CHARACTERISATION AND AGONIST REGULATION

OF THE HUMAN PLATELET β-ADRENOCEPTOR

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

by

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Now, here you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that.

from Through the Looking Glass

by Lewis Carroll.

TABLE OF CONTENTS

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PUBLICATIONS

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CHAPTER I.

General Introduction

I.1 HUMAN PLATELETS

I.1.1 Platelet Formation

Human platelets are derived from megakaryocytes, which originate from multipotent stem cells in the bone marrow. Committed megakaryocytic stem cells differentiate and proliferate to result in mature megakaryocytes. Fragments of cytoplasm are then pinched off to form platelets which are released from the bone marrow into the circulation at the rate of about 3.4×10^7 platelets/ml/day. (Pennington, 1981). Some mature megakaryocytes (20-50%) also pass into the circulation and are thought to lodge in the venous microcirculation of the lungs where they can release platelets (Kaufman et al., 1965).

The normal circulating platelet count is maintained within a narrow range 150-400 x 10^6/ml, indicating that platelet production is a regulated process. Removal or destruction of platelets has been shown to stimulate platelet production, whereas transfusion of viable platelets inhibits the process (de Gabriele and Pennington, 1967). The precise mechanism of this regulation remains unknown (Burstein and Harker, 1983), although there is increasing evidence to suggest that a stimulatory humoral factor generally termed "thrombopoietin" is involved (Pennington, 1981). In addition a humoral inhibitor of platelet production has been reported in certain patients (Shreiner et al., 1980). Thus platelet production may be controlled by humoral stimulatory and inhibitory activites. Platelets remain in the circul-

ation for an average of 7 to 10 days and are then removed by the reticuloendothelial system in spleen and liver. The mechanism by which platelets are recognised as ready for sequestration is unknown.

Circulating platelets exhibit heterogeneity in size and density (see Frojmovic and Milton, 1982). Certain workers suggest that platelets become smaller and denser as they age (Corash et al., 1977), whereas others maintain that heterogeneity is megakaryocytic in origin. Until platelet separation according to age is technically viable, it is unlikely that the factors responsible for platelet heterogeneity will be unravelled.

I.1.2 Platelet Structure

Platelets normally (i.e. in a non-activated state) circulate as anucleate biconvex discs 2-5um in diameter. Details of platelet ultrastructure as revealed by electron microscopy are shown diagrammatically in figure I.1.

The outer coat or glycocalyx, rich in glycoproteins gives the platelet a smooth outline, although it appears as a fuzzy surface in electron micrographs. Its chemical constituents provide the receptors for stimuli triggering platelet activation and are therefore important in adhesion-aggregation reactions. Immediately below the plasma membrane which has a trilaminar structure characteristic of mammalian cell membranes, lies a band of microtubules which form a marginal bundle

