STUDIES ON THE PHARMACOLOGY OF RAT

ADIPOCYTE BETA-ADRENOCEPTORS

Thesis submitted to the University of Leicester for the degree of Doctor of Philosophy in the Faculty of Medicine

by

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Summary

The pharmacological characteristics of the rat adipocyte beta-adrenoceptor have been examined using radioligand binding techniques as well as analysis of biochemical and functional effector responses. For ligand binding studies, the binding characteristics of $^{125}$I cyanopindolol ($^{125}$ICYP), a newly developed ligand for beta-adrenoceptors, have been compared to those of $^3$H dihydroalprenolol ($^3$H DHA) in rat lung membranes to validate its further use in adipocytes. These studies demonstrated that $^{125}$ICYP binding was stereospecific, saturable and quantitatively similar to that of $^3$H DHA. Furthermore, inhibition of $^{125}$ICYP binding by selective and non-selective beta-adrenoceptor antagonists indicated that $^{125}$ICYP bound to sites with the pharmacological specificity of beta-adrenoceptors.

The ligand binding properties of beta-adrenoceptors on isolated adipocyte membranes have been examined and compared to those on rat lung and whole fat pad membranes. The results showed that $^{125}$ICYP bound with high affinity to isolated adipocytes and could be displaced by classical agonists with the rank order of potency associated with a beta$_1$ adrenoceptor. On the other hand, inhibition of binding by agonists in lung and whole fat pad gave a rank order of potency that was more typical of a beta$_2$ adrenoceptor classification. When agents selective for beta$_1$ or beta$_2$ adrenoceptor subtypes were used to inhibit binding, the resultant inhibition curves were biphasic in all three preparations, indicative of receptor heterogeneity. Analysis of the inhibition curves by computer curve fitting revealed that the majority of binding sites in isolated adipocytes could be identified as beta$_1$ adrenoceptors whereas lung and whole fat pad contained a predominance of beta$_2$ adrenoceptors.
The beta-adrenoceptor mediating the lipolytic response has been examined. These studies demonstrated that irrespective of whether lipolysis was stimulated by a beta\textsubscript{1} or beta\textsubscript{2} adrenoceptor selective agonist, the pA\textsubscript{2} values of the beta\textsubscript{1} selective antagonist betaxolol and the beta\textsubscript{2} selective antagonist ICI 118.551 were much lower than the values expected at typical beta\textsubscript{1} or beta\textsubscript{2} adrenoceptors. These results suggested that an atypical beta-adrenoceptor is coupled to the functional response. Analysis of cAMP accumulation in whole cells showed that while the rank order of potency of agonists at stimulating cAMP production was suggestive of a beta\textsubscript{1} adrenoceptor, the pA\textsubscript{2} values for betaxolol and ICI 118.551 against isoprenaline stimulation were similar to those obtained for antagonism of lipolysis. In addition, the isomers of the non-selective beta adrenoceptor antagonist propranolol displayed a lower stereoselectivity to that expected at typical beta adrenoceptors.

The pharmacology of the rat adipocyte beta adrenoceptor has been further investigated by examination of the membrane bound adenylate cyclase. The results from these studies showed that the rank order of potency of classical agonists at stimulating the cyclase was again indicative of a beta\textsubscript{1} adrenoceptor subtype. However, the pA\textsubscript{2} or K\textsubscript{i} values of a range of beta adrenoceptor antagonists at inhibiting the isoprenaline stimulated enzyme showed a poor correlation with their affinities at inhibiting \textsuperscript{125}I-CYP binding to beta\textsubscript{1} adrenoceptors. In contrast, a good correlation was found between the affinities of antagonists at inhibiting adenylate cyclase in rat reticulocyte membranes with their affinities at typical beta adrenoceptors as derived from binding studies. These findings further suggested that an atypical beta adrenoceptor which was coupled to adenylate cyclase was responsible for all of the effector responses studied. In order to determine whether typical beta\textsubscript{1}
adrenoceptors could be separated from atypical beta-adrenoceptors, an irreversible beta-adrenoceptor antagonist has been employed to selectively block typical but not atypical beta-adrenoceptors. These studies showed that whereas irreversible inhibition of beta-adrenoceptor binding sites led to a parallel decrease in the isoprenaline-stimulated adenylate cyclase activity in reticulocytes, the adenylate cyclase activity was unaffected in adipocytes. This indicated that typical beta_1 adrenoceptors were not involved in the cyclase response in fat cells and further strengthened the proposal that an atypical beta-adrenoceptor was responsible for the lipolytic effect of catecholamines.
Publications

The following publications have resulted from work described in this thesis:


CHAPTER 1

Introduction
1:1 Historical aspects of beta-adrenoceptor classification

1:1:1 General introduction

Many tissues undergo biochemical or physiological changes upon the interaction of hormones or neurotransmitters with specific cell surface receptors. This concept had its beginnings in 1878 when Langley investigated the interplay of atropine and pilocarpine on salivary flow in cats. In 1905, Langley proposed the existence of 'receptive substances' to explain the mutual competition of nicotine and curare at the neuromuscular junction. Furthermore, the notion of the receptive substance included its action as an acceptor for a stimulus in its transduction to an effector response. The term receptor was introduced by Ehrlich in 1913 and it was on the conceptual basis of this operational unit that receptor theory developed. Indeed, only comparatively recently has it been demonstrated that receptors are discreet molecular entities which interact with drugs in a manner not dissimilar to that proposed by Clark in 1926. This chapter deals with the historical development of beta-adrenoceptor classification followed by a review on the biochemical aspects of beta-adrenergic effects on fat cell lipolysis. The aim of this thesis has been to investigate the possibility of a third type of beta-adrenoceptor located on adipose tissue, thereby extending the concept that there are only two beta-adrenoceptor subtypes.

1:1:2 Anatomical aspects of adrenoceptor classification

The classification of adrenoceptors stems from the anatomical subdivision of the nervous system. The sympathetic nervous system which is part of the autonomic or involuntary nervous system, has outflowing nerves mainly in the intermediolateral region of the spinal cord between the eighth cervical and second or third lumbar
These axons synapse with sympathetic ganglia which are outside the cerebrospinal axis and the efferent postganglionic sympathetic fibres innervate and ramify a wide range of tissues. Chemical transmission from nerve to the effector organ is mediated by the neurotransmitter noradrenaline which interacts with adrenergic receptors located in the tissue. The sympathetic nervous system can also regulate bodily function by means of hormonal release of adrenaline from the adrenal medulla. This is circulated in the blood stream where it can produce adrenoceptor mediated responses in innervated and non-innervated tissues. The term adrenoceptor (= adrenergic receptor) stems from the early observations of researchers who found that extracts from the adrenal medulla were able to produce responses in sympathetically innervated tissues.

1:1:3 Alpha- and beta-adrenoceptors

The first evidence for two different types of adrenergic receptors was provided by Dale in 1906. He recognised that adrenaline interacted with a 'receptive mechanism for adrenaline' and demonstrated that ergot alkaloids had selective actions in blocking the effects of most excitatory but not inhibitory adrenergic responses. These studies were complicated by the uncertainty of the neurotransmitter located in sympathetic nerves and in the following years, the predominant theory that developed was that by Cannon and Rosenblueth (1937) who suggested that the inhibitory and excitatory actions of the sympathetic nervous system were attributed to two kinds of neurotransmitter, named sympathin I and E respectively.

The first major step forward towards the differentiation of adrenergic receptors came when Ahlquist in 1948 presented evidence for the existence of two classes of adrenoceptors based upon the
rank order of potency of six sympathomimetic amines. Thus the
adrenoceptors which mediated vasoconstriction, contraction of the
uterus, ureter and nictitating membrane, pupil dilation and gut
relaxation were termed alpha, while those that mediated vasodila-
tion, uterine relaxation and myocardial stimulation were termed
beta. Further evidence for Ahlquist's proposal was provided when it
was demonstrated that classical adrenergic blocking drugs such as
phenoxybenzamine, dibenamine and dibozane were fully capable of
antagonising alpha- but not beta-adrenoceptor mediated responses
(Nickerson 1949, Levy and Ahlquist 1960, Levy and Ahlquist 1961,
Moran and Perkins 1961). Although this hypothesis was at first slow
to gain acceptance, the cornerstone for this classification and
direct evidence for the existence of alpha- and beta-adrenoceptors
came with the development of dichloroisoprenaline, a specific beta-
adrenoceptor antagonist (Powell and Slater 1958, Moran and Perkins
1958). This antagonist decreased the inotropic and chronotropic
effects of adrenaline on the heart and blocked adrenergically
induced vasodilation. With the further development of drugs such as
pronethalol (Black and Stephenson 1962) and propranolol (Black et
al 1964), beta-blockers achieved clinical importance as anti-
anginal agents.

1:1:4 The dual beta-adrenoceptor hypothesis

Initial suggestions that beta-adrenoceptors could be further
subdivided came from studies on alpha-substituted derivatives of
methoxamine. Thus N-isopropyl methoxamine (Levy 1964, Burns et al
1964, Salvador et al 1964) reduced hyperglycaemia, free fatty acid
increase and uterine relaxation to agonists while butoxamine (Levy
1966a) and dimethyl isopropyl methoxamine (Levy 1966b) blocked
beta-adrenoceptors in the canine intestine, peripheral blood vessels
and rat uterus. These drugs had no effect, however, on the action of catecholamines on the heart. In 1967, Lands and co-workers proposed that beta-adrenoceptors could be further subdivided into two groups. This suggestion was based upon the rank orders of potency of a series of beta-sympathomimetic amines. Thus, adrenoceptors mediating cardiac stimulation and lipolysis in adipocytes where the general rank order of potency was isoprenaline > noradrenaline > adrenaline were named \( \beta_1 \), while those mediating vasodilation and tracheal smooth muscle relaxation where the rank order was isoprenaline > adrenaline > noradrenaline were termed \( \beta_2 \). Direct support for the dual beta-adrenoceptor concept came with the advent of drugs selective for each receptor subtype. Early antagonists of which practolol (Dunlop and Shanks 1968) was the prototype were apparently \( \beta_1 \) selective, antagonising beta-adrenoceptors in the heart selectively over those in the respiratory system. The rapid developmental expansion of drugs in this field has led to a vast array of selective agents, the most notable examples being the \( \beta_1 \) selective antagonist betaxolol (Boudot et al 1979), the \( \beta_2 \) selective antagonist ICI 118.551 (Bilski et al 1980) and the \( \beta_2 \) selective agonist procaterol (Yabuuchi et al 1977). In addition to the receptor selective drugs, other agonists such as salbutamol (Brittain et al 1968) and prenalterol (Carlsson et al 1977) which have been termed \( \beta_2 \) and \( \beta_1 \) selective respectively, have been shown to be selective by virtue of a selective efficacy at producing a response rather than selectivity at the receptor itself (Minneman et al 1979c, Hedberg and Mattsson 1981).

At present, there is definitive evidence for only two subtypes of mammalian beta-adrenoceptor (Minneman et al 1979b). Recently, Lefkowitz, Caron and co-workers have examined the molecular properties of both subtypes with photoaffinity labelling techniques.
Lavin et al 1982, Stiles et al 1983a, Benovic et al 1983). These studies have indicated that beta\textsubscript{1} and beta\textsubscript{2} adrenoceptor binding subunits reside on a peptide of molecular weight 62 - 64,000. Further investigations by Stiles et al (1983b) using peptide mapping approaches have shown that the peptide fragments of beta\textsubscript{1} and beta\textsubscript{2} adrenoceptors are different indicating that they are different proteins. Interestingly enough, whereas the peptide structure of the beta\textsubscript{2} adrenoceptor appears to be well conserved in a range of species, there are slight interspecies variations in the structure of the beta\textsubscript{1} adrenoceptor.

In this study, an attempt has been made to characterise the beta-adrenoceptor on adipocytes with the use of several selective agonists and antagonists to determine whether this receptor can be accommodated within the dual beta-adrenoceptor hypothesis or whether it represents a novel beta-adrenoceptor subtype responsible for the lipolytic action of catecholamines (see later).

1:1:5 The coexistence of beta\textsubscript{1} and beta\textsubscript{2} adrenoceptors in tissues

With the development of the dual beta-adrenoceptor hypothesis (see Arnold et al 1968, Arnold and McAuliff 1968, Lands et al 1969, Arnold 1972), it was generally accepted that tissue responses were mediated by a single receptor subtype. Evidence against this was first put forward by Carlsson et al (1972) who demonstrated differential blockade of chronotropic effects in the cat heart by selective beta\textsubscript{1} and beta\textsubscript{2} adrenoceptor antagonists depending upon the subtype selective agonist used. These results were interpreted on the assumption that beta\textsubscript{1} and beta\textsubscript{2} adrenoceptors coexisted in the heart and mediated the same functional response. Further evidence for this concept was provided by Ablad et al (1974), Furchgott and Wakade (1975) and Furchgott (1976) and has been
reviewed by Daly and Levy (1979). As a result of these and more recent studies, it has now been accepted that a variety of tissues have responses mediated by both beta-adrenoceptor subtypes. Therefore experiments described in this thesis have been performed to investigate whether a single beta-adrenoceptor subtype mediates the lipolytic response or whether it can be mediated by both beta_1 and beta_2 subtypes.

1:2 Biochemical and pharmacological aspects of lipolysis in adipocytes

The prime function of adipose tissue is the uptake, synthesis, storage and release of free fatty acids. Fatty acids are a major source of energy to the organism and can supply about half of the oxidisable substrate used by the body (Carlson 1968, Campbell and Hales 1976). They are stored in adipose tissue as esters mainly with glycerol in the form of immobile triglycerides. Upon release, they bind to plasma albumin, circulate in the blood stream and taken up by tissues where they are reesterified, oxidised or in the liver, converted to ketone bodies.

The control of lipolysis is under neurohormonal and metabolic control. Adipose tissue has a dense sympathetic innervation (Diculescu and Stoica 1970) and the role of the adrenergic system in the control of lipolysis has been well documented (Himms-Hagen 1967, Fredholm 1970, Scow and Chernick 1970). In addition to neuronal modulation, adipose tissue is responsive to exogenously applied adrenaline (Dole 1956, Gordon and Cherkes 1956, Gordon and Cherkes 1958). The fact that catecholamines have lipolytic effects in most species led Scow and Chernick (1970) to suggest that lipolysis is under dominant adrenergic control. In addition to catecholamine receptors, adipocytes are also responsive to other agents
depending upon the species examined. These include adrenocorticotrophic hormone, growth hormone, glucagon, vasoactive intestinal peptide, parathyroid hormone, 5-hydroxytryptamine, secretin, thyroid stimulating hormone, vasopressin, neurophysins, lipotropin, alpha and beta melanotropin as well as 'permissive' hormones such as glucocorticoids, thyroid hormones and antilipolytic agents such as insulin, prostaglandins and adenosine. These have been reviewed elsewhere (Steinberg 1976, Hales et al 1978, Fain 1982) and the general opinion is that the only other hormonal factor which has significant physiological actions is insulin.

The biochemical pathway between agonist-receptor occupation and activation of lipolysis is well documented. The subject has been critically reviewed by Robison et al (1971) and Steinberg (1976) and a simplified scheme is shown in figure 1:1. The major factor controlling the activity of hormone sensitive lipase is cyclic 3'5'adenosine monophosphate (cAMP), a detailed summary of its involvement in the lipolytic response of which is given in 1:2:2.

Occupation of beta-adrenoceptors by an agonist results in the activation of the membrane bound adenylate cyclase and the catalytic conversion of ATP to cAMP which is believed to be the second messenger in the transduction of stimulus to a lipolytic response. The cAMP binds to the regulatory subunit of the protein kinase dimer which leads to dissociation of the complex to liberate the active catalytic subunit. Phosphorylation of hormone sensitive lipase by protein kinase then results in the accelerated hydrolysis of triglycerides which is believed to be the rate limiting step in the reaction. In addition to triglyceride lipase activity, other acyl hydrolase activities are also present which can liberate free fatty acids from mono- and diglycerides and cholesterol ester. Deactivation of hormone sensitive lipase is achieved by two
independent mechanisms. There is present a lipase phosphatase which can rapidly deactivate the active lipase and cAMP phosphodiesterase which converts cAMP to 5'AMP which is inactive. These processes enable the lipase activity to be finely controlled enabling the adipocyte to be rapidly responsive to metabolic changes.

On the basis of the above pathway, experiments have been performed to investigate the characteristics of the beta-adrenoceptor on adipocytes. These studies have included analysis of the release of free fatty acids - the functional response, measurements of intracellular cAMP accumulation and of adenylate cyclase activity in membrane preparations.

1:2:1 Classification of the beta-adrenoceptor mediating lipolysis

The control of lipolysis in adipose tissue is mediated primarily by beta-adrenoceptors (Wenke 1966, Fain et al 1966, Fain 1967,
In their classical subdivision, Lands et al (1967) subclassified the rat adipocyte beta-adrenoceptor as a $\beta_1$ subtype on the basis of agonist rank orders of potency. This classification was later confirmed by Lefkowitz (1975) who examined adenylate cyclase and Williams et al (1976) and Malbon et al (1978) who demonstrated the same rank orders of potency at inhibiting specific $[^3\text{H}]DHA$ binding to fat cell membranes. This classification, however, has proved somewhat confusing when attempts have been made to delineate the beta-adrenoceptor with the use of antagonists. Stanton (1972) showed that in vivo, the non-selective antagonist propranolol and the $\beta_1$ selective practolol were ineffective at inhibiting the isoprenaline induced release of free fatty acids while they were capable of reducing the concomitantly produced chronotropic effect. These results were confirmed and further extended by Zaagsma and co-workers (Harms et al 1974, De Vente et al 1980) who clearly demonstrated that antagonists were weak at blocking the catecholamine-stimulated lipolytic response in vitro compared to their expected affinities at $\beta_1$ or $\beta_2$ adrenoceptors. These studies indicated that the rat adipocyte beta-adrenoceptor has a dualistic nature where the binding site for the aromatic part of arylethanolamines and aryloxypropanolamines has $\beta_2$ characteristics while the site that interacts with the ethanolamine side chain has $\beta_1$ characteristics. Further evidence for an atypical beta-adrenoceptor was provided by Harms et al (1977) who examined the stereoselectivities of several beta-adrenoceptor antagonists at inhibiting beta-adrenergic responses in adipocytes, atrium ($\beta_1$) and diaphragm ($\beta_2$). These results demonstrated that the ratio of affinities of each isomer was lower in adipocytes than the other tissues suggesting that the adipocyte beta-adrenoceptor was neither $\beta_1$ or $\beta_2$ in nature. Other studies by
Jolly et al (1978) have indicated a lipolytic response which appears to be beta_2 adrenoceptor mediated based upon the observation that the beta_2 selective agonist salbutamol was a full lipolytic agent while tazolol, a beta_1 selective agonist, had no effect. Clearly, there is a requirement for definitive experiments in order to classify the beta-adrenoceptor on adipocytes, particularly when a potentially different subtype may be involved. Therefore, several experimental approaches have been used to identify the beta-adrenoceptor, these include attempts to directly label the receptor using high affinity ligand binding techniques and to examine the various effector responses in the presence of selective and non-selective beta-adrenoceptor antagonists.

1:2:2 The role of cAMP in the lipolytic response

Since the discovery of cAMP by Rall and Sutherland (1958), it has been accepted that cAMP is the second messenger in the transduction of a hormonal or neuronal stimulus to an effector response in many tissues and species (Robison et al 1971). In adipocytes, the finding that adrenaline with ATP or cAMP on its own could activate lipase in a cell free lipolytic enzyme system (Rizak 1964), suggested that cAMP was involved in the lipolytic response. A substantial amount of evidence has accumulated to suggest that this is indeed the case:

(i) sympathomimetic amines activate adenylate cyclase in membrane preparations (Klainer et al 1962, Butcher and Sutherland 1967, Rodbell et al 1968)

(ii) cAMP activates protein kinase and hormone sensitive lipase in fat cell preparations (Rizak 1964, Soderling et al 1973), furthermore, dibutyryl cAMP is an effective lipolytic agent
(Aulich et al 1967, Blecher et al 1968)

(iii) lipolytic hormones increase intracellular cAMP levels
(Butcher et al 1968)

(iv) agents which increase cAMP through receptor independent mechanisms are also lipolytic. These include methylxanthines such as theophylline which inhibits cAMP phosphodiesterase and antilipolytic effects of adenosine (Schwabe and Ebert 1974, Hjemdahl and Fredholm 1976) and forskolin which directly stimulates the adenylate cyclase (Litosch et al 1982, Schimmel 1984)

(v) beta-adrenoceptor antagonists inhibit catecholamine-stimulated adenylate cyclase and lipolysis (Fain 1967, Birnbaumer and Rodbell 1969)

(vi) agents which attenuate cAMP accumulation also inhibit lipolysis, these include adenosine (Schwabe et al 1975), PGE$_1$ (Steinberg et al 1963, Butcher and Baird 1968) and insulin (Jungas and Ball 1963, Butcher et al 1968)

Although the above evidence would suggest a causal relationship between cAMP and lipolysis, there are doubts whether hormonal regulation of lipolysis is achieved solely through cAMP. These have developed as a result of observations which did not fit into the expected pattern of events, these are outlined below:

(i) there is a dissociation between the levels of cAMP and lipolysis which can continue at an accelerated rate even after cAMP levels have returned to basal (Manganiello et al 1971, Birnbaum and Goodman 1977)

(ii) a further dissociation occurs between cAMP and lipolysis with inhibitory agents. Adenosine and adenosine analogues can decrease cAMP levels but have little or no effect on lipolysis
Trost and Stock 1977, Fain et al 1979). Furthermore, the inhibitory effect of insulin on lipolysis cannot be accounted for by the small attenuation of cAMP levels in adipocytes (Kono and Barham 1973).

(iii) 5-Hydroxytryptamine can elevate cAMP levels and activate cAMP dependent protein kinase but has no effect on lipolysis (Honeyman et al 1979)

(iv) hormone sensitive lipase has different substrate specificities in homogenates exposed to adrenaline than cAMP dependent protein kinase (Wise and Jungas 1978)

(v) when forskolin is used to stimulate lipolysis, higher intracellular concentrations of cAMP appear to be necessary to produce the functional response as compared to isoprenaline stimulation through beta-adrenoceptors (Litosch et al 1982, Schimmel 1984)

(vi) an analogue of ACTH (adrenocorticotropin-(7-24)-octadecapeptide) was found to be effective at increasing cAMP levels in adipocytes but did not produce a concomitant effect on lipolysis (Lang et al 1976)

(vii) phosphatidic acid is able to inhibit cAMP accumulation at concentrations that have no effect on lipolysis (Schimmel et al 1980a)

These findings have led to two possible explanations. Firstly, lipolysis may occur through cAMP dependent and independent processes (Wise and Jungas 1978, Fain et al 1979, Schimmel 1984) and secondly, the compartmentalisation of cAMP and cAMP dependent protein kinases into specific pools may occur (Earp and Steiner 1978, Litosch et al 1982, Schimmel 1984). If only specific pools are necessary for the lipolytic response, the divergent effects of cAMP and lipolysis could be accounted for.

It is clear that the intracellular events leading from receptor activation to lipolysis are complicated by the above data. Since
cAMP is involved in the lipolytic response and most beta-adrenoceptors are linked to adenylate cyclase, it was pertinent to use this system in order to investigate the pharmacological characteristics of adipocyte beta-adrenoceptors at a site less distal than the lipolytic effector response. The experiments performed in this thesis therefore have involved the analysis of cAMP accumulation in whole cells and production in membranes where the role of compartmentalisation would be expected to be absent.

1.3 Current concepts of receptor-adenylate cyclase coupling

Rapid progress in the field of receptor-cyclase coupling has been made since Sutherland and Rall (1960) reported that catecholamines and other hormones may exert their effects by activation of adenylate cyclase. Initial concepts centred around the possibility that hormones interacted with receptors which were intimately linked to the catalytic component (Robison et al 1967). It has become apparent however, that this model was too simplistic and work in the early 1970s by Rodbell and co-workers (see Rodbell et al 1975) led to the proposal of a three component model consisting of receptor, guanine nucleotide regulatory protein and catalytic unit of adenylate cyclase. To date, several different approaches have been used to examine the interactions of these components during a hormonal stimulus, these have included:

(i) solubilisation and reconstitution studies (Pfeuffer 1977)
(ii) cell fusion approaches (Orly and Schramm 1976)
(iii) experiments on cells genetically deficient in the coupling components (Ross and Gilman 1977)
(iv) kinetic analysis of receptor-cyclase interactions (Tolkowski and Levitzki 1978)
(v) radioligand binding techniques (De Lean et al 1980)
(vi) target site analysis (Houslay et al 1977)

The above references are only representative examples of a very large body of evidence and fuller accounts of the techniques used and their implications have been the subject of several reviews (Hoffmann and Lefkowitz 1980, Rodbell 1980, Swillens and Dumont 1980, Limbird 1981, Stadel et al 1982). Stadel et al (1982) have put forward a general unifying model based upon much of the evidence to date (figure 1:2).

An agonist (H) binds to the receptor (R) which transiently leads to association with the GDP liganded guanine nucleotide regulatory protein (N). This complex has a low affinity for GDP resulting in its release and the formation of a high affinity ternary complex HRN. Upon interaction with GTP, the ternary complex dissociates to form HR and N.GTP complexes. The HR complex is of low affinity and may dissociate while the GTP liganded N protein has a high affinity for the inactive catalytic unit (C) to form GTP.NC which is the active state of adenylate cyclase. The activation is terminated by GTP hydrolysis to GDP and dissociation of NC. This model is applicable to the events found in binding studies, namely, the high and low affinity forms of agonist binding and conversion to a homogenous low affinity state in the presence of guanine nucleotides (De Lean et al 1980, Kent et al 1980).

It must be stressed, however, that this model is not without its anomalies. It cannot explain the reversal of the GppNHp-promoted persistently activated state of the enzyme with agonist and added nucleotide (Cassel and Selinger 1977) or the enhancement of agonist-promoted release of GDP from N in the presence of added nucleotide (Cassel and Selinger 1978). The former mechanism implies that the
(1) Hormone binding, (2) formation of high affinity state, 
(3) breakdown of high affinity state, (4) enzyme activation, 
(5) enzyme deactivation.

Figure 1:2 Proposed mechanism of hormonal activation of adenylate cyclase (from Stadel et al 1982).

HR complex can react with the guanine nucleotide liganded NC complex while the latter mechanism can be interpreted on the assumption that the guanine nucleotide regulatory protein has a dimeric or oligomeric nature. Furthermore, the model predicts complex kinetic events involved in the cyclase activation whereas Tolkovski and Levitzki (1978) have clearly shown that a collision coupling model which follows first order kinetics of activation can be applied. This model requires only the transient coupling of the HR complex with the catalytic unit in order for cyclase activation to occur. Although the collision coupling model accommodates the guanine
nucleotide regulatory component in the activation of adenylate cyclase, it does not explain the agonist formation of a high affinity state of binding which is believed to be the ternary complex (De Lean et al 1980, Stadel et al 1980). Other studies have also suggested that R and N units may exist as oligomers (Schlegel et al 1979, Rodbell 1980) or that several guanine nucleotide regulatory proteins may be involved (Svoboda et al 1978, Gill and Meren 1983).

The situation has clearly not been resolved, however in view of the fact that the ternary complex model as outlined by Stadel et al (1982) appears to be the most valid from the standpoint of ligand binding studies, it was held as the operative model during the course of this thesis. Experiments have been performed to investigate agonist displacement of radiolabelled antagonist binding and its modulation by guanine nucleotides to assess the possible coupling of the adipocyte beta-adrenoceptor to adenylate cyclase.

1:4 Aims of this thesis

The aim of this study has been to identify and classify the beta-adrenoceptor located on rat adipocytes. This investigation has had the following approaches:

(i) To perform a comparative study between the binding characteristics of two radiolabelled beta-adrenoceptor antagonists, the newly developed ligand [125I]cyanopindolol and the well established radiolabel [3H]dihydroalprenolol. These experiments were performed in order to validate the use of [125I]cyanopindolol as a ligand to delineate beta-adrenoceptor subtypes and examine agonist binding properties in adipocytes.

(ii) Identification of beta-adrenoceptors in membrane preparations of whole fat pad and isolated adipocytes with the use of high
affinity ligand binding techniques. Furthermore, attempts have been made to subclassify and quantify beta-adrenoceptor subtypes with the use of agents selective for beta_1 and beta_2 adrenoceptors.

(iii) Classification of the beta-adrenoceptor mediating lipolysis and cAMP accumulation in whole cells. Effector responses have been examined by the use of selective and non-selective agonists and antagonists to investigate whether a novel beta-adrenoceptor mediates these responses or whether beta_1 and beta_2 adrenoceptors coexist in adipocytes.

(iv) Examination of the beta-adrenoceptor linked to adenylate cyclase in washed membrane preparations. These studies have also been performed on rat reticulocyte, a cell type with typical beta-adrenoceptors for comparative reasons and hamster adipocyte to examine species variation in the adipocyte beta-adrenoceptor. As an extension of these studies, attempts have been made to use an irreversible antagonist to determine whether beta-adrenoceptors identified by ligand binding studies are identical to those identified in adenylate cyclase experiments.
CHAPTER 2

Basic concepts and methods used to investigate drug-receptor interactions
2:1 Introduction

Receptors may be classified in two ways, examination of the rank order of potency of a series of agonists and blockade by antagonists. It was by the first method that Ahlquist (1948) proposed the existence of alpha- and beta-adrenoceptors and Lands et al (1967) provided evidence for two subclasses of beta-adrenoceptors. There can be, however, problems associated with the use of agonists to classify receptors (discussed later) and the most commonly used quantitative method has been analysis of competitive antagonism of the receptor. These studies are based upon the notion that the affinity of an antagonist for a receptor will remain constant irrespective of any factors such as the use of different agonists to stimulate through the same receptor or the tissue where the receptor is located.

Two general methods can be used to estimate drug affinities, inhibition of radiolabelled ligand binding and antagonism of an effector response produced by an agonist. Each of these methods is discussed specifically later in the chapter, however, certain common elements are present when analysing drug-receptor interactions which have developed primarily from Clark's occupation theory of receptor activation (Clark 1926). In this model, the reaction of a drug (D) with receptor (R) may be represented as:

\[ D + R \rightleftharpoons DR \]  \[1\]

Assumptions made for the model are that the reaction is reversible, association and dissociation of components follows second and first order kinetics respectively and the drug does not discriminate between different receptors. Since the model was believed to obey the law of mass action, the dissociation constant (\(K_D\)) of drug for
the DR complex may be represented as:

$$K_D = \frac{[D][R]}{[DR]}$$

The model introduced the concept of affinity of a drug for a receptor and has been robust enough to be applied to radioligand binding studies and expanded further in the case of effector response studies. With ligand binding techniques, where no effect is measured beyond the DR complex formation, the experiments can easily be performed in a test tube at a simple molecular level. When measuring effector responses, experiments can be performed biochemically (i.e., adenylate cyclase assays) or in intact tissues. In the following sections, only methods of receptor analysis and classification pertaining to this study will be examined.

