aggregation (Hamilton et al, 1986b). Thus, the usefulness of the $alpha_2$ adrenoceptor in platelets as a model for $alpha_2$ adrenoceptors in other organs and tissues remains equivocal. In addition to $alpha_2$ adrenoceptor subtypes, drug efficacy, differential accessibility of the receptor to drugs and hormones and differences in the rate of metabolism of the receptor by the cells on which they are situated could all affect the rate of desensitisation and down regulation and contribute to

apparent tissue selectivity.

Tissue specific alterations in beta adrenoceptor density were also demonstrated in these investigations. Long term administration of both adrenaline and noradrenaline caused striking reductions in beta adrenoceptor density in heart and lung (Table 8.2), both these tissues containing a majority of $beta_1$ receptors in the rabbit (chapter three). No decrease in lymphocyte $beta_2$ adrenoceptor number was found during long term treatment with either adrenaline or noradrenaline. These results were surprising considering that the desensitisation and down regulation of lymphocyte $beta_2$ receptors in response to chronic agonist treatment in asthmatic patients has been well documented (Connolly and Greenacre, 1976; Galant et al,1978; Tashkin et al, 1982). In the present studies it is possible that infusion of a higher dose of adrenaline could have produced a decrease in lymphocyte $beta_2$ adrenoceptor number. In a separate study utilising the present model, Deighton et al (1987) found the $beta_2$ adrenoceptors in rabbit skeletal muscle to be down regulated in response to chronic adrenaline infusion but not noradrenaline infusion. These results were, as expected, considering that adrenaline has potent $beta_2$ adrenoceptor activity and noradrenaline is relatively selective for $beta_1$ receptors (Minneman et al, 1981). Thus, down regulation of $beta_2$ receptors following long term exposure to adrenaline may be tissue specific. Several investigators have, however acknowledged that the $beta_2$ adrenoceptor could be less susceptible than the $beta_1$ receptor to agonist promoted down

Table 8.2

Comparison of changes in beta adrenoceptor number following chronic adrenaline and noradrenaline infusions

	CONTROL	ADRENALINE	CONTROL	NORADRENALINE
LYMPHOCYTE	5 ± 2	6 ± 3	5 ± 1	4 ± 0.4
HEART	18 ± 5	*9 ± 3	16 ± 4	*4 ± 2
LUNG	84 ± 13	*46 ± 10	75 ± 18	*40 ± 19

* $P < 0.01$

Bmax was expressed fmoles/mg protein and each group represents the mean ± standard deviation for six or more animals.

regulation. Indeed, the resistance of $beta_2$ adrenoceptors to down regulation has been found in tissues such as rat brain (Minneman et al, 1979), vascular smooth muscle (Cohen and Schenck, 1987) and mesenteric artery (Tsujimoto and Hoffman, 1984). These examples, together with several others, have been summarised in Table 8.3. This proposed independent regulation of $beta_1$ and $beta_2$ adrenoceptors is compatible with reports which suggest that the two receptor subtypes may be differently coupled to adenylate cyclase (Dickinson and Nahorski, 1983; Gille et al, 1985). Moreover, functional studies carried out by Broadley and co-workers reveal that $beta_1$ adrenoceptors may be innervated receptors while $beta_2$ adrenoceptors are non-innervated (possibly hormonal) receptors.

In the present studies, it would have been interesting to assess the relative proportions of both $beta_1$ and $beta_2$ adrenoceptors in heart and lung in control and adrenaline treated animals. The extent of agonist induced changes in $beta_1$ and $beta_2$ receptors within the same tissue may then be determined. Alterations in the balance of these two receptor subtypes in the affected tissues could produce alterations in the tissue selectivity of $beta_1$ and $beta_2$ subtype selective agonists and antagonists.

Before leaving the subject of beta adrenoceptor down regulation, the usefulness of the peripheral $beta_2$ adrenoceptors in blood lymphocytes as a model for beta adrenoceptor changes in less easily accessible tissues must be considered. Studies undertaken in man have examined the validity of using lymphocyte $beta_2$ receptors as an index of changes in beta adrenoceptor

Table 8.3

Evidence that $beta_1$ and $beta_2$ adrenoceptors can be independently regulated

Regulatory factor	Receptor subtype		References
Decreased noradrenaline levels	$Beta_1$	$Beta_2$	
- 6-OH Dopamine	Increase	No change	Minneman et al, 1979
- Reserpine	Increase	No change	Broadley et al, 1986
Increase noradrenaline levels			
- Desipramine	Decrease	No change	Minneman et al, 1979
- Phaeochromocytoma implants	Decrease	No change	Snavely et al, 1983
Adrenaline infusion	Decrease	Decrease or no change	Tsujimoto and Hoffman, 1984
Isoprenaline infusion	Decrease	No change	Cohen and Schenck, 1987
Thyroxine	Increase	Decrease or no change	Scarpace and Abrass, 1981
Methimazole	Decrease	No change	O'Donnell et al, 1987
Ageing	Increase or no change	Decrease	Pittman et al, 1980

Reference has been made to both functional studies and radioligand binding studies.