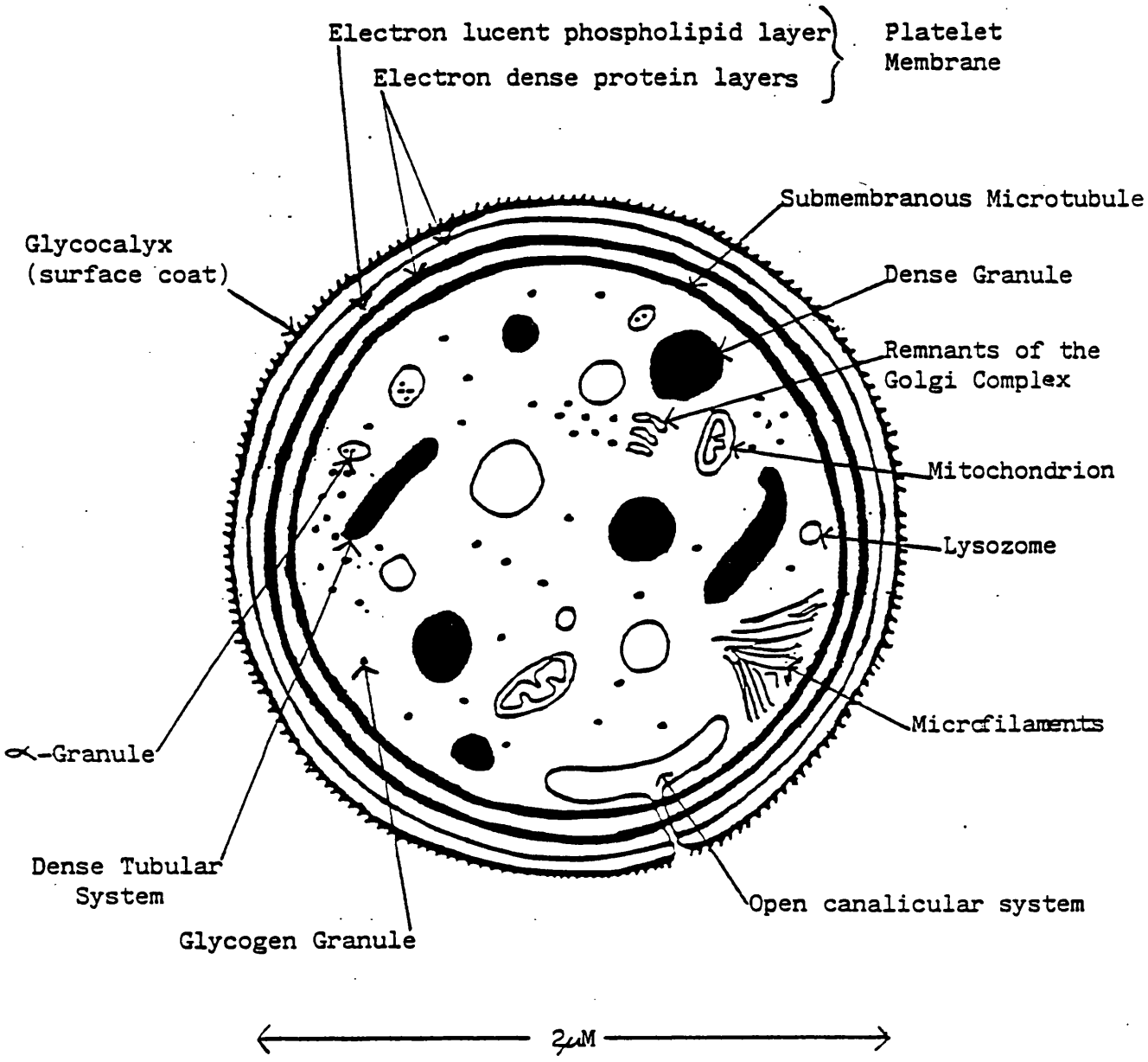


Figure I.1. A Diagrammatic representation of platelet ultrastructure

encircling the platelet. These have a cytoskeletal function, to maintain the characteristic discoid shape of quiescent platelets and cannot themselves contract (Gordon, 1981). In addition to microtubules, platelets contain finer microfilaments arranged in bundles in an extensive network. They are believed to contain actin filaments which interact with myosin rods following phosphorylation of myosin light chains under the influence of calmodulin (Harris, 1981), and may be important in maintaining the discoid shape of unaltered platelets and provide a contractile system involved in shape change, pseudopod extension, internal contraction and secretion (Nachmias, 1980; White, 1983).

In direct continuity with the plasma membrane is the surface-connected open canalicular system. This network of invaginations enlarges the surface area of the platelet accessible to plasma factors, serves as a channel for release of platelet products during secretion, and may be externalised during activation (White, 1983). The dense tubular system immediately associated with the open canalicular system is the major storage site for calcium (White, 1973), and is an important site of arachidonic acid metabolism in the platelet (Gerrard et al., 1981).

Platelets contain a number of different organelles distributed throughout the cytoplasm. Mitochondria and glycogen storage granules are both present, enabling aerobic and anaerobic respiration. Dense granules (dense bodies) contain high molecular weight aggregates of adenosine nucleotides, serotonin and calcium (Chesterman et al., 1982) and smaller amounts of histamine and catecholamines. Alpha granules

are heterogenous in nature and contain a range of proteins which include : platelet factor 4 and β-thromboglobulin (both are anti-heparin proteins), fibronetin, fibrinogen and thrombospondin (aggregation cofactors), platelet derived growth factor, platelet factor VIII related antigen, platelet factor V, Von Willebrand factor, albumin and protease inhibitors (see Ogston, 1983). The lysosomes contain a range of enzymes including acid phosphatase and β-N-acetylglucosaminidase. These organelles lose their random distribution during platelet activation and become centralised.

