ADRENOCEPTOR STATUS IN BRONCHIAL ASTHMA

Thesis submitted for the

Degree of

DOCTOR OF PHILOSOPHY

by

Saad Jaber Titinchi

Department of Pathological Biochemistry

Western Infirmary

University of Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Publications

Trope, G.E., Clark, B. and Titinchi, S.J.S. (1982) Identification of beta-adrenergic receptors in the pigmented mammalian iris-ciliary body diaphragm.
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XI International Congress of Allergology and Clinical Immunology. London 17 - 22 October 1982.

Titinchi, S., Al-Shamma, M., Patel, K.R., Kerr, J.W. and Clark, B. Circadian variation in number and affinity of $beta_2$ adrenoceptors in lymphocytes of asthmatic patients.
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Titinchi, S., Al-Shamma, M., Patel, K.R., Kerr, J.W. and Clark, B. (1984) Circadian variation in number and affinity of $beta_2$-adrenoceptors in lymphocytes of asthmatic patients.
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Clark, B., Kelman, A.W., Trope, G.E. and Titinchi, S.J. (1984) RECFIT: A microcomputer-based nonlinear regression curve-fitting package for the quantitative resolution of beta-adrenoceptor subtypes and estimation of nonspecific binding.
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SUMMARY

The aim of this thesis was to investigate a long-standing hypothesis (Szentivanyi, 1968) that the underlying aetiology of atopic diseases (particularly bronchial asthma) may have a component related to beta-adrenoceptor defects. Later (Szentivanyi, 1979) this hypothesis was modified to include a possible imbalance of the alpha:beta adrenoceptor ratio.

Patients with asthma have marked circadian variation in bronchomotor tone, often demonstrated as the "morning dip" in FEV_1. In terms of the above hypothesis, this could result from changes in adrenoceptor kinetics. Equally, the "dip" might be related to other humoral circadian rhythms.

Owing to problems in obtaining human lung tissue this study, in accord with many others, used human lymphocytes as the target tissue for adrenoceptor investigations. $Beta_2$-adrenoceptor kinetics were studied using radioligand binding techniques in both normals and asthmatic patients (extrinsic) at 0800 h and 1800 h. In addition, the effects of orally administered salbutamol were followed at similar times. Any disparity in the adrenoceptor kinetics of the control/asthmatic groups would support Szentivanyi's hypothesis.

A significant circadian variation in $beta_2$-adrenoceptor number was observed in both groups. The expected down-regulation of the receptors occurred in both groups on administration of salbutamol, but the circadian rhythm persisted although its magnitude was reduced. Both groups appeared to compensate the down-regulation by

increasing receptor affinity. At no point in the study were the results for the two groups statistically significantly different.

In a second study, both $beta_2$- and $alpha_2$-adrenoceptor kinetics were studied under similar conditions to the first study. In addition, the ratio of $alpha_2$:$beta_2$ adrenoceptors was followed to see if any shifts occurred, particularly in the asthmatic group. The $beta_2$ results were statistically similar to the first study. The kinetics of the $alpha_2$-adrenoceptors remained constant throughout the study, i.e. they did not show a circadian variation and were not affected by salbutamol administration. Although there was a significant difference in the $alpha_2$:$beta_2$ ratio at 0800 h and 1800 h, this related solely to the circadian variation and/or down-regulation of the $beta_2$-adrenoceptors.

In conclusion, this study produces no evidence to support Szentivanyi's hypothesis. Receptor function in the asthmatic groups was remarkably similar to that of the control group. Neither was there any evidence for a beta to alpha shift in asthmatic patients.

INTRODUCTION

1. DEFINITION OF ADRENOCEPTORS

The autonomic nervous system is the part of the nervous system concerned with the regulation of visceral activities and is comprised of the nerve cells and fibres that are distributed to smooth muscle, cardiac muscle, and glands. The efferent autonomic nervous system is divided, on the basis of the anatomy of the outflow of nerves from the central nervous system, into two portions designated sympathetic and parasympathetic (Fig. 1).

The term " sympathetic " is an anatomical term referring to the neural pathways that originate from neurons with their cell bodies in the thoracolumbar segments of the spinal cord. The parasympathetic pathways originate from neurons that have their cell bodies in the midbrain, the medulla, or the sacral portion of the spinal cord.

The sympathetic pathways consist of three major components - preganglionic neurons, postganglionic neurons and responsive tissues. The preganglionic neurons, which originate in the spinal cord, synapse with the postganglionic neurons in the sympathetic trunk ganglia or in specialised collateral ganglia. The postganglionic neurons then terminate at nerve endings on the responsive organs (Fig. 2).

Most, but not all, postganglionic sympathetic fibres release catecholamines from their endings on the responsive organs and are designated as adrenergic neurons.

Thus, the term "adrenergic" refers to the neurons that release a particular type of transmitter. The end organ receptors for adrenergic transmitters have been termed "adrenoceptors", and the responses elicited by stimulation of adrenergic nerves are termed "adrenergic responses".

FIG. 1

Classification of autonomic responses. The division between parasympathetic and sympathetic responses is based on the anatomical location of the nerve cell bodies. Adrenergic and cholinergic responses are distinguished by the chemical nature of the neurotransmitter released at the end organ. Beta and alpha responses are distinguished by the pharmacological specificity of the responses (Williams and Lefkowitz, 1978a).

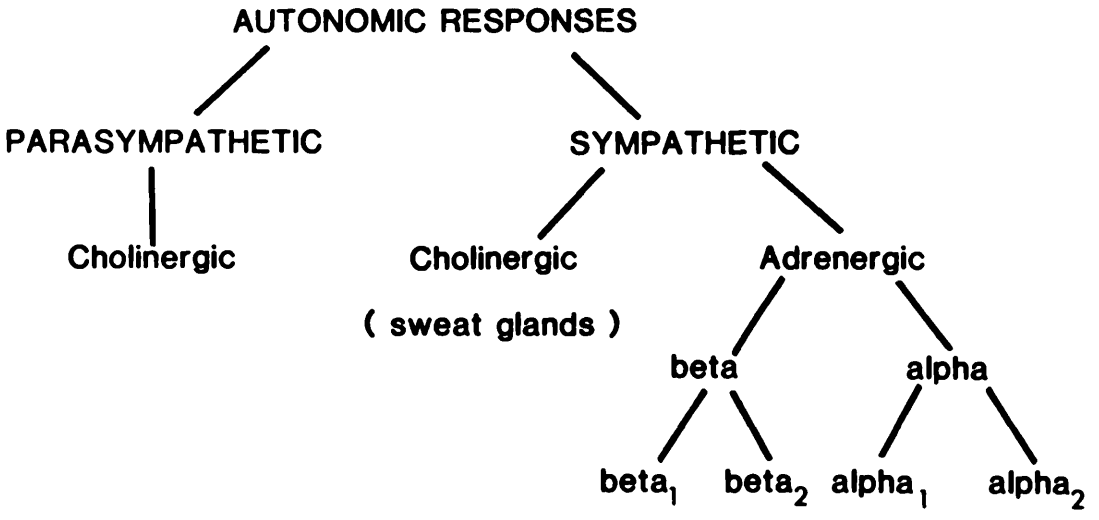
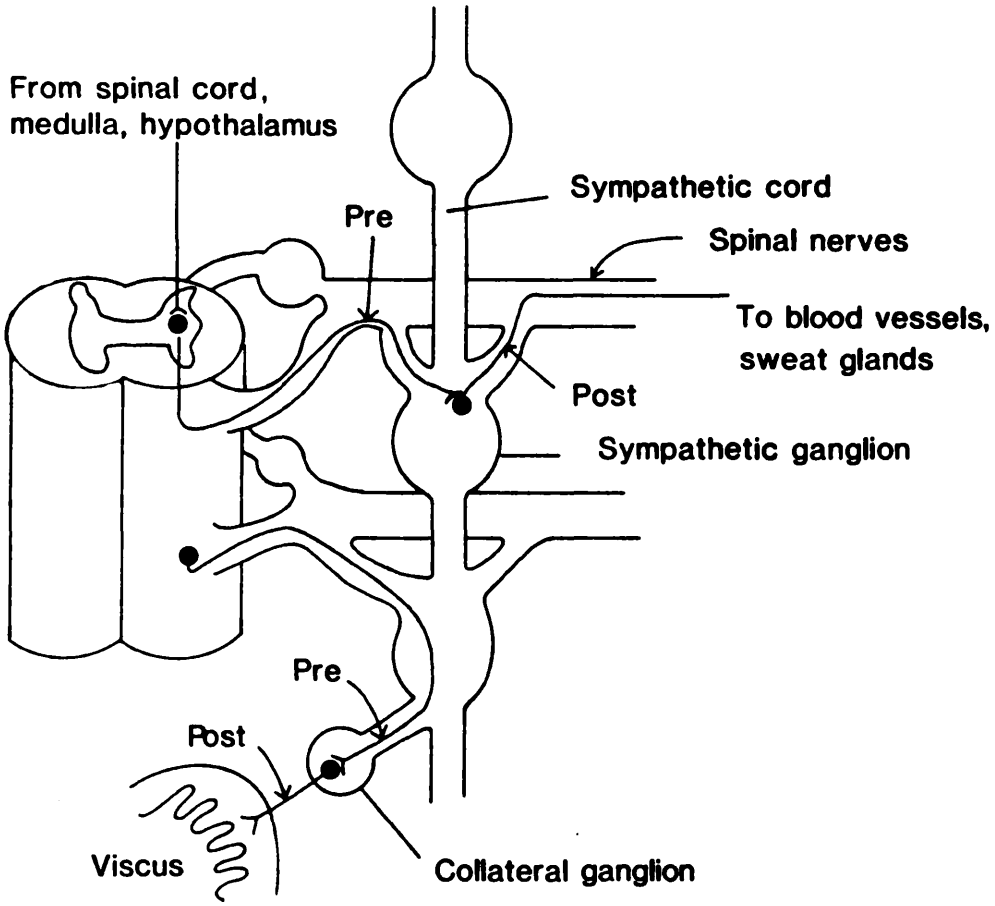

AUTONOMIC RESPONSES
PARASYMPATHETIC
SYMPATHETIC
Cholinergic
Cholinergic
(sweat glands)
Adrenergic
beta
alpha
beta1
beta2
alpha1
alpha2

FIG. 2

Autonomic nervous system. Pre, preganglionic neuron; Post, postganglionic neuron (Ganong, 1981).

SYMPATHETIC DIVISION



In vivo, these responses can be elicited by:

(a) Catecholamine transmitters (especially noradrenaline) released at the nerve ending as a result of adrenergic nerve stimulation.

(b) Catecholamines, especially adrenaline, released into the circulation from the adrenal medulla.

(c) Parenterally administered catecholamines or related adrenergic agents.

In vitro, adrenergic responses can be elicited by exogenous catecholamines in denervated or in innervated preparations, thereby indicating that the nerve ending per se is not required for the response. The specialised part of the effector cell through which adrenergic compounds act to evoke a characteristic response is defined as the adrenoceptor.

2. PHARMACOLOGICAL CLASSIFICATION OF ADRENOCEPTORS

The biological effects of catecholamines are among the most diverse and important of any known class of chemical agents. Thus it is not surprising that over the past several decades much research has been directed towards the goal of a better understanding of the physiological and biochemical basis for their actions.

An important concept that has helped direct a great deal of this effort was first put forward by Ahlquist (1948) who proposed, on the basis of a series of physiological experiments, that there were two distinct types of receptors for catecholamines. He called these alpha- and beta-adrenoceptors. These receptors were defined in terms of the relative potency series for several catecholamines and related drugs for stimulating a variety of physiological responses.

Alpha-adrenoceptors, such as those that mediate smooth muscle contraction and platelet aggregation, were characterised by the potency series adrenaline > noradrenaline > isoprenaline. In contrast for the beta-adrenoceptor responses, which include smooth muscle relaxation, the positive inotropic and chronotropic cardiac responses, and metabolic responses such as lipolysis, an almost opposite relative potency series was found with isoprenaline > adrenaline > noradrenaline (Fig. 3).

Although very few adrenergic antagonists were available at the time that Ahlquist did his original studies, he did find that the naturally occurring ergot alkaloids were capable of blocking the alpha-adrenergic responses whereas the beta-adrenergic responses were unaffected. Similarly, haloalkylamines such as phenoxybenzamine could block alpha- but not beta-adrenergic responses. This classification scheme was not well accepted until some years later when specific beta-adrenergic antagonists, such as propranolol, were developed.

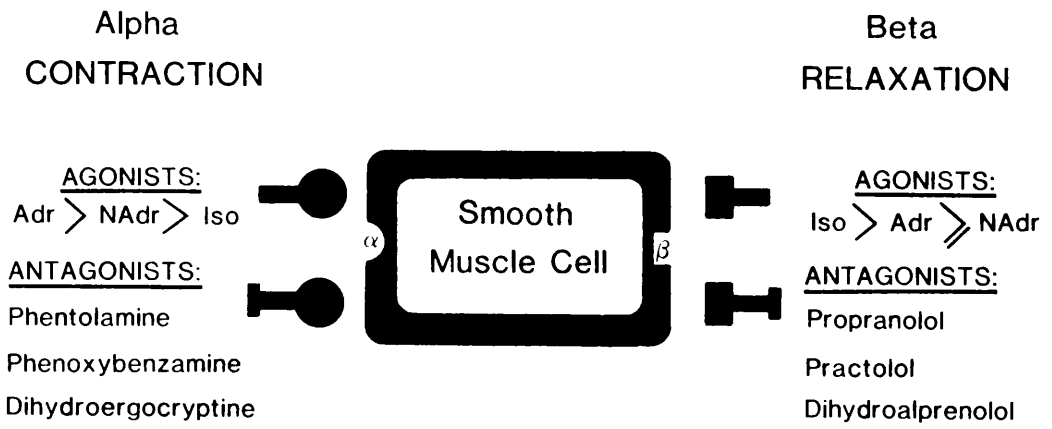
As more was learned about the adrenoceptors from physiological studies, it became clear that although the original classification remained useful, it required further refinement. In particular, there appeared to be subtypes of both alpha- and beta-adrenoceptors. Two major subtypes of each of the adrenoceptors have been described so far.

2.1. BETA-ADRENOCEPTOR SUBTYPES

In 1967, Lands and co-workers identified two subtypes of beta-adrenoceptors which were designated "beta$_1$" and "beta$_2$". These subtypes were defined by comparing the potency of adrenaline and noradrenaline at various beta-adrenoceptors. In the first subtype,

FIG. 3

Adrenergic responses of a smooth muscle cell. Alpha- and beta-adrenoceptors are indicated by the indentation on the outer surface of the cell. Adr=adrenaline; NAdr=noradrenaline; Iso=isoprenaline (Lefkowitz, 1979).


Alpha
CONTRACTION
AGONISTS:
Adr > NAdr > Iso
ANTAGONISTS:
Phentolamine
Phenoxybenzamine
Dihydroergocryptine
α
Smooth
Muscle Cell
β
Beta
RELAXATION
AGONISTS:
Iso > Adr >> NAdr
ANTAGONISTS:
Propranolol
Practolol
Dihydroalprenolol

$beta_1$, the catecholamines adrenaline and noradrenaline are approximately equipotent in eliciting a response, whereas in the second subtype, $beta_2$, adrenaline is considerably more potent than noradrenaline. Thus, the potency series for $beta_1$-adrenoceptors is isoprenaline > adrenaline = noradrenaline; whereas for the $beta_2$-adrenoceptors it is isoprenaline > adrenaline > noradrenaline. The group of $beta_1$ responses includes the positive inotropic effect on the heart, the lipolytic response of adipose tissue and the inhibition of intestinal motility. Typical $beta_2$ responses include bronchodilation, glycogenolysis in skeletal muscle, and relaxation of the uterus. Although several beta-adrenoceptor blocking drugs (e.g., propranolol, practolol and dihydroalprenolol) have been developed over the years which may have slightly greater affinity for one or the other subtype, such as practolol that have a somewhat greater affinity for $beta_1$-adrenoceptors than for $beta_2$-adrenoceptors, highly selective beta-adrenergic antagonists have not yet been developed (Fig. 4).

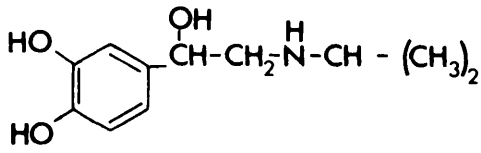
2.2. ALPHA-ADRENOCEPTOR SUBTYPES

More recently, subtypes of alpha-adrenoceptors have been described as well. The initial development of this concept occurred in the early 1970's (Langer, 1974) and was based on the observation that noradrenaline and other catecholamine agonists were capable of mediating feed back inhibition of noradrenaline release from sympathetic nerve terminals. This feed back mechanism appeared to be an alpha-adrenoceptor - mediated process and was blocked by typical alpha-adrenergic antagonists. However, as additional work was done, it became clear that the detailed pharmacological properties of these so-called "presynaptic" alpha-adrenoceptors was

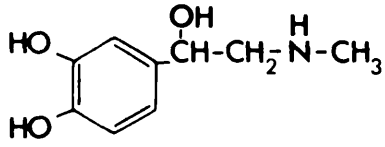
FIG. 4

Structures of some beta-adrenergic agonists and antagonists.

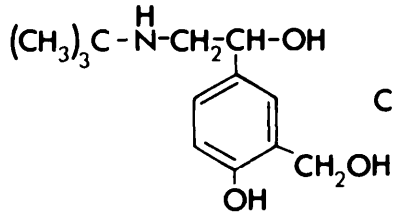
AGONISTS



Isoprenaline

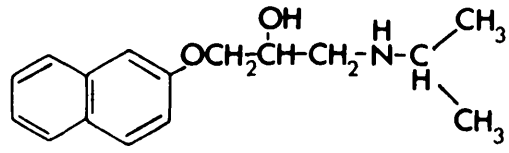


Adrenaline

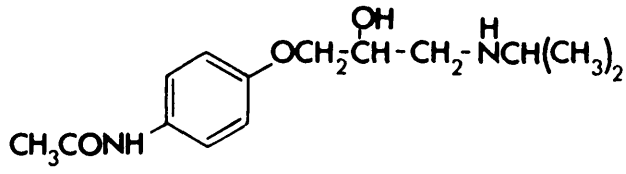


Salbutamol

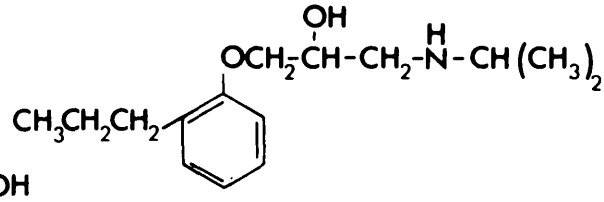
ANTAGONISTS



Propranolol



Practolol



Dihydroalprenolol

somewhat different from that of the typical "postsynaptic" alpha-adrenoceptors mediating the usual physiological effects of catecholamines (Langer, 1974, 1976; Borowski et al, 1977; Starke, 1977; Westfall, 1977).

A wide variety of drugs have been found to have selectively greater potency at either presynaptic or postsynaptic alpha-adrenoceptors, in much the same way that some beta-adrenergically active compounds have selectively greater potency at either $beta_1$- or $beta_2$-adrenoceptors.

A further evolution of this scheme has occurred in the past few years as it has become increasingly clear that alpha-adrenoceptors possessing many of the characteristics of the "presynaptic" receptors can also be found in "postsynaptic" locations. Thus, typical "presynaptic" receptors have been demonstrated on human platelets (Hoffman et al, 1979), in several cultured cell lines (Sabol and Nirenberg, 1979), and in frog skin (Pettinger, 1977). Thus, the terminology of "postsynaptic and presynaptic" should be replaced by the designations "$alpha_1$"- and "$alpha_2$"-adrenoceptors, respectively (Berthelsen and Pettinger, 1977).

$Alpha_1$-adrenoceptors include typical postsynaptic alpha-adrenoceptors mediating smooth-muscle contraction. $Alpha_2$-adrenoceptors include not only all known presynaptic autoregulatory alpha-adrenoceptors but also some less typical postsynaptic receptors existing on, for example, human platelets (Hoffman et al, 1979; Wood et al, 1979). Thus far, no succinct definition of $alpha_1$- and $alpha_2$-adrenoceptors analogous to that used in defining $beta_1$- and $beta_2$-adrenoceptor subtypes has emerged.

Many drugs have appeared to be putatively $alpha_1$ or $alpha_2$ selective in a number of isolated tissues from various animals.

However, in many cases generalities about the selectivity of a drug are not possible because of the great variability of responses to it from one tissue to another. For agonists it has been suggested that methoxamine and phenylephrine are alpha$_1$ selective, that clonidine is alpha$_2$ selective, and that adrenaline and noradrenaline, having approximately equal potency at alpha$_1$- and alpha$_2$-adrenoceptors, are nonselective (Berthelsen and Pettinger, 1977). Among alpha-adrenergic antagonists, prazosin (Cambridge et al, 1977; Cavero et al, 1977; Doxey et al, 1977) and phenoxybenzamine (Doxey et al, 1977) are considered alpha$_1$ selective, yohimbine (a plant alkaloid) alpha$_2$ selective (Doxey et al, 1977), and phentolamine nonselective (Cavero et al, 1977), (Fig. 5). These designations of alpha$_1$ and alpha$_2$ selectivity have generally been determined in experiments in which effects on alpha$_1$-adrenoceptors were determined in terms of smooth - muscle contraction and effects on alpha$_2$-adrenoceptors determined through assessment of noradrenaline release from nerve terminals.

At present, one of the most useful and widely applied criteria for distinguishing pharmacologically between alpha$_1$- and alpha$_2$-adrenoceptors is the relative potencies of the alpha antagonists prazosin and yohimbine. Prazosin is much more potent than yohimbine at alpha$_1$-adrenoceptors, whereas yohimbine is generally somewhat more potent at alpha$_2$-adrenoceptors (Hoffman et al, 1979).

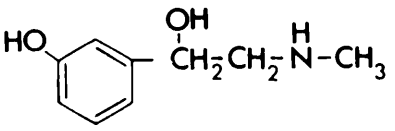
3. HISTORICAL PERSPECTIVE OF RADIOLIGAND BINDING STUDIES

The first receptors studied by radioligand binding techniques were the polypeptide hormone and nicotinic cholinergic receptors. Prior to the use of radioactive ligands, the approach to the study of hormone and neurotransmitter receptors was to infer characteristics

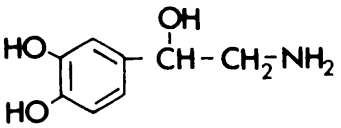
FIG. 5

Structures of some alpha-adrenergic agonists and antagonists.

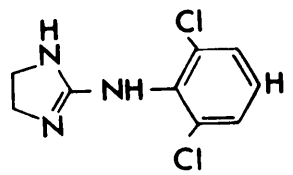
AGONISTS



Phenylephrine

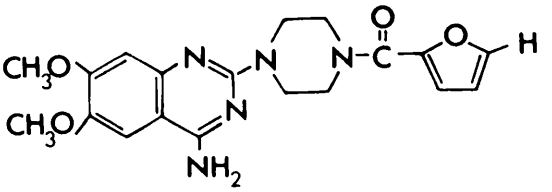


Noradrenaline

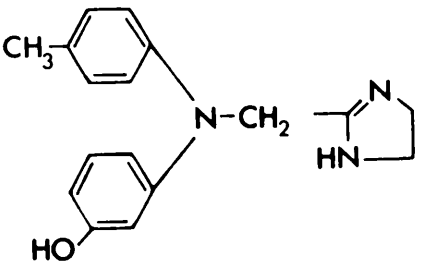


Clonidine

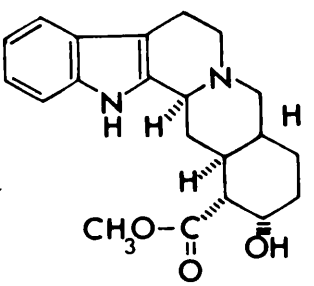
ANTAGONISTS



Prazosin



Phentolamine



Yohimbine

of the receptors from an examination of the physiological response to the active agents. The specificity of the hormone - receptor interaction was delineated by measuring the biological response to structural analogs of the hormone. Alterations of the biological responses to hormones induced by pathological or pharmacological manipulations were often attributed to changes in the number or function of receptors, but these changes could never be documented. Hence the interpretation of many early physiological studies of hormone receptors was limited by the necessity of making observations of the biological response, a response that is several steps distal to the actual interaction of hormone with receptor. It was not until 1969 that the interaction of a hormone with its receptor was studied directly. In that year two groups, using [^{125}I]adrenocorticotrophic hormone (ACTH) and [^{125}I]angiotensin, respectively, demonstrated that radioactively labelled hormones could be used to study directly the interaction of hormones with specific membrane binding sites (Lefkowitz et al, 1970; Lin and Goodfriend, 1970). In the next few years the approach was extended to the study of a variety of peptide hormone and nicotinic cholinergic receptors as reviewed elsewhere (Kahn, 1975; Lefkowitz et al, 1976a; Karlin, 1974).

3.1. BETA-ADRENOCEPTORS

Although extensive pharmacological studies of adrenoceptors had been performed for three decades, direct binding studies of adrenoceptors lagged behind those of polypeptide and cholinergic receptors. During the 1950's and 1960's, a wealth of pharmacological data on adrenergic responses appeared. Studies such as those of Ariens (1967) documented in detail the structure -

activity relationships responsible for eliciting adrenergic responses. The apparently competitive nature of the antagonism of beta-adrenergic responses by antagonists such as dichlorisoprenaline and propranolol was also demonstrated.

The studies of Ahlquist (1948) had demonstrated the existence of at least two distinct types of adrenoceptors. Despite these advances in the understanding of adrenergic pharmacology, by the late 1960's the limitations of classic pharmacological methods were becoming apparent, as was the fact that further information about the receptors could only be obtained by new techniques, such as radioligand binding studies.

One of the earliest attempts at using a radioactive compound to study beta-adrenoceptors was that of Potter (1967). It was found that the radioactive antagonist [^{3}H]propranolol bound to pieces of atrial muscle *in vitro* and to subcellular fragments derived from such tissue. However, under the conditions utilized, the binding appeared to be nonsaturable and did not have the characteristics one would expect of binding to beta-adrenoceptors (see section 6). Subsequently a number of investigators attempted to use [^{3}H]propranolol and [^{14}C]propranolol to perform binding studies on fractions derived from brain (De Robertis and Fiszer de Plazas, 1969), heart (Vatner and Lefkowitz, 1974), bronchial tissue (Furchgott *et al*, 1973), and other tissues (Huunan-Seppala, 1972). In none of these studies were sites labelled that had the appropriate characteristics of beta-adrenoceptors (see section 6). With hindsight, it is now clear that the specific radioactivity of [^{14}C]propranolol was much too low to have permitted accurate identification of the small number of beta-adrenoceptors present in most tissues.

During the same period (1967 - 1974) several groups began to explore the possibility of using radiolabelled beta- adrenergic agonists such as tritiated isoprenaline, adrenaline, and noradrenaline to identify the receptors. The binding of these radioactively labelled catecholamines was studied in membrane fractions derived from liver (Tomasi et al, 1970; Marinetti et al, 1969), heart (Lefkowitz and Haber, 1971), adipose tissue (Jarett et al, 1974), erythrocytes (Bilezikian and Aurbach, 1973) and other tissues (Fiszer de Plazas and De Robertis, 1972).

The binding sites labelled in these studies also failed to demonstrate the binding characteristics expected of beta-adrenoceptors. The binding did not demonstrate stereospecificity, and the competitive beta-adrenergic antagonist propranolol inhibited binding only at very high, non-physiological concentrations. In addition, compounds such as catechol, which are devoid of beta-adrenergic biological activity, significantly inhibited the binding. Thus it became increasingly apparent that in these studies the sites labelled by catecholamines were not beta-adrenoceptors.

It was proposed that this catecholamine binding was to the catechol - O - methyl transferase system (Cuatrecasas et al, 1974) or to a catecholamine oxidizing system. However, the exact nature of these "nonreceptor" binding sites remains unknown. Some evidence exists for the covalent binding of the labelled catecholamines to the receptor sites as a result of an oxidative process (Maguire et al, 1974; Wolfe et al, 1974). Such binding cannot be used to characterise beta-adrenoceptors. Thus by 1974, there had been no reports of successful identification of beta-adrenoceptors using radioactive ligands. In that year, three

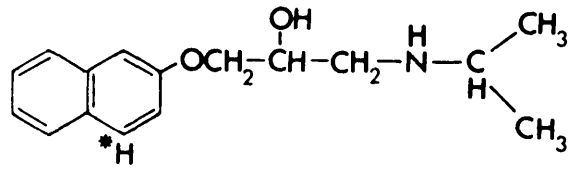
groups independently developed methods that appeared to permit the direct study of beta-adrenoceptors. In each case the radioligand used was a potent beta-adrenergic antagonist. Levitzki et al (1974) used [^{3}H]propranolol, Lefkowitz et al (1974) used (-) [^{3}H]dihydroalprenolol ([^{3}H]DHA), and Aurbach et al (1974) used [^{125}I]hydroxybenzylpindolol ([^{125}I]HYP), (Fig. 6). Latterly other groups have worked with beta-adrenoceptor agonists and for convenience, the differing approaches will be discussed separately.

3.1.1. Radioligand studies using beta-adrenoceptor antagonists

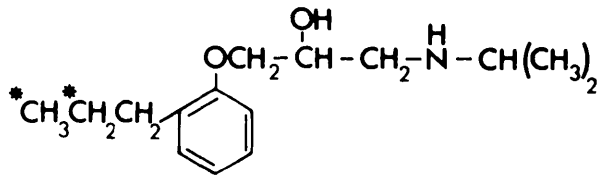
During the following two years the technique using radioactively labelled beta-adrenergic antagonists was applied to a variety of catecholamine-sensitive systems (Williams et al, 1976a,b; Mukherjee et al, 1975a; Alexander et al, 1975a,b; Zatz et al, 1976; Maguire et al, 1976a; Bylund and Snyder, 1976) and also proved useful in studying solubilization of the receptor (Caron and Lefkowitz, 1976), site - site interactions among receptors (Limbird and Lefkowitz, 1976), alterations of receptor number caused by a variety of physiological manipulations (Mukherjee et al, 1975b; Mickey, et al, 1975; Mukherjee, et al, 1976; Mukherjee and Lefkowitz, 1976a; Kebabian et al, 1975; Harden et al, 1976; Williams et al 1977a; Shear et al, 1976), alteration of the receptor affinity caused by nucleotides (Lefkowitz et al, 1976b), the identification of ectopic beta-adrenoceptors (Williams et al, 1977b), and the genetic regulation of receptors in various cell clones (Maguire et al, 1976a). The success of studies using radioactively labelled antagonists to identify beta-adrenoceptors can be attributed to a variety of factors, including the availability of high-affinity antagonists labelled to high specific

FIG. 6

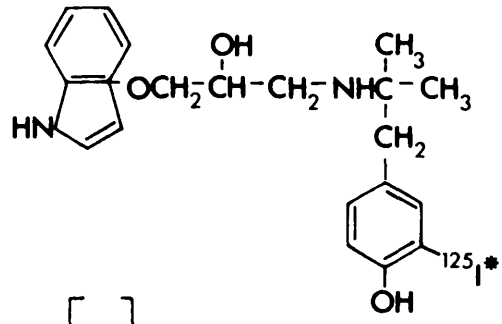
Structures of radioactively labelled beta-adrenergic antagonists. Sites of specific radioactive labelling are indicated by an asterisk.



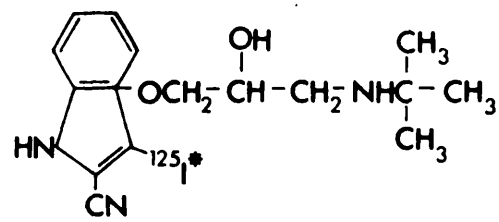
[^{3}H] Propranolol



[^{3}H] Dihydroalprenolol



[^{125}I] Iodohydroxybenzylpindolol



[^{125}I] Iodocyanopindolol

radioactivity and an increased awareness of the appropriate technical conditions for detection of beta-adrenoceptors.

From 1974 to 1976 the technical aspects of measuring binding were improved to such an extent that the assays could be routinely performed using a wide variety of tissues. Hence by 1976 the use of radioactively labelled antagonists had become well established as a valuable tool for the direct study of beta-adrenoceptors.

Two antagonist ligands have been widely used, namely, (-) [^{3}H]DHA and (±)[^{125}I]HYP. Both ligands have been validated in different tissues and appear to give fairly comparable results (Insel and Stoolman, 1978). (-)[^{3}H]DHA has the advantages of being a pure (-) isomer compound, having a very long physical half-life (12 years) and is not readily susceptible to radioactive decomposition. Its binding has a high specificity which leads to a low non-specific binding. Its specific radioactivity however is not very high (90 - 120 Ci/mmol) and it is necessary to use large amounts of membrane proteins bearing the beta-adrenoceptors to get measurable results.

In contrast, [^{125}I]HYP has the advantage of higher specific radioactivity of about 2200 Ci/mmol and higher affinity for beta-adrenoceptors than its [^{3}H] counterparts. These properties allow the use of much smaller amounts of protein in binding studies. However, as a consequence of the phenethylamine side chain (Fig. 6), [^{125}I]HYP binds to alpha-adrenoceptors (Aggerbeck *et al*, 1979) and 5-HT (5-hydroxytryptamine) receptors (Dickinson *et al*, 1981). Furthermore, depending on the reaction conditions, the iodination procedure for hydroxybenzylpindolol leads to the formation of 2 different iodination products (Bearer *et al*, 1980), moreover [^{125}I]HYP is a racemic compound.

Neither compound discriminates between the $beta_1$- and $beta_2$-adrenoceptors; i.e., in a membrane or cell suspension containing both $beta_1$- and $beta_2$-adrenoceptor sites these ligands would be expected to label both with equal affinity (Hancock et al, 1979; Minneman et al, 1979).

Recently, an alternative ligand [^{125}I]cyanopindolol ([^{125}I]CYP) has been used to study beta-adrenoceptors (Engel, 1980; Davies and Lefkowitz, 1983). [^{125}I]CYP has the advantage of higher specificity for beta-adrenoceptors due to a lack of affinity for alpha-adrenoceptors and 5-HT receptors as compared with [^{125}I]HYP (Engel, 1980). Unfortunately [^{125}I]CYP was unavailable commercially at the commencement of this study.

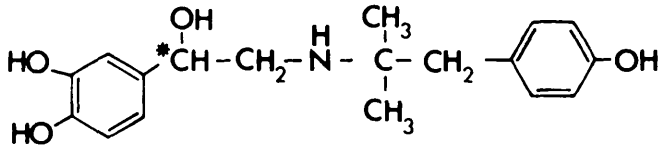
3.1.2. Radioligand studies using beta-adrenoceptor agonists

At the same time, two beta-adrenergic agonists have been used as radioligands, i.e. [^{3}H]hydroxybenzylisoprenaline (Lefkowitz and Williams, 1977) and [^{3}H]adrenaline (U'Prichard et al, 1978), (Fig. 7). (±)[^{3}H] hydroxybenzylisoprenaline has a higher affinity for $beta_2$- than $beta_1$-adrenoceptors (Lefkowitz and Williams, 1977; Williams and Lefkowitz, 1977c). It is approximately equipotent with isoprenaline at $beta_1$-adrenoceptors and generally about 10 times more potent than isoprenaline at $beta_2$-adrenoceptors.

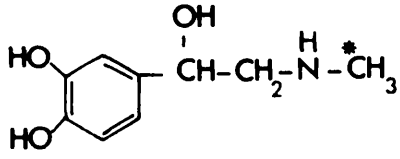
Interpretation of agonist binding to adrenoceptors, however, is much more complicated than interpretation of antagonist binding. Several points need to be developed in order to provide a clear picture of the significance of agonist binding. It now appears that a fundamental property of agonists acting at adenylate cyclase - coupled beta-adrenoceptors is the ability to induce or stabilise a high affinity form of the beta-adrenoceptor (Kent et al, 1980).

FIG. 7

Structures of radioactively labelled beta-adrenergic agonists. Sites of specific radioactive labelling are indicated by an asterisk.



[^{3}H] Hydroxybenzylisoprenaline

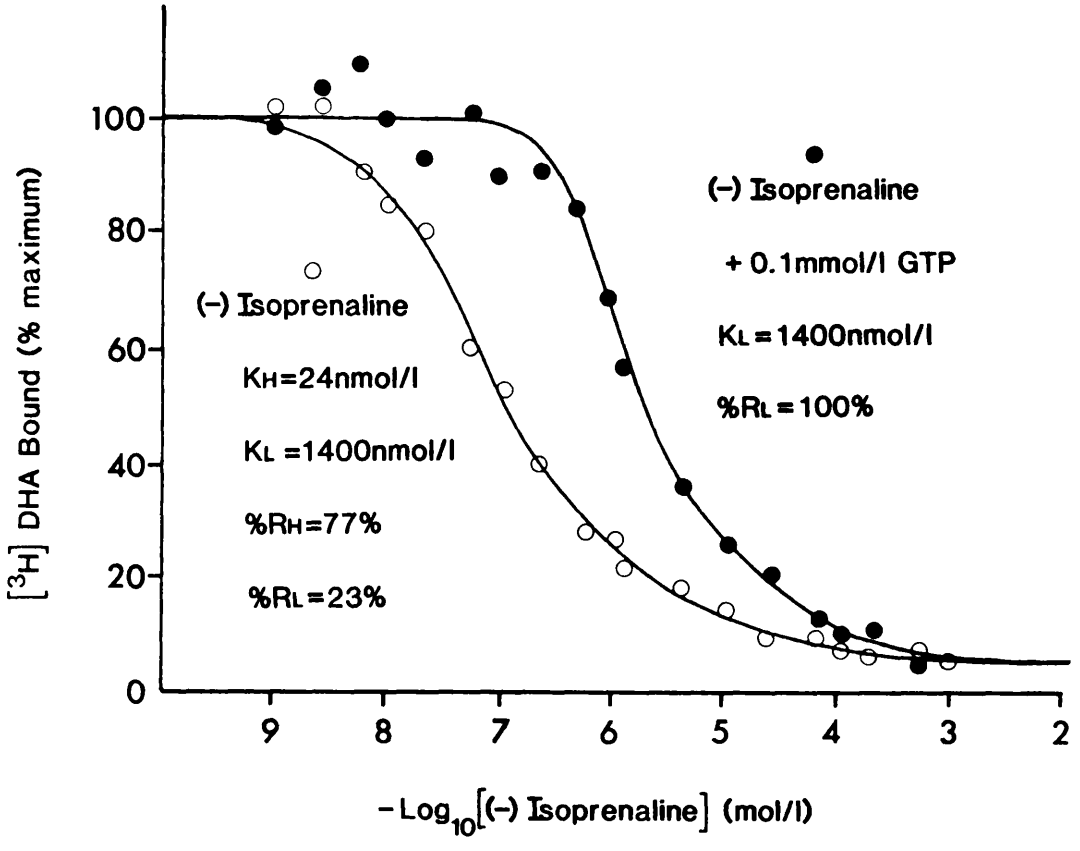


[^{3}H] Adrenaline

This form of the receptor does not display preferential affinity for antagonistic drugs. Thus, in the presence of a beta-adrenergic agonist, the beta-adrenoceptors will exist in two affinity states, one of higher and one of lower affinity. It has been shown that the ability to form the high affinity state is directly correlated with the intrinsic activity or efficacy of the beta-adrenergic drug (Kent et al, 1980). Guanine nucleotides such as guanosine triphosphate (GTP) which are virtually required for catecholamine stimulation of adenylate cyclase (Williams and Lefkowitz, 1977c) appear to mediate a transition of the high affinity state of the receptor to its low affinity state (Kent et al, 1980; Lefkowitz et al, 1976b; Maguire et al, 1976b; Malbon et al, 1978). Thus, in the presence of guanine nucleotides, all of the beta-adrenoceptors in the presence of agonist will be in the lower of the two affinity forms. This can be demonstrated experimentally as a nucleotide - promoted rightward shift in an agonist displacement curve of a radioligand such as [^{3}H]DHA (Fig. 8) or [^{125}I]HYP. In addition to shifting to the right, the competition curve steepens since now, rather than two affinity states of the receptor, in the presence of nucleotide there is only the one of lower affinity. Antagonist displacement curves are not shifted by nucleotides since they label only a single affinity state of homogeneous affinity (Kent et al, 1980) in the presence or absence of nucleotides. In general, for full agonists the affinity of the higher affinity state is 50 to 100 times higher than that of the lower affinity state (Kent et al, 1980). Thus, when an agonist ligand such as [^{3}H]hydroxybenzylisoprenaline is used, there is preferential, if not exclusive, labelling of only the high affinity state of the receptor. The low affinity state, in general, cannot be directly

FIG. 8

Computer-modelled competition curve for inhibition of [^{3}H]DHA binding by (-)isoprenaline in the presence and absence of guanosine triphosphate (GTP). Frog erythrocyte membranes were incubated with 2.1nmol/l [^{3}H]DHA in competition with increasing concentrations of the beta-adrenergic agonist (-)isoprenaline, in both the absence and presence of 0.1mmol/l GTP. K_H and K_L denote the dissociation constants for binding of the drug to the high-affinity and low-affinity forms of the receptor, respectively, and R_H and R_L the proportions of the receptor in each form (Kent *et al*, 1980).


[3H] DHA Bound (% maximum)
100
80
60
40
20
0
9
8
7
6
5
4
3
2
-Log10[(-) Isoprenaline] (mol/l)
(-) Isoprenaline
KH=24nmol/l
KL =1400nmol/l
%RH=77%
%RL=23%
(-) Isoprenaline
+ 0.1mmol/l GTP
KL=1400nmol/l
%RL=100%

determined with a ligand such as [^{3}H]hydroxybenzylisoprenaline. It should be stressed that since only the high affinity form of the receptors can be directly labelled with low concentrations of an agonist such as [^{3}H]hydroxybenzylisoprenaline, the total number of binding sites observed with an agonist such as [^{3}H]hydroxybenzylisoprenaline may be fewer than that observed with an antagonist ligand such as [^{3}H]DHA. Moreover, the relationship of these two values will depend critically on variables such as, for example, the concentration of endogenous guanine nucleotides contaminating a membrane preparation. Thus, the greater the amount of nucleotides present, the greater the extent to which high affinity state receptors will be converted to low affinity receptors, and the greater the discrepancy will become between the number of beta-adrenoceptors labelled with [^{3}H] hydroxybenzylisoprenaline as opposed to an antagonist ligand (Kent et al, 1980). Thus, with beta-adrenergic agonist ligands such as [^{3}H]hydroxybenzylisoprenaline or [^{3}H]adrenaline, it must be borne in mind that only a fraction of the total beta-adrenoceptor pool may be labelled. Theoretically, the lower affinity state of the beta-adrenoceptors could be labelled with high concentrations of [^{3}H]hydroxybenzylisoprenaline, but in practice this is not possible to carry out experimentally because of problems with non-specific binding at very high concentrations of [^{3}H] hydroxybenzylisoprenaline.

In purified plasma membranes from frog erythrocytes, in which endogenous guanine nucleotide contamination is presumably quite low, up to 90 to 95% of the $beta_2$-adrenoceptors that can be labelled with [^{3}H]DHA are also labelled with [^{3}H]hydroxybenzylisoprenaline (Kent et al, 1980). However, in

less purified preparations only 60 to 65% of the sites are labelled with the agonist (Kent et al, 1980).

3.2. ALPHA-ADRENOCEPTORS

Prior to 1976 there had been no reports of the successful identification of alpha-adrenoceptors by radioligand binding studies. A number of early studies had demonstrated the binding of alpha-adrenergic radioligands to high - capacity low - affinity sites that did not have the specificity of alpha- adrenoceptors (Moran et al, 1967; Yong and Nickerson, 1973; Terner et al, 1971; Fiszer de Plazas and De Robertis, 1972). In retrospect, these studies were probably unsuccessful for several reasons:

1. In general, these attempts used radioactively labelled ligands of relatively low specific radioactivity (25 to 50 mCi/mmol) in comparison with those currently available (30 - 90 Ci/mmol). Thus in order to identify the very small number of true receptors, high concentrations of ligand were required. At these high concentrations, non-specific binding of ligands to high-capacity low-affinity nonreceptor sites presumably obscured binding to the small number of physiological high - affinity receptors.
2. In early attempts to identify alpha-adrenoceptors by radioligand binding, intact strips of tissue were used as a source of receptors (Moran et al, 1967; Yong and Nickerson, 1973). The use of membrane preparations probably allows the attainment of higher receptor site concentrations and may also eliminate some of the drug and hormone uptake processes that occur in intact tissue.

The first reported successful identification of alpha-adrenoceptors

by radioligand binding techniques was in 1976 (Williams and Lefkowitz, 1976; Williams et al, 1976c; Williams and Lefkowitz, 1977a,b). In these studies, [^{3}H]dihydroergocryptine, a tritiated alpha-adrenergic antagonist with a high specific radioactivity (23 Ci/mmol), was used to label binding sites in membrane preparations from rabbit uterus, a tissue that contracts on alpha-adrenergic stimulation. This ligand was subsequently used to identify alpha-adrenoceptors in the rat parotid gland (Strittmatter et al, 1977) and in human platelets (Newman et al, 1978). By contrast, [^{3}H]dihydroergocryptine - binding to rat brain preparations did not have the specificity characteristics expected of binding to alpha-adrenoceptors (Strittmatter et al, 1977). Other ligands have been useful in studying alpha- adrenoceptors in the central nervous system. U'Prichard and Snyder (1977) have reported the use of an alpha-adrenergic antagonist, [^{3}H]WB4101, and the alpha-adrenergic agonists [^{3}H]clonidine, [^{3}H]adrenaline, and [^{3}H]noradrenaline to directly identify alpha-adrenoceptors in brain membranes. Another ligand, [^{3}H]dihydroazapetine, was also used in an attempt to label alpha-adrenergic binding sites, although the sites that were labelled did not appear to have an affinity for alpha-adrenergic agonists (Ruffolo et al, 1976).

In analogy with the beta-adrenoceptors discussed above, some of the alpha-adrenoceptors are also capable of existing in high and low affinity states (Hoffman et al, 1980a). The high affinity state is uniquely promoted by agonists and is convertible to the lower affinity state by guanine nucleotides (Hoffman et al, 1980a; Tsai and Lefkowitz, 1979). However, only the alpha$_2$-adrenoceptors and not the alpha$_1$-adrenoceptors display such heterogeneity of binding modulated by guanine nucleotides (Hoffman et al, 1980a).

Alpha-adrenoceptors can now be directly identified using antagonist radioligands, such as [^{3}H]dihydroergocryptine ([^{3}H]DHE) which labels both alpha$_1$- and alpha$_2$-adrenoceptors (Williams and Lefkowitz, 1976), [^{3}H]prazosin which selectively labels alpha$_1$-adrenoceptors (Barnes et al, 1979), and [^{3}H]yohimbine which selectively labels alpha$_2$-adrenoceptors (Motulsky et al, 1980; Hoffman et al, 1981; Daiguji et al, 1981), (Fig. 9).