2:2 Radioligand binding studies

The advantages of radioligand binding studies to estimate drug affinities are that assays can be performed in homogenates of tissue thereby enabling problems of accessibility and distribution of drugs to be largely overcome. The experiments are simple to perform and no account has to be taken of effector response characteristics such as receptor reserve or partial agonism. In some cases, binding studies can provide more information about receptors than functional studies such as the concentration of receptors in a particular tissue. However, when studying a specific type of receptor with ligand binding techniques, the results should be validated by showing good correlations with antagonist affinities obtained from functional studies. Analysis of binding data takes three basic forms, kinetic analysis of association and dissociation,
saturation experiments and competition experiments with cold drugs.

2:2:1 Kinetic studies

Since the binding is treated as a simple bimolecular reaction, it should be possible to examine the rates of association and dissociation of a drug with a receptor, as shown by:

\[ D + R \rightleftharpoons DR \]

where \( k_1 \) is the second order rate of association and \( k_{-1} \) the first order rate of dissociation. Estimation of the association rate is performed by measuring the amount of ligand bound to the receptor at various times before equilibrium is achieved. The reaction can be made to follow pseudo-first order kinetics if the amount of bound ligand is maintained below 10% of the total added ligand. This enables the kinetic rates to be easily measured by graphical analysis (see Bennett 1978 for derivations). The apparent rate of association (\( k_{obs} \)) can be calculated from the slope of the plot \( \ln(X_{eq}/X_{eq} - X) \) versus time where \( X_{eq} \) is the amount of ligand bound at equilibrium and \( X \) the amount of ligand bound at time \( t \). The rate of dissociation can be calculated simply by prevention of the forward reaction after equilibrium has been attained. This can be performed either by the addition of an excess of cold displacing agent or dilution of the reaction mixture. The dissociation rate is calculated from the negative slope of the plot \( \ln(X/X_{eq}) \). As the apparent rate of association (\( k_{obs} \)) includes a contributory factor related to the concomitant dissociation present, the true rate of association can be calculated from:
\[ k_1 = \frac{k_{\text{obs}} - k_{-1}}{[D]} \]

The dissociation constant of the drug can then be calculated from the kinetic rates by the equation \( K_D = \frac{k_{-1}}{k_1} \). Kinetic analyses are usually used to confirm the value of the dissociation constant which is normally obtained by Scatchard analysis. In addition, if a reaction does not follow the law of mass action, the kinetics of dissociation become complex and the first order plot will be biphasic.

2:2:2 Saturation analysis

Saturation experiments involve the incubation of increasing concentrations of ligand with a fixed concentration of membrane resulting in concentration dependent increases in specific binding until saturation is obtained. The relationship between bound and free ligand can be analysed by a Scatchard plot (Scatchard 1949). This graphical representation was designed to be linear if a single set of non-interacting sites conforming to law of mass action behaviour are involved. The data is plotted as bound (DR) versus bound/free (DR/D) radiolabel from which two parameters may be obtained, the maximal binding capacity (\( B_{\text{max}} \)) on the bound intercept and the dissociation constant from the slope of the regression line. Any deviation from a straight line Scatchard plot is an indication that cooperativity of binding occurs or multiple affinity states are present, data evaluation of which is complex. Saturation data is also frequently analysed by the Hill (1910) plot (\( \log \left( \frac{DR}{B_{\text{max}} - DR} \right) \) versus \( \log [D] \)). This plot requires the value of \( B_{\text{max}} \) and for Clark's model, the slope of the regression line will be one. The dissociation constant can be calculated at
log \( \frac{DR}{B_{max}} - DR \) = 0, ie at 50% receptor occupancy. The Hill plot is a sensitive indicator of deviation from law of mass action behaviour and the Hill slope or coefficient \((nH)\) is often used to indicate if the ligand is binding in a bimolecular fashion. A Hill coefficient of greater than one indicates that positive cooperativity of binding is present and if less than unity may indicate either negative cooperativity or multiple affinity states. A treatise by Boeynaems and Dumont (1975) has efficiently summarised the use of these and other graphical analyses used in binding studies and possible artifacts that can be associated with their interpretation.

2.2.3 Analysis of inhibition curves

When competitive agents are introduced into binding studies, additional components to the model in equation 1 have to be introduced as shown below:

\[
\begin{align*}
D + R &\rightleftharpoons DR \\
I + R &\rightleftharpoons IR
\end{align*}
\]

[2]

where \(I\) is the competitive agent and \(K_I\) the dissociation constant of \(I\) for receptor \(R\). Since all the reactions are reversible, it is predicted that \(I\) and \(D\) will compete for the receptor \(R\). Two factors will govern the respective complex formation, the concentration and affinity of each ligand for the receptor. This relationship results in an inhibition curve if the concentration of ligand \(D\) is fixed and the concentration of competitor \(I\) varied. As the ligand \(D\) only acts as a tracer, the inhibition curve by \(I\) should conform to law of mass action binding. This can be verified by analysis of
inhibition curves with the Hill plot (log X/100-X versus log [I]) where X is the percentage inhibition of specific radioligand binding. The Hill plot can also be used to calculate the IC$_{50}$ of the inhibitor when log (X/100-X) = 0, being the concentration of I necessary to produce a 50% inhibition of the DR complex. The affinity of I can be calculated from the IC$_{50}$ using the Cheng and Prussoff (1973) equation if the values of [D], K$_D$ and IC$_{50}$ are known:

\[
K_I = \frac{IC_{50}}{1 + [D]/K_D}
\]

This equation is necessary in order to take into account the contribution of the ligand D to the overall inhibition curve. This equation has been validated for use in ligand binding studies by Weiland et al (1980).

All the reactions above have involved the interaction of one or two drugs with one receptor. In the situation where two receptor populations are involved, the equation [2] must be expanded further as shown below:

\[
\begin{align*}
D + R_1 &\rightleftharpoons DR_1 \\
D + R_2 &\rightleftharpoons DR_2 \\
I + R_1 &\rightleftharpoons IR_1 \\
I + R_2 &\rightleftharpoons IR_2
\end{align*}
\]
where $K_H$ and $K_L$ are high and low affinity dissociation constants for $R_1$ and $R_2$ respectively. In this example, $I$ has been arbitrarily chosen as a drug selective for $R_1$. The above situation can arise when either a selective antagonist is used to inhibit binding in a heterogeneous population of receptors or an agonist is used where high and low affinity states of binding may occur. Analysis of the data is made easier by the choice of a non-selective radioligand such that $K_D = K_D$. When a selective agent is used to inhibit binding in such a heterogeneous system, the resultant inhibition curve is shallow or biphasic. Several methods can be used to analyse this data. In this study, the inhibition curves were analysed by fitting the data to two sites using a computer iterative curve fitting routine (Appendix 6). The program is designed to fit a curve to two non-interacting sites based upon law of mass action binding parameters. When the best fit has been achieved, the relative proportions of receptor and $IC_{50}$ values are given. $K_I$ values of the drug for each site can then be determined from the $IC_{50}$ values using the Cheng and Prussoff equation.

2:3 Effector response studies

2:3:1 Studies with agonists

Whereas ligand binding studies may be used to estimate drug affinities by competitive antagonism, there are no clear means of accurately estimating the ability of an agent to produce a functional response. An advantage of measuring effector responses is that the direct occupation of a receptor by a drug can identify it as an agonist or antagonist. The production of a response is a parameter not included in the previous equations and must be added to the other factor determining receptor occupancy, namely the
affinity:

\[ D + R \xrightarrow{\text{DR}} \text{effect} \]

The first of the major developments in Clark's occupancy theory was by Ariëns in 1954 who introduced the concept of intrinsic activity of an agonist. This property was related to the capacity of a drug to produce a functional response. Up to and including the work of Ariëns, several assumptions were made relating to the action of agonists. These were:

(i) occupation of receptors by an agonist was directly proportional to the response with a maximal response occurring only when all of the receptors were occupied

(ii) binding of a drug to a receptor followed law of mass action behaviour

(iii) the amount of drug bound to the receptor was negligible relative to the total added drug.

In 1956, Stephenson further expanded the occupation theory by demonstrating that not all of the muscarinic receptors in guinea pig ileum were required for a full agonist response. As a result, the occupation theory was broadened to include:

(i) maximal effects by an agonist could be produced when only a small proportion of receptors were occupied

(ii) the response was not linearly proportional to the number of receptors occupied

(iii) different agonists had different capacities to produce a response and thus occupied different proportions of receptors when
producing equal responses.

Stephenson's work introduced a further parameter into the nature of receptor-effector interactions which was termed the efficacy of a drug. Further support for this concept was provided by Nickerson (1956) who demonstrated that irreversible blockade of histamine receptors initially produced a parallel shift to the right of the histamine dose response curve before a reduction in the maximal response was observed. The term 'receptor reserve' was applied on the grounds that only a fraction of the total receptor population was needed to subserve the effector response. It was therefore clear that it was difficult to estimate the affinity of an agonist since additional factors dictated the position of the dose response curve.

The next major development in receptor theory was by Furchgott in 1966 who extended the term efficacy to include two factors, the receptor selective intrinsic efficacy of the drug (E) and the organ selective efficacy (e). This can best be demonstrated by the following equation:

\[
D + R \underset{\text{DR}}{\xrightarrow{\text{Dr}}} DR^* \xrightarrow{\text{e}} \text{effect}
\]

The receptor selective intrinsic efficacy is defined as the degree to which an agonist is capable of stimulating an effector response by virtue of its action through the receptor only. Thus in the equation, E is the degree of efficacy that an agonist can confer to DR to transform it into the activated state DR*. A full agonist is capable of transforming all the complex into the active state, whereas an antagonist has no effect. Partial agonists are
only able to convert a fraction of the DR complex into the active form.

The organ selective efficacy (e) is a factor dependent upon the tissue and not the receptive mechanism. Thus factors beyond the formation of DR will be included in this term such as the density of receptors in a particular tissue, receptor reserve, etc. It is clear therefore, that both full and partial agonists with respect to ε could be full agonists at a functional response if there was a sufficient receptor reserve. However, the partial agonist would have to occupy more receptors in order to produce the same response as a full agonist. On the basis of this concept, it is clear that tissues with different receptor reserves would have quite different response characteristics with respect to full and partial agonists. Indeed, Kenakin (1984) has shown that it is even possible for the rank order of potency of full and partial agonists to reverse depending on the degree of receptor reserve. Since the rank order of potency may not be reliable, this type of analysis to identify receptors should be treated with caution.

A further problem with receptor identification by agonists is that even apparently selective agonists may produce a response through a receptor for which they are not selective. This has been demonstrated by Kenakin (1982) and Mattsson et al (1982) who found that the apparently selective beta₁ agonist prenalterol could relax the rat uterus by stimulating beta₂ adrenoceptors. The explanation for this phenomenon was that even though prenalterol had a very low receptor selective intrinsic efficacy for beta₂ adrenoceptors, there was a sufficiently large receptor reserve to amplify the stimulus and produce a full relaxation in the tissue. It must therefore be borne in mind that true agonist selectivity may not be related to the apparent selectivity of partial agonists and that
it may be more appropriate to term these agents 'organ selective' in order to avoid confusion.

2:3:2 Studies with antagonists

In view of the complexities of examining solely the action of agonists to delineate receptors, the use of antagonists has been employed in this study to identify the adipocyte beta-adrenoceptor. The relationship of antagonist binding to a receptor does not have to take into account effector response parameters and performs identically in theory to the binding equations described earlier. An agonist is used to provide the response and since the same agonist is used throughout the experiment and identical responses are measured in the absence and presence of the antagonist, the effector response parameters \( \xi \) and \( \epsilon \) remain constant and need not be included in the calculations. This enables antagonist affinities to be compared in different tissues irrespective of the efficacy of agonist, receptor reserve, etc.

Two methods can be used for estimation of antagonist affinities at a receptor. The first method involves the production of a response using a single concentration of agonist and its inhibition by varying concentrations of antagonist. From the resultant inhibition curve, analogous to an inhibition curve found in binding studies, the IC\(_{50}\) can be obtained and the corresponding K\(_I\) value calculated using the Cheng and Prussoff equation if the affinity of the agonist is known. This method can only be applied if there is no receptor reserve at the effector response. Therefore, this method can be applied to estimate the affinities of drugs in adeny-late cyclase assays (see Minneman et al 1979c) and an alternative method has to be applied where functional responses are examined. The second method involves the incubation of a tissue with...
antagonist and reversal of the competitive blockade by increasing concentrations of agonist. The ratio of agonist concentrations needed to produce an identical response before and after the addition of antagonist is termed the dose-ratio. Several methods for estimating the affinity of an antagonist are available on the basis of dose ratios (see Furchgott 1955, Furchgott 1964 for examples). The estimation of drug affinity in this study was based upon the pA scale as originally defined by Schild (1949) and extended by Arunlakshana and Schild (1959). For this type of analysis, several concentrations of antagonist are used to produce a series of agonist dose-response curves. The data is then plotted using the Schild plot \( \log (\text{dose ratio} - 1) \) versus \( \log [I] \), where \([I]\) is the concentration of competitive antagonist used. According to theory, the points in such a plot should fit a straight line with a slope of unity if a bimolecular reaction is involved. The pA\(_2\) value, obtained by regression of the points to \( \log (\text{dose ratio} - 1) = 0 \), is a measure of the affinity of the antagonist and is equivalent to \(-\log [K_I]\) if the Schild slope is one. This type of plot holds for single receptor interactions. If two receptors are present which mediate the same response, the use of an antagonist selective for one receptor subtype will give a low slope. No calculations can be made for receptor heterogeneity using antagonists in this way. O'Donnell and Wanstall (1979b) have shown that this problem can be overcome by the use of agonists selective for each receptor subtype. Effectively, in theory at least, the functional response is then mediated through only the agonist selected receptor and the antagonist should behave according to the law of mass action. Each receptor can therefore be examined at the effector response and affinities calculated accordingly.

Detailed explanations of specific events relating to the methods of data analysis are discussed in the relevant chapters.
CHAPTER 3

Methods
3:1 Animals

Male Sprague Dawley and Wistar rats weighing 150-200 g for lipolysis studies and experiments on lung membranes and 300-350 g for all other experiments were obtained from Bantin and Kingman plc. or bred from stock animals. No qualitative or quantitative strain differences were found in the experiments. Male Syrian hamsters weighing 110-150 g were obtained as above. All the animals had free access to food and water and were maintained on a twelve hour light/dark cycle at room temperature.

3:2 Animal treatment - induction of reticulosis in rats

Rats were injected intra-peritoneally with a sodium bicarbonate-neutralised saline solution of phenylhydrazine HCl according to the following dosing regimen: - days 1, 3 and 5, 50 mg/Kg. Blood was collected by cardiac puncture under halothane anaesthesia on day 8. The dosing regime produced a red blood cell population which was >95% reticulocytes as determined by staining diluted aliquots of blood with brilliant cresyl blue.

3:3 Preparation of isolated adipocytes

Rats or hamsters were killed by a blow to the head followed by decapitation. The epididymal fat pads were removed and rinsed in saline. Human subcutaneous adipose tissue was obtained from patients undergoing abdominal surgery and quickly transported to the laboratory. No attempt was made to discriminate between age, sex, weight or pathological condition of patients. The adipose tissue was minced with scissors and isolated adipocytes prepared by the method of Rodbell (1964). Minced tissue was incubated with shaking for 60 minutes at 37°C under 95% O₂, 5% CO₂ in three volumes of Krebs-bicarbonate buffer (pH 7.4, 1.25 mM CaCl₂)
containing 1 mg/ml collagenase and 4% BSA. The cells were filtered through one layer of cheesecloth and washed twice in Krebs-BSA by centrifugation at 400 x g for 5 minutes at room temperature.

3:4 Binding studies

3:4:1 Membrane preparation

(A) Rat lung membranes

Rats were killed by a blow to the head followed by decapitation. The lungs were removed, rinsed in ice cold saline and dissected free of major bronchi. They were then homogenised in five volumes of ice cold 25 mM Tris-HCl pH 7.8, 0.154 M NaCl (Tris-isosaline) using a 'Willems' polytron (3 x 5 sec bursts at speed 5). The homogenate was then filtered through a double layer of cheesecloth and centrifuged at 50,000 x g for 15 minutes at 4° C. The supernatant was discarded and the pellet washed three times in Tris-isosaline at 50,000 x g. The final pellet was taken up in Tris-isosaline at a protein concentration of 1-2 mg/ml, rapidly frozen and stored at -50°C until use.

Some experiments dictated the use of different buffers for membrane preparation (chapter 4). In these cases, the Tris-isosaline was replaced either with 50 mM Tris-HCl pH 7.8 throughout or in the case of 'EDTA treated membranes', 50 mM Tris-HCl pH 7.8, 1 mM EDTA at all steps except final resuspension of pellet which is described in text.

(B) Whole fat pad membranes

Rats were killed by a blow to the head followed by decapitation. The epididymal fat pads were removed and rinsed in saline. They were then minced with scissors and homogenised in four volumes of ice cold 0.25 M sucrose, 10 mM Tris-HCl pH 7.4
using the polytron (3 x 5 sec bursts at speed 6). The homogenate was then centrifuged at 50,000 x g for 15 minutes at 4°C. The fat 'cake' and supernatant were discarded and the pellet washed three times in Tris-isosaline at 50,000 x g. The final pellet was resuspended in Tris-isosaline at a protein concentration of 1-2 mg/ml and stored at -50°C.

(C) Isolated adipocyte membranes

Isolated adipocytes were prepared as in 3:3. The adipocytes were then homogenised and membranes prepared as for whole fat pad membranes.

3:4:2 Preparation of radioligands

(A) Iodination of (+)cyanopindolol

(+)-Cyanopindolol (CYP) was iodinated according to the method of Engel et al (1981). 10 μl of 13.5 mM HCl containing 20 μg CYP, 20 μl 0.3 M potassium phosphate buffer pH 7.6, 2 mCi Na$^{125}$I and 20 μg aqueous solution of chloramine T (0.34 mg/ml) were combined in that order, in a polypropylene test tube at room temperature and allowed to stand for five minutes. The reaction was stopped by addition of 300 μl of an aqueous sodium thiosulphate solution (1 mg/ml), followed by addition of 10 μl 1 M sodium hydroxide. The iodinated product was then extracted four times with 300 μl of ethyl acetate containing 0.01% phenol. The four washes were combined and spotted on a strip of Whatman 3 MM paper and chromatographed in a descending manner at 4°C with 0.1 M ammonium formate pH 8.5, 0.01% phenol. The paper was removed when the solvent front had almost reached the end of the paper and then cut into 1 cm strips which were each placed into 5 mls of methanol. [$^{125}$I]CYP was located as a single peak with $R_F$ of 0.09-0.10, placed into glass vials and stored at -20°C.
(B) Iodination of (-)pindolol

(-)Pindolol (PIN) was iodinated according to the method of Barovski and Brooker (1980). 5 µl of 10 mM PIN in ethyl acetate was evaporated to dryness under a stream of nitrogen. To this was added 1-2 mCi Na$^{125}$I followed by 40 µl of 0.3 M potassium phosphate buffer pH 7.5 and 5 µl aqueous solution of chloramine T (0.17 mg/ml) in a polypropylene test tube at room temperature and allowed to stand for three minutes. The reaction was stopped by addition of 500 µl of 1 mg/ml sodium metabisulphite in acetic acid followed by the addition of 2 M NaOH until the mixture was adjusted to pH 10. The iodinated product was then extracted seven times with 300 µl ethyl acetate. After the addition of 5 µl of 1% phenol in ethyl acetate the volume was reduced by drying the extract under a stream of nitrogen, spotted on a strip of Whatman 3 MM paper and chromatographed in a descending manner at 4°C with 1.0 M ammonium formate (pH 8.5) : methanol, 10:1 (v:v). The paper was removed when the solvent front had almost reached the end of the paper and then cut into 1 cm strips which were placed into 5 mls of ethyl acetate containing 2% (v:v) diethylamine followed by the addition of 5 µl of 1% phenol in ethyl acetate. [$^{125}$I]PIN was located as a single peak with $R_f$ of 0.20-0.30, placed into glass vials and stored at -20°C.

3:4:3 Assay of radioligand binding

Frozen membranes were found to be capable of retaining their binding capacity for up to one year if stored at -50°C. When ready to be used, they were thawed, diluted in respective assay buffer and homogenised using the polytron (2 x 5 sec bursts at speed 5) to disaggregate particles.
(A) [³H]DHA binding assay

100 µl of membrane suspension (approximately 100 µg protein) was added to 25 mM Tris-HCl, pH 7.8, 0.154 M NaCl, 1.1 mM ascorbic acid (assay buffer) except where mentioned in text, containing [³H]DHA, buffer or competing drug in a final volume of 250 µl. Incubations were normally carried out at room temperature (22°C) for 30 minutes after which the reaction was stopped by addition of 3 ml of ice cold assay buffer followed by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. The filters were washed with 3 x 5 ml of assay buffer at room temperature, placed into insert vials to which 4 mls of scintillation fluid was added. The vials were left overnight for the mixture to extract and then the radioactivity associated with the filters was counted in a Packard Tricarb liquid scintillation spectrometer at an efficiency of 38%.

(B) [¹²⁵I]CYP binding assay

100 µl of membrane suspension (6-100 µg protein) was added to 25 mM Tris-HCl pH 7.8, 0.154 M NaCl, 1.1 mM ascorbic acid (assay buffer) except where indicated in text, containing [¹²⁵I]CYP, buffer or competing drug in a final volume of 250 µl. Incubations were carried out at 37°C for 60 minutes after which the reaction was stopped by addition of 3 ml of ice cold assay buffer followed by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. The filters were washed with 2 x 10 ml of assay buffer at room temperature, placed into small test tubes and radioactivity on the filters counted in a Packard or LKB gamma counter at efficiencies of 70%.

(C) [¹²⁵I]PIN binding assay

100 µl of membrane suspension (10-100 µg protein) was added to 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 1.1 mM ascorbic
acid, containing $[^{125}\text{I}]$PIN, buffer or competing drug in a final volume of 250 µl. Incubations were carried out at 37°C for 15 minutes after which the reaction was stopped by addition of 3 ml of ice cold 25 mM Tris-HCl pH 7.8, 0.154 M NaCl (wash buffer) followed by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. The filters were washed with 3 x 5 ml of wash buffer at room temperature, placed into small test tubes and counted as for $[^{125}\text{I}]$CYP.

All binding assays were performed in duplicate except saturation assays which were performed in triplicate.

(D) Non specific binding determination and validation of use

Non specific binding was determined with all the ligands used by incubation of ligand and membranes in the presence of 200 µM (-)isoprenaline which displaced all the radioligand bound to the receptor. Specific binding was defined as the total binding minus the non specific binding. Since some antagonists can give anomalous results when used to define non specific binding (Bylund 1978, Nahorski and Richardson 1979, Mendel and Almon 1979, Dax and Partilla 1982) which may result in incorrect estimations of $K_D$ and $B_{\text{max}}$, the binding characteristics of increasing concentrations of $[^{125}\text{I}]$CYP have been examined in boiled isolated adipocyte membranes in the presence and absence of 200 µM (-)isoprenaline. Figure 3:1 shows clearly that isoprenaline has no effect on the displacement of $[^{125}\text{I}]$CYP from non receptor sites. Dax and Partilla (1982) showed that the degree to which drugs could displace radiolabel from non specific sites was based on their octanol : water partition coefficient, therefore the use of a hydrophilic agonist such as isoprenaline would minimise any errors that could result.
Figure 3:1  Binding of $[^{125}\text{I}]$CYP to isolated adipocyte membranes after thermal inactivation of beta-adrenoceptors. Membranes were boiled for 10 minutes followed by cooling and incubation with $[^{125}\text{I}]$CYP in the absence (○) and presence (▲) of 200 μM (-)isoprenaline. Results are expressed as mean ± SEM of three experiments performed in duplicate.
3:5 Pharmacological experiments on isolated adipocytes

Rat isolated adipocytes were used for estimates of lipolysis and cAMP accumulation. Adipocytes were prepared as described in 3:3 except that 1% of demineralised BSA was used throughout for lipolysis and 1% BSA fraction V for cAMP accumulation experiments. The adipocytes, final concentrations 30,000-60,000/ml for lipolysis and 100,000-200,000/ml for cAMP accumulation were incubated with shaking at 1-2 cycles/second in volumes of 3 and 1 ml respectively containing Krebs-bicarbonate, 1% BSA, 50 μg/ml sodium metabisulphite (to prevent oxidation of agonists) and appropriate drugs at 37°C and gassed with 95% O₂, 5% CO₂. Teflon pots were used for each incubation to prevent adhesion of adipocytes to vessel walls and subsequent rupture. The incubations were terminated after 90 minutes for lipolysis by solvent extraction and 8 minutes for cAMP accumulation by heating in a boiling water bath for 5 minutes. Assays for lipolysis are described in 3:6 and cAMP in 3:10. In all cases, antagonists were allowed to preincubate for 10 minutes before the addition of agonist to start the response. Separate incubations were performed in the absence of drugs to determine basal stimulations and in the presence of isoprenaline to determine the maximal isoprenaline response. All assays were performed in duplicate.

3:6 Measurement of lipolysis

The release of free fatty acids by adipocytes was measured by the method of Ko and Royer (1967). Incubations were terminated by the addition of 3 mls of extraction medium (Appendix 3) followed by vortexing for 10-15 seconds. The organic and aqueous layers were separated by centrifugation and 0.5 ml aliquots of the organic layer transferred into glass test tubes. Each tube was
then titrated with tetra n-butyl ammonium hydroxide solution (Appendix 3) under an atmosphere of nitrogen using thymolptalein as indicator.

3:7 Protein binding of antagonists

Binding of betaxolol and ICI 118.551 to 1% bovine serum albumin was studied by equilibrium dialysis for 16-18 hours at 20°C using Teflon compartments separated by cellulose acetate membranes (Technicon nr. 105-110). Samples were taken from the albumin-free compartment and analysed fluorimetrically using a Perkin-Elmer 300 spectrofluorimeter. Excitation wavelengths were 271 and 272 nm, with the emission peaks appearing at 299 and 301 nm with betaxolol and ICI 118.551 respectively.

3:8 Experiments analysing adenylate cyclase activity

3:8:1 Membrane preparation

(A) Adipocyte ghosts

Fat cell ghosts were prepared by lysing the isolated adipocytes (prepared as in 3:3) in 20 volumes of ice cold 2 mM Tris-maleate pH 7.4, 2 mM EGTA (lysing buffer), followed by centrifugation at 50,000 x g for 15 minutes at 4°C. The fat cake and supernatant were discarded and the pellet resuspended using a glass-Teflon homogeniser in 80 mM Tris-maleate pH 7.4, 4 mM MgSO₄, 0.2 mM EDTA, 1 mM IBMX (assay buffer) except where indicated, followed by centrifugation. The final pellet was then resuspended in assay buffer at a protein concentration of 1-2 mg/ml and stored at -50°C.

(B) Reticulocyte membranes

Blood (approximately 10 ml) was collected and
placed into tubes containing 0.6 ml of 0.2 M EDTA pH 7.4 as anticoagulant. The blood was centrifuged at 500 x g for 10 minutes at 4°C after which plasma was removed by aspiration. The cells were then washed in ice cold saline and recentrifuged at 500 x g. The supernatant was discarded and the cells washed a further three times in saline by centrifugation at 1500 x g for 10 minutes at 4°C. The buffy coat layer was removed during the course of the washing. 1 ml aliquots of packed cells were then lysed in 40 volumes of ice cold lysing buffer. After 10 minutes, the lysate was centrifuged at 50,000 x g for 15 minutes at 4°C. The pellet was resuspended in assay buffer and recentrifuged at 50,000 x g. The resulting pellet was then taken up in assay buffer at a protein concentration of 1-2 mg/ml and stored at -50°C.

3:8:2 Adenylate cyclase assay

(A) Reticulocyte and rat adipocyte membranes

Adenylate cyclase activity was determined by incubating membranes (10-20 μg protein) in assay buffer containing 1 mM ATP, 100 μM GTP, 1mM ascorbic acid and an ATP regenerating system consisting of 20 mM phosphocreatine and 100 U/ml creatine phosphokinase in a total volume of 250 μl for 10 minutes at 37°C. The reaction was stopped by placing the tubes in a boiling water bath for 5 minutes, followed by centrifugation and cyclic AMP produced determined using the protein binding assay in 3:10:1. Where antagonists were added, these were allowed to preincubate with membrane for 10 minutes prior to addition of ATP. Separate incubations were performed without drugs in the absence and presence of isoprenaline to determine basal and maximal isoprenaline stimulated activities. All experiments were performed in duplicate.
(B) Hamster adipocyte membranes

Experiments were conducted as above except that membrane preparation and assay were performed in assay buffer containing 0.154 M NaCl.

3.9 Irreversible inhibition of beta-adrenoceptors

Membranes from reticulocytes and adipocytes were prepared as in 3.8 except that they were washed and frozen in 80 mM Tris-maleate pH 7.4, 0.154 M NaCl, 4 mM MgSO₄, 0.2 mM EDTA, 1 mM IBMX (assay buffer). The membranes were thawed and homogenised using a motor driven glass-Teflon homogeniser, then 0.8 ml aliquots were placed into small centrifuge tubes to which was then added 200 μl of assay buffer or para-amo benzyl carazolol (PABC) at the required concentration followed by vortexing. After 10 minutes at room temperature, the tubes were centrifuged at 20,000 x g for 5 minutes at 4°C. The supernatants were removed by aspiration and the membranes each resuspended in 1 ml sodium phosphate buffer pH 6.0 followed by addition of 20 μl dimethylsulphoxide (DMSO) for control and some treated membranes or 5 mM N-succinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (SANAH) in DMSO. After 10 minutes at 25°C in the dark, the reaction was quenched by the addition of 20 μl 1 M glycine and the samples either photolysed using a Hanovia medium pressure mercury vapour lamp in the case of SANAH treated membranes or left to stand at 22°C for 30 minutes. After this time, the samples were washed by centrifugation and resuspension in assay buffer followed by incubation for 10 minutes at 37°C to aid dissociation of unbound ligand. The membranes were then washed and the incubation repeated with fresh buffer. After the final centrifugation, the pellets were each resuspended in an equal volume of assay buffer followed by inclusion either in the
radioligand binding (3:4:3B) or adenylate cyclase (3:8:2) assays. Both assays were performed in the assay buffer mentioned above in the presence of 1 mM ascorbic acid to maintain as constant conditions as possible.