I.1.3 Biological responses and reactions of platelets

The main physiological function of platelets is to initiate the formation of a haemostatic plug at the site of vascular injury by ensuring efficient coagulation, and promoting subsequent wound healing. Platelets have also been implicated in the maintenance of endothelial integrity, phagocytosis, vascular permeability changes, pathological thrombosis and atherosclerosis, inflammatory reactions and transplant rejection, these functions will not be detailed here (reviewed by Gordon and Milner, 1976).

Discoid platelets in the blood do not readily adhere to any surface and this is essential for the maintenance of blood flow. Vascular injury however, results in the exposure of subendothelial collagen, which brings about platelet activation. Once activated, platelets immediately adhere to the vascular collagen, release constituents such as serotonin, ADP, arachidonic acid metabolites and calcium, all of

which are platelet activators which act to recruit other platelets in the immediate vicinity to clump together. At the same time, the blood coagulation system is activated following the unmasking of the procoagulant phospholipid - platelet factor 3, and activation of coagulation factor XI, at the platelet surface. The coagulation cascade then proceeds with the release of procoagulant proteins such as fibrinogen, platelet factor 4 and β-thromboglobulin, culminating in the formation of a haemostatic plug (Gordon, 1981).

The haemostatic role of platelets is dependent on the wide array of morphological responses that occur when platelets become activated. These responses include shape change, adhesion, aggregation, secretion of granule contents and eicosanoid synthesis and secretion.

Shape change : discoid platelets are characterised by a marginal bundle of microtubules. When platelets are activated, microtubule organisation is disrupted and the submembraneous microfilament networks interact, leading to a dramatic and rapid change in platelet shape. This shape change consists of centralisation of organelles and extrusion of long thin pseudopodia, which effectively increase the platelet surface area and facilitate the platelet to platelet contact essential for aggregation (Born, 1970; Gordon, 1981). Shape changes can be monitored in vitro by measuring transmission and/or scattering of light through platelet rich plasma or washed platelet suspensions, and is characterised by an increase in optical density i.e. a decrease in light transmission (Michal and Born, 1971).

Adhesion : Once activated, platelets will readily adhere to certain biological polymers and a variety of foreign surfaces. The mechanisms involved in platelet adhesion however are not well understood since attempts to correlate physical and chemical properties of artificial surfaces with their attractiveness to platelets have not been successful. In vitro adhesion has been quantitated by a variety of techniques (see Weiss, 1983).

Aggregation : Platelet aggregation (i.e. attachment of platelets to each other, as distinct from their attachment to foreign surfaces (adhesion)) can be induced by a variety of stimuli. Aggregation of platelets is normally preceded by shape change. Adrenaline however is unusual in causing a qualitatively distinct change in platelet shape without formation of pseudopodia. This has been described as 'mild' shape change and occurs in the absence of changes in light transmission (Gerrard et al., 1981). Reversible or 'primary' aggregation can be induced by low agonist concentrations. Secondary aggregation can be induced by higher agonist concentrations and is associated with secretion of granule contents and prostanoid formation, and is essentially irreversible (Smith and Willis, 1971). Aggregation is dependent on exposure of fibrinogen receptors (Peerschke, 1984) and the degree to which agents expose these receptors may relate directly to platelet aggregability. Aggregation may be monitored in platelet rich plasma or washed platelet suspensions by measuring increases in light transmission through stirred samples (Born, 1962), or by platelet counting, where numbers of single platelets are counted before and after addition of an aggregating agent (Lumley and Humphrey, 1981).

Aggregation may be monitored in whole blood by the latter platelet counting method or by an electrical impedence method (Cardinal and Flower, 1980).

Secretion : a) Degranulation : platelet secretion, also referred to as the platelet release reaction, involves the specific discharge of platelet granules to the outside of the cell. The quantity and type of substance secreted depends on the nature and concentration of the stimulatory agent. ADP or adrenaline induce secretion from the dense granules only (Mills et al., 1968) while stronger stimuli e.g. high concentrations of thrombin and collagen evoke in addition, secretion from the α-granules. Dense granule contents (with the exception of ATP) are pro-aggregatory and their main function is presumably to promote the growth of a haemostatic plug along with alpha granule constituents e.g. fibrinogen and the antiheparin factors, which promote coagulation. Secretion of dense granule contents can be monitored by measuring released ATP by luciferin-luciferase luminescence (Feinman et al., 1977) or by a radio-isotopic (^{14}C or ^{3}H-serotonin) prelabelling method.

b) Release of newly synthesized consituents : prostaglandins are synthesized from membrane phospholipids via the activation of two enzymes - phospholipase C and phospholipase A_2. This results in the formation of thromoxane A_2 which by virtue of being a very potent stimulator of both platelet aggregation and the release reaction (Hamberg et al., 1975) results in further amplification of the aggregatory process.