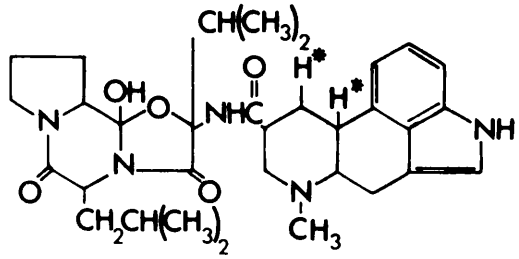
4. ADRENOCEPTOR FUNCTION

The binding of hormones, catecholamines or drugs to adrenoceptors brings about a variety of biological responses such as a change in the rate or strength of cardiac beating or change in the tone of vascular smooth muscle.

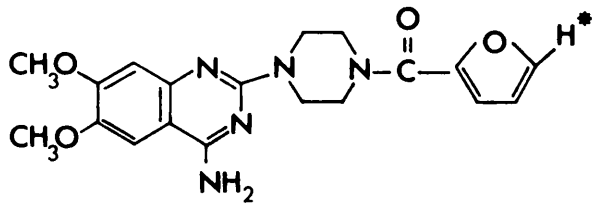
In case of beta-adrenoceptors, the hormone-receptor interaction results in activation of the membrane - bound enzyme adenylate cyclase. It is suggested that binding of hormonal agonists to adrenoceptors changes the fluidity of the cell membrane so that the hormone - receptor complex diffuses laterally along the membrane surface until it couples with and thereby activates adenylate cyclase (Hirata et al, 1979). A second protein appears to be involved in the activation of adenylate cyclase that results from the hormone - receptor interaction. This protein, which has been called guanine nucleotide regulatory protein (N) (Ross et al, 1978; Rodbell, 1980; Limbird, 1981; Spiegel and Downs, 1981) interacts with both the receptor and the cyclase. Interaction of the hormone with the receptor affects N in such a way that it binds GTP, the N-GTP then apparently activates the cyclase (Pfeuffer, 1977). Adenylate cyclase, after its activation by the hormone - receptor complex, stimulates conversion of adenosine triphosphate (ATP) to

FIG. 9

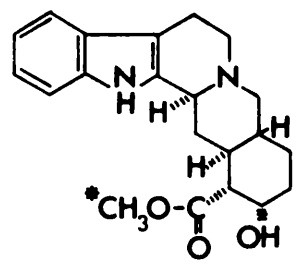
Structures of radioactively labelled alpha-adrenergic antagonists. Sites of specific radioactive labelling are indicated by an asterisk.



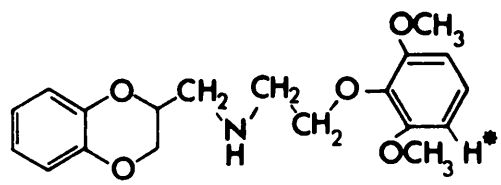
[^{3}H] Dihydroergocryptine



[^{3}H] Prazosin



[^{3}H] Yohimbine



[^{3}H] WB-4101

adenosine 3'-5'-monophosphate (cyclic AMP), which serves as the "second messenger" for the hormone (Sutherland and Rall, 1960) and activates intracellular protein kinases (Nimmo and Cohen, 1977; Greengard, 1978). These in turn cause phosphorylation of enzymes that lead to the characteristic cellular response, such as relaxation of smooth muscle cells.

In case of alpha-adrenoceptors, one popular hypothesis is that the hormone - receptor interaction activates the membrane - bound enzyme guanylate cyclase and possibly thereby increases cellular levels of guanosine 3'-5'-monophosphate (cyclic GMP) by converting guanosine triphosphate (GTP) to cyclic GMP (Goldberg and Haddox, 1977), which serves as the "second messenger" for the hormone (Lees, 1981). The cyclic GMP generated by the cyclase is then thought to activate the cyclic GMP - dependent protein kinases (Lefkowitz, 1976; Baxter and Funder, 1979) and ultimately produce the observed biological effect of the hormone. However, it was observed that $alpha_2$-adrenoceptors (Tsai and Lefkowitz, 1979; Sabol and Nirenberg, 1979) but not $alpha_1$-adrenoceptors are coupled to adenylate cyclase, and it appear that $alpha_2$-adrenoceptors inhibit adenylate cyclase activity and therefore reduce cyclic AMP levels (Hoffman and Lefkowitz, 1980b). $Alpha_1$-adrenoceptors do not affect the adenylate cyclase system and probably have a direct effect on calcium ion flux across the cell membrane (Butcher, 1978).

5. REGULATION OF ADRENOCEPTORS

Measurements of both receptor number and affinity by direct radioligand binding methods have greatly advanced the understanding of various factors that regulate adrenoceptors. It is now apparent that a wide variety of hormones, drugs and physiological and

pathological conditions may change adrenoceptor number and therefore sensitivity of tissues to catecholamines (Lefkowitz, 1979). Many of these findings have important clinical implications.

Regulation of adrenoceptors has been classified as "homologous" and "heterologous". Homologous regulation is the regulation of the adrenoceptors by the hormones or agonists that normally interact with those adrenoceptors. By contrast, heterologous regulation refers to all other influences that regulate the adrenoceptors, such as other types of hormones or drugs that do not normally interact directly with the adrenoceptor (Lefkowitz, 1979).

Two additional terms are commonly used to describe adrenoceptor regulation. The terms "down regulation" and "up regulation" refer to directional changes in the number of adrenoceptors under a particular set of physiological or pathophysiological circumstances. Thus an intervention that leads to a reduction in the number of adrenoceptors on cells would be said to "down regulate" the adrenoceptors, whereas an increase in adrenoceptors would be referred to as "up regulation" (Lefkowitz, 1979).

5.1. HOMOLOGOUS REGULATION

Homologous regulation is regulation of the receptors by hormones or drugs that normally interact with those receptors.

It became apparent that a wide variety of hormones and drugs have the ability to regulate the concentration of their own receptors in tissues. In general, the pattern that has been observed is of an inverse relation between the ambient concentration of the biologically active molecule and the number of its receptors (Tell *et al*, 1978). Thus, high concentrations of

hormone or drug seem to "down regulate" their corresponding receptor number. In contrast, when hormone concentrations are lowered, their corresponding receptor concentrations may increase ("up regulation").

With adrenoceptors, these hormone- and drug- related changes in adrenoceptor concentration appear to mediate real changes in tissue sensitivity or responsiveness to catecholamines (Lefkowitz, 1979).

5.1.1. Down regulation

Continued exposure to increased concentrations of catecholamine hormones or agonists often reduces adrenoceptor numbers, leading to a fall in tissue responsiveness to that agonist (Wood, 1980). Such diminished responsiveness occurring as a result of chronic exposure to a high level of the agonist is often referred to as tachyphylaxis, desensitisation, refractoriness or tolerance (Lefkowitz, 1980; Motulsky and Insel, 1982a).

Several different cellular mechanisms are involved in these processes. All have in common that they are promoted by agonists but not antagonists and ultimately lead to the translocation of the surface receptors to an intracellular location (Lefkowitz and Michel, 1983). In some cases the intracellular transport is accomplished by special endocytotic vesicles that arise at the cell surface (Goldstein et al, 1979). Once within the cell the receptors may be degraded, processed, or recycled back to the cell surface. Agonist - promoted internalisation of receptors may serve a number of very distinct functions. In some cases it serves to attenuate cellular sensitivity to further hormonal or agonist stimulation. This may be accomplished by reduction of the number of functional receptors at the cell surface and by their

sequestration within the cell in compartments physically removed from the normal effector units. This is one of a number of mechanisms responsible for tachyphylaxis or desensitisation to drug and hormone actions. Alternatively, internalisation of the hormone - receptor complex may serve as an essential pathway in the activation of the biological response (Lefkowitz and Michel, 1983).

5.1.2. Up regulation

By contrast experimental sympathetic denervation (either surgically or pharmacologically with guanethidine or 6-hydroxydopamine) removes the tonic release of neurotransmitter noradrenaline, leaving adrenoceptors unoccupied. This results in an increase in adrenoceptor number leading to increased adrenergic responsiveness or denervation hypersensitivity (Sporn et al, 1976; Glaubiger et al, 1978). Similarly, chronic blockade of adrenoceptors by specific antagonists causes an increase in receptor number. In an animal model, chronic treatment with the adrenoceptor antagonist propranolol leads to an increase in beta-adrenoceptor number (Glaubiger and Lefkowitz, 1977). In healthy volunteers, administration of 160 mg of propranolol daily for seven days increased lymphocyte beta-adrenoceptor number by 30 to 50% (Aarons et al, 1980). After withdrawal of propranolol, the number of beta-adrenoceptors decreased to normal over four days, but no propranolol was detectable in the plasma after the first day; thus, for several days these subjects had an increased number of receptors in the absence of the beta-adrenoceptor blocking drug.

People with hypertension often have abnormal sympathetic-nervous-system responses, which resemble a hyperadrenergic state, yet is associated with only slightly increased concentrations of

plasma catecholamines (Goldstein, 1981); investigators have looked for possible changes in adrenoceptors. Studies in animals using radioligand binding techniques have supported the notion that adrenoceptors are altered in experimental hypertension (Woodcock et al, 1978; Woodcock and Johnston, 1980; Yamada et al, 1980; Limas and Limas, 1978,1979; Pettinger et al, 1982). In humans, Kafka et al (1979) found no difference in [^{3}H]DHA binding to lymphocytes of hypertensive subjects, and reported that the number of alpha-adrenoceptors (using the radioligand [^{3}H]DHE) on the platelet membranes of men (but not women) with hypertension was normal. Motulsky et al (1983), using the alpha$_2$-selective radioligand [^{3}H]yohimbine, performed a study on normal and hypertensive men and found no difference between hypertensive subjects and normals in the number of alpha$_2$-adrenoceptors on intact platelets or in the affinity (dissociation constant) of these sites for [^{3}H]yohimbine or adrenaline. Furthermore, anti-hypertensive treatment with the alpha$_2$-agonist guanabenz or the beta-antagonist propranolol did not change the number or affinity of platelet alpha$_2$-adrenoceptors.

5.2. HETEROLOGOUS REGULATION

Not only do the catecholamines themselves regulate their receptors, but various hormones and other influences also control receptor numbers and properties. One of the more interesting examples is thyroid hormones. Clinicians have thought that many of the symptoms of clinical hyperthyroidism are similar to what would be expected for a "hyper-beta-adrenergic" state. These include such manifestations as tachycardia, hyperdynamic circulation, increased pulse pressure, hyperhidrosis, anxiety and tremor. This impression has been strengthened by the finding that propranolol, a beta-

adrenoceptor antagonist, provides relief in many patients with symptomatic hyperthyroidism (Lefkowitz, 1979). Nonetheless, clear-cut evidence of increased sensitivity to beta-adrenoceptor catecholamines in patients with hyperthyroidism has been difficult to document (Levey, 1971). It seems clear that tissue and serum levels of catecholamines are not elevated in patients with hyperthyroidism (Christensen, 1972). The possibility that thyroid hormones might alter the concentration of beta-adrenoceptors in tissues has been explored in rats rendered hyperthyroid by injection of thyroxine or triiodothyronine. There was a highly statistically significant increase in the number of beta-adrenoceptors in the hearts of these hyperthyroid animals without any change in the affinity of binding of the antagonist [^{3}H]DHA to these receptors (Williams *et al*, 1977a). Conversely, Banerjee and Kung (1977) have shown that the number of beta-adrenoceptors is decreased in the hearts of hypothyroid rats.

6. METHODOLOGICAL APPROACH TO RADIOLIGAND BINDING STUDIES OF ADRENOCEPTORS

6.1. GENERAL CONSIDERATIONS

The goal of radioligand binding experiments is to study the physicochemical interaction between radioactively labelled ligands and adrenoceptors. The basic technical requirements for adrenoceptor studies are a radioactively labelled, biologically active adrenergic ligand (agonist or antagonist), a tissue preparation containing adrenoceptors, and a valid method for separation of bound and free ligand. However, merely demonstrating the presence of bound ligand is not sufficient to prove that the observed binding is in fact to a receptor site. In order to establish that the binding measurements truly reflect the interaction of ligand with physiologically relevant receptors, several criteria must be satisfied:

1. The radioligand must be physiologically active and chemically pure. The presence of even a small percent of radioactive impurity might complicate the interpretation of binding data.
2. Since there should only be a finite number of receptor sites, the binding of radioligand to receptor preparations must demonstrate saturability at the receptors.
3. The concentration range over which the ligand occupies the receptors should be comparable to the concentration range over which it elicits a biological response.
4. The binding sites labelled by the radioligand must exhibit the specificity and stereospecificity of the adrenergic response. The order of potency of agonists in eliciting the biological

response should be reflected in their order of potency in competing for the binding sites. The biologically active (-) stereoisomers of adrenergic agonists should be more potent in competing for the binding sites than the less active (+) stereoisomers. Specific antagonists that inhibit the biological adrenergic response should block the binding of radioligand.

5. The kinetics of binding should reflect the kinetics of the adrenergic ligand in eliciting or antagonising a biological response.

In general, if these criteria are satisfied, it is likely that the measured binding of radioligand represents binding to adrenoceptors in the tissue preparations under investigation.

6.2. RADIOLIGAND

During the last two decades hundreds of compounds have been synthesised that interact as either agonists or antagonists with adrenoceptors (Table 1). The large number of these compounds and the wealth of pharmacological data on their actions have provided a virtually limitless source of ligands that can potentially be radioactively labelled. For specific studies the choice of radioligand depends on several considerations, including whether an agonist or antagonist is desirable and whether a tritiated or iodinated ligand is most suitable. However, there are a number of characteristics that any currently available or new adrenergic radioligand should have.

1. The radioligand should have demonstrable biological activity as an agonist or antagonist.
2. Since the absolute number of adrenoceptor sites in most

TABLE 1

List of commonly used agonists and antagonists, with radiolabelled derivatives used in binding studies

	Non-selective action	$Alpha_1$	$Alpha_2$	$Beta_1$	$Beta_2$
Alpha-adrenoceptor agonists	Adrenaline, Noradrenaline	Methoxamine, Phenylephrine	Clonidine		
Beta-adrenoceptor agonists	Adrenaline, Isoprenaline			Noradrenaline	Salbutamol, Terbutaline
Alpha-adrenoceptor antagonists	Dihydroergocryptine, Phentolamine	Phenoxybenzamine, Prazosin, WB 4101	Rauwolscine, Yohimbine		
Beta-adrenoceptor antagonists	Alprenolol, Dihydroalprenolol, Pindolol, Propranolol, Timolol			Atenolol, Betaxolol, Metoprolol, Practolol	Butoxamine, ICI 118,551, IPS-339
Radiolabelled alpha-adrenoceptor antagonists	[^{3}H]Dihyroergocryptine	[^{3}H]Prazosin, [^{3}H]WB 4101	[^{3}H]Yohimbine		
Radiolabelled beta-adrenoceptor antagonists	[^{3}H]Dihydroalprenolol, [^{125}I]Hydroxybenzylpindolol, [^{125}I]Cyanopindolol				

tissues is small, the radioligand should have a high specific radioactivity. In most cases it is very difficult to work with radioligands having a specific radioactivity of less than 5 or 10 Ci/mmol.

3. The radioligand should have a high affinity for the receptor sites, thus permitting the use of the radioligand at low concentrations and reducing the likelihood of binding to non-specific sites. In addition, the rate of dissociation of the radioligand from the receptor sites should be sufficiently slow to allow separation of bound and free radioligand using currently available techniques.
4. The radioligand should be chemically stable under the assay conditions employed.
5. Ideally the radioligand should specifically interact with only one type of receptor site under the assay conditions used. Thus the use of ligands (e.g. [^{3}H]phenoxybenzamine) with the potential to bind to several receptor types would give a complex binding pattern that would be difficult to interpret.

Both tritiated and iodinated ligands have been used for binding studies of adrenoceptors. Each type of isotopic labelling has its advantages. Tritiated ligands offer the advantages of a long radioactive half-life and no significant alteration of the chemical structure of the ligand because of the presence of the isotope. The principle advantage of iodinated ligands is the high specific radioactivity, for example, the incorporation of one atom ^{125}I per molecule of ligand would give > 2000 Ci/mmol. The disadvantages of using iodinated ligands for binding studies are the relatively short half-life (60 days for ^{125}I) and the potential alteration of biological activity caused by iodination. However, for studies of

systems in which the amount of biological material is very limited, the use of high specific activity iodinated ligands may significantly enhance their feasibility.

Currently available radioligands have been used primarily in three types of experiments: kinetic studies, saturation assays and competitive binding experiments.

Kinetic experiments determine how rapidly a radioligand binds to a receptor and dissociates from it; these studies yield the rate constants characterising the radioligand-receptor interaction. Kinetic experiments also give practical information: how long it takes for the binding reaction to reach equilibrium and how much radioligand dissociates from the receptors during the time required to separate bound from unbound radioligand.

Saturation assays quantitate the concentration of receptors and the affinity of these receptors for the radioligand and have been the principal type of experiment to study adrenoceptors in human tissues. These assays are performed by incubating a constant concentration of tissue adrenoceptors with various concentrations of the radioligand. Radioligands typically bind to adrenoceptors in a saturable manner. The specific binding approaches a maximum (B_{max}) that represents the total quantity of adrenoceptors present. The concentration of radioligand that binds to half this number of adrenoceptors is the dissociation constant (K_d) and is a measure of the affinity of the adrenoceptors for the radioligand. A simple rearrangement of the specific binding data into a Scatchard (1949) plot (B/F vs. B, where B and F refer to the amounts of bound and free ligand respectively), a transformation that usually yields straight lines for radioligand binding to adrenoceptors and is commonly used to display and analyse the results.

Competitive binding experiments determine the ability of various compounds to compete with the radioligand in binding to adrenoceptors. These are carried out by incubating a constant concentration of tissue adrenoceptors and radioligand with various concentrations of the competing compound. As the concentration of the competitor increases, more adrenoceptors are occupied by it, and fewer are occupied by the radioligand. The affinity of the adrenoceptors for the competitor (expressed as the dissociation constant K_d) is determined from the competitive binding curve.

6.3. TISSUE PREPARATIONS

Since many organs are responsive to alpha- or beta- adrenergic stimulation, or both, there are many potential sources of tissue for adrenergic binding studies. In the ideal situation, the tissue studied would consist of a single cell type, since interpretation of binding data is complicated if the cell type responsible for the observed binding is unknown. In addition it would be advantageous to use a tissue that is highly enriched in adrenoceptors, by analogy with the electroplax (electric organ of the electric eel) tissue that has been extremely useful for the study of cholinergic receptors. Unfortunately to date no human tissue have been found that is particularly enriched with alpha- or beta-adrenoceptors. Tissues can be processed in a variety of ways in preparation for binding studies. Binding of adrenergic ligands has been measured in isolated intact cells (Brown *et al*, 1976; Mukherjee and Lefkowitz, 1976b; Insel *et al*, 1977), in particulate membrane fractions (Levitzki *et al*, 1974; Lefkowitz *et al*, 1974; Aurbach *et al*, 1974; Alexander *et al*, 1975a,b; Williams *et al*, 1976a,b; Zatz *et al*, 1976; Maguire *et al*, 1976a; Bylund and Snyder, 1976), and in

soluble preparations (Caron and Lefkowitz, 1976). The measurement of specific binding in intact microscopic pieces of tissue is difficult because of the low density of adrenoceptors and the high non-specific retention of ligand in the tissue. Performing binding studies using isolated intact cells, in contrast with studies on membrane preparations or solubilised tissue, offers the obvious advantage of maintaining a structural and metabolic state closer to that present *in vivo*. However, the preparation of membrane fractions often allows a partial purification and concentration of the specific type of membrane material (usually plasma membrane) containing the greatest density of receptors.

In general, receptor binding sites are somewhat labile as can be observed by the partial loss of binding activity when the preparations are stored (unfrozen) for several hours. However, the lability of the binding sites is in most cases not nearly as great as that of the adenylate cyclase catalytic activity. In general quick freezing of membrane preparations permits preservation of both binding and adenylate cyclase activities. However in some systems freezing and subsequent thawing of tissue cause a slight increase in the level of non-specific binding of radioligand.

6.4. METHODS OF ASSAYING SPECIFIC RADIOLIGAND BINDING

A variety of methods have been used to quantify specific radioligand binding. In general these methods require a total or partial physical separation of bound and free ligand. They include filtration and centrifugation.

Filtration

The most rapid and widely used technique for separating bound and free ligand has been filtration. In this method a membrane

preparation is incubated with radioligand for an appropriate length of time. Then this incubation solution or a portion of it is rapidly filtered under suction through a horizontal 2.4 cm filter composed of paper or other appropriate matrix, and the amount of particulate bound radioactivity retained on the filter is measured. If a small volume is used for the incubation, it is advantageous to rapidly dilute the incubation in buffer to a volume that can homogeneously cover the filter to minimize sample loss on transfer of the tube contents. After filtration, the filters are washed rapidly with buffer (5 to 30 ml) to remove non-specifically trapped ligand. The optimum volume of washing should be the amount that gives the maximal reduction in non-specific binding without reducing the level of specific binding. The most important principle involved in this assay procedure is that the amount of measured binding should reflect the amount of binding in the incubation just prior to separation of bound and free ligand. Hence the separation method must be sufficiently rapid that an appreciable amount of association or dissociation of ligand does not occur during the assay procedure.

Centrifugation

In the centrifugation method, a membrane preparation is incubated with radioligand for an appropriate length of time. After centrifugation, the amount of particulate bound radioactivity is determined by simply measuring the radioactivity in the pellet. The speed in the centrifugation method is achieved by the use of "table top" centrifuges, which allow rapid pelleting of small aliquots (100 to 200 µl).

The main disadvantage in the centrifugation technique, is that the background level of non-specific binding (possibly because of

the trapping of free ligand in the pellet) is higher than with other methods.

6.5. INCUBATION CONDITIONS

The incubation of radioligand and receptor preparation should be at a physiological pH, temperature, and ionic composition. Within the broad constraints of these conditions, there are very few absolute requirements that must be met in the incubation conditions. Several types of buffer systems, including Hepes, phosphate and Tris-HCL, have been used in adrenergic radioligand binding studies, which are usually carried out at pH 7.4 - 7.6. The concentration of radioligand in the incubation mixture is obviously an important variable, since the fraction of total binding of ligand that is non-specific and non-saturable is highest at high radioligand concentrations, it is a technical advantage if assays can be performed at relatively low concentrations of radioligand.

6.6. NON-SPECIFIC BINDING

Radioactively labelled ligands, like many other compounds, non-specifically adsorb to glass, paper, cellular membranes, and other substances. Thus when measuring the amount of radioactivity bound to a receptor preparation, it is important to subtract the amount of radioactive "background" that represents non receptor binding. In practice, "non-specific" binding is defined as the amount of measured radioactivity not displaced by adrenergic agonists and antagonists. The levels of non-specific binding depend on a variety of factors including the tissue preparation, the assay method, and the radioligand used. The nature of non-specific binding is unknown. It is usually non-saturable and generally

occurs instantaneously. A portion of non-specific binding may represent physical trapping of radioligand.

6.7. VALIDITY OF IN VITRO ESTIMATIONS OF ADENYLATE CYCLASE COUPLED BETA-ADRENOCEPTORS

Sutherland and his colleagues developed the now widely accepted theory that the link between receptor occupancy and biological response is often in the form of so-called "second messengers" such as cyclic 3', 5'- adenosine monophosphate (cAMP) and other cyclic nucleotides (Sutherland and Rall, 1960). They were the first to demonstrate that stimulation of myocardial and hepatic adenylate cyclase by catecholamines in broken cell preparations was mediated by a beta-adrenoceptor. The order of potency of agonists for enzyme stimulation was isoprenaline > adrenaline > noradrenaline; and stimulation was antagonised by the beta-adrenergic antagonist dichlorisoprenaline (Murad *et al*, 1962).

Subsequently, a number of investigators have studied, in some detail, the pharmacological characteristics of the adenylate cyclase coupled beta-adrenoceptors. In all respects the specificity of the receptor/cyclase complex determined *in vitro* is identical to the specificity of the complex as determined by pharmacological techniques on intact tissues (Rosen *et al*, 1970; Mayer, 1972; Grunfeld *et al*, 1974; Kaumann and Birnbaumer, 1974; Lefkowitz, 1975; Mukherjee *et al*, 1975a).

A number of characteristics of adenylate cyclase stimulation by catecholamines provide evidence that the beta-adrenoceptor is mediating these effects. When studied *in vitro*, catecholamine stimulation of the receptor/cyclase complex is competitively antagonised by beta-adrenergic antagonists; effects of agonists and

antagonists on the complex display the same marked stereospecificity that is apparent for their physiological actions on intact tissues, i.e. (-) isomers are considerably more potent than (+) isomers (Mukherjee et al, 1975a; Tell and Cuatrecasas, 1974); the ability of beta- adrenergic agents to stimulate or competitively antagonise stimulation of the adenylate cyclase enzyme directly parallels their efficacy at physiological beta-adrenoceptors. Moreover, the differences among beta-adrenoceptors in intact tissues (e.g. $beta_1$, $beta_2$) are closely mirrored by the specificity of the adenylate cyclase - coupled beta-adrenoceptors (Lefkowitz, 1975). Studies of adenylate cyclase activation in broken cell preparations have demonstrated that the beta-adrenoceptors of heart and adipose tissue are $beta_1$ (Mayer, 1972; Kaumann and Birnbaumer, 1974; Lefkowitz, 1975), whereas those of liver, skeletal muscle and lung are $beta_2$ (Mayer, 1972; Lefkowitz, 1975; Burges and Blackburn, 1972). These findings support the contention that both $beta_1$ and $beta_2$ adrenoceptors coupled to adenylate cyclase are the physiologically relevant beta-adrenoceptors; agonist occupancy of these receptors stimulates adenylate cyclase thereby increasing cellular levels of adenosine 3', 5' monophosphate (cyclic AMP).

Recently it has been observed that $alpha_2$-adrenoceptors (Tsai and Lefkowitz, 1979; Sabol and Nirenberg, 1979), but not $alpha_1$ receptors are coupled to adenylate cyclase, they inhibit adenylate cyclase activity and in turn decrease cellular cyclic AMP levels.

7. ADRENOCEPTORS IN DISEASE

7.1. GENERAL CONSIDERATIONS

There is a growing awareness of the need to study disease mechanisms at a cellular level. Work with isolated organs or tissue slices represent moves in that direction, though many artifacts are inherent in their preparation, and their application to man is very limited. Cellular elements of peripheral blood have therefore a great heuristic appeal since they possess receptors specific to a number of endogenous compounds, and they can be isolated repeatedly and with relative ease, even during periods of severe illness. They therefore provide a useful tool for studying a number of cellular mechanisms. Following the discovery of adenylate cyclase in sonicated leucocytes (Scott, 1970) and the presence of beta-adrenoceptors in peripheral blood leucocytes (Smith and Parker, 1970), it has been found that these cells possess histamine receptors (Bourne et al, 1971) and prostaglandin E_1 receptors (Bourne and Melmon, 1971). Insulin (Gavin et al, 1972; Gavin et al, 1974) and growth hormone receptors (Archer et al, 1973) have also been described.

Since the section on regulation of adrenoceptors (see section 5) has described various adrenoceptor disorders in diseases, the present discussion will concentrate on adrenoceptor dysfunction in bronchial asthma, which is the subject of this thesis.

7.2. SZENTIVANYI HYPOTHESIS

Szentivanyi (1968) postulated that the atopic state and bronchial hyper-reactivity in asthma results from a reduced beta-adrenoceptor function and a relative increase in alpha- adrenergic activity. This contention was based on observations of reduced

beta$_2$ metabolic responses such as hyperglycaemia and peripheral vasodilation in asthmatic patients following administration of beta agonists as compared to these responses in non-atopic normal subjects (Cookson and Reed, 1963; Middleton and Finke, 1968).

Other workers showed that beta adrenergic blockade enhances bronchial reactivity to inhaled allergens (Ouellette and Reed, 1967) or to methacholine (McGeady et al, 1968) in patients with seasonal allergic rhinitis without a previous history of bronchial asthma. By progressively increasing doses of the beta blocker, an acute airway obstruction could be produced which seemed to reach an intensity that may be found in patients with bronchial asthma (McGeady et al, 1968). Administration of beta blockers has also been reported (McNeill, 1964) to aggravate already existent asthmatic conditions and to cause precipitous and prolonged falls in Forced Expiratory Volume in 1 second (FEV_1). Blockade of beta-adrenoceptors was found to increase significantly the bronchial sensitivity of asthmatic subjects to methacholine, whereas no such effect was detectable in normal human subjects as judged by the change in FEV_1 (Zaid and Beall, 1966).

This hypothesis has therefore attracted considerable attention and provoked a great deal of research. Several groups (Logsdon et al, 1972; Parker and Smith, 1973; Alston et al, 1974) have studied asthmatic patients using leucocyte beta-adrenoceptors. They have reported depressed beta-adrenoceptor function, at least during the active phases of the disease and have interpreted these data as supporting Szentivanyi's hypothesis. However, other workers (Grieco et al, 1968; Zaid et al, 1968; Gillespie et al, 1974) were unable to find a significant difference between normal subjects and most asthmatic patients. Conolly (1980), rejected Szentivanyi's

hypothesis believing that the depressed beta-adrenoceptor activity found in such patients can better be explained as a consequence of prolonged exposure to high levels of exogenous or endogenous beta-adrenergic agonists (Greenacre and Conolly, 1978).

7.3. ALPHA-ADRENOCEPTORS IN ASTHMA

Increased alpha-adrenergic responsiveness has been described in asthmatic patients (Henderson et al, 1979) although little is known about the role of alpha-adrenoceptors in human asthma, alpha receptor antagonists have been shown to modify exercise and allergen-induced asthma (Patel and Kerr, 1975; Patel et al,1976). In an animal model of chronic asthma a small decrease in pulmonary beta-adrenoceptor numbers accompanied by a pronounced increase in alpha-adrenoceptor numbers has been shown (Barnes et al, 1980a). In human lung there is some evidence for increased alpha-adrenoceptors in lungs from patients with airways obstruction (Barnes et al, 1980b). Previous studies using [^{3}H]DHE were able to identify alpha-adrenoceptors in human lymphocytes (Sano et al, 1981a,b), but the subtype was not defined, and the preliminary evidence has indicated that the lymphocytes of patients with asthma have elevated numbers of [^{3}H]DHE-binding sites, which are presumed to indicate increased numbers of alpha-adrenoceptors.

7.4. BRONCHIAL ASTHMA

Bronchial asthma is a condition characterised by episodic and variable breathlessness. This breathlessness results from wide - spread narrowing of intrapulmonary airways and varies in severity over short periods of time, either spontaneously or with treatment. Variable narrowing of the peripheral airways is due to one or all

of the following:

1. Contraction of bronchial smooth muscle (bronchoconstriction)
2. Oedema of the mucous membrane
3. Mucus within the lumen

Bronchoconstriction may be related to exposure to environmental factors, especially inhaled substances in concentrations that do not affect the majority of persons, or may occur without apparent external cause. Detectable factors include specific antigen-antibody (IgE) reactions, usually to inhaled antigens, hyper-reactivity of the airways to physical and chemical stimuli, and exercise.

Resting bronchial 'tone' can be demonstrated in bronchial musculature by a decrease in airway resistance after administration of bronchodilator drugs.

In the last fifteen years it has been recognized that the airways in normal subjects are under the control of the autonomic nervous system and constrict reflexly following many chemical and mechanical stimuli, although this narrowing usually does not produce symptoms or signs. The dominant symptom in bronchial asthma is breathlessness - an unpleasant awareness of difficulty in breathing which may be sensed not only in expiration but also in inspiration especially when there is marked hyper-inflation. Tightness in the chest is then also a component of the dyspnoea. Wheezing usually accompanies both inspiration and expiration unless the asthma is so severe that the reduced air flow is unable to create the sound. The pattern of wheezy breathing varies considerably, it may be episodic in which the episodes are short or long or it may persist for very long periods.

There is now a wide choice of tests for assessing airway

function. Tests based on the forced expiratory manoeuvre are repeatable and for many purposes measurement of peak expiratory flow (PEF) or forced expiratory volume in one second (FEV_1) is adequate. Pulmonary function tests in asthma have three main uses; first, to make the diagnosis; second, to assist in the assessment of the severity of an asthmatic attack; third, to monitor the course and its modification by treatment.

Some regard this bronchial hyper-reactivity as the central abnormality in the asthmatic subjects. The precise causes are uncertain. The amount of muscle in the bronchi of asthmatic subjects is increased, but it has not been shown to be more reactive *in vitro*. Both nervous and humoral factors have been postulated in the pathogenesis of bronchial reactivity. There is dispute as to whether a stimulus causes an abnormal acute response (increased release of humoral mediators or abnormal firing of a nervous reflex) or whether the enhanced reactivity is due to a normal acute response superimposed on increased resting bronchial tone. Abnormal release of mediators has not been demonstrated but temporary hyper-reactivity develops in normal subjects with viral infections, possibly due to abnormal stimulation of irritant receptors in the bronchial mucosa. There is no doubt that clinical and functional evidence of hyper-reactivity is greatly enhanced when there is pre-existing airway narrowing. There are at least three reasons for this effect of airway narrowing. First, a given absolute change in airway diameter will produce a larger increase in airflow resistance if initial diameter is reduced; second, increases in bronchial tone may potentiate the stimulation of irritant receptors and the initiation of reflex bronchoconstriction; third, increases in tone potentiate the

effects of bronchoconstrictor drugs.

Asthma is broadly divisible into two groups: extrinsic and intrinsic.

Extrinsic or atopic asthma

This refers to the large group in whom asthma is due to IgE-mediated hypersensitivity reactions to inhaled antigens commonly present in the air. Many of them also have seasonal or perennial allergic rhinitis. Most of them start having wheezy breathlessness early in life, in some after infantile eczema. In some the early episodes are prolonged, and masquerade initially as attacks of bronchitis and bouts of coughing. The special liability which these patients show to develop IgE antibodies as a result of minor exposure to common environmental antigens has been estimated to affect about 10% of people and is presumably genetic. It has been called atopy. In the name used for this sort of asthma, 'extrinsic' implies that asthma is precipitated by contact with environmental antigens and 'atopic' refers to the sort of hypersensitivity reaction that is concerned.

Intrinsic asthma

With intrinsic asthma on the other hand wheezy breathlessness although episodic at first tends to be much more persistent. The illness usually starts later in life, often in the late 20s or 30s, but no age is exempt. A frank allergic background is not found but perennial rhinitis is not uncommon. Aspirin sensitivity is sometimes a feature and nasal polyps not an infrequent finding. The onset of intrinsic asthma is often related to an acute respiratory infection and persistence of infection is a serious matter.

Bronchodilators

Bronchodilator drugs are defined as drugs which relax bronchial smooth muscle and decrease bronchial tone. In animals, the effect may be demonstrated on isolated bronchial smooth muscle and in intact animal preparations, by inhibiting bronchoconstriction induced by histamine and acetylcholine administration. In man, the effect may be demonstrated by the relief of 'spontaneous' bronchospasm or that induced by exercise or other challenge.

At present, bronchodilator drugs, as defined above, are either anti-cholinergics, adrenergics or xanthine derivatives. In addition to relaxing the smooth muscle of the bronchus, they have other actions which may be of importance in relieving airways obstruction. They are known to influence small blood vessel function and so may reduce oedema and in addition can exert a prophylactic effect by inhibiting mediator release from the lung mast cells. They can be used for the relief of the acute attack or they may be taken continuously, where they exert a 'prophylactic' effect. Presumably, their effectiveness when given chronically is partly due to inhibition of mediator release but may also be due to suppressing the bronchoconstrictor effect of the released mediators on the bronchial smooth muscle.

Adrenergic drugs are extremely useful in the management of asthma, whereas anticholinergics have a limited role. It may be that despite the poor innervation there are numerous beta-adrenoceptors which can respond to circulating adrenaline or to adrenergic drugs used in therapy. Stimulation of adrenoceptors produces bronchodilatation in animals and a decrease in airways resistance in man. Recently, evidence has accumulated to suggest that there are alpha-adrenoceptors in the bronchi and these could,

when stimulated under certain circumstances, produce bronchoconstriction. Normally, however, the beta-adrenoceptors (bronchial relaxant) are the predominant adrenoceptors in airways smooth muscle. Adrenergic bronchodilator drugs must therefore stimulate beta-adrenoceptors.

Classification of bronchodilator drugs is based on the autonomic pharmacology of bronchial smooth muscle. Bronchodilatation is produced by beta-adrenoceptor stimulation and bronchoconstriction, by stimulation of cholinergic receptors and possibly too, alpha-adrenoceptors. The available drugs can be classified as:

1. Anticholinergic drugs, e.g. atropine
2. Sympathomimetic (adrenergic) drugs
   a) with alpha and beta effects, e.g. adrenaline, ephedrine
   b) with $beta_1$ and $beta_2$ effects but no alpha effects, e.g. isoprenaline
   c) with selective $beta_2$ effects, e.g. salbutamol, terbutaline
3. Others, methylxanthines, e.g. aminophylline and choline theophyllinate.

Glucocorticoids

These should be reserved for more severe or protracted asthma that does not respond to the proper use of bronchodilators. Their mode of action in the relief of the airway obstruction is unknown. However, the effects are not immediate, and as much as 6 hours may be needed before any benefit can be demonstrated. Consequently, in acute situations, vigorous bronchodilator therapy should be continued while steroids are being administered. In addition, there is some suggestion that glucocorticoids can restore beta-adrenergic responsiveness in both normal and asthmatic subjects

(Ellul-Micallef and Fenech, 1975; Holgate et al, 1977; Shenfield et al, 1975).

8. CIRCADIAN RHYTHM (DIURNAL VARIATION)

8.1. GENERAL CONSIDERATIONS

Although research in the field of human 24 - hour rhythms has a history of about 150 years, it is only during the last 15 years that real progress has been made towards a better understanding of the mechanisms involved. A few generalisations can now be made (Aschoff and Wever, 1976):

1. Nearly all structures and functions seem to undergo regular changes from day to night. These diverse rhythms keep distinct phase relationships to each other under normal conditions, representing temporal order within the organism.
2. In subjects kept in isolation and deprived of periodic (24-hour) inputs from the environment, the rhythms continue with periods slightly different from 24 hours, i.e. the rhythms are "free-running", like autonomous self-sustained oscillations, and hence belong to the class of circadian rhythms.
3. The human circadian system consists of a multiplicity of oscillators that are usually coupled to each other but may change their phase relationship depending on conditions, and may even become desynchronized, free-running with different frequencies.

8.2. DIURNAL VARIATION IN ASTHMA

It is well known that certain features of asthma show a marked diurnal variation. Large diurnal swings in peak expiratory flow rate (PEFR) and early morning wheezing are commonly seen in asthma

patients and may be related to the high incidence of sudden asthma deaths in hospital in the early morning (Cochrane and Clark, 1975) as some have been known to have marked diurnal swings in peak expiratory flow before their death. Also patients with airways obstruction commonly complain that their wheeze varies in severity from time to time, often consistently at certain times of the 24 hours. This was first documented in 1960 (Lewinsohn et al, 1960), but little is known of the prevalence and nature of such variation in asthmatics and in other patients with airways obstruction (Turner-Warwick, 1977).

Corticosteroid drugs do not reduce diurnal variation in PEFR (Soutar et al, 1975), although they may well produce better subsequent control of the attack. The best pharmacological solution to the problem appears to be to ensure that adequate therapeutic levels of sympathomimetic drugs are maintained throughout the night, although they reduce diurnal variation but do not abolish them.

8.3. CIRCADIAN RHYTHM OF ALPHA- AND BETA-ADRENOCEPTORS

Recently, it was shown that there is a diurnal variation in the number of specific beta-adrenergic binding sites in rat pineal gland (Romero et al, 1975), with the highest number at 1800 h and the lowest at 0600 h. Also circadian rhythms in the numbers of alpha- and beta-adrenoceptors exist in the rat forebrain and hypothalamus (Kafka et al, 1981). It has also been reported that in asthmatic subjects, plasma cyclic AMP showed a significant circadian variation with peak values at 1600 h and trough values at 0400 h (Barnes et al, 1982). In normals, plasma cyclic AMP concentrations also demonstrated a circadian variation with the

maximum concentration at 1400 h and the minimum at 0200 h (Mikuni et al, 1978).

9. LYMPHOCYTES AS A TOOL FOR RECEPTOR STUDIES IN ASTHMATICS

There are difficulties that hinder direct investigation of cellular mechanisms in human lung. A simple solution to this problem, first suggested by Smith and Parker (1970), has been to use peripheral blood leucocytes, which themselves possess beta-adrenoceptors. This approach presupposes a pharmacological identity between the lung and leucocyte receptors. There has been considerable enthusiasm for the use of leucocytes, either as a mixed population of cells or as purified lymphocytes.

Several studies (Logsdon et al, 1972; Parker and Smith, 1973; Alston et al, 1974; Gillespie et al, 1974) have been reported. These authors have all assumed that the beta-adrenoceptor of the leucocyte is identical with that in the lung. Conolly and Greenacre (1977), showed that the lymphocyte beta-adrenoceptor is readily blocked by (±) propranolol, but appears to be far less sensitive to the effect of practolol. The same pattern of susceptibility to the different beta-blockers is seen in lung tissue. Both propranolol and practolol have been shown in other tissues to exert only a simple competitive antagonism, and studies with another beta blocking drug, alprenolol, in human lymphocytes (Williams et al, 1976b) indicate that beta blockade is a reversible competitive antagonism in this cell model. Salbutamol stimulated the lymphocyte beta-adrenoceptor, being about one tenth as active as isoprenaline.

On the basis of these studies the lymphocyte adrenoceptor may according to Land's classification (Lands et al, 1967) be

categorised as $beta_2$ and in this respect is valid as a model for studying beta-adrenoceptor function in asthma.

In summary, the following workers have used lymphocytes in the search for adrenoceptor disorders in asthma: Conolly and Greenacre, 1976; Kariman and Lefkowitz, 1977; Greenacre et al, 1978; Brooks et al, 1979; Szentivanyi et al, 1979; Sano et al, 1981b; Meurs et al, 1982; Tashkin et al, 1982; Van den Berg et al, 1982.

10. THE THESIS

Szentivanyi's (1968) hypothesis that an inherent defect of beta-adrenoceptors might underlie a range of atopic diseases, including bronchial asthma has stimulated a great deal of research. Several reports (Haddock et al, 1975; Kariman and Lefkowitz, 1977; Brooks et al, 1979) appeared to support this idea, however, others (Zaid et al, 1968; Gillespie et al, 1974) failed to confirm these findings. It has also been suggested by others (Conolly and Greenacre, 1976; Galant et al, 1980) that the receptor abnormality is drug induced.

Patients with asthma have marked variations in breathing over 24 h which is not seen in non-asthmatic subjects. This variation is thought to be due to changes in control of the bronchial muscle tone and mediated via the receptors in the bronchial smooth muscle, for the sympathetic nervous system.

This circadian variation in the bronchomotor tone, often demonstrated as the morning dip in FEV_1 could be due to changes in the numbers and/or the sensitivity of beta-adrenoceptors or related to humoral circadian rhythms.

Blood lymphocytes have similar beta-adrenoceptors ($beta_2$) and this study uses these lymphocyte receptors as a model of events

taking place in the bronchial smooth muscle over a 24 h period.

The limitation of previous work were:

1. No indication of sample time.
2. No attempt to investigate receptor number and receptor sensitivity at different times of the day.

These criteria are important because in asthmatic patients lung function is only impaired at a certain time during the day, and obviously the time for collecting blood sample is very important.

11. AIMS OF THIS THESIS

1. Validation of previous beta-adrenoceptor methodology in human lymphocytes.
2. To investigate beta-adrenoceptor function in normals and asthmatic patients at different times of the day and night. Many asthmatic patients have nocturnal asthma around midnight and a morning dip in FEV_1. These changes in ventilation may be a consequence of a diurnal rhythm in the number and/or the sensitivity of the beta-adrenoceptors.
3. To assess the effect of orally administered beta-agonist (salbutamol) on beta-adrenoceptor function and to see whether, in accordance with the hypothesis of Szentivanyi there was some apparent receptor dysfunction in extrinsic asthmatics and if exposure to a $beta_2$-agonist induced tachyphylaxis.
4. To establish an assay for estimating lymphocyte $alpha_2$-adrenoceptors.
5. To compare the alpha:beta adrenoceptor ratio. Alpha-adrenoceptors have recently been identified in human lymphocytes and preliminary evidence indicate increased

number of alpha-adrenoceptors in lymphocytes of asthmatic patients. Therefore comparing the alpha:beta receptor ratio in both normals and asthmatic patients may be important.

MATERIALS AND METHODS

MATERIALS

Listing of all reagents and consumables used in this thesis can be found in Appendix A.

METHODS

1. RAT LUNG STUDIES

1.1. PREPARATION OF RAT-LUNG

Male Sprague-Dawley rats (200-250 g) were killed by cervical dislocation. Lungs were homogenised using an ultra-turrax (6 x 10 sec) in 20 volumes of 0.25 mol/l sucrose containing 50 mmol/l Tris, 1 mmol/l $MgCl_2$ pH 7.4, followed by 10 strokes using a motor driven teflon-pestle; the homogenate was filtered through three layers of gauze. After centrifugation (1000g, 10 min, 4C) the crude nuclear pellet was discarded. The supernatant was centrifuged (10000g, 20 min, 4C) and the crude mitochondrial pellet discarded. The supernatant was recentrifuged (70000g, 60 min, 4C) to obtain the microsomal pellet which was resuspended in assay buffer (50 mmol/l Tris, 5 mmol/l $MgCl_2$ pH 7.8). The microsomal pellet (stored on ice) was used in all experiments.

1.2. SATURATION STUDIES WITH [^{3}H]DHA

In the saturation assay, membrane suspension (0.3-0.35 mg protein) was incubated at 30C with [^{3}H]DHA (1-12 nmol/l), and non-specific binding was determined in the presence of 10 µmol/l (-) propranolol in a final volume of 250 µl. After 20 min, 2.5 ml ice-cold assay buffer was added to each tube and filtered under reduced pressure through Whatman GFC glass microfibre filters (pre-wetted in assay buffer), then the filters were washed twice with 8 ml ice-cold

assay buffer (filtering and washing took less than 15 sec). The filters were dried at room temperature, then shaken with 10 ml toluene : Triton X-100 (2 : 1) scintillator and the radioactivity determined (efficiency 50%). The total binding at each point was determined by measuring the radioactivity obtained when incubations were performed in the absence of the antagonist (-) propranolol. The specific binding at each point was defined as the difference between total and non-specific binding. Saturation assays were carried out in polystyrene tubes (12 x 75 mm). Protein concentration was estimated using the commercially available Bio-Rad kit based on dye-binding assay (Coomassie Brilliant Blue G-250) as described by Bradford (1976).

1.3. EQUILIBRIUM BINDING TIME

Tubes with lung membrane suspension (0.3-0.35 mg protein) in assay buffer were set up on ice. At different time intervals starting from zero time, [^{3}H]DHA (4 nmol/l) was added to the tubes. Non-specific binding was determined in the presence of 10 µmol/l (-) propranolol for each time; the final volume was 250 µl. The tubes were incubated at 30C for various intervals (15 sec, 30 sec, 1 min, 2 min, 4 min, ... 60 min). Then 2.5 ml of ice-cold assay buffer was added to each tube, filtering and drying as before. Total and non-specific binding were determined in parallel duplicate samples and at each time interval. Assays were carried out in polystyrene tubes (12 x 75 mm).