number in the heart. One group (Michel et al, 1986) concluded that changes in lymphocyte $beta_2$ adrenoceptors can be taken as representative for changes in $beta_1$ and $beta_2$ receptors in solid tissues only when these changes are caused by non-selective beta adrenergic agents (e.g. isoprenaline or propranolol). However, if subtype selective drugs are applied, changes in lymphocyte $beta_2$ adrenoceptors mirror precisely changes in $beta_2$ adrenoceptors in solid tissues but only very slightly changes in $beta_1$ adrenoceptors. In contrast, however, human studies by Hausen et al (1983) and animal studies by Jones et al (1985b) have both demonstrated no correlation between changes in receptor density in lymphocyte and cardiac membranes. In the present investigations, changes in the concentration of lymphocyte $beta_2$ receptors could be used as a marker of changes in solid tissues following chronic noradrenaline infusions, however caution is advised when extrapolating binding data from lymphocytes to solid tissues. Finally, it is important to consider that lymphocytes are composed of B cells and different subsets of T cells which contain different densities of $beta_2$ adrenoceptors (Landman et al, 1984). Therefore, it is possible that changes in lymphocyte $beta_2$ adrenoceptors could be caused by a shift in the distribution of the different subsets of cells and not by (patho) physiological or pharmacological influences.

There were no long term changes in either the blood pressure or the heart rate during intravenous administration of adrenaline and noradrenaline in these studies although acute infusions of both agonists evoked an initial rise in the mean arterial pressure which could be attributed to vasoconstriction due to

stimulation of postsynaptic alpha$_1$ and alpha$_2$ adrenoceptors. Fifty percent of these animals recovered baseline blood pressures within one hour. In the chronic agonist infusion studies, there were no differences in blood pressure between ascorbate and agonist treated animals after twenty four hours or ten days of infusion (sections 5.3.2 and 6.3.2). It is likely that prolonged exposure to adrenaline and noradrenaline resulted in a desensitisation and down regulation of vascular adrenoceptors. Thus, measurement of adrenoceptor density in isolated blood vessels under these conditions would be an interesting subject of further research. In contrast, Tsujimoto et al (1984) observed increases in blood pressure in rats implanted with phaeochromocytoma. However this hypertensive effect was only recorded at plasma noradrenaline levels considerably greater (forty fold) than those achieved in the present study.

In conclusion, differences in the regulation of alpha$_2$ adrenergic receptors in different tissues may be consistent with the presence of further subtypes of this adrenoceptor in rabbit platelet and kidney. Furthermore, tissue selective changes in beta adrenoceptor density during chronic agonist infusion demonstrate that down regulation of beta adrenoceptor subtypes does not always run in parallel.

Further studies are warranted to elucidate further the observed regulatory phenomena and gain more knowledge of the underlying mechanisms of diseases associated with abnormal catecholamine levels.

SUMMARY OF CONCLUSIONS

The first set of investigations (chapter three) demonstrated that the platelet pro-aggregatory response to adrenaline is mediated through activation of $alpha_2$ adrenoceptors in the rabbit. [^{3}H] Yohimbine was shown to bind to sites corresponding to $alpha_2$ adrenoceptors in rabbit platelets and kidney. Displacement studies using [^{125}I] ICYP revealed that rabbit platelets and lymphocytes contain largely $beta_2$ adrenoceptors while the heart and lung were both predominantly $beta_1$ adrenoceptor in type.

Acute administration of the agonists alpha methylnoradrenaline and adrenaline led to a rapid dose and time dependent desensitisation of platelet and vascular $alpha_2$ adrenoceptors without any change in the density of $alpha_2$ receptors in platelets (chapter four). These results may be consistent with the rapid dose and time dependent internalisation of adrenoceptors which has been reported in beta adrenoceptor systems.

Ten day infusions of adrenaline (chapter five) were associated with decreases in platelet $alpha_2$ adrenoceptor pro-aggregatory responses to adrenaline and platelet $alpha_2$ receptor number without any alteration in the number of $alpha_2$ receptors in kidney. There were opposing findings following ten day infusions of noradrenaline (chapter six); reductions in $alpha_2$ adrenoceptor density being confined to the kidney only. Contrasting results for these two agonists may be consistent with the presence of further subtypes of $alpha_2$ adrenoceptor in rabbit platelet and kidney.

Both chronic adrenaline and chronic noradrenaline infusions caused significant falls in beta adrenoceptor density in rabbit heart and lung without concurrent changes in lymphocyte $beta_2$ receptor number. Thus, in this rabbit model the $beta_2$ adrenoceptor appears to be more resistant to agonist promoted down regulation.

The final set of studies (chapter seven) present evidence to suggest that there may be differences in the rates of $alpha_2$ and beta adrenoceptor down regulation in response to adrenaline infusion. Platelet $alpha_2$ adrenoceptors were found to be more resistant than the beta adrenoceptors of heart and lung to adrenaline promoted down regulation. Thus, in tissues harbouring both types of adrenoceptor, long term treatment with agonists may affect the ratio of $alpha_2$ and beta adrenoceptors which in turn would modify tissue responses.

PRESENTATIONS AND PUBLICATIONS CONTAINING THE WORK UNDERTAKEN FOR THIS THESIS

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