Platelet function may be altered by a wide variety of biological agents. These compounds (agonists) are diverse in nature and may act to stimulate platelet activation e.g. thrombin, ADP, serotonin, catecholamines, thromboxanes, platelet activating factor, collagen and immune complexes; or to abolish or attenuate activation e.g. certain prostaglandins, adenosine and its derivatives.

Receptors for many of the above agonists have been identified by radioligand binding studies. Their precise location is unknown but presumed to be the platelet surface - although intracellular receptors may exist for certain lipophilic compounds such as thromboxane A_2 or platelet activating factor. Thus, transduction mechanisms must be present in the platelet to convey the external agonist - receptor interaction signal into a modification of cellular responsiveness. Several such mechanisms have been proposed to occur in the platelet, those considered to be of major importance at present involve intracellular second messengers. Platelet activation appears to be associated with agonist-induced elevations in the intracellular concentrations of 1,2-diacylglycerol, inositol 1,4,5-trisphosphate, and calcium (Kaibuchi et al., 1983; O'Rourke et al., 1985; Rink et al., 1982), whereas, platelet inhibition is associated with agonist-induced elevation of intracellular cyclic AMP (Haslam et al., 1978). It is not clear at present how many of these events occur following agonist occupancy of a single type of receptor. Agonists can also influence platelet responsiveness by other mechanisms such as exposure of fibrinogen receptors (Peershke, 1984) and production of substances

within the platelet which are themselves agonists e.g. thromboxane A_2 (for review see Huang and Detwiler, 1986). The situation is further complicated by the observation that most (if not all) platelet agonists are capable of acting in a synergistic manner, in that, addition of two or more agonists can result in a response (i.e. aggregation or dense granule secretion) that is significantly greater than the sum of the individual responses. To date, despite extensive investigations, the molecular mechanisms underlying the control of platelet function remain unclear. Platelets however provide an easily accessible homogenous population of cells that are particularly useful for the study of receptors, agonist-receptor interactions, and the consequences of such interactions in terms of stimulus-response coupling pathways, and receptor regulation. Such studies may be performed with a view to understanding and controlling platelet function for therapeutic purposes, or may, as in the present study, employ this easily accessible human cell as a model for exploring receptor mechanisms that may have wide implications for other cell types, in particular other less accessible human cell types. The receptors of interest in the present study were human platelet adrenoceptors (see section I.2.3. for more detail).

I.2. RECEPTORS

I.2.1. Introduction

The concept of a 'receptor' was first introduced to explain the specificity of response of a system to added agents of interest. As

early as 1878, Langley referred to a constituent of the cell - the 'receptive substance' whose function he proposed was to receive stimuli from drugs or hormones acting upon it, and transfer these stimuli to the effector organ. The term 'receptor' was introduced later by Ehrlich (1913) to describe the site of initial recognition to which "agents must be bound if they are to act." Clark subsequently proposed that drug - receptor interactions could be described by a mathematical relationship. In 1926, he put forward his "occupancy theory" which postulated that response was directly proportional to number of receptors occupied. It soon became apparent however that such a simple relationship was not applicable in all situatons. For example, Ariens (1954) noted that the occupancy theory did not account for the ability of 'drugs' to elicit a response subsequent to binding, and thus proposed that drug-receptor interactions involved two separate and independent parameters : a) affinity - the recognition and binding of a ligand by the receptor (governed by the law of mass action - Clark, 1926) and b) intrinsic activity - the ability of a drug to elicit a biological response. Thus agonist drugs - analogous to endogenous hormones and neurotransmitters in the sense that they elicit a biological response, be it stimulatory or inhibitory, would have affinity and intrinsic activity, whereas antagonist drugs - which block receptor mediated effects elicited by hormones, neurotransmitters or agonist drugs by competing for receptor occupancy, would have affinity for the receptor, but lack the necessary intrinsic activity to elicit a response.