1.4. DISSOCIATION OF [^{3}H]DHA FROM LUNG MEMBRANES

Dissociation of specifically bound [^{3}H]DHA from receptor sites was evaluated by incubating membrane suspension (0.3-0.35 mg protein)

at 30C with [^{3}H]DHA (4 nmol/l) to equilibrium. Then 10 µmol/l (-) propranolol was added, and specific binding was estimated at varying time intervals (10 sec, 20 sec, 30 sec, 45 sec, 1 min, 2 min, ... 10 min).

1.5. COMPETITIVE BINDING STUDIES

Membrane suspension (0.3-0.35 mg protein) in assay buffer was incubated at 30C with [^{3}H]DHA (4 nmol/l) and varying concentrations of (+) or (-) propranolol in a final volume of 250 µl. After 20 min the samples were filtered and dried as before. Total binding was determined by measuring the amount of [^{3}H]DHA bound when incubations were performed in the absence of (+) or (-) propranolol. Other drug displacement curves were obtained similarly by substituting the appropriate drug for propranolol.

2. LYMPHOCYTE STUDIES

2.1. LYMPHOCYTE ISOLATION

Lymphocytes were prepared from heparinised blood by a modification of the technique of Harris and Ukaejiofo (1970), which produced a cell population of approximately 90-95% lymphocytes, the remainder of the cells were monocytes, and recoveries of 90-94%.

Blood (50 ml) was collected into Evans heparinised bottles, 8 ml/bottle, gently mixed to distribute heparin and centrifuged (250g, 20 min, 15C). The platelet-rich plasma was aspirated and discarded. The pellet was diluted 1 : 3 (V/V) with buffer A. Buffer A was prepared by adding 0.1% calf serum and 20 mmol/l Hepes to Earle's Balanced Salt Solution (without sodium bicarbonate) adjusted to pH 7.6. 8 ml portions of this suspension of erythrocytes and leucocytes were carefully layered onto 12 ml of

lymphocyte separation medium, density 1.077 ± 0.001 g ml^{-1}, in 30 ml universal containers and centrifuged (250g, 25 min, 15C). The milky layer of lymphocytes was harvested from the interface using a pasteur pipette, washed by diluting 1:3 with buffer A, centrifuged (250g, 15 min, 15C) and the supernatant discarded. The pellet was resuspended in 20 ml of buffer A and gently disaggregated.

The addition of 0.1% calf serum to the medium reduced lymphocyte aggregation and ensured an adequate yield (90-94%) of cells. Cells were counted in a Coulter Cell Counter; final cell concentrations were usually in the range of 2.5-3.0 x 10^6 lymphocytes/ml. Cell viability was assessed by trypan blue exclusion and was found to be greater than 95% during all the procedures described above.

2.2. LYMPHOCYTE COUNTING AND VIABILITY

2.2.1. Viable cell count

The use of trypan blue stain is based on the ability of viable cells to exclude the dye (Merchant et al, 1960).

0.1 ml of 0.4% trypan blue (0.4 g trypan blue in BSS) were added to 0.5 ml of lymphocyte suspension in a polystyrene tube (12 x 75 mm). The contents were mixed and allowed to stand for 5 min. Cells were counted using an "improved Neubauer" counting chamber at a magnification of 40 to give the total lymphocyte count and percentage of unstained lymphocytes; unstained cells are assumed to be viable. Results are expressed as % viable lymphocytes.

2.2.2. Coulter counter settings for white blood cell counting

The instrument was set up with a 100 μm orifice, 0.5 ml manometer volume and the attenuation switch (which sets the sensitivity of

the Coulter Counter amplifier) to position 0.707. The aperture current switch (which controls the amount of current flowing between the two electrodes) was set to position 16. The threshold control (which determines the size level above which the Model ZF counts; the instrument will count all of the pulse tips which reach or exceed the given threshold value, but none below) was set to position 10. These were standard settings currently in use in the Haematology Dept. for lymphocyte counting.

2.2.3. White blood cell counting

40 µl of lymphocyte suspension were diluted with 20 ml ISOTON II. To obtain white blood cell counts the use of a stromatolysing agent was necessary. 6 drops of ZAPONIN (a fast acting lytic agent for the complete lysis of red cells) was added and after mixing by inversion the red cells lysed immediately as confirmed by microscopy. The Coulter Counter was set up as described (2.2.2.) and the cells counted as follows: the vial containing the white cell dilution was placed on the beaker platform, making sure that the orifice wafer and external electrode were fully immersed. The control tap was opened to zero the counter mechanism, and activate the two electrodes. The control tap was then closed and the white cell count read directly from the display as cell count per microlitre of solution. The number of white blood cells in the blood and in the sample were estimated each time, and the percentage of lymphocytes was estimated by preparing a blood film stained with Romanowsky stains. These stains vary in complexity but depend for their effects on the interaction of a basic dye (methylene blue) and an acidic dye (eosin).

2.3. LYMPHOCYTE BETA-ADRENOCEPTORS

2.3.1. Saturation analysis

The ligand [^{125}I]hydroxybenzylpindolol ([^{125}I]HYP), which has a high affinity for beta-adrenoceptors (Aurbach et al, 1974) was used.

The saturation assays were done in polypropylene tubes (12 x 75 mm) in a final volume of 500 µl comprising for the total binding, lymphocyte suspension (250-350 µg protein) and [^{125}I]HYP in a range of concentrations (50-800 pmol/l) in buffer A. Tubes used to determine non-specific binding contained (-) propranolol (0.3 µmol/l). Totals and non-specific binding were estimated in duplicate at each [^{125}I]HYP concentration. The tubes were set up on ice and [^{125}I]HYP added to start the reaction followed by incubation at 30C in the dark (to minimise photolysis of the [^{125}I]HYP) for 45 min. The reaction was stopped by the addition of 2.5 ml buffer B (50 mmol/l Tris (pH 7.6), 0.9% sodium chloride and 0.1 mmol/l (±) propranolol at 37C). The samples were then allowed to stand for 45 sec before filtering through Whatman GFC glass microfibre filters (presoaked for 60 min in buffer B) by applying a vacuum of 3-4 psi, and the filters were rinsed with 3 x 10 ml of 50 mmol/l Tris (pH 7.6) and 0.9% sodium chloride at 37C. Filtering and rinsing were complete within 12 sec. The filters were air dried, shaken with 10 ml of emulsifier scintillator 299 and the radioactivity was determined (30% efficiency).

Protein concentration was estimated using the commercially available Bio-Rad kit.

2.3.2. Kinetic analysis of [^{125}I]HYP binding

Tubes with lymphocyte suspension (250-350 µg protein) in buffer A

were set up on ice. At different time intervals starting from zero time, [^{125}I]HYP at appropriate concentration (0.4 nmol/l) was added to the tubes; and non-specific binding was determined in the presence of 0.3 µmol/l (-) propranolol in a final volume of 500 µl. The tubes were incubated at 30C for various intervals (1 min, 2 min, 4 min, 8 min, ... 60 min), then 2.5 ml of buffer B was added to each tube, filtering and drying as before. Total and non-specific binding were estimated in parallel duplicates at each time interval.

Dissociation of specifically bound [^{125}I]HYP was evaluated by incubating lymphocyte suspension (250-350 µg protein) in buffer A at 30C with [^{125}I]HYP (0.4 nmol/l) for 45 min to allow equilibrium, then 0.3 µmol/l (-) propranolol was added, and specific binding was estimated at varying time intervals (1 min, 2 min, 4 min, 10 min, 20 min, ... 60 min), as above. Assays were performed in polypropylene tubes (12 x 75mm).

2.3.3. Competitive binding studies

To demonstrate stereospecificity of the beta-adrenoceptors, lymphocyte suspension (250-350 µg protein) in buffer A was incubated at 30C with a fixed concentration of [^{125}I]HYP (0.4 nmol/l) in the presence of varying concentrations of the (+) or (-) stereoisomers of propranolol (10 pmol/l to 1 mmol/l) in a final volume of 500 µl. After 45 min, the samples were filtered and dried as before. Total binding was determined by measuring the amount of [^{125}I]HYP bound when incubations were performed in the absence of (+) or (-) propranolol. The stereospecific assays were performed in duplicate in polypropylene tubes. Other drug displacement curves were obtained similarly by substituting the

appropriate drug for propranolol.

2.4. LYMPHOCYTE ALPHA$_2$-ADRENOCEPTORS

2.4.1. Saturation analysis

The ligand [^{3}H]yohimbine, a potent and selective pharmacological antagonist with high affinity for alpha$_2$-adrenoceptors (Motulsky et al, 1980; Daiguji et al, 1981) was used.

The saturation assays were done in polypropylene tubes (12 x 75mm) in a final volume of 500 µl comprising for the total binding, lymphocyte suspension (250-350 µg protein) and [^{3}H]yohimbine in a range of concentrations (1-20 nmol/l) in buffer A. In tubes used to determine non-specific binding, phentolamine (10 µmol/l) was also added. Total and non-specific binding were estimated in duplicate at each [^{3}H]yohimbine concentration.

The assay mixtures were prepared on ice and then incubated for 25 min at 25C. The reaction was stopped by the addition of 2.5 ml of buffer C (50 mmol/l Tris and 0.9% sodium chloride, pH 7.6) at 25C. The samples were then filtered through Whatman GFC glass microfibre filters (pre-wetted in buffer C) by applying a vacuum of 3-4 psi, and the filters were rinsed with 2 x 10 ml of buffer C at 25C. Filtering and rinsing were complete within 12 sec. The filters were air dried, shaken with 10 ml of emulsifier scintillator 299 and the radioactivity was determined (50% efficiency). Protein concentration was estimated using the commercially available Bio-Rad kit.

2.4.2. Kinetic analysis of [^{3}H]yohimbine binding

Tubes with lymphocyte suspension (250-350 µg protein) in buffer A were set up on ice. At different time intervals starting from zero

time, [^{3}H]yohimbine (5 nmol/l) was added to the tubes; and non-specific binding was determined in the presence of 10 µmol/l phentolamine in a final volume of 500 µl. The tubes were incubated at 25C for various intervals (0.25, 0.5, 1.0, 2.0, 4.0, ... 30.0 min), then 2.5 ml of buffer C was added to each tube, filtering and drying as before. Total and non-specific binding were estimated in duplicate at each time interval.

Dissociation of specifically bound [^{3}H]yohimbine was evaluated by incubating lymphocyte suspension (250-350 µg protein) in buffer A at 25C with [^{3}H]yohimbine (5 nmol/l) for 25 min to allow equilibrium, then 10 µmol/l phentolamine was added, and specific binding was estimated at varying time intervals (0.5, 1.0, 2.0, 4.0, 10.0, ... 60.0 min) same as above. Assays were done in polypropylene tubes (12 x 75mm).

2.4.3. Competitive binding studies

To demonstrate stereospecificity of the alpha$_2$-adrenoceptor sites, lymphocyte suspension (250-350 µg protein) in buffer A was incubated at 25C with [^{3}H]yohimbine (5 nmol/l) in the presence of varying concentrations of (+) or (-) stereoisomers of adrenaline (10 pmol/l to 1 mmol/l) with the addition of 0.8 mmol/l ascorbic acid (to inhibit oxidation of the catecholamine), in a final volume of 500 µl. After 25 min, the samples were filtered and dried as before. Total binding was determined by measuring the amount of [^{3}H]yohimbine bound when incubations were performed in the absence of (+) or (-) adrenaline. Total and non-specific binding were estimated in duplicate using polypropylene tubes. Other drug displacement curves were performed similarly by substituting the appropriate drug for adrenaline.

3. CLINICAL STUDIES

3.1. STUDY I

Subjects

Five patients with extrinsic asthma and five normal volunteers were studied. The asthmatic patients were in remission and were all known to have reversible airflow obstruction. All sympathomimetic drugs including salbutamol and theophylline compounds were stopped and the patients maintained on ipratropium bromide as necessary for seven days before commencing the study, so that the beta-adrenoceptors will have recovered from any beta-agonist induced desensitisation. None of the patients was on inhaled or oral steroid therapy. The patients gave informed consent and the study was approved by the Medical Ethics Committee of the hospital.

Protocol

Studies were performed in the respiratory unit and the asthmatic patients were admitted at least 2 days before the study was commenced so as to become accustomed to the surroundings. Both groups were familiar with having blood samples taken.

The following sequence of tests was performed on each patient or volunteer. On day 1, 50 ml of blood was withdrawn at 1800 h for estimation of plasma cortisol and isolation of lymphocytes for beta-adrenoceptor estimation. On day 2, 50 ml of blood was withdrawn as above at 0800 h and then 4 mg of salbutamol was administered orally at 4 h intervals. At 1800 h, a further blood sample was taken and the dosage of salbutamol continued. On day 3 (0800 h), 50 ml of blood was withdrawn as above, the salbutamol stopped and previous therapy resumed. FEV_1 and FVC were determined

after taking each blood sample.

3.2. STUDY II

Subjects

Four patients with extrinsic asthma and five normal volunteers were studied. The asthmatic patients were in remission and were all known to have reversible airflow obstruction. They were taken off all inhaled and oral sympathomimetic drugs and maintained on inhaled ipratropium bromide as required for seven days before commencing the study. None of the patients was on inhaled or oral steroid therapy. The patients gave informed consent and the study was approved by the hospital Medical Ethical Committee.

Protocol

Studies were performed in the respiratory unit and the asthmatic patients were admitted at least 2 days before the study was commenced. Both groups were familiar with having blood samples taken. The subjects were treated according to the protocol described above with the addition of alpha$_2$-adrenoceptor estimation.

In both studies, plasma cortisol was estimated using Cortisol [^{125}I] Radioimmunoassay kit in the MRC-Blood Pressure Unit, Western Infirmary.

3.3. DETERMINATION OF FEV_1 AND FVC

Forced Expiratory Volume in one second (FEV_1) and Forced Vital Capacity (FVC) were measured using a Vitalograph dry wedge spirometer (Cotes, 1975). The best of three attempts was used for analysis and volume corrected to body temperature and pressure

saturated (BTPS).

4. TECHNIQUES OF RECEPTOR ANALYSIS

4.1. THEORY OF LIGAND - RECEPTOR INTERACTIONS

Although the exact physicochemical nature of the interaction of adrenergic agents with receptor sites is unknown, the use of thermodynamic principles allows the derivation of simple equations that appear to describe the behaviour of adrenergic drug - receptor systems. These calculations can be applied to the analysis of binding data from studies using radioactive adrenergic ligands. Most of the concepts presented here are derived from the mass action law (Hill, 1909), from the pharmacological theory of A.J. Clark (1933), and from the classic Michaelis - Menton analysis of enzyme substrate kinetics. The analysis presented here depends on a number of stated assumptions, and outlines the specific experimental conditions required if the analysis is to be applied to radioligand binding studies.

4.1.1. Law of mass action

The simplest mechanistic assumption that can be made about the interaction of a radioactive ligand, L, with a receptor, R, is that a single molecule of L reacts reversibly with a single receptor molecule, R, to form a complex RL, as shown in Eq. (1):

$$L + R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} RL \qquad \ldots.(1)$$

Each molecular interaction is assumed to be independent of other interactions. Then at equilibrium, a direct consequence of the law of mass action is

$$\frac{[R][L]}{[RL]} = K_d = 1/K_a \qquad \ldots.(2)$$

where [R], [L], and [RL] represent the concentrations of free receptor sites, unbound ligand, and ligand-receptor complex respectively. K_1 and K_{-1} are the kinetic constants for association and dissociation (rate constants) and K_d and K_a are the equilibrium dissociation and association constants. The equilibrium and kinetic constants are related, such that:

$$K_d = K_{-1}/K_1$$

Then the total concentration of receptor sites $[R_t]$ is equal to [R] + [RL] and

$$\frac{[(R_t)-(RL)][L]}{[RL]} = K_d \qquad \ldots.(3)$$

which can be rearranged to

$$\frac{[RL]}{[R_t]} = \frac{[L]}{K_d + [L]} \qquad \ldots.(4)$$

The ratio $[RL]/[R_t]$ represents the fraction of total receptors occupied by ligand. At half maximal occupancy of the receptor, $[RL]/[R_t] = 1/2$ and $K_d = [L]$. Hence the concentration of L required for half - maximal occupancy of the receptor is equal to K_d. An additional rearrangement of Eq. (4) gives:

$$[RL] = \frac{[R_t][L]}{K_d + [L]} \qquad \ldots.(5)$$

Equation (5) is the familiar hyperbolic function in which [RL] = 0 when [L] = 0, and [RL] approaches $[R_t]$ when [L] is very large. The derivation of this equation is completely analogous to that for the classic Michaelis - Menton equation and is mathematically similar to the derivation of the adsorption isotherm originally derived by Langmuir for the adsorption of gases to a surface.

Equation (5) is particularly useful in binding studies in deriving the K_d and the number of binding sites R_t. Experimentally the radioligand is added over a range of concentrations to a fixed concentration of receptors. The level of binding approached asymptotically at high ligand concentrations is R_t, and the concentration of free ligand that elicits a level of binding equal to $[R_t]/2$ represents the K_d (Fig. 10).

The assumptions that have been made for this analysis are that the reaction is a simple bimolecular reaction Eq. (1) and that the measurements of binding are made at equilibrium. It should be emphasised that in Eq. (5) and in Fig. (10) the concentrations of ligand [L] refer to the concentrations of "free" L. If only a small fraction of L is bound, then the concentration of total L can be used to approximate the concentration of free L. This condition is usually met when the total binding site concentration is considerably lower than the K_d of L for the specific sites.

4.1.2. Scatchard analysis

A useful rearrangement of Eq. (3) is

$$\frac{[RL]}{[L]} = [(R_t) - (RL)]\, K_d^{-1} \qquad \ldots(6)$$

which is the form of the equation derived by Scatchard (1949). Thus a plot of the ratio of bound to free ligand, [RL]/[L], versus the concentration of bound ligand, [RL], gives a straight line with a slope of K_d^{-1} and an intercept with the abscissa of $[R_t]$ (Fig. 11). The main advantage of Scatchard analysis (Fig. 11) over the analysis using Eq. (5) (Fig. 10) is that the Scatchard plot linearises the data, something particularly valuable in binding systems that have a high level of non-specific binding. In these

FIG. 10

Plot of the relationship of $(RL)/(R_t)$ to (L). (RL), concentration of ligand-receptor complex; (R_t), concentration of total receptor sites; (L), concentration of free ligand; K_d, the equilibrium dissociation constant.

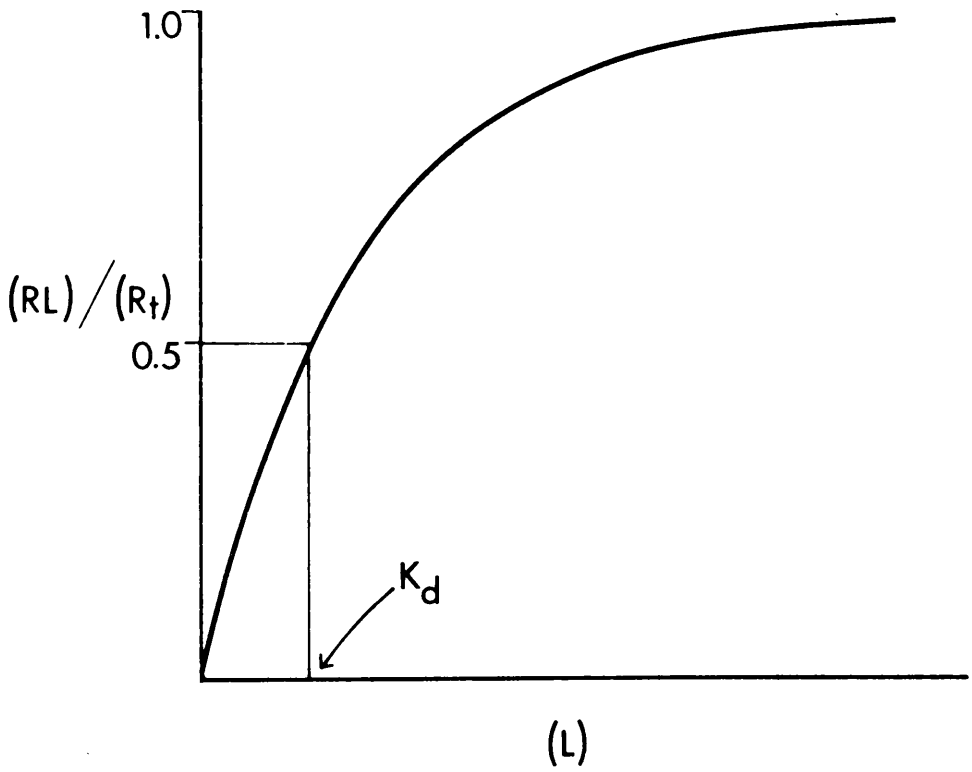
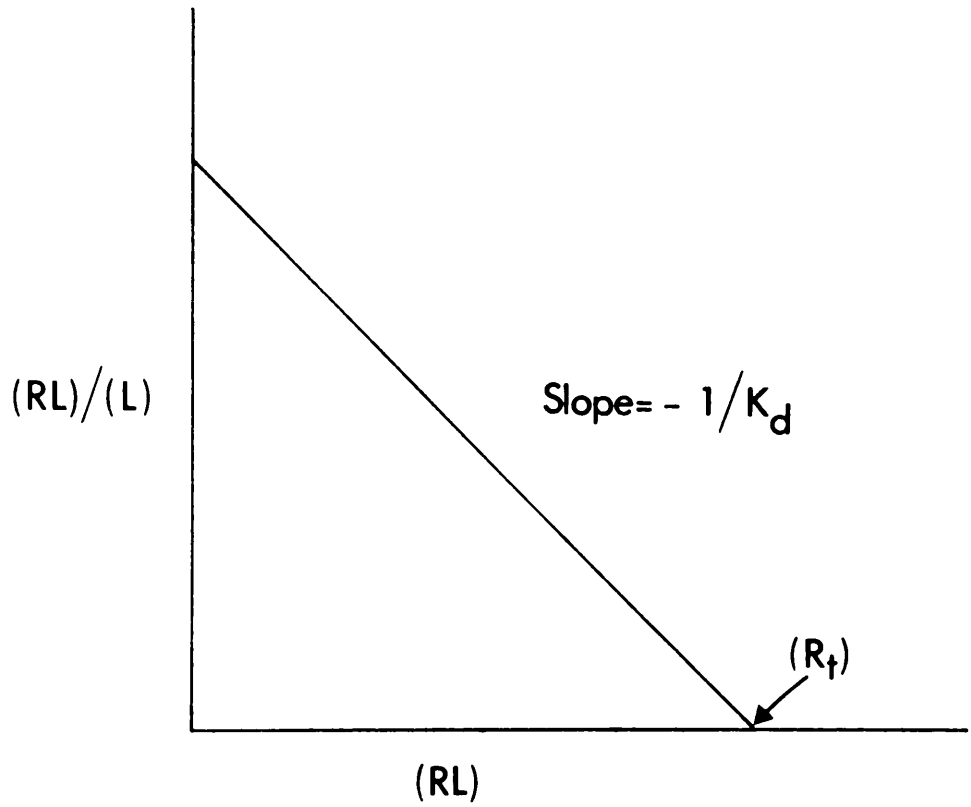

1.0
(RL) / (Rt)
0.5
Kd
(L)

FIG. 11

Scatchard plot. (R_t) refers to the concentration of the total number of binding sites; (RL), concentration of ligand-receptor complex; (L), concentration of free ligand. The slope of the line is the negative reciprocal of the equilibrium dissociation constant (K_d).


(RL)/(L)
Slope= - 1/K_d
(R_t)
(RL)

systems, measurements of binding are difficult at high concentrations of radioligand because the relative contribution of nonsaturable, non-specific "background" binding is highest at high ligand concentrations, thus making the estimation of R_t (and hence K_d) difficult using Eq. (5) and Fig.(10). By contrast, using Scatchard analysis data can be collected at low or intermediate concentrations of L and the linear plot can be extrapolated to estimate R_t and K_d.

Artifactual interpretation (Williams and Lefkowitz, 1978a) of Scatchard plots will occur when:

1. The values used for [RL] and [L] are not true measures for specifically bound and free hormone, respectively.
2. The measurements are made when the system is not at a true state of equilibrium for all concentrations of ligand tested. In order to demonstrate that a system is at true equilibrium, it is necessary to show that the final concentrations of R, L, and RL in the equilibrium $R + L \rightleftharpoons RL$ can be reached from either direction, i.e. by starting either from excess RL or from excess R + L. It should be noted that a system at steady state level of binding (i.e. the amount of binding does not change with time) is not necessarily at equilibrium.
3. The receptor-containing preparation is contaminated by enzymes that inactivate the ligand.
4. The separation of bound and free hormone is not complete.
5. Binding to low-affinity sites dissociates during the separation of bound and free hormones.
6. There is a difference in affinity or kinetic constants between native ligand and its labelled analog.

If the above methodological complications can be avoided, the shape

of the Scatchard plot can provide useful information about receptor - ligand interaction. For example a curved Scatchard plot, can be indicative of co-operative interactions among receptors and can be also obtained if the ligand is binding to two or more sites with different affinities.

4.1.3. Competitive binding studies

In investigations of the specificity of receptor binding sites, the ability of an unlabelled adrenergic agent, I, to compete with the radioligand, L, for the binding site is often measured. If the competing agent, I, is assumed to be a competitive inhibitor of the binding of L, then the receptor is in equilibrium with both L and I:

$$I + R \rightleftharpoons RI$$

$$L + R \rightleftharpoons RL$$

From these equilibria, two simultaneous equations of the form of Eq. (2) can be written and solved for RL to give:

$$[RL] = \frac{[L][R_t]}{K_d\,(1 + I/K_I) + [L]} \qquad \ldots.(7)$$

where [I] is the concentration of free unlabelled competing ligand, and K_I is the equilibrium dissociation constant for the interaction of I with the receptor:

$$K_I = \frac{[R][I]}{[RI]}$$

when there is no competing ligand present [(I) = 0], then the amount of binding $[RL]_0$ which is the concentration of the drug-receptor complex just prior to dilution or addition of a competing ligand is given by:

$$[RL]_0 = \frac{[L][R_t]}{K_d + [L]} \quad \ldots.(8)$$

which is as expected from Eq. (5). When the competing ligand is added at a concentration $[I_{50}]$ at which the value of binding $[RL]_{50}$ is reduced to 50% of the binding in the absence of competing ligand, then Eq. (7) can be written as

$$[RL]_{50} = \frac{[L][R_t]}{K_d\,(1 + I_{50}/K_I) + [L]} \quad \ldots.(9)$$

Since when $I = I_{50}$, $[RL]_{50} = [RL]_0/2$, Eqs. (8) and (9) give

$$\frac{2[L][R_t]}{K_d\,(1 + I_{50}/K_I) + [L]} = \frac{[L][R_t]}{K_d + [L]} \quad \ldots.(10)$$

which simplifies to

$$K_I = \frac{I_{50}}{1 + [L]/K_d} \quad \ldots.(11)$$

Thus using Eq. (11) the equilibrium dissociation constant of an unlabelled competing ligand, I, can be determined by measuring the concentration $[I_{50}]$ of I which half-maximally inhibits the binding of the radioligand present at the concentration [L]. The derivation of this equation is after that of Cheng and Prusoff (1973) for competitive inhibitors of enzymes. Again, it should be emphasised that the concentrations of I and L in these equations refer to the concentrations of free I and L at equilibrium. Experimentally the concentration of free I may be difficult to ascertain and can be approximated by the value for the concentration of total I if the amount of I bound is low compared to K_I. This condition is usually satisfied if $R_t \ll K_I$. If $R_t > K_I$, the value of K_I from Eq. (11) will be an overestimate of the

true value of K_I.

4.1.4. Competitive binding studies with multiple binding sites

Until recently, it has been believed that individual tissues contain only one of the beta-adrenoceptor subtypes. However, pharmacological studies by Carlsson et al, (1972) demonstrated a mixture of $beta_1$ and $beta_2$ adrenoceptors in kitten, but not rat heart. Similar physiological techniques have indicated that frog myocardium might contain a small $beta_1$ component in addition to a predominant population of $beta_2$ adrenoceptors (Stene-Larsen and Helle, 1978). Using radiolabelled ligand techniques, Barnett et al, (1978) demonstrated a mixture of 25% $beta_1$ and 75% $beta_2$ adrenoceptors in rat lung, but rat heart studies demonstrated only one class of sites ($beta_1$). Nahorski et al, (1979) found in rat spleen membranes, the co-existence of both $beta_1$- and $beta_2$-adrenoceptor sites (30-35% $beta_1$; 65-70% $beta_2$). Minneman et al (1979b) examined rat heart, lung and five regions of brain, and found that each of these tissues contain both $beta_1$- and $beta_2$-adrenoceptors in differing ratios.

There are two basic approaches that have been developed using the binding assays to quantitate receptor subtypes.

The first approach uses a radioligand that is selective for one receptor subtype. If one has a radioligand that is 100% selective (i.e. it binds to only one subtype) then it is only necessary to characterise the binding of the ligand to this receptor to define the properties of the receptor subtype. However, typically radioligands will bind to more than one subtype. Therefore, it is usually necessary to use a more involved mathematical analysis to determine the properties and densities of the receptor subtypes

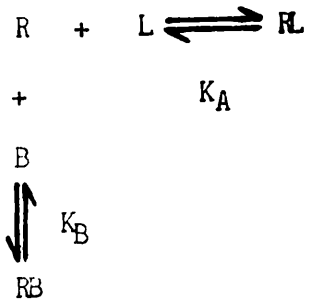
present in a tissue. For a partially selective radioligand a saturation curve is determined and the data is then usually transformed by the method of Scatchard (1949). Scatchard analysis of the binding of a partially selective radioligand will yield a curvilinear plot in the presence of receptor subtypes.

A second approach to studying receptor subtypes involves the use of a selective competing ligand. A displacement curve for a selective competing drug is generated and the data analysed by the method of Eadie (1952) and Hofstee (1952), again generating curvilinear plots in the presence of receptor subtypes.

Early work by Barnett et al (1978) had used a manual graphical method derived from the classical Scatchard (Scatchard, 1949) data analysis (pseudo-Scatchard) to assess the relative proportions of receptor subtypes. Hancock et al (1979) have shown that this method provides less accurate predictions than a non-linear computer model, and have given an analysis of the errors involved.

4.1.5. Computer modelling

For the competitive reaction between the free concentrations of a receptor R, the radiolabelled ligand L and a beta-adrenoceptor blocking agent B, the following equations are valid as described by Wenke (1971) for a competitive reaction scheme:



K_A and K_B represent the dissociation constants of L and B.

Under steady state conditions, assuming a constant number of beta-

adrenoceptors R_T

$$R_T = R + RB + RL$$

the concentration of bound radioligand is

$$RL = R_T \Big/ \left[1 + \frac{K_A}{L}\left(1 + \frac{B}{K_B}\right) \right] \qquad ....(12)$$

If two types of beta-adrenoceptors are present in the membrane fraction and each component obeys the simple mass action law without any interference, then equation (12) can be generalised to

$$RL = \sum_{i=1}^{2} \frac{R_{Ti}}{1 + \frac{K_A}{L}\left(1 + \frac{B}{K_{Bi}}\right)} \qquad ....(13)$$

where i = 1, 2 or 3 classes of binding sites.

In equation (13) it is further assumed that the radioligand L binds with equal affinity to $beta_1$- and $beta_2$-adrenoceptors, whereas B possesses a different affinity to each receptor subclass.

Existing packages SCATFIT (Rodbard, 1973), SAAM 26 (Engel, 1980) and LIGAND (Munson and Rodbard, 1980) provide the necessary algorithms for the least squares analysis of receptor subtypes. In addition Clark developed a non-linear regression based curve fitting package (RECFIT), (Clark et al, 1984) for quantitative analysis of beta-adrenoceptor subtypes which has the additional feature of simultaneous estimation of non-specific binding.

5. STATISTICAL METHODS

Statistical analysis was done with paired Student's t-tests (comparisons on the same subject group) and two-sample t-tests (comparison between patient and volunteer groups) as appropriate on an ICL-2976 computer using the package MINITAB (Ryan et al, 1976).

Computer assisted analysis of alpha- and beta-adrenoceptor saturation assays was carried out by Scatchard analysis (see section 4.1.2., Clark et al, 1983).

Analysis of beta-adrenoceptor sub-populations was performed using a least squares curve-fitting package (RECFIT), (Clark et al, 1984). Because of the current limitations of RECFIT when using selective radioligands, $alpha_2$-adrenoceptor sub-populations were analysed by the method of Cheng and Prusoff (1973), see section (4.1.3.).

All data shown are the mean ± standard deviation where indicated.

DEVELOPMENT OF METHODS

1. INITIAL RECEPTOR FAMILIARISATION STUDIES

The initial aim of the project was to become familiar with the techniques for isolating and characterising beta-adrenoceptors, prior to commencing a study of the changes in beta-adrenoceptors in asthma, where the target tissue for study would be the lymphocyte.

The initial familiarisation studies were performed on the rat lung, with the advantage that not only has the rat lung beta-adrenoceptor been characterised by other workers (Barnett et al, 1977, 1978; Minneman et al, 1979a,b) but the tissue was easily obtained and relatively inexpensive.

1.1. RAT-LUNG CELL MEMBRANE RECEPTORS

Rat-lung cell membranes were prepared as described (Materials and Methods, 1.1.). The first part of the work showed that approximately 95% of cell receptors in the crude homogenate were recovered in the various fractions (i.e. nuclear:5-10%, mitochondrial:18-20% and microsomal:67-70%) and the maximum yield was in the microsomal fraction with a 9 ± 2 fold purification of the beta-adrenoceptors indicating that the receptors were membrane bound. Therefore the microsomal fraction was routinely used in the work. The receptors were not soluble since none could be detected in the 70000g supernatant.

Whatman GFC glass microfibre filters gave the highest recoveries (95%) of beta-adrenoceptors based on a comparison with estimating beta-adrenoceptors in the crude homogenate. Whatman GFB glass microfibre filters were inferior in this respect, giving only 86% recovery, and greater scatter in the experimental results, possibly the much lower rate of filtration allowed the binding equilibrium to be disturbed. 0.45 μm Millipore filters as

routinely used for Radioimmunoassay and competitive protein binding assays were not suitable; they clog too easily, with an adverse effect on the results similar to the GFB filters.

Freezing both the crude homogenate and microsomal fraction at -20C for 24 h resulted in a decrease of approximately 50% in beta-adrenoceptor number. Because of this adverse effect all preparations were assayed immediately.

1.2. KINETIC ANALYSIS OF [^{3}H]DHA BINDING

The next stage was to characterise the lung beta-adrenoceptor. When characterising receptors it is essential to demonstrate that the binding of the chosen radioactive ligand is specific, saturable, stereoselective and reversible.

Kinetic analyses were performed and repeated twice as described (Materials and Methods, 1.3. and 1.4.). Binding of [^{3}H]DHA to rat lung membranes at 30C was a rapid process, with half-maximal specific binding ($t_{1/2}$) of 0.4-0.5 min (Fig. 12), reaching equilibrium within 18-20 min. Binding remained stable for 60 min at 30C. An incubation time of 20 min was chosen to represent equilibrium binding in subsequent experiments.

The initial rate constant (K_{ob}) for the association reaction obtained from the slope (Fig. 12, inset) was 0.748 min^{-1} for 4 nmol/l [^{3}H]DHA. Dissociation of bound [^{3}H]DHA at 30C was determined by incubating rat lung membranes to equilibrium, and then adding 10 µmol/l (-) propranolol (final concentration) at time zero and measuring residual specific binding at subsequent time intervals (Fig. 13). Dissociation was rapid with a $t_{1/2}$ of 5-6 min, and the dissociation rate constant (K_2) was 0.126 ± 0.011 min^{-1} (Fig. 13,B).

FIG. 12

Time course of [^{3}H]DHA binding to rat-lung membranes. Rat-lung membranes in assay buffer were incubated with [^{3}H]DHA (4nmol/l) in the absence and presence of 10μmol/l (-) propranolol. At the indicated times, the samples were diluted with ice-cold assay buffer and filtered. Specific binding was determined in duplicate at each time interval. (Inset) the regression line (r = 0.99) was determined by the plot of ln $[B_{eq}/(B_{eq} - B_t)]$ vs. time, where B_{eq} = binding at equilibrium and B_t = binding at time t. K_{ob} is equal to the slope of the line.

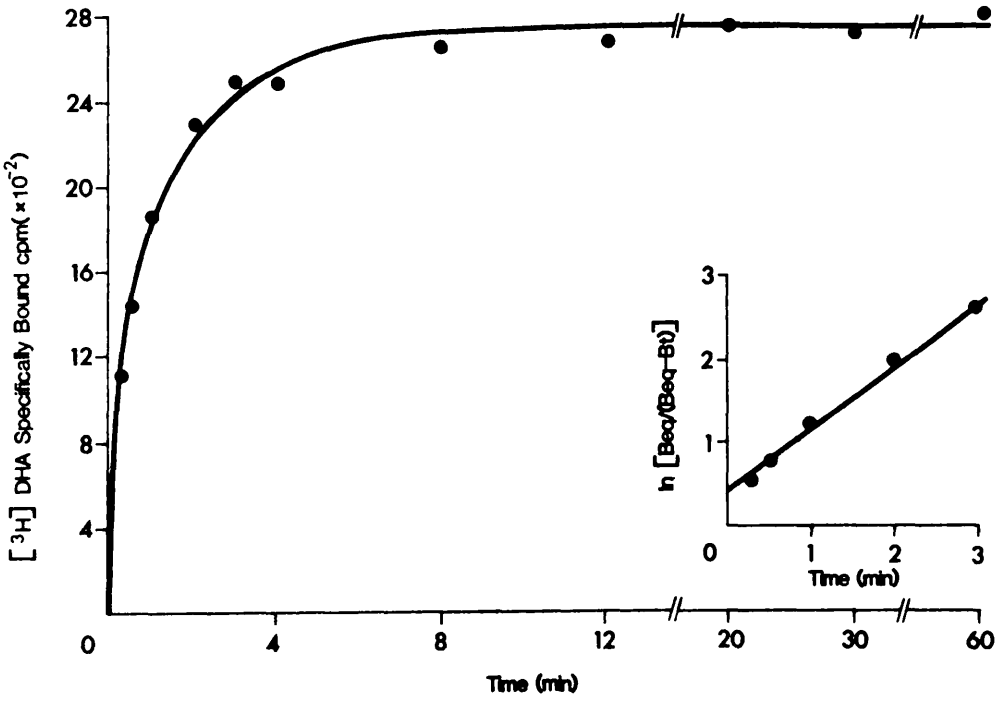
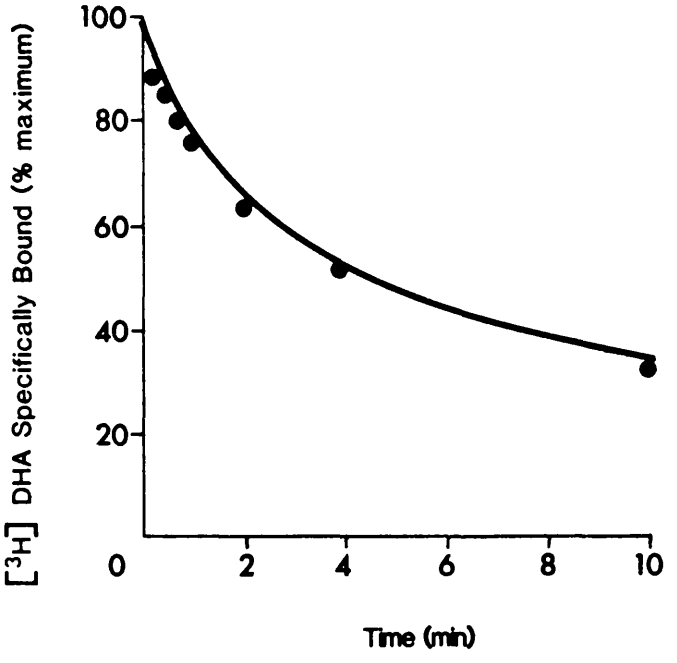
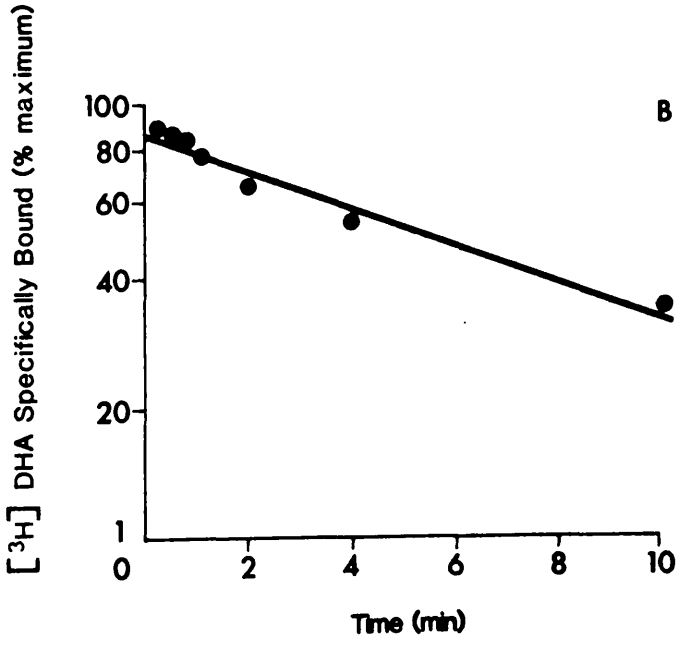

[^{3}H] DHA Specifically Bound cpm($\times 10^{-2}$)
28
24
20
16
12
8
4
0
4
8
12
20
30
60
Time (min)
ln [Beq/(Beq-Bt)]
3
2
1
0
1
2
3
Time (min)

FIG. 13

Time course for dissociation of [^{3}H]DHA binding to rat-lung membranes. Rat-lung membranes in assay buffer were incubated with [^{3}H]DHA (4nmol/l) for 20 min to allow equilibrium. At zero time, 10μmol/l (-) propranolol (final concentration) was added to the samples and specific binding was determined at various time intervals. 100% binding refers to the specific binding just prior to the addition of (-) propranolol. B. First-order rate plot of dissociation of [^{3}H]DHA binding. K_2 is equal to the slope of the line.


[3H] DHA Specifically Bound (% maximum)
100
80
60
40
20
0
2
4
6
8
10
Time (min)


B
[3H] DHA Specifically Bound (% maximum)
100
80
60
40
20
1
0
2
4
6
8
10
Time (min)

The association rate constant (K_1) of [^{3}H]DHA binding was then calculated from the equation (Bennett, 1978): $K_1 = (K_{ob} - K_2)/[DHA]$, where [DHA] is equal to the concentration of [^{3}H]DHA in the assay (4 nmol/l), K_1 was $1.555 \times 10^8 \pm 0.027 \times 10^8$ M^{-1} min^{-1}.

The equilibrium dissociation constant (K_d), determined from the ratio K_2/K_1, was 0.71 ± 0.1 nmol/l.

1.3. SATURABILITY OF [^{3}H]DHA BINDING

The studies were carried out as described (Materials and Methods, 1.2.). The binding characteristics of [^{3}H]DHA to rat-lung membranes is shown in Fig. (14). Specific binding of [^{3}H]DHA (the binding that could be displaced by 10 μmol/l (-) propranolol) was clearly saturable and of high affinity. Apparent saturation of binding sites seemed to occur at [^{3}H]DHA concentrations of 4-6 nmol/l, with half maximal binding occuring at about 0.4-0.5 nmol/l.

Scatchard analysis (Fig. 15) of the saturation data yielded a straight line, indicating that [^{3}H]DHA bound to a single class of high affinity receptors. The total number of binding sites (B_{max}) was 120 ± 19 fmol/mg protein, and the dissociation constant (K_d) of the binding was 0.53 ± 0.07 nmol/l (10 experiments).

1.4. DISCUSSION OF KINETIC STUDIES

These results demonstrated that the binding of [^{3}H]DHA was rapid and reversible at 30C. The specific binding of [^{3}H]DHA represented 80-85% of the total binding and was clearly saturable with a dissociation constant (K_d) of 0.53 ± 0.07 nmol/l. This is similar to that observed by other workers (Barnett et al, 1977). This K_d (0.53 ± 0.07 nmol/l), which was established from equilibrium data was also similar to the K_d of 0.71 ± 0.1 nmol/l determined by

FIG. 14

Specific binding of [^{3}H]DHA to rat-lung membranes as a function of concentration of [^{3}H]DHA. Rat-lung membranes in assay buffer were incubated with various concentrations of [^{3}H]DHA in the presence and absence of 10μmol/l (-) propranolol. Specific binding was determined at each concentration of [^{3}H]DHA.