3:10 Assays for cAMP

3:10:1 Protein binding assay

One of the assays used for cAMP was the protein binding assay of Brown et al (1971). This method is based upon the binding of radiolabelled cAMP to a protein kinase from bovine adrenals which can be competitively displaced by cold cAMP. Construction of a standard curve enables the measurement of cAMP in unknown samples to be determined.

(A) Preparation of adrenal binding protein

Bovine adrenals were obtained from the local abattoir and transported to the laboratory on ice. The adrenals (maintained at 4°C) were trimmed of surrounding fat and the medulla and the cortices homogenised in a Waring blender in 2 volumes of Littlefields medium (Appendix 4). The homogenate was centrifuged at 2000 x g for 5 minutes at 4°C and the pellet discarded. The supernatant was recentrifuged at 50,000 x g for 15 minutes at 4°C and divided into 1 ml aliquots after which it was frozen at -20°C until use. Throughout the procedure, care was taken to remove any fat which floated on the surface of the medium.

For assay standardisation, the binding of [³H]cAMP was examined at different protein dilutions and the chosen dilution was that which bound approximately 40% of the total added [³H]cAMP (approx 0.5 pmoles). This dilution (approximately 1:10) resulted in a high degree of specific binding and an assay sensitivity with minimal
detectable limit of 100 fmoles cAMP.

(B) Assay of cAMP

All reagents and protein were diluted in assay buffer which consisted of 50 mM Tris-HCl pH 7.4, 4 mM EDTA. For standard curve tubes 100 μl of boiled buffer was added with [³H]cAMP (approx 25,000 cpm.), adrenal binding protein and standard to a final volume of 400 μl. With unknown samples, 100 μl of unknown was combined with [³H]cAMP, binding protein and buffer and all samples vortexed. Non specific binding of [³H]cAMP was determined by separate incubations in the presence of 8 μM cold cAMP and was routinely about 10% of total binding. The tubes were then incubated overnight at 4°C to reach equilibrium. The incubation was terminated by the addition of charcoal/BSA suspension (Appendix 4) at 4°C and mixing. The mixture was left to stand for 25 minutes followed by centrifugation at 2500 x g for 25 minutes at 4°C. The supernatant was decanted into scintillation vials followed by addition of 4.5 mls of scintillation fluid. After the vials were vortexed, they were counted in a Packard liquid scintillation spectrometer at an efficiency of 38%.

3:10:2 Radioimmunoassay of cAMP

The other assay for cAMP used was a modification of the radioimmunoassay of Skomedal et al (1980). Antiserum found to have a low cross reactivity with other nucleotides was a gift from Dr K Siddle, Cambridge University. Two factors distinguish this radioimmunoassay from other conventional assays, since [³H]cAMP is used as radiolabel, high titres of antibody were necessary and the radiolabel had to be acetylated. [³H]cAMP itself was found to have a low affinity for the antibody and since in order to raise antibodies, the cAMP had to be succinylated to a hapten, it appears
that the recognition site is at the level of the cross linkage. Therefore, the acetylation of \(^3\text{H}\)cAMP was found to increase the affinity to workable levels. A titre (final assay concentration) of approximately 1:2000 was found to bind in the order of 30% of the total added \(^3\text{H}\)cAMP (0.2-0.25 pmoles). Again a high degree of specific binding was obtained with a minimal detectable limit of 20 fmoles cAMP.

Reagents and antibody were diluted in assay buffer (50 mM Na-acetate pH 6.0). Assays were performed essentially as for the protein binding assay except that the final assay volume was 310 µl and the mixture contained 0.01% BSA. Non specific binding was determined as for the protein binding assay. Before the addition of antibody, unknown and standard samples together with radiolabel were acetylated by the addition of 10 µl acetic anhydride/triethylamine (2:1 v:v). After addition of antibody, the mixture was left to equilibrate overnight and the incubation terminated as for the protein binding assay.

3:10:3 Analysis of data

Data was analysed by a computer assisted curve fitting routine developed by R.A Ferguson (ICI Pharmaceuticals plc). The model upon which this data was analysed was based upon the equation:

\[
Y = \frac{A - D}{1 + (X/C)^B} + D
\]

where \(Y\) is the response, \(X\) the dose, \(A\) the total binding, \(D\) the non specific binding, \(C\) the \(ED_{50}\) and \(B\) the slope factor. The programme estimated the curve of best fit by a least squares iterative fitting routine estimating the 95% confidence limits for each sample.
3:11 Measurement of protein

Protein was determined by the method of Lowry et al (1951). All samples and standards were prepared in buffer used. Standard curves were set up using BSA fraction V (0-300 µg/ml) and following incubation with the Lowry reagents (Appendix 5), the absorbance was measured at a wavelength of 750 nm by a Pye-Unicam SP6-500 U.V. Spectrophotometer. Standard curves were determined using a linear least squares regression fit and sample values determined using the equation:

\[ X = \frac{Y - C}{M} \]

where \( X \) is unknown sample concentration, \( Y \) the absorbance, \( C \) the value of \( Y \) at \( X = 0 \) and \( M \) the slope of the regression line.

3:12 Statistical treatment of results

In all cases, mean values \( \pm \) standard errors of mean are given. Significancies between values were tested using one of the tests shown below:

(i) Student's \( t \) test for two sample groups

(ii) Paired \( t \) test for comparisons between control and test groups

(ii) \( t \) Distribution for comparing slopes different from unity.

Differences were considered to be significant with probabilities of 95% or greater.
CHAPTER 4

Comparison of $[^3H]$dihydroalprenolol and $[^{125}I]$cyanopindolol binding to membrane preparations of rat lung
4:1 Introduction

In order to identify beta-adrenoceptors using ligand binding techniques, several criteria must be fulfilled, these are:

(i) Kinetics

The binding of ligand to a receptor should be at least as fast as the appearance of a biological response and in the case of a competitive ligand, the binding should be reversible.

(ii) Saturability

The number of receptors in a tissue should be finite, therefore specific binding of a radioligand should saturate with increasing ligand concentrations.

(iii) Distribution

Receptor binding sites should only be located in tissues or cells where there is a functional response to agonists.

(iv) Pharmacology

Inhibition of binding should follow all of the characteristics of pharmacologically active antagonists. Thus stereoselectivity should be observed and there should be reasonable correlations between the affinity constants of drugs at inhibiting binding to those at a functional response mediated by beta-adrenoceptors. In view of the problems of assessing the rank order of potency of agonists and their affinities at a functional response (see chapter 2), these relationships may not hold true for ligand binding studies.

Initial attempts to identify beta-adrenoceptors using ligand binding techniques were performed with tritiated agonists in membranes known to have a catecholamine responsive adenylate cyclase (Tomasi et al 1970, Dunnick and Marinetti 1971, Lefkowitz
and Haber 1971). Problems associated with these initial binding studies have been extensively reviewed by Haber and Wrenn (1976) and Lefkowitz et al (1976a). In summary, these results were unsuccessful as the binding sites were not stereoselective and were inhibited with low affinity by beta-adrenoceptor antagonists, furthermore, catechol containing compounds which were ineffective at beta-adrenoceptors were inhibitors of binding.

The first successful ligand binding studies were performed with the use of radiolabelled antagonists such as \(^{3}\text{H}\)dihydroalprenolol \((^{3}\text{H}\text{DHA}, \text{Lefkowitz et al} 1974)\), \(^{3}\text{H}\)propranolol \((\text{Atlas et al} 1974)\) and \(^{125}\text{I}\)hydroxybenzylpindolol \((^{125}\text{I}\text{HYP}, \text{Aurbach et al} 1974)\). These antagonists had a higher affinity than agonists for the putative receptor sites which corresponded closely to their affinities at inhibiting catecholamine stimulated adenylate cyclase, in addition, their binding characteristics satisfied all the criteria necessary for receptor binding.

The most commonly used ligands to date have been \(^{3}\text{H}\text{DHA}\) and \(^{125}\text{I}\text{HYP}\), \(^{3}\text{H}\)propranolol binding displayed a high degree of non specific binding in many systems and coupled with its low specific activity (<20 Ci/m mole), provided no advantage over the other two ligands.

\(^{3}\text{H}\text{DHA}\) and \(^{125}\text{I}\text{HYP}\) possess somewhat different chemical properties which give certain advantages and disadvantages to each ligand, these are described below:

\[^{3}\text{H}\text{DHA}\]

The main advantage of \(^{3}\text{H}\text{DHA}\) has been the high degree of specificity of the radioligand for the beta-adrenoceptor and a low degree of non specific receptor binding in many membrane preparations. This has resulted in its successful use at
identifying beta-adrenoceptors in a wide range of tissues (see Nahorski 1981 for review). In addition, \(^3\)H]DHA is available as the optically active (-) isomer which has been shown to be preferrable in use to a racemate (Bürgisser et al 1981). The main disadvantage of \(^3\)H]DHA has been its low specific activity (50-100 Ci/mmole) which necessitates the use of high amounts of tissue protein for receptors to be assayed.

\(^{125}\)I]HYP

\(^{125}\)I]HYP has an advantage over \(^3\)H]DHA due to the high specific activity of the \(^{125}\)I isotope (2175 Ci/mmole). In conjunction with the greater efficiency of counting \(^{125}\)I over \(^3\)H, much lower quantities of receptors may be assayed, this advantage is apparent in tissue where there is a low beta-adrenoceptor density or the availability of tissue is low. This advantage has overridden several major disadvantages of \(^{125}\)I]HYP such as the large amount of non specific binding in some tissues which has required for its reduction the inclusion of phentolamine (Sporn and Molinoff 1976, Pittmann et al 1980) or d-isoprenaline (Harden et al 1976) in the incubation or terminating the reaction in the presence of propranolol followed by washing at 37°C (Maguire et al 1976). Further problems with \(^{125}\)I]HYP have related to its radioactive stability (Maguire et al 1977) and the uncertainty of the iodination reaction which may result in multiple iodination products (Bearer et al 1980). \(^{125}\)I]HYP may not be as specific for beta-adrenoceptors as \(^3\)H]DHA and has been reported to also bind to 5HT receptors (Willcocks and Nahorski 1983) precluding its use where these are present. The racemic nature of \(^{125}\)I]HYP may also lead to certain quantitative errors in the estimation of the dissociation constants for radioligand and competing agents (Bürgisser et al 1981).
Recently, the development of a new iodinated ligand (±)[\textsuperscript{125}I]-cyanopindolol was reported by Engel et al (1981). [\textsuperscript{125}I]CYP was found to label beta-adrenoceptors in guinea pig heart and lung with high affinity and possessed a smaller non specific binding component than [\textsuperscript{125}I]HYP. [\textsuperscript{125}I]CYP appeared to be more specific for the beta-adrenoceptor therefore its use was chosen and in this study, the characteristics of [\textsuperscript{125}I]CYP binding in rat lung membranes were compared to those of [\textsuperscript{3}H]DHA. The purposes of this study were three fold, to examine whether both ligands labelled the same number of sites, whether [\textsuperscript{125}I]CYP could be used to examine receptor heterogeneity and whether agonist inhibition of binding and its modulation by guanine nucleotides could be observed. In view of the different incubation conditions and buffers that have been used for [\textsuperscript{3}H]DHA and [\textsuperscript{125}I]CYP binding experiments, a limited study was performed to examine the effects of sodium chloride, Mg\textsuperscript{++}, incubation time and temperature on isoprenaline inhibition of [\textsuperscript{3}H]DHA binding in the presence and absence of guanine nucleotides to assess the contribution of each variable for the differences in agonist binding observed with the two radioligands.

4:2 Results

4:2:1 [\textsuperscript{3}H]DHA binding

(A) Kinetics

The specific binding of [\textsuperscript{3}H]DHA to rat lung membranes was rapid and reversible at 22°C (fig 4:1), equilibrium of binding being reached within ten minutes. The kinetics of association and dissociation were calculated from linear transformations of pseudo-first order and first order interactions
Figure 4:1 Kinetics of specific [³H]DHA binding to rat lung membranes. (a) Time courses of specific [³H]DHA association (●) and dissociation (▲). Dissociation was initiated by the addition of 200 μM (-)isoprenaline after equilibrium had been reached (30 min). (b) Linear transformation showing pseudo first order kinetics of association giving $K_{obs} = 0.271 \text{ min}^{-1}$. (c) First order kinetic plot of [³H]DHA dissociation where $K_{-1} = 0.086 \text{ min}^{-1}$. Concentration of [³H]DHA used was 1.02 nM therefore $K_{+1} = 1.814 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ and $K_D = 0.474 \text{ nM}$. Results are expressed as mean ± SEM of four experiments performed in duplicate.
Figure 4:2  
(a) Saturation of $[^3\text{H}]$DHA binding in rat lung membranes. Increasing concentrations of $[^3\text{H}]$DHA were incubated with membranes and binding defined as in text. Results are expressed as mean ± SEM of three experiments performed in triplicate. (b) Scatchard analysis of same data giving $B_{\text{max}} = 350 \pm 19$ fmoles/mg protein and $K_D = 0.31 \pm 0.02$ nM as determined by linear regression of the points.
Analysis of the derived rate constants enabled the dissociation constant \( (K_D) \) to be calculated \( (K_{-1}/K_{+1} = 0.47 \text{ nM}) \).

**(B) Saturability**

Specific \( \left[^3\text{H}\right] \) DHA binding was defined as the total minus non-specific binding. Whereas non-specific binding increased linearly with concentration, the specific binding was saturable (fig 4:2). Scatchard analysis of the data revealed a homogeneous population of binding sites with an equilibrium dissociation constant of 0.31 nM which is in excellent agreement with the kinetically derived \( K_D \). The maximal binding capacity \( (B_{\text{max}}) \) of rat lung membranes was 350 ± 19 fmoles/mg protein which agrees closely with other reports for this tissue (Rugg et al 1978, Ashton et al 1981). Hill analysis of the data (not shown) did not reveal the presence of any cooperative interactions \( (n_H = 1.14 ± 0.11) \).

**4:2:2 \( \left[^{125}\text{I}\right] \) CYP binding**

**(A) Kinetics**

Specific binding of \( \left[^{125}\text{I}\right] \) CYP to rat lung membranes was slower at association and dissociation compared to \( \left[^3\text{H}\right] \) DHA. Since equilibrium of binding could not be attained after 60 minutes at room temperature (not shown), experiments were performed throughout at 37°C. As can be seen in fig 4:3, \( \left[^{125}\text{I}\right] \) CYP reached equilibrium of binding after 55 minutes. Kinetic analysis of the data gave a \( K_D \) value of 3.53 pM.

**(B) Saturability**

Specific binding of \( \left[^{125}\text{I}\right] \) CYP was saturable whereas non-specific binding increased linearly with radioligand concentration (fig 4:4). Scatchard plots revealed again a homogeneous class of binding sites with \( K_D \) of 17.3 ± 1.3 pM which
Figure 4:3 Kinetics of specific $^{125}$I CYP binding to rat lung membranes. (a) Time courses of association (○) and dissociation (▲). Dissociation was induced by the addition of 200 μM (-)-isoprenaline after 60 min of incubation. (b) Pseudo first order kinetic plot of association giving $K_{obs} = 0.057 \text{ min}^{-1}$ and (c) first order plot of dissociation giving $K_{-1} = 0.00509 \text{ min}^{-1}$. $^{125}$I CYP concentration was 36.0 pM therefore $K_+ = 1.442 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$ and $K_D = 3.53$ pM. Results are mean ± SEM of three to four experiments performed in duplicate.
Figure 4.4  (a) Saturation of \(^{125}\)I-CYP binding in rat lung membranes. Results are expressed as mean ± SEM of four experiments. (b) Same data analysed by Scatchard. Linear regression gave values of \(B_{\text{max}} = 400 \pm 9\) fmoles/mg protein and \(K_D = 17.3 \pm 1.3\) pM.
was slightly higher than the kinetically derived $K_d$. $[^{125}\text{I}]CYP$ was a more potent ligand than $[^3\text{H}]DHA$ having a more than ten fold greater affinity for beta-adrenoceptors. The $B_{\text{max}}$ (400 ± 9 fmoles/mg protein) was not significantly different to that obtained using $[^3\text{H}]DHA$ and Hill coefficients were close to unity (0.91 ± 0.02) suggesting no cooperative interactions.

4:2:3 Comparison of antagonist affinities for $[^3\text{H}]DHA$ and $[^{125}\text{I}]$-CYP binding

(A) Propranolol stereoisomers

Propranolol inhibition of $[^3\text{H}]DHA$ and $[^{125}\text{I}]CYP$ binding was stereoselective, in each case the (-) isomer was approximately two orders of magnitude more potent than the (+) isomer, however, both isomers were slightly more potent against $[^3\text{H}]DHA$ binding as compared to that of $[^{125}\text{I}]CYP$ (fig 4:5a). In all cases Hill coefficients were close to unity indicating no cooperative interaction and law of mass action behaviour.

(B) Selective antagonists

Inhibition curves of either $[^3\text{H}]DHA$ or $[^{125}\text{I}]CYP$ binding by selective antagonists yielded curves which deviated from law of mass action behaviour (fig 4:5b). The competition curves were biphasic with Hill slopes less than unity, analysis of the data by computer iterative curve fitting gave best fit parameters for a two site model in each case. Irrespective of the radiolabel used, the beta$_1$ selective antagonist betaxolol (Boudot et al 1979) and the beta$_2$ selective antagonist ICI 118.551 (Bilski et al 1980) were able to identify the presence of a heterogeneous population of beta-adrenoceptors consisting of approximately 20% beta$_1$ and 80% beta$_2$ subtypes which agrees closely with other reports for the same tissue (Rugg et al 1978, Minneman et al 1979a,
Figure 4:5  (a) Inhibition of specific \[^3\text{H}\]DHA (●, ○) and \[^{125}\text{I}\]CYP (▲, △) binding in rat lung by the stereoisomers of propranolol. (-)Propranolol (closed symbols) and (+)propranolol (open symbols) were incubated with radioligand and membranes as described in methods. IC\text{50} values and Hill coefficients were calculated by analysing Hill plots and K\text{I} values calculated using the Cheng and Prussoff equation. The results are expressed as mean ± SEM of three to four experiments performed in duplicate.

(b) Inhibition of specific \[^3\text{H}\]DHA (●, ○) and \[^{125}\text{I}\]CYP (▲, △) binding in rat lung by the beta\text{1} selective antagonist betaxolol (open symbols) and the beta\text{2} selective antagonist ICI 118.551 (closed symbols). Curves were analysed by computer iterative curve fitting to two sites giving IC\text{50} values and relative proportions (in parentheses) of each site. Nanomolar K\text{I} values were calculated using the Cheng and Prussoff equation. K\text{I} beta\text{1} = inhibition constant at beta\text{1} adrenoceptors, K\text{I} beta\text{2} = inhibition constant at beta\text{2} adrenoceptors. Results are expressed as mean of three to five experiments performed in duplicate and standard errors are excluded for clarity but were less than ± 4%.
Figure 4:5

(a) $K_i$ (nM)      nH

- (●) $0.199 \pm 0.004$  $0.86 \pm 0.02$
- (▲) $0.949 \pm 0.014$  $1.13 \pm 0.04$
- (○) $28.9 \pm 1.6$     $1.17 \pm 0.05$
- (△) $65.2 \pm 5.9$     $1.16 \pm 0.05$

(b) $K_i_{beta_1}$      $K_i_{beta_2}$

- (●) $56.4$ (17%)     $2.24$ (83%)
- (▲) $142$ (17%)      $3.63$ (83%)
- (○) $1.84$ (18%)     $212$ (82%)
- (△) $3.39$ (23%)     $297$ (77%)
Dickinson et al 1981). In addition, the affinities of the antagonists at beta_1 and beta_2 receptors respectively were similar when either radioligand was used.

All the antagonists had affinities at binding which were comparable to their affinities at inhibiting a beta-adrenoceptor mediated functional response (discussed in chapter 5).

4:2:4 Effect of NaCl on $[^{125}\text{I}]\text{CYP}$ binding

Binding of $[^{125}\text{I}]\text{CYP}$ to membranes has been reported mainly with the use of a buffer containing saline (Engel et al 1981). Therefore, the binding comparisons between $[^{3}\text{H}]\text{DHA}$ and $[^{125}\text{I}]\text{CYP}$ were performed in 25 mM Tris-HCl pH 7.8, 0.154 M NaCl (Tris-saline) to maintain consistency of experimentation. However, in this laboratory and elsewhere, $[^{3}\text{H}]\text{DHA}$ binding assays have been performed in simple hypotonic Tris buffers (Rugg et al 1978, Mukherjee et al 1976). Thus, the binding characteristics of $[^{125}\text{I}]\text{CYP}$ were examined using Tris-saline or 50 mM Tris-HCl pH 7.8 (Tris) as incubation buffers.

Scatchard analysis of the data revealed that while the $K_D$ for $[^{125}\text{I}]\text{CYP}$ was similar in both buffers, the $B_{\text{max}}$ using Tris buffer was reduced by 46% when compared to the Tris-saline incubated membranes (table 4:1). No deviation from law of mass action binding was apparent since Hill slopes were both close to unity. These experiments were performed at the normal incubation conditions for $[^{125}\text{I}]\text{CYP}$, saturation analysis of $[^{3}\text{H}]\text{DHA}$ binding was unaffected between incubation buffers (not shown). However, when membranes were incubated in Tris with $[^{3}\text{H}]\text{DHA}$ for 60 minutes at 37°C, there was a reduction in specific binding compared to membranes incubated under standard conditions (not shown).
Table 4.1 Comparison of $^{125}$I]CYP binding in Tris and Tris-isosaline buffers. Membranes were incubated with increasing concentrations of $^{125}$I]CYP and respective buffer for 60 minutes at 37°C after which incubations were terminated and specific $^{125}$I]CYP binding assessed. $K_D$ and $B_{max}$ values were estimated by Scatchard analysis and Hill coefficients from Hill plots. Results are expressed as mean ± SEM of four to five experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>$B_{max}$ (fmoles/mg protein)</th>
<th>$K_D$ (pM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-incubated</td>
<td>215 ± 28</td>
<td>30.8 ± 1.6</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>Tris-isosaline incubated</td>
<td>400 ± 9</td>
<td>17.3 ± 1.3</td>
<td>0.91 ± 0.02</td>
</tr>
</tbody>
</table>

4:2:5 NaCl effects on agonist binding

Preliminary experiments showed that isoprenaline displacement of $[^3H]$DHA and $[^125$I]CYP binding was relatively weak compared to earlier experiments in this laboratory using Tris buffers. Therefore, the inhibition of $[^3H]$DHA by isoprenaline was examined using Tris and Tris-isosaline buffers in the incubation. Isoprenaline produced shallow inhibition curves with low Hill slopes in both cases. Computer curve fitting of the data revealed the presence of both high and low affinity states of binding as has been reported for the beta-adrenoceptor (Kent et al 1980), however, the inhibition curve in the presence of NaCl was approximately one order of magnitude to the right and parallel to the curve produced in Tris buffer (fig 4:6). These comparisons could not be performed with $[^125$I]CYP since specific binding decreased with Tris incubations (see 4:2:4).
Figure 4:6. Inhibition of specific $[^3]H$DHA binding by isoprenaline in Tris (●) and Tris-isosaline (▲) buffers. Results are expressed as mean ± SEM of four experiments performed in duplicate. The IC$_{50}$ values and relative proportions of sites (in parentheses) were obtained by computer assisted curve fitting to two sites. $K_i$ values were calculated from IC$_{50}$ values using the Cheng and Prussoff equation and Hill coefficients were obtained from Hill plots.
Effect of EDTA, Mg$^{++}$ and guanine nucleotides on agonist binding

The inhibition of $[^3H]$DHA binding by isoprenaline was examined in Mg$^{++}$ depleted membranes by prior membrane preparation in EDTA containing buffer. The classical shallow displacement curve by isoprenaline was abolished, instead the inhibition curve was steep with a Hill coefficient of 0.88 and an affinity corresponding closely to the low affinity state of agonist binding (fig 4:7a). The presence of the non-hydrolysable analogue of GTP, GppNHp, had only a minor effect on agonist binding, raising the Hill coefficient to a value of 0.99 and decreasing the overall isoprenaline affinity by a factor of two.

Addition of 10 mM MgCl$_2$ to the incubation buffer (fig 4:7b) reintroduced the presence of a high affinity component of agonist binding, resulting in shallow inhibition curves with low Hill slopes which were similar to those obtained in non EDTA treated membranes (fig 4:6). GppNHp produced a shift to the right of the isoprenaline inhibition curve with a resultant steepening of the Hill slope to unity consistent with the conversion of high and low affinity states to a homogeneous low affinity state of binding which appeared to be similar to that obtained with EDTA washed membranes in the absence of Mg$^{++}$.

GTP did not produce a shift of the isoprenaline inhibition curve in the same manner as GppNHp (fig 4:7c). The shift to the right was much smaller and the Hill coefficient of the binding curve was increased to only 0.77 suggesting that the high and low affinity states did not totally convert to a homogeneous low affinity population. Multiple affinity states could not be analysed with GTP modified inhibition curves due to limitations in accurately modelling the data, therefore the overall affinity
Figure 4:7 (a) Isoprenaline inhibition of specific $[^3H]DHA$ binding in EDTA prepared membranes. Membranes were incubated with Tris-HCl containing no Mg$^{++}$ in the absence (●) and presence (▲) of $10^{-4}$ M GppNHp. Inhibition curves were analysed using Hill plots and $K_i$ values calculated from the overall IC$_{50}$ value. Results are mean ± SEM of four experiments. (b) Experiments as above except that 10 mM MgCl$_2$ was included in the incubation medium. The low slope for isoprenaline was analysed by two site curve fitting as described in text. Results are mean ± SEM of four experiments. (c) Isoprenaline inhibition of $[^3H]DHA$ binding in EDTA prepared membranes incubated with 10 mM MgCl$_2$. Isoprenaline was incubated in the absence (●) and presence (▲) of $10^{-4}$ M GTP and data analysed as above. Results are mean ± SEM of three experiments performed in duplicate.
Figure 4:7

(a) $K_i = 56.5 \pm 6.0 \text{ nM}$, $nH = 0.88 \pm 0.02$

(b) $K_L = 81.5 \text{ nM (64\%)}$, $K_i = 111 \pm 16 \text{ nM}$, $nH = 0.67 \pm 0.03$

(c) $K_L = 67.9 \text{ nM (60\%)}$, $K_i = 37.0 \pm 4.5 \text{ nM}$, $nH = 0.65 \pm 0.02$, $K_H = 1.26 \text{ nM (40\%)}$, $nH = 0.77 \pm 0.01$
is shown. Clearly, this is in between the high and low affinity states and further supports the suggestion that GTP is not able to fully convert all the high affinity states of binding. These effects of GTP were also observed in membranes not pretreated with EDTA (not shown) and are contrary to the effects reported for this nucleotide by other workers (Lefkowitz et al 1976b, Ross et al 1977).

4:2:7 NaCl effects on GppNHp modulation of agonist binding

EDTA prepared membranes were incubated in Tris-saline buffer containing 10 mM MgCl₂ using the same incubation conditions as those that gave a good guanine nucleotide 'shift' in the absence of saline (see 4:2:6). Isoprenaline inhibited [³H]DHA binding with a shallow displacement curve and lower potency than that observed in membranes incubated in Tris-10 mM Mg⁺⁺ alone (fig 4:8). In addition, curve fitting to two sites did not result in an adequate fit of the data suggestive of more than two affinity states present. Analysis of the data was made complex due to the existence of a shoulder at low concentrations of isoprenaline, the major component of the inhibition curve still had however a Hill coefficient of less than one. GppNHp was not able to convert the agonist inhibition curve to a homogeneous affinity state with a Hill slope of one contrary to the results obtained in the absence of sodium chloride, the Hill coefficient only being increased to a value of 0.78.

4:2:8 Effects of incubation time and temperature on agonist modulation of binding by GppNHp

EDTA washed membranes were incubated in Tris containing 10 mM Mg⁺⁺, conditions found to be optimal at analysing the effects of
Figure 4:8 Inhibition of \(^{3}\text{H}\)DHA binding in EDTA prepared rat lung membranes by isoprenaline in the absence (●) and presence of \(10^{-4}\) M GppNHp (▲). Membranes were incubated in Tris-isosaline containing 10 mM MgCl\(_2\) and binding described as in text. Computer curve fitting of the isoprenaline inhibition curve to a two site model gave a better fit than to one site, however a poor fit was still obtained suggestive of multiple affinity states. GppNHp modified curves were analysed using Hill plots and overall \(K_i\) values calculated using the Cheng and Prussoff equation. Results are expressed as mean ± SEM of three experiments performed in duplicate.

Figure 4:9 (overleaf) Effects of incubation time and temperature on isoprenaline inhibition of specific \(^{3}\text{H}\)DHA binding and modulation by GppNHp. EDTA washed membranes were incubated in Tris buffer containing 10 mM MgCl\(_2\) using the following conditions: (a) 10 min 22°C; (b) 60 min, 22°C; (c) 60 min, 37°C. Isoprenaline curves were analysed by two site curve fitting and GppNHp modified curves by Hill analysis. Results are mean ± SEM of three to four experiments.
Figure 4:9

(a) $K_L = 62.3 \text{ nM (60\%)}$

\begin{align*}
\text{nH} & = 0.71 \pm 0.02 \\
K_i & = 91.6 \pm 18 \text{ nM} \\
\text{nH} & = 1.04 \pm 0.06
\end{align*}

(b) $K_L = 125 \text{ nM (48\%)}$

\begin{align*}
\text{nH} & = 0.61 \pm 0.01 \\
K_i & = 82.0 \pm 18.7 \text{ nM} \\
\text{nH} & = 0.78 \pm 0.03
\end{align*}

(c) $K_L = 232 \text{ nM (63\%)}$

\begin{align*}
\text{nH} & = 0.57 \pm 0.001 \\
K_i & = 152 \pm 5 \text{ nM} \\
\text{nH} & = 0.84 \pm 0.02
\end{align*}
GppNHp (see 4:2:6). The effects of incubation time were marked only after long incubation periods, after a 10 minute incubation (the shortest time taken for $[^3H]$DHA to reach equilibrium), the isoprenaline inhibition curve and the shift caused by GppNHp were similar to those obtained after 30 minute incubations (fig 4:9a). The low isoprenaline slope was steepped by the nucleotide resulting in a Hill coefficient of unity. After a 60 minute incubation at room temperature however, GppNHp failed to produce a maximal shift, the Hill slope being increased to only 0.78 indicating that heterogeneity of agonist binding sites was still present (fig 4:9b). The isoprenaline inhibition curve also appeared shallower, caused by a slight reduction in potency of the low affinity component as compared to the equivalent component after 10 or 30 minute incubations (fig 4:7b).