Many theories of drug receptor interactions have since evolved in

response to experimental findings (see Ruffolo, 1982; Hollenberg, 1985), some dealing with traditional pharmacological observations such as dose-response curves, others in answer to the development of more direct methods of investigating drug-receptor interactions that have provided more specific information about receptors, such as their molecular weights, subunit composition, and amino acid sequence e.g. β-adrenoeptors (see I.2.2.1.). In this respect "receptors" are no longer hypothetical entities, but distinct cellular macromolecules, believed to perform at least two basic functions : recognition of a hormone or drug and initiation of events leading to a cellular response.

I.2.2 Classification of adrenoceptors

The physiological effects of adrenaline and noradrenaline are exerted via their interaction with a variety of cellular receptors called adrenoceptors (adrenergic receptors).

Initial evidence for the existence of multiple types of adrenoceptors arose from the work of Dale. In 1906, he demonstrated that excitatory responses of various organs to adrenaline were blocked by ergot alkaloids, while inhibitory responses remained unaffected. Ahlquist, (1948) later divided adrenergic responses into two major categories on the basis of their pharmacological specificity i.e. order of potency for a series of sympathomimetic amines. His results showed that excitation of smooth muscle in the vasculature, uterus, nictitating membrane and dilator pupillae exhibited a potency order of adrenaline >

noradrenaline > α-methylnoradrenaline > α-methyladrenaline > isoprenaline, while the order of potency for inhibition of smooth muscle in the vasculature, bronchi and uterus, and excitation of the heart was isoprenaline > adrenaline > α-methyladrenaline > α-methylnoradrenaline > noradrenaline. It was concluded that two types of adrenoceptors were involved and these were named α- and β-respectively. Support for Ahlquist's adrenoceptor subdivision came with the introduction of selective adrenergic blocking drugs e.g. initially, dibenamine and phenoxybenzamine (Nickerson, 1949) to block the α-adrenoceptor effects, and later dichloroisoprenaline - a specific β-adrenoceptor antagonist (Powell and Slater, 1958; Moran and Perkins, 1958).

There is now considerable evidence to suggest that both α- and β-adrenoceptors can be further divided into two distinct subtypes known as α_1, α_2 and β_1, β_2 respectively.

I.2.2.1. β_1 and β_2 - adrenoceptors

In 1967, Lands and co-workers provided evidence for the existence of more than one type of β-adrenoceptor by comparing the rank order of potency of catecholamines on β-adrenoceptor mediated responses. The receptor involved in cardiac stimulation and fatty acid mobilization which exhibited a potency order of isoprenaline > adrenaline = noradrenaline was called β_1, whereas that involved in bronchodilation and vasodilation which exhibited a potency order of isoprenaline > adrenaline > noradrenaline was called β2. Direct support for this

β_1/β_2 subclassification came with the advent of synthetic drugs selective for either receptor subtype. From the prototype, practolol - β_1 selective antagonist (Dunlop and Shanks, 1968) has arisen a vast array of subtype selective agents. Some are receptor selective e.g. betaxolol - β_1 selective antagonist (Boudot et al., 1979), ICI 118,551 - β_2 selective antagonist (Bilski et al., 1980) and have been used in the present study to characterise the human platelet β-adrenoceptor. Others are supposedly selective by virtue of a selective efficacy at producing a response e.g. salbutamol (Brittain et al., 1968) and prenalterol (Carlsson et al., 1977) which have been called β_2 and β_1 selective agonists respectively.

With the discovery of adenylate cyclase (Sutherland and Rall, 1960) came the opportunity to study a more proximal consequence of drug-receptor interaction, namely the activation of the enzyme. Numerous studies exploring catecholamine stimulated adenylate cyclase activity have since demonstrated that both β_1- and β_2 -adrenoceptors stimulate enzyme activity, and that β-adrenoceptor subtype heterogeneity was apparent at the level of adenylate cyclase activation (Burges and Blackburn, 1972; Lefkowitz, 1975).

Until 1972, it was generally accepted however that tissue responses were mediated by a single receptor subtype. Evidence to the contrary was first introduced by Carlsson et al., (1972) who demonstrated differential blockade of chronotropic effects in the cat heart by selective β_1 and β_2-adrenoceptor antagonists depending upon the subtype selective agonist used. Their results led them to propose that β_1- and

β_2-adrenoceptors may co-exist within a single organ or tissue and under certain circumstances even mediate the same functional response. This has subsequently been demonstrated for a number of responses mediated by catecholamines (see review by Daly and Levy, 1979).