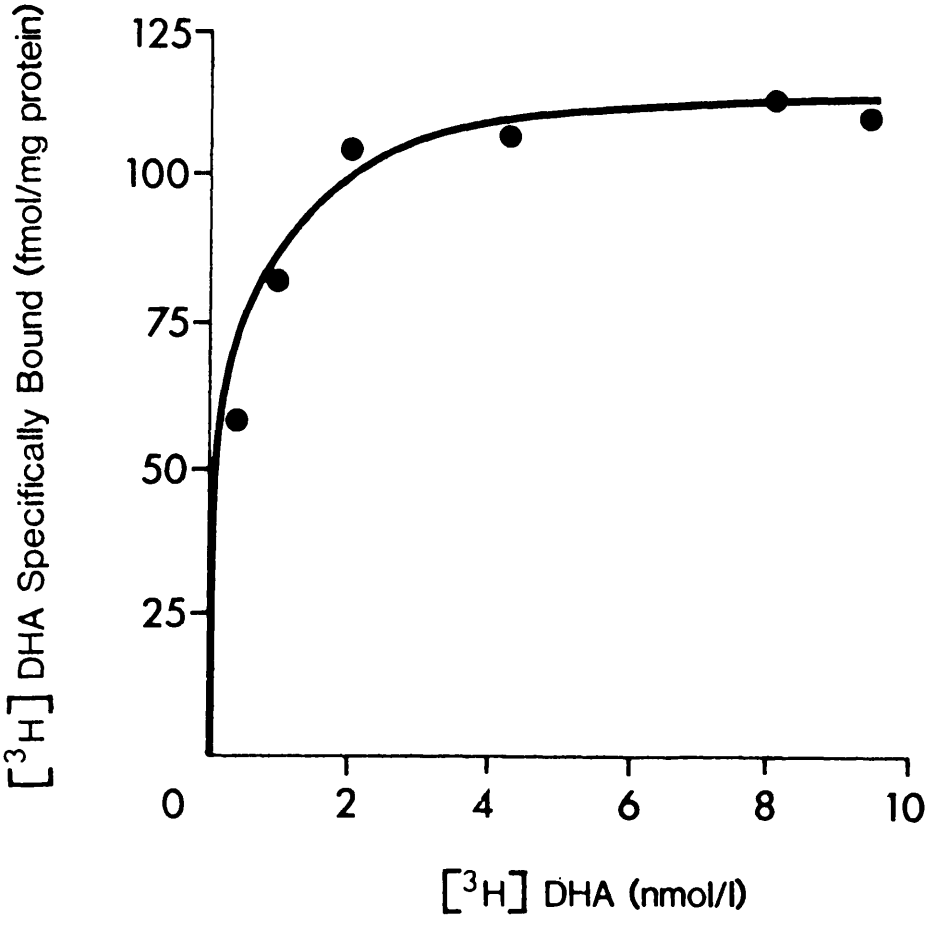
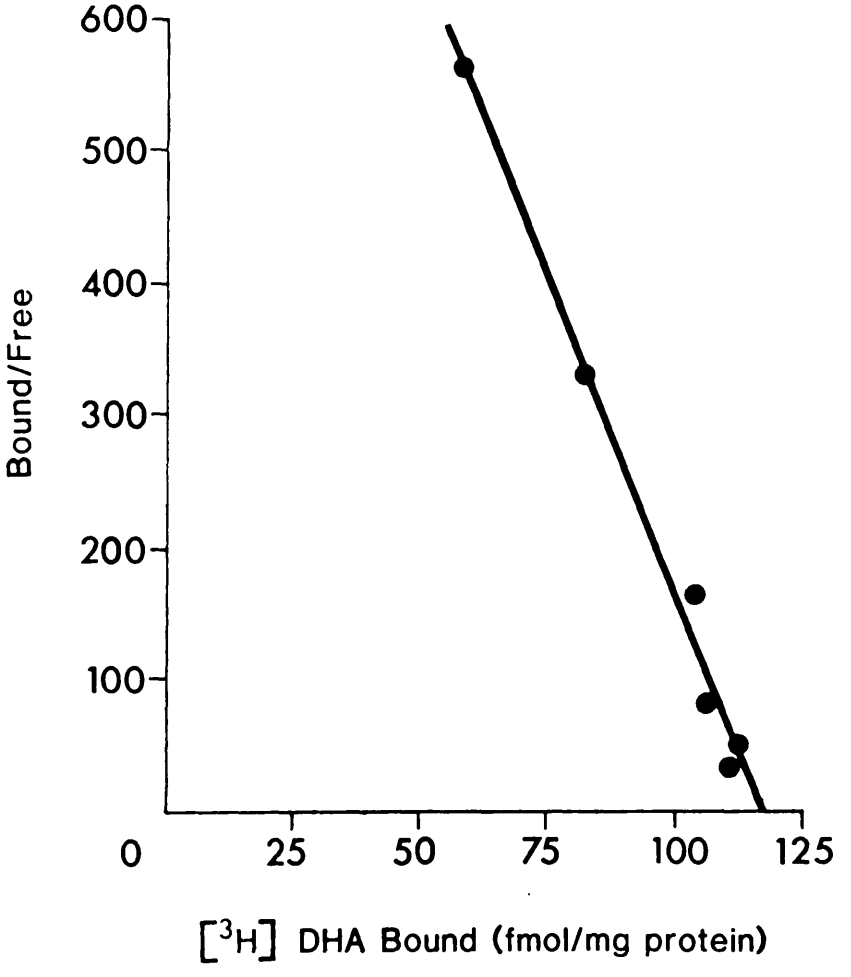

[3H] DHA Specifically Bound (fmol/mg protein)
125
100
75
50
25
0
2
4
6
8
10
[3H] DHA (nmol/l)

FIG. 15

Scatchard analysis of the binding data (Fig. 14) yielding a K_d of 0.49 nmol/l and the total number of binding sites was 117 fmol/mg protein.


600
500
400
300
200
100
0
25
50
75
100
125
Bound/Free
[3H] DHA Bound (fmol/mg protein)

kinetic methods (i.e. ratio of association and dissociation rate constants).

1.5. COMPETITION FOR [^{3}H]DHA BINDING SITES BY ADRENERGIC COMPOUNDS

Competition studies were performed and repeated twice as described (Materials and Methods, 1.5.). Beta-adrenoceptors typically exhibit stereoselectivity in the expression of pharmacological activity. As Fig. (16) shows, (-) propranolol is approximately 100 times more potent in inhibiting binding of [^{3}H]DHA than (+) propranolol (Table 2). Moreover, the relative potencies of a number of drugs (Table 2) suggests that the beta-adrenoceptors in rat-lung are predominantly of the beta$_2$ subtype. For example, H35/25 (1-(4-methylphenyl)-2-isopropylamino-propranolol), a non-specific beta-antagonist and salbutamol, a relatively specific beta$_2$ agonist, more potently inhibited [^{3}H]DHA binding than (±) practolol, beta$_1$ antagonist, with K_d values of 0.38, 1.10 and 12.6 μmol/l for H35/25, salbutamol and practolol respectively (Fig. 17). Table 2 summarises the results which are in accord with those of Barnett *et al* (1977).

The preliminary work has shown that the results for the lung beta-adrenoceptor satisfy the requirements laid down for verifying the existence of a receptor and indicate that the majority of receptors detected were beta$_2$-adrenoceptors.

2. DEVELOPMENT OF LYMPHOCYTE ISOLATION

2.1. AIMS

Since it was intended to perform multiple lymphocyte receptor studies on patients and volunteers, the aims of this work were to develop a technique which gives a high yield and purity of

FIG. 16

Stereoselectivity of [^{3}H]DHA binding sites in rat-lung membranes. Various concentrations of (-) propranolol (●) and (+) propranolol (▲) were incubated with rat-lung membranes and [^{3}H]DHA (4nmol/l) for 20 min. The samples were then diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

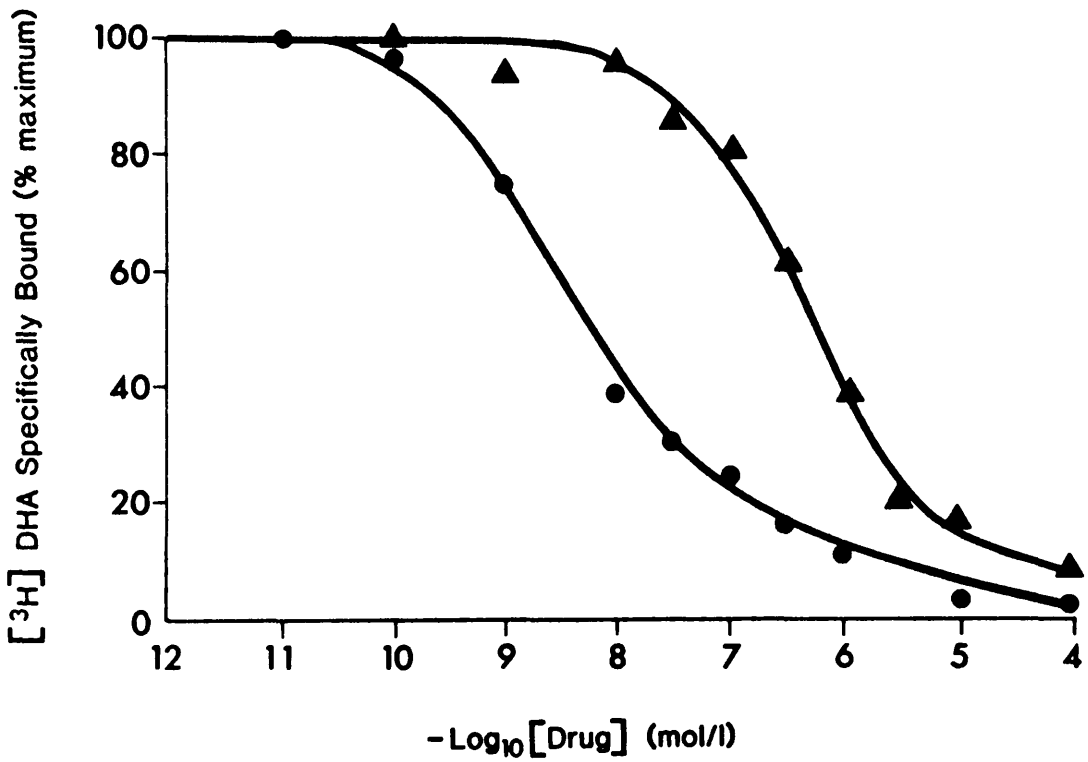

[3H] DHA Specifically Bound (% maximum)
100
80
60
40
20
0
12
11
10
9
8
7
6
5
4
-Log10[Drug] (mol/l)

TABLE 2

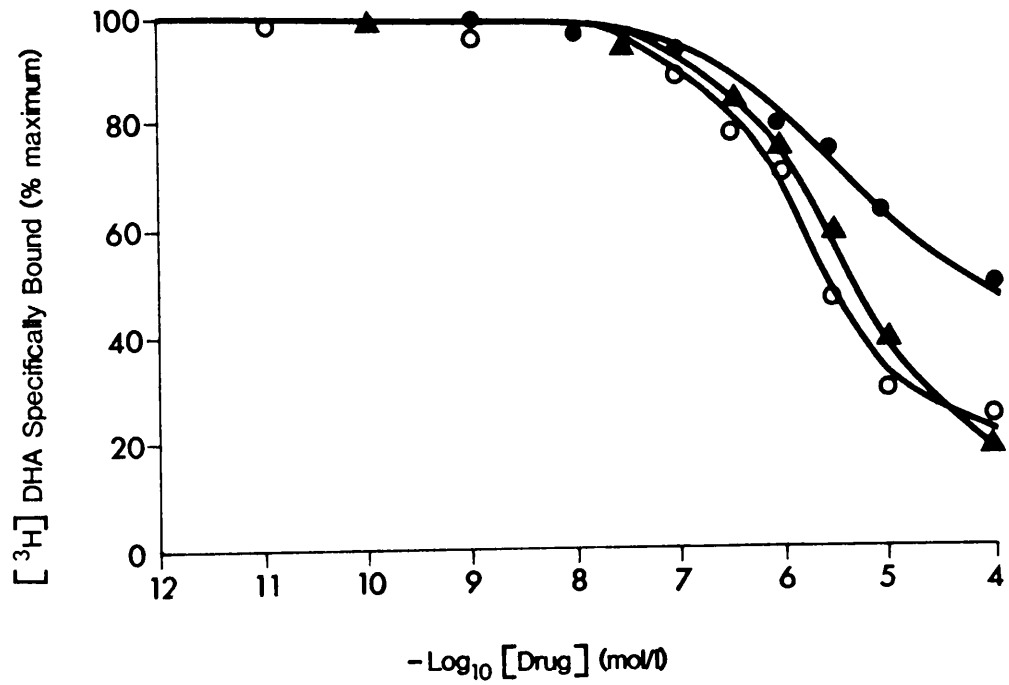
The dissociation constants (K_d) of drugs determined by competition for [^{3}H]DHA binding

Drug	Dissociation Constant (K_d)
(-)Propranolol	$4.07 \pm 1.09 \times 10^{-10}$
(+)Propranolol	$3.98 \pm 0.96 \times 10^{-8}$
H 35/25	$3.82 \pm 0.33 \times 10^{-7}$
Salbutamol	$1.10 \pm 0.15 \times 10^{-6}$
Practolol	$1.26 \pm 0.23 \times 10^{-5}$

The dissociation constant (K_d) was calculated using a least squares curve-fitting package (RECFIT), (Clark et al, 1984). Results are expressed as the Mean ± SD (3 experiments).

FIG. 17

Competition for [^{3}H]DHA binding sites by adrenergic drugs in rat-lung membranes. Various concentrations of H 35/25 (O), salbutamol (▲) and practolol (●) were incubated with rat-lung membranes and [^{3}H]DHA (4nmol/l) for 20 min. The samples were then diluted and filtered. Specific binding was determined in duplicate at each drug concentration.


[^{3}H] DHA Specifically Bound (% maximum)
100
80
60
40
20
0
12
11
10
9
8
7
6
5
4
-Log$_{10}$ [Drug] (mol/l)

lymphocytes as well as a high cell viability while using the least possible blood volume.

2.2. EXISTING METHODS

Initially using the recommended methods (Conolly and Greenacre, 1976; Williams et al, 1976b) problems were encountered with poor recoveries (30-40%) of lymphocytes and aggregation of cells was frequently observed.

By a process of elimination it transpired that the type of buffer, the presence of sodium bicarbonate ($NaHCO_3$), the presence of added protein, the dilution of cells prior to density centrifugation, the gravitational force (g) used to harvest washed cells, and the buffer pH were all critical factors. Details of these studies are set out below.

2.3. DEVELOPMENT OF THE PRESENT METHOD

Earle's Balanced Salt Solution (without $NaHCO_3$) with the supplements outlined in Table 3 gave best results, particularly when the recommended phosphate buffered saline (PBS) was replaced by Earle's medium (without $NaHCO_3$). The presence of $NaHCO_3$ in the buffers, gassed with 95% air and 5% CO_2 resulted in unstable pH values (pH 7.2-7.8) in the open test tubes leading to a fall in viability. Hepes buffer at pH 7.6 was optimal for good viability. $NaHCO_3$ itself seemed to have a deleterious effect; with 0.22% $NaHCO_3$ (recommended concentration) in the presence of Hepes at pH 7.6, recoveries fell to 30% (Table 3).

Initial studies were performed using a 10% calf serum supplement as widely reported in the literature (Harris and Ukaejiofo, 1970; Conolly and Greenacre, 1977) but the results were

TABLE 3

Isolation of human lymphocytes using different mediums

Isolation medium	Washing buffer	Recovery	Viability after resuspension	Viability after 60 min at 30 C		
				No additions	With (-) propranolol	With (+) propranolol
1. Hanks medium (with $NaHCO_3$)	PBS	44%	95%	92%	88%	90%
2. Hanks medium (without $NaHCO_3$)	PBS	50%	90%	80%	80%	80%
3. Earle's medium (with $NaHCO_3$)	PBS	30%	92%	92%	90%	90%
4. Earle's medium (without $NaHCO_3$)	PBS	70%	95%	93%	92%	92%
	Earle's medium (4)	94%	98%	96%	96%	96%

The isolation of lymphocytes was done as described (Materials and Methods, 2.1.). All the above mediums were at pH 7.6 supplemented with 20 mmol/l Hepes and 0.1% calf serum.

PBS = phosphate buffered saline.

markedly inferior to those listed above, with recoveries as low as 30-40% accompanied by marked aggregation of the lymphocytes. 0.1% calf serum added to the medium was optimal in reducing lymphocyte aggregation and essential to ensure an adequate yield of cells. Aggregation during isolation of lymphocytes was further reduced by removing the platelet-rich plasma from the initial centrifugation of whole blood and replacing it with Earle's medium, simultaneously diluting the packed cells to three times the original blood volume.

The ratio of diluted blood (suspension of erythrocytes and leucocytes) to the lymphocyte separation medium was also critical, 8 ml diluted blood to 12 ml separation medium in a 30 ml container gave minimal clumping; increasing the blood:medium ratio led to increased aggregation. After harvesting the lymphocytes it was necessary to wash them free of the viscous separation medium. Use of phosphate buffered saline or Tris buffer gave rise to aggregation and Hepes buffer was chosen in preference. It was also necessary to harvest the cells at 250g and not 1000g as recommended, to further reduce aggregation.

Since beta-adrenoceptor estimations were to be performed at 30C in the presence of various drugs, the effects of extended exposure to (+) or (-) propranolol on lymphocyte viability were also investigated for each of the culture media detailed in Table 3. In summary, Earle's medium (without $NaHCO_3$) which gave best yield and viability during lymphocyte isolation, also gave the highest viability during a 60 min incubation at 30C. The presence of (+) or (-) propranolol had no deleterious effects on lymphocyte viability (Table 3).

3. DEVELOPMENT OF LIGAND BINDING TECHNIQUES

3.1. [^{3}H]DHA BINDING ASSAY

[^{3}H]DHA has been used as a radioligand for beta-adrenoceptor binding studies in lymphocytes and polymorphonuclear cells (Williams et al, 1976b; Kariman and Lefkowitz, 1977; Brooks et al, 1979; Bismopric et al, 1980; Galant et al, 1980). However, in this study saturation analysis in human lymphocytes using [^{3}H]DHA was very difficult to perform because very low specifically bound counts were achieved and marked scatter was observed on Scatchard analysis. Of more concern is the fact that some of the above workers (Brooks et al, 1979; Galant et al, 1980) were using very high concentrations of [^{3}H]DHA (30 nmol/l, 48 nmol/l or 60 nmol/l) in their assays but during the course of this study, it has been found with tissues from rat lung is that at concentrations of [^{3}H]DHA above 20 nmol/l, this ligand appears either to solubilise in the cell membrane or to partition into membrane micelles (i.e. cannot be displaced by the degree of washing used here). This mechanism appears to be blocked by the propranolol used in the non-specific binding tubes and so the trapped ligand does not appear as non-specific binding (Clark et al, 1985).

The major disadvantage of using [^{3}H]DHA involves sensitivity. The specific activity of preparations is usually in the range of 30-70 Ci/mmol, therefore in typical membrane fractions that contain receptor binding activity at 100 fmol/mg protein, 1 mg protein would be required to obtain 2,500 cpm bound when the ligand is present at a concentration equal to its K_d (dissociation constant).

Maguire et al (1977) using [^{125}I]HYP have shown that two orders of magnitude less protein are required to obtain the same specifically bound counts as compared with [^{3}H]DHA. It has also

been confirmed that using [^{125}I]HYP in binding studies for both granulocytes and mononuclear cells offer the advantage of requiring fewer cells and therefore smaller quantities of blood (Motulsky and Insel, 1982a). Therefore for the studies in this thesis, [^{125}I]HYP offer a positive advantage when repeated estimations are to be made on a given subject.

3.2. [^{125}I]HYP BINDING ASSAY CONDITIONS

3.2.1. Assay tubes

The ligand [^{125}I]HYP adsorbs to both glass and polystyrene tubes and variability of binding from tube to tube was particularly high with the latter type. In addition, polystyrene is soluble in ethyl acetate, the solvent in which [^{125}I]HYP is stored (Maguire *et al*, 1977). Polypropylene tubes seem to be the best alternative as these retain the lowest counts of all tubes tested (glass, polystyrene and polypropylene).

3.2.2. Minimising non-specific binding

One of the problems of using [^{125}I]HYP was the lack of reproducibility and poor coefficients of variation on quadruplicate samples (Table 4, option 1). This was traced to adsorption of [^{125}I]HYP to the filters and to variability in the amount of non-specifically bound [^{125}I]HYP lost on filtering.

To overcome this the following approach was used. The addition of 0.1 mmol/l (±) propranolol to buffer B encourages the non-specifically bound [^{125}I]HYP to leave the membrane while not significantly affecting specifically bound ligand (Maguire *et al*, 1976a) and also to eliminate non-specific retention of counts on the filters and thus to equalise samples incubated with ligand

TABLE 4

Minimising non-specific binding

	Pre-soaked filter papers	Buffer B with propranolol	Pre-rinse filter papers	Filtering pressure (psi)	Total cpm ± SD	Total CV.	NSB cpm ± SD	NSB CV.
1.	N	N	N	3-4	4492 ± 448	10.0%	2534 ± 362	14.3%
2.	Y	N	N	3-4	4313 ± 749	17.0%	2622 ± 128	5.0%
3.	N	Y	N	3-4	2152 ± 240	11.0%	932 ± 142	15.3%
4.	Y	Y	N	3-4	1919 ± 26	1.3%	827 ± 45	5.4%
5.	Y	Y	Y	3-4	2089 ± 203	9.7%	899 ± 83	9.2%
6.	Y	Y	N	20	1905 ± 111	5.8%	955 ± 79	8.2%

Pre-soaked refers to presoaking the filter papers with buffer B for 60 min prior to start the assay, buffer B refers to whether or not the buffer used to terminate the reactions contained (±) propranolol, pre-rinse indicates if the filter papers were wetted with the washing buffer before filtering the samples. Y = yes, N = no, NSB = non-specific binding, cpm = count per min, SD = standard deviation, CV = coefficient of variation.

alone or radioligand plus competitors of binding (Insel and Stoolman, 1978).

Since [^{125}I]HYP also binds to the filters, these are pre-soaked for 60 min in buffer B; the (±) propranolol blocks non-specific binding of radioactivity to the filters (Galant and Allred, 1981). Also leaving the samples to stand for 45 sec before filtering allowed non-specifically bound or occluded ligand to dissociate (Sporn and Molinoff, 1976).

The results in Table 4 were obtained by setting up [^{125}I]HYP binding assays in quadruplicate to represent "total" binding and "non-specific" binding (NSB) with 0.3 µmol/l (-) propranolol. The concentration of [^{125}I]HYP was (0.4 nmol/l). The assays were terminated under permutations of the following:

1. Pre-soaked filters.
2. Buffer B contains propranolol.
3. Filter papers wetted just before filtering to see if accidentally wetting the filters interfered with the reproducibility of the assays.

The questions to be answered were:

1. Is pre-soaking filter papers with propranolol beneficial.
2. Is propranolol in buffer B benficial.
3. Is a combination better.
4. Does wetting the pre-soaked filter papers with rinsing buffer (which does not contain propranolol) have an adverse effect.
5. Is filtering at 3-4 psi better than 20 psi.

The criteria used for this crucial experiment were:

1. A low coefficient of variation for the quadruplicate observations.
2. The lowest non-specific binding (NSB).

Option 4 in Table 4, (Y, Y, N), was chosen as satisfying the above criteria, giving optimum reproducibility coupled with a good Total:NSB difference.

Other workers performed their assays in the presence of phentolamine (Pochet et al, 1979; Tohmeh and Cryer, 1980; Aarons et al, 1980) or in the presence of catechol and phentolamine (Galant et al, 1978a; Galant and Allred, 1981) to reduce non-specific binding without affecting specific binding. But at least in one case, rat hypothalamus membranes, phentolamine was found to displace [^{125}I]HYP from beta-adrenoceptors (Sporn and Molinoff, 1976). It was also noted that phentolamine, at the concentration range of 10 µmol/l to 0.1 mmol/l, can block the isoprenaline-dependent adenylate cyclase activation in neuroblastoma-glyoma hybrids (Atlas et al, 1979).

The use of phentolamine or catechol and phentolamine in the present assay system had no significant effect on the results. This is in agreement with Pochet et al (1979), who also found that the addition of phentolamine to intact lymphocyte binding assays was not crucial, as did Atlas et al (1979) who found that the effect of phentolamine is marginal and so phentolamine can be omitted from the assay.

3.2.3. Intact cells vs cell membranes

Some investigators (Williams et al, 1976b; Brooks et al, 1979; Aarons et al, 1980; Galant et al, 1980; Tohmeh and Cryer, 1980)

studied beta-adrenoceptors in lymphocyte fragments (membranes), while others (Conolly and Greenacre, 1976; Insel and Stoolman, 1978; Pochet et al, 1979; Galant and Allred, 1981; Hui et al, 1981) used intact lymphocytes.

A comparison of intact cells with cell membranes was made. A preparation of lymphocytes was halved and cell membranes were prepared using a mini-bomb cell disruption chamber. 15 ml of intact lymphocyte suspension were placed inside the disruption chamber (which was precooled by immersion in an ice bath) and exposed to 500 psi nitrogen for 45 min. During this time nitrogen is taken into individual cells. The pressure was slowly released and as the pressurised suspension was exposed to atmospheric pressure the gas expands and ruptures the cells. This adiabatic expansion was sufficient to lyse greater than 90% of the lymphocytes. The suspension was centrifuged (400g, 10 min, 4C) to remove intact cells and nuclei, and then the cell membranes were harvested from the supernatant (78000g, 30 min, 4C) and B_{max} and K_d estimated as described (Materials and Methods, 2.3.1.) for both the membranes and the original intact lymphocytes. The results are summarised in Table 5. These results show no significant difference between the number (B_{max}) and affinity (K_d) of beta-adrenoceptors for intact and disrupted lymphocytes ($p>0.1$). However, Insel and Stoolman (1978) found that the disruption of S49 lymphoma cells decreases the response of adenylate cyclase to isoprenaline and other hormones, also the coupling between receptors and cyclase appears to be altered. Another disadvantage, is that ions and nucleotides (e.g. GTP) are important modulators of receptor function, but they are largely washed away when "membranes" are prepared (Motulsky and Insel, 1982a).

TABLE 5

B_{max} and K_d values for binding of [^{125}I]HYP to intact and disrupted lymphocytes

	B_{max} (fmol/10^7 lymphocytes)	K_d (nmol/l)
Intact lymphocytes	6.25 ± 0.53	0.36 ± 0.05
Disrupted lymphocytes	6.03 ± 0.64	0.29 ± 0.06

B_{max} = total number of binding sites.

K_d = dissociation constant.

Data represent Mean ± SD (5 experiments).

Finally, the intact cell model may permit a better appreciation of the mechanism of altered beta-adrenoceptor function in several clinical entities in man than has been possible with plasma membrane preparations, which lack a comparable physiological component (Galant and Allred, 1981). Therefore intact lymphocytes were chosen for subsequent studies.

4. CHARACTERISATION OF THE LYMPHOCYTE BETA-ADRENOCEPTOR PROPERTIES

Lymphocytes were prepared as described (Materials and Methods, 2.1.) using Earle's Balanced Salt Solution (without sodium bicarbonate) supplemented with 0.1% calf serum and 20 mmol/l Hepes at pH 7.6 which gave best yield.

Final cell concentrations were usually 2.5-3.0 x 10^6 lymphocytes/ml with cell populations of approximately 90-95% lymphocytes, the remainder being monocytes. Cell viability remained greater than 95% during all the described procedures as determined by trypan blue exclusion.

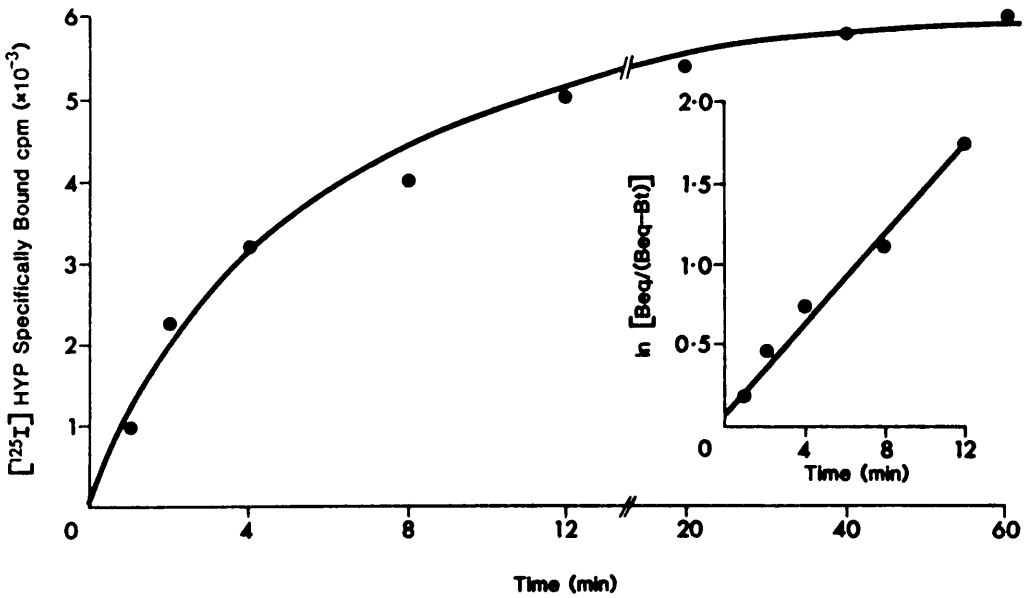
4.1. KINETIC ANALYSIS OF [^{125}I]HYP BINDING

The interaction of beta-adrenergic agents with beta-adrenoceptors is a relatively rapid and reversible process. Accordingly, a reasonable expectation is that the binding interaction of a radiolabelled adrenergic ligand with beta-adrenoceptors will be similarly rapid and reversible.

Kinetics of binding were performed as described (Materials and Methods, 2.3.2.). Specific binding of [^{125}I]HYP to human lymphocytes at 30C achieved equilibrium within 30-40 min, with half-maximal specific binding ($t_{1/2}$) of 3-4 min (Fig. 18). Binding remained stable for 60 min at 30C. A 45 min incubation time was

FIG. 18

Time course of [^{125}I]HYP binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{125}I]HYP (0.4nmol/l) in the absence and presence of 0.3µmol/l (-) propranolol. At the indicated times, the samples were diluted with buffer B and filtered. Specific binding was determined in duplicate at each time interval. (Inset) the regression line ($r = 0.98$) was determined by the plot of ln $[B_{eq}/(B_{eq} - B_t)]$ vs. time, where B_{eq} = binding at equilibrium and B_t = binding at time t. K_{ob} is equal to the slope of the line.


[125I] HYP Specifically Bound cpm (×10-3)
6
5
4
3
2
1
0
4
8
12
20
40
60
Time (min)
ln [Beq/(Beq-Bt)]
2·0
1·5
1·0
0·5
0
4
8
12
Time (min)

routinely used to represent equilibrium in subsequent binding experiments.

The initial rate constant (K_{ob}) for the association reaction obtained from the slope (Fig. 18, inset) was 0.14 min^{-1} for 0.4 nmol/l [^{125}I]HYP. Dissociation of bound [^{125}I]HYP at 30C was determined by incubating lymphocyte suspension to equilibrium, then adding 0.3 µmol/l (-) propranolol (final concentration) at time zero and measuring residual specific binding at subsequent time intervals (Fig. 19). Dissociation was rapid with a ($t_{1/2}$) of 8-10 min, and the dissociation rate constant (K_2) was 0.077 ± 0.0085 min^{-1} (Fig. 19, inset).

The association rate constant (K_1) of [^{125}I]HYP binding was then calculated from the equation: $K_1 = (K_{ob} - K_2)/[IHYP]$, where [IHYP] is equal to the concentration of [^{125}I]HYP in the assay (0.4 nmol/l), K_1 was 1.566 x 10^8 ± 0.21 x 10^8 M^{-1} min^{-1}. The equilibrium dissociation constant (K_d), determined from the ratio K_2/K_1, was 0.47 ± 0.11 nmol/l (2 experiments).

4.2. SATURABILITY OF [^{125}I]HYP BINDING

Saturation studies were carried out as described (Materials and Methods, 2.3.1.). With increasing concentrations of [^{125}I]HYP, specific binding (the binding that could be displaced by 0.3 µmol/l (-) propranolol) reached saturation at 500-600 pmol/l (Fig. 20), with half maximal binding occuring at about 150-250 pmol/l.

Scatchard analysis (Fig. 21) of the saturation data yielded a straight line, indicating that [^{125}I]HYP bound to a single class of high affinity receptors in intact lymphocytes. The total number of binding sites (B_{max}) was 6.42 ± 0.88 fmol/10^7 lymphocytes, and the dissociation constant (K_d) of the binding was 0.33 ± 0.07 nmol/l (8

FIG. 19

Time course for dissociation of [^{125}I]HYP binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{125}I]HYP (0.4nmol/l) for 45 min to allow equilibrium. At zero time, 0.3μmol/l (-) propranolol (final concentration) was added to the samples and specific binding was determined at various time intervals. 100% binding refers to the specific binding just prior to the addition of (-) propranolol. (Inset) first-order rate plot of dissociation of [^{125}I]HYP binding. K_2 is equal to the slope of the line.

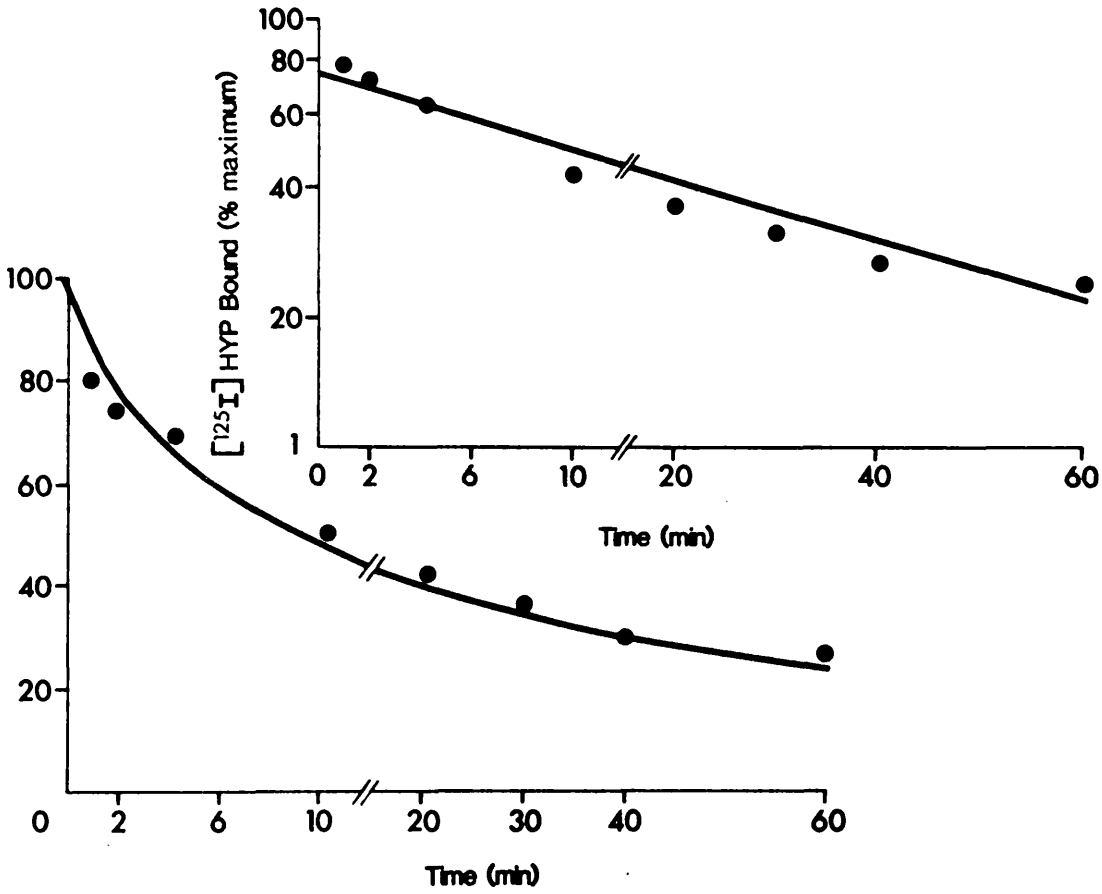

100
80
60
40
20
1
[125I] HYP Bound (% maximum)
0
2
6
10
20
40
60
Time (min)
100
80
60
40
20
0
2
6
10
20
30
40
60
Time (min)

FIG. 20

Specific binding of [^{125}I]HYP to intact human lymphocytes as a function of concentration of [^{125}I]HYP. Lymphocytes in buffer A were incubated with various concentrations of [^{125}I]HYP in the presence and absence of 0.3µmol/l (-) propranolol. Specific binding was determined at each concentration of [^{125}I]HYP.

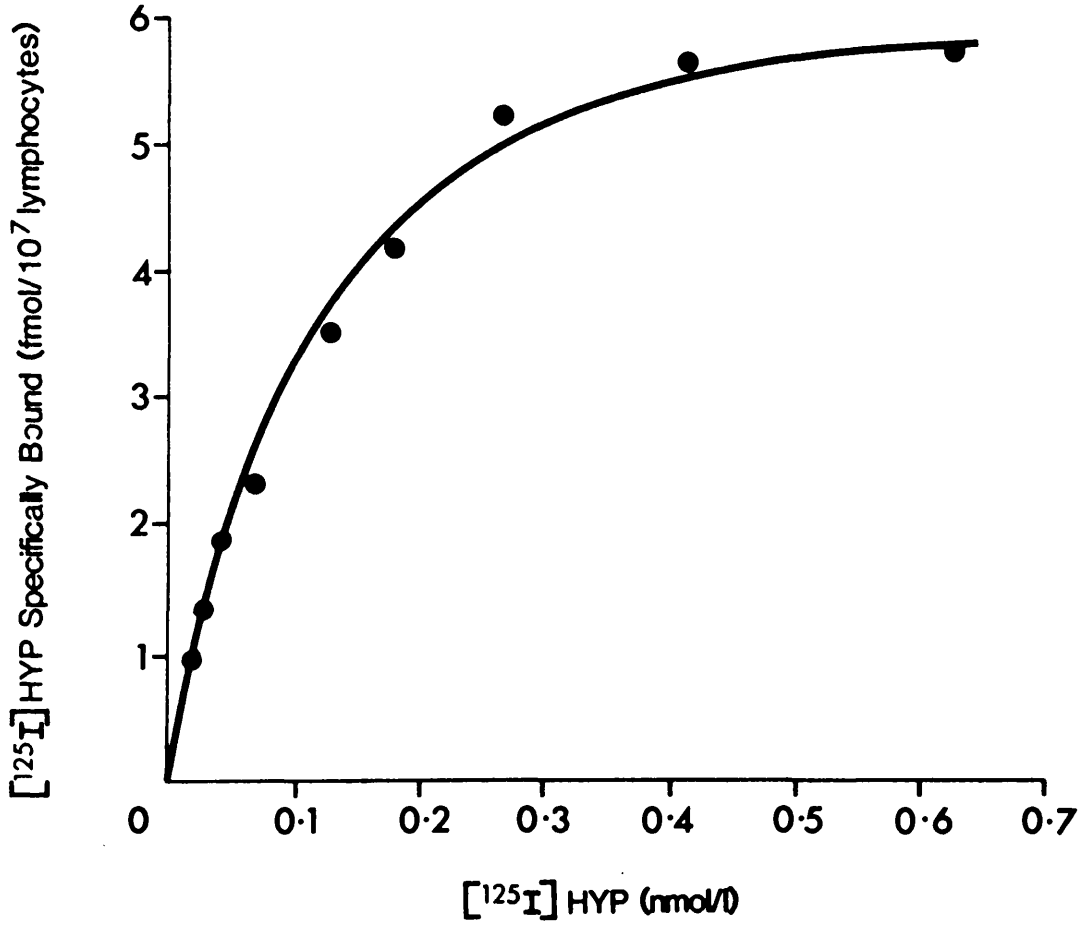
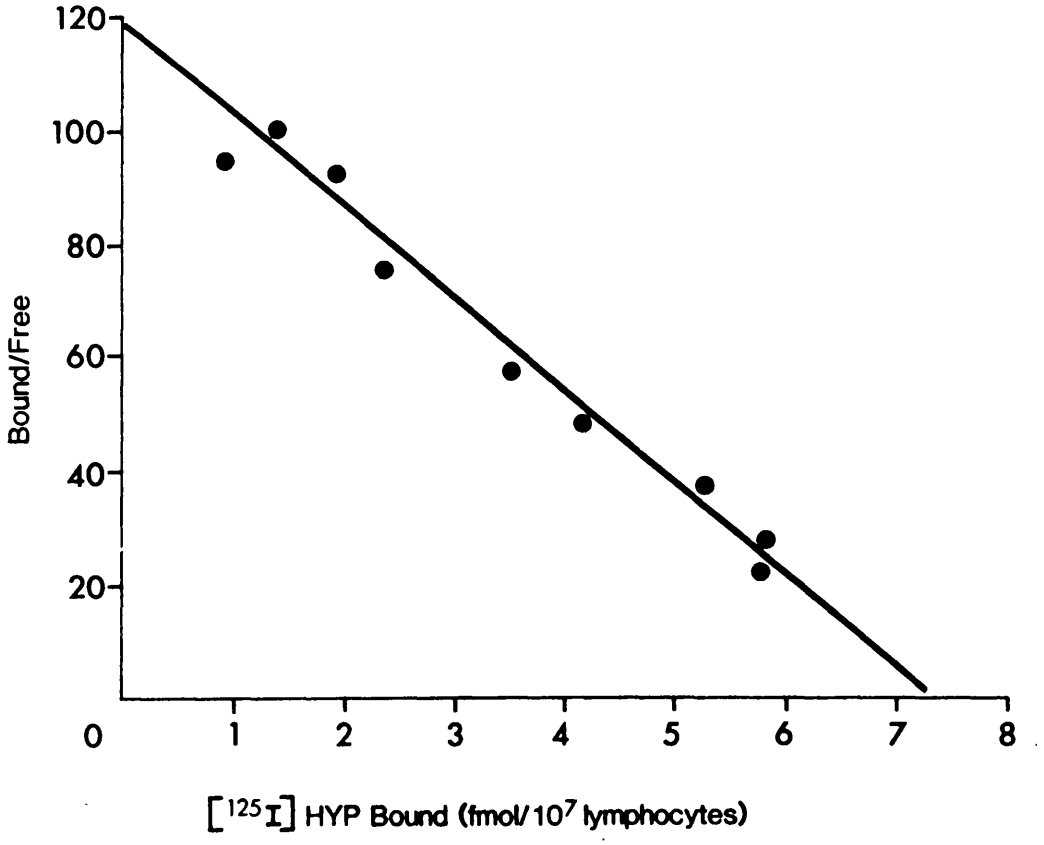

[125I]HYP Specifically Bound (fmol/10^7 lymphocytes)
6
5
4
3
2
1
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
[125I] HYP (nmol/l)

FIG. 21

Scatchard analysis of the binding data (Fig. 20) yielding a K_d of 0.27nmol/l and the total number of beta-adrenoceptors was 7.3 fmol/10^7 lymphocytes.


120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Bound/Free
[125I] HYP Bound (fmol/10^7 lymphocytes)

experiments).

4.3. DISCUSSION OF KINETIC STUDIES

The results indicated that the specific binding of [^{125}I]HYP, which represented 70-75% of the total binding, to intact human lymphocytes was consistant with the criteria expected of ligand interaction at a beta-adrenoceptor. [^{125}I]HYP binding was rapid, reversible and saturable with a K_d, estimated from equilibrium, of 0.33 ± 0.07 nmol/l which was compatible with those of other workers (Paietta et al, 1982) using [^{125}I]HYP as binding ligand and also in agreement with the K_d of 0.47 ± 0.11 nmol/l calculated from kinetic data.

4.4. COMPETITION FOR [^{125}I]HYP BINDING SITES BY ADRENERGIC COMPOUNDS

Competition of beta-adrenergic compounds with [^{125}I]HYP were performed and repeated three times as described (Materials and Methods, 2.3.3.). These studies indicated both stereoselectivity (Fig. 22), where the (-) isomer of propranolol was two orders of magnitude more potent in inhibiting [^{125}I]HYP binding than the (+) isomer, and a rank order of competitors typical of beta$_2$-adrenoceptors. Beta$_2$ specificity was shown by rank order potency of [^{125}I]HYP inhibition by salbutamol, a relatively specific beta$_2$-adrenergic agonist, which was about 25-fold more potent than practolol, a beta$_1$-adrenergic antagonist (Fig. 22, Table 6). The dissociation constants (K_d) for the inhibition of [^{125}I]HYP binding for the various competing drugs were calculated using a non-linear least squares curve fitting package (RECFIT, Clark et al, 1984). The results indicated only a one site fit (i.e. beta$_2$), for both

FIG. 22

Competition for [^{125}I]HYP binding sites by adrenergic drugs in intact human lymphocytes. Various concentrations of (-) propranolol (●), (+) propranolol (▲), salbutamol (■) and practolol (○) were incubated with lymphocytes and [^{125}I]HYP (0.4nmol/l) for 45 min. The samples were then diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

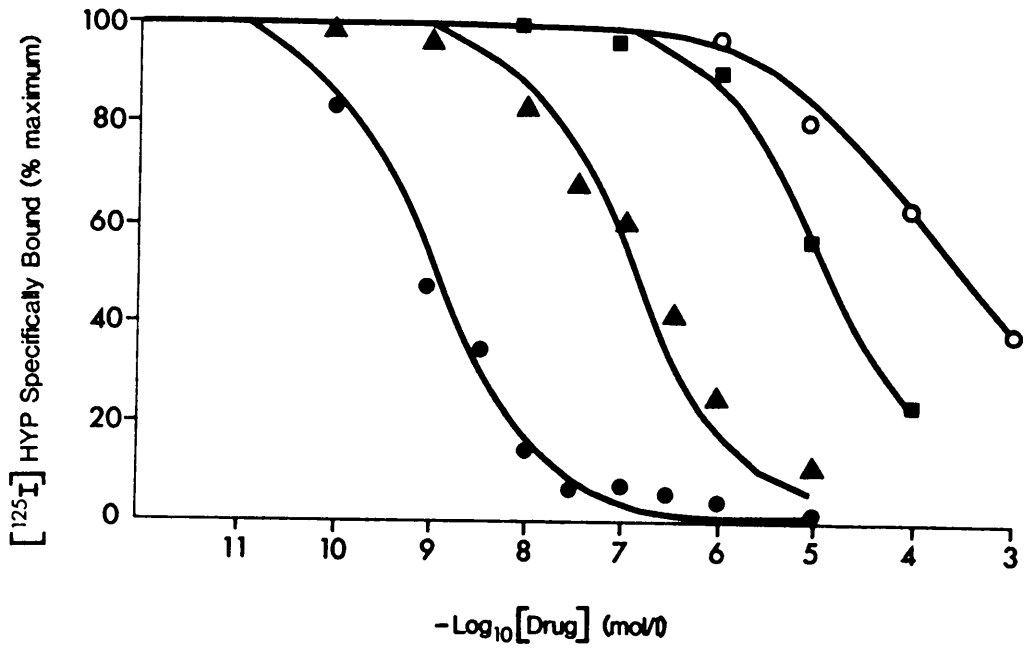

100
80
60
40
20
0
[125I] HYP Specifically Bound (% maximum)
11
10
9
8
7
6
5
4
3
-Log10[Drug] (mol/l)

TABLE 6

The dissociation constants (K_d) of drugs determined by competition for [^{125}I]HYP binding

Drug	Dissociation Constant (K_d)
(-)Propranolol	$5.45 \pm 1.66 \times 10^{-10}$
(+)Propranolol	$6.68 \pm 1.84 \times 10^{-8}$
Salbutamol	$5.68 \pm 1.69 \times 10^{-6}$
Practolol	$1.42 \pm 0.22 \times 10^{-4}$

The dissociation constant (K_d) was calculated using a least squares curve-fitting package (RECFIT), (Clark *et al*, 1984). Results are expressed as the Mean ± SD (4 experiments).

salbutamol and practolol, with no detectable contamination from beta$_1$-adrenoceptors. The derivation of this confirmation is explained elsewhere (Clark et al, 1984).

5.CHARACTERISATION OF THE LYMPHOCYTE ALPHA-ADRENOCEPTOR PROPERTIES

Radioligands have been successfully used to label alpha-adrenoceptors in different tissues (Williams and Lefkowitz, 1978b; Begin-Heick et al, 1979; Holck et al, 1979; Tsai and Lefkowitz, 1979; Carman-Krzan, 1980). In the last few years it become clear that there are two subtypes of alpha-adrenoceptors, termed alpha$_1$ and alpha$_2$ receptors (Berthelsen and Pettinger,1977).

Using radioligand binding techniques, two different approaches have been applied to characterise the presence of alpha$_1$ and alpha$_2$ receptors. The first involves the use of a non-selective radioligand such as [^{3}H]dihydroergocryptine ([^{3}H]DHE) to label the entire alpha receptor population, then distinguishing between the alpha receptor subtypes by constructing competition curves using antagonist compounds which have selectively greater potency for one or the other alpha subtype (Miach et al, 1978; Hoffman et al, 1979; Hoffman and Lefkowitz, 1980a; Hoffman et al, 1980a,b).