Incubation of the membranes at 37°C for 60 minutes had a greater effect on the isoprenaline displacement curve which clearly showed biphasic characteristics (fig 4:9c). GppNHp in a similar fashion to the 60 minute incubation at room temperature failed to convert the heterotropic binding to a single low affinity component, the Hill coefficient being 0.84. It is worthy of note to mention that the high affinity agonist state of binding appeared unchanged irrespective of the incubation conditions, the affinity being identical throughout. The low affinity state of binding appeared to decrease in potency after 60 minute incubations at which stage GppNHp also failed to produce the classical shift of agonist binding associated with guanine nucleotides.

4:2:9 Guanine nucleotide effects on isoprenaline inhibition of $[^{125}\text{I}]$CYP binding

Isoprenaline inhibition of $[^{125}\text{I}]$CYP binding produced a shallow
biphasic inhibition curve which could not be fitted accurately to a one or two site model (fig 4:10). At low isoprenaline concentrations, a shoulder was present similar to that shown in fig 4:8. The guanine nucleotides GTP and GppNHp abolished this component but with apparently little effect on the rest of the inhibition curve. However, neither nucleotide was able to steepen the curves to a Hill slope of one. This observation is similar to that seen for agonist binding with \(^3\)H\(DHA\) after long incubation periods at 37°C except that an even smaller effect of guanine nucleotides was observed.

4:3 Discussion

Comparative experiments of \(^{125}\)I\(CYP\) with \(^3\)H\(DHA\) were necessary in order to examine if the ligand and associated factors such as the composition of buffers or incubation conditions could adversely influence the binding characteristics of agonists or antagonists. \(^{125}\)I\(CYP\) displayed kinetics of binding which were slower compared to \(^3\)H\(DHA\). The association rate is governed by two factors, the concentration of ligand and receptor, since the ligand has a high affinity and is iodinated, low concentrations of both reactants are present resulting in a correspondingly slow rate of association as has been reported for \(^{125}\)I\(HYP\) (Harden et al 1976, Maguire et al 1977). Dissociation of a ligand is independent of these factors and was very slow for \(^{125}\)I\(CYP\) \(t_\frac{1}{2} = 136\) minutes) following first order kinetics contrary to the observation of Engel et al (1981) who showed a biphasic dissociation of \(^{125}\)I\(CYP\) from guinea pig lung membranes. These authors suggested that either two forms of interconvertible receptors existed or that the enantiomers of \(^{125}\)I\(CYP\) dissociated with different rates. These results should be interpreted with caution
Figure 4:10 Isoprenaline inhibition of $[^{125}\text{I}]$CYP binding and modulation by guanine nucleotides in rat lung membranes. Membranes were incubated in Tris-isosaline with $[^{125}\text{I}]$CYP and isoprenaline in the absence (●) and presence of $10^{-4}$ M GTP (▲) or $10^{-4}$ M GppNHp (■). No affinity values could be calculated for isoprenaline since the curve could not be fitted to a one or two site model. Hill coefficients and overall $K_i$ values for GTP and GppNHp modified curves were calculated from Hill plots. Results are means of three to four experiments and standard errors are excluded for clarity but were less than ± 3%. 

(▲) $K_i = 219 \pm 29$ nM

$nH = 0.75 \pm 0.03$

(■) $K_i = 285 \pm 11$ nM

$nH = 0.82 \pm 0.04$
since propranolol was used to estimate the specific binding. In view of the observation that propranolol may displace radiolabelled antagonist binding from non receptor sites (Nahorski and Richardson 1979, Dax and Partilla 1982), the biphasic dissociation could also be accounted for by the dissociation of ligand from receptors and a lower affinity non specific binding component. As no biphasic dissociation was seen when isoprenaline was used to assess non specific binding in this study (see methods), the latter suggestion may be an alternative explanation for these findings.

The interpretation of kinetic data when using $^{125}$I]CYP should also be viewed with caution on two grounds:

(i) The very high affinity of $^{125}$I]CYP may result in more than 10% of the total added ligand to be bound to the receptor thereby affecting the pseudo-first order association.

(ii) Dissociation of ligand is hampered by long incubation periods resulting in visible oxidation of isoprenaline and possible incorrect estimation of non specific binding.

With these points in mind, the kinetically derived $K_D$ for $^{3}$H]DHA resulted in a good correlation with the equilibrium determined $K_D$ while the kinetic values for $^{125}$I]CYP gave a $K_D$ estimation that was of a slightly higher affinity than the $K_D$ determined by equilibrium analysis. Mention should also be made of the racemic nature of $^{125}$I]CYP which would lead to errors in assessing the $K_D$ of the active isomer. Bürgisser et al (1981) have clearly shown that the $K_D$ of a racemic ligand may vary depending on the receptor concentration and that the apparent $K_D$ is a hybrid of the $K_D$s for the (+) and (-) isomers respectively. This additional factor may also explain differences in $K_D$ estimations with these
two commonly used methods.

Both $[^3]$H]DHA and $[^125]$I]CYP labelled similar proportions of sites which were clearly identified as beta-adrenoceptors since stereoselectivity was shown in both cases and selective antagonists were able to delineate beta$_1$ and beta$_2$ adrenoceptors. $[^125]$I]CYP therefore could be used to quantitatively and qualitatively identify beta-adrenoceptors and has been used to identify beta-adrenoceptors on adipocytes (chapter 5). Small differences were still apparent with respect to antagonist affinities and all the drugs exhibited greater potencies at the beta-adrenoceptor when using $[^3]$H]DHA compared to $[^125]$I]CYP. This problem has been resolved experimentally and theoretically by Bürgisser et al (1981), however the differences were considered to be acceptable and at the time of writing no suitable alternative ligand was available. The future use of (−) enantiomers of potent antagonists as radioligands would be an advantage in minimising any sources of error that may at present exist.

The thermal stability of beta-adrenoceptors is a field which has received little attention and may be of importance when receptors are subjected to long incubations at physiological temperatures. Baker and Potter (1981) have shown that canine heart beta-adrenoceptors could be almost totally lost after incubation at 50°C for one hour in a standard Tris buffer. Occupation of the receptors with agonists but not antagonists resulted in no loss of receptors following the same treatment and prompted the discussion that altered receptor conformations may drastically affect the stability to temperature degradation. Relating these findings to the effects observed with $[^125]$I]CYP together with the dilute amount of protein involved in an assay, a significant proportion of receptors could be lost during the course of the
incubation. Inclusion of sodium chloride appeared to stabilise the receptors at the incubation conditions used such that the same density was observed as that found under normal $[^3H]$DHA binding conditions (Rugg et al 1978, Ashton et al 1981). Stabilisation of soluble beta-adrenoceptors by sodium chloride has been reported by Caron et al (1979) and Dickinson (1982), furthermore, the latter author showed that the protective nature of sodium chloride was directly correlated to the ionic strength of the buffers and suggested that the 'salting in' of proteins may conformationally favour receptor stability. The inclusion of sodium chloride to maintain receptor stability using $[^{125}\text{I}]$CYP is therefore an absolute requirement.

The binding of antagonists to beta-adrenoceptors appears not to be influenced by ionic conditions (Williams et al 1978, U'Pritchard et al 1978), however, the effects of ions can profoundly influence the binding characteristics of agonists. As sodium chloride is required for $[^{125}\text{I}]$CYP binding the effects of the salt were examined with respect to agonists affinities and is discussed later under the appropriate conditions, the effects on guanine nucleotide modulation of agonist binding. Sodium chloride has been shown to decrease agonist affinities at beta-adrenoceptors (U'Pritchard et al 1978, Heidenreich et al 1980) as well as opiate receptors (Pert and Snyder 1974, Pasternak and Snyder 1975), alpha-adrenoceptors (Greenberg et al 1978, Tsai and Lefkowitz 1978, Gonzalez et al 1981), histamine receptors (Chang and Snyder 1980) and muscarinic receptors (Birdsall et al 1979). The mechanism of decreased affinity has been reported to be an increased dissociation rate of agonist from the receptor as determined using $[^3\text{H}]$hydroxybenzylisoprenaline, an agonist (Heidenreich et al 1980). The effect of sodium has been ascribed to the hydrated radius of
the ion since ions of similar size such as Li$^+$ and NH$_4^+$ had comparable effects (U'Pritchard et al 1978). The site of action at present is unknown although the effects of sodium on alpha-adrenoceptors appears to be direct as they can still be demonstrated with solubilised receptors that are divorced from guanine nucleotide binding proteins (Limbird and Speck 1983).

Experiments analysing the effects of Mg$^{++}$ on agonist binding were performed because initial experiments failed to show large effects of the guanine nucleotide GTP to alter the binding properties of isoprenaline as has been reported elsewhere (Lefkowitz et al 1976b, Maguire et al 1976). In addition, Mg$^{++}$ and sodium chloride have been reported to have opposing effects with respect to agonist affinities at beta-adrenoceptors (Heidenreich et al 1980). In view of the complexities of interpreting the data, the effects of Mg$^{++}$ on agonist binding were analysed first followed by the effects of sodium chloride at influencing the binding properties in the absence and presence of guanine nucleotide.

The requirement of Mg$^{++}$ for the expression of high affinity agonist binding at beta-adrenoceptors has been demonstrated in several tissues including rat reticulocytes (Shane et al 1981), turkey erythrocytes (Shane et al 1981, Vauquelin et al 1982), frog erythrocytes (Williams et al 1978), murine S49 lymphoma (Bird and Maguire 1978), rat lung (Heidenreich et al 1980) and rabbit lung (Dickinson and Nahorski 1983). The effects of Mg$^{++}$ also extend to other receptors and increased agonist affinities have been reported at alpha-adrenoceptors (Tsai and Lefkowitz 1978, U'Pritchard and Snyder 1980), opiate receptors (Pasternak et al 1975), PGE$_1$ receptors (Williams et al 1978) and oxytocin receptors (Pearlmutter and Soloff 1979). The presence of Mg$^{++}$ for activation of receptors by agonists would thus appear essential since the
most widely accepted theory of receptor-effector coupling involves the formation of a ternary complex (De Lean et al 1980) through which the high affinity agonist state of binding is mediated (see Stadel et al 1982). The removal of Mg\(^{++}\) by EDTA pretreatment abolished the high affinity agonist binding state confirming the data of Williams et al (1978) where agonist inhibition curves became steep in the absence of Mg\(^{++}\) (or another divalent cation). As GppNHp had a minor effect on the isoprenaline inhibition curve, it is possible that even after the EDTA treatment used in this study, trace amounts of a divalent cation are still present to influence the overall inhibition curve. In view of the fact that membranes which were not EDTA treated but extensively washed still showed biphasic agonist inhibition curves, it appears that divalent cations are tightly bound and retained after washing to produce a maximal degree of high affinity binding. Since other membranes may not retain sufficient divalent cations to produce a maximal amount of high affinity binding, experiments should be performed in the presence of Mg\(^{++}\) to enable guanine nucleotide shifts to be observed. The supposition that sufficient Mg\(^{++}\) is retained in rat lung membranes is supported by the observation that addition of 10 mM Mg\(^{++}\) to EDTA washed membranes reintroduced the high affinity component and resulted in inhibition curves similar to the control membrane population. The use of 10 mM Mg\(^{++}\) was chosen since the Mg\(^{++}\) requirement for expression of high affinity binding has been shown to be half maximal at 2-3 mM Mg\(^{++}\) in S49 lymphoma membranes (Bird and Maguire 1978).

The inability of GTP to decrease agonist affinities may be related to the hydrolysis of GTP since the non-hydrolysable GppNHp had the effect of fully converting the heterotropic binding. The lack of effect by GTP is not due to the concentration of
nucleotide used since maximal effects have been shown at 30 μM (Maguire et al. 1976, Bird and Maguire 1978). GTP insensitive states of agonist binding have been also observed in rabbit lung (Dickinson and Nahorski 1983), however the agonist inhibition curve was steepened by GTP to a Hill slope of one when the membranes were incubated at 37°C in the presence of Mg^{++} and EDTA. The major effect of Mg^{++} was to allow GTP inhibition by isoprenaline to be observed rather than an increase in high affinity agonist binding. These effects are not easily explained by the perhaps simplistic assumptions above and alternative explanations have included the proposal that EDTA may clear the guanine nucleotide regulatory protein of GDP or remove an inactive divalent cation (Shane et al. 1981), the effects of Mg^{++} may be to facilitate GDP/GTP exchange (Cassel and Selinger 1978) or enhance the effects of GTP by forming Mg-GTP complex. However in the present study, the effects of EDTA washing and reintroduction of Mg^{++} did not enable GTP to produce a 'GTP shift'. Future experiments using GTP regenerating systems may answer some of the questions posed. As GppNHp produced a classical guanine nucleotide 'shift' it was used to assess the modulation of agonist binding in the presence of sodium chloride and at different incubation conditions.

Inhibition of [\(^3\)H]DHA binding in Tris-isosaline and its modulation by GppNHp was complex and was quite different to the observation in sodium chloride free buffer. No real estimations can be made of multiple affinity states since no models as yet exist to explain the effects reported. Heidenreich et al. (1980) using [\(^3\)H]hydroxybenzylisoprenaline reported that sodium decreased the agonist affinity and could be overcome by 9 mM Mg^{++}. This effect was not seen since the membranes incubated in sodium
chloride containing buffer still showed a lower overall affinity for agonists than Tris incubated membranes although a shoulder appeared at low agonist concentrations which might be attributed to the reversal effect shown by Heidenreich et al (1980). Divalent cations have also been shown to reverse sodium chloride effects at alpha-adrenoceptors (U'Pritchard and Snyder 1980), these effects may point to a possible site of action of sodium chloride at the guanine nucleotide regulatory protein as the effects of Mg^{++} are clearly mediated through this since the mutant cell lines of S49 lymphoma CYC^- and UNC which are deficient in a functional N protein show no Mg^{++} effects on agonist binding (Bird and Maguire 1978).

The inability of GppNHp to produce a normal shift may be the result of an interfering effect of sodium either at the receptor or N protein, the effect of GppNHp has also been shown by Heidenreich et al (1980) where the nucleotide shifted the agonist Hill slope from 0.55 to 0.88. No comment was made on this effect although it should also be reported that Minneman et al (1979a) have demonstrated no deleterious effects of sodium chloride on GTP modulation of agonist binding. It should be emphasised that most ligand binding studies are performed in simple hypotonic buffers which bear no resemblance to a physiological medium. In view of the differences observed with nucleotide modulation of agonist binding in Tris and Tris-isosaline buffers, it may be more pertinent to perform the studies in physiological solutions thus attempting to mimic in-situ receptor interactions. It may be possible that the biochemical observations in simple buffers are atypical of true coupling phenomena and these questions remain to be answered.

Another factor which may influence agonist binding that appears
to have been overlooked is the effect of incubation time. Most studies have been performed at the normal incubation conditions determined by the radioligand used. In this study, there is a clearly decreased ability of GppNHp to induce a shift to a law of mass action curve after 60 minutes of incubation. Changes in agonist affinities at different temperatures may lead to errors of interpretation since thermodynamic parameters may cause these to change (Weiland et al 1979, Weiland et al 1980), however at 22°C, the isoprenaline inhibition curve appeared to be of slightly lower overall affinity compared to the 10 or 30 minute incubations. The interpretation of this data is again complicated by a lack of suitable models to explain the observation. It is possible that this is a mechanism of receptor desensitisation by a decreased ability of the active ternary complex to be dissociated by guanine nucleotides and activate adenylate cyclase. It should be noted that Ross et al (1977) using $[^{125}\text{I}]$HYP in S49 lymphoma membranes at incubation times of 30 minutes at 37°C showed that GppNHp increased the Hill coefficient for isoprenaline from 0.52 to only 0.83. It is possible that the reduced shift in affinity has not been carefully studied and consequently not discussed. Numerous schemes of desensitisation have been proposed and include a decrease in the total beta-adrenoceptor density (Mickey et al 1975) as well as a decrease in the proportion of high affinity agonist binding sites (Wessels et al 1979). Hoffman and Lefkowitz (1980) have suggested that the high affinity form of agonist binding may be a crucial intermediate for desensitisation and a functional N protein is necessary since the guanine nucleotide deficient S49 mutant cell line CYC does not show down regulation (Shear et al 1976). Stadel and Lefkowitz (1979) have shown that agonist occupancy by itself is not sufficient to initiate desensitisation
thus a functional N protein may be necessary. The results presented here although intriguing should be cautiously viewed since it is also possible that the guanine nucleotide regulatory protein or indeed the process involved with the association and dissociation of the ternary complex may be labile and thus show impaired binding properties after long incubations. Clearly, further experiments by analysing agonist occupied and unoccupied receptor incubations may be useful in elucidating whether this phenomenon has a functional role or is simply an artifact of the incubation conditions used.

The analysis of $[^{125}\text{I}]$CYP binding with respect to agonist inhibition and modulation by guanine nucleotides is thus impossible to interpret. The binding characteristics are clearly different to those reported for other ligands and can from the work in this study be attributed to two factors:

(i) The necessary inclusion of sodium chloride to protect the receptors from inactivation.

(ii) Long incubations are necessary to achieve equilibrium of binding.

$[^{125}\text{I}]$CYP therefore from these studies can be successfully used to analyse receptor heterogeneity and has been used to identify beta-adrenoceptors on fat cells (chapter 5). The analysis of agonist receptor interactions would be best achieved with another ligand which has rapid kinetics thus enabling experiments to be performed without the constraints imposed above.
CHAPTER 5

Identification and classification of beta-adrenoceptors in rat lung, whole fat pad and isolated adipocyte membranes
5:1 Introduction

The development of radioligand binding techniques and use of drugs selective for beta_1 or beta_2 adrenoceptors has provided a powerful method by which beta-adrenoceptors can be directly identified and subclassified (Minneman et al 1981, Nahorski 1981). From these types of study it has essentially been shown that in mammalian species at least, only two subtypes of beta-adrenoceptor are present in a wide range of tissues and species (Minneman et al 1979b). Furthermore, beta-adrenoceptor subtypes have also been shown to coexist in a variety of tissues (Nahorski 1981).

The precise classification of the rat adipocyte beta-adrenoceptor is unclear. Results from functional lipolytic studies have suggested a beta_1-adrenoceptor (Lands et al 1967), beta_2-adrenoceptor (Jolly et al 1978) and an atypical or hybrid beta-adrenoceptor (Harms et al 1974, Harms et al 1977, De Vente et al 1980). Clearly, the use of ligand binding studies to identify and subclassify beta-adrenoceptors on adipocyte membranes would be a direct approach to clarify the present uncertainty of classification and has been performed in this study.

Early attempts to identify beta-adrenoceptors on adipocyte membranes were unsuccessful since agonists such as [^3H]adrenaline (Jarrett et al 1974, Lesko and Marinetti 1975) and [^3H]noradrenaline (Aprille et al 1974, Koretz and Marinetti 1974, Giudicelli et al 1977) were used to label the putative receptor sites. The problems of the use of agonists in ligand binding studies have already been outlined in chapter 4 and in adipocyte membranes few of the criteria for receptor binding were satisfied. Furthermore, binding was present in subcellular fractions other than plasma membrane where the beta-adrenoceptor was thought to be located.

The first report of ligand binding which appeared to fulfil
receptor binding criteria, at least in part, was by Williams et al (1976) using $[^3H]$DHA and subsequently confirmed by other workers (Giudicelli and Pecquery 1978, Malbon et al 1978, Giudicelli et al 1979a, Cabelli and Malbon 1979). These initial studies although encouraging disclosed certain problems in the use of $[^3H]$DHA as binding did not follow law of mass action behaviour since Scatchard plots were curvilinear with upward concavity which could be interpreted as negative cooperativity or multiple affinity states. The only subclassification attempted was with agonists where the rank order of potency suggested a beta$_1$-adrenoceptor (Williams et al 1976, Malbon et al 1978, Cabelli and Malbon 1979).

With respect to receptor classification, the use of selective antagonists has also enabled atypical beta-adrenoceptors to be identified in non mammalian species such as avians and amphibians from both binding (Minneman et al 1980, Dickinson and Nahorski 1981) and functional studies (O'Donnell and Wanstall 1982). Therefore, the aims of this study were to identify beta-adrenoceptors on whole fat pad and isolated adipocytes using the newly developed radioligand $[^{125}\text{I}]$CYP and to perform a comparative study with rat lung, a tissue with a well defined population of beta$_1$ and beta$_2$ adrenoceptors (Rugg et al 1978, Minneman et al 1979a, Dickinson et al 1981).

5.2 Results

All binding studies were performed after 60 minutes of incubation at 37°C as preliminary experiments showed that equilibrium of binding had been reached at these incubation conditions (not shown). In view of the slow dissociation rate of $[^{125}\text{I}]$CYP and oxidation of isoprenaline no kinetic experiments
were performed.

5:2:1 Saturability of specific $^{125}$I CYP binding to whole fat pad and isolated adipocyte membranes

Specific binding of $^{125}$I CYP determined as binding in the absence and presence of 200 µM (-)isoprenaline was saturable in both membrane preparations whereas non specific binding increased linearly with radioligand concentration (fig 5:1). Scatchard analysis of the specific binding data revealed a homogeneous population of binding sites in both cases with dissociation constants for whole fat pad and isolated adipocyte of 33.5 ± 3.9 pM and 29.1 ± 1.8 pM respectively, these values agreeing closely with that already obtained in rat lung membranes (chapter 4). The maximal binding capacities were 66.3 ± 3.0 fmoles/mg protein for whole fat pad and 53.3 ± 1.4 fmoles/mg protein for isolated adipocytes. The maximal binding capacity for isolated adipocytes reported here is about five times lower than those reported by other workers using $[^3]$H DHA as radioligand (Williams et al 1976, Giudicelli and Pecquery 1978, Dax et al 1981b). Hill plots of the data could not be performed as in each membrane preparation one point on the Scatchard plot was above the determined $B_{\text{max}}$ value.

5:2:2 Pharmacological characterisation of $^{125}$I CYP binding sites on lung, whole fat pad and isolated adipocytes

(A) Agonists

Competition experiments with natural and synthetic agonists were performed in order to characterise the binding sites on lung, whole fat pad and isolated adipocytes as originally defined by Lands et al (1967). Fig 5:2 shows that the potencies of agonists at inhibiting binding in each membrane preparation
Figure 5.1  Saturation of $[^{125}\text{I}]$CYP binding in (a) whole fat pad and (b) isolated adipocyte membranes. Membranes were incubated with increasing concentrations of $[^{125}\text{I}]$CYP and specific binding determined as described in methods. The results are expressed as mean ± SEM of five experiments performed in triplicate. (c) Scatchard analysis of the above data from which $B_{\text{max}}$ and $K_D$ values were determined. Values were: Whole fat pad $K_D = 33.5 \pm 3.9$ pM, $B_{\text{max}} = 66.3 \pm 3.0$ fmoles/mg protein; isolated adipocyte $K_D = 29.1 \pm 1.8$ pM, $B_{\text{max}} = 53.3 \pm 1.4$ fmoles/mg protein. Hill plots could not be constructed as one point was above the calculated $B_{\text{max}}$ value.
Figure 5:1
Figure 5:2 Inhibition of specific $^{125}$I-CYP binding to (a) rat lung, (b) whole fat pad and (c) isolated adipocyte membranes by the adrenergic agonists (●) (-)isoprenaline, (▲) (-)adrenaline and (■) (-)noradrenaline. Membranes were incubated with $^{125}$I-CYP and agonist for 60 minutes at 37°C and specific binding determined as described in text. Results are expressed as mean ± SEM of three to four experiments performed in duplicate.
were different indicating possible differences in receptor subtype. The rank order of potency at the $IC_{50}$ level in rat lung and whole fat pad was (-)isoprenaline > (-)adrenaline > (-)noradrenaline which is suggestive of a beta$_2$ adrenoceptor while for isolated adipocytes the rank order of potency was (-)isoprenaline > (-)noradrenaline > (-)adrenaline which is more characteristic of a beta$_1$ adrenoceptor subtype (Lands et al 1967) and which compares to the order found from lipolytic experiments on intact adipocytes. The use of agonists to delineate receptor subtypes can be somewhat misleading due to the presence of high and low affinity states of binding (Kent et al 1980) which resulted in inhibition curves with low slopes. A more correct approach would be to examine rank orders of potency in the presence of a guanine nucleotide to abolish heterotrophic binding, however this could not be performed using $^{125}\text{I}]$CYP for the reasons discussed in chapter 4. In addition, the quantification of receptor subtypes with agonists in the event of a heterogeneous receptor population would be impossible due to low or absent selectivity of the agonists used.

**(B) Antagonists**

Inhibition of $^{125}\text{I}]$CYP binding by the stereoisomers of propranolol was stereoselective in all three membrane preparations, in all cases the (-) isomer was approximately two orders of magnitude more potent than the (+) isomer (table 5:1). The affinity constants of both isomers of propranolol compared very favourably with affinities reported for the antagonists derived from functional studies (Harms et al 1977). Detailed comparisons of the inhibition curves revealed that while the inhibition constants for (-)propranolol were not significantly different between lung and isolated adipocyte membranes ($K_i = 0.949 \pm 0.014$ nM and 2.33 ± 0.29 nM respectively), the (+) isomer of propranolol
Table 5: Inhibition constants and Hill coefficients for the stereoisomers of propranolol, H35/25 and salbutamol determined from $^{[125]}$ICYP inhibition in rat lung, whole fat pad and isolated adipocyte membranes.

<table>
<thead>
<tr>
<th>drug</th>
<th>lung</th>
<th>whole fat pad</th>
<th>isolated adipocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_1$</td>
<td>nH</td>
<td>$K_1$</td>
</tr>
<tr>
<td>(-)propranolol</td>
<td>0.949 ± 0.014</td>
<td>1.13 ± 0.04</td>
<td>2.28 ± 0.18</td>
</tr>
<tr>
<td>(+)propranolol</td>
<td>65.2 ± 5.9</td>
<td>1.16 ± 0.05</td>
<td>116 ± 8</td>
</tr>
<tr>
<td>H35/25</td>
<td>657 ± 29</td>
<td>0.96 ± 0.02</td>
<td>1320 ± 1.14</td>
</tr>
<tr>
<td>salbutamol</td>
<td>2300 ± 320</td>
<td>0.97 ± 0.03</td>
<td>-</td>
</tr>
</tbody>
</table>

IC$_{50}$ values and Hill coefficients were calculated from Hill plots and $K_1$ values (nM) calculated from IC$_{50}$s using the Cheng and Prussoff equation. Results are expressed as mean ± SEM of three to five determinations performed in duplicate.
was significantly less potent at adipocyte ($K_i = 306 \pm 16$ nM)
than lung membranes ($K_i = 65.2 \pm 5.9$ nM, $p < 0.01$ Student's t test).
Therefore, the degree of stereoselectivity in adipocytes (131 fold)
was higher than in rat lung membranes (69 fold).

The inhibition curves of selective beta$_1$ adrenoceptor antag-
onists in rat lung membranes were shallow with Hill coefficients
less than unity suggesting negative cooperativity or multiple
affinity states (fig 5:3). All the curves were clearly biphasic
and similar in appearance with approximately 20-30% of binding
being of high affinity. Inhibition of binding by the beta$_2$ 'select-
tive' agents did not result in a uniform set of inhibition curves,
the antagonist H35/25 (Levy 1967) and agonist salbutamol (Brittain
et al 1968) inhibited binding with Hill slopes close to unity
(fig 5:3 and table 5:1) while the antagonist ICI 118.551 and agonist
procaterol (Yabuuchi et al 1977) inhibited binding in a biphasic
manner with curves that appeared to be mirror images of those
produced by the beta$_1$ selective antagonists. Analysis of the
biphasic inhibition curves produced by the selective agents using
the computerised iterative curve fitting routine described in
chapter 2 produced best fit parameters for a two site model
(Appendix 6). The relative proportions of sites of the combined
data were 78 ± 2% beta$_2$ adrenoceptors and 22 ± 2% beta$_1$ adreno-
ceptors (table 5:2) which agrees well with previous reports for
this tissue (Rugg et al 1978).

Inhibition of [125I]CYP binding by selective agents to whole
fat pad (fig 5:4) and isolated adipocyte membranes (fig 5:5)
resulted in curves which deviated from law of mass action type
behaviour in both cases. The shapes of the curves were different
to those obtained with rat lung and with each membrane preparation,
the inhibition curve with a beta$_1$ selective antagonist was
Figure 5:3 Inhibition of specific $[^{125}\text{I}]\text{CYP}$ binding in rat lung by (a) the beta$_{1}$ selective antagonists (•) betaxolol, (▲) metoprolol, (■) atenolol and (O) practolol and (b) by the beta$_{2}$ selective agents (•) ICI 118.551, (▲) procaterol, (■) H35/25 and (O) salbutamol. $[^{125}\text{I}]\text{CYP}$ and respective drug were incubated with membranes for 60 minutes at 37°C and specific binding determined as described in methods. Results are expressed as mean ± SEM of three to eight experiments performed in duplicate.
Figure 5:4 Inhibition of specific $^{125}$I CYP binding to whole fat pad membranes by (a) the selective beta$_1$-adrenoceptor antagonists betaxolol (●), metoprolol (▲), atenolol (■) and practolol (O) and (b) the selective beta$_2$-adrenoceptor antagonist ICI 118.551. $^{125}$I CYP (approx. 50 pM), respective antagonist and membranes were incubated for 60 minutes at 37°C and specific binding determined as described in methods. Results are expressed as mean ± SEM of three to five experiments performed in duplicate.
Figure 5:5 Inhibition of specific $[^{125}\text{I}]$CYP binding to isolated adipocyte membranes by (a) the beta$_1$-adrenoceptor selective antagonists betaxolol (●), metoprolol (▲), atenolol (■) and practolol (O) and (b) by the selective beta$_2$-adrenoceptor agents ICI 118.551 (●) and procaterol (▲). $[^{125}\text{I}]$CYP was incubated with respective drug and membranes for 60 minutes at 37°C and specific binding determined as described in methods. Results are expressed as mean ± SEM of three to six determinations performed in duplicate.
Table 5:2  
Inhibition constants of selective adrenergic agents at beta-adrenoceptor subtypes of rat lung, whole fat pad and isolated adipocyte membranes.