Further support for the existence of only two β-adrenoceptor subtypes in mammalian tissues, that correspond to pharmacologically defined β_1- and β_2-adrenoceptors, has come from radioligand binding studies. High affinity ligand binding assays have been employed to successfully delineate β-adrenoceptor subtypes in a wide variety of tissues (Nahorski, 1981; Minneman, 1979), and in some cases both subtypes have been found within the same cell (Homburger et al., 1981).

Recently, the availability of techniques for the purification of β-adrenoceptors, and of specific affinity and photoaffinity probes capable of identifying the receptor binding subunits, has resulted in the elucidation of several biochemical and structural features of mammalian β_1- and β_2-adrenoceptors. Although there is a general agreement that both β_1- and β_2-adrenoceptor binding subunits reside on polypeptide chains of molecular weights 60,000-65,000 (Shorr et al., 1985), peptide mapping studies have revealed conflicting results. Stiles et al., (1983) in obtaining both similarities and dissimilarities between the two subtypes, concluded that they were different proteins, whereas Graziano et al., (1985) showed that the partial proteolytic digests obtained for both receptor subtypes were essentially identical. In addition, immunological approaches have also yielded conflicting results, with Venter, (1984) observing differences

between the subtypes and Moxham et al., (1986) finding a high degree of intersubtype homology. The need for more specific techniques was thus apparent. Several workers had previously employed a molecular genetic approach to successfully elucidate the structure of several receptors (Hollenberg, 1987). These complementary DNA (cDNA) cloning techniques have subsequently been employed for the study of β-adrenoceptor structure. During the last two years genes and cDNA for several β-adrenoceptors, including hamster lung β_2-adrenoceptor (Dixon et al., 1986) and turkey erythrocyte β_1-adrenoceptor (Kobilka et al., 1987), have been cloned, and the entire amino acid sequence of these receptors elucidated. All these receptors appear to share some amino acid sequence homology with each other and with other receptors such as rhodopsin (Hargrave, 1982), and the muscarinic cholinergic receptor (Kubo et al., 1986), particularly in seven hydrophobic regions believed to represent transmembrane helices (Nathans and Hogness, 1983).

The basis for the pharmacological differences in binding of ligands to these receptors, and in particular to β_1- and β_2-adrenoceptors, at present remains unknown. Dixon et al., (1987) employing deletion mutants of the β_2-adrenoceptor have shown however, that the proposed extracellular hydrophilic receptor domains are not directly involved in the binding of agonists or antagonists to the receptor, and that possibly both internal and external domains of the β_2-adrenoceptor may modulate ligand binding. Undoubtedly, rapid advances in this field will occur in the near future. To date, no definitive evidence for the presence of more than two mammalian β-adrenoceptor subtypes exists.

I.2.2.2. α_1 and α_2 - adrenoceptors

α-adrenoceptors were originally classified by Langer, (1974), according to their anatomical locations, with the classical smooth muscle postsynaptic receptor being called α_1, and those located on presynaptic terminals being called α_2. However, as α_2-adrenoceptors were also found to be postsynaptic, this terminology was inappropriate. Berthelsen and Pettinger, (1977), then suggested that α-adrenoceptors be classified on the basis of their function; α_1-adrenoceptors being excitatory and α_2-adrenoceptors, inhibitory. This classification also seemed inappropriate, since certain α_2 responses could be considered excitatory, rather than inhibitory.

The third attempt to subclassify α-adrenoceptors was based on a biochemical approach, which suggested that α_1-adrenoceptor mediated effects were secondary to intracellular calcium elevation and involved an increased turnover of phosphatidylinositol, while α_2-adrenoceptor effects were mediated by the inhibition of adenylate cyclase (Wikberg, 1979; Fain and Garcia-Sainz, 1980). Although it seems likely that α_1- and α_2-adrenoceptors do possess different mechanisms for signal transduction, so that a biochemical subdivision may be correct, the most widely accepted subclassification of α-adrenoceptors at present is made with reference to pharmacological selectivity only (Starke, 1981). Selective agents include : methoxamine and phenylephrine (α_1 agonists); prazosin and corynanthine (α_1 antagonists); clonidine and UK 14304 (α_2 agonists); and yohimbine and rauwolscine (α_2 antagonists).Non selective agents include : adrenaline and noradrenaline (