The second, the more useful and widely applied criterion for distinguishing between alpha receptor subtypes, is by using radioligands which themselves selectively label either alpha$_1$ or alpha$_2$ receptors (Barnes et al, 1979; Greengrass and Bremner, 1979; Karliner et al, 1979; Daiguji et al, 1981; Motulsky and Insel, 1982b; Hoffman et al, 1981; Tharp et al, 1981; Mukherjee, 1981; Motulsky et al, 1980; Engfeldt et al, 1983). [^{3}H]prazosin is selective for alpha$_1$ receptors whereas [^{3}H]yohimbine is selective

for alpha$_2$ receptors. Because no other worker have characterised alpha$_2$-adrenoceptors on human lymphocytes, the following methods were developed.

5.1. KINETIC ANALYSIS OF [^{3}H]YOHIMBINE BINDING

Kinetics of binding were performed as described (Materials and Methods, 2.4.2.). Binding of [^{3}H]yohimbine to human lymphocytes at 25C was rapid, with half-maximal specific binding ($t_{1/2}$) of 1.5-2.0 min (Fig. 23), reaching equilibrium within 16-20 min.

Binding remained stable for 30 min at 25C. An incubation time of 25 min was chosen to represent equilibrium binding in subsequent experiments. The initial rate constant (K_{ob}) for the association reaction obtained from the slope (Fig. 23, inset) was 0.32 min^{-1} for 5 nmol/l [^{3}H]yohimbine. Dissociation of bound [^{3}H]yohimbine at 25C was determined by incubating lymphocytes to equilibrium and then adding 10 µmol/l phentolamine (final concentration) at time zero and measuring residual specific binding at subsequent time intervals (Fig. 24). Dissociation was rapid with a $t_{1/2}$ of 5.0-6.0 min (Fig. 24) and the dissociation rate constant (K_2) was 0.127 ± 0.016 min^{-1} (Fig. 24, inset).

The association rate constant (K_1) was then calculated from the equation: $K_1 = (K_{ob} - K_2)/[\text{yohimbine}]$, where [yohimbine] is equal to the concentration of [^{3}H]yohimbine in the assay (5 nmol/l), K_1 was 0.386 x 10^8 ± 0.027 x 10^8 M^{-1} min^{-1}. The equilibrium dissociation constant (K_d), determined from the ratio of K_2/K_1, was 3.49 ± 0.39 nmol/l (3 experiments).

FIG. 23

Time course of [^{3}H]yohimbine binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{3}H]yohimbine (5nmol/l) in the absence and presence of 10µmol/l phentolamine. At the indicated times, the samples were diluted with buffer C and filtered. Specific binding was determined in duplicate at each time interval. (Inset) the regression line ($r = 0.99$) was determined by the plot of $\ln [B_{eq}/(B_{eq} - B_t)]$ vs. time, where B_{eq} = binding at equilibrium and B_t = binding at time t. K_{ob} is equal to the slope of the line.

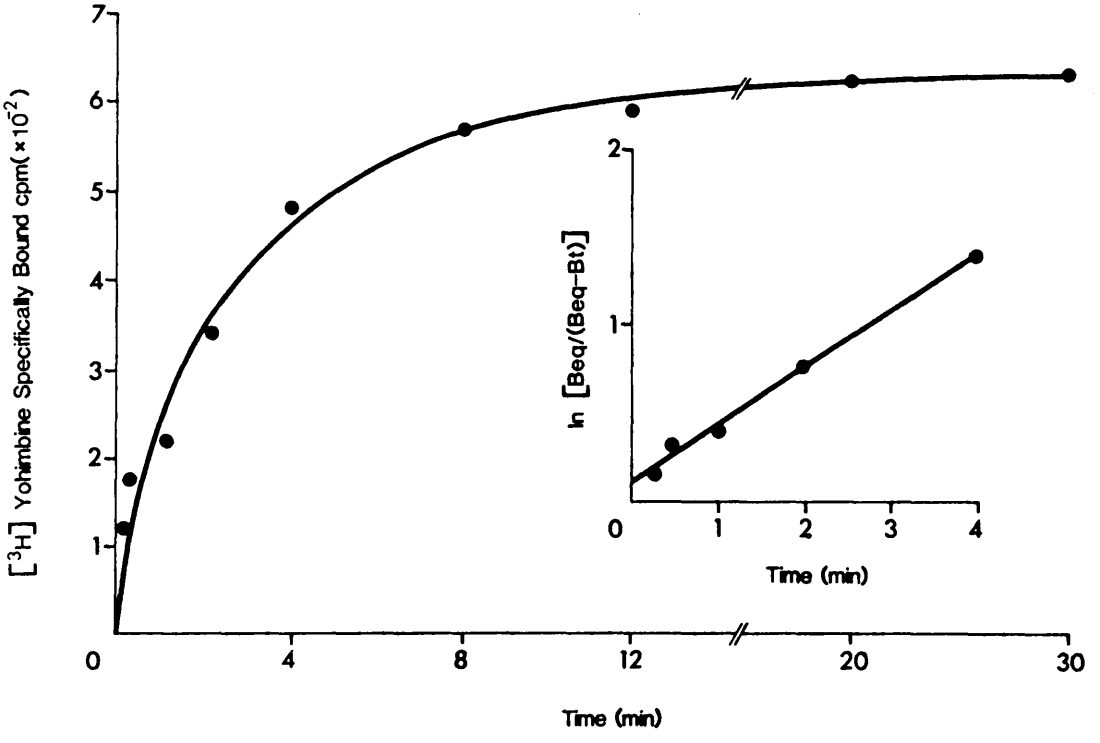
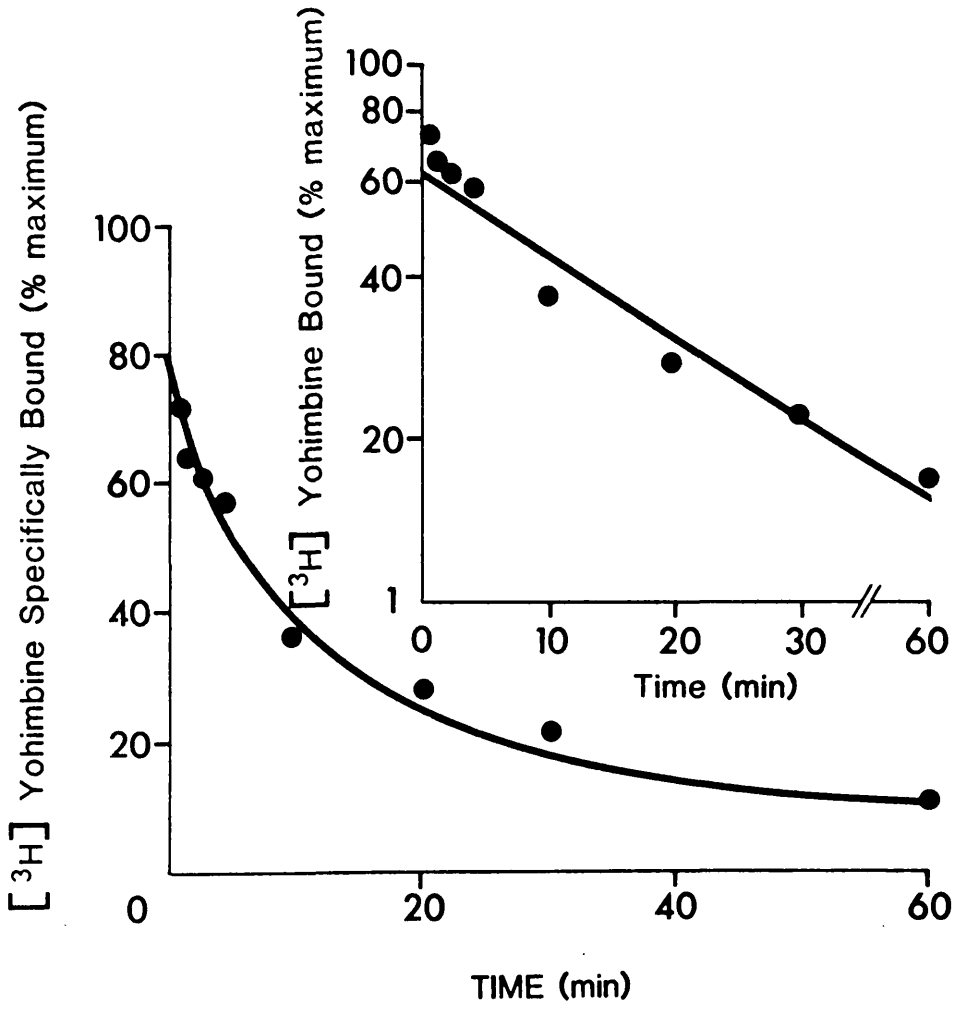

[3H] Yohimbine Specifically Bound cpm(×10-2)
7
6
5
4
3
2
1
0
4
8
12
20
30
Time (min)
ln [Beq/(Beq-Bt)]
2
1
0
1
2
3
4
Time (min)

FIG. 24

Time course for dissociation of [^{3}H]yohimbine binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{3}H]yohimbine (5nmol/l) for 25 min to allow equilibrium. At zero time, 10μmol/l phentolamine (final concentration) was added to the samples and specific binding was determined at various time intervals. 100% binding refers to the specific binding just prior to the addition of phentolamine. (Inset) first-order rate plot of dissociation of [^{3}H]yohimbine binding. K_2 is equal to the slope of the line.


[3H] Yohimbine Specifically Bound (% maximum)
100
80
60
40
20
0
20
40
60
TIME (min)
[3H] Yohimbine Bound (% maximum)
100
80
60
40
20
1
0
10
20
30
60
Time (min)

5.2. SATURABILITY OF [^{3}H]YOHIMBINE BINDING

Saturation studies were carried out as described (Materials and Methods, 2.4.1.). The binding characteristics of [^{3}H]yohimbine to intact human lymphocytes is shown in Fig. 25. Specific binding of [^{3}H]yohimbine (the binding that could be displaced by 10 μmol/l phentolamine) was clearly saturable and of high affinity.

Apparent saturation of binding sites seemed to occur at [^{3}H]yohimbine concentration of 14-16 nmol/l, with half-maximal binding occuring at about 2-3 nmol/l. Scatchard analysis (Fig. 25, inset) of the saturation data yielded a straight line, indicating that [^{3}H]yohimbine bound to a single class of receptors. The dissociation constant (K_d) of binding was 3.7 ± 0.86 nmol/l (8 experiments) and the total number of binding sites was 19.9 ± 5.3 fmol/10^7 lymphocytes.

5.3. DISCUSSION OF KINETIC STUDIES

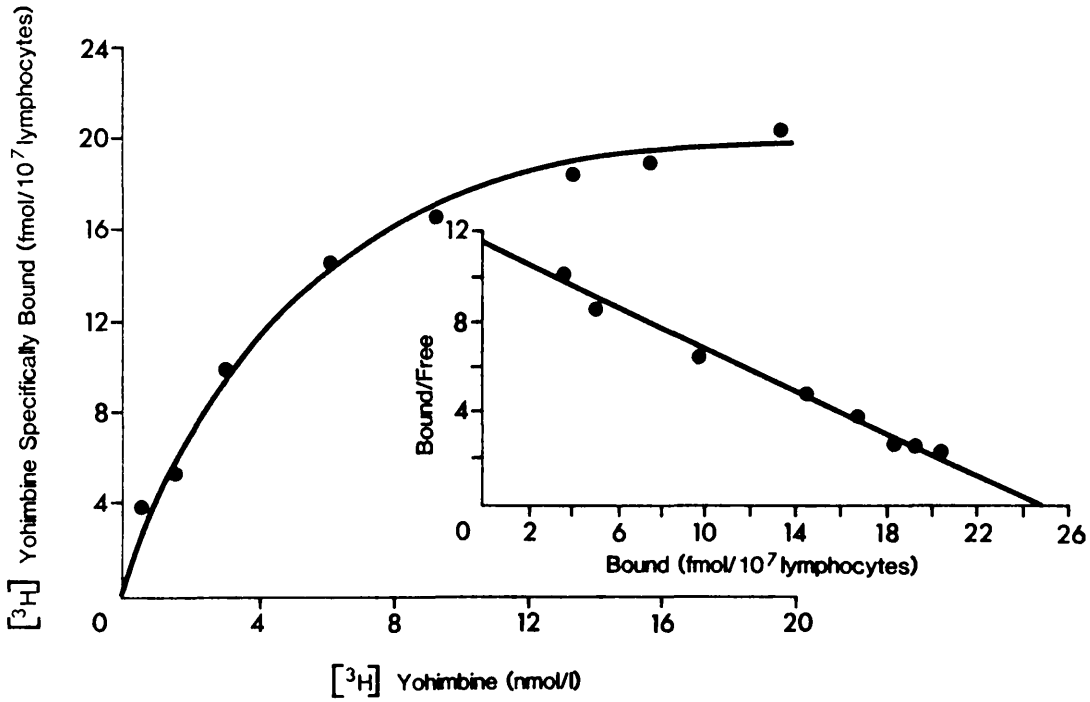
The results indicated that the specific binding of [^{3}H]yohimbine, which represented 70-80% of the total binding, was rapid, reversible and saturable. The dissociation constant of [^{3}H]yohimbine (3.7 ± 0.86 nmol/l) established at equilibrium was in agreement with the K_d of 3.49 ± 0.39 nmol/l determined from kinetic data (i.e. ratio of association and dissociation rate constants). Similar dissociation constants have been observed for human platelets (Motulsky *et al*, 1980) and for human adipocyte membranes (Tharp *et al*, 1981).

5.4. COMPETITION FOR [^{3}H]YOHIMBINE BINDING SITES BY ADRENERGIC COMPOUNDS

Competition studies of adrenergic compounds with [^{3}H]yohimbine were

FIG. 25

Specific binding of [^{3}H]yohimbine to intact human lymphocytes as a function of concentration of [^{3}H]yohimbine. Lymphocytes in buffer A were incubated with various concentrations of [^{3}H]yohimbine in the presence and absence of 10μmol/l phentolamine. Specific binding was determined at each concentration of [^{3}H]yohimbine. (Inset) Scatchard analysis of the binding data yielding a K_d of 4.24nmol/l and the total number of receptors was 24.8 fmol/10^7 lymphocytes.


[3H] Yohimbine Specifically Bound (fmol/10^7 lymphocytes)
24
20
16
12
8
4
0
4
8
12
16
20
[3H] Yohimbine (nmol/l)
Bound/Free
12
8
4
0
2
6
10
14
18
22
26
Bound (fmol/10^7 lymphocytes)

performed and repeated three times as described (Materials and Methods, 2.4.3.).

Binding of [^{3}H]yohimbine was stereospecifically inhibited by adrenaline (Table 7); the (-) isomer was more potent than the (+) stereoisomer. Adrenergic agonists competed for [^{3}H]yohimbine binding sites with a potency order of clonidine > (-) adrenaline > (-) noradrenaline > (+) adrenaline >> (-) isoprenaline, with K_d values of 0.28, 1.50, 6.74 and 16.00 µmol/l for clonidine, (-) adrenaline, (-) noradrenaline and (+) adrenaline respectively (Fig. 26 and Table 7). The beta-adrenergic agonist, (-) isoprenaline, was a very weak inhibitor of [^{3}H]yohimbine binding (K_d = 126.00 µmol/l, Fig. 26 and Table 7).

The alpha-adrenergic antagonists phentolamine, yohimbine and prazosin competed for [^{3}H]yohimbine binding sites with a potency order of yohimbine > phentolamine > prazosin (Fig. 27 and Table 7). Yohimbine, an alpha$_2$-adrenergic antagonist competed for the binding sites with a K_d of 0.01 µmol/l. Phentolamine, a non-selective alpha-adrenergic antagonist competed with a K_d of 0.03 µmol/l. Prazosin, a specific alpha$_1$-adrenergic antagonist, competed with a K_d of 2.00 µmol/l.

The dissociation constants (K_d) for the interaction of these various competing unlabelled adrenergic agents with [^{3}H]yohimbine binding sites were calculated from the concentration of these agents required to inhibit 50% of the specific [^{3}H]yohimbine binding based on the equation of Cheng and Prusoff (1973), taking into account the concentration of the ligand [^{3}H]yohimbine in the assay and the dissociation constant of [^{3}H]yohimbine determined from binding experiments. Moreover, the dissociation constants for these adrenergic compounds (Table 7) were in accord with data

TABLE 7

The dissociation constants (K_d) of drugs determined by competition for [^{3}H]yohimbine binding.

Drug	Dissociation Constant (K_d, μmol/l)
Clonidine	0.28 ± 0.027
(-)Adrenaline	1.51 ± 0.15
(+)Adrenaline	16.20 ± 1.3
(-)Noradrenaline	6.74 ± 0.65
(-)Isoprenaline	126.00 ± 12.0
Yohimbine	0.01 ± 0.0011
Phentolamine	0.03 ± 0.0028
Prazosin	2.10 ± 0.2

The dissociation constant (K_d) was calculated according to the equation of Cheng and Prusoff (1973). Results are expressed as the Mean ± SD (4 experiments).

FIG. 26

Competition for [^{3}H]yohimbine binding sites by adrenergic agonists in intact human lymphocytes. Various concentrations of clonidine (▲), (-) adrenaline (O), (-) noradrenaline (Δ) and (-) isoprenaline (●) were incubated with lymphocytes and [^{3}H]yohimbine (5nmol/l) for 25 min. The samples were then diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

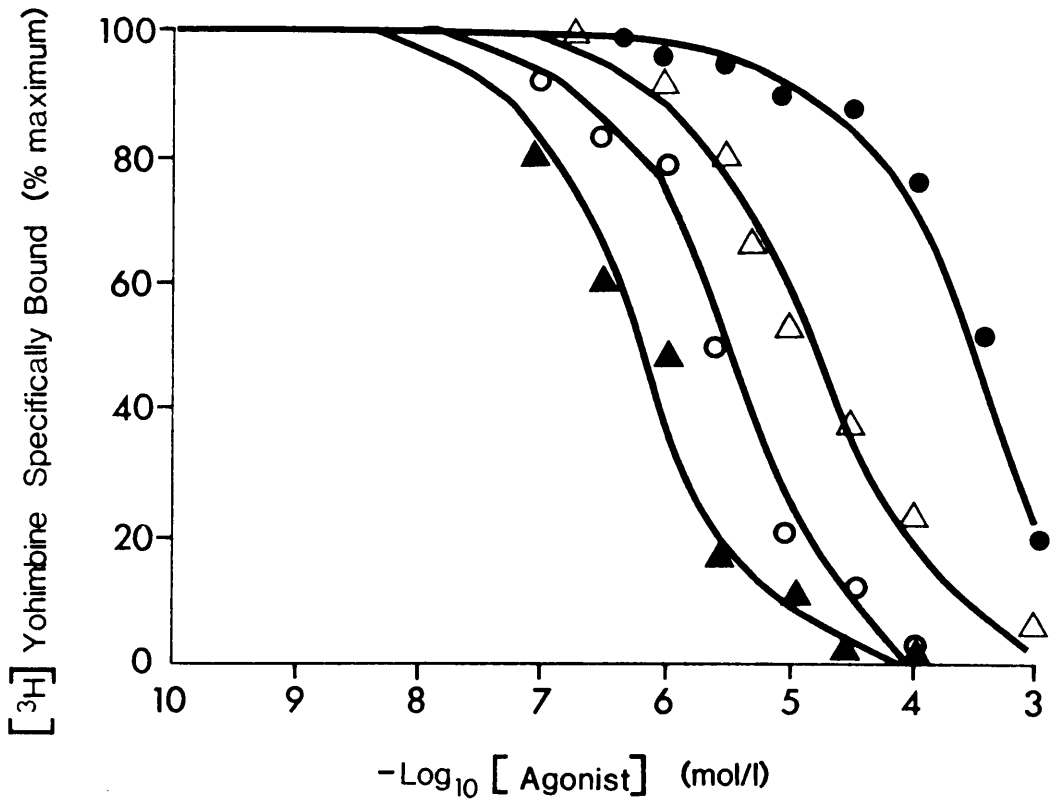
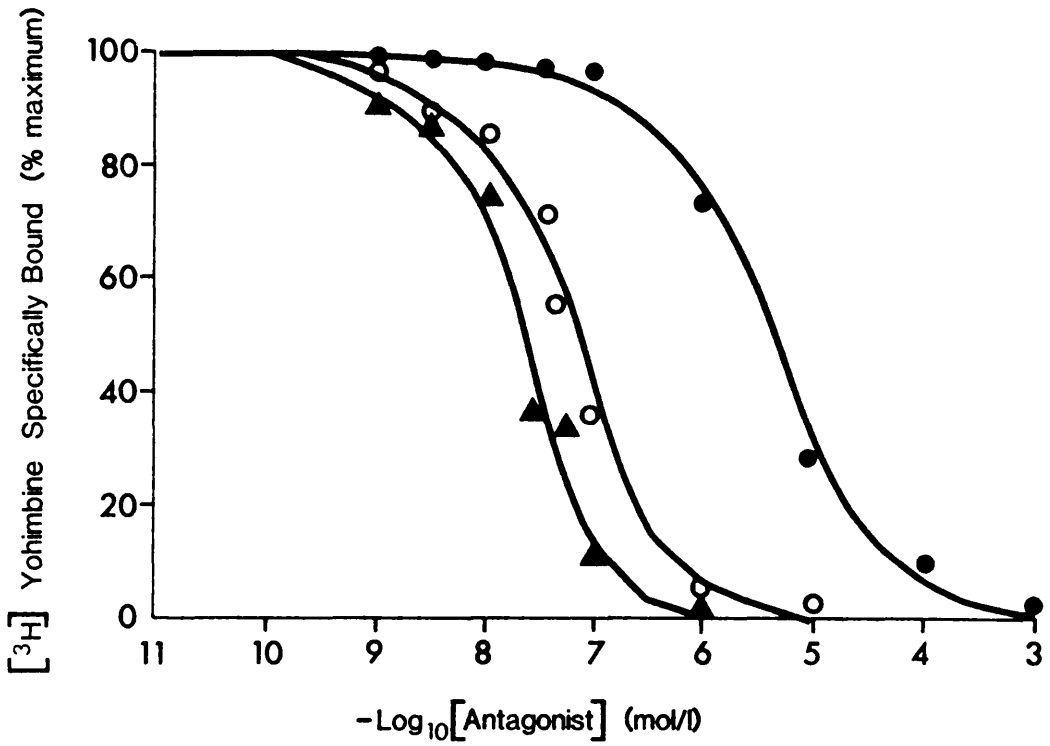

[3H] Yohimbine Specifically Bound (% maximum)
100
80
60
40
20
0
10
9
8
7
6
5
4
3
-Log10 [Agonist] (mol/l)

FIG. 27

Competition for [^{3}H]yohimbine binding sites by adrenergic antagonists in intact human lymphocytes. Various concentrations of yohimbine (▲), phentolamine (O) and prazosin (●) were incubated with lymphocytes and [^{3}H]yohimbine (5nmol/l) for 25 min. The samples were then diluted and filtered. Specific binding was determined in duplicate at each drug concentration.


[3H] Yohimbine Specifically Bound (% maximum)
100
80
60
40
20
0
11
10
9
8
7
6
5
4
3
-Log10[Antagonist] (mol/l)

reported by Motulsky *et al* (1980) for human platelets.

Therefore these observations indicate the presence of putative alpha$_2$-adrenoceptors on human lymphocytes.

RESULTS OF THE CLINICAL STUDIES

Beta-adrenoceptor number (B_{max})

Both normal and asthmatic groups not on salbutamol showed a marked circadian variation in the number (B_{max}) of $beta_2$-adrenoceptors found on lymphocytes ($p<0.05$, Table 8) with the bigest number of receptors at 1800 h. This circadian variation is not abolished on exposure to salbutamol. Representative saturation curves for a normal subject and an asthmatic patient, both on salbutamol, are shown in Figs 28 and 29 with the corresponding Scatchard analyses in Figs 30 and 31.

In both groups, the number of receptors at 0800 h on salbutamol is significantly less than found when salbutamol was not being taken ($p<0.05$) showing that "down regulation" of the receptors is induced by salbutamol. This "down regulation" is not observed for the 1800 h samples.

For comparable regimens (i.e. off or on salbutamol/time combinations), no significant difference in receptor number was observed between asthmatic patients and normals.

$Beta_2$-adrenoceptor number (B_{max}) has been expressed in terms of fmol/10^7 lymphocytes instead of fmol/mg protein, where although there was a correlation (corr = -35.9%, $p<0.05$, Fig. 32) between the amount of protein in lymphocytes compared with lymphocyte numbers in pooled data from all observations, there was a large degree of scatter which is not acceptable when evaluating individual results. This emphasises the importance of expressing results in terms of cell numbers rather than cell protein as is often done.

TABLE 8

Mean values (± SD) of beta-adrenoceptor number (B_{max}) in five control and five asthmatic subjects

	Time (h)	B_{max} (fmol/10^7 lymphocytes)	
Control (5)	1800	9.02 ± 2.21	*
Not on salbutamol	0800	5.40 ± 1.16	
On salbutamol	1800	7.18 ± 2.01	**
	0800	2.01 ± 1.74	
Asthmatic (5)	1800	6.86 ± 2.72	**
Not on salbutamol	0800	3.40 ± 1.50	
On salbutamol	1800	5.12 ± 1.48	**
	0800	1.28 ± 0.41	

* $p<0.05$

** $p<0.01$

In addition, the comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference.

FIG. 28

[^{125}I]HYP binding to intact lymphocytes of a control subject while on the salbutamol regimen at 1800 h (day 2,●) and 0800 h (day 3,▲).

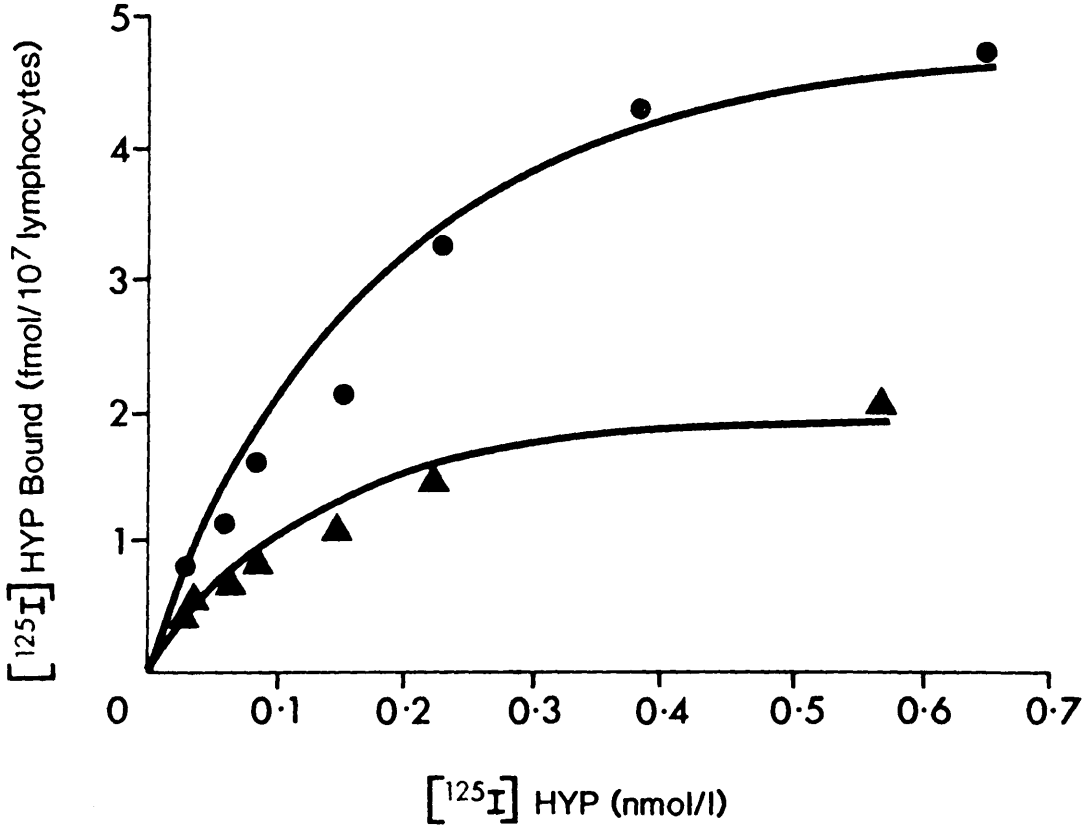

[125I] HYP Bound (fmol/10^7 lymphocytes)
5
4
3
2
1
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
[125I] HYP (nmol/l)

FIG. 29

[^{125}I]HYP binding to intact lymphocytes of an asthmatic patient while on the salbutamol regimen at 1800 h (day 2,●) and 0800 h (day 3,▲).

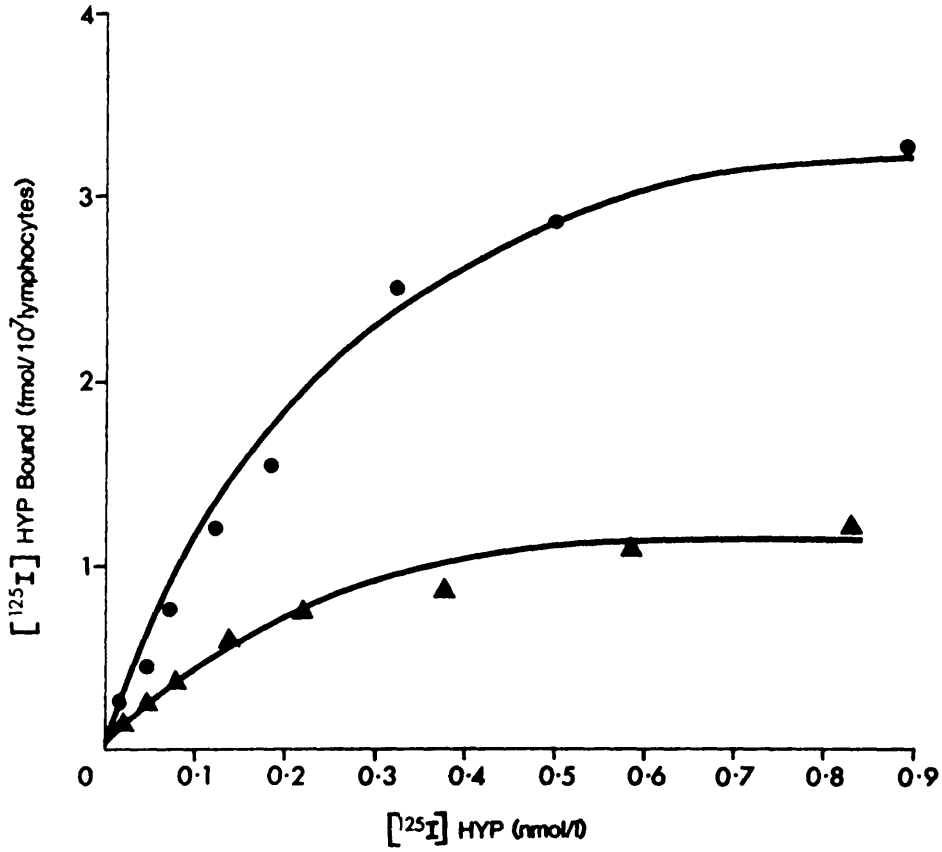

4
3
2
1
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
[125I] HYP Bound (fmol/10⁷lymphocytes)
[125I] HYP (nmol/l)

FIG. 30

Scatchard analysis of the saturation data from Fig. 28: 1800 h (day 2,●) and 0800 h (day 3,▲).

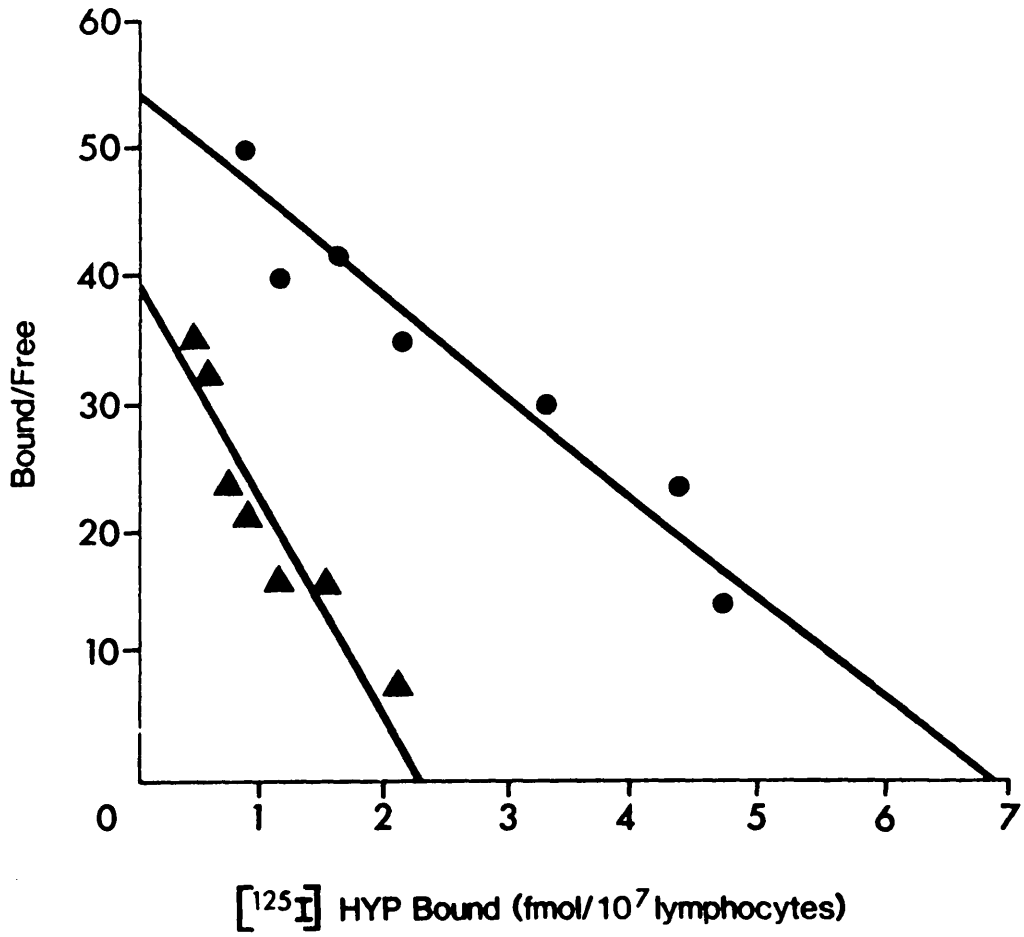

60
50
40
30
20
10
0
1
2
3
4
5
6
7
Bound/Free
[125I] HYP Bound (fmol/10^7 lymphocytes)

FIG. 31

Scatchard analysis of the saturation data from Fig. 29: 1800 h (day 2,●) and 0800 h (day 3,▲).

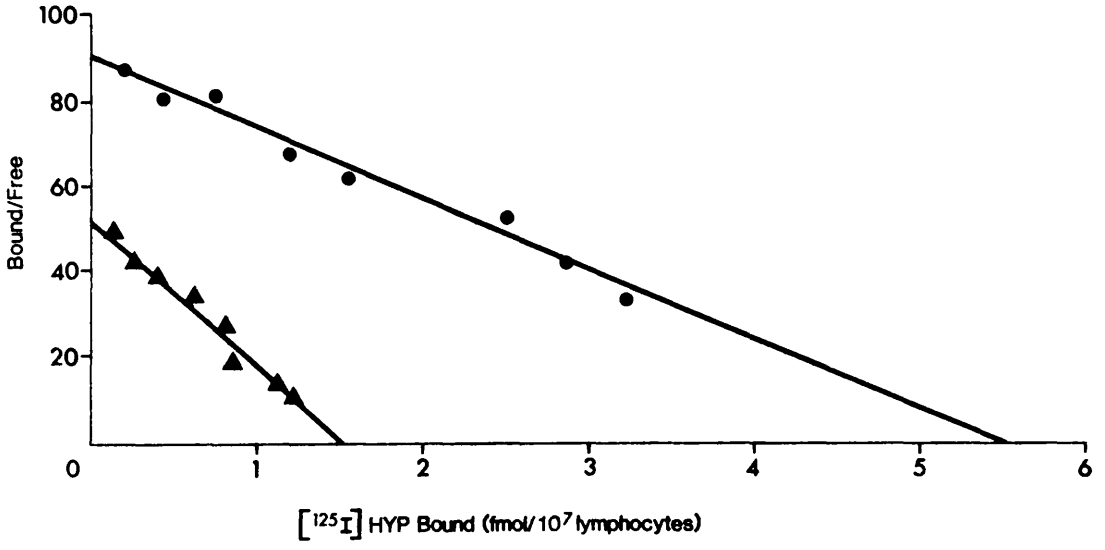
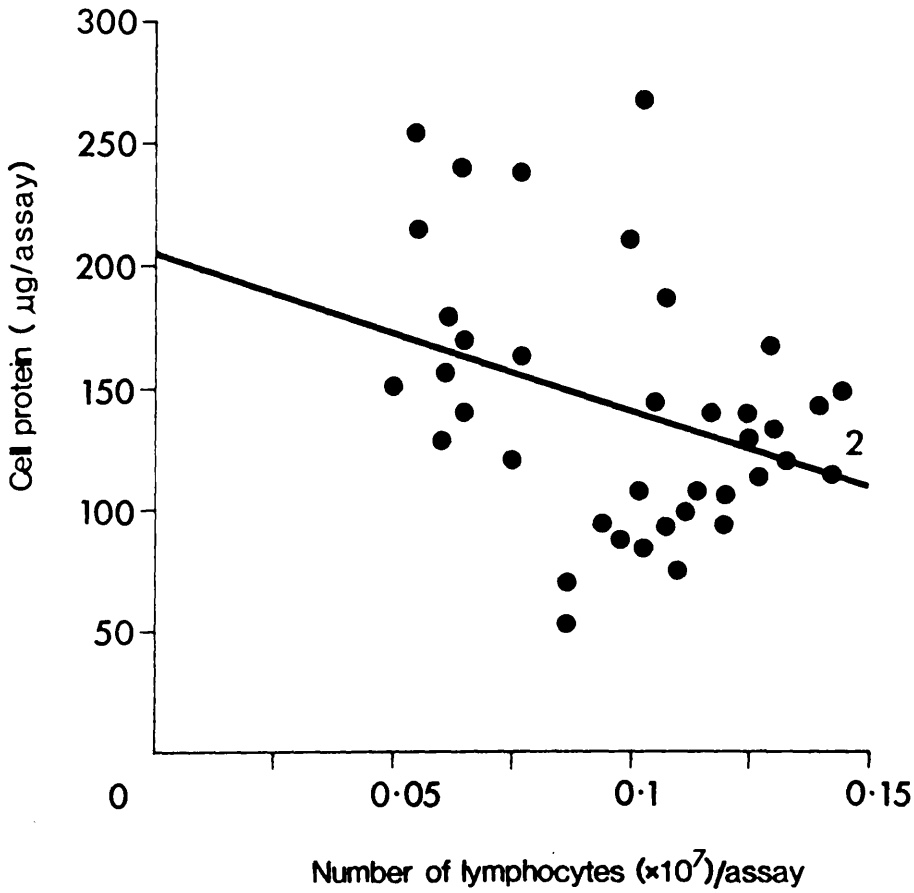

100
80
60
40
20
0
1
2
3
4
5
6
Bound/Free
[125I] HYP Bound (fmol/10^7 lymphocytes)

FIG. 32

Plot of lymphocyte protein vs. lymphocyte number.


300
250
200
150
100
50
0
0·05
0·1
0·15
Cell protein (µg/assay)
Number of lymphocytes (×10⁷)/assay
2

Beta-adrenoceptor affinity (K_d)

In normals, there was no significant difference in the receptor affinity (K_d) between 1800 h and 0800 h while off salbutamol, but on salbutamol a circadian variation appeared with the K_d significantly lower ($p<0.05$, Fig. 30) (i.e. the receptor is more sensitive) at 0800 h. Receptor affinity at 1800 h was not significantly affected by the administration of salbutamol.

In asthmatic patients, a circadian variation in K_d was present both off and on salbutamol ($p<0.05$). The administration of salbutamol neither significantly altered receptor affinity at 0800h nor at 1800 h nor abolishes the existing circadian variation.

With the exception of K_d estimation at 0800 h off salbutamol, where the K_d for asthmatic patients was significantly lower ($p<0.01$, Table 9) than normals, the K_d's of both groups at comparable times were not significantly different.

FEV_1/FVC ratio

In the normal group no circadian variation in FEV_1/FVC ratio was observed, (Table 10) either off or on salbutamol therapy. Asthmatic patients not receiving salbutamol showed the expected circadian variation in FEV_1/FVC ratio, giving rise to the "morning-dip" in FEV_1/FVC ratio ($p<0.05$) which was abolished on giving salbutamol therapy.

For comparable regimens, there was no significant difference in FEV_1/FVC ratios between the two groups except at 0800 h off salbutamol when the FEV_1/FVC ratio for asthmatic patients was significantly lower than the normals ($p<0.001$), this corresponding to the "morning-dip" in the asthmatic patients.

TABLE 9

Mean values (± SD) of beta-adrenoceptor affinity (K_d) in five control and five asthmatic subjects

	Time (h)	K_d (nmol/l)	
Control (5)	1800	0.49 ± 0.23	n.s.
Not on salbutamol	0800	0.35 ± 0.11	
On salbutamol	1800	0.46 ± 0.14	*
	0800	0.21 ± 0.19	
Asthmatic (5)	1800	0.31 ± 0.04	**
Not on salbutamol	0800	0.14 ± 0.06	
On salbutamol	1800	0.42 ± 0.15	*
	0800	0.23 ± 0.11	

* $p<0.05$

** $p<0.01$

n.s., not significant

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference except at 0800 h off salbutamol.

TABLE 10

Mean values (± SD) of FEV_1/FVC (%) in five control and five asthmatic subjects

	Time (h)	FEV_1/FVC (%)	
Control (5)	1800	83.2 ± 7.2	n.s.
Not on salbutamol	0800	82.9 ± 7.9	
On salbutamol	1800	86.2 ± 6.8	n.s.
	0800	83.9 ± 5.8	
Asthmatic (5)	1800	69.8 ± 14.1	*
Not on salbutamol	0800	51.9 ± 10.3	
On salbutamol	1800	71.8 ± 14.4	n.s.
	0800	67.5 ± 19.4	

* $p<0.05$

n.s., not significant

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference except at 0800 h off salbutamol.

Plasma cortisol concentrations

Plasma cortisol concentrations showed the expected circadian variation in both normals and asthmatic patients ($p<0.05$, Table 11) off and on salbutamol. Administration of salbutamol did not significantly affect plasma cortisol concentrations in either group.

For comparable regimens there was no significant difference in plasma cortisol concentrations between the normal and asthmatic groups.

STUDY II

Alpha$_2$-adrenoceptor number (B_{max})

No circadian variation in alpha$_2$-adrenoceptor number was observed in either normals or asthmatic patients whether off or on salbutamol therapy (Table 12). Administration of salbutamol did not significantly affect the number of alpha$_2$-adrenoceptors in either group.

For comparable regimens (i.e. off or on salbutamol/time combinations) there was no significant difference in alpha$_2$-adrenoceptor number between normals and asthmatic patients.

Alpha$_2$-adrenoceptor affinity (K_d)

In both normals and asthmatic patients, there was no significant difference in the alpha$_2$-adrenoceptor affinity between 1800 h and 0800 h while off or on salbutamol (Table 13), and again administration of salbutamol did not significantly alter receptor affinity at either times.

For comparable regimens, no significant difference was observed in alpha$_2$-adrenoceptor affinity between normals and asthmatic

TABLE 11

Mean values (± SD) of cortisol concentrations in five control and five asthmatic subjects

	Time (h)	Cortisol (µg/dl)	
Control (5)	1800	8.3 ± 1.7	*
Not on salbutamol	0800	15.1 ± 3.3	
On salbutamol	1800	9.8 ± 3.3	*
	0800	18.1 ± 2.7	
Asthmatic (5)	1800	11.8 ± 5.7	**
Not on salbutamol	0800	21.0 ± 8.2	
On salbutamol	1800	9.4 ± 5.9	*
	0800	22.1 ± 9.0	

* $p<0.05$

** $p<0.01$

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference. Similarly salbutamol did not have any significant effect on plasma cortisol concentrations at the respective times.

TABLE 12

Mean values (± SD) of alpha$_2$-adrenoceptor number (B_{max}) in five control and four asthmatic subjects

	Time (h)	B_{max} (fmol/10^7 lymphocytes)	
Control (5)	1800	20.91 ± 0.91	n.s.
Not on salbutamol	0800	20.26 ± 4.22	
On salbutamol	1800	22.30 ± 3.33	n.s.
	0800	22.25 ± 1.56	
Asthmatic (4)	1800	21.73 ± 1.83	n.s.
Not on salbutamol	0800	21.70 ± 4.07	
On salbutamol	1800	22.61 ± 2.33	n.s.
	0800	20.15 ± 2.84	

n.s., not significant

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference.

TABLE 13

Mean values (± SD) of alpha$_2$-adrenoceptor affinity (K_d) in five control and four asthmatic subjects

	Time (h)	K_d (nmol/l)	
Control (5)	1800	4.23 ± 0.47	n.s.
Not on salbutamol	0800	3.97 ± 0.77	
On salbutamol	1800	4.21 ± 0.58	n.s.
	0800	4.42 ± 1.02	
Asthmatic (4)	1800	4.32 ± 0.32	n.s.
Not on salbutamol	0800	4.38 ± 0.10	
On salbutamol	1800	4.29 ± 0.41	n.s.
	0800	4.23 ± 1.07	

n.s., not significant

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference.

patients.

Beta-adrenoceptor number (B_{max})

Both normal and asthmatic groups showed a significant circadian variation in the number of $beta_2$-adrenoceptors (B_{max}) with the bigest number of receptors at 1800 h ($p<0.05$, Table 14) which was not abolished on treatment with salbutamol. On salbutamol, "down-regulation" of beta-adrenoceptor number occurred in both groups in the 0800 h samples ($p<0.05$) and no evidence for "down-regulation" was found in the 1800 h samples.

No significant difference in beta-adrenoceptor number was observed either at 0800h or 1800h between the normal and asthmatic groups whether off or on salbutamol.

Beta-adrenoceptor affinity (K_d)

No significant difference in the receptor affinity (K_d) was observed between 1800 h and 0800 h in normal subjects while off salbutamol, but on salbutamol a circadian variation appeared in K_d with lower values at 0800 h ($p<0.05$). Asthmatic patients had a circadian variation in K_d both off and on salbutamol ($p<0.05$), with the lower K_d at 0800 h.

For comparable regimens, no significant difference in the receptor affinity between normal and asthmatic subjects was observed except at 0800 h off salbutamol ($p<0.05$, Table 15).

$Alpha_2$: $beta_2$ receptor ratio

Both normal and asthmatic groups showed a significant difference in the $alpha_2$: $beta_2$ ratio between 1800 h and 0800 h off and on salbutamol with the ratio significantly higher at 0800 h ($p<0.05$,

TABLE 14

Mean values (± SD) of beta-adrenoceptor number (B_{max}) in five control and four asthmatic subjects

	Time (h)	B_{max} (fmol/10^7 lymphocytes)	
Control (5)	1800	8.87 ± 1.45	*
Not on salbutamol	0800	5.41 ± 0.83	
On salbutamol	1800	7.31 ± 1.76	**
	0800	2.79 ± 0.85	
Asthmatic (4)	1800	6.94 ± 1.82	*
Not on salbutamol	0800	3.91 ± 1.06	
On salbutamol	1800	5.11 ± 1.71	*
	0800	1.93 ± 0.21	

* $p<0.05$

** $p<0.01$

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference.