<table>
<thead>
<tr>
<th>beta&lt;sub&gt;1&lt;/sub&gt; selective antagonists</th>
<th>lung K&lt;sub&gt;i&lt;/sub&gt; (M)</th>
<th>whole fat pad K&lt;sub&gt;i&lt;/sub&gt; (M)</th>
<th>isolated adipocyte K&lt;sub&gt;i&lt;/sub&gt; (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(t)atenolol</td>
<td>1.78×10&lt;sup&gt;-7&lt;/sup&gt; (19%)</td>
<td>1.05×10&lt;sup&gt;-5&lt;/sup&gt; (81%)</td>
<td>6.32×10&lt;sup&gt;-7&lt;/sup&gt; (40%)</td>
</tr>
<tr>
<td>(t)metoprolol</td>
<td>2.41×10&lt;sup&gt;-8&lt;/sup&gt; (28%)</td>
<td>9.12×10&lt;sup&gt;-7&lt;/sup&gt; (72%)</td>
<td>1.33×10&lt;sup&gt;-7&lt;/sup&gt; (49%)</td>
</tr>
<tr>
<td>(t)practolol</td>
<td>2.44×10&lt;sup&gt;-7&lt;/sup&gt; (19%)</td>
<td>4.24×10&lt;sup&gt;-5&lt;/sup&gt; (81%)</td>
<td>7.51×10&lt;sup&gt;-7&lt;/sup&gt; (35%)</td>
</tr>
<tr>
<td>(t)betaxolol</td>
<td>3.39×10&lt;sup&gt;-9&lt;/sup&gt; (23%)</td>
<td>2.97×10&lt;sup&gt;-7&lt;/sup&gt; (77%)</td>
<td>9.08×10&lt;sup&gt;-9&lt;/sup&gt; (36%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>beta&lt;sub&gt;2&lt;/sub&gt; selective agents</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(t)ICI 118.551</td>
<td>1.42×10&lt;sup&gt;-7&lt;/sup&gt; (17%)</td>
<td>3.63×10&lt;sup&gt;-9&lt;/sup&gt; (83%)</td>
<td>1.69×10&lt;sup&gt;-7&lt;/sup&gt; (60%)</td>
</tr>
<tr>
<td>(t)procaterol</td>
<td>5.15×10&lt;sup&gt;-6&lt;/sup&gt; (24%)</td>
<td>1.99×10&lt;sup&gt;-7&lt;/sup&gt; (76%)</td>
<td>-</td>
</tr>
</tbody>
</table>

| mean relative proportions           | 22 ± 2%          | 78 ± 2%          | 40 ± 2%          |

K<sub>i</sub> values were calculated using the Cheng and Prussoff equation. IC<sub>50</sub>s and relative proportions of sites (in parentheses) were determined by two-site curve fitting analysis and affinity constants shown are overall apparent K<sub>i</sub> values from meaned curves.
approximately a mirror image of that obtained with a beta$_2$ selective
agent. Curve fitting of the data gave again best fit parameters
for a two site model, the overall receptor proportions being
60 ± 2% beta$_2$, 40 ± 2% beta$_1$ adrenoceptors for whole fat pad and
20 ± 2% beta$_2$, 80 ± 2% beta$_1$ adrenoceptors for isolated adipocytes
(table 5:2). This suggests that in whole fat pad, a large propor­
tion of beta$_2$ adrenoceptors are present which are not located on
adipocytes but on other cell types. In view of the large beta$_2$
receptor population, it is possible that contamination during
isolated adipocyte preparation may account for the small proportion
of beta$_2$ receptors on isolated adipocyte membranes although the
possibility of beta-adrenoceptor heterogeneity on adipocytes them­
selves cannot be discounted.

The use of curve fitting analysis to quantify beta$_1$ and beta$_2$
adrenoceptor subtypes can be validated since irrespective of the
membrane preparation used, the inhibition constants of selective
drugs were similar at the respective beta$_1$ and beta$_2$ adrenoceptors
(see also 5:2:3 for detailed analysis of the beta$_1$ adrenoceptor
on isolated adipocytes). Furthermore, the inhibition constants
are directly correlated to the mean affinities of the drugs at each
receptor subtype as determined from functional studies (slope =
0.82, r = 0.90 for affinities at beta$_1$ adrenoceptors, slope = 1.05,
r = 0.99 for affinities at beta$_2$ adrenoceptors, table 5:3).

5:2:3 Correlation of inhibition constants of drugs for beta$_1$
adrenoceptors between lung and isolated adipocyte membranes

In order to confirm the proposal that the beta-adrenoceptor
present on adipocytes is beta$_1$ in nature, the inhibition constants
of non selective antagonists and selective agents at beta$_1$ adreno­
ceptors were compared between rat lung and isolated adipocytes
Table 5:3 Comparison of pA₂ values of selective antagonists at beta₁ (atrial) and beta₂ (tracheal or uterine) adrenoceptors derived from functional studies with -log Kᵢ values derived from binding studies in lung, whole fat pad and isolated adipocyte membranes.

<table>
<thead>
<tr>
<th>drug</th>
<th>beta₁ adrenoceptors</th>
<th>beta₂ adrenoceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pA₂</td>
<td>-log Kᵢ</td>
</tr>
<tr>
<td>(±)atenolol</td>
<td>7.68ᵃ</td>
<td>7.27ᵇ</td>
</tr>
<tr>
<td>(±)betaxolol</td>
<td>8.29ᵃ</td>
<td>5.51ᵈ</td>
</tr>
<tr>
<td>(±)H35/25*</td>
<td>7.17ᵉ</td>
<td>6.96ᶠ</td>
</tr>
<tr>
<td>(±)ICI 118.551</td>
<td>7.45ᵃ</td>
<td>7.43ᶜ</td>
</tr>
<tr>
<td>(±)metoprolol</td>
<td>7.30ᵃ</td>
<td>6.49ᵇ</td>
</tr>
</tbody>
</table>

-log Kᵢ values are expressed as mean ± SEM and are pooled values from data in tables 5:1 and 5:2. pA₂ values were obtained from the following references: (a) Boudot et al 1979, (b) Barrett et al 1973, (c) Harms 1976a, (d) O'Donnell and Wanstall 1979a, (e) Bilski et al 1980, (f) O'Donnell and Wanstall 1980.

* As H35/25 did not adequately fit a two site model, -log Kᵢ values quoted are those obtained by one site analysis in rat lung (overall beta₂ adrenoceptor) and isolated adipocyte (overall beta₁ adrenoceptor).
Figure 5:6 Correlation of inhibition constants of selective and non-selective drugs at rat lung (abcissa) and isolated adipocyte (ordinate) beta$_1$-adrenoceptors. Inhibition constants for non-selective drugs were determined from overall inhibition curves using Hill plots and for selective drugs were calculated from the two site curve fitting routine. Linear regression of the points gave values of slope $= 0.97$ and correlation coefficient $= 0.98$. 

(a) atenolol  
(b) betaxolol  
(H) H35/25  
(I) ICI 118.551  
(m) metoprolol  
(pra) practolol  
(pro) procaterol  
((+)p) (+)propranolol  
((-)p) (-)propranolol
This type of analysis has been used by Minneman et al (1980) and Dickinson and Nahorski (1981) to distinguish typical mammalian beta1 and beta2 adrenoceptors from atypical avian and amphibian beta-adrenoceptors. If the same type of beta1 adrenoceptor was present on both membrane preparations, a slope of one and correlation of one would be expected. Linear regression of the points gave values of slope = 0.97, r = 0.98 which clearly identifies the adipocyte beta-adrenoceptor as beta1 in subtype.

Discussion

Over the past few years there has been some controversy over the strict subclassification of beta-adrenoceptors into beta1 and beta2 subtypes. Although Lands et al (1967) subdivided beta-adrenoceptors into beta1 and beta2 classes, other workers have suggested either a single beta-adrenoceptor (Leclerc et al 1981) or multiple subtypes of beta-adrenoceptor (Bristow et al 1970, Boissier et al 1971, Levy 1973, Ahlquist 1976). As the evidence for multiple beta-adrenoceptors has been derived from functional studies, the differences quoted should be carefully interpreted since in order to perform accurate quantitative analysis, several criteria have to be taken into consideration (Furchgott 1970). In view of these points, O'Donnell and Wanstall (1976) have demonstrated that apparent differences between the beta-adrenoceptors of respiratory and vascular smooth muscle can be accounted for by extraneuronal uptake of agonists.

Since the use of ligand binding studies has indicated only two subtypes of beta-adrenoceptor in mammalian species (Minneman et al 1979b), the present studies were performed with this technique to accurately quantify and compare the pharmacological characteristics of the adipocyte beta-adrenoceptors to those of rat lung.
From these studies a binding site has been identified which possesses all the properties of a beta-adrenoceptor in both whole fat pad and isolated adipocyte membranes. Most previous reports on \[^{3}\text{H}]\text{DHA}\) binding to rat adipocyte (Williams et al 1976, Giudicelli and Pecquery 1978, Goswami and Rosenberg 1978, Malbon et al 1978, Giudicelli et al 1979a) have indicated a much larger density of receptor sites than reported in this study. Furthermore, the dissociation constants reported for \[^{3}\text{H}]\text{DHA}\) in these studies were much higher (15-25 nM) than those reported in other tissues such as rat lung (Dickinson et al 1981), heart (Cheniieux-Guicheney et al 1978), brain (Nahorski 1978) and liver (Munnich et al 1981). In one study (Cabelli and Malbon 1979) \[^{3}\text{H}]\text{DHA}\) binding was found not to saturate at 100 nM in whole adipocytes and the phenomenon of low affinity / high capacity binding has also been observed in mouse and rabbit adipocytes (Fan and Ho 1980) but not in human (Burns et al 1981, Engfeldt et al 1982) or hamster brown adipocytes (Svoboda et al 1979). An associated factor with the low affinity binding in rat adipocytes was the apparent negative cooperativity or heterogeneous nature of the binding. The former suggestion was excluded by Malbon and Cabelli (1978) and Giudicelli et al (1979a) after applying the direct kinetic method of De Meyts et al (1973) and the proposal that high and low affinity states of binding existed confirmed (Giudicelli et al 1979a, Dax et al 1981b, Dax et al 1982). Furthermore, Dax et al (1982) demonstrated that the low affinity of binding was not stereoselective and suggested that this was a non receptor site. Problems with the incorrect estimation of specific binding have been attributed to the use of propranolol to define non specific binding in these experiments. These effects of propranolol have been observed in bovine lung (Nahorski and Richardson 1979), guinea pig brain (Bylund 1978), rat kidney
(Woodcock and Johnston 1980) and rat brain stem (Stone and U'Pritchard 1981). Mendel and Almon (1979) and Dax and Partilla (1982) have both shown that the degree to which certain antagonists displace $[^3H]$DHA binding from non specific binding sites was directly related to the lipophilicity of the drug, thus the very lipophilic antagonist propranolol which had been used at high concentrations could be the root of the problem associated with the early binding studies. Clearly the use of a hydrophilic agent such as isoprenaline to determine specific binding would be of advantage and was used in the present study (see chapter 3 for validation of use in isolated adipocyte membranes).

The problem of incorrect estimation of specific binding may have far reaching consequences. In simpler terms, the most obvious errors would be an overestimation of the maximal binding capacity and dissociation constant of the radioligand. A more complex situation exists where Dax et al (1981a) have shown that the magnitude of non-stereospecific binding which is displaceable by propranolol in rat liver varies depending on the physiological state of the animal used. With this important factor in mind, it is possible that many previous reports on changes in beta-adrenoceptor density by influences such as temperature acclimatization (Kurahashi and Kuroshima 1979), fasting (Giudicelli et al 1982), cell size and ageing (Giudicelli and Pecquery 1978), thyroid hormones (Ciaraldi and Marinetti 1978), thyroidectomy (Giudicelli 1978) and an unique antagonist promoted desensitisation (Giudicelli et al 1979b) may be misleading and simply attributed to non receptor events.

The use of isoprenaline to define specific binding in rat adipocyte has been shown to result in linear Scatchard plots with dissociation constants of 2-4 nM (Dax et al 1982) which approaches
the values found in other tissues. The maximal binding capacity (approx. 100 fmoles/mg protein) was still approximately double of that reported in this study, a possible explanation being that even isoprenaline may give incorrect estimates of $[^3H]DHA$ specific binding. Indeed, in this study, a small degree of curvilinearity was still apparent when analysing Scatchard plots of $[^{125}\text{I}]CYP$ binding.

The rank orders of potency of a series of agonists at inhibiting $[^{125}\text{I}]CYP$ binding correlated closely with the orders expected at functional responses (Lands et al 1967). Thus the lung appeared to possess the overall profile of a $\beta_2$ receptor population while isolated adipocytes appeared to be of the $\beta_1$ subtype. Further support for these classifications was obtained with agents selective for $\beta_1$ or $\beta_2$ adrenoceptors. The direct subclassification of beta-adrenoceptors by selective agents was first shown in rat and rabbit lung by Rugg et al (1978) who demonstrated that inhibition curves deviated from law of mass action behaviour. This was interpreted on the assumption that the tissues in question had a heterogeneous population of beta-adrenoceptors and that the drugs had a greater affinity for one receptor subtype. These and other workers (Minneman et al 1979a) analysed the data by a modified type of Scatchard analysis, the Hofstee (1952) plot to linearly interpolate the inhibition curves from which inhibition constants and proportions of sites could be calculated. Although this plot is accurate for highly selective drugs, when drugs of relatively low selectivity are used the data cannot be easily evaluated (Minneman and Molinoff 1980). More recently, the use of iterative curve fitting programmes which do not linearly transform the data and which can evaluate the binding according to law of mass action parameters have been described (Hancock et al 1979, Munson and
Rodbard 1980, Dickinson et al 1981, De Lean et al 1982). These methods do not suffer the problem of introducing error into the data during a transformation and are therefore a much more accurate means of analysis. The curve fitting routine described by Dickinson et al (1981) was used in this study although a more powerful programme, 'LIGAND' (Munson and Rodbard 1980) has been developed which can pool data from individual curves and contains a comprehensive statistical package. The curve fitting routine used in this study is accurate if the data is 'tight' since mean curves were analysed which would introduce a small error in the calculation.

Inhibition curves which have low slopes can be attributed to heterogeneity of binding or negative cooperative interaction. The evidence in favour of the former suggestion is based on the following:

(i) Only selective agents inhibited binding in a biphasic manner since the non selective antagonist propranolol inhibited binding with Hill slopes close to unity.

(ii) Antagonists selective for beta\textsubscript{1} adrenoceptors produced curves which approximated mirror images of those produced by beta\textsubscript{2} selective agents.

(iii) The affinities of drugs at beta\textsubscript{1} and beta\textsubscript{2} adrenoceptors as determined by curve fitting correlated very closely with their respective affinities at beta-adrenoceptors determined from functional studies.

Two 'beta\textsubscript{2} selective' agents did not produce biphasic inhibition curves. Salbutamol has already been shown not to be receptor selective and is an agonist at beta\textsubscript{2} receptors by virtue of a
greater efficiency of coupling to presumably form a ternary complex with a guanine nucleotide binding protein (Minneman et al 1979c). The 'beta_2 selective' antagonist H35/25 has also been shown not to be selective in binding studies (Minneman et al 1979b,c) and in this study appeared to be only three fold selective as judged by the inhibition constants at lung (mainly beta_2) and adipocyte (mainly beta_1) adrenoceptors. As the selectivity was low, it would be anticipated for the Hill slopes to be close to unity as compared to the more selective drugs which show receptor heterogeneity more clearly.

The present study confirms the results of earlier reports (Rugg et al 1978, Minneman et al 1979a, Dickinson et al 1981) that rat lung possesses a heterogeneous population of beta-adrenoceptors consisting of approximately 20% beta_1 and 80% beta_2 adrenoceptors. This study also indicates that the predominant adrenoceptor subtype on adipocytes is beta_1, the presence of a small proportion of beta_2 adrenoceptors on isolated adipocytes is probably not indicative of the coexistence of beta_1 and beta_2 adrenoceptors on the same cell as has been reported for C6 glioma (Homburger et al 1981) and glial cell primary cultures (Ebersolt et al 1981) but more likely relates to contamination from cell types such as capillary, endothelial, mast, macrophage and epithelial cells since whole fat pad membranes possess a relatively high density of beta_2 adrenoceptors.

With respect to the adipocyte classification reported here, cultured 3T3-L1 preadipocytes also appear to possess a beta_1 adrenoceptor (Lai et al 1982), however when the cells were exposed to dexamethasone and IBMX, the receptor subtype changed to beta_2 and the adipocytes became catecholamine responsive. Interpretation of these results is difficult relative to this study and may
reflect a phenomenon specific for the embryonic cell line used and not observed under natural conditions.

The question therefore arises whether the typical beta_1 adrenoceptor identified in this study is the one mediating catecholamine induced lipolysis in the rat adipocyte. Since the beta-adrenoceptor is almost always coupled to adenylate cyclase (see Maguire and Erdos 1980, Erdos et al 1981 for exceptions) it is difficult to argue that the typical beta_1 adrenoceptor present on adipocytes is not the one responsible for the action of catecholamines in stimulating lipolysis. This poses the question as to why the adipocyte beta-adrenoceptor appears atypical at a functional response. Indeed, it is possible that if a very low affinity atypical beta-adrenoceptor was present, it may not be detectable with high affinity ligand binding techniques. Recently Cubero and Malbon (1984) have solubilised and purified the adipocyte beta_1 adrenoceptor, if the atypical beta-adrenoceptor exists it would not have been detected since [^3H]DHA and an alprenolol affinity column were used both of which would have a low affinity for the atypical beta-adrenoceptor (Harms et al 1977).

An alternative explanation for the atypical beta-adrenoceptor is that various physicochemical properties, protein binding or artifacts may influence the apparent affinity of antagonists at a lipolytic response. These questions could only be answered by analysis of various effector responses such as lipolysis and cAMP accumulation in whole cells (chapter 6) and adenylate cyclase activity in membranes (chapter 7).

In this study, the only difference that indicates non-conformity of the adipocyte beta-adrenoceptor is that of stereospecificity of propranolol isomers. The degree of stereospecificity was greater in adipocytes, the difference relating to the affinity
of (+)propranolol. This possibly indicates that there are differences at the site on the receptor which interacts with the beta-hydroxy moiety, however this point would have to be resolved further in view of the observations that the (+) isomer of adrenergic drugs contributes little or nothing to the affinity for beta-adrenoceptors (Howe and Shanks 1966, Shonk et al 1971).
CHAPTER 6

Studies on the atypical nature of the rat adipocyte beta-adrenoceptor in whole fat cells
6:1 Introduction

The classification of the rat adipocyte beta-adrenoceptor at present is unclear. Lands et al (1967) originally proposed a $\beta_1$ subtype based upon the rank order of potency of a series of sympathomimetic amines to stimulate lipolysis. More recently however, a growing amount of evidence has suggested that adipocyte beta-adrenoceptors differ from $\beta_1$ adrenoceptors in the heart and indeed $\beta_2$ adrenoceptors in other tissues. Thus it was found that a number of beta-adrenoceptor antagonists were much weaker at inhibiting beta-adrenoceptor stimulated lipolysis than was expected when compared to tissues with responses mediated by classical $\beta_1$ and $\beta_2$ adrenoceptors (Grana et al 1972, Stanton 1972, Harms et al 1974, Harms and Van der Meer 1975, Harms 1976b, Harms et al 1977, De Vente et al 1980, Tan and Curtis-Prior 1983). Furthermore, a novel series of beta-adrenoceptor agonists has been reported which had potencies and rank orders not expected at $\beta_1$ or $\beta_2$ adrenoceptors (Wilson et al 1984). Collectively these results suggest that a hybrid or atypical beta-adrenoceptor is present on adipocytes which mediates the functional response to agonists.

Radioligand binding studies on the other hand have clearly revealed that the receptor subtype is $\beta_1$ in nature (chapter 5). However, since one cannot equivocally ascertain from binding studies whether a receptor is functionally linked to a response, it is possible that the $\beta_1$ adrenoceptor is not involved in lipolysis but instead is driven through a low affinity atypical beta-adrenoceptor which because of its low affinity for antagonists was not identified in binding studies.

In view of the observation that more than one beta-adrenoceptor subtype may mediate the same functional response in some tissues
(Carlsson et al 1972, Ablad et al 1974, Carswell and Nahorski 1983, Zaagsma et al 1983), it is possible that $\beta_1$ and $\beta_2$ adrenoceptors coexist on adipocytes (as this was not entirely excluded by ligand binding studies) and that this phenomenon may explain the apparent low affinity of selective antagonists for inhibition at the lipolytic beta-adrenoceptor. Receptor subtypes can be distinguished on a functional level by the use of agonists and antagonists selective for each subtype (see O'Donnell and Wanstall 1979a,b, O'Donnell and Wanstall 1980, Carswell and Nahorski 1983, Zaagsma et al 1983), this approach therefore has been used to investigate the lipolytic beta-adrenoceptor.

The atypical beta-adrenoceptor has only been identified from lipolytic experiments, as there is a close association between lipolysis and cAMP production in adipocytes (Butcher et al 1965, Robison et al 1971), the ability of beta-adrenoceptor antagonists to inhibit isoprenaline stimulated cAMP accumulation in whole adipocytes has been studied to see whether the atypical beta-adrenoceptor could be identified at an effector response more proximal to the receptor.

An alternative explanation for the apparently atypical beta-adrenoceptor is that endogenous mediators such as adenosine or prostanoids may influence the apparent affinity of antagonists. Experiments examining the inhibition of isoprenaline-stimulated cAMP accumulation have thus been performed when the production and action of these mediators has been suppressed.
6:2 Results

6:2:1 Inhibition of noradrenaline or fenoterol stimulated lipolysis by the selective beta-adrenoceptor antagonists betaxolol and ICI 118.551

(A) Dose response curves

The beta₁ selective agonist noradrenaline (Furchgott 1976) and the beta₂ selective agonist fenoterol (O'Donnell 1970) were full lipolytic agents with respect to the maximum isoprenaline response, pD₂ values determined for the agonists were: noradrenaline 7.15 ± 0.09 (n=8) and fenoterol 6.29 ± 0.12 (n=8).

Both betaxolol (beta₁ selective antagonist) and ICI 118.551 (beta₂ selective antagonist) induced dose dependent shifts to the right of either noradrenaline or fenoterol dose response curves. Figure 6:1a shows a representative set of dose response curves for noradrenaline in the absence and presence of four different concentrations of betaxolol. In all cases, high concentrations of antagonists had to be used to shift dose response curves to the right. At very high concentrations of antagonist, the maximal lipolytic response was usually depressed with dose response curves becoming shallower, therefore the EC₅₀ for the agonist was calculated to be the concentration required to achieve 50% of the new maximal response.

(B) Protein binding of betaxolol and ICI 118.551

The binding of betaxolol and ICI 118.551 to 1% BSA was examined in order to assess the amount of bound and therefore pharmacologically inactive drug present in each assay. Both antagonists exhibited protein binding which was concentration dependent (figure 6:1b). The relative percentage bound over
Figure 6:1  
(a) Dose response curves of noradrenaline stimulated lipolysis in adipocytes in the absence (●) and presence of 3 x 10^-6 M (▲), 10^-5 M (■), 3 x 10^-5 M (○) and 10^-4 M (△) betaxolol. Adipocytes were incubated with drugs for 90 minutes and free fatty acids released were measured as described in methods. Results are expressed as mean ± SEM of four experiments performed in duplicate. (b) Protein binding of the antagonists ICI 118.551 (●) and betaxolol (▲) to 1% BSA (Organon Teknika) in Krebs-bicarbonate buffer. Binding was determined by equilibrium dialysis as described in methods and results are expressed as mean ± SEM of three experiments performed in duplicate.
total drug added was greater at low drug concentrations and decreased when higher concentrations were examined. ICI 118.551 was bound to a greater extent than betaxolol, linear regression of the points gave the following values: betaxolol, \( y = 12.50x - 47.19 \); ICI 118.551, \( y = 16.91x - 62.13 \). From these values corrected free drug concentrations could be calculated for all the additions in the lipolysis experiments.

(C) Schild analysis of lipolytic dose response curves

Schild analysis of the data in figure 6:1a as shown in figure 6:2 was performed for betaxolol corrected and uncorrected for binding to 1% BSA. Clearly, the \( pA_2 \) calculated after protein binding was accounted for (6.16 ± 0.10) was higher than that obtained for uncorrected values (6.02 ± 0.10). Table 6:1 gives the \( pA_2 \) and slope values for betaxolol and ICI 118.551 when either noradrenaline or fenoterol were used as lipolytic agonists.

Irrespective of the agonist used, the \( pA_2 \) values for each antagonist used were similar. The \( pA_2 \) values obtained are neither indicative of a \( \beta_1 \) or \( \beta_2 \) adrenoceptor as both antagonists inhibited lipolysis with low affinity, betaxolol being approximately two orders of magnitude weaker than its affinity at typical \( \beta_1 \) adrenoceptors (Boudot et al 1979, chapter 5) and ICI 118.551 more than one hundred times weaker than at \( \beta_2 \) adrenoceptors (O'Donnell and Wanstall 1980, Dickinson et al 1981, Carswell and Nahorski 1983, chapter 5). The slopes of the Schild plots after correction for protein binding while not significantly different from unity for ICI 118.551 were significantly different for betaxolol which does not suggest a simple competitive relationship.
Figure 6:2  Schild plot of data in figure 6:1a. Concentrations of betaxolol shown are those uncorrected (●) and corrected (▲) for binding to 1% BSA. Linear regression of the four points gave the following values; uncorrected for binding to BSA, pA₂ = 6.02 ± 0.10, slope = 0.77* ± 0.05; corrected for binding to BSA, pA₂ = 6.16 ± 0.10, slope = 0.73* ± 0.05. Values quoted are expressed as mean ± SEM of four experiments performed in duplicate. (* slopes significantly different from unity p < 0.05).
Table 6.1  pA₂ values and Schild slopes (in parenthesis) of betaxolol and ICI 118.551 against noradrenaline (NA) or fenoterol (FEN) stimulated lipolysis in adipocytes.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Betaxolol (beta₁ selective)</th>
<th>ICI 118.551 (beta₂ selective)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD₂</td>
<td>pD₂</td>
</tr>
<tr>
<td>NA (beta₁ selective)</td>
<td>6.96 ± 0.07</td>
<td>7.34 ± 0.10 5.76 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.77* ± 0.05)</td>
<td>(1.31 ns ± 0.13)</td>
</tr>
<tr>
<td>Corrected for protein</td>
<td>6.16 ± 0.10</td>
<td>5.96 ± 0.06 1.19 ns ± 0.12</td>
</tr>
<tr>
<td>binding</td>
<td>(0.73* ± 0.05)</td>
<td></td>
</tr>
<tr>
<td>FEN (beta₂ selective)</td>
<td>6.28 ± 0.12</td>
<td>6.31 ± 0.22 5.67 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>(0.90* ± 0.03)</td>
<td>(1.45* ± 0.11)</td>
</tr>
<tr>
<td>Corrected for protein</td>
<td>5.88 ± 0.21</td>
<td>5.85 ± 0.15 1.31 ns ± 0.09</td>
</tr>
<tr>
<td>binding</td>
<td>(0.85* ± 0.03)</td>
<td></td>
</tr>
</tbody>
</table>

pA₂'s and slopes were calculated by linear regression of four points on the log (dose-ratio - 1) versus log [antagonist] plot. Results are expressed as mean ± SEM of four experiments performed in duplicate (ns = not significantly different from unity, * p < 0.05, 't' distribution).
6:2:2 cAMP accumulation in adipocytes

(A) Time course

Figure 6:3a shows the time course of cAMP accumulation in adipocytes in the presence and absence of $10^{-5}$ M isoprenaline. cAMP levels were elevated by isoprenaline almost maximally at the first time point measured. Prior boiling of adipocytes (not shown) produced no increase in cAMP levels after isoprenaline addition. Both basal and isoprenaline stimulated levels remained at a constant level up to the last time point tested (20 minutes) showing no transient peaking in levels as has been reported by other workers (Ho and Sutherland 1971, Manganiello et al 1971, Birnbaum and Goodman 1977). Since basal and stimulated levels were constant, all cAMP accumulation experiments except where stated were performed after 8 minutes of incubation.

(B) Agonists

The agonists isoprenaline, noradrenaline and adrenaline were all full agonists with respect to cAMP accumulation in adipocytes (figure 6:3b). The rank order of potency was isoprenaline > noradrenaline = adrenaline which is suggestive of a beta_{1} adrenoceptor subtype as originally defined by Lands et al (1967). Fenoterol was not able to fully stimulate cAMP levels relative to isoprenaline.

(C) Inhibition of isoprenaline stimulated cAMP accumulation by selective and non selective antagonists

All the antagonists used produced dose dependent shifts to the right of the isoprenaline dose response curve. A representative example is shown in figure 6:4a where high concentrations of betaxolol were necessary to produce the parallel shifts. The corresponding Schild plot (figure 6:4b) indicates a pA_{2} value for betaxolol of 6.15 ± 0.20 (6.30 ± 0.21 corrected for
Figure 6:3 (a) Time course of isoprenaline stimulated cAMP accumulation in isolated adipocytes. Adipocytes were incubated at 37°C in the absence (▲) and presence (●) of $10^{-5}$M isoprenaline and after the required times, incubations were terminated by boiling and cAMP assayed as described in text. Results are expressed as mean ± SEM of five experiments performed in duplicate. (b) Stimulation of cAMP accumulation by the agonists isoprenaline (●), adrenaline (▲), noradrenaline (■) and fenoterol (○). Adipocytes were incubated with agonists for 8 minutes and cAMP determined as described in methods. Results are expressed as mean of three to five experiments performed in duplicate. SEMs were less than ± 16%.
Figure 6:4  
(a) Dose response curves of isoprenaline stimulated cAMP accumulation in adipocytes in the absence (●) and presence of $3 \times 10^{-6}$ M (▲), $10^{-5}$ M (■), $3 \times 10^{-5}$ M (○) and $10^{-4}$ M (△) betaxolol. Adipocytes were incubated with drugs for 8 minutes and cAMP assayed as described in text. Results are expressed as mean ± SEM of four experiments performed in duplicate. 
(b) Schild plot of the same data from which $pA_2$ and slope were calculated. Values were; $pA_2 = 6.15 \pm 0.20$, slope = $0.84 \pm 0.12$ (not significantly different from unity).
protein binding) which is in excellent agreement with the results obtained from lipolysis experiments. Betaxolol therefore was still approximately two orders of magnitude weaker than its affinity at typical beta\textsubscript{1} adrenoceptors. Data in table 6:2 shows the results obtained with the other antagonists used. ICI 118.551 had a pA\textsubscript{2} value of 6.59 ± 0.26 which again is in good agreement with data obtained from lipolysis experiments and which is approximately one hundred fold weaker than its affinity at typical beta\textsubscript{2} adrenoceptors. Stereoselectivity was displayed by the stereoisomers of propranolol, the (-) isomer being approximately ten times more potent than the (+) isomer. The pA\textsubscript{2} values are in close agreement to those obtained from lipolytic studies in rat adipocyte but generally lower than expected at typical beta-adrenoceptors (Harms et al 1977).

6:2:3 Effects of adenosine deaminase, theophylline and indomethacin on basal and isoprenaline stimulated cAMP accumulation

(A) Basal stimulated cAMP accumulation

None of the agents used had any significant effects on basal stimulated cAMP accumulation in adipocytes (figure 6:5). For adenosine deaminase, these results are in disagreement to those of Fain (1973a) and Schwabe et al (1975) who found that adenosine deaminase increased basal cAMP levels. With respect to theophylline, these results are in accordance with Kuo and De Renzo (1969), Fain (1973b) and Pairault and Laudat (1974) but in disagreement with Dalton et al (1974), Schwabe and Ebert (1974) and Hjemdahl and Fredholm (1976) who reported theophylline induced increases in cAMP accumulation.

(B) Time courses of isoprenaline stimulated cAMP accumulation

None of the agents when used either alone or in
Table 6:2  \( pA_2 \) values and Schild slopes (in parentheses) of beta-adrenoceptor antagonists against isoprenaline stimulated cAMP accumulation in adipocytes.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>( pA_2 )</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>betaxolol (beta(_1) selective)</td>
<td>6.15 ± 0.20</td>
<td>0.84(^{\text{ns}}) ± 0.12</td>
</tr>
<tr>
<td>corrected for protein binding</td>
<td>6.30 ± 0.21</td>
<td>0.79(^{\text{ns}}) ± 0.11</td>
</tr>
<tr>
<td>ICI 118.551 (beta(_2) selective)</td>
<td>6.35 ± 0.24</td>
<td>1.11(^{\text{ns}}) ± 0.08</td>
</tr>
<tr>
<td>corrected for protein binding</td>
<td>6.59 ± 0.26</td>
<td>1.01(^{\text{ns}}) ± 0.07</td>
</tr>
<tr>
<td>(-)propranolol</td>
<td>7.35 ± 0.23</td>
<td>1.22(^{\text{ns}}) ± 0.18</td>
</tr>
<tr>
<td>(+)propranolol</td>
<td>6.39 ± 0.27</td>
<td>0.98(^{\text{ns}}) ± 0.11</td>
</tr>
</tbody>
</table>

\( pA_2 \) values and slopes were calculated by linear regression of points from Schild plots. Results are expressed as mean ± SEM of four to six experiments performed in duplicate (\( \text{ns} = \) slopes not significantly different from unity).
Figure 6:5 Effects of (a) adenosine deaminase (ADA), (b) theophylline and (c) indomethacin on cAMP accumulation in adipocytes. Cells were incubated with respective compound for 8 minutes at 37°C and cAMP assayed as described in methods. Results are expressed as mean ± SEM of three experiments performed in duplicate.
combination with each other had any effects on the time course of basally stimulated cAMP accumulation. Figure 6:6a shows the effects of adenosine deaminase and theophylline singly and in combination on isoprenaline stimulated cAMP accumulation. Adenosine deaminase (1 μg/ml) and theophylline (1 mM) markedly elevated cAMP levels above the normal isoprenaline response. When the two agents were combined the effect was additive, at 10 minutes the amount of cAMP produced was approximately six times greater than for isoprenaline alone. The inclusion of indomethacin (10^{-5} M) to the assay had only a small effect on isoprenaline stimulated cAMP accumulation but a significantly greater potentiation was found when adipocytes were incubated with, rather than without, indomethacin in the presence of isoprenaline, adenosine deaminase and theophylline (figure 6:6b).

6:2:4 Antagonism of isoprenaline stimulated cAMP accumulation by betaxolol in the presence of adenosine deaminase, theophylline and indomethacin

(A) Isoprenaline dose response curves

Isoprenaline stimulated cAMP production in adipocytes in the presence of adenosine deaminase and theophylline with high potency, the pD$_2$ value being 7.05 ± 0.16 (n=4). This is in contrast to the ten fold lower potency observed in the absence of adenosine deaminase and theophylline (pD$_2$ = 6.07 ± 0.07, n=23 not shown). When indomethacin was included in the assays, the pD$_2$ for isoprenaline was not greatly different to that without (pD$_2$ = 7.18 ± 0.10, n=3).

(B) Inhibition of isoprenaline stimulated cAMP accumulation by betaxolol

The β$_1$ selective antagonist betaxolol shifted
Figure 6:6 Time courses of basal and isoprenaline ($10^{-4}$ M) stimulated cAMP accumulation in adipocytes in the presence of adenosine deaminase, theophylline or indomethacin. (a) Adipocytes were incubated in the absence of isoprenaline with or without modulators (♦) and in the presence of (●) isoprenaline, (▲) isoprenaline + adenosine deaminase (1 µg/ml), (■) isoprenaline + theophylline ($10^{-3}$ M) and (○) isoprenaline + adenosine deaminase + theophylline. Basal stimulations shown by the symbol (♦) included the following incubations; no additions, adenosine deaminase, theophylline and adenosine deaminase + theophylline. These incubations had no effect on basal values and error bars shown are extreme values for pooled data. (b) Adipocytes were incubated in the absence of isoprenaline with or without modulators (♦) and in the presence of (●) isoprenaline, (▲) isoprenaline + indomethacin ($10^{-5}$ M), (○) isoprenaline + adenosine deaminase + theophylline and (■) isoprenaline + adenosine deaminase + theophylline + indomethacin. Basal stimulations shown by the symbol (♦) included the following incubations; no additions, indomethacin, adenosine deaminase + theophylline and adenosine deaminase + theophylline + indomethacin. These incubations had no effect on basal values and error bars shown are extreme values for pooled data. Results are expressed as mean ± SEM of three experiments performed in duplicate. (* significantly different from isoprenaline + adenosine deaminase + theophylline incubation p < 0.05 paired 't' test).
Figure 6:6
the isoprenaline dose response curve to the right in the presence of adenosine deaminase and theophylline at concentrations that were much lower than those producing a shift in the absence of the two modulating agents (figure 6:7a). Initially the shift to the right was quite large but further shifts with increasing concentrations of antagonist were relatively slight. Examination of Schild plots of the data (figure 6:7b) revealed that the slope was low and appeared to be biphasic. Clearly, extrapolation of the points to give a pA₂ value is inappropriate due to the large error involved in regressing the line to the x axis. However a greater than two fold shift of the dose response curve was obtained at a concentration of 10⁻⁷ M betaxolol and therefore approaches the expected shift for this antagonist at typical beta₁ adrenoceptors (Boudot et al 1979, chapter 5).

In the presence additionally of indomethacin, the slope of the Schild plot was not increased but was similar to that obtained with the first three points obtained for betaxolol with adenosine deaminase and theophylline alone. A two fold shift of the dose response curve would have been obtained at a concentration below the lowest examined (3 x 10⁻⁸ M). The low Schild slopes could be possibly interpreted as due to the presence of multiple affinity states of receptors which mediate the same effector response.

6:3 Discussion

Detailed investigation of the rat lipolytic beta-adrenoceptor by the use of selective agonists and antagonists has confirmed and extended previous proposals that the beta-adrenoceptor appears to be atypical belonging neither to a beta₁ or beta₂ subtype classification. The use of selective agents to delineate beta-adrenoceptors functionally was initially proposed by Carlsson et
Figure 6:7  (a) Dose response curves of isoprenaline stimulated cAMP accumulation in adipocytes in the absence (●) and presence of $10^{-7} \text{M} (▲)$, $3 \times 10^{-7} \text{M} ( ■)$, $10^{-6} \text{M} ( ○)$ and $3 \times 10^{-6} \text{M} ( △)$ betaxolol. Adipocytes were incubated in the presence of adenosine deaminase (1μg/ml), theophylline ($10^{-3} \text{M}$) and respective drugs for 10 minutes at 37°C and cAMP produced was determined as described in methods. Results are mean of three experiments and standard errors are excluded for clarity. (b) Schild plot of the above data (●) and from dose response curves in the presence of adenosine deaminase, theophylline and $10^{-5} \text{M}$ indomethacin (▲). pA2 values were not calculated due to low slopes which were $0.52 \pm 0.09$ for experiments in the presence of adenosine deaminase and theophylline and $0.31 \pm 0.10$ for experiments performed with the addition of indomethacin. Results are expressed as mean ± SEM of three experiments performed in duplicate.
Figure 6:7
al (1972) who demonstrated that the potencies of selective antagonists against the beta-adrenoceptor stimulated chronotropic effect on isolated cat heart was dependent on the particular agonist used. This type of analysis has successfully been used to identify receptors which are functionally heterogeneous or homogeneous (see O'Donnell and Wanstell 1979b) and therefore offers a powerful qualitative method by which receptors can be identified. As outlined by O'Donnell and Wanstell (1979b), interpretation of the data depends on three principles which determine the pA\textsubscript{2} of a competitive antagonist providing that experimental conditions are optimal, only a single receptor subtype is involved and that antagonism is purely competitive:

(i) the pA\textsubscript{2} value for an antagonist should be a measure of the affinity of the antagonist for that receptor

(ii) the pA\textsubscript{2} value for an antagonist should be the same whatever agonist is used

(iii) identical receptors in different tissues should give rise to the same pA\textsubscript{2} value for a given antagonist.

From this it is apparent that if the pA\textsubscript{2} value for an antagonist varies with the agonist used, then more than one receptor is involved in the response and if pA\textsubscript{2} values for an antagonist are not the same then the receptor populations in the two tissues are not identical.

With these points in mind, irrespective of whether noradrenaline or fenoterol were used to stimulate lipolysis, the pA\textsubscript{2} values for each antagonist were similar thus suggesting that a single receptor subtype is involved in the lipolytic response. However, this receptor (or indeed receptors if the selective agents could not
distinguish between them) cannot be classified as either $\beta_1$ or $\beta_2$ in subtype since the $pA_2$ values for betaxolol or ICI 118.551 were much lower than that selectively expressed at each subtype (O'Donnell and Wanstall 1980, Dickinson et al 1981, Zaagsma et al 1983, chapter 5). De Vente et al (1980) have also demonstrated that when noradrenaline or salbutamol were used to stimulate lipolysis, no difference in $pA_2$ values were observed for the selective antagonists practolol or H35/25 further strengthening the proposal made in this study that the adipocyte beta-adrenoceptor is atypical. It should be mentioned that although weak selective antagonist affinities may be explained by the coexistence of $\beta_1$ and $\beta_2$ adrenoceptors in adipocytes, this possibility would not be able to explain the low affinity of non selective antagonists at inhibiting lipolysis (Harms et al 1977).

To date no critical evaluation of the competitive nature of antagonists for the adipocyte beta-adrenoceptor has been performed. Previously $pA_2$ values were established on the basis of one or two shifts of the dose response curve to agonists. In this study, a fuller examination has been performed where inhibition by antagonists was examined using the log (dose ratio -1) versus log [antagonist] plot (Arunlakshana and Schild 1959). Since this type of analysis not only reveals the $pA_2$ value but also the slope of the regression line, a simple bimolecular competitive interaction can be assessed if the slope of the plot is unity.

Before assessing the $pA_2$ values, it is important to take into account the binding of drugs to BSA which may influence the free drug concentration in the assay. The inclusion of BSA in isolated adipocyte preparation is a necessity. Albumin acts as a free fatty acid receptor and addition of lipolytic agents to cells incubated in the absence of albumin has been shown to result in a high
accumulation of intracellular free fatty acids but an impaired lipolytic response (Angel et al 1971). Binding to BSA has been demonstrated by Zaagsma et al (1977) to have profound effects on the apparent affinity of certain antagonists and the contribution of protein binding must be taken into account. Neither of the antagonists were bound to BSA to a great extent (<35%) and after correction for protein binding the $pA_2$ values for the antagonists were only slightly increased. However, greater effects were observed on the Schild slopes resulting in the slope for ICI 118.551 not significantly differing from unity suggesting a simple competitive interaction while the slopes for betaxolol were both significantly different from one. This phenomenon may be explained on the following grounds if it is assumed that in addition to an atypical beta-adrenoceptor there is in rat adipocytes a minor $\beta_1$ adrenoceptor component. The $pA_2$ for ICI 118.551 at the atypical beta-adrenoceptor is approximately ten fold lower as that found at typical $\beta_1$ adrenoceptors (see table 5:3). If indeed the $\beta_1$ component is minor (since betaxolol still has a low $pA_2$ value against noradrenaline stimulated lipolysis) then ICI 118.551 may not appear selective enough between the two components and thus antagonised lipolysis with a slope of unity. Betaxolol on the other hand is approximately two orders of magnitude more selective for $\beta_1$ adrenoceptors than the atypical beta-adrenoceptors. As a result it is possible that noradrenaline and fenoterol may stimulate both receptor subtypes and as a result a relatively low Schild slope would be anticipated (Furchgott 1976, Harper et al 1978).

It should be considered that in this study, noradrenaline and fenoterol, apparently selective agents for $\beta_1$ and $\beta_2$ adrenoceptors respectively, were full stimulators of lipolysis. Lands et al (1967) classified the rat adipocyte beta-adrenoceptor as
$\beta_1$ based upon the rank order of potency of a series of agonists to stimulate lipolysis. It therefore appears that the atypical beta-adrenoceptor has the same rank order of potency as the $\beta_1$ adrenoceptor when conventional agonists are used. The recent development of a series of arylethanolamine beta-adrenoceptor agonists (Wilson et al 1984) has enabled a distinction to be observed in the rank order of potency between typical and atypical beta-adrenoceptors in white (Wilson et al 1984) and brown adipocytes (Arch et al 1984). The ability of $\beta_2$ adrenoceptor selective agonists to stimulate lipolysis is well documented. Salbutamol (Grana et al 1972, Fain et al 1973), hexoprenaline (Lipshitz and Vinik 1978) and fenoterol (Wilson et al 1984) have all been reported to stimulate lipolysis. Beta$_1$ selective agonists on the other hand such as prenalterol (Wilson et al 1984) or tazolol (Jolly et al 1978) were ineffective. On this basis, Jolly et al (1978) incorrectly suggested that the adipocyte beta-adrenoceptor was $\beta_2$ in subtype. Kenakin (1982) has clearly demonstrated that 'selective' agonists may in fact be tissue selective and not necessarily receptor selective. Thus prenalterol a $\beta_1$ selective agent was found to be capable of stimulating $\beta_2$ adrenoceptor mediated relaxation in rat uterus. It is therefore likely that the $\beta_2$ adrenoceptor selective agonists are all organ selective for the adipocyte and not stimulating lipolysis via $\beta_2$ adrenoceptors. This is further substantiated by the observation that fenoterol was not a full agonist relative to cAMP accumulation suggesting that it is a partial agonist at the receptor level but the tissue contains a sufficient receptor reserve to elicit a full lipolytic response to this agonist.

An atypical beta-adrenoceptor has also been reported in rat brown adipocytes (Arch et al 1984), guinea pig (Bertholet et al...
1981) and human adipocyte (Goldberg et al 1975, Harms and Van der Meer 1975). In addition there is evidence for the atypical beta-adrenoceptor in hamster brown (Mohell et al 1983) and white adipocytes (Hittelman et al 1973) as antagonist potencies appeared to be considerably lower than expected at typical beta-adrenoceptors. It is apparent however that in dog adipocytes, at least, there is evidence for the coexistence of beta\(_1\) and beta\(_2\) adrenoceptors mediating the lipolytic response (Belfrage and Fredholm 1978) and more recently the same has been proposed for hamster adipocytes (Bjorgell and Belfrage 1982) although the results could in the opinion of the author also be interpreted on the basis of an atypical beta-adrenoceptor.

In order to obtain a clearer picture of the apparently atypical beta-adrenoceptor, it was necessary to examine an effector response less distal to the receptor, namely cAMP accumulation. The relationship between cAMP and the lipolytic response has been well established. Butcher et al (1965) first showed that cAMP was associated with lipolysis and more recently it has been clearly demonstrated that cAMP dependent protein kinases directly activate hormone sensitive lipase (Khoo et al 1976) and that the protein kinase in turn has been shown to be activated in adipocytes exposed to lipolytic hormones (Corbin et al 1975, see also Steinberg 1976, Hales et al 1978 for reviews). As lipolysis is mediated via cAMP generation it would be anticipated that the atypical beta-adrenoceptor could be identified from cAMP accumulation experiments. In agreement with initial lipolysis experiments (Lands et al 1967) the rank order of potency of agonists suggested a beta\(_1\) classification. The antagonist pA\(_2\) values closely correlated with those observed from lipolytic experiments and furthermore the pA\(_2\) values for the stereoisomers of propranolol showed a
decreased stereoselectivity as was observed by Harms et al (1977). The atypical beta-adrenoceptor therefore appears to be linked to cAMP generation in adipocytes.

There are several possible explanations for the apparently atypical beta-adrenoceptor. Firstly, it is possible that the characteristics of the atypical beta-adrenoceptor are a real effect and that the observations noted here and reported elsewhere simply reflect the interrelationship of agonists and antagonists through a novel beta-adrenoceptor. This is the most plausible explanation. On the other hand it is possible that endogenous mediators such as adenosine, prostanoids and free fatty acids may in some manner invoke the appearance of an atypical beta-adrenoceptor when in fact the receptor is typical. The affinity of an antagonist for a receptor should be constant irrespective of outside influences. However, it may appear to change if the agonist reference dose-response curve shifted due to the formation of mediators in the presence of the antagonist such that the dose-ratio may change artifactualy. This would provide an incorrect \( PA_2 \). This situation would be impossible to assess directly therefore experiments were performed in the presence of adenosine deaminase, theophylline and indomethacin to remove the contribution, if any, of adenosine and prostanoids.

Adenosine (Schwabe et al 1973) and prostaglandins (Shaw and Ramwell 1968) are produced by adipocytes and have direct effects on lipolysis. Adenosine has been shown to act at two distinct sites, an external receptor termed the R site and an internal P site which is possibly located on the cyclase unit (Londos and Wolff 1977). Activation of either site with reactive adenosine analogues leads to an inhibition of cAMP accumulation in the intact adipocyte (Fain et al 1972, Fain 1973a, Fain and Weiser 1975, see also Fain
Furthermore, the prostaglandins $E_1$ (Steinberg et al 1963, Bergström and Carlson 1965), $H_2$ (Gorman et al 1975, Gorman et al 1976), $E_2$ (Dalton and Hope 1973) and $I_2$ (Fredholm et al 1980) have all been shown to have inhibitory effects on lipolysis or adenylate cyclase in the fat cell. Although the evidence for adenosine as an endogenous modulator is quite strong, that for prostaglandins is more tenuous (Fredholm 1978). In this study, there is strong evidence to suggest that adenosine is present in the assays as removal by adenosine deaminase or blockade of R sites by theophylline (Trost and Stock 1977) elevated isoprenaline stimulated cAMP levels above that produced by isoprenaline alone. Therefore it is suggested that endogenous adenosine may control the extent of cAMP produced within the adipocyte. The additive effect observed when theophylline and adenosine deaminase were used together is somewhat difficult to explain unless it is assumed that at the concentrations used neither substance was able totally to eliminate the effects of adenosine or that theophylline also contributes to cAMP elevation by the inhibition of cAMP phosphodiesterase.

The small effect of indomethacin observed after adenosine effects had been suppressed may suggest that a prostanoid may have minor effects on inhibiting cAMP accumulation although it is currently held that prostaglandins are of minor importance as feedback inhibitors (Fredholm and Hedqvist 1975, Fredholm 1978).

After the adenosine effects had been repressed it was intriguing to observe that isoprenaline was approximately ten times more potent at stimulating cAMP accumulation than in the absence of adenosine deaminase and theophylline. The isoprenaline maximum response was much higher in the presence of adenosine deaminase and theophylline which may predict a lower receptor reserve.
In this case it would be expected for the isoprenaline dose response curve to be shifted to the right and not the left of the control dose response curve. The data presented here may suggest that isoprenaline induces the formation of adenosine which in turn suppresses cAMP accumulation resulting in greater isoprenaline concentrations to be necessary to overcome the blockade (in effect this could be likened to functional antagonism). The resultant isoprenaline dose response curve would be of low potency as observed and the proposal is further substantiated by the fact that the isoprenaline dose response curve was much steeper in the presence of adenosine deaminase and theophylline than without (not shown). There is no evidence for adenosine formation by agonists in adipocytes (Schwabe et al 1973, Fain 1979), therefore as yet there is no evidence to support the present proposal. Interestingly enough a recent report by Wilson et al (1984) has also shown a shift to the left of the isoprenaline lipolytic dose response curve in the presence of adenosine deaminase confirming the above observations.

The Schild plots for betaxolol against isoprenaline induced cAMP formation in the presence of adenosine deaminase and theophylline are intriguing. In the absence of adenosine effects, betaxolol was a more potent antagonist with more than two fold shifts present at concentrations that had no effect previously. However, since the slopes were very low, the antagonism is clearly not a simple competitive one and suggests that the blockade through beta-adrenoceptors is not mediated solely through one receptor subtype. The slope was not increased by indomethacin and therefore suggests that prostaglandins and adenosine at least are not responsible for the appearance of the atypical beta-adrenoceptor. The greater potency of betaxolol initially may suggest that part of the
response could be mediated by $\beta_1$ adrenoceptors. The earlier suggestion that a minor $\beta_1$ component was present in lipolysis can be extended here by the possibility that adenosine may suppress the expression of the $\beta_1$ adrenoceptor. Therefore in the presence of adenosine, the atypical beta-adrenoceptor is in dominant control of the effector response while when adenosine is suppressed, the coexistence of $\beta_1$ and atypical beta-adrenoceptors is more pronounced resulting in a low Schild slope. Further experiments using selective and non-selective antagonists may reveal an answer to this suggestion. It is also possible to explain the leftward shift of the isoprenaline dose response curve with this concept.

Results presented in chapter 8 from adenylate cyclase assays demonstrate that the potency of isoprenaline as assessed by the $K_{\text{act}}$ value is greater at typical beta than atypical beta-adrenoceptors by a factor of approximately ten. Thus if typical $\beta_1$ adrenoceptors were activated by isoprenaline in the absence of adenosine effects then the potency of isoprenaline may alter, resulting in the shift to the left of the dose response curve. Clearly, these results are very intriguing and further investigation may clarify the present complexities that exist in the adipocyte.

The results in this study are in agreement with previous suggestions that the adipocyte beta-adrenoceptor is atypical and that it is associated with cAMP accumulation in adipocytes. Moreover, the use of conditions where the effects of endogenous mediators have been suppressed has suggested the coexistence of $\beta_1$ and atypical beta-adrenoceptors, therefore the next series of experiments were aimed at examining the beta-adrenoceptors in a relatively simpler environment, that of activation of adenylate cyclase in washed membrane preparations.
CHAPTER 7

Identification and subclassification of beta-adrenoceptors linked to adenylate cyclase in rat reticulocyte, rat adipocyte and hamster adipocyte membranes
7:1 Introduction

The rat adipocyte beta-adrenoceptor has been identified by both ligand binding techniques (chapter 5) and analysis of lipolytic and cAMP accumulation effector responses (chapter 6). However, whereas the beta-adrenoceptor was identified as a beta\textsubscript{1} subtype by binding studies, analysis of the effector responses revealed that the receptor appeared to be atypical. The precise identification of the beta-adrenoceptor was made even more difficult by the complex effects of adenosine deaminase and theophylline on the apparent affinity of betaxolol which led to the speculation that a minor component of the cAMP accumulation response may be mediated by beta\textsubscript{1} adrenoceptors. Therefore, to clarify the situation further, experiments have been carried out on membrane preparations of rat adipocytes to investigate the pharmacology of the membrane bound adenylate cyclase.

The first report of antagonism of catecholamine-stimulated adenylate cyclase was by Murad et al (1962) who showed that antagonists such as dichloroisoprenaline blocked the stimulatory action of catecholamines in dog heart particles. Later work by Burges and Blackburn (1972) and Mayer (1972) demonstrated that beta\textsubscript{1} and beta\textsubscript{2} adrenoceptors could be identified at the cyclase level and more recently, comprehensive studies have been performed by several workers examining the effects of agonists and antagonists at beta-adrenoceptors (see Kaumann and Birnbaumer 1974, Lefkowitz 1975, Coleman and Somerville 1977, Minneman et al 1979c for examples). These and other studies have clearly shown that the pharmacological properties of beta-adrenoceptors were maintained at the tissue homogenate level. In accordance, good correlations have been shown between the affinities of antagonists at inhibiting binding and adenylate cyclase stimulated by agonists (Mukherjee et al 1976,
Brown et al 1976a,b, Caron et al 1978, Minneman et al 1979c). Thus, analysis of the membrane bound adenylate cyclase has provided a simple and effective method of beta-adrenoceptor classification. As the beta-adrenoceptor on rat adipocytes appears to be atypical, its pharmacology by the use of selective and non selective agonists and antagonists was compared to that of rat reticulocytes, a cell type with typical beta$_2$ adrenoceptors and hamster adipocytes whose beta-adrenoceptor classification as yet is poorly understood.

7:2 Results

7:2:1 Time courses of cAMP production in membrane preparations of rat adipocyte, lung, reticulocyte and hamster adipocyte

Figure 7:1 shows the time course of basal and maximal (3 x 10$^{-4}$ M) isoprenaline stimulated adenylate cyclase activity in rat adipocyte membranes. Generation of cAMP was a linear function of time in all the membranes examined up to the last time point investigated (10 minutes). There were profound differences however, between the tissues with respect to their basal and stimulated activities (table 7:1). Rat reticulocyte had the highest adenylate cyclase activity (646 ± 52 pmoles/mg prot/10 min) and also the most favourable signal to noise ratio (approx 27 fold stimulation over basal). Tissues such as rat lung on the other hand had a much lower isoprenaline stimulation (80 ± 8 pmoles/mg prot/10 min) which was only 51% greater than the basal stimulation. In view of the poor stimulation obtained in rat lung, rat reticulocyte was chosen as a reference membrane preparation to compare typical with putatively atypical beta-adrenoceptor mediated adenylate cyclase responses.
Figure 7:1  Time course of basal (●) and isoprenaline stimulated (▲) adenylate cyclase in rat adipocyte membranes. Membranes were incubated in buffer containing ATP, regenerating system with or without 3 x 10^{-4} M isoprenaline. After the required time, the incubations were terminated by boiling and cAMP assayed as described in methods. Results are expressed as mean ± SEM of four experiments performed in duplicate.

Table 7:1  Maximal isoprenaline stimulated and basal adenylate cyclase activities in membrane preparations of rat adipocyte, rat lung, rat reticulocyte and hamster adipocyte. Values shown are those obtained from linear regression analysis of time courses as shown in figure 7:1. Results are expressed as mean ± SEM of four experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$V_{max}$ (pmoles/mg prot/10 min)</th>
<th>Basal (pmoles/mg prot/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat adipocyte</td>
<td>288 ± 74</td>
<td>58 ± 11</td>
</tr>
<tr>
<td>Hamster adipocyte</td>
<td>207 ± 76</td>
<td>115 ± 40</td>
</tr>
<tr>
<td>Rat lung</td>
<td>80 ± 8</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>Rat reticulocyte</td>
<td>646 ± 52</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>
Effect of various agents on the basal and maximal isoprenaline stimulated adenylate cyclase activity of rat adipocyte, reticulocyte and hamster adipocyte membranes

(A) Guanine nucleotides

Both GTP (10^{-4} M) and GppNHp (10^{-4} M) elevated basal stimulated adenylate cyclase in all three membrane preparations (table 7:2a) which is consistent with the effects of these nucleotides on membranes possessing the enzyme (Rodbell et al 1971, Londos et al 1974). Stimulation by GppNHp was approximately six fold over basal while stimulation by GTP was approximately two fold. Besides elevating basal stimulated adenylate cyclase activity, GTP also enhanced the response to isoprenaline in all membranes by a factor of three which is in accordance with the effects of GTP in other systems (Rodbell et al 1971, Krishna et al 1972, Wolff and Cook 1973).

(B) NaF

Sodium fluoride (10 mM) was capable of stimulating adenylate cyclase in all the membranes studied (table 7:2a). Addition of GTP had no further effect in rat adipocyte membranes.

(C) Adenosine deaminase and indomethacin

As the suppression of adenosine effects was found to influence the apparent pA_2 value of betaxolol in cAMP accumulation experiments (chapter 6), the effects of adenosine deaminase and indomethacin to inhibit prostaglandin synthesis were examined with respect to basal and stimulated adenylate cyclase activities. Neither substance had any effect irrespective of the combination of stimulating agents used (table 7:2a,b) suggesting that at the incubation conditions used, the action of adenosine and prostaglandins are negligible.
Table 7:2  Effects of various agents on basal and maximal isoprenaline stimulated adenylate cyclase in rat adipocyte, reticulocyte and hamster adipocyte membranes. Agents were incubated with membranes and assay reagents for 10 minutes at 37°C after which the reaction was terminated by boiling and cAMP assayed as described in methods. Results shown in (a) are separate from those in (b). The values shown are percentages of the maximal stimulation obtained in the presence of isoprenaline + GTP and are expressed as mean ± SEM of three to four experiments performed in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>Rat adipocyte</th>
<th>Hamster adipocyte</th>
<th>Rat reticulocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>basal</td>
<td>10.2 ± 2.5</td>
<td>21.4 ± 0.5</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>GTP (10^{-4} M)</td>
<td>19.5 ± 0.3</td>
<td>41.7 ± 1.3</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>ISO</td>
<td>34.9 ± 6.6</td>
<td>33.8 ± 1.1</td>
<td>25.7 ± 2.1</td>
</tr>
<tr>
<td>ISO + GTP</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GppNHp (10^{-4} M)</td>
<td>65.9 ± 6.9</td>
<td>142 ± 9</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>NaF (10^{-2} M)</td>
<td>47.0 ± 2.3</td>
<td>183 ± 9</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>NaF + GTP</td>
<td>50.7 ± 7.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADA (1 U/ml)</td>
<td>8.7 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADA + GTP</td>
<td>19.1 ± 2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ISO + ADA</td>
<td>26.3 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ISO + ADA + GTP</td>
<td>102.9 ± 6.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaF + ADA</td>
<td>46.4 ± 2.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaF + ADA + GTP</td>
<td>43.0 ± 3.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GppNHp + ADA</td>
<td>65.5 ± 2.7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(b) GTP (10^{-4} M) included in all tubes

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td>basal</td>
<td>13.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>ISO</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>indomethacin (10^{-5} M)</td>
<td>13.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>ISO + indomethacin</td>
<td>104.4 ± 15.3</td>
<td></td>
</tr>
<tr>
<td>ADA (1 U/ml)</td>
<td>12.0 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>ISO + ADA</td>
<td>99.3 ± 11.5</td>
<td></td>
</tr>
<tr>
<td>ISO + ADA + indomethacin</td>
<td>92.1 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>ADA + indomethacin</td>
<td>11.7 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations used: ISO = isoprenaline, ADA = adenosine deaminase.
7:2:3 Pharmacological characterisation of beta-adrenoceptor stimulated adenylate cyclase

(A) Agonists

The agonists isoprenaline, noradrenaline and adrenaline were full stimulators of adenylate cyclase in rat adipocyte and reticulocyte membranes (figure 7:2). The rank order of potency in rat adipocyte was isoprenaline > noradrenaline = adrenaline which is indicative of a beta\(_1\) adrenoceptor (Lands et al 1967) and which agrees with the results obtained from cAMP accumulation experiments (chapter 6). The reticulocyte rank order of potency was isoprenaline > adrenaline > noradrenaline suggestive of a beta\(_2\) adrenoceptor subtype. With respect to hamster adipocyte membranes, adrenaline and noradrenaline did not behave as full agonists with reference to isoprenaline, adrenaline showing the greater depressed response. The rank order of potency therefore could not be used to estimate the receptor subtype involved in the cyclase response.

(B) Antagonists

(Bi) Schild analysis of adenylate cyclase dose response curves in rat adipocyte

Antagonism of isoprenaline stimulated adenylate cyclase was performed using the methods in chapter 6 where dose response curves to isoprenaline were performed in the absence and presence of four concentrations of antagonists. Four antagonists were used, the beta\(_1\) selective antagonist betaxolol, the beta\(_2\) selective antagonist ICI 118.551 and the (+) and (-) isomers of propranolol. Figure 7:3 shows the dose response curves with betaxolol together with the corresponding Schild plot. Betaxolol produced dose-dependent shifts to the right of the isoprenaline dose response curve, although the maximal stimulation for cAMP generation
Figure 7:2  Stimulation of adenylate cyclase in (a) rat adipocyte, (b) rat reticulocyte and (c) hamster adipocyte membranes by beta-adrenoceptor agonists. Isoprenaline (●), adrenaline (▲), noradrenaline (■) and fenoterol (O) were incubated with assay reagents and membranes for 10 minutes at 37°C followed by boiling and assay of cAMP. Results are expressed as mean ± SEM of three to six experiments performed in duplicate.
Figure 7:2
Figure 7:3  (a) Dose response curves of isoprenaline stimulated adenylate cyclase activity in the absence (●) and presence of $10^{-6}$ M (○), $3 \times 10^{-6}$ M (■), $10^{-5}$ M (□) and $3 \times 10^{-5}$ M (▲) betaxolol. The results shown are the means of three separate experiments and standard errors are excluded for clarity but were less than ±10%. (b) Schild plot of the above data which gave the following values; $pA_2 = 6.15 \pm 0.15$, slope = $0.82 \pm 0.10$ (not significantly different from unity).
appeared to decrease somewhat with higher antagonist concentrations, the dose response curves remained parallel to one another and the EC$_{50}$ of isoprenaline calculated was the concentration required to reach 50% of the reference isoprenaline dose response curve. Similar results were obtained with the other antagonists.

The corresponding Schild plot for betaxolol yielded a pA$_2$ value that was atypical for the beta$_1$ adrenoceptor but which correlated very closely with the values obtained from lipolysis and cAMP accumulation experiments (see chapter 6). Table 7:3 shows the pA$_2$ values and Schild slopes for all the antagonists used. In all cases, the values are in close agreement with the results obtained from cAMP accumulation experiments and again do not correspond to either a classical beta$_1$ or beta$_2$ adrenoceptor. The Schild slopes for (+) and (-)propranolol were both significantly less than unity suggestive of deviation from law of mass action behaviour.

(Bii) Inhibition curves of isoprenaline stimulated adenylate cyclase

Antagonism of isoprenaline stimulated adenylate cyclase was examined by incubating a range of antagonist concentrations with membranes in the presence of a fixed concentration (10$^{-4}$ M for adipocytes, 10$^{-5}$ M for reticulocytes) of isoprenaline. The inhibition curves obtained for rat reticulocyte and hamster adipocyte are shown in figure 7:4 and calculated inhibition constants in table 7:4. Inhibition of rat reticulocyte and hamster adipocyte adenylate cyclase was stereoselective, the (-) isomer of propranolol being more potent than the (+) isomer in both cases. However, whereas the inhibition constants for the propranolol isomers in reticulocyte membranes correlated favourably with those found at typical beta-adrenoceptors (see chapter 5), the potencies of propranolol isomers were much lower in hamster adipocytes
Figure 7:4  Inhibition of isoprenaline stimulated cyclase by beta-adrenoceptor antagonists in (a) rat reticulo­cyte and (b) hamster adipocyte membranes. (●) (-)propranolol, (O) (+)propranolol, (△) ICI 118.551, (□) betaxolol, (▲) metoprolol, (■) atenolol or (♦) practolol were incubated with membranes in the presence of isoprenaline (10⁻⁵ M for reticulocytes, 10⁻⁴ M for adipocytes) and assay reagents for 10 minutes after which the reaction was terminated by boiling and cAMP assayed as described in methods. Results are expressed as mean ± SEM (where shown) of three to five experiments performed in duplicate. Standard errors were excluded for hamster adipocyte but were less than ± 11%.
Table 7:3  pA₂ values and Schild slopes of selective and non selective beta-adrenoceptor antagonists against isoprenaline stimulated adenylate cyclase in rat adipocyte membranes. Results are expressed as mean ± SEM of three to five experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>pA₂</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>betaxolol</td>
<td>6.15 ± 0.15</td>
<td>0.82\textsuperscript{ns} ± 0.10</td>
</tr>
<tr>
<td>ICI 118.551</td>
<td>6.43 ± 0.16</td>
<td>0.91\textsuperscript{ns} ± 0.15</td>
</tr>
<tr>
<td>(-)propranolol</td>
<td>7.81 ± 0.27</td>
<td>0.68\textsuperscript{*} ± 0.11</td>
</tr>
<tr>
<td>(+)propranolol</td>
<td>6.78 ± 0.17</td>
<td>0.50\textsuperscript{**} ± 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{ns} = slope not significantly different from unity, \textsuperscript{*} p < 0.05, \textsuperscript{**} p < 0.01, 't' test.

Table 7:4 Inhibition constants of selective and non selective antagonists at inhibiting isoprenaline stimulated adenylate cyclase in rat adipocyte, reticulocyte and hamster adipocyte membranes. Kᵢ values (nM) were calculated from IC\textsubscript{50} values using the Cheng and Prussoff equation. Results are expressed as mean ± SEM of three to five experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>rat adipocyte</th>
<th>rat reticulocyte</th>
<th>hamster adipocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>atenolol</td>
<td>46000 ± 1200</td>
<td>20400 ± 1900</td>
<td>-</td>
</tr>
<tr>
<td>betaxolol</td>
<td>-</td>
<td>592 ± 90</td>
<td>7430 ± 1150</td>
</tr>
<tr>
<td>ICI 118.551</td>
<td>-</td>
<td>3.78 ± 0.18</td>
<td>2340 ± 330</td>
</tr>
<tr>
<td>metoprolol</td>
<td>11300 ± 500</td>
<td>1920 ± 250</td>
<td>-</td>
</tr>
<tr>
<td>practolol</td>
<td>70200 ± 14200</td>
<td>43500 ± 8000</td>
<td>-</td>
</tr>
<tr>
<td>(-)propranolol</td>
<td>-</td>
<td>0.941 ± 0.118</td>
<td>598 ± 276</td>
</tr>
<tr>
<td>(+)propranolol</td>
<td>-</td>
<td>120 ± 14</td>
<td>5250 ± 480</td>
</tr>
</tbody>
</table>
indicative of an atypical beta-adrenoceptor. Furthermore, the
degree of stereoselectivity was lower in hamster adipocytes (approx
9) compared to typical beta-adrenoceptors on reticulocytes (approx
128).

Antagonism of isoprenaline stimulated adenylate cyclase by
selective antagonists in reticulocytes revealed that the beta_2
selective antagonist ICI 118.551 was a potent inhibitor whereas the
beta_1 selective antagonists were relatively weak. Analysis of the
inhibition constants showed that the values agreed closely with
the affinities of the drugs at typical beta_2 adrenoceptors (see
7:2:4).

The inhibition constants of betaxolol and ICI 118.551 at hamster
adipocyte beta-adrenoceptors did not indicate the presence of beta_1
or beta_2 adrenoceptors since both drugs were weak at antagonising
the effector response. Furthermore, the antagonist potencies were
lower in hamster adipocytes than those observed in rat adipocytes.

Further studies on the inhibition of rat adipocyte adenylate
cyclase by the beta_1 selective antagonists metoprolol, atenolol
and practolol further demonstrated that the affinities of cardio-
selective antagonists were low in adipocytes than those expected
at conventional beta_1 adrenoceptors. Correlations of binding and
adenylate cyclase are shown in the next section.

7:2:4 Correlation of antagonist affinities at [^{125}I]CYP binding
sites and pA_2 or K_1 values against isoprenaline stimulated adenylate
cyclase in rat adipocyte, reticulocyte and hamster adipocyte
membranes

The inhibition constants against [^{125}I]CYP binding of non
selective and selective antagonists at rat lung beta_2 adrenoceptors
for reticulocytes and beta_1 adrenoceptors for adipocytes were
compared with the $K_i$ or $pA_2$ values obtained in adenylate cyclase experiments (figure 7:5). Linear regression of the points revealed that whereas the antagonist affinities at reticulocyte membranes showed a good correlation ($r = 0.997$), no such correlation was observed for rat adipocyte ($r = 0.691$) or hamster adipocyte ($r = 0.576$) membranes. This indicates that whereas the receptor coupled to adenylate cyclase in reticulocytes can be identified as a beta$_2$ subtype, the receptor on rat or hamster adipocytes cannot be identified as beta$_1$.

7:3 Discussion

The measurement of adenylate cyclase activity provides several advantages over the use of whole cell systems. Cell disruption and membrane preparation can potentially decrease complications due to the selective uptake and unequal access of drug to the receptor. Furthermore with respect to adipocytes in particular, other factors such as protein binding and partitioning into lipids would be obviated and any feedback modulators produced in whole cells might not be expected to be formed to influence the effector response at the membrane level.

All the membrane preparations studied demonstrated adenylate cyclase activity responsive to isoprenaline. In addition, several agents known to modulate the activity of the enzyme had effects in the three membranes studied. Thus, a GTP requirement for the full expression of isoprenaline-stimulated activity (Rodbell 1980, Ross and Gilman 1980, Limbird 1981) was demonstrated while GppNHp (Londos et al 1974, Spiegel and Aurbach 1974, Lefkowitz 1974) and NaF (Sutherland et al 1962, Birnbaumer et al 1969, Birnbaumer et al 1971) were stimulators of the enzyme.

The lack of adenosine deaminase and indomethacin effects
Figure 7:5 Correlation of inhibition constants of selective and non selective beta-adrenoceptor antagonists at inhibition of $[^{125}\text{I}]$CYP binding and isoprenaline stimulated adenylate cyclase in rat adipocyte (△), reticulocyte (●) and hamster adipocyte (■) membranes. For adipocytes, the pA$_2$ or K$_i$ of the antagonist at adenylate cyclase was compared to the K$_i$ values of the drugs at inhibiting the beta$_1$ adrenoceptor present on rat adipocytes (chapter 5). With reticulocytes, the affinities of the drugs at inhibiting adenylate cyclase were compared to their K$_i$ values at the beta$_2$ adrenoceptor present on rat lung membranes. Correlation coefficients were as follows; rat adipocyte, $r = 0.691$, rat reticulocyte, $r = 0.997$, hamster adipocyte, $r = 0.576$. 

(a) atenolol  
(b) betaxolol  
(I) ICI 118.551  
(m) metoprolol  
(pra) practolol  
((+)p) (+)propranolol  
((-)p) (-)propranolol
indicated that in the rat adipocyte membranes examined, there were probably no significant actions of adenosine or prostaglandins on basal or isoprenaline-stimulated adenylate cyclase. This therefore suggests that in membranes, the production of these agents was minimal and their contribution to an observed effector-response would be probably insignificant.

Pharmacological characterisation of beta-adrenoceptors by examining the agonist rank orders of potency confirmed the report by Lands et al (1967) that the rat adipocyte appears to possess a beta_1_ adrenoceptor. As mentioned in chapter 6 however, the same rank order of potency is applicable to the apparently atypical beta-adrenoceptor in this tissue, therefore this classification would not distinguish between the two receptor subtypes. Rat reticulocytes on the other hand conformed to the beta_2_ adrenoceptor classification as clearly demonstrated by Bilezikian et al (1977) and Delhaye et al (1983). With respect to hamster adipocyte membranes, the lack of a full agonist response by adrenaline and noradrenaline could be explained by the dualistic action of these agonists on beta and alpha_2_ adrenoceptors, the latter subtype being negatively coupled to adenylate cyclase (Aktories et al 1979, Aktories et al 1980). No further agonist experiments were performed in this cell type but any analysis of beta-adrenergic responses by stimulants of alpha and beta-adrenoceptors would have to be performed in the presence of alpha-adrenoceptor antagonist drugs.

The use of antagonists to delineate receptor subtypes was performed using both Schild analysis and single inhibition curves. The Schild analysis to examine beta-adrenoceptor antagonists at the level of adenylate cyclase has been demonstrated by several workers (Kaumann and Birnbaumer 1974, Coleman and Somerville 1977, Caron et al 1978) where it was shown that antagonism appeared to
be identical to that found in whole tissues, thus competitive blockade through a single receptor gave a slope of unity. This has enabled a more detailed survey to be performed than that available with a single inhibition curve. The results in this study confirm previous reports (see chapter 6) indicating that the rat adipocyte beta-adrenoceptor is atypical and further show that this receptor is coupled to adenylate cyclase. Although previous experiments on the inhibition of adipocyte adenylate cyclase by antagonists had been performed (Birnbaumer and Rodbell 1969, Yamamura et al 1976), no accurate quantitative data on antagonist potencies was available. The data presented here clearly show that the $pA_2$ values of the antagonists studied approximated those found from experiments in whole adipocytes (chapter 6). Furthermore, the Schild slopes for betaxolol and ICI 118.551 at least, were not significantly different from unity indicating that the antagonism appeared to be competitive at a single receptor subtype. The reason for the low slopes obtained with propranolol stereoisomers is not clear, a possible interpretation being that more than one receptor subtype is involved in the effector response. This however is not easy to deduce in view of the fact that selective antagonists had Schild slopes not significantly different from unity. On the other hand, it must be stressed that the antagonists were used at high concentrations and the possible influence of non specific membrane effects on the effector response cannot be excluded. Indeed, Mendel and Almon (1979) have clearly demonstrated that high concentrations of propranolol can depress fluoride stimulated adenylate cyclase activity. Therefore, further studies are necessary to resolve the effects of non specific contributory factors on the apparent competitive antagonism of beta-blocking agents before any suggestions can be made on the existence of multiple receptor subtypes.
Another problem encountered when analysing the dose response curves was the decrease in maximal isoprenaline stimulated adenylate cyclase with increasing antagonist concentrations. This may be attributed to the effect that Mendel and Almon (1979) have shown as discussed above. However, as the dose response curves remained parallel, it is possible that interference may have only occurred at the high agonist/antagonist concentrations used in the assay. This is a plausible explanation since if the decrease in maximal response was accounted for (ie taking the ED$_{50}$ as 50% of each response instead of 50% of the reference isoprenaline response), the Schild slopes were very low and no meaningful data could be obtained.

Further experiments with single inhibition curves to selective beta$_1$ adrenoceptor antagonists in adipocytes confirmed the above findings that beta-adrenoceptor antagonists were weak at inhibiting isoprenaline stimulated adenylate cyclase and analysis of the correlation plot clearly showed that the beta-adrenoceptors identified by adenylate cyclase measurements were not similar to beta-adrenoceptors identified by ligand binding studies.

Comparison of antagonist affinities at adenylate cyclase in rat reticulocytes was found to be very well correlated with the affinities of drugs at beta$_2$ adrenoceptors which confirms previous reports for this cell type (Dickinson et al 1981, Delhaye et al 1983). This at least is reassuring in respect to any technical difficulties associated with the assay and strongly suggests that the rat adipocyte beta-adrenoceptor is indeed atypical under these experimental conditions.

Hamster adipocytes also appeared to have an atypical beta-adrenoceptor. Bjorgell and Belfrage (1982) suggested that lipolysis in hamster adipocytes was mediated by a heterogeneous population
of beta_1 and beta_2 adrenoceptors. This was based upon the observa-
tion that antagonism of lipolysis stimulated by isoprenaline or
selective agonists was blocked by both beta_1 and beta_2 selective
antagonists. However since propranolol inhibited lipolysis with
low affinity (IC_{50} approx 2 \times 10^{-7} M) the beta-adrenoceptor may in
fact be atypical. Careful investigation of other work on hamster
adipocytes (Hittelman et al 1973, Rosak and Hittleman 1977) reveal-
ed that propranolol was weak at antagonising lipolysis and cAMP
accumulation, further supporting the suggestion that this beta-
adrenoceptor is also atypical. The atypical beta-adrenoceptor is
not restricted to the rat adipocyte and as mentioned in chapter 6,
other species and adipocyte types have atypical beta-adrenoceptor
mediated lipolytic responses. With regard to adenylate cyclase,
both murine (Begin-Heick 1981) and human (Kather and Simon 1977)
adipocyte beta-adrenoceptors appear atypical where antagonists had
to be used at high concentrations to inhibit the stimulated enzyme.
However, no calculations were made regarding the affinities of drugs
for the receptors therefore no comparison could be made with the
above results.

In order to further strengthen the proposal that the hamster
adipocyte beta-adrenoceptor was atypical, experiments were perform-
ed in the presence of 0.154 M NaCl to investigate inhibitory
alpha_2 adrenoceptors. Hamster but not rat adipocytes possess
alpha_2 adrenoceptors (Carpene et al 1983), which have opposite
effects to beta-adrenergic stimulation, ie are antilipolytic and
attenuate cAMP accumulation (Carpene et al 1980, Schimmel et al
1980b). As Na^+ has been shown to be a necessary requirement for the
inhibition of adenylate cyclase activity in most but not all systems
(Aktories et al 1979, Blume et al 1979, Londos et al 1981), it was
included in all the experiments on hamster adipocytes. The purposes
of this study were to have an internal control where, in the same membrane preparation, typical alpha<sub>2</sub> adrenoceptors could be identified with atypical beta-adrenoceptors. Unfortunately, due to the low degree of stimulation and poor reproducibility, these experiments could not be performed under the experimental conditions used and would have to await the use of possibly the <sup>32</sup>P-ATP assay (Salomon et al 1974) which measures the direct conversion of ATP to cAMP thereby eliminating the protein binding assay where experimental errors are compounded.

This study poses several intriguing questions about the role of beta-adrenoceptors in adipose tissue. The first question is why do rat adipocytes possess typical beta<sub>1</sub> adrenoceptor binding sites (chapter 5) but atypical beta-adrenoceptors which appear to mediate the effector response? A possible answer is that the beta<sub>1</sub> adrenoceptors are very poorly coupled or indeed not coupled to adenylate cyclase as suggested by the above data. Recent evidence for coupled and non-coupled beta-adrenoceptors has been shown from studies on the human heart (Robberecht et al 1983, Waelbroeck et al 1983) where it was clearly shown that human heart possesses both beta<sub>1</sub> and beta<sub>2</sub> adrenoceptors in equal proportions as determined by binding studies whereas apparently only the beta<sub>2</sub> adrenoceptor was coupled to adenylate cyclase. These authors concluded that either the beta<sub>1</sub> adrenoceptors were poorly coupled so as not to be noticeable or that they were linked to a second messenger system different to cAMP production. Relating the above findings to the observations in this study, it is possible that the beta<sub>1</sub> adrenoceptors in rat adipocytes are not involved in the effector response under normal conditions.

A further complication relates to the observation that hamster adipocyte beta-adrenoceptors appear more atypical than those in
rat adipocytes. There are at least two possibilities to consider, either hamster adipocyte beta-adrenoceptors are a different subtype which is more atypical than that of the rat or that the atypical beta-adrenoceptor response is a hybrid of a beta1 and atypical beta-adrenoceptor mediated response. The suggestion that beta1 and atypical beta-adrenoceptors may both be involved in the effector response was made in chapter 6. Indeed even with the adenylate cyclase experiments a Schild slope of 0.82 for betaxolol was observed which although not significantly different from unity may suggest a small degree of heterogeneity. The small contribution of beta1 adrenoceptors at the effector response although not noticeable may affect the pA2 value slightly and make it deviate towards a higher value. With these facts in mind, table 7:5 shows the Bmax values obtained with [125I]CYP in membranes of human, rat and hamster adipocytes. Although no evaluation can be made whether the beta-adrenoceptors identified by [125I]CYP are functional, it is interesting to note that hamster adipocytes do not appear to possess any [125I]CYP binding sites and also have a very atypical effector response. On the other hand, human adipocytes which have the highest density of beta-adrenoceptors have a lipolytic response more characteristic of a typical beta-adrenoceptor (Harms 1976b). It is therefore a possibility that the ratio of typical beta1 to atypical beta-adrenoceptors may determine the overall 'beta-adrenergic response' and that the techniques used here cannot resolve the two components. This explanation may at least explain in part the anomalies found relating to low Schild slopes.

The classification of the rat adipocyte beta-adrenoceptor is complicated in view of the points discussed above. Nevertheless, strong evidence has been provided for the existence of atypical beta-adrenoceptors which are coupled to adenylate cyclase. As
Table 7:5  Maximal binding capacities and equilibrium dissociation constants for $[^{125}\text{I}]$CYP in human, rat and hamster adipocyte membranes. Membranes were incubated with increasing concentrations of $[^{125}\text{I}]$CYP and specific binding assessed as described in text. $B_{\text{max}}$ and $K_D$ values were determined by Scatchard analysis of the specific binding. Results are expressed as mean ± SEM of four to five experiments performed in duplicate.

<table>
<thead>
<tr>
<th>Species</th>
<th>$B_{\text{max}}$ (fmoles/mg protein)</th>
<th>$K_D$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>100 ± 9</td>
<td>29.5 ± 1.8</td>
</tr>
<tr>
<td>rat</td>
<td>53.3 ± 1.4</td>
<td>29.1 ± 1.8</td>
</tr>
<tr>
<td>hamster</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

ND = no detectable binding was observed.

$\beta_1$ and atypical beta-adrenoceptors coexist on rat adipocytes, further experiments attempting to distinguish between the two beta-adrenoceptors have been performed using an irreversible beta-adrenoceptor antagonist, these are described in the following chapter.
Further studies on the atypical nature of the adipocyte beta-adrenoceptor: use of the apparently irreversible antagonist para amino benzyl carazolol
8:1 Introduction

There is substantial evidence to suggest that an atypical beta-adrenoceptor appears to mediate catecholamine stimulated lipolysis, cAMP accumulation and adenylate cyclase activity in the rat adipocyte (chapters 6 and 7). However, typical beta\textsubscript{1} adrenoceptors have also been identified on rat adipocytes with the use of high affinity ligand binding assays (chapter 5). Although the typical beta\textsubscript{1} adrenoceptor appears to be uncoupled or at most is poorly coupled to adenylate cyclase, positive evidence for the coexistence of two potentially different beta-adrenoceptors would be provided if they could be separated from each other. In this study therefore, attempts have been made to selectively suppress the high affinity beta\textsubscript{1} adrenoceptors with an irreversible antagonist in order to examine whether alteration in the beta\textsubscript{1} adrenoceptor population would influence the catecholamine-stimulated cyclase response.

The use of irreversible and slowly reversible antagonists for beta-adrenoceptors has resulted in a comprehensive series of studies which have included the following:


(ii) estimation of the mode of coupling between receptor and adenylate cyclase (Tolkovski and Levitzki 1978)

(iii) determination of receptor reserves in isolated tissue preparations (Takayanagi et al 1976, Avner and Wilson 1979, Terasaki et al 1979, Venter 1979)

(iv) investigation of the rate of receptor synthesis following blockade in vitro and in vivo (Lucas et al 1979, Fraser and Venter
In this study, a novel use for irreversible antagonists has been attempted. This has been based upon the observation that the atypical adipocyte beta-adrenoceptor has generally a lower affinity than typical beta-adrenoceptors for beta-adrenoceptor antagonists, therefore, a selectivity of irreversible antagonism should be possible. The antagonist chosen was the high affinity non-selective blocker para-amino benzyl carazolol which has recently been used as a photoaffinity label to successfully identify beta-adrenoceptor subunits (Shorr et al 1982). The advantage of using a photoaffinity label is that in the absence of ultra violet irradiation, the antagonist acts as a competitive agent thereby enabling accurate estimates of affinity to be made prior to covalent attachment with the receptor.

In order to investigate the effects of irreversible antagonism on beta-adrenoceptors, it is necessary to assess the presence of a receptor reserve at the level of adenylate cyclase. Therefore an analysis of the potencies of isoprenaline at binding and cyclase has also been made in this study.

Since the adipocyte appears to possess atypical beta-adrenoceptor mediated responses, comparative studies have also been performed on rat reticulocytes which have well characterised adenylate cyclase coupled beta\textsubscript{2} adrenoceptors (chapter 7).

8:2 Results

8:2:1 Comparison of affinities of PABC at binding and adenylate cyclase in rat reticulocyte and adipocyte membranes

Figure 8:1 shows the ability of PABC to inhibit the specific binding of [\textsuperscript{125}I]CYP and isoprenaline stimulated (10\textsuperscript{-5} M for
Figure 8:1 Inhibition of specific $^{125}$I CYP binding (●) and isoprenaline stimulated adenylate cyclase (▲) in (a) reticulocyte and (b) adipocyte membranes. Assays were performed as described in methods and $IC_{50}$ values calculated either by analysing Hill plots for binding experiments or graphically for cyclase experiments and $K_i$ values calculated from $IC_{50}$ values using the Cheng and Prusoff equation. Results are expressed as mean ± SEM of three to five experiments performed in duplicate.
reticulocytes, $10^{-4}$ M for adipocytes) adenylate cyclase activity in both membrane preparations. The antagonist inhibited $[^{125}\text{I}]{\text{CYP}}$ binding with similar affinity in both membranes ($0.618 \pm 0.057$ nM in reticulocytes and $0.671 \pm 0.015$ nM in adipocytes). Furthermore, the ability of PABC to inhibit isoprenaline stimulated adenylate cyclase in reticulocytes was very similar to its affinity in binding assays in the same tissue ($K_i$ cyclase / $K_i$ binding = 2.5). On the other hand, there was significant selectivity of PABC to inhibit ligand binding in the adipocyte ($K_i$ cyclase / $K_i$ binding = 35) reflecting the much lower affinity exhibited by this antagonist against isoprenaline stimulated adenylate cyclase in the adipocyte. This confirms the results found in chapter 7 that the adipocyte in contrast to the reticulocyte appears to have an atypical beta-adrenoceptor with respect to antagonists and indicates the possibility of using PABC as an irreversible antagonist to selectively inactivate typical beta-adrenoceptors.

8:2:2 Comparison of inhibition constants for isoprenaline against $[^{125}\text{I}]{\text{CYP}}$ binding with $K_{act}$ values at stimulating adenylate cyclase in reticulocyte and adipocyte membranes

In order to investigate the possibility of a receptor reserve at adenylate cyclase in the two membranes, the affinity of isoprenaline as determined by binding studies was compared to that observed at adenylate cyclase stimulation. Inhibition of $[^{125}\text{I}]{\text{CYP}}$ binding by isoprenaline in both membranes was fitted to a two site model which indicated that the majority of sites were in the low affinity state (figure 8:2). Stimulation of adenylate cyclase by isoprenaline in reticulocytes indicated that the $K_{act}$ closely corresponded to the low affinity state of binding suggesting that the receptor identified from adenylate cyclase measurements was
Figure 8:2 Inhibition of specific $[^{125}\text{I}]$CYP binding (●) and stimulation of adenylate cyclase (▲) by isoprenaline in (a) reticulocyte and (b) adipocyte membranes. For adenylate cyclase experiments, $K_{\text{act}}$ values were calculated using 'ALLFIT' and for binding assays, curves were analysed by computer iterative curve fitting to two sites giving IC$_{50}$ values and relative proportions (in parentheses). $K_i$ values were calculated from IC$_{50}$ values using the Cheng and Prussoff equation. Results are expressed as mean ± SEM of three to four experiments performed in duplicate.
probably the same receptor identified by binding studies and that no receptor reserve was apparent. In contrast, the $K_{act}$ for isoprenaline in adipocyte membranes was approximately one order of magnitude higher than the low affinity state of binding suggesting that the receptor identified by adenylate cyclase was not the same as that seen with binding studies.

8:2:3 Irreversible inhibition of beta-adrenoceptors by PABC in reticulocyte and adipocyte membranes

(A) Binding studies

Figure 8:3 shows a representative experiment where preincubation of reticulocyte membranes with $10^{-8}$ M PABC resulted in a decrease in the total specific binding capacity for $[^{125}\text{I}]\text{CYP}$. Scatchard analysis of the data revealed that although the $B_{\text{max}}$ was reduced to 42% of control, the $K_D$ of $[^{125}\text{I}]\text{CYP}$ was only altered slightly indicating non-competitive interactions. The effect of different concentrations of PABC on the $B_{\text{max}}$ of both reticulocyte (figure 8:4a) and adipocyte (figure 8:5a) membranes was also examined and demonstrated that increasing PABC concentrations resulted in a dose-dependent decrease in $B_{\text{max}}$ in both preparations. Somewhat surprisingly, identical results were also obtained in membranes that were also subjected to SANAH and photolysis treatment. In order to assess the competitive nature of PABC at different concentrations, the $K_D$ values of $[^{125}\text{I}]\text{CYP}$ at each concentration of PABC were expressed as a ratio of the control $K_D$ value for both membrane preparations (table 8:1).

(B) Adenylate cyclase assays

Incubation of control membranes with increasing concentrations of isoprenaline resulted in stimulation of adenylate cyclase in reticulocytes (figure 8:4b) and in adipocytes (figure
Figure 8:3  (a) Saturation analysis of specific $[^{125}\text{I}]\text{CYP}$ binding to rat reticulocyte membranes in the absence (●) and presence of $10^{-8}\text{M PABC} (\Delta)$. Membranes were treated as described in methods and incubated with increasing concentrations of $[^{125}\text{I}]\text{CYP}$ for 60 minutes at 37°C. (b) Scatchard analysis of same data. $K_D$ and $B_{\text{max}}$ values were determined by linear regression using the method of least squares. The data shown are from a single experiment but similar results were obtained on three other occasions.
Figure 8:4 Irreversible inhibition of beta-adrenoceptors by PABC in reticulocyte membranes. (a) Inhibition of specific $^{125}$I-CYP binding after membranes were treated without (●) and with SANAH and photolysis (▲). $B_{max}$ values were determined as a percentage of control values using Scatchard analysis. Results are expressed as mean ± SEM of three to four experiments performed in duplicate. (b) Stimulation of adenylate cyclase by isoprenaline after PABC treatment. Membranes were treated in the absence (●) or presence of $10^{-9}$M (▲), $10^{-8}$M (■), $10^{-7}$M (○) or $10^{-6}$M (△) PABC and adenylate cyclase assayed as described in methods. Results are expressed as mean ± SEM of four experiments performed in duplicate. Values were considered to be significantly different from control using the paired t test (*p<0.05, **p<0.01, ***p<0.001).
Irreversible inhibition of beta-adrenoceptors by PABC in adipocyte membranes. (a) Inhibition of specific $[^{125}\text{I}]$CYP binding after membranes were treated without (●) and with SANAH and photolysis (▲). $B_{\text{max}}$ values were determined as a percentage of control membranes using Scatchard analysis. Results are expressed as mean ± SEM of three to four experiments performed in duplicate. (b) Stimulation of adenylate cyclase by isoprenaline after PABC treatment. Membranes were treated in the absence (●) or presence of $10^{-8}\text{M}$ (▲), $10^{-7}\text{M}$ (■) or $10^{-6}\text{M}$ (○) PABC and adenylate cyclase assayed as described in methods. Results are expressed as means of three experiments and standard errors were excluded for clarity but were less than ± 10%. Values were considered to be significantly different from control using the paired t test (*p<.05, **p<.01)
When reticulocyte membranes were treated with increasing concentrations of PABC, the maximal isoprenaline stimulated adenylate cyclase activity was decreased in a dose-dependent manner which correlated favourably with the reduction in binding sites observed with \(^{125}\text{I}]\text{CYP. Analysis of the dose response curves using the iterative dose response curve analysis programme 'ALLFIT' (De Lean et al 1978) enabled the } K_d^{\text{act}} \text{ values for each of the dose response curves to be calculated (table 8:2). Clearly, although the maximal isoprenaline stimulated adenylate cyclase activity was decreased after PABC treatment, the } K_d^{\text{act}} \text{ values were similar up to } 10^{-7} \text{ M PABC indicating the non-competitive nature of interaction with beta-adrenoceptors.}

In contrast, irreversible inhibition of rat adipocyte beta\(_1\) adrenoceptors by PABC had no effect on the isoprenaline stimulated adenylate cyclase activity except at high concentrations (10\(^{-6}\) M) where the isoprenaline dose response curve was slightly shifted to the right.

### Table 8:1

<table>
<thead>
<tr>
<th>PABC (M)</th>
<th>reticulocyte</th>
<th>adipocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>10(^{-9})</td>
<td>1.01 ± 0.08</td>
<td>0.90 ± 0.08</td>
</tr>
<tr>
<td>10(^{-8})</td>
<td>1.34 ± 0.13</td>
<td>1.68 ± 0.20</td>
</tr>
<tr>
<td>10(^{-7})</td>
<td>3.14 ± 0.31</td>
<td>6.10 ± 2.65</td>
</tr>
<tr>
<td>10(^{-8})</td>
<td>1.45 ± 0.13</td>
<td>2.36 ± 0.31</td>
</tr>
<tr>
<td>10(^{-7})</td>
<td>3.67 ± 0.23</td>
<td>7.02 ± 2.87</td>
</tr>
</tbody>
</table>

\(K_d/K_d^{\text{control}}\) values for \(^{125}\text{I}]\text{CYP in reticulocyte and adipocyte membranes treated with PABC expressed as a ratio over control values. } K_d \text{ values were calculated by Scatchard analysis and results are expressed as mean ± SEM of three to four experiments performed in duplicate.}
Table 8:2 $V_{\text{max}}$, slope factors and $K_{\text{act}}$ values for isoprenaline stimulated adenylate cyclase in membranes treated with PABC. $V_{\text{max}}$ is expressed as a percentage of control and curves fitted using 'ALLFIT'. Results are expressed as mean ± SEM of three to four experiments performed in duplicate.

<table>
<thead>
<tr>
<th>PABC (M)</th>
<th>$V_{\text{max}}$ (%)</th>
<th>slope</th>
<th>$K_{\text{act}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>reticulocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.83 ± 0.04</td>
<td>1.01 ± 0.10</td>
</tr>
<tr>
<td>10^{-9}</td>
<td>102 ± 6</td>
<td>0.86 ± 0.03</td>
<td>0.73 ± 0.10</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>42.8 ± 5.9</td>
<td>0.90 ± 0.04</td>
<td>1.51 ± 0.22</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>20.8 ± 2.0</td>
<td>1.23 ± 0.20</td>
<td>4.97 ± 1.43</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>14.7 ± 1.5</td>
<td>1.21 ± 0.21</td>
<td>35.6 ± 8.0</td>
</tr>
<tr>
<td>adipocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.71 ± 0.01</td>
<td>12.6 ± 2.5</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>107 ± 6</td>
<td>0.84 ± 0.09</td>
<td>13.8 ± 2.6</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>109 ± 1</td>
<td>0.88 ± 0.02</td>
<td>13.6 ± 3.2</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>105 ± 4</td>
<td>0.98 ± 0.11</td>
<td>44.5 ± 15.4</td>
</tr>
</tbody>
</table>

The diterpene forskolin which can stimulate adenylate cyclase by a receptor independent mechanism (Seamon and Daly 1981) was found to retain its capacity to stimulate the enzyme identically irrespective of the degree of irreversible beta-adrenoceptor blockade (not shown).

8:3 Discussion

There are several 'irreversible' beta-adrenoceptor antagonists currently available. These include N-(2-hydroxy-3-napthoxypropyl)-N'-bromoacetyl-ethylenediamine (Atlas et al 1976), acebutolol azide (Wrenn and Homcy 1980), aminobenzyl propranolol (Terasaki et al 1979), bromoacetylated alprenolol menthane (Baker and Pitha 1982), cyanopindolol azide (Burgermeister et al 1982) and propranolol-SH (Gozlan et al 1980). Each of these compounds has been successfully used to irreversibly block beta-adrenoceptors. However, it was
anticipated that the photoaffinity label PABC would be a suitable agent to distinguish typical from atypical beta-adrenoceptors as it was of high affinity and could also be examined under reversible conditions prior to covalent incorporation in the presence of SANAH (a bifunctional cross linking agent) and photolysis. The present results strongly suggest that PABC is a relatively selective agent for typical beta-adrenoceptors and has a greater than thirty fold selectivity in suppressing specific $[^{125}\text{I}]$CYP binding than isoprenaline-stimulated adenylate cyclase activity in adipocytes. This suggested that PABC could be used at concentrations occupying the majority of beta$_1$ adrenoceptors but only a small proportion of apparent atypical sites.

In order to examine the effects of irreversible antagonism of beta-adrenoceptors, it is necessary to estimate the level of receptor reserve at the adenylate cyclase. This has been performed by comparing the $K_{act}$ for isoprenaline with the affinity of the agonist as determined from ligand binding studies. In the reticulocyte, the $K_{act}$ and $K_L$ values were in excellent agreement which suggests that no receptor reserve was present (see Ruffolo 1982). Accordingly, it is predicted that all the beta-adrenoceptors must be occupied by the agonist to elicit a maximal response. Therefore, irreversible inhibition of these beta-adrenoceptors would be expected to result in a reduction in the maximal isoprenaline responsiveness (see Nickerson 1956, Furchgott 1966, Ruffolo 1982 for examples). In adipocytes on the other hand, the $K_{act}$ for isoprenaline was approximately ten times higher than the $K_L$ at typical beta$_1$ adrenoceptors. This intriguing result suggests that the adenylate cyclase coupled beta-adrenoceptor is not identical to that seen in binding studies. Furthermore, these observations reflect that agonist potencies at the atypical beta-adrenoceptor
are lower than at beta_1 or beta_2 adrenoceptors. If the same is true for the agonists adrenaline and noradrenaline, then a clear distinction can be made between typical and atypical beta-adrenoceptors on the basis of agonist affinities although the rank order of potency between beta_1 and atypical beta-adrenoceptors is the same.

Preincubation of membranes with PABC produced a dose-dependent irreversible inhibition of [^{125}I]CYP binding to beta-adrenoceptors in both membrane preparations. However, the degree of 'irreversible' inhibition was greater in reticulocytes which can be explained in two possible ways. Firstly, the reticulocyte is a cell type with a very high population of beta-adrenoceptors (Kaiser et al 1978, Dickinson et al 1981). This has enabled binding to be examined under conditions of very low non-specific binding. As a result, irreversible blockade of beta-adrenoceptors could be easily examined without the influence of a major non-specific binding component.

In contrast, the adipocyte has a relatively low density of beta-adrenoceptors and a moderately high degree of non-specific binding with [^{125}I]CYP (chapter 5). Furthermore, the Scatchard plot for [^{125}I]CYP binding in adipocytes displays a small degree of curvilinearity at high radioligand concentrations. Curvilinear Scatchard plots with adipocyte membranes have been demonstrated by several workers (see chapter 5) and as discussed in the same chapter, the effects seen were probably due to the use of inappropriate drugs to assess non-specific binding. It is still possible that a small degree of curvilinearity may occur even with the use of 200 µM (-)isoprenaline to assess the non-specific binding (see figure 5:1c). This could have serious consequences on the quality of data since irreversible inhibition of beta-adrenoceptors in adipocytes would amplify the error introduced by the low affinity component. This would result in significant overestimation of the K_D and B_{max}
values. It is therefore possible that the real degree of irreversible antagonism is better reflected by the results observed in reticulocytes.

Another possibility which could account for the observed differences is that PABC is not a truly irreversible antagonist but a very slowly reversible one. This is indeed supported by the fact that the apparent effectiveness of PABC in suppressing the binding capacity was not reduced in the absence of SANAH and photolysis. As photoaffinity labeling of the beta-adrenoceptor by PABC was reported to only involve 0.5 - 1.5% incorporation of the bound ligand (Shorr et al 1982), it is likely that the small degree of covalent attachment was overshadowed by the larger amount of ligand slowly reversibly bound to the receptors. It remains possible that PABC is a slowly reversible antagonist but that the dissociation from beta$_1$ adrenoceptors is greater during washing than at beta$_2$ sites on reticulocytes. This is unlikely since the affinity of PABC at reticulocyte (beta$_2$) and adipocyte (mainly beta$_1$) adrenoceptors was identical which would predict similar kinetic off-rates.

Indeed, if PABC is a slowly reversible antagonist, this property could have been of great advantage regarding the selectivity of the drug. Since the affinity of a drug for a receptor is governed primarily by its rate of dissociation, it can be expected for PABC to have a greater dissociation rate from atypical then typical beta-adrenoceptors resulting in typical beta-adrenoceptors still occupied after the washing procedure but the atypical beta-adrenoceptors unoccupied and free to accept an agonist molecule. This is a likely explanation in view of the fact that PABC concentrations up to $10^{-7}$ M were used which would have occupied the majority of atypical beta-adrenoceptors as well.

Whatever the mechanism of action, during the period of experi-
mentation, PABC was effective in reducing the density of \[^{125}\text{I}]\text{CYP}\) binding sites but had quite different effects on the isoprenaline-stimulated adenylate cyclase activity in the two membranes. In reticulocytes, there was a parallel loss of isoprenaline-stimulated adenylate cyclase with the maximal \[^{125}\text{I}]\text{CYP}\) binding capacity indicating that the same beta-adrenergceptors were probably identified by the two techniques. In marked contrast, the reduction in receptor density in adipocytes did not alter the adenylate cyclase stimulation by isoprenaline which suggests that isoprenaline stimulated adenylate cyclase may be independent of beta\(_1\) adrenoceptor binding sites in rat adipocytes and is probably mediated by a low affinity atypical beta-adrenoceptor. However, it should be taken as a caveat that due to technical difficulties, it was not possible to irreversibly inhibit all of the adipocyte beta\(_1\) adrenoceptors and therefore it cannot be assessed whether these adrenoceptors are coupled to some extent to the cyclase. Only under conditions of total irreversible blockade of beta\(_1\) adrenoceptors and the maintenance of a maximal isoprenaline response would it be possible to categorically suggest that the beta\(_1\) adrenoceptors are not coupled to adenylate cyclase. It is therefore possible that a small component of the cyclase response may be attributed to coupling with beta\(_1\) adrenoceptors but under the experimental conditions used, this component was too small to be detectable, otherwise a small reduction in the isoprenaline maximal response would have been observed.

In conclusion, these results suggest that most if not all of the isoprenaline stimulated adenylate cyclase is independent of beta\(_1\) adrenoceptor binding sites in rat adipocytes and is probably mediated by a low affinity atypical beta-adrenoceptor.
CHAPTER 9

Concluding Discussion
The present studies were undertaken in an attempt to identify and classify the rat adipocyte beta-adrenoceptor using several different experimental approaches. As a result, a number of interesting observations have been made relating to the nature of the receptor which mediates the functional lipolytic response.

High affinity ligand binding assays have been frequently and successfully used to delineate beta-adrenoceptor subtypes in a wide variety of tissues (see Nahorski 1981, Minneman et al 1981). Using this approach, which was successful because of the use of $^{125}$I CYP as ligand, typical beta$_1$ adrenoceptors could be identified on isolated adipocyte membranes. These studies could not reveal, however, the functional significance of these binding sites. In view of the observations in this thesis which have indicated that an atypical beta-adrenoceptor mediates all of the effector responses studied, a criticism may be made as to the validity of binding studies to identify functionally relevant beta-adrenoceptors in adipocytes. This criticism may be of some importance when it is considered that a substantial amount of effort has been devoted to the effects of hormones or metabolic changes on the binding properties of typical beta-adrenoceptors (see Fain 1982, chapter 5 discussion). The results obtained with high affinity ligand binding studies may bear no relation to the events occurring at the atypical beta-adrenoceptor. In this context therefore, it may be necessary to make a full re-evaluation of all the beta-adrenoceptor binding studies performed in fat cells. It could now be considered naive to have performed the ligand binding studies in this thesis as the atypical beta-adrenoceptor would not have been identified. Indeed, in hamster adipocyte membranes, no binding sites were found and yet the tissue possessed an adenylate cyclase that was responsive to catecholamines. However, the binding studies do pose a question as
to the functional role, if any, of the typical β₁ adrenoceptor. There are tentative suggestions in this study that the β₁ adrenoceptor may in fact be poorly coupled to the functional response. This proposal is made on the grounds that the Schild slope for betaxolol inhibition of noradrenaline stimulated lipolysis was significantly less than unity. Furthermore, betaxolol, at a concentration approaching its affinity for typical β₁ adrenoceptors, produced an initially large shift of the isoprenaline stimulated cAMP accumulation dose-response curve, when adenosine effects were suppressed.

One approach which can be used to estimate the possible coupling of the typical β₁ adrenoceptor to adenylate cyclase would be to examine the inhibition of ligand binding by an agonist and its modulation by guanine nucleotides. This aspect could not be examined with [¹²⁵I]CYP as the long incubation times and inclusion of sodium chloride in the buffer had deleterious effects on guanine nucleotide shifts of the agonist inhibition curve. More recent attempts at tackling this problem have been made in this laboratory using the radioligand [¹²⁵I]PIN (Barovski and Brooker 1980, Ezrailson et al 1981). Preliminary experiments with this ligand had indicated rapid kinetics of association which allowed its use at short incubation times in the absence of sodium chloride. These conditions were deemed necessary for the investigation of guanine nucleotide effects on agonist binding (chapter 4). The results of experiments performed with [¹²⁵I]PIN are presented here. When isoprenaline was used to inhibit [¹²⁵I]PIN binding in adipocyte membranes (figure 9:1b), the inhibition curves were of low slope indicating the presence of high and low affinity states of binding (Kent et al 1980). As the high affinity state is believed to be the ternary complex and the intermediate step in cyclase activation (Stadel et al 1980), it could be
Figure 9:1  Inhibition of specific $[^{125}\text{I}]$PIN binding by isoprenaline in the absence (●) and presence of $10^{-4}$ M GppNHp (▲). Rat lung (a) and adipocyte (b) membranes were incubated with $[^{125}\text{I}]$PIN and isoprenaline for 15 min at 37°C and specific binding determined as described in methods. Curves were analysed by computer curve fitting to two sites giving IC$_{50}$ values and relative proportions (in parentheses) of each site. Inhibition constants were calculated from IC$_{50}$ values using the Cheng and Prussoff equation and Hill coefficients from Hill plots. Results are expressed as mean ± SEM of four experiments performed in duplicate.
inferred that the beta$_1$ adrenoceptor does demonstrate coupling to the adenylate cyclase.

However, these studies were not without complications as GppNHp failed to convert the heterotrophic binding to a homogeneous low affinity state. The reason for this observation is unclear but since a similar result was obtained in rat lung (figure 9:1a) which usually shows a good guanine nucleotide shift, there may be problems associated with the assay technique. These difficulties may relate to the use of $[^{125}\text{I}]$PIN but no explanations according to current models are available to account for this phenomenon. As no guanine nucleotide shifts could be observed, it is not possible to determine whether the receptor is really coupled to a guanine nucleotide protein or the high affinity state is simply an artifact. These studies have therefore failed to provide evidence for beta$_1$ adrenoceptor coupling to the cyclase. In several reports where $[^3\text{H}]$DHA has been used as ligand, guanine nucleotide shifts of agonist binding have been demonstrated (Giudicelli et al 1979a, Malbon 1980, Giudicelli et al 1982). These reports may therefore be the only evidence to suggest that the beta$_1$ adrenoceptor exhibits linkage to adenylate cyclase.

Besides the minor and questionable role of the beta$_1$ adrenoceptor in the lipolytic response, it can be speculated that the receptor performs other functions in the cell. Since specific pools of cAMP are believed to be present in fat cells (Litosch et al 1982, Schimmel 1984), it is conceivable for the pools to be independently regulated. It is therefore possible that the beta$_1$-adrenoceptor may act via lipolysis independent pools and control other cAMP dependent pathways such as the regulation of glycogen synthetase and phosphorylase activity. An alternative possibility is that the typical beta$_1$-adrenoceptor may act through cAMP independent
processes as has been reported for beta-adrenoceptors in murine S49 lymphoma cells where they regulate Mg^{++} transport (Erdos et al 1981). A recent report by Kashiwaigi et al (1983) has shown that isoprenaline can stimulate the glucose transport mechanism in adipocytes to which cAMP is inhibitory. It may therefore be prudent to investigate the pharmacological characteristics of these effector mechanisms to delineate the receptor subtype involved in each response.

The atypical beta-adrenoceptor has been identified down to the level of adenylate cyclase in washed membrane preparations. This has been essential due to the many variable factors which may have influenced the apparent affinity of a drug in whole cell experiments. The elimination of these factors would clearly exclude them as causative to the atypical characteristics of the beta-adrenoceptor. This study has therefore provided strong evidence for the existence of an atypical beta-adrenoceptor.

The next steps towards positive identification of this novel adrenoceptor must come with the development of selective drugs for this receptor subtype. The newly developed arylethanolamine beta-adrenoceptor agonists which have been developed as lipolytic and thermogenic agents (Arch et al 1984, Wilson et al 1984) may well provide clues as to the structure activity relationships of the atypical beta-adrenoceptor. The affinities of these drugs could easily be examined with adenylate cyclase assays. These future studies may lead to the discovery of an agent with a sufficiently high affinity to be developed as a radioligand. The consequences of this would be the direct labelling of the atypical beta-adrenoceptor. This would open up the possibility of examining the effects of metabolic state, hormones, guanine nucleotides, etc on the binding of the functionally relevant lipolytic adrenoceptor. Furthermore,
as an extension of ligand binding techniques, the atypical beta-adrenoceptor could be solubilised and its hydrodynamic properties analysed (see Haga et al 1977) as well as its peptide structure using peptide mapping techniques (Stiles et al 1983b).

In the light of findings presented in this study, it may be necessary to reassess previous studies which have suggested the existence of more than two beta-adrenoceptor subtypes. Thus some tissues appear to have functional responses which cannot be explained on the basis of $\beta_1$ or $\beta_2$ adrenoceptors. These include rat fundus (Bristow et al 1970, Wasserman and Levy 1972), rat oesophagus (Buckner and Christopheson 1974), rabbit small intestine (Furchgott 1972), human placenta (Tulenko and High 1976) and bovine iris sphincter (Patil et al 1971). It is improbable that the atypical beta-adrenoceptor is solely located in adipose tissue and it is possible that the same or another atypical beta-adrenoceptor may be responsible for some or all of the functional responses in the above tissues.

At present it is thought that only four types of adrenoceptor mediate all the effects of catecholamines. In their review, Ariëns and Simonis (1983) have suggested that $\alpha_1$ and $\beta_1$ adrenoceptors may be considered as innervated or postjunctional receptors which are responsive to noradrenaline whereas $\alpha_2$ and $\beta_2$ adrenoceptors which are also located in non-innervated tissues and cell types can be considered as hormonal or extrajunctional receptors which are recipients for circulating adrenaline. This hypothesis only requires the existence of two subtypes of alpha- and beta-adrenoceptors. An explanation relating to the lipolytic adrenoceptor is to in fact question its classification as a beta-adrenoceptor. The argument in favour of this classification is that isoprenaline, adrenaline and noradrenaline are all full agonists with isoprenaline
being the most potent of the three catecholamines. However, the stronger argument lies with respect to antagonist affinities. Propranolol which is non-selective between beta₁ and beta₂ adrenoceptors is between ten and one hundred times less potent at the lipolytic adrenoceptor. Moreover, the ratio of stereoisomer affinities is much lower in adipocytes than in tissues with beta₁ or beta₂ adrenoceptors (Harms et al 1977). These results indicate that the atypical beta-adrenoceptor is markedly different to beta₁ or beta₂ subtypes. It may be more pertinent therefore to consider classifying the lipolytic adrenoceptor outside the alpha/beta receptor classification. The existence of gamma- and delta-adrenoceptors was proposed by Furchgott in 1959 to explain the effects of sympathomimetic amines on hepatic glycogenolysis and intestinal smooth muscle relaxation. It is now clear that the above functional responses are not mediated by different adrenoceptors and that the misinterpretation was due to the coexistence of alpha- and beta-adrenoceptors or the uptake and metabolism of antagonist (Ahlquist and Levy 1959, Furchgott 1960, Kunos et al 1983). In addition, Neild and co-workers (see Neild and Zelder 1982) have proposed the existence of a gamma-adrenoceptor which apparently mediates the contractile response in vascular smooth muscle produced by sympathetic nerve stimulation. At present, there is some controversy as to the existence of this receptor (see Bevan 1984) and this emphasises the problem that without direct evidence for a novel adrenoceptor, it is possible for a plethora of adrenoceptors to develop with a resultant confusion in nomenclature. It is therefore of the author's opinion that in future, the assignation of a new receptor subtype can only occur after the receptor has been identified by both functional and ligand binding studies. With this point in mind, future research into the molecular pharmacology of the catecholamine lipolytic receptor may eventually lead to its designation as a third class of adrenoceptor.
APPENDICES
Appendix 1  Chemicals, drugs, enzymes, proteins and radiochemicals

Chemicals

All chemicals were of analytical reagent grade. The following compounds were purchased from the indicated companies: cAMP, GTP, GppNHp, phosphocreatine, phenylhydrazine-HCl (Sigma); ATP (Boehringer Mannheim); Norit GSX charcoal (BDH Biochemicals); N-succinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (Pierce chemical Co).

Drugs

(-)Adrenaline bitartrate, (-)isoprenaline bitartrate, (-)noradrenaline bitartrate, 3-isobutyl-1-methyl xanthine, indomethacin (Sigma); (±)H35/25 (Hassle); (±)atenolol, (±)practolol, (±)ICI 118.551, (-) and (+)propranolol (ICI Pharmaceuticals); (±)metoprolol (Astra); (±)salbutamol (Allen and Hanburys); (±)fenoterol (Boehringer Ingleheim); (±)betaxolol (Synthelabo); (±)procaterol (Otsuka), theophylline (BDH Biochemicals); (±)cyanopindolol, (-)pindolol (Sandoz); para amino benzyl carazolol (Dr KEJ Dickinson, Duke University).

Enzymes and proteins

Adenosine deaminase (240 U/mg protein), collagenase type II, bovine serum albumin fraction V, creatine phosphokinase (Sigma); crude bacterial collagenase (Worthington); demineralised bovine serum albumin (Organon Teknika).

Radiochemicals

(-)propyl-2-3-[\(^3\)H]dihydroalprenolol (62-97 Ci/mmole), 5'-8-[\(^3\)H]cyclic AMP (45-62 Ci/mmole), Na\(^{125}\) (carrier free), (Amersham International).
# Appendix 2  
## Krebs-bicarbonate Buffer

<table>
<thead>
<tr>
<th>Salt</th>
<th>Composition (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.0</td>
</tr>
<tr>
<td>KCl</td>
<td>4.7</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>1.2</td>
</tr>
<tr>
<td>CaCl$_2$.6H$_2$O</td>
<td>1.25</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.2</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>25.0</td>
</tr>
</tbody>
</table>

All the salts except CaCl$_2$ were dissolved in distilled water and the solution gassed with 95% O$_2$/ 5% CO$_2$ for 30 minutes prior to the addition of CaCl$_2$. 
Appendix 3  Reagents for free fatty acid assay

(A) Extraction medium (v/v)

2-propanol  40
n-heptane  20
0.5 M $\text{H}_2\text{SO}_4$  1

(B) Tetra n-butyl ammonium hydroxide solution: 20 mls.

2 ml  0.1 M tetra n-butyl ammonium hydroxide in 2-propanol/methanol
18 ml  2-propanol

(C) Thymolphtalein indicator

Thymolphtalein was dissolved in acetone and 100 μl added to each tube. The end point of titration was indicated by the appearance of a blue colouring in the assay tube.
Appendix 4 Reagents for cAMP assay

(A) Littlefields medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>250 mM</td>
</tr>
<tr>
<td>Tris</td>
<td>50 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>25 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>5 mM</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>6 mM</td>
</tr>
<tr>
<td>Theophylline</td>
<td>8 mM</td>
</tr>
</tbody>
</table>

pH is adjusted to 7.4 with HCl

(B) Charcoal separation

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norit GSX</td>
<td>0.25 g</td>
</tr>
<tr>
<td>BSA fraction V</td>
<td>0.13 g</td>
</tr>
</tbody>
</table>

50 mM Tris-HCl pH 7.4, 4 mM EDTA or
50 mM Na-acetate pH 6.0, 50 mls.

The suspension is stirred vigorously and kept at 4°C.
Appendix 5  Reagents for Lowry protein assay

A  2% NaCO$_3$ in 0.1 M NaOH

B$_1$  1% CuSO$_4$$\cdot$5H$_2$O
B$_2$  2% Sodium potassium tartrate

C  prepared fresh:
   100 ml solution A
   1 ml solution B$_1$
   1 ml solution B$_2$

D  Folin-Ciocalteau reagent diluted 1:3

E  BSA standards (12.5 - 300 µg/ml) prepared in same buffer as samples to be assayed.

Method

Add to 0.5 ml of standard, blank or sample, 2.5 ml of reagent C and allow to stand for 10 minutes. Then add 250 µl of Folin-Ciocalteau reagent, vortex and allow to stand for 30 minutes after which absorbance is measured.
Appendix 6 Two site curve fitting analysis

Inhibition curves which did not fit to a one site model were analysed using a computer iterative curve fitting program (NAG library, E04FBF, NAG Ltd, Oxford 1977) which arrived at the best fit parameters by the method of least sum of squares. The mathematical model for the two site, non-interacting system was:

\[ B = \frac{B_{\text{max}_1} [I]}{[I] + IC_{50_1}} + \frac{B_{\text{max}_2} [I]}{[I] + IC_{50_2}} \]

where \( B \) is the percentage of binding displaced, \([I]\) the concentration of inhibitor, \( IC_{50_1} , IC_{50_2} \) the concentration of ligand producing 50% inhibition at each site, \( B_{\text{max}_1} , B_{\text{max}_2} \) the proportions of each site. 'Goodness of fit' was estimated by use of the error sum of squares which was defined by:

\[ \text{error s.o.s} = \frac{\text{residual s.o.s}}{(M - N)} \] (from Richardson 1979)

where \( M \) is the number of observations and \( N \) the number of parameters.
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The pharmacology of the rat adipocyte beta-adrenoceptor has been examined using radioligand binding techniques as well as analysis of biochemical and functional effector responses. The ligand binding properties of beta-adrenoceptors on isolated adipocyte membranes have been compared to those in rat lung and whole fat pad membranes. This study demonstrated that binding sites could be identified on adipocytes which had all the characteristics expected of beta-adrenoceptors. Furthermore, inhibition of binding by agents selective for beta, or beta\textsubscript{2} adrenoceptors indicated that the receptor subtype present on adipocytes was predominantly beta\textsubscript{1}, whereas lung and whole fat pad membranes contained mainly beta\textsubscript{2} adrenoceptors.

The characteristics of the beta-adrenoceptor mediating lipolysis have also been examined. Evidence is presented which showed that inhibition of lipolysis stimulated through beta-adrenoceptors was inhibited by selective beta-adrenoceptor antagonists with a lower potency than that expected at beta\textsubscript{1} or beta\textsubscript{2} adrenoceptors. This suggested that an atypical beta-adrenoceptor was responsible for mediating the lipolytic response.

The apparently atypical beta-adrenoceptor has been further investigated by analysis of cAMP accumulation in whole cells and adenylate cyclase activity in membranes. These studies confirmed the results from lipolysis studies where it was again shown that antagonists had low potencies. In addition, the stereospecificity displayed by antagonist isomers was lower to that expected at typical beta-adrenoceptors.

In order to investigate whether typical beta\textsubscript{1} and atypical beta-adrenoceptors could be separated, an irreversible beta-adrenoceptor antagonist has been used to selectively inhibit typical beta-adrenoceptors. The results demonstrated that whereas beta\textsubscript{1} binding sites could be irreversibly blocked, the beta-adrenoceptor-stimulated adenylate cyclase activity was unaffected. This further suggested that the receptors identified by binding studies are not the same as those mediating the lipolytic response to catecholamines.