TABLE 15

Mean values (± SD) of beta-adrenoceptor affinity (K_d) in five control and four asthmatic subjects

	Time (h)	K_d (nmol/l)	
Control (5)	1800	0.50 ± 0.12	n.s.
Not on salbutamol	0800	0.40 ± 0.12	
On salbutamol	1800	0.39 ± 0.08	*
	0800	0.22 ± 0.08	
Asthmatic (4)	1800	0.33 ± 0.07	*
Not on salbutamol	0800	0.16 ± 0.04	
On salbutamol	1800	0.41 ± 0.06	*
	0800	0.24 ± 0.07	

* $p<0.05$

n.s., not significant

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference except at 0800 h off salbutamol.

Table 16). In normals and asthmatic patients, the administration of salbutamol significantly increased the ratio in the 0800 h samples ($p<0.05$), while no significant difference was observed in the ratio for the 1800 h samples.

For comparable regimens, there was no significant difference in the $alpha_2$: $beta_2$ ratio between the normal and asthmatic groups.

FEV_1/FVC ratio

Asthmatic patients not receiving salbutamol showed the expected circadian variation in FEV_1/FVC ratio, giving rise to the "morning dip" ($p<0.05$, Table 17). This was abolished on giving salbutamol therapy. In normal subjects, no circadian variation in FEV_1/FVC ratio was observed either off or on salbutamol.

There was no significant difference between the two groups except at 0800 h off salbutamol when the FEV_1/FVC ratio for asthmatic patients was significantly lower than the normals ($p<0.002$).

Plasma cortisol concentrations

Plasma cortisol concentrations showed the expected circadian variation in both normals and asthmatic patients ($p<0.05$, Table 18), off and on salbutamol; the concentrations in neither group were significantly affected by the administration of salbutamol.

No significant difference in plasma cortisol concentrations was observed between the normal and asthmatic groups.

TABLE 16

Mean values (± SD) of $alpha_2$:$beta_2$ adrenoceptor ratio in five control and four asthmatic subjects

	Time (h)	$Alpha_2$:$beta_2$ ratio	
Control (5)	1800	2.4 ± 0.35	*
Not on salbutamol	0800	3.7 ± 0.48	
On salbutamol	1800	3.2 ± 1.12	*
	0800	8.7 ± 3.65	
Asthmatic (4)	1800	3.0 ± 0.73	*
Not on salbutamol	0800	5.3 ± 1.50	
On salbutamol	1800	5.1 ± 1.84	**
	0800	10.6 ± 0.71	

* $p<0.05$

** $p<0.01$

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference.

TABLE 17

Mean values (± SD) of FEV_1/FVC (%) in five control and four asthmatic subjects

	Time (h)	FEV_1/FVC (%)	
Control (5)	1800	83.9 ± 6.9	n.s.
Not on salbutamol	0800	82.3 ± 3.8	
On salbutamol	1800	85.6 ± 4.1	n.s.
	0800	84.5 ± 2.2	
Asthmatic (4)	1800	73.2 ± 8.3	*
Not on salbutamol	0800	56.0 ± 6.0	
On salbutamol	1800	78.7 ± 4.9	n.s.
	0800	79.7 ± 5.5	

* $p<0.05$

n.s., not significant

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference except at 0800 h off salbutamol.

TABLE 18

Mean values (± SD) of cortisol concentrations in five control and four asthmatic subjects

	Time (h)	Cortisol (µg/dl)	
Control (5)	1800	8.5 ± 2.08	**
Not on salbutamol	0800	16.0 ± 0.81	
On salbutamol	1800	9.2 ± 2.21	*
	0800	17.7 ± 3.68	
Asthmatic (4)	1800	7.0 ± 1.41	*
Not on salbutamol	0800	19.0 ± 6.37	
On salbutamol	1800	9.7 ± 3.20	*
	0800	22.2 ± 8.50	

* $p<0.05$

** $p<0.01$

The comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference.

DISCUSSION

The aim of the studies described in this thesis was to investigate if there is an inherent defect in beta-adrenoceptors that might be involved in the development of bronchial asthma. The target tissue of choice for studying these adrenoceptors is the lung, but there are obvious constraints that hinder the investigation of beta-adrenoceptors in human lung, particularly the difficulty of obtaining isolated healthy lung tissues if repeated studies within an individual subject or patient are required. For these reasons, alternative tissues have been examined by several authors (Parker and Smith, 1973; Conolly and Greenacre, 1976, 1977; Kariman and Lefkowitz, 1977; Greenacre et al, 1978; Brooks et al, 1979; Szentivanyi et al, 1979; Sano et al, 1979; Meurs et al, 1982; Tashkin et al, 1982; Van den Berg et al, 1982) and in particular the peripheral blood lymphocytes, as a readily accessible source of beta-adrenoceptors, has received special attention. Before such a model could be accepted, it was necessary to compare and contrast as extensively as possible the lymphocyte and lung beta-adrenoceptors.

Earlier studies on human lymphocytes (Conolly and Greenacre, 1977) showed that the lymphocyte beta-adrenoceptor was readily blocked by propranolol (non-selective) but appear to be far less sensitive to the effect of practolol ($beta_1$ selective); the same pattern of susceptibility to the different beta-blockers was seen in lung tissue. Also the pA_2 values for both propranolol and practolol as derived by Conolly and Greenacre (1977) corresponded closely to those obtained by other workers (Hedges and Turner, 1971; Barrett et al, 1973) and conform to the pattern which would be predicted for a tissue possessing a $beta_2$-adrenoceptor. Moreover, salbutamol stimulated the lymphocyte beta-adrenoceptor,

being about one tenth as active as isoprenaline and these findings corresponded closely to those of Cullum et al (1969) who reported a 10:1 potency ratio between isoprenaline and salbutamol on guinea-pig tracheal chain. Therefore, on the basis of those findings Conolly and Greenacre (1977) stated that the lymphocyte adrenoceptor may be categorised as $beta_2$ supporting the validity of using lymphocytes as a model to study beta-adrenoceptor function in bronchial asthma.

Recently, Davis et al (1980) have compared the cyclic AMP response to different beta-agonists and antagonists in lymphocytes and in human lung parenchyma with the results of some beta-agonists that cause 50% inhibition (relaxation) in human bronchial muscle. They concluded that the beta-adrenoceptors of the lymphocyte, bronchial smooth muscle and lung parenchyma have $beta_2$ selectivity although they differ somewhat quantitatively from each other.

Another potential obstacle to the use of lymphocytes has been the presence of subpopulations of lymphocytes (B and T cells), as differences in adrenoceptor number or cyclic AMP responsiveness of lymphocytes from patients with specific diseases might be attributable to differences in the relative numbers of B and T lymphocytes. Therefore Bishopric et al (1980) carried out a study to compare B and T cells obtained from the same population of peripheral blood lymphocytes for direct beta-adrenoceptor binding characteristics as well as their biological response to beta-adrenoceptor agonists. They found that the number of beta-adrenoceptors of B cells did not differ significantly from the number found in T cells. Similarly, there was no significant difference in their dissociation constants. In addition, assays performed on mixed unseparated lymphocytes revealed no significant

differences in number or affinity from the purified B and T populations. Also they found that B and T lymphocytes had comparable stimulation of cyclic AMP accumulation by isoprenaline. Bishopric et al (1980) suggest that variations in adrenoceptor content found among whole lymphocyte populations in different disease states could not be attributable to variations in the proportion of B and T subpopulations and would therefore reflect changes intrinsic to the disease state.

Consequently, along with many other workers, the use of lymphocyte beta-adrenoceptor as a model for the lung beta-adrenoceptor seems justified.

Other potential problems with lymphocytes might conceivably arise from the use of calf serum in the isolation of lymphocytes. The presence of thymic factors in the calf serum may well affect the results of the assays, since these thymic factors may induce the conversion of "null" cells to T-lymphocytes (Byrom et al, 1978). However this assay used calf serum at a concentration of only 0.1%, in contrast to the concentration of 10% used by other workers (Harris and Ukaejiofo, 1970; Conolly and Greenacre, 1977). Furthermore, the calf serum all came from the same batch so it was reasonable to assume that, were any thymic factors interacting with the receptors, these should be constant in their effects. Work by Thomson et al (1978) and Saraclar et al (1977) has shown that foetal calf serum has no apparent effect on B- and T- lymphocyte cell counts in asthmatic patients or on their response to doses of concanavalin A or phytohaemagglutinin. Therefore, to improve lymphocyte recoveries, the use of calf serum seems justified particularly since its concentration is so low and the volume of blood samples available was limited.

STUDY I

This study was undertaken to investigate the number (B_{max}) and sensitivity (K_d) of the lymphocyte beta$_2$-adrenoceptor with a view to explaining the 'morning dip' observed in patients with extrinsic asthma. Each patient or volunteer sample was characterised using 7-9 concentrations of the radioligand so that the B_{max} and the K_d could be determined from Scatchard analysis (Scatchard, 1949), rather than from just 1 or 2 concentrations (Kariman and Lefkowitz, 1977; Van den Berg *et al*, 1982). Characterisation of cell adrenoceptors at only one or two ligand concentrations provides little information about the status of the adrenoceptors and does not detect a change in adrenoceptor affinity for binding ligands. Also there is no real assurance that an accurate B_{max} is being obtained. It is interesting to note that Lefkowitz has recently reversed his opinions on the use of a single standard (Lefkowitz and Michel, 1983) and changed to using full characterisation in agreement with the findings of this thesis.

The asthmatic patients were in remission and were all known to have reversible airflow obstruction. All sympathomimetic drugs including theophylline compounds were stopped for at least 7 days before commencing the study to allow recovery of any desensitisation which may have occurred. It should be realised, of course, that asthma is a variable disease and some patients do require bronchodilator therapy for relief of acute symptoms. Patients were allowed to use the anticholinergic agent ipratropium bromide given as an aerosol (80 mgm) as required. It is generally accepted by clinicians (Dr Patel, personal communication) that ipratropium bromide, unlike sympathomimetic agents has no direct

effect on the alpha- or beta-adrenoceptors, although no real documentary evidence to this effect could be found.

This is the first time that such an extensive study of the effects of circadian variation on adrenoceptor kinetics has been undertaken in the human lymphocyte. Since the lymphocyte is used by many workers as a model for the lung beta$_2$-adrenoceptor (Brooks et al, 1979; Kariman, 1980; Sano et al, 1981b; Meurs et al, 1982) this study is of particular value, as many workers neglect to mention the time of sampling. One limitation of this present work is the fact that samples were only determined at 0800 h and 1800 h. Sampling times were affected by several external constraints such as the need for sequential studies which restricted the amount of blood which could be withdrawn from each subject and the time required to analyse the samples to avoid undue delay in processing the blood samples. The chosen times were the most practical.

A significant circadian variation was demonstrated in the number of beta$_2$-adrenoceptors (B_{max}) in both normals and asthmatic patients, with the lesser number of adrenoceptors in the early (0800 h) sample. This variation persisted when both groups were given salbutamol though 'down-regulation' of adrenoceptor numbers occurred in both groups in the 0800 h sample. This accords with the findings of others (Galant et al, 1978b; Tashkin et al, 1982), assuming that their samples were taken in the morning. No evidence for 'down-regulation' was found in the late (1800 h) sample and as yet no explanation for this can be offered. This could be further investigated.

No significant difference in adrenoceptor numbers was observed either at 0800 h or 1800 h between the normal and asthmatic groups whether off or on salbutamol and this contrasts with other reported

results (Kariman and Lefkowitz, 1977; Brooks et al, 1979; Sano et al, 1979) that asthmatic patients generally have significantly less beta-adrenoceptors than normals. However, most of these workers either do not mention the time of sampling or else only used one or two concentrations of radioligand to obtain the B_{max}. It may well be that the results in this thesis differ because the B_{max} data reported here were derived from full saturation analysis and not a single estimation. It would be expected that the former would give the more reliable results.

With regard to the observed circadian variations, it has also been found that plasma cAMP concentrations in normals show a circadian variation with the maximum concentration at 1400 h and the minimum at 0200 h (Mikuni et al, 1978). In asthmatic patients, plasma cAMP also showed a circadian variation similar to that in normals with trough values at 0400 h, which are significantly ($p<0.01$) lower than the peak values recorded at 1600 h (Barnes et al, 1980c, 1982).

Generally, other workers appear to have accepted (Galant et al, 1980; Meurs et al, 1982) that there is no difference in adrenoceptor affinity (K_d) between normals and asthmatic patients. In this study it is reported that in fact asthmatic patients do seem to have a significantly lower K_d ($p<0.01$) than normals at 0800 h, when they are not taking salbutamol. In support of these results, the K_d's measured here are compatible with those of other workers using [^{125}I]HYP as a binding ligand (Sano et al, 1981b; Paietta et al, 1982). Also, in normal subjects on salbutamol a circadian variation appears in K_d, with lower values (i.e. increased sensitivity) at 0800 h appearing with the lower adrenoceptor numbers. This would almost seem to be some form of

compensatory mechanism with the increased sensitivity compensating for the reduced adrenoceptor number. It would be expected that low cAMP levels would correlate with reduced receptor activity. Since B_{max} is decreased, it might be reasonable to expect that less cAMP would be produced. If the shift in K_d was compensatory then possibly circulating concentrations of cAMP should not be affected. However, it must be remembered that circulating catecholamines are also lowest in the morning and so stimulation of the receptor is probably submaximal. However, other workers (Mikuni et al, 1978) have shown that plasma cAMP levels are lowest in the morning, and so this observation cannot satisfactorily be explained, but may serve to minimise the fall in cAMP in asthmatic patients.

To investigate the cause of the modulation of beta-adrenoceptor numbers, plasma cortisol concentrations were assayed in the frozen plasma samples. Several workers have found that administration of exogenous cortisol leads to an increase in beta-adrenoceptor numbers both in humans (Sano et al, 1980) and rodents (Mano et al, 1979; Handslip et al, 1981). Other work with polymorphonuclear leucocytes (PMN) has shown that asthmatic patients on long-term steroid therapy have a normal number of beta-adrenoceptors (Galant et al, 1980). In this study, the number of adrenoceptors vary inversely with plasma cortisol; the receptor number is lower in the morning when plasma cortisol is higher. Also, Davies and Lefkowitz (1980) have shown that administration of cortisone acetate modulates both receptor number (B_{max}) and affinity (K_d) in both PMN and mononuclear leucocytes. This is in agreement with the results in this thesis, where B_{max} appears to vary inversely with endogenous plasma cortisol concentrations in both groups. The role of cortisol in the regulation of beta-adrenoceptor function needs

further investigation.

Fraser et al (1981a) concluded that there is a reciprocal correlation between circulating plasma adrenaline concentrations and lymphocyte beta-adrenoceptor density. This study did not estimate plasma adrenaline concentrations but the results would appear to contradict those of Fraser et al (1981a), as the receptor density observed was lower in the morning when plasma adrenaline is low. However, the conclusions reached by Fraser et al (1981a) are true only if adrenaline is the sole controlling factor; the observation that two biological rhythms correlate with each other chronologically is not sufficient proof that one causes the other (Clark and Hetzel, 1980). The inter-relationships of circulating catecholamine and lymphocyte beta-adrenoceptor density are presently not well resolved and require further investigation.

STUDY II

In the last few years, there has been some evidence for alpha-adrenoceptor dysfunction in asthma. Henderson et al (1979) demonstrated abnormal alpha-adrenergic sensitivity of the pupillary dilator and vasoconstrictor muscles in subjects with asthma. Others have shown that there may be an increased number of alpha-adrenoceptors in the lungs (Szentivanyi, 1979) and the lymphocytes (Szentivanyi, 1979; Sano et al, 1981a,b) of asthmatic patients.

Alpha$_2$-adrenoceptors appear to be intrinsically linked to the adenylate cyclase system, whereas alpha$_1$-adrenoceptors appear to be coupled to processes that regulate cellular calcium-ion fluxes (Lefkowitz et al, 1984). Alpha$_2$-adrenoceptors were studied as these seemed to be the next logical candidates to antagonise beta-adrenoceptor function and to be involved in any alpha-adrenoceptor

dysfunction in asthma. Also a high specific radioactivity alpha$_2$ ligand ([^{3}H]yohimbine, 70-90 Ci/mmol) was readily available whereas the ligands which are either non-selective or alpha$_1$ selective were of low specific radioactivity ([^{3}H]dihydroergocryptine, 20-50 Ci/mmol; [^{3}H]prazosin, 10-30 Ci/mmol respectively). Consequently, for the initial studies, investigation of alpha$_2$-adrenoceptor function was chosen.

Having successfully developed a method for alpha$_2$-adrenoceptor estimation in lymphocytes (Titinchi and Clark, 1984), the next phase of the study could now be proceeded with.

This study was carried out for 2 reasons

1) To see if the circadian variation in beta$_2$-adrenoceptor observed in "Study I" could actually be re-observed in a repeat study.

2) To investigate the number (B_{max}) and sensitivity (K_d) of the lymphocyte alpha$_2$-adrenoceptor to see if there is a circadian variation in alpha$_2$-adrenoceptor similar to that found in beta$_2$-adrenoceptor and moreover to see whether, in accordance with the finding of other workers (Szentivanyi et al, 1979; Szentivanyi, 1980) there is a shift in the relative numbers of adrenoceptors from beta to alpha in lymphocytes of asthmatic patients. It would also be vital to compare the ratio of alpha$_2$:beta$_2$ adrenoceptors in normals and asthmatic patients to verify results produced by other workers who looked at the general alpha-adrenoceptor population.

Again each patient or volunteer sample was characterised using 7-9 concentrations of the radioligand for both alpha$_2$ and beta$_2$ adrenceptor estimations and the respective B_{max} and the K_d's were determined from Scatchard analysis (Scatchard, 1949). One

limitation of this study was that the samples were only determined at 0800 h and 1800 h as sampling times were again affected by several external constraints including the amount of blood which could be withdrawn from each subject and the time required to analyse the samples.

The results observed for the $beta_2$-adrenoceptors in this second study were extremely interesting, because for comparable combinations of time and drug therapy there was no significant difference between the B_{max} and K_d results observed for "Study I" and "Study II" either for the normal or the asthmatic groups. Thus the discussion of the beta-adrenoceptor results under "Study I" is equally applicable to "Study II" and no further insight would be gained by repeating this discussion here.

The fact that these two independent studies give similar results is a valuable observation and strengthens the argument that a genuine phenomenon (i.e. the circadian variation in receptor function) is being observed which is not based on artefacts arising from the methodology.

Since the completion of the practical aspects of this study an extremely important observation has been published by 2 groups of workers (Bertouch et al, 1983; Ritchie et al, 1983). They have observed diurnal variation in the total number of lymphocytes. This is reflected in a diurnal variation in lymphocyte subsets (both B and T cells). At first sight these results appear to have important implications for the interpretation of the adrenoceptor function results discussed for "Studies I and II".

However, it should be remembered that quite fortuitously adrenoceptor kinetics have been expressed with reference to 10^7 lymphocytes. This means that the results are independent of the

number of circulating lymphocytes. Secondly, it has been repeatedly shown that the beta-adrenoceptor function is essentially identical on lymphocyte subsets (i.e. B and T cells) so that even if the ratio of B to T cells changes during the day (which has not been suggested either by Bertouch *et al*, 1983 or Ritchie *et al*, 1983) then this would not significantly affect the results reported here. As a corollary to this, a significant correlation ($p<0.05$) was found between lymphocyte numbers and lymphocyte protein estimations in this study, but as can be seen from Fig 32, there is a large degree of scatter. From the above, workers in this field would therefore be well advised to express their adrenoceptor kinetic results in terms of fmol/10^7 lymphocytes rather than fmol/mg protein. Such results then have much more relevance to the patient studies as several variables are essentially eliminated.

No significant difference was observed in the number of alpha$_2$-adrenoceptors (B_{max}) between 1800 h and 0800 h in both normals and asthmatic patients while off salbutamol. Administration of salbutamol did not significantly affect alpha$_2$-adrenoceptor number at 1800 h or 0800 h in either group. Other studies have also found that the number of alpha$_2$-adrenoceptors ([^{3}H]yohimbine-binding sites) on human platelets is not subject to "down-regulation". Karliner *et al* (1982) found that incubation of platelets with adrenaline *in vitro* did not decrease the number of [^{3}H]yohimbine binding sites. Furthermore, Snavely *et al* (1982) and Pfeifer *et al* (1982) found normal number of alpha$_2$-adrenoceptors on the platelets of patients with catecholamine-secreting pheochromocytoma, the clinical setting in which "down-regulation" would be most evident.

No significant difference in alpha$_2$-adrenoceptor number was observed at 0800 h or 1800 h between the normal and asthmatic

groups whether off or on salbutamol. This contrasts with other reported results (Szentivanyi et al, 1979; Sano et al, 1981b) that asthmatic patients generally have significantly more alpha-adrenoceptors than normals. However, some of these workers only used one concentration of the radioligand to obtain the B_{max} and, as discussed earlier, the B_{max} calculated from full saturation analysis would give more reliable results than a single estimation.

Concerning the alpha$_2$-adrenoceptor affinity (K_d), there was no significant difference between 1800 h and 0800 h in both normals and asthmatic patients whether off or on salbutamol. At the same time, no significant difference was observed between the two groups at either times whether off or on salbutamol. This is in accord with Sano et al (1981b) who also found that there was no significant change in K_d between normals and asthmatic patients.

Davis and Lieberman (1982) found that platelet alpha$_2$-adrenergic cAMP responses were normal in subjects with asthma. They studied alpha$_2$-adrenergic responses in platelets from normal subjects and patients with asthma who had taken no medication prior to the study, alpha$_2$-adrenergic inhibition of prostaglandin E_1-stimulated cAMP accumulation was normal in platelets from subjects with asthma. The time course of inhibition, the extent of inhibition, and the dose-response relationship did not differ between the groups. Also, Barnes et al (1980a) found that there was no difference in the K_d values of [^{3}H]prazosin (alpha$_1$ selective) in lung membrane homogenates of an animal model (guinea-pig) of chronic asthma and those from controls.

Regarding the alpha$_2$:beta$_2$ ratio, there was a significant difference in the ratio between 1800 h and 0800 h off salbutamol in both normals and asthmatic patients with the higher ratio at 0800h.

This significant difference is not due to a shift in the number of $beta_2$-adrenoceptors to $alpha_2$-adrenoceptors, but is in fact related to the circadian variation in the number of $beta_2$-adrenoceptors between 1800 h and 0800 h in both groups while off salbutamol with the lesser number of $beta_2$-adrenoceptors at 0800 h. This significant difference in the $alpha_2$:$beta_2$ ratio between 1800 h and 0800 h persisted even when both groups were given salbutamol and again this was due to persistence of the circadian variation in $beta_2$-adrenoceptor number in both normals and asthmatic patients on salbutamol therapy.

In both groups, the $alpha_2$:$beta_2$ ratio at 0800 h on salbutamol was significantly higher than at 0800 h not on salbutamol and this is due to the "down-regulation" of $beta_2$-adrenoceptors induced by salbutamol. The ratio at 1800 h was not significantly different off or on salbutamol in either normals or asthmatic patients.

No significant differences in the ratio were observed between normals and asthmatic patients, and this contrast with other reported results (Sano et al, 1981b) that asthmatic patients have significantly higher alpha:beta ratio than normals.

At present, the mechanism of the observed circadian variation in beta-adrenoceptor numbers in either groups has not been investigated. In both studies, the observation that beta-adrenoceptors are reciprocally correlated to plasma cortisol levels are in agreement with Davies and Lefkowitz (1980), but apparent contradictions exist in the literature where other workers have found that steroids increase lymphocyte beta-adrenoceptor number (Sano et al, 1980). The role of cortisol remains to be investigated in much more detail. The lowest number of beta-adrenoceptors corresponds to the observation of 'morning dip' in asthmatic

patients, and this diminishing of receptor number may well be a predisposing factor to the fall in FEV_1/FVC ratio. The 'defect' may in fact be external to the beta-adrenoceptor as one report (Fraser et al, 1981b) has suggested that circulating auto-antibodies may interfere with the functional binding of catecholamines to the beta-adrenoceptor in some patients with asthma. It is possible therefore, that there is no intrinsic beta-adrenoceptor defect. Certainly the results reported in this thesis contain no evidence that the beta-adrenoceptors of asthmatic patients are functionally different from those of normal subjects; both display a circadian variation in receptor number, both are down-regulated by salbutamol administration and the number of receptors does not differ significantly between the two groups.

Neither do the results contain any evidence for $alpha_2$-adrenoceptor dysfunction in asthmatic patients. The circadian variation in $alpha_2$:$beta_2$ ratio is related only to the circadian variation in $beta_2$-adrenoceptors. This does not preclude alpha-adrenoceptors having a functional role in asthma. The increase in $alpha_2$:$beta_2$ ratio in the morning may well be a predisposing factor to the fall in FEV_1/FVC ratio (morning dip) in asthmatic patients.

In conclusion, the results reported here would seem to be in agreement with other work published during the course of this thesis: that Szentivanyi's hypothsis should once and for all be rejected (Conolly, 1980). That is, there is no intrinsic defect either in the beta-adrenoceptors or the alpha-adrenoceptors of patients with extrinsic asthma, and no apparent shift from beta- to alpha-adrenoceptors occur. The cause of dyspnoea in extrinsic asthma patients must therefore be looked for elsewhere.

APPENDIX A

General Materials

The components of materials marked '*' will be found at the end of the Appendix.

1. CHEMICALS AND ASSAY KITS

Aseptic calf serum	Gibco Bio-Cult Ltd. Paisley, Scotland
Catechol (O-dihydroxybenzene)	BDH Chemicals Ltd. Poole, England
Dulbecco's Phosphate Buffered Saline (IX) (PBS) *	Gibco Bio-Cult Ltd. Paisley, Scotland
Earle's Balanced Salt Solution (10X) without Sodium Bicarbonate (EBSS) *	Gibco Bio-Cult Ltd Paisley, Scotland
Earle's Balanced Salt Solution (IX) (EBSS) *	Gibco Bio-Cult Ltd. Paisley, Scotland
Emulsifier scintillator 299™ Cat. No. 6013079 *	Packard Instruments Ltd. Berks, England
Hank's Balanced Salt Solution (10X) without Sodium Bicarbonate (HBSS) *	Gibco Bio-Cult Ltd. Paisley, Scotland
Hank's Balanced Salt Solution (IX) (HBSS) *	Gibco Bio-Cult Ltd. Paisley, Scotland
Hepes buffer powder (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)	Flow Laboratories Ltd. Ayrshire, Scotland
(-) Isoprenaline bitartrate (Fig. 4)	Sigma Chemical Co. Ltd. Poole, England
Isoton II	Coulter Electronics Ltd. Luton, Beds., England
Lymphocyte separation medium *	Flow Laboratories Ltd. Ayrshire, Scotland

Magnesium chloride	Sigma Chemical Co. Ltd. Poole, England
(-) Noradrenaline bitartrate (Fig. 5)	Sigma Chemical Co. Ltd. Poole, England
Omnifluor * Cat. No. NEF-906	New England Nuclear Massachusetts, USA
Sodium chloride 'AnalaR'	BDH Chemicals Ltd. Poole, England
Sucrose 'AnalaR'	BDH Chemicals Ltd. Poole, England
Toluene 'AnalaR'	BDH Chemicals Ltd. Poole, England
Triton X-100	BDH Chemicals Ltd. Poole, England
Trizma base (2-amino-2-hydroxymethyl- propane-1,3-diol)	Sigma Chemical Co. Ltd. Poole, England
Trypan blue stain, 0.4%	Gibco Bio-Cult Ltd. Paisley, Scotland
Yohimbine hydrochloride (Fig. 5)	Sigma Chemical Co. Ltd. Poole, England
Zaponin	Coulter Electronics Ltd. Luton, Beds., England
Bio-Rad protein assay kit Cat. No. 500-0001	Bio-Rad Laboratories Watford, England
IMMO PHASE^{TM} [^{125}I] Cortisol Radioimmunoassay kit	Corning Medical and Scientific, Essex, England

2. CONSUMABLES AND DISPOSABLES

Evans heparinised bottles	Evans Medical Ltd. Liverpool, England

Glass microfibre filters grade (GFC), size (2.5 cm)	Whatman LabSales Maidstone, England
Glass microfibre filters grade (GFB), size (2.5 cm)	Whatman LabSales Maidstone, England
Polypropylene tubes (12 x 75 mm) Cat. No. 526	Sarstedt Ltd. Leicester, England
Polystyrene tubes (12 x 75 mm)	MacFarlane Robson Ltd. Glasgow, Scotland
Soda glass tubes (12 x 75 mm)	MacFarlane Robson Ltd. Glasgow, Scotland
Universal containers (30 ml)	Sterilin Ltd. Middlesex, England

3. RADIOCHEMICALS

1-[propyl-2,3-^{3}H] dihydroalprenolol Specific Radioactivity 30 - 60 Ci/mmol (Fig. 6)	The Radiochemical Centre, Amersham, England
[^{125}I] iodohydroxy-benzylpindolol, Specific Radioactivity 2200 Ci/mmol (Fig. 6)	New England Nuclear Dreieich, W. Germany
[methyl-^{3}H]-yohimbine Specific Radioactivity 70-90Ci/mmol (Fig. 9)	New England Nuclear Dreieich, W. Germany

4. DRUGS

Clonidine hydrochloride (Catapres ampoules, 0.15mg/ml) (Fig. 5)	Boehringer Ingelheim Berks., England
Ipratropium bromide (Atrovent inhaler)	Boehringer Ingelheim Berks., England
Phentolamine mesylate (Rogitine ampoules, 10mg/ml) (Fig. 5)	Ciba Laboratories West Sussex, England

Salbutamol-as sulphate (Ventolin tablets, 2mg) (Fig. 4)	Allen & Hanburys Ltd. Middlesex, England

The following drugs were kindly donated by the sources indicated:

(+) Adrenaline bitartrate (Fig. 4)	Kodak Ltd. Industrial and Research Chemical Sales, Liverpool England
(-) Adrenaline B.P.	Smith and Nephew Pharmaceuticals, England
H 35/25	Hassle Molndal, Sweden
Practolol (Fig. 4)	Imperial Chemical Industries (ICI) Ltd. Pharmaceuticals Division Cheshire
Prazosin hydrochloride (Fig. 5)	Pfizer Ltd., England
(±) Propranolol hydrochloride B.P. (Fig. 4)	ICI
(-) Propranolol hydrochloride	ICI
(+) Propranolol hydrochloride	ICI

5. INSTRUMENTS

Automatic Tri-Carb Liquid Scintillation Spectrometer Model 3330	Packard Instrument Ltd. Berks, England
Coulter counter Model ZF	Coulter Electronics Ltd. Herts., England
Millipore filtration apparatus	Millipore (U.K.) Ltd. Millipore House Middlesex, England

Mini-bomb cell disruption chamber, cat. No. K-881455	Kontes Glass Co. Vineland, N.J., USA
MSE Mistral 4L refrigerated centrifuge	MSE Scientific Instruments, England
Ultracentrifuge Model L5-65	Beckman Instruments INC. California, USA
Ultra-Turrax Type TP 18/10	Janke and Kunkel IKA-Products, Belmont, Surrey
Vitalograph dry wedge spirometer	Vitalograph Ltd. Buckingham, England

Components	Dulbeccos Phosphate Buffered Saline	Earles Balanced Salt Solution (without $NaHCO_3$)	Earles Balanced Salt Solution	Hanks Balanced Salt Solution (without $NaHCO_3$)	Hanks Balanced Salt Solution
	mg/l	mg/l	mg/l	mg/l	mg/l
Inorganic salts					
NaCl	8000.00	6800.00	6800.00	8000.00	8000.00
KCl	200.00	400.00	400.00	400.00	400.00
$MgSO_4\ 7H_2O$	-----	200.00	200.00	100.00	100.00
$NaH_2PO_4\ 2H_2O$	-----	158.00	158.00	-----	-----
Na_2HPO_4 (anhy)	1150.00	-----	-----	48.00	48.00
$CaCl_2\ 2H_2O$	132.00	264.00	264.00	185.00	185.00
$NaHCO_3$	-----	-----	2200.00	-----	350.00
KH_2PO_4	200.00	-----	-----	60.00	60.00
$MgCl_2\ 6H_2O$	100.00	-----	-----	100.00	100.00
Other components					
Glucose	-----	1000.00	1000.00	1000.00	1000.00
Phenol red	-----	10.00	10.00	10.00	10.00

Lymphocyte separation medium

Lymphocyte separation medium is an aqueous solution of a high density sucrose-epichlorohydrin polymer (Ficoll 400) and sodium diatrizoate.

Omnifluor

Omnifluor is a precisely-blended scintillator powder composed of 98% PPO (2,5-diphenyloxazole) and 2% bis-MSB (1,4-Di-(2-methyl-styryl)-benzene).

Emulsifier scintillator 299

Emulsifier scintillator 299 is a xylene based, complete liquid scintillation cocktail for aqueous and non-aqueous samples.

COMPONENTS OF BUFFERS

Buffer A - pH 7.6

Earles Balanced Salt Solution (without sodium bicarbonate)

20 mmol/l Hepes

0.1% calf serum

Buffer B - pH 7.6

50 mmol/l Tris

0.9% sodium chloride

0.1 mmol/l (±)propranolol

Buffer C - pH 7.6

50 mmol/l Tris

0.9% sodium chloride

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Circadian variation in number and affinity of β_2-adrenoceptors in lymphocytes of asthmatic patients

S. TITINCHI*, M. AL SHAMMA†, K. R. PATEL†, J. W. KERR† AND B. CLARK*

**Department of Biochemistry and † Department of Respiratory Medicine, Western Infirmary, Glasgow, Scotland, U.K.*

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Summary

1. To determine whether circadian variation in adrenoceptor function might underlie the 'morning dip' in peak expiratory flow (PEF) rate and its abolition by salbutamol we measured indices of β-adrenoceptor function ($B_{max.}$ and K_d), the ratio FEV_1/FVC, and plasma cortisol at 08.00 and 18.00 hours on and off salbutamol (4 mg given orally every 4 h) in five extrinsic asthmatic patients and five normal volunteers.

2. There was a significant circadian variation in receptor numbers ($B_{max.}$) in both the control and asthmatic groups which was not abolished on treatment with salbutamol.

3. Both groups appeared to compensate for loss of receptor number induced by salbutamol administration by increasing receptor affinity.

4. For comparable combinations of drug/time, there was no significant difference between the control and asthmatic groups.

5. We conclude that the 'morning dip' observed in asthmatic patients cannot simply be explained by changes in cell receptor number or affinity, as our results suggest that both groups have intact β-adrenoceptor function. Nevertheless, our observations of the normal circadian rhythm has important implications for future studies of β-adrenoceptors in asthmatic patients.

Key words: β-adrenoceptors, circadian variation, extrinsic asthma, lymphocytes.

Abbreviations: FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; HYP, iodohydroxybenzylpindolol; PEF, peak expiratory flow.

Correspondence: Dr Barry Clark, Department of Pathological Biochemistry, Western Infirmary, Glasgow G11 6NT, Scotland, U.K.

Introduction

Bronchial hyper-reactivity is now recognized as a major factor in the development of bronchial asthma and in atopic disease. Szentivanyi [1] has postulated a defect in the β-adrenoceptors. Several reports [2–5] seem to support this hypothesis; however, others [6–8] were unable to find a significant difference between normal and asthmatic subjects. It has also been suggested by others [9] that the receptor abnormality is drug induced. Patients with asthma have marked variations in bronchomotor tone over 24 h which contrasts with the lack of variability in normals. This circadian variation in the bronchomotor tone, often demonstrated as the 'morning dip' [10] in FEV_1, could be due to changes in the numbers and/or the affinity of β-adrenoceptors or related to humoral circadian rhythms.

The aim of this study was to investigate β-adrenoceptor function in normal and asthmatic subjects at different times, to assess the effect of orally administered β_2-agonist (salbutamol) and to see whether, in accordance with the hypothesis of Szentivanyi, there was some apparent receptor dysfunction in extrinsic asthmatic patients and finally if exposure to a β_2-agonist produced tachyphylaxis.

In this study, lymphocyte β-adrenoceptors (β_2) were used as a model of events taking place in the bronchial smooth muscle over a 24 h period [11].

Methods

Subjects

Five patients with extrinsic asthma and five normal volunteers were studied. The asthmatic patients were in remission and were all known to have reversible airflow obstruction. All sympatho-

mimetic drugs, including salbutamol and theophylline compounds, were stopped and the patients maintained on ipratropium bromide as necessary for 7 days before commencing the study, so that the β-adrenoceptors will have recovered from any β-agonist induced desensitization. None of the patients was on inhaled or oral steroid therapy. The subjects gave informed consent and the study was approved by the Medical Ethics Committee of the hospital.

Protocol

Studies were performed in the respiratory unit and the asthmatic patients were admitted at least 2 days before the study was commenced so as to become accustomed to the surroundings. Both groups were familiar with having blood samples taken.

The following sequence of tests was performed on each patient or volunteer. On day 1, 50 ml of blood was withdrawn at 18.00 hours for estimation of plasma cortisol and isolation of lymphocytes for β-adrenoceptor estimation. On day 2, 50 ml of blood was withdrawn as above at 08.00 hours and then 4 mg of salbutamol was orally administered to the patient or volunteer at 4 hourly intervals. At 18.00 hours, a further blood sample was taken and the dosage of salbutamol continued. On day 3 (08.00 hours), 50 ml of blood was withdrawn as above, the salbutamol stopped and previous therapy resumed. FEV_1/FVC ratio was determined after taking each blood sample.

Isolation of lymphocytes

Lymphocytes were prepared from heparinized blood by our modification of the technique of Harris & Ukaejiofo [12], which produced cell populations of approximately 90–95% lymphocytes with the remainder as monocytes and recoveries of 90–94%. Blood (50 ml) was collected into Evans heparinized bottles and centrifuged ($250\,g_{max.}$, 20 min, 15°C). The platelet-rich plasma was aspirated and stored frozen at −20°C for subsequent plasma cortisol estimations. The pellet was diluted 1:3 (v/v) with Earle's Balanced Salt Solution (Gibco Bio-Cult Ltd, Paisley, Scotland) minus sodium bicarbonate and supplemented with 0.1% calf serum and 20 mmol/l Hepes (Flow Laboratories Ltd, Ayrshire, Scotland), adjusted to pH 7.6 (buffer A). Portions (8 ml) of the dilution were layered on 12 ml of Lymphocyte Separation Medium (Flow Laboratories Ltd) and centrifuged ($250\,g_{max.}$, 250 min, 15°C). The milky layer of lymphocytes was harvested from the interface, washed by diluting 1:3 with buffer A, centrifuged ($250\,g_{max.}$, 15 min, 15°C) and the supernatant discarded. The pellet was resuspended in 20 ml of buffer A and gently disaggregated. The addition of calf serum reduced lymphocyte clumping and ensured an adequate yield of cells. Cells were counted in a Coulter Cell Counter; final cell concentrations were usually $2.5–3.0 \times 10^9$ lymphocytes/l. Cell viability remained greater than 95% during all the described procedures as determined by trypan blue exclusion.

Receptor saturation analysis

The saturation assays were done in polypropylene tubes (Sarsted, 12 mm × 75 mm) in a final volume of 500 μl, comprising, for the total binding, lymphocyte suspension (250–350 μg of protein) and [^{125}I]iodohydroxybenzylpindolol (^{125}I-HYP, New England Nuclear, West Germany) at appropriate concentrations (50–800 pmol/l) in buffer A. In tubes used to determine non-specific binding, (−)propranolol (3×10^{-7} mol/l) was also added. Total and non-specific binding was estimated in duplicate at each ^{125}I-HYP concentration. The tubes were incubated at 30°C in the dark (to minimize photolysis of the ^{125}I-HYP) for 45 min. The reaction was stopped by the addition of a 'stopping solution' of 2.5 ml of 50 mmol/l Tris (pH 7.6), 0.9% sodium chloride and 0.1 mmol/l (±)propranolol at 37°C. The samples were allowed to stand for 45 s before being filtered through Whatman GFC fibre-glass filters (presoaked for 60 min in 'stopping solution'), by applying a vacuum of 3–4 psi, and the filters were rinsed with 3 × 10 ml of 50 mmol/l Tris (pH 7.6) and 0.9% sodium chloride at 37°C. Filtering and rinsing were complete within 12 s. The filters were air dried, shaken with 10 ml of Scintillator 299 (Packard) and the radioactivity was determined. Reproducibility of the assay was as follows for five determinations: $B_{max.}$, 6.05 ± 0.64 fmol/10^7 lymphocytes; K_d, 0.36 ± 0.05 nmol/l.

Other estimations

Plasma cortisol was estimated by using Cortisol (^{125}I) Radioimmunoassay Kit (Corning). The FEV_1/FVC ratio was measured with a standard Vitalograph.

Statistical methods

Statistical analysis was done with paired Student's *t*-tests (comparisons on the same subject group) and two-sample *t*-tests (comparison

between patient and volunteer groups) as appropriate on an ICL-2976 computer using the package MINITAB (Ryan, Joiner & Ryan, Pennsylvania State University).

Results

FEV_1/FVC ratio

In the normal group no circadian variation in FEV_1/FVC ratio was observed (Table 1), either on or off salbutamol therapy. Asthmatic patients not receiving sulbutamol showed the expected circadian variation in FEV_1/FVC ratio, giving rise to the 'morning dip' ($P<0.05$), which was abolished on giving salbutamol therapy.

For comparable regimens (i.e. salbutamol/time combinations), there was no significance between the two groups except at 08.00 hours off salbutamol, when the FEV_1/FVC ratio for asthmatic patients was significantly lower than that for the normals ($P<0.001$), this corresponding to the 'morning dip' in the asthmatic group.

Plasma cortisol concentrations

Plasma cortisol concentrations showed the expected circadian variation in both normal and asthmatic subjects ($P<0.05$, Table 1), on and off salbutamol and administration of salbutamol did not significantly affect plasma cortisol concentrations in either group. For comparable regimens there was no significant difference in plasma cortisol concentrations between the normal and asthmatic groups.

β-Adrenoceptor kinetics

Receptor number ($B_{max.}$). Both normal and asthmatic groups not on salbutamol show a marked circadian variation in the number ($B_{max.}$) of β_2-adrenoceptors found on the lymphocyte ($P<0.05$, Table 1) with the greater number of receptors at 18.00 hours. This circadian variation is not abolished on exposure to salbutamol. Representative saturation curves for a normal subject on salbutamol are shown in Fig. 1 with the corresponding Scatchard analyses in Fig. 2.

In both groups, the number of receptors at 08.00 hours on salbutamol is also significantly less

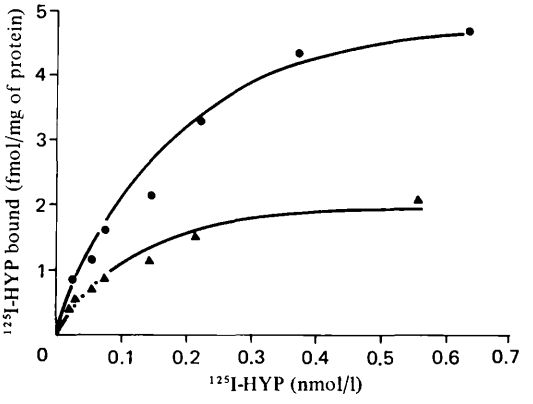

FIG. 1. ^{125}I-HYP binding to lymphocytes of a typical control subject while on the salbutamol regimen at 18.00 hours (day 2, ●) and 08.00 hours (day 3, ▲).

TABLE 1. *Mean values (± SD) of $B_{max.}$, K_d, cortisol concentrations and FEV_1/FVC (%) in five control and five asthmatic subjects*

$^*P<0.05$; $^{**}P<0.01$. n.s., not significant. In addition, the comparison of controls with asthmatic patients with and without oral salbutamol did not reveal any significant difference. Similarly salbutamol did not have any significant effect on plasma cortisol concentrations at the respective times.

	Time (hours)	$B_{max.}$ (fmol/10^7lymphocytes	K_d (nmol/l)	Cortisol (μg/dl)	FEV_1/FVC (%)
Control (5)	18.00	9.02 ± 2.21	0.49 ± 0.23	8.3 ± 1.7	83.2 ± 7.2
		*	n.s.	*	n.s.
Not on salbutamol	08.00	5.40 ± 1.16	0.35 ± 0.11	15.1 ± 3.3	82.9 ± 7.9
On salbutamol	18.00	7.18 ± 2.01	0.46 ± 0.14	9.8 ± 3.3	86.2 ± 6.8
		**	*	*	n.s.
	08.00	2.01 ± 1.74	0.21 ± 0.19	18.1 ± 2.7	83.9 ± 5.8
Asthmatic (5)	18.00	6.86 ± 2.72	0.31 ± 0.04	11.8 ± 5.7	69.8 ± 14.1
		**	**	**	*
Not on salburamol	08.00	3.40 ± 1.50	0.14 ± 0.06	21.0 ± 8.2	51.9 ± 10.3
On salbutamol	18.00	5.12 ± 1.48	0.42 ± 0.15	9.4 ± 5.9	71.8 ± 14.4
		**	*	*	n.s.
	08.00	1.28 ± 0.41	0.23 ± 0.11	22.1 ± 9.0	67.5 ± 19.4

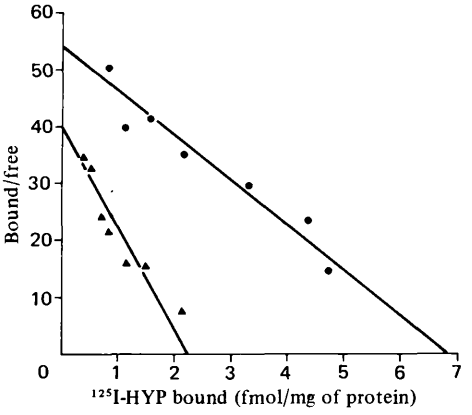

FIG. 2. Scatchard analysis of the saturation data from Fig. 1: 18.00 hours (day 2, ●) and 08.00 hours (day 3, ▲).

than at 08.00 hours not on salbutamol ($P < 0.05$), showing that 'down regulation' of the receptors is induced by salbutamol. This 'down regulation' is not observed for the 18.00 hour samples.

For comparable regimens no significant difference in receptor number was observed between asthmatic and normal groups.

Receptor affinity (K_d). In normals there is no significant difference in the receptor affinity (K_d) between 18.00 hours and 08.00 hours while off salbutamol, but on salbutamol a circadian variation appears, with the K_d significantly lower ($P < 0.05$, Fig. 2) (i.e. the receptor is more sensitive) at 08.00 hours. This compares with receptor affinity at 18.00 hours, which is not significantly different on or off salbutamol.

In asthmatic patients, a circadian variation in K_d is present both on and off salbutamol ($P < 0.05$) but the administration of salbutamol does not significantly alter receptor affinity at either 08.00 or 18.00 hours.

No significant differences in receptor affinity between normal and asthmatic subjects were observed except at 08.00 hours off salbutamol ($P < 0.01$, Table 1).

Discussion

The problems of using the lymphocyte β_2-adrenoceptor as a model for the lung β-adrenoceptor has not been extensively discussed. However, Davis *et al.* [13] have stated that the β-adrenoceptors of the lymphocyte, bronchial smooth muscle and lung parenchyma have β_2 selectivity, although they differ quantitatively from each other. Bishopric *et al.* [14] have shown that β-adrenoceptor function on B- and T-lymphocytes does not differ significantly. Consequently we, along with many others, feel justified in using the lymphocyte β-adrenoceptor as a model for the lung.

This study was undertaken to investigate the number ($B_{max.}$) and sensitivity (K_d) of the lymphocyte β_2-adrenoceptors with a view to explaining the 'morning dip' observed in patients with extrinsic asthma. Each patient sample was characterized with seven to nine standards, so that the $B_{max.}$ and the K_d could be determined from Scatchard analysis [15], rather than from just one or two standards [5, 16]. To our knowledge, this is the first time that such an extensive study of the effects of carcadian variation on adrenoceptor kinetics has been undertaken in the human lymphocyte. Since the lymphocyte is used by many workers as a model for the lung β_2-adrenoceptor [17-20], this study is of particular value, since many workers neglect to mention the time of sampling. One limitation of this present work is the fact that samples were only determined at 08.00 and 18.00 hours. However, the need for sequential studies restricted the amount of blood which could be withdrawn. Measurement of cell adrenoceptor numbers at only one or two ligand concentrations [5, 16] is not adequate to allow adrenoceptor characterization, particularly since adrenoceptor sensitivity might also be a factor in explaining the 'morning dip' observed in asthmatic subjects.

A significant circadian variation was demonstrated in the number of β_2-adrenoceptors ($B_{max.}$) in both normal and asthmatic subjects, with the lesser number of adrenoceptors in the early (08.00 hours) sample. This variation persisted when both groups were given salbutamol, though 'down-regulation' of adrenoceptor numbers occurred in both groups in the 08.00 hours sample. This accords with the findings of others [21, 22], assuming that their samples were taken in the morning. Interestingly, no evidence for 'down-regulation' was found in the late (18.00 hours) sample and as yet we can offer no explanation for this.

No significant difference in adrenoceptor numbers was observed either at 08.00 or 18.00 hours between the normal and asthmatic groups whether on or off salbutamol, and this contrasts with other reported results [5, 17, 23] that asthmatic patients generally have significantly less β-adrenoceptors than normal subjects. However, most of these workers either do not mention the time of sampling or else used only one or two concentrations of radioligand to obtain the $B_{max.}$. It may well be that our results differ because the $B_{max.}$ data reported here were derived from full

saturation analysis and not a single estimation. It would be expected that the former would give the more reliable results.

With regard to the observed circadian variations, it has also been found that plasma cAMP concentrations in normal subjects demonstrated a circadian variation with the maximum concentration at 14.00 and the minimum at 02.00 hours [24]. In asthmatic patients, plasma cAMP also showed a circadian variation similar to that in normals with trough values at 04.00 hours, significantly ($P < 0.01$) lower than the peak values recorded at 16.00 hours [25, 26].

Generally, other workers appear to have accepted [20, 27] that there was no difference in adrenoceptor affinity (K_d) between normal and asthmatic subjects. In this study it is reported that in fact asthmatic patients do seem to have a significantly lower K_d ($P < 0.01$) than normals at 08.00 hours, not on salbutamol. In support of these results, the K_d values measured in our assay are compatible with those of other workers with ^{125}I-HYP used as binding ligand [19, 28]. Also, in normal subjects on salbutamol a circadian variation appears in K_d, with lower values at 08.00 hours appearing with the lower adrenoceptor numbers. This would almost seem to be some form of compensatory mechanism with the increased sensitivity compensating for the reduced adrenoceptor number. However, other workers [24] have shown that plasma cAMP levels are lowest in the morning, and so this observation cannot satisfactorily be explained. Asthmatic patients have a circadian variation in K_d both on and off salbutamol, with the lower K_d in the morning, but again, as pointed out by others [25, 26] the plasma cAMP is also lower in the morning.

To investigate the cause of the modulation of β-adrenoceptor numbers, plasma cortisol concentrations were assayed. Several workers have found that administration of exogenous cortisol leads to an increase in β-adrenoceptor numbers, both in humans [29] and rodents [30, 31]. Other work with polymorphonuclear leucocytes has shown that asthmatic patients on long-term steroid therapy have a normal number of β-adrenoceptors [27]. In our study, the number of adrenoceptors appears to vary inversely with plasma cortisol; the receptor number is lower in the morning when plasma cortisol is higher. Our results fall short of significance and so cortisol is unlikely to be the mediator of this effect. However, Davies & Lefkowitz [32] have also shown that administration of cortisone acetate modulates both receptor $B_{max.}$ and K_d in both polymorphonuclear and mononuclear leucocytes. This is in agreement with our results, and so the role of cortisol in the regulation of β-adrenoceptor function needs further investigation.

It has been shown [33] that there is a reciprocal correlation between circulating plasma adrenaline concentrations and lymphocyte β-adrenoceptor density. Our study did not estimate plasma adrenaline concentrations but our results would appear to contradict those results [33] as the receptor density was lower in the morning when plasma adrenaline is low. However, the conclusions reached [33] are true only if adrenaline is the sole controlling factor; the observation that two biological rhythms correlate with each other in time is not sufficient proof that one causes the other [34]. Adrenaline infusion has no effect on lymphocyte β-adrenoceptors [35] and so the interrelationships are presently unclear.

At present, we have no explanation for the observed circadian variation in β-adrenoceptor numbers in either group. The lowest number of β-adrenoceptors corresponds to the observation of 'morning dip' in asthmatic patients, and this diminishing of receptor number may well be a predisposing factor to the fall in FEV_1/FVC ratio. One report [36] has suggested that circulating auto-antibodies may interfere with the functional binding of catecholamines to the β-adrenoceptor in some patients with asthma. This is a more likely explanation of the β-adrenoceptor defect in asthmatic patients as our data contain no evidence that their β-adrenoceptors are functionally different from those of normal subjects; both display a circadian variation in receptor number, both are down-regulated by salbutamol administration and the number of receptors does not differ significantly between the two groups.

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RECFIT: A Microcomputer-Based Nonlinear Regression Curve-Fitting Package for the Quantitative Resolution of β-Adrenoceptor Subtypes and Estimation of Nonspecific Binding

BARRY CLARK,* ANDREW W. KELMAN,† GRAHAM E. TROPE,‡ AND SA'AD J. TITINCHI*

*Department of Pathological Biochemistry, Western Infirmary, Glasgow, G11 6NT; †West of Scotland Health Boards, Department of Clinical Physics and Bioengineering, 11 West Graham Street, and Department of Nuclear Medicine, Stobhill General Hospital, Glasgow; ‡Tennent Institute of Opthalmology, Western Infirmary, Glasgow, Scotland

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A dedicated nonlinear regression based curve-fitting packages has been developed for quantitative analysis of β-adrenoceptor subtypes. A feature of this package is the provision to obtain initial parameter estimates using a conversational graphical technique where the user is prompted for parameter estimates, and the resulting curve is displayed over the data. Data are then fitted to a one- or two-binding site model with user-selected weighting and constraints on the parameters. A novel feature is the provision to estimate the nonspecific binding component in the assays as a parameter in the model. The printout for each model consists of the parameters and their standard deviations, estimates of the goodness of fit, an analysis of residuals, and a graph of the data points overlayed with the fitted curve. The nonlinear regression algorithm is based on that of Gauss–Newton. When unweighted, the values determined by RECFIT are essentially the same as those found using the BMDPAR programs on an ICL 2976 mainframe computer. The implementation is reasonably efficient; a typical run for a two-site fit, using nine data points and estimating nonspecific binding, took a total time of about 8 min, excluding data entry and derivation of initial estimates, 4 min for the fitting, 30 sec for graph generation, and 2–3 min for printing of the graph and data.

INTRODUCTION

When developing receptor assays in new tissues, it is essential to have analytical tools which will allow detection of receptor subtypes if such are present. Recently, Trope and Clark (*1, 2*) have developed a system for the detection of β-adrenergic receptors in pigmented ciliary processes; the latter are thought to be implicated in the development of glaucoma, a serious eye condition affecting many people (*3*). Some empirical evidence (*4*) suggested that the main receptors present were β_2-adrenergic receptors; difficulties in the dissection procedures for obtaining ciliary processes might lead to some contamination with blood vessels, whose receptors are type β_1. A sensitive technique for quantifying receptor subtypes was thus essential. Additionally, Titinchi and Clark (*5*)

0010-4809/84 $3.00

have recently characterized lymphocyte alpha receptors using similar techniques with tritiated ligands with identical requirements for analysis. Early work by Barnett *et al.* (*6*) had used a graphical method derived from the classical Scatchard (*7*) data analysis ("pseudo Scatchard") to assess the relative proportions of receptor subtypes. Hancock *et al.* (*8*) have shown that this method provides less accurate predictions than a nonlinear computer model, and have given an analysis of the errors involved.

Existing packages (SCATFIT (*8*) and SAAM26 (*9*)) provide the necessary algorithms for the least squares analysis of receptor subtypes but the former is written in PL/1 and both are only available on certain mini- or mainframe computers. The initial approach adopted was to provide suitable model equations as subroutines to one of the general nonlinear regression curve-fitting packages available on the Glasgow University ICL 2976 mainframe computer (BMDPAR (*10*)) interfaced to a custom-written graphics program based on the GHOST (*11*) graphical output routines but this approach had several limitations; users were required to be familiar with the complex ICL operating system and text editor, and access to the system was only by means of a (slow) remote 300-baud line. The printer and plotter were also a considerable distance from the Department, making the whole procedure exceptionally tedious.

One of the problems associated with cell-receptor studies is the assessment of ligand which is bound nonspecifically (nonspecific binding, NSB) to the tissue under analysis. The classic way of estimating this is to add sufficient unlabeled drug to saturate all the specific receptors and then determine how much labeled ligand is still bound to the tissue membrane. Since the equations used to describe the ligand–receptor interactions are dependent on how much ligand is specifically bound the data obtained from the assays must be transformed (specific = total − NSB) as only total binding and nonspecific binding can be directly determined in the assays. Estimation of NSB is therefore vitally important and it was decided to fit this component as an additional parameter in the model equations. Consequently, a nonlinear least squares algorithm which allows multiple parameters to be fitted simultaneously was essential. NSB can now be estimated as a parameter in the fit, providing verification of the data obtained experimentally and giving greater confidence in the results.

The advent on an Apple II microcomputer into the department lead to the development of a package specifically tailored to receptor analysis utilizing the inbuilt graphics capability to display the results of the fit.

This paper describes a comprehensive menu-driven program package for cell–receptor analysis; it is called RECFIT, and runs on an Apple II Europlus microcomputer.

Methods

Computer Software

Most of the package is written in compiled Microsoft FORTRAN 66. This language was chosen because, unlike Apple FORTRAN 77, it supports double-

precision variables, thus increasing the accuracy of the matrix operations. It also has the advantage of compiling directly to fast Z-80 machine code whereas FORTRAN 77 is run by a relatively slow p-code interpreter under the Apple PASCAL operating system. Double-precision variables are used sparingly, because they substantially slow the curve-fitting procedures. The lack of text handling in FORTRAN 66 was compensated for by use of "the STRING 80 bit" (Keybits Inc., P.O. Box 592293, Miami, Fla.) routines. These routines also give direct program access to the CP/M operating system. This allows, for example, the disk directory to be retrieved and listed or the presence of a file or disk to be verified before attempts are made to access it. The graphics programs are written in Microsoft BASIC 80, as FORTRAN 66 does not support high-resolution graphics. These interpretative programs are compiled using Microsoft's BASCOM compiler so that both languages run in a common environment (CP/M "COM" files).

The Package

(*a*) *The model.* The equations fitted by the program are those described by Wenke (*13*) for the competitive reaction between the free concentrations of a receptor R, the radiolabeled ligand [^{125}I]HYP ([^{125}I]hydroxybenzylpindolol) and a β-adrenoceptor blocking agent B.

$$\begin{array}{l} R \; + \; [^{125}\text{I}]\text{HYP} \underset{K_A}{\rightleftharpoons} R \cdot [^{125}\text{I}]\text{HYP} \\ + \\ B \\ \downarrow\uparrow \; K_B \\ \text{RB} \end{array}$$

K_A and K_B represent the dissociation constants of [^{125}I]HYP and B.

Under steady state conditions, assuming a constant number of β-adrenoceptors R_T

$$R_T = R + RB + R \cdot [^{125}\text{I}]\text{HYP},$$

the concentration of bound radioligand is

$$R \cdot [^{125}\text{I}]\text{HYP} = R_T \Big/ \left[1 + \frac{K_A}{[^{125}\text{I}]\text{HYP}} \left(1 + \frac{B}{K_B}\right)\right] \qquad [1]$$

This equation [1] deals with specifically bound drug (R · [^{125}I]HYP). To avoid transformation of the observed data (total drug bound) by subtraction of an estimated amount of nonspecifically bound drug (NSB) we can modify the left-hand side of Eq. [1]:

$$R \cdot [^{125}\text{I}]\text{HYP}_{\text{total}} - \text{NSB} = R_T \Big/ \left[1 + \frac{K_A}{[^{125}\text{I}]\text{HYP}} \left(1 + \frac{B}{K_B}\right)\right] \qquad [2]$$

NSB thus becomes a parameter to the equation. If two types of β-adrenoceptors are present in the tissue under analysis and if each component obeys

simple mass action law without any interference, then Eq. [2] can be further generalized to

$$R \cdot [^{125}\text{I}]\text{HYP}_{\text{total}} - \text{NSB} = \sum_{i=1}^{2} \frac{R_{T_i}}{1 + (K_A/[^{125}\text{I}]\text{HYP})(1 + B/K_{B_i})} \quad [3]$$

In Eq. [3] it is further assumed that the radioligand [^{125}I]HYP binds with equal affinity to β_1- and β_2-adrenoceptors, whereas B possesses a different affinity for each receptor subclass. The validity of these assumptions has been discussed by Engel (*14*) for [^{125}I]CYP ([^{125}I]cyanopindolol) and applies equally to [^{125}I]HYP. We have used both ligands in our studies.

Two constants, the concentration of [^{125}I]HYP ([^{125}I]CYP) in the assay and K_A (the affinity of the radioligand for the receptor population under study) are required by Eq. [3]. The former is obtained by counting the standard used in the assay, the latter is obtained in a separate experiment, using saturation analysis and the data-reduction method of Scatchard (*7*). To simplify the latter procedure a set of programs written in Applesoft BASIC is used. The computer is equipped with a light pen for interactive editing of the regression line and details have been published elsewhere by Clark *et al.* (*15*) in a simplified form for similar analyses on steroid receptors.

The data from each measurement of the displacement curve are repeatedly fitted according to Eq. [3], using the model of i = one or two classes of binding sites using an algorithm based on the Gauss–Newton method (*16*) which supports simultaneous estimation of up to 10 parameters. If NSB has been estimated separately in the assay as a measurement it can either be subtracted from the total counts at each point and the data fitted with and without attempting to solve for NSB as a parameter, or the observed value for NSB can be supplied simply as an initial starting value without transforming the data. Using either method, the observed NSB can be compared with the theoretical NSB for the assay.

For the unknown parameters the program provides the best estimates together with their standard deviations and the sum of squares of the residuals. The goodness of fit is estimated using the general linear test (*21*) since the models for one- and two-site fit can be considered to form an hierarchical series. By F-test analysis the goodness of fit between the models can be compared and the most appropriate model selected.

(*b*) *Program structure*. The package is also designed to provide all the necessary facilities for file and data handling, so that it is not just another isolated nonlinear least squares regression program. The options available from the main menu are detailed below.

(1) *Input raw counts*. The results of the receptor assays are in the form of cpm (counts per minute) from a gamma counter, along with the concentration of membrane protein in the assay. This program takes the data and converts them into moles of drug bound/milligrams protein. If using ^{125}I-labeled drugs, correction is made for the age of the isotope. At this stage, NSB may be

subtracted from the data if required. The results are output to a workfile—WORK.DAT—which is used by all the other programs in the package.

(2) *Manual input.* Data available from earlier experiments in the form of drug concentration and corresponding amount of ^{125}I ligand bound may be entered into the workfile using this program.

(3) *Edit/review.* Data in the workfile may be listed out on the screen for verification or sets or data (drug concentration and ^{125}I bound) may be added or deleted. Before modifying the data set, the user should use option 7 to take a copy of the initial workfile.

(4) *Initial estimates.* The algorithm used for the nonlinear least squares requires that reasonable initial estimates are given for the fitting parameters. This can be done by various means such as serendipity or based on experience. The method chosen here is to use a graphical technique where the user is shown a graph of the data points and then asked if a one- or two-site fit is required. The program then prompts for estimates of the various parameters and then sketches in the curve corresponding to the supplied parameters. A typical session is shown in Fig. 1 where the user is obtaining initial estimates for a two-site fit of the IPS-339 data shown in Table I. Before leaving the program a summary of the final estimates is given on the screen in confirmation.

(5) *Fit to 1 or 2 Sites.* Once initial estimates have been obtained the user may proceed to the nonlinear curve fitting. Again, data entry is conversational and the assay constants and initial estimates required by the program are entered. The user is then prompted for the constraints to be imposed on the parameters and the program then proceeds to fit the data, printing out the final estimates, their standard deviations, and estimates of the goodness of fit and an analysis of

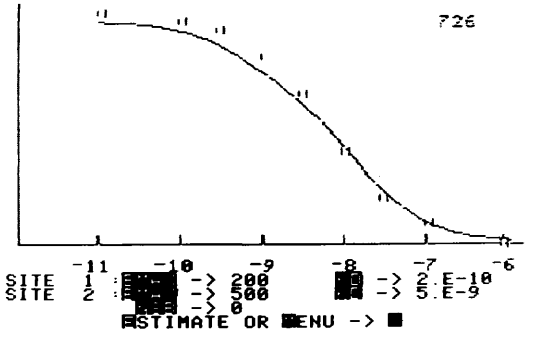

FIG. 1. Photograph of Apple screen while obtaining initial estimates for a two-site fit on the IPS-339 data in Table I. The number in the top right-hand corner of the screen is the maximum estimated number of receptors (fmole/mg protein) and remains there at all times to prompt the user. The user then enters estimates for the number of receptors (BMAX, corresponds to R_{T_i} in Eq. [3]) and receptor affinity (KD, corresponds to K_{B_i} in Eq. [3]) for each site and the program then draws the estimated line through the data points. Note that the sum of the two BMAXs approximates to the estimated figure of 726. In this example the estimates are reasonable enough to proceed to the curve-fitting option.

TABLE I

SAMPLE DATA FOR IPS-339 WITH THE ASSAY CONSTANTS AND INITIAL ESTIMATES AND CONSTRAINTS USED FOR THE FITTING PROCEDURE

Concentration of IPS-339 (mole/liter)	[^{125}I]CYP bound (fmole/mg protein)
0.0	460.5
1.0E-11	461.0
1.0E-10	442.2
3.0E-10	424.1
1.0E-09	371.5
3.0E-09	297.2
1.0E-08	182.5
3.0E-08	89.3
1.0E-07	38.2
1.0E-06	4.85

Assay constants
K_A = 410 pmole/liter.
Concentration of [^{125}I]CYP in assay = 710 pmole/liter.
Initial estimates and constraints
Site 1: RT_1 = 200 fmole/mg protein (50.0 to 300.0)
K_{B1} = 2.0E-10 mole/liter (1.0E-11 to 1.0E-09)
Site 2: RT_2 = 500 fmole/mg protein (300.0 to 800.0)
K_{B2} = 5.0E-09 mole/liter (1.0E-10 to 1.0E-08)
NSB: 2.0 (−20.0 to 20.0)
Weighting factor: 0 (unweighted fit)

Note. The final parameters obtained after fitting this data to a two-site fit are given in the results, with the corresponding graphical display in Fig. 2.

residuals. If required the fit may then be repeated. The constraints may be altered manually, or left unchanged. On completion, the final parameter estimates are written out to disk. The progress of the fit is continuously updated on the screen and the fitting process may be terminated at any time by simply pressing any key on the keyboard; the current values of the parameters are then printed out as above.

(6) *Graphical display of fit.* This program takes the final parameter estimates from disk and the data in the workfile and produces a fully annotated plot as in Fig. 2. This plot can also be output to the printer for hard copy. Should inspection of the plot reveal any obviously erroneous points, the corresponding data set can be removed from the file using the EDIT option and the fitting process repeated.

(7) *Disc utilities.* These provide the package with full file handling facilities and is menu driven. The workfile may be copied to a named file or previous data recovered. Saved files may be listed or deleted. Use of the powerful STRING 80 BIT routines means that an alphabetically sorted disk directory

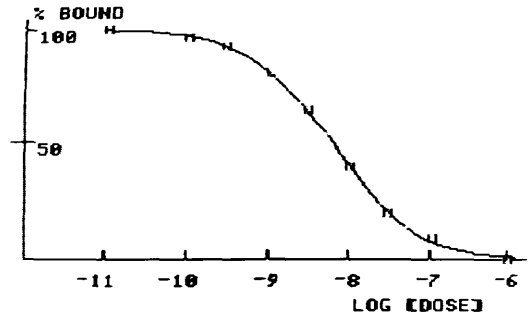

FIG. 2. Photograph of Apple screen showing the graphical result of the two-site curve fitting. The plot is fully annotated and can be output to the printer for hard copy. Note that goodness of fit (i.e., one- or two-site) is assessed only by use of the general linear test and not by visual inspection of this plot.

can be held inside the program, so that attempts to recover a nonexistant file, or to overwrite an already existing file, can be detected and the user alerted.

(8) *General linear test.* This option holds an F table at the 5% level (*22*) and advises on whether a one- or two-site fit is more appropriate. Since the models form a hierarchy of models, using the one-site model as a reduced (R) model, and a two-site model as a full (F) model then:

$$\left(\frac{SSQ_R - SSQ_F}{(df_R - df_F)}\right) \div \left(\frac{SSQ_F}{df_F}\right)$$

is F distributed with $df_R - df_F$, df_F degrees of freedom. This program prompts the user for the residual sum of squares (SSQ) and the appropriate degrees of freedom, evaluates the above equation, looks up the theoretical F value in its table and informs the user which model gives the best fit.

(9) *Return to CP/M.* This option returns the user to the CP/M operating system so that other programs may be run.

Cell receptor displacement curves. Cell receptors estimations were done as previously described (*1, 2, 5*).

RESULTS AND DISCUSSION

Both real and contrived data were fit to one- and two-binding-site models. Typical real data was obtained using the β_2 drug IPS-339 (*23*) (a gift from Dr. G. LeClerc) to displace [^{125}I]HYP from a preparation of ciliary process membranes. Contrived data was generated using a short BASIC program fed with theoretical parameters equipped with a random number generator to add errors in a specified percentage range. When fitting the data without weighting, RECFIT gave closely similar values to the mainframe package BMDPAR (*10*). The real data, from which the observed NSB had already been subtracted, fit best to a two-site fit; site 1: $R_{T1} = 175.55 \pm 66.25$ fmoles/mg protein, $K_{B1} = 0.34 \pm 0.18$ nM; site 2: $R_{T2} = 541.31 \pm 65.33$ fmoles/mg protein, $K_{B2} = 3.68 \pm 0.67$

n*M*. The NSB evaluated to be -3.44 ± 4.28 (i.e., essentially zero) showing that the classical way of determining NSB (*17*) was acceptable for IPS-339 in this assay system; the expected value for NSB was 0. The graphical output of the final fit is shown in Fig. 2; an exact facsimile of the computer screen may be sent to the printer for future reference or to simplify the work of medical illustration in producing diagrams for papers, theses, etc. The two-site fit with estimation of NSB took 4 min for the fitting, 30 sec for the graph generation and 2–3 min to print the data and graph.

The results from this particular data set were of interest in that they displayed a biphasic displacement curve when only one phase would have been expected. The cause of this was investigated as it may have meant that, for example, this particular preparation was contaminated with β_1 receptors or that IPS-339 was displacing [^{125}I]HYP off other receptors (e.g., alpha or histamine (*14*)). Repeat experiments gave only one-site fits, so it was suspected that this preparation of ciliary processes was faulty. Apart from routine data processing therefore, this package has very real application as a research and diagnostic tool.

The nonlinear regression algorithm used in this fitting is that of Gauss–Newton (*16*) with the provision for setting constraints on the parameters and appears to be very robust. The initial development lacked these constraints and the program often failed on fitting difficult data sets due to arithmetic overflow as the parameters were adjusted in the initial iterations.

The use of nonlinear regression algorithms on microcomputers is still a fairly new area of development. General programs are available for the PET microcomputer (*18*), the Texas TI-59 programmable calculator (*19*) and a more restricted one by Duggleby (*12*) but these lack full file handling and graphics capability. Recently, Greco *et al.* (*20*) have produced a dedicated graphics package (ROSFIT) for enzyme kinetics. Since RECFIT is intended to be used by clinicians and clinical biochemists a dedicated approach is required for the cell–receptor assays as many of these users are not familiar with computers. Because of this it is important to help such users develop a "feel" for their data. To this end, the initial estimates for the regression are obtained graphically. Users have generally reported that this facility is indeed helpful, and provides insight both into the curve-fitting process and also aids in understanding the final printout. By adopting a dedicated approach, it is possible to provide comprehensive error checking on data input, and to annotate the graphs with meaningful legends. The provision of full file-handling facilities means that data files can be preserved and past results are available for instant recall and reprocessing.

In summary, a comprehensive package to enable the quantification of cell receptor subtypes has been implemented on an Apple II microcomputer. This has considerable advantages over the mainframe approach; although the actual fitting is slower, the microcomputer is available at bench level, 24 hr a day. The package is dedicated and easy to use, with fully annotated graphical output. Printout is available immediately without the need to go outside the depart-

ment. This package has been successfully used with α_2-receptor assays and, indeed should be capable of use with any receptor system which needs quantitative assessment of receptor subpopulations providing they meet with the limitations and assumptions outlined for the beta-receptor system (*13, 14*).

RECFIT has been designed to run on an Apple II microcomputer extended by the addition of a 16K Apple Language card, Grappler Printer Card (Orange-Micro, 3150 E. La Palma, Suite G, Anaheim, Calif.) to allow both text and graphics to be output to a Paper Tiger 560 printer (2K graphics buffer, Integeral Data Systems, Milford, N.H. 03055), a Z-80 CP/M card to give access to the CP/M 2.22 56K operating system (Microsoft Consumer Products, 400 108th Ave. NE, Suite 200, Bellevue, Wash. 98004), a 12-in. B/W monitor and 2 Apple disk drives. Users interested in the software should contact the principal author in the first instance.

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ALPHA$_2$-ADRENOCEPTORS IN HUMAN LYMPHOCYTES: DIRECT CHARACTERISATION BY [^{3}H]YOHIMBINE BINDING

Sa'ad Titinchi and Barry Clark*

Department of Pathological Biochemistry, Western Infirmary, Glasgow, G11 6NT, Scotland, UK

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SUMMARY: [^{3}H]yohimbine, a potent and selective alpha$_2$-adrenergic antagonist was used to label alpha-adrenoceptors in intact human lymphocytes. Binding of [^{3}H]yohimbine was rapid ($t_{1/2}$ 1.5 -2.0 min) and readily reversed by 10uM phentolamine ($t_{1/2}$ = 5 - 6 min) and of high affinity (K_d = 3.7 + 0.86nM). At saturation, the total number of binding sites was 19.9 + 5.3 fmol/10^7 lymphocytes. Adrenergic agonists competed for [^{3}H]yohimbine binding sites with an order of potency: clonidine > (-) epinephrine > (-) norepinephrine > (+) epinephrine >> (-) isoproterenol; adrenergic antagonists with a potency order of yohimbine > phentolamine > prazosin. These results indicate the presence of alpha$_2$-adrenoceptors in human lymphocytes.

INTRODUCTION: Radioligands have been successfully used to label alpha-adrenoceptors in different tissues (1-5). In the last few years it became clear that there are two subtypes of alpha receptors, termed alpha$_1$ and alpha$_2$ receptors (6). Alpha$_1$ receptors include typical postsynaptic alpha receptors mediating smooth muscle contraction. alpha$_2$ receptors include not only all known presynaptic autoregulatory alpha receptors but also some less typical postsynaptic receptors existing on, for example, human platelets. Using radioligand binding techniques, two different approaches have been applied to determine the presence of alpha$_1$ and alpha$_2$ receptors. The first involves the use of a non-selective radioligand such as [^{3}H] dihydroergocryptine ([^{3}H] DHE) to label the entire alpha receptor population. The alpha receptor subtypes are distinguished by constructing competition curves using antagonist compounds which have selectively greater potency for one or the other alpha receptor subtype (7-11), for example, the antagonists prazosin (alpha$_1$ selective) and yohimbine (alpha$_2$ selective). The second, the most useful and widely applied criterion for distinguishing between alpha receptor subtypes, is by using radioligands which themselves selectively label either alpha$_1$ or alpha$_2$ receptors (12-21). [^{3}H]prazosin is selective for alpha$_1$ receptors whereas [^{3}H]yohimbine is selective for alpha$_2$ receptors. Previous studies using [^{3}H] DHE were able to identify alpha-adrenoceptors in human lymphocytes (22), but the subtype was not defined.

The purpose of this study was to characterize the alpha adrenoceptor subtypes in intact human lymphocytes.

MATERIALS AND METHODS: [^{3}H]yohimbine (84.5 Ci/mmol) was obtained from New England Nuclear, West Germany. The following drugs were kindly donated by the sources indicated: prazosin hydrochloride (Pfizer, UK); (-) epinephrine

*To whom correspondence should be addressed.

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BP (Smith and Nephew, UK); and (+) epinephrine bitartrate (Kodak, UK). Other drugs and chemicals were obtained from commercial sources.
Lymphocytes were isolated as described elsewhere (23).

Alpha$_2$-Adrenoceptor Assay - The saturation assays were done in polypropylene tubes (Sarsted, 12 x 75mm) in a final volume of 500 ul, comprising for the total binding: lymphocyte suspension (250-350 ug protein) and [^{3}H]yohimbine at appropriate concentrations (1-20 nM) in Buffer A (Earle's Balanced Salt Solution minus sodium bicarbonate and supplemented with 0.1% calf serum and 20 mM Hepes at pH 7.6). In tubes used to determine non-specific binding, 10 uM phentolamine was also added. Total and non-specific binding were estimated in duplicate at each [^{3}H]yohimbine concentration. The tubes were incubated at 25C for 25 minutes. The reaction was stopped by the addition of 2.5 ml Buffer B (50 mM Tris and 0.9% sodium chloride, pH 7.6) at 25C. The samples were then filtered through Whatman GFC fibre-glass filters (pre-wetted in Buffer B) by applying a vacuum of 3-4 psi, and the filters were rinsed with 2 x 10 ml of Buffer B at 25C. Filtering and rinsing were complete within 12 s. The filters were air dried, shaken with 10 ml scintillator 299 (Packard) and the radioactivity determined (50% efficiency).

Data Analysis - All experiments were performed in duplicate and replicated at least twice. The number (B_{max}) and sensitivity (K_d) of the lymphocyte alpha$_2$-adrenoceptors were determined from Scatchard analysis (24). The dissociation constant (K_d) of various competing drugs for binding sites was calculated from the equation of Cheng and Prusoff (25).
All data shown are the mean $\pm$ standard deviation where indicated.

RESULTS:

Kinetic Analysis of Binding - Binding of [^{3}H]yohimbine to human lymphocytes at 25C was rapid, with half - maximal specific binding ($t_{1/2}$) of 1.5-2.0 min (Fig.1), reaching equilibrium within 16-20 min. Binding remained stable for

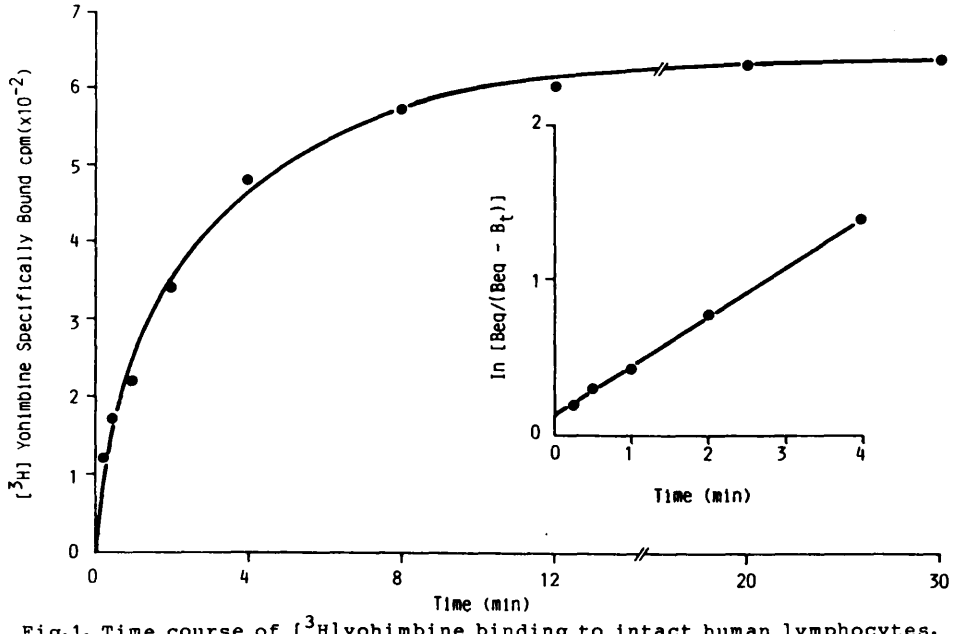

Fig.1. Time course of [^{3}H]yohimbine binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{3}H]yohimbine (5nM) in the absence and presence of 10uM phentolamine. At the indicated times, the samples were diluted with buffer B and filtered. Specific binding was determined in duplicate at each time interval. (Inset) the regression line (r=0.99) was determined by the plot of ln [$B_{eq}/(B_{eq}-B_t)$] vs. time, where B_{eq} = binding at equilibrium and B_t = binding at time t. K_{ob} is equal to the slope of the line.

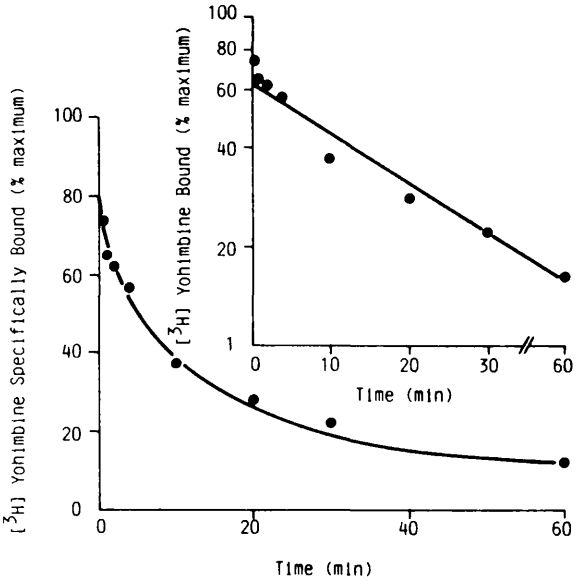

Fig.2. Time course for dissociation of [^{3}H]yohimbine binding to intact human lymphocytes. Lymphocytes in buffer A were incubated with [^{3}H]yohimbine (5nM) for 25 min to allow equilibrium. At zero time, 10uM phentolamine was added to the samples and specific binding was determined at various time intervals. 100% binding refers to the specific binding just prior to the addition of phentolamine at zero time. (Inset) first-order rate plot of dissociation of [^{3}H]yohimbine binding. K_2 is equal to the slope of the line.

30 min at 25C. An incubation time of 25 min was chosen to represent equilibrium binding in subsequent experiments.

The initial rate constant (K_{ob}) for the association reaction obtained from the slope (Fig.1,inset) was 0.32 min^{-1} for 5nM [^{3}H]yohimbine. The dissociation of bound [^{3}H]yohimbine at 25C was determined by incubating lymphocytes to equilibrium and then adding 10uM phentolamine at time zero and measuring residual specific binding at subsequent time intervals (Fig.2). Dissociation was rapid with a $t_{1/2}$ of 5.0-6.0 min (Fig.2) and the first - order dissociation rate constant (K_2) was 0.138 min^{-1} (Fig.2, inset).

The second - order association rate constant (K_1) was then calculated from the equation (26): $K_1 = (K_{ob} - K_2)/[yohimbine]$, where [yohimbine] is equal to the concentration of [^{3}H]yohimbine in the assay (5nM), K_1 was 0.364 x 10^8 M^{-1} min^{-1}. The equilibrium dissociation constant (K_d), determined from the ratio of K_2/K_1, was 3.79nM.

Saturability of Binding - The binding characteristics of [^{3}H]yohimbine to intact human lymphocytes is described in Fig.3. Specific binding of [^{3}H]yoimbine (the binding that could be displaced by 10uM phentolamine) was clearly saturable and of high affinity. Apparent saturation of binding sites seemed to occur at [^{3}H] yohimbine concentration of 14-16nM, with half maximal binding occuring at about 2-3nM. Scatchard analysis (Fig.3, inset) of the saturation data yielded a straight line, indicating that

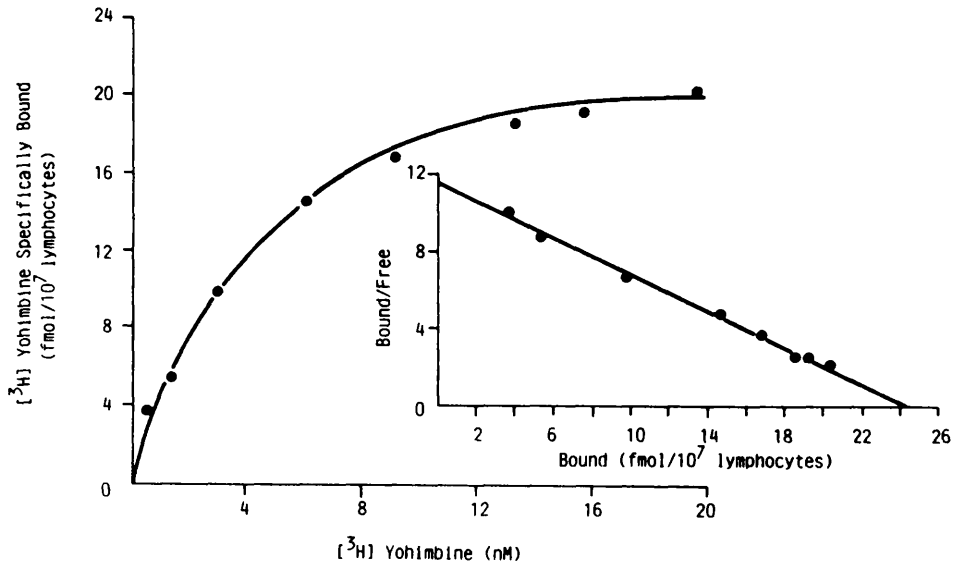

Fig 3. Specific binding of [^{3}H]yohimbine to intact human lymphocytes as a function of concentration of [^{3}H]yohimbine. Lymphocytes in buffer A were incubated with various concentrations of [^{3}H]yohimbine in the presence and absence of 10uM phentolamine. Specific binding was determined at each concentration of [^{3}H]yohimbine. (Inset) Scatchard analysis of the binding data yielding a K_d of 4.24nM and the total number of receptors was 24.8 fmol/10^7 lymphocytes.

[^{3}H]yohimbine bound to a single class of receptors. The dissociation constant (K_d) of binding was 3.7 ± 0.86nM (8 experiments) and the total number of binding sites was 19.9 ± 5.3 fmol/10^7 lymphocytes.

Inhibition of [^{3}H]Yohimbine Binding by Competing Ligands - Binding of [^{3}H]yohimbine was stereospecifically inhibited by epinephrine (Table 1); the (-) isomer was more potent than the (+) stereoisomer. Adrenergic agonists competed for [^{3}H]yohimbine binding sites with a potency order of clonidine >

Table 1

The dissociation constants (K_d) of drugs determined by competition for [^{3}H]yohimbine binding

Drug	Dissociation Constant (K_d,uM)
Clonidine	0.28 ± 0.027
(-)Epinephrine	1.51 ± 0.15
(+)Epinephrine	16.20 ± 1.3
(-)Norepinephrine	6.74 ± 0.65
(-)Isoproterenol	126.00 ± 12.0
Yohimbine	0.01 ± 0.0011
Phentolamine	0.03 ± 0.0028
Prazosin	2.10 ± 0.2

The dissociation constant (K_d) was calculated according to the equation of Cheng and Prusoff (25). Results are expressed as the Mean ± SD (4 expts).

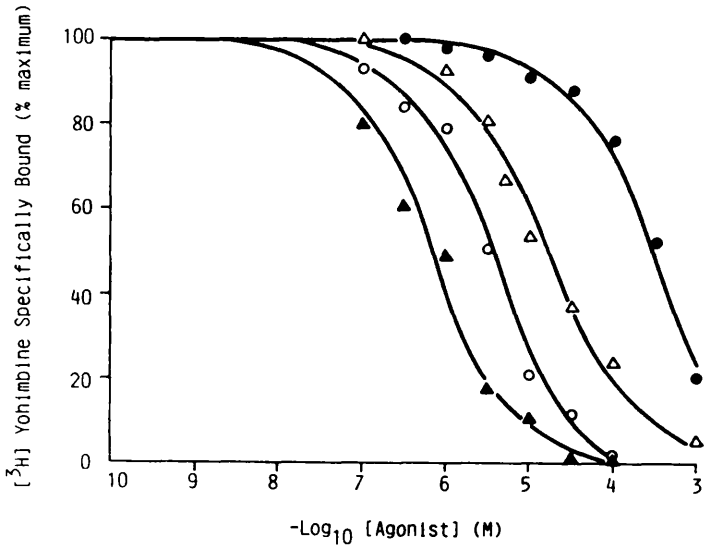

Fig.4. Competition for [^{3}H]yohimbine binding sites by adrenergic agonists in intact human lymphocytes. Various concentrations of clonidine (▲), (-) epinephrine (○), (-)norepinephrine (△) and (-)isoproterenol (●) were incubated with lymphocytes and [^{3}H]yohimbine (5nM) for 25 min. Then, the samples were diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

(-) epinephrine > (-) norepinephrine > (+) epinephrine >> (-) isoproterenol, with K_d values of 0.28, 1.50, 6.74, and 16.00 uM for clonidine, (-) epinephrine, (-) norepinephrine and (+) epinephrine respectively (Fig.4 and Table 1). The beta-adrenergic agonist, (-) isoproterenol, was a very weak inhibitor of [^{3}H]yohimbine binding (K_d = 126.00 uM, Fig.4 and Table 1).

The alpha-adrenergic antagonists phentolamine, yohimbine and prazosin competed for [^{3}H]yohimbine binding sites with a potency order of yohimbine > phentolamine > prazosin (Fig.5 and Table 1). Yohimbine, an $alpha_2$ adrenergic antagonist competed for the binding sites with a K_d of 0.01 uM. Phentolamine, a non-selective alpha-adrenergic antagonist competed with a K_d of 0.03 uM. Prazosin, a specific $alpha_1$-adrenergic antagonist, competed with a K_d of 2.00 uM.

DISCUSSION: Earlier studies by us (23) have indicated the presence of a circadian variation in $beta_2$-adrenoceptor number and affinity in lymphocytes of both normals and asthmatic patients. To continue these studies it was essential to investigate if there was a similar variation in $alpha_2/beta_2$ receptor ratios in one or other (or both) of the groups as other workers have described differences between the alpha/ beta ratios in normals and asthmatic patients (22) but have not defined the receptor subtypes. These workers used [^{3}H] DHE to look at the general alpha receptor population. Perhaps as a result of the low specific activity of commercially available preparations of [^{3}H]DHE we were unable to reproduce the method satisfactorily, and so developed the method described here using the much higher activity ligand [^{3}H]yohimbine.

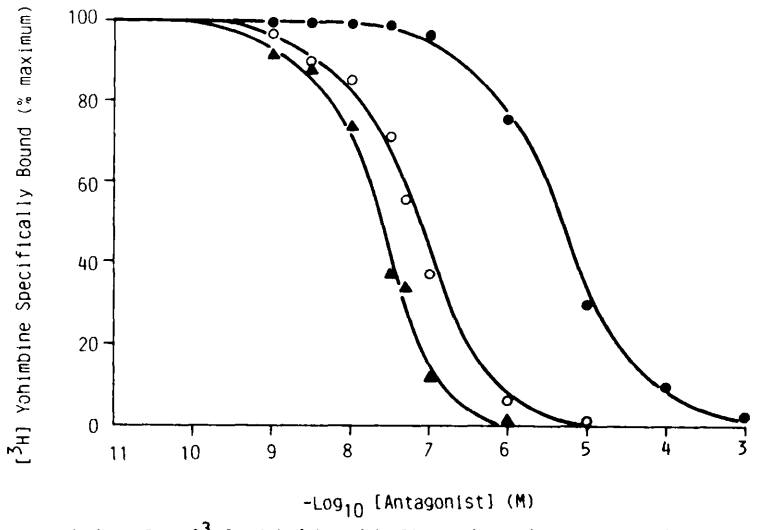

Fig.5. Competition for [^{3}H]yohimbine binding sites by adrenergic antagonists in intact human lymphocytes. Various concentrations of yohimbine (▲), phentolamine (○) and prazosin (●) were incubated with lymphocytes and [^{3}H]yohimbine (5nM) for 25 min. Then, the samples were diluted and filtered. Specific binding was determined in duplicate at each drug concentration.

[^{3}H]yohimbine has been used to label alpha$_2$-adrenoceptors in a variety of tissues (17-21). Other workers (15,16) using [^{3}H]yohimbine to label alpha$_2$ adrenoceptors in human platelets have shown that this radioligand appears to have a number of advantages as compared to other radioligands, including lower non-specific binding, higher specific activity and fewer cells required. Accordingly, we have used [^{3}H]yohimbine in our present study to demonstrate the existence of binding sites for this ligand on intact human lymphocytes. Our results indicate that [^{3}H]yohimbine binding was rapid and reversible. Specific binding was high (60-80%) and saturable with high affinity.

The dissociation constant of [^{3}H]yohimbine of 3.7 $\pm$ 0.86 nM established at equilibrium was very similar to that of 3.31 $\pm$ 0.49 nM determined by kinetic data (i.e., ratio of association and dissociation rate constants). Similar dissociation constants have been observed for human platelets (15) and for human adipocyte membranes (18).

The stereospecificity of the receptor was shown by (-) epinephrine displacing [^{3}H]yohimbine more potently than (+) epinephrine. Binding of [^{3}H]yohimbine was inhibited by adrenergic agonists with the potency order of clonidine > (-) epinephrine > (-) norepinephrine >> (-) isoproterenol and by adrenergic antagonists with a potency series of yohimbine > phentolamine > prazosin. The dissociation constants for these adrenergic drugs (Table 1) are in accord with data reported by Motulsky et al (15) for human platelets.

Therefore these observations indicate the presence of putative alpha$_2$-adrenoceptors on human lymphocytes.

Finally to our knowledge, this is the first report to characterize alpha$_2$-adrenoceptors in intact human lymphocytes using the radioligand [^{3}H]yohimbine.

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LETTER TO THE EDITORS

Identification of Beta-Adrenergic Receptors in the Pigmented Mammalian Iris–Ciliary Body Diaphragm

Beta-adrenergic receptors have been recently identified in the animal iris–ciliary body diaphragm (Neufield and Page, 1977) and ciliary processes (Bromberg, Gregory and Sears, 1980). The presence of melanin in non-albino animal ciliary bodies has prevented us from applying these ligand binding techniques to the study of beta-receptors and their potential role in intra-ocular pressure control, despite a recent publication to the contrary (Bhargava, Makman and Katzman, 1980). As a result we have developed a simple technique to identify these receptors in the pigmented iris–ciliary body diaphragm. Iris ciliary bodies are isolated using an operating microscope and beta-receptors in the melanin-free membranes are estimated using 125I-iodohydroxybenzylpindolol (125I-HYP) in a ligand binding assay (modified from Insel and Stoolman, 1978).

Eyes from freshly slaughtered rams were immediately enucleated and placed on ice. The corneas were removed with a 6–8 mm rim of sclera under the operating microscope. The iris–ciliary body diaphragm was then dissected off the underlying vitreous under direct vision. Within 1 hr of death the iris–ciliary body diaphragm was freed and placed into cold Hartman's solution at 4°C. Tissue from each eye was homogenized separately in 3 ml of a buffer solution consisting of 50 mM-Tris (pH 7·5) and 1 mM-ascorbic acid (Buffer A). Tissue was homogenized initially with an Ultraturrax (75% full speed, 5 × 10 sec, 4°C) and then 20 strokes in a ground glass hand homogenizer. One ampoule of Hyalase (1500 i.u.) was added to the pooled homogenate, mixed and allowed to stand for 1 min. The mixture was then centrifuged (3000 ***g***, 20 min, 4°C). The pellet was resuspended in 3 ml of buffer A and further homogenized with the Ultraturrax (25% full speed, 2 × 10 sec, 4°C). The homogenate was loaded onto a discontinuous sucrose gradient of 5%:75% and centrifuged (131000 ***g***, 18 hr, 4°C) in a Beckman SW27 rotor. This process successfully removed all melanin to the bottom of the tube and the left membrane particles at the interface. Electron microscopy has confirmed the presence of melanin-free membrane at the 5%:75% interface (Fig. 1). The interface was collected, diluted in buffer A and then re-centrifuged (131000 ***g***, 3 hr, 4°C). The pellet was then resuspended by gentle hand homogenization in a buffer containing 20 mM-Hepes (pH 7·6), Earles Balanced Salt Solution (Flow Laboratories) and 0·3 mM-catechol (BDH) (Assay Buffer). All procedures were performed at 4°C.

To detect the presence of beta-adrenergic receptors on the melanin-free membranes the ligand 125I-HYP, which has a high affinity for these receptors (Brown, Aurbach, Hauser and Trexler, 1976) was used. In these preliminary studies two fundamental properties of such receptors, namely saturability and stereospecificity, were used to study how the ligand binds to our membrane preparations.

When studying the saturability of receptors it is essential to consider only the specifically bound 125I-HYP since this ligand also binds non-specifically to the cell membranes. To do this, total binding is estimated for a range of 125I-HYP concentrations (see Fig. 2) and then repeated in the presence of 3×10^{-6} M-propranolol. This concentration will displace 125I-HYP only from the beta-receptors (see

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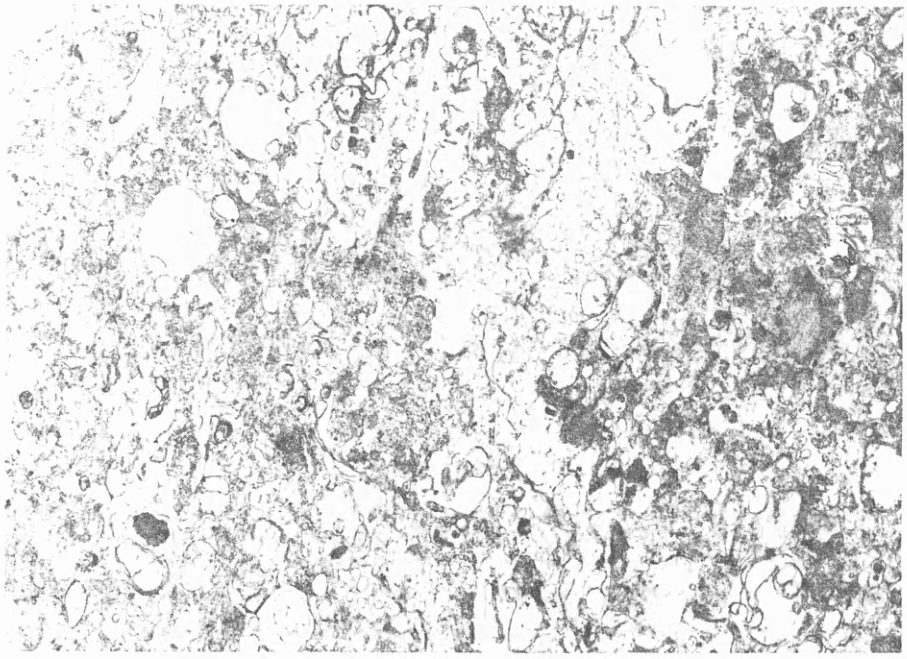

FIG. 1. Electron micrograph showing melanin free membrane particles isolated from the 5%:75% sucrose density gradient interface. Magnification × 8000.

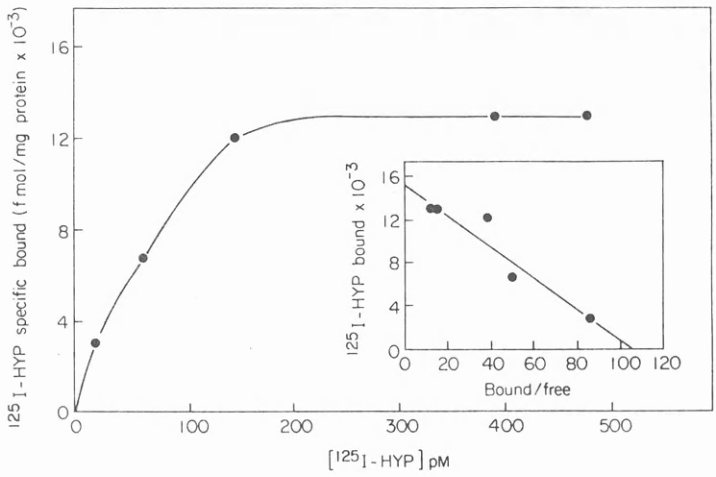

FIG. 2. Saturation binding curve and Scatchard analysis (inset).

Fig. 3). Any 125I-HYP which remains bound is non-specific binding (NSB). Specific binding = total binding − NSB.

The saturation assays were done in polypropylene tubes (Sarstedt, part no. 526, 12 × 75 mm) in a final volume of 250 μl comprising (final concentrations in parenthesis) for the total binding: membrane suspension (5–10 μg protein) and 125I-HYP (New England Nuclear) at appropriate concentrations (0–500 pM). In tubes used to

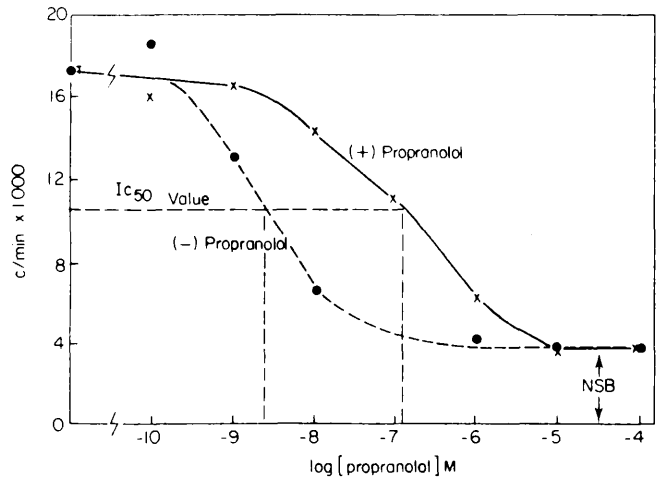

FIG. 3. Relative potencies of (+) and (−) propranolol in displacing 125I−HYP from the receptors. [T = Total binding. NSB = non-specific binding. IC50 value = T−NSB/2).]

determine NSB (−) propranolol (3×10^{-6} M) was also added. Totals and NSB were estimated in duplicate at each 125I-HYP concentration.

To demonstrate stereospecificity of the receptors, membrane suspensions are incubated with a fixed concentration of 125I-HYP in the presence of increasing amounts of the (+) and (−) stereoisomers of propranolol (1×10^{-10} M to 1×10^{-4} M, see Fig. 3). If a functional receptor is present the relative potencies at displacing 125I-HYP from the receptor should be (−) ≫ (+).

Displacement assays were done in duplicate in polypropylene tubes in a final volume of 250 μl comprising membrane suspension (5–10 μg protein), 125I-HYP (150 pM) and (+) or (−) propranolol (1×10^{-10} M to 1×10^{-4} M) as appropriate (Fig. 3). To determine the total binding, propranolol was omitted from the assay.

For both experiments the assay mixtures were prepared on ice and incubation was commenced by transfer of the tubes to a 36°C water bath, at 1 min intervals. When transfer was completed, the bath was covered and the samples incubated in the dark (to minimize photolysis of the 125I-HYP) for 45 min.

The reaction was stopped by adding 2·5 ml of a 'stopping solution' of 50 mM-Tris (pH 7·6), 0·9% sodium chloride and 0·1 mM (±) propranolol at 37°C. The samples were then allowed to stand for 45 sec before filtering through Whatman GFC fibreglass filters by applying a vacuum of 3–4 psi. Membranes were retained on the filters and were rinsed with 3 × 10 ml of 50 mM-Tris (pH 7·6), 0·9% sodium chloride at 37°C. Filtering and rinsing was complete within 12 sec.

This 'stopping procedure' has several effects. Firstly the dilution minimizes sample loss on transfer of the tube contents to the filter. Secondly, the 125I-HYP concentrations fall by a factor of 11 and since the kinetics of binding of the non-specifically bound ligand, are much more rapid than those of specifically bound ligand (Maguire, Wiklund, Anderson and Gilman, 1976), there is a tendency for the non-specifically bound ligand to leave the membrane. Thirdly, this process is assisted by the use of propranolol at 0·1 mM which, for similar reasons, encourages the non-specifically bound 125I-HYP to leave the membrane while not significantly affecting specifically bound ligand. Since 125I-HYP also binds avidly to GFC filters, these are pre-soaked

(90 min) in 'stopping solution': the propranolol saturates the non-specific sites on the filters.

All these measures and the 3×10 ml rinse, significantly reduce non-specifically bound counts trapped on the filters (either directly or on the cell membranes) and improve the reproducibility and sensitivity of the assay. NSB is usually 20–40 % of the total binding.

The filters were air-dried overnight at room temperature and the associated radioactivity was determined in a Packard Auto Gamma Scintillation Spectrometer. Membrane protein concentration was estimated using the commercially available Bio-Rad kit(Bio-Rad part no. 1401).

Figure 2 shows that 125I-HYP binding specifically to the membrane preparations does saturate and as such is amenable to saturation analysis after the method of Scatchard (1949). The inset to Fig. 2 shows such an analysis of the data. The correlation coefficient of this line is 95 % with $P < 0{\cdot}05$ indicating a single class of receptor binding sites with no interference from non-specific binding. The Ka of binding in this representative experiment is 50 pM with a B_{max} of 1500 fmol/mg protein.

Figure 3 demonstrates the stereospecificity of the beta-receptor sites, (−) propranolol being much more potent than (+) propranolol—Kd, the displacement constant, is $4{\cdot}36 \times 10^{-10}$ M (−) and $3{\cdot}28 \times 10^{-8}$ M (+ propranolol)—by a factor of 75. This figure is typical of a functional stereospecific beta-receptor. The Kd values are calculated from the equation $Kd = IC50/(1 + S/Ka)$ where IC50 is the concentration of drug which displaces 50 % of the total specific binding (specific = T-NSB, see Fig. 3) and S is the concentration of 125I-HYP used in the displacement assays.

We conclude that the membrane fractions isolated by the above procedures demonstrate the presence of putative beta-adrenergic receptors as classified by the criterion of saturability of specific binding, a sensitive Ka (50 pM) and stereospecificity towards (+) and (−) propranolol. This method of identifying beta-receptors in pigmented eye tissue is of great value. In contrast to other workers (Bhargava et al. 1980) working with different species, we find that if beta-receptors are estimated in crude preparations of sheep eyes, significant and variable amounts of the added 125I-HYP (30–60 %) will be bound to melanin, in contrast to the cell membrane which binds approximately 1–2 %, thus significantly affecting the results of the assay, not only by altering the final concentration of 125I-HYP in the assay, but also by giving very high non-specifically bound counts on filtration, to the detriment of the reproducibility and sensitivity of the assay.

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Tennent Institute of Opthalmology,
University of Glasgow,
Scotland
GRAHAM E. TROPE,

Department of Pathological Biochemistry,
Western Infirmary,
Glasgow,
Scotland
BARRY CLARK
AND S. J. S. TITINCHI

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Letter to the editor

Beta-adrenergic receptor subtypes iris-ciliary body of rabbits

C.J. Schmitt et al., Graefe's Arch Clin Exp Ophthalmol (1984) 221:167–170

B. Clark,[1] * G.E. Trope,[2] and S.J.S. Titinchi [1]

[1] Department of Pathological Biochemistry, Western Infirmary, Glasgow G11 6NT, UK
[2] Department of Opthalmology, Toronto General Hospital, 101 College St. Ontario, Canada, M5G 1L7

Dear Sir, – We read the above article with interest. As workers who have developed a technique for investigating the presence of beta-adrenoceptor subtypes in pigmented mammalian eyes [1] and published articles on the predominance of the beta-2 adrenoceptor subtypes in iris-ciliary body diaphragms and ciliary processes [2–5], we are concerned about several aspects of this study.

1) The use of [^{3}H]-DHA is unusual in this kind of study where the normal aims are for maximum sensitivity and minimum tissue requirements as discussed elsewhere [6]. The use of 500–800 µg membrane protein *per assay tube* appears excessive; with intact lymphocytes we used only 200–300 µg protein per assay tube [7] and only 5–10 µg membrane protein for eye work [1]. Our concern is that other workers could have difficulty in extending this work owing to the large number of animals (or amount of tissue) required. It is currently preferable to use [^{125}I]-HYP or [^{125}I]-CYP as the ligand. We estimate that the amount of protein used here gave 7,000–8,000 cpm specifically bound for the highest standard (100 nM) used; these counts could have been achieved with only 10–20 µg protein if using [^{125}I]-HYP or [^{125}I]-CYP.

2) A more serious concern relates to the Scatchard analysis of [^{3}H]-DHA binding. No saturation curves are presented and no statistics as to assay repeatability are quoted. It is usual to publish 'B_{max} and $K_d \pm$ SD (n experiments)'.

We are concernced too that 'percentage specific binding' is only quoted at 1 nM [^{3}H]-DHA. What is it at 100 nM? Our experience with tissues from rat lung and rat and guinea pig hearts is that at concentrations of [^{3}H]-DHA above 20 nM, this ligand appears either to solubilise in the cell membrane or to partition into membrane micelles (i.e. cannot be displaced by the degree of washing used here). This mechanism appears to be blocked by the propranolol or alprenolol used in the non-specific binding tubes and so the trapped ligand does not appear as non-specific binding. Obviously this will seriously distort the Scatchard analysis and, in the absence of any graphical analysis, we are concerned that the observed binding may not truly reflect adrenoceptor sites. In view of this, it would have been valuable to see evidence that the stereospecificity of the receptor had been preserved.

3) In the section on "Determination in atria and trachea", the equation incorporating EC50 and Bmax is neither described nor referred to. Further, fitting two parameters by non-linear least squares empirically requires a minimum of 8 data points for any valid statistical inferences to be drawn. In our own work, we would be concerned about conclusions drawn on only 5 points, preferring to use 7–9.

4) The method of characterising receptor subtypes by plotting PA2 vs pK_i seems rather archaic, considering that modern methods are available for quantifying receptor subtypes using mathematical models. This approach has been extensively discussed and validated [8] and also adapted by us for use on a personal microcomputer [9]. The method described here could not easily be used routinely on samples prepared from different sections of the eye and, in addition to requiring large amounts of tissue, does not give a real indication of the actual proportions of receptor subtypes present. We have shown [2] that greater than 75% of the beta-adrenoceptor subtypes in pigmented ciliary processes are of type beta-2.

Would the authors care to comment?

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* Corresponding author

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APPENDIX B

Healthy, non-atopic subjects (five men) with no history of asthma or respiratory disease (medical and laboratory personnel) participated in Study 1 as controls. Their ages were 29,31,35,31 and 35 years. All subjects were drug free and non-smokers. The subjects gave informed consent.

Brief clinical details of asthmatic patients involved in Study 1 FEV_1 and FVC are expressed as percentage of predicted given in brackets.

Subject 1 S.M. (F), age 26 years. This patient developed asthma when she was 8 years old. Patient was on steroid but it was stopped 3 months before the study.

FEV_1, 2.53 (83%). FVC, 3.25 (93%).

Skin prick test: strong reaction to house dust mite, cat fur, feathers, grass pollen.

RAST IgE test: very high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug t.i.d.), sodium cromoglycate (20mg q.i.d.) and occasional aminophylline suppositories.

Subject 2 K.L. (F), age 19 years. This patient developed asthma in childhood.

FEV_1, 1.74 (55%). FVC, 2.59 (67%)

Skin prick test: strong reaction to house dust mite, feathers, grass pollen.

RAST IgE test: high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug t.i.d.) and beclomethasone dipropionate (100ug q.i.d.).

Subject 3 M.S. (F), age 58 years. This patient developed asthma when she was 12 years old.

FEV_1, 0.77 (38%). FVC, 1.71 (57%).

Skin prick test: strong reaction to dust, house dust mite, feathers.

RAST IgE test: moderate antibody titre to house dust mite, cat epithelium, dog dander.

Therapy: inhaled salbutamol (200ug q.i.d.), inhaled betamethasone valerate (100ug q.i.d.).

Subject 4 J.M. (M), age 34 years. This patient developed asthma when he was 11 years old.

FEV_1, 1.51 (37%). FVC, 3.38 (68%).

Skin prick test: strong reaction to dust, house dust mite, cat fur, dog hair, feathers, grass pollen.

RAST IgE test: high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug t.i.d.) and beclomethasone dipropionate (100ug q.i.d.).

Subject 5 J.S. (M), age 23 years. This patient developed asthma in childhood.

FEV_1, 1.57 (49%). FVC, 2.23 (54%).

Skin prick test: strong reaction to dust, house dust mite, feathers, grass pollen.

RAST IgE test: moderate antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug t.i.d.) and beclomethasone dipropionate (100ug q.i.d.).

APPENDIX C

Healthy, non-atopic subjects (four men, one woman) with no history of asthma or respiratory disease (medical and laboratory personnel) participated in Study II as controls. Their ages were 30,32,35,30 and 32 years.

All subjects were drug free and non-smokers. The subjects gave informed consent.

Brief clinical details of asthmatic patients involved in Study II. FEV_1 and FVC are expressed as percentage of predicted given in brackets.

Subject 1 K.S. (F), age 18 years. This patient developed asthma when she was 11 years old.

FEV_1, 2.58 (94%). FVC, 3.31 (93%).

Skin prick test: strong reaction to dust, house dust mite, dog hair, horses, grass pollen.

RAST IgE test: high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug t.i.d.) and inhaled ipratropium bromide (80ug q.i.d.).

Subject 2 M.M. (F), age 44 years. This patient developed asthma when she was 6 years old.

FEV_1, 2.31 (84%). FVC, 3.65 (92%).

Skin prick test: strong reaction to dust, house dust mite, feathers.

RAST IgE test: high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug q.i.d.) and sodium cromoglycate (20mg q.i.d.).

Subject 3 J.M. (M), age 35 years. This patient developed asthma in childhood.

FEV_1, 1.5 (39%). FVC, 3.4 (73%).

Skin prick test: strong reaction to dust, house dust mite, feathers, grass pollen.

RAST IgE test: high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug q.i.d.) and beclomethasone dipropionate (100ug q.i.d.).

Subject 4 R.M. (F), age 35 years. This patient developed asthma when she was 5 years old.

FEV_1, 2.1 (76%). FVC, 3.15 (95%).

Skin prick test: strong reaction to dust, house dust mite, dog hair, cat fur, feathers, grass pollen.

RAST IgE test: very high antibody titre to house dust mite.

Therapy: inhaled salbutamol (200ug q.i.d.).

ADDENDUM

Since the completion of writing this thesis, there has been progress, in asthma and adrenoceptor research which is now reviewed.

BRONCHIAL ASTHMA

Autonomic regulation of the airways

Airways obstruction in asthma is due not only to an increase in tone and bulk of airway smooth muscle, but may also be due to bronchial mucosal edema (resulting from increased microvascular leakage) and from plugging of airways by mucus secretions. These events may be brought about by the release of inflammatory mediators such as histamine, leukotrienes and prostaglandins derived from mast cells and other inflammatory cells in the asthmatic airway (Nadel and Barnes, 1984).

The airways are innervated by a sympathetic, a parasympathetic and a third (perhaps vasoactive intestinal peptide (VIP) releasing) nervous system (Nadel and Barnes, 1984). These modulate smooth muscle tone and submucosal gland secretion and possibly other cell systems in the airways. It is therefore possible that autonomic nervous mechanisms may be responsible for diurnal changes in bronchomotor tone (Barnes, 1984a).

Human airways are densely innervated by cholinergic nerves, which upon activation produce bronchoconstriction. By contrast, the sympathetic nerve supply to human airways is very sparse, with no direct innervation of airway smooth muscle (Richardson, 1979). Few, if any, adrenergic nerve fibres directly supply bronchial muscle (Sheppard *et al*, 1983) and there is no evidence for functional adrenergic innervation (Zaagsma *et al*, 1983).

Circulating adrenaline

However, beta-adrenoceptor agonists potently relax human airway smooth muscle *in vivo* and *in vitro* (Barnes *et al*, 1984) and autoradiographic

studies in human lung (Carstairs et al, 1985) revealed that beta-adrenoceptors were widely distributed, with dense labelling over airway epithelium, alveolar walls and submucosal glands, and a lower density over airway and vascular smooth muscle.

The beta-adrenoceptors of airway smooth muscle, airway epithelium and vascular smooth muscle were of the $beta_2$ subtype. In bronchial submucosal glands and alveolar walls, both $beta_1$ and $beta_2$ receptor subtypes appeared to coexist.

Thus, in the absence of a functional sympathetic nervous system, circulating catecholamines (particularly adrenaline) may be important in the physiological regulation of airway tone (Barnes, 1983).

The normal fall in plasma adrenaline at night is closely related to the timing of increased bronchoconstriction and is inversely related to a rise in plasma histamine concentration (Barnes et al, 1980). Moreover, an infusion of adrenaline in low concentration at the time of maximal bronchoconstriction reduces the plasma histamine, suggesting that circulating adrenaline might act indirectly on the airways by having a stabilizing effect on airway mast cells. At night, the normal fall in circulating adrenaline might then result in mediator release from abnormal asthmatic mast cells.

Further evidence in support of this is that in some asthmatics plasma histamine concentrations rose during an infusion of propranolol (Ind et al, 1982). In vitro adrenaline has been shown to potently inhibit the release of histamine by antigen in passively sensitised human lung fragments (Butchers et al, 1980). In asthmatics who develop exercise-induced bronchoconstriction, plasma adrenaline fails to rise on exercise (but there was a blunted rise in plasma noradrenaline which is sufficient to cause bronchoconstriction) compared with a marked rise in matched normal subjects (Warren et al, 1982). The responses to histamine infusion (Ind et al, 1983) and to more severe exercise (Larsson et al, 1982) were normal.

Recently Ind et al (1985) found that in acute severe asthma plasma noradrenaline concentrations were increased while plasma adrenaline concentrations failed to increase. These findings suggest that there may be a defect in the release of catecholamine present in asthma, although the mechanism remains uncertain, and this might contribute to bronchial hyperreactivity.

Thus, endogenous catecholamines may play a protective role in asthma, although they are unlikely to play a major role in pathogenesis, since normal subjects do not develop bronchial hyperreactivity after beta-adrenergic blockade or adrenalectomy (Barnes, 1984b).

Cholinergic mechanisms

Human airway smooth muscle is densely innervated by the vagus nerve, and stimulation of the vagus causes bronchoconstriction that is mediated by cholinergic receptors and can be blocked by atropine (Nadel and Barnes, 1984). Autoradiography confirmed a high density of muscarinic receptors in smooth muscle of large airways (Barnes et al, 1983; Basbaum et al, 1983), but the density of these receptors decreases as the airways become smaller. It is possible that vagal tone might increase at night, resulting in bronchoconstriction. Inflammatory mediators such as histamine, secreted at night might stimulate airway irritant receptors leading to reflex bronchoconstriction (Barnes, 1985). In addition, gastroesophageal reflux may initiate vagal reflex bronchoconstriction, and the fall in plasma adrenaline level may lead to increased cholinergic neurotransmission. It has not yet been possible to measure vagal tone in the airways at night, but recordings of heart rate, which may reflect general vagal tone, have demonstrated a fall in heart rate overnight (Clarke et al, 1976), the time course of which is similar to that of peak flow (Barnes et al, 1982). There is preliminary evidence that anticholinergic drugs may significantly reduce nocturnal wheezing in some patients, implicating the involvement of cholinergic mechanisms (Coe and Barnes, 1985).

Coincidence of rhythms

It seems likely that nocturnal asthma is best understood in terms of endogenous circadian rhythms, with an exaggeration of the normal 24-hour change in airway tone.

Thus, the fall in circulating adrenaline level may occur at the same time as an increase in vagal tone, and both may be regulated by a central hypothalamic "clock" (Moore-Ede et al, 1983). In addition, the delayed effects of the fall in plasma cortisol level (which precedes that of adrenaline by some four hours) may all lead to bronchoconstriction at night and in the early morning (Barnes, 1984a, 1985).

In normal subjects, these rhythms have only a small, clinically insignificant, effect on airway function. In asthmatic patients, however, these same changes produce pronounced bronchoconstriction, because of bronchial hyperreactivity. The effect of these rhythms may also be further amplified in asthmatics by mediator release from "leaky" mast cells (Barnes, 1985).

Nonadrenergic noncholinergic regulation

A third autonomic nervous system, which is nonadrenergic and noncholinergic, is well described in the gastrointestinal tract, and more recently was described in lung (Richardson, 1981). This nervous system is inhibitory for airway smooth muscle and, therefore, defective function of this system could contribute to bronchial hyperreactivity in asthma. In the absence of functionalsympathetic innervation of airway smooth muscle, this nonadrenergic inhibitory system is the only nerve-mediated means of relaxation in human airway smooth muscle (Davis et al, 1982).

Nonadrenergic noncholinergic bronchodilatation has been demonstrated in vivo in guinea pigs and in cats by stimulation of the vagus nerve in the presence of muscarinic and beta-adrenergic blockade (Irvin et al, 1980; Yip et al, 1981). Nonadrenergic noncholinergic nerves may also supply

submucosal glands, since electrical stimulation in the presence of muscarinic and adrenergic blockade produces secretion of mucus in isolated ferret tracheal segments (Borson et al, 1982).

Studies of this system are hampered by the uncertainty about the neurotransmitter and the lack of specific blockers. Although there is some evidence in the gastrointestinal tract that one of the neurotransmitters may be ATP or a related purine (Burnstock, 1981). A more likely candidate for neurotransmitter is vasoactive intestinal peptide (VIP). In human lung, VIP nerves are closely associated with airway smooth muscle, submucosal glands, and pulmonary vessels, and are present in airway ganglia (Dey et al, 1981). In animal and human airways, VIP relaxes airway smooth muscle in vitro (Davis et al, 1982; Kitamura et al, 1980), and in animals it protects against histamine-and prostaglandin-induced bronchoconstriction in vivo (Said, 1982).

VIP acts through specific receptors that, like beta-adrenoceptors, activate adenylate cyclase to increase intracellular cyclic AMP content (Kitamura et al, 1980). Immunocytochemical studies show that VIP stimulates cyclic AMP formation in tracheal epithelium and in submucosal glands (Lazarus et al, 1983). Thus, VIP appears to be a promising candidate for the neurotransmitter, although the case is not proven. Until a specific antagonist becomes available, the role of the nonadrenergic noncholinergic nervous system and VIP nerves in the regulation of airway function will remain uncertain (Nadel and Barnes, 1984).

Axon reflexes

More recently, it was proposed that axon reflexes with release of potent sensory neuropeptides may be involved in the pathophysiological features of asthma (Barnes , 1986). In asthma, damage to airway epithelium, possibly caused by eosinophil products such as major basic protein and

eosinophil cationic protein, which are very toxic to airway epithelial cells (Frigas et al, 1980) and are detectable in the sputum of asthmatics (Gleich et al, 1985), exposes C-fibre afferent nerve endings. Stimulation of these endings by inflammatory mediators such as bradykinin may result in an axon (local) reflex with antidromic conduction down afferent nerve collaterals and release of sensory neuropeptides such as substance P (SP), SP has been found in human airways (Polak and Bloom, 1982), neurokinin A, which is also present in sensory nerves and even more potent than SP (Karlsson et al, 1984), and calcitonin gene-related peptide which has also been localised to nerves in airways of several species including man (Palmer et al, 1985) and is probably co-localised with SP in sensory nerves (Lundberg et al, 1985).

These peptides are potent inducers of airway smooth muscle contraction, bronchial oedema, extravasation of plasma, mucus hypersecretion, and possibly inflammatory cell infiltration and secretion. Thus, axon reflexes could account for at least some of the pathophysiology of asthma and this concept might lead to new strategies for treatment.

ADRENOCEPTORS

Recently developed methods permit the study of the structure of the adrenergic and other plasma-membrane receptors. The two most useful approaches are affinity chromatography (Cribnau et al, 1982) and photoaffinity labelling (Guillory and Jeng, 1983).

In affinity chromatography a drug or hormone analogue that combines with a receptor is covalently linked to a solid support such as Sepharose beads, usually by a hydrocarbon side chain. When a detergent-solubilized extract of the cell membrane is passed over such a column, the receptors are adsorbed to the column by means of their binding interaction with the immobilized drug. Other proteins pass through. After appropriate washing

steps, the receptors can be biospecifically eluted in highly purified form with specific drugs (agonist or antagonist ligands) with which they bind. It is not uncommon for several thousand fold purification to be achieved in a single such step (Lefkowitz and Caron, 1985).

Columns of the beta-antagonist alprenolol bound to Sepharose beads, for example, have been useful in purifying the beta-adrenoceptors. When coupled to other conventional methods of protein purification, such as high-preformance liquid chromatography, these techniques can yield receptor preparations purified essentially to homogeneity (Lefkowitz et al, 1983).

The other approach used to study aspects of membrane-receptor structure is photoaffinity labelling. This is accomplished by synthesising a drug or hormone analogue that contains a photoactivatable group in its structure. The photoactivatable compound must retain high affinity for binding specifically to the receptor. An example would be an azide moiety ($-N_3$), which upon ultraviolet irradiation breaks down to the very reactive free radical nitrene (Lefkowitz and Caron, 1985). Such methods have been successfully applied to both subtypes of beta-adrenoceptors ($beta_1$ and $beta_2$) (Lefkowitz et al, 1983) and $alpha_1$-adrenoceptors (Leeb-Lundberg et al, 1984). In each case a ^{125}I-tagged high-affinity receptor antagonist was synthesised that contained an arylazide group. After the drug is allowed to bind to the receptors in membrane-bound or purified form, non-specific binding interactions can be disrupted by washing, the preparation is subjected to ultraviolet irradiation. The resulting formation of the chemically reactive species (nitrene) leads to covalent incorporation of the drug into the receptor macromolecule.

As in other types of approaches to ligand binding, validation of the method is based on the specificity of the receptor. Thus, it is necessary to document that a series of drugs block covalent incorporation of the photoaffinity label into the receptor with potencies comparable to those with which they occupy the receptors. In the case of the adrenoceptors, it would be expected (for example) that (-) isomers of agonists and

antagonists would be more effective than (+) isomers in blocking such covalent incorporation into the receptors.

After incorporation, the covalently labelled receptor can be visualised by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) followed by autoradiography. It appears that $beta_1$ and $beta_2$ adrenoceptor subtypes consist of peptides of similar molecular mass (Stiles et al, 1983). $Alpha_2$-adrenoceptors also appear to have a mol mass similar to that of the beta-adrenoceptors, i.e. 60-64 000 daltons (Regan et al, 1984). In contrast, the $alpha_1$-adrenoceptors have mol masses of 80 000 (Leeb-Lundberg et al, 1984).

It is of interest that a variety of adenylate cyclase coupled receptors have now been visualised by photoaffinity or photoaffinity crosslinking experiments (Herberg et al, 1984; Coltrera et al, 1981; Landmann et al, 1983). All appear to have mol mass of 60 000-70 000 daltons. This suggests that their structures may be similar indeed (Lefkowitz and Caron, 1985). These receptors all appear to be intrinsic membrane glycoproteins. Beyond that, however, very little is known about the details of their molecular architecture. Another point worthy of emphasis is that the receptors appear to consist of only a single peptide (Lefkowitz and Caron, 1985). This contrasts with the more complicated pattern observed for example with the nicotinic cholinergic receptor (Noda et al, 1983) which has multiple subunits, or for the insulin receptor which has two distinct subunits (Ullrich et al, 1985).

Molecular mechanisms of adrenoceptors desensitisation

Desensitisation, the tendency of biological responses to wane over time despite the continuous presence of a stimulus of constant intensity, is observed in organisms as diverse as bacteria and mammals (Sibley and Lefkowitz, 1985). Recently, new insights into the molecular mechanisms underlying these phenomena have emerged from the study of the receptors

coupled to the second messenger-generating system adenylate cyclase. These mechanisms involve sequestration or down-regulation of the receptors from the cell surface as well as functionally significant covalent modifications of the receptors and/or guanine nucleotide regulatory proteins.

Among the receptor-effector systems that mediate the effects of many hormones and drugs in man and other animals is the adenylate cyclase system, which synthesises the second messenger cyclic AMP. Persistent stimulation of the system by catecholamines or by synthetic analogues leades to rapid desensitisation of the cAMP response with consequent blunting of physiological responses, for example, the bronchodilator effect of adrenaline in the treatment of asthma or the inotropic effect of adrenergic agents in the treatment of congestive heart failure.

Hormone-induced desensitisation has usually been divided into two general categories referred to as agonist-specific or homologous desensitisation and agonist-nonspecific or heterologous desensitisation. The term "homologous" is used to designate that form of desensitisation in which only the subsequent response to the desensitising hormone is attenuated while the efficacy of other hormone activators is unimpaired. Conversely, heterologous desensitisation indicates that incubation with one agonist attenuates the response to multiple, different agonists operating through distinct receptors (Sibley and Lefkowtiz, 1985).

Homologous desensitisation

Homologous desensitisation in cultured mammalian cells exhibits a complex phenomenon consisting of multiple sequential events (Perkins, 1983; Harden, 1983; Hertel and Perkins, 1984). An initial step involves a rapid 'uncoupling' of the receptors from the other components of the adenylate cyclase system, which may involve sequestration of receptors

from the cell surface. They may reside in endocytotic vesicles or they may be in a compartment of membrane that remains contiguous with the plasma membrane (Strader et al, 1984).

Stadel et al (1983) showed that the beta-adrenoceptors that were located in the cytosolic fraction after desensitisation in frog erythrocytes were sedimentable at 158 000g, indicating that these receptors were located in light membrane particles or vesicles. These were devoid of adenylate cyclase activity and the guanine nucleotide regulatory protein. Comparable findings have been obtained using the rat lung as an in vivo model of homologous desensitisation (Strasser et al, 1984).

Others (Waldo et al, 1983; Toews et al, 1984) have also provided results consistent with sequestration of receptors during homologous desensitisation in astrocytoma cells. Sucrose-density gradient centrifugation of control astrocytoma cell lysates resulted in a single peak of beta-adrenoceptor binding activity located with enzyme markers for plasma membranes including adenylate cyclase activity. In contrast, after desensitisation to isoprenaline, the beta-adrenoceptors were present not only in the plasma membrane peak, but also in a peak sedimenting at light sucrose densities devoid of adenylate cyclase activity or other plasma membrane or cell surface markers. Similar findings have been reported using C6 glioma cells (Hertel et al, 1983a,b).

Strulovici et al (1983) have tested the functional activity of the sequestered receptors that can be recovered in light membrane particles in frog erythrocytes and were shown to be functionally active. Similar conclusions have been reached by Clark et al (1985) in characterising the sequestered receptors found in S49 cell light membrane fractions. Nonetheless they are desensitised. Thus, the desensitisation appears to be due to their sequestration away from their normal effector guanine nucleotide regulatory protein and catalytic components rather than to any inherent functional alteration in the receptors (Lefkowitz and Caron, 1985). Recently it has been found that preincubation of intact

human mononuclear leucocytes with isoprenaline reduced the number of surface beta-adrenoceptors, measured by radioligand binding at 4C, by about 70% without affecting receptor affinity (De Blasi et al, 1986). This was caused not by receptor degradation but more likely by their internalisation to a cellular compartment not accessible to the radioligand. In fact, the same receptor number was found in control and desensitised cells when binding was measured at 37C.

A subsequent event involves a down-regulation of receptor number exhibited by a decrease in the ability to detect beta-adrenoceptors with radiolabelled antagonists (Hertel et al, 1983a,b).
Down-regulation occurs more slowly than the initial sequestration process in most cell types, and the exact fate of these beta-adrenoceptors is not known with certainty. In some cases it seems clear that the receptors are proteolytically degraded and new receptor synthesis is required to regenerate them (Frederich et al, 1983). In others, the number of beta-adrenoceptors can return to control levels even in the absence of new protein synthesis (Doss et al, 1981). This suggests that these down-regulated receptors can regain their binding activity with time. Although beta-adrenoceptor-guanine nucleotide regulatory protein coupling seems to be necessary for maximal down-regulation to occur, activation of adenylate cyclase and the generation of cAMP is not required (Mahan et al, 1985).

Other workers (Snavely et al, 1985) found that infusion of adrenaline or noradrenaline elevated plasma levels of each catecholamine 10- to 20-fold and decreased rat renal cortical $alpha_1$-adrenoceptor number about 50% without changing $alpha_2$-receptor number. Isoprenaline infusion raised plasma levels of this catecholamine, but had no effect on the number of either $alpha_1$- or $alpha_2$-adrenoceptors. Renal cortical beta-adrenoceptor number was decreased by infusion of all three catecholamines. However, the $beta_1$- and $beta_2$-adrenoceptors were altered selectively by

the different agonists. Infusion of noradrenaline decreased both $beta_1$- and $beta_2$-receptor number, but was more effective for the $beta_1$-receptors. The decrease in beta-receptor number due to adrenaline infusion was largely due to loss of the renal cortical $beta_2$-receptors. Infusion of isoprenaline decreased the number of both $beta_1$- and $beta_2$-receptors (69% and 75% respectively).

These results indicate that adrenoceptor subtypes are differentially down-regulated by elevated levels of circulating catecholamines and that this differential loss of receptors depends on the nature of the receptor subtype, the agonist, and perhaps also whether catecholamines are infused rather than increased by pheochromocytoma.

It has concluded by others (Brodde et al, 1985) that glucocorticoids as well as ketotifen, an antianaphylactic drug, can accelerate recovery of density and responsiveness of lymphocyte $beta_2$-adrenoceptors desensitised by long-term treatment with $beta_2$-agonists. Such an effect may have clinical implications for preventing tachyphylaxis of asthmatic patients against therapy with beta-agonists.

These processes seem to occur in all normal cells that exhibit homologous desensitisation, although their kinetics can vary considerably. Homologous desensitisation is thus associated with alterations occurring at the receptor level of the adenylate cyclase system (Sibley and Lefkowitz, 1985). It has also been demonstrated that acute desensitisation of $alpha_2$-adrenoceptors on human platelets occur after short term and long term exposure to catecholamines, which is not mediated through mechanisms of receptor internalisation and reductions in receptor number (Jones et al, 1985, 1986). The present findings suggest that the coupling of the platelet $alpha_2$-adrenoceptor to adenylate cyclase may be altered after exposure to agonists.

The biochemical mechanisms involved in homologous desensitisation are not yet known with certainty. Studies of homologous desensitisation in

cell-free systems have indicated a requirement for phosphorylating conditions (Salomon et al, 1981). Recently, Sibley et al (1985) have shown directly that homologous desensitisation of adenylate cyclase in frog erythrocytes is associated with phosphorylation of the beta-adrenoceptor. This phosphorylation is induced in a stereospecific fashion by isoprenaline and is blocked by propranolol. The phosphorylation state of the receptor stoichiometrically increases about three fold on desensitisation. Interestingly, prostaglandin E_1 (PGE_1) does not promote beta-adrenoceptor phosphorylation despite the fact that PGE_1 activates adenylate cyclase in these cells (Sibley et al, 1985). This observation agrees well with the notion that homologous desensitisation is not cAMP-mediated. The nature of the protein kinase that phosphorylates the beta-adrenoceptor during homologous desensitisation is unknown.

There are at least two potential mechanisms by which receptor phosphorylation could contribute to the homologous desensitisation. One possibility is that the phosphorylation induces or triggers the sequestration of the receptor from the cell surface. Another possibility is that the phosphorylation results in a functional modification in the receptor protein such that it is less efficacious in activating adenylate cyclase (Sibley and Lefkowitz, 1985).

Heterologous desensitisation

Heterologous forms of desensitisation of adenylate cyclase occur in many tissues and cell types. This form of desensitisation represents a broad pattern of refractoriness in which the response to multiple hormones and sometimes nonhormonal effectors is impaired. In contrast to homologous desensitisation (which may be unimechanistic), heterologous desensitisation certainly occurs by more than one mechanism. In many cell types, heterologous desensitisation occurs in addition to the homologous form of desensitisation, thus making its analysis difficult. In general, however, the heterologous

response occurs with a slower onset than the homologous one, suggesting that heterologous desensitisation represents an adaptive response to relatively prolonged stimulation (Sibley and Lefkowitz, 1985).

Heterologous desensitisation has been extensively studied in simple avian erythrocyte systems that possess beta-adrenoceptors and adenylate cyclase (Stiles et al, 1984). Heterologous desensitisation in avian erythrocytes is associated with modifications in the adenylate cyclase system at both the level of the receptor and the guanine nucleotide regulatory proteins. It should be pointed out that structural and/or functional alterations of the enzyme catalytic unit cannot be excluded although there is no evidence for this at present.

It seems reasonable to propose that the mechanisms of heterologous desensitisation elucidated in erythrocytes are not unique to this cell type. Thus, one major means of achieving heterologous desensitisation would be modification(s) in the guanine nucleotide regulatory proteins. The nature of these modifications is not yet known, although one hypothesis suggests that a phosphorylation event is involved. Although, in some systems, cAMP seems to induce the guanine nucleotide regulatory proteins modification (Stadel et al, 1981), in others it seems not to be involved (Rich et al, 1984; Noda et al, 1984).

Another major pathway of heterologous desensitisation is modification of the receptor proteins. In this case the effect is not receptor sequestration or down-regulation as in homologous desensitisation but a functional uncoupling of the receptor from adenylate cyclase. Evidence from avian erythrocytes indicates that phosphorylation is the probable mechanism for producing the functional modification.

Moreover, because of the heterologous nature of the desensitisation it is reasonable to suppose that all the receptors coupled to adenylate cyclase would probably be modified in this way, that is, phosphorylated (Sibley and Lefkowitz, 1985).

Radioligand binding techniques

Beta-adrenoceptors have been defined and characterised in membranes of many tissues as well as on intact cells by using radiolabelled agonists and antagonists.

In contrast with studies on membrane preparations, studies on living cells often suffer from a very high proportion of non-specific low affinity binding when hydrophobic radiolabelled ligands such as ^{3}H-DHA and ^{125}I-CYP are used (Porzig et al, 1981; Andre et al, 1981; Meurs et al, 1982). These binding sites not coupled with the biological response may mimic receptor binding with e.g. rapid (reversible) association time course and displacement by antagonists (Kerry and Scrutton, 1983).
Therefore the main problem in studies of beta-adrenoceptor properties of intact cells using radiolabelled ligands is to discriminate between stereospecific low capacity binding of these ligands to receptors and non-stereospecific high capacity binding to cellular membranes and possibly to intracellular structures. Dax and Partilla (1982) have shown that non-specific low affinity binding was at least in part directly related to the hydrophobicity of the ligand.

Others (Maloteaux et al, 1983) suggested that such a displaceable binding could represent a possibility of the diffusion of labelled ligand through the cell membrane and its accumulation in the lysosomal compartment.

Recently, Rademaker et al (1985) found that there were two types of saturable binding, besides the well-known non-specific binding, when hydrophobic ligands were used for investigating the vesiculisation of beta-adrenoceptors on cultured HeLa and Chang liver cells. The first (compartment I) representing beta-adrenoceptors with high affinity (^{3}H-DHA 0.8nmol/1, ^{125}I-CYP 27pmol/1) and low capacity (10-20fmol/mg protein), the second (compartment II) had a rather high affinity (^{3}H-DHA 400nmol/1, ^{125}I-CYP 30nmol/1) and a very high capacity (20 000-25 000fmol/mg protein). The affinity of adrenergic agents for compartment II correlates very well with the calculated hydrophobicity. It is concluded that these

types of binding sites might interfere with the determination of adrenoceptor binding sites when hydrophobic ligands are used. When using hydrophobic ligands like these special care should be taken to avoid such interference.

Future studies

In conclusion, the results reported in this thesis showed that lymphocyte adrenoceptor function in the asthmatic group was similar to that of the control group.

At present, one of the concerns is that it is not clear how well the lymphocyte adrenoceptors resemble those on the cells of target organs. The extent to which the regulatory properties of the adrenoceptors in these cells will directly correlate with those in the less accessible, but more physiologically relevant receptor sites such as the lung remains to be determined.

Therefore, future studies should answer the following:

1. What are the effects of administered beta-agonists on lung adrenoceptors function in vitro?
2. How does the status and activity of the lung adrenoceptors compare with the lymphocyte adrenoceptors from the same subjects?
3. Is there a circadian variation in adrenoceptor function on the lung membranes of animal models (rats)?

Recently developed techniques for purification of adrenoceptors (affinity chromatography and photoaffinity labelling) should permit the following studies:

a. Can antibodies be raised to the purified receptor protein?
b. Could antibodies be used for future adrenoceptor studies?

Finally, it is important to continue the investigation of the molecular mechanisms involved in the effects of drugs on receptors, because receptors represent the molecular entity through which many pharmacologically active

agents interact, and also because a variety of disease processes may involve changes in the density of catecholamine receptors. It is also worth noting that changes in catecholamine receptors occur as a consequence of the administration of agents widely used in the treatment of diseases ranging from hypertension to depression. An understanding of the molecular properties of catecholamine receptors may thus lead to significant improvement in our ability to treat various diseases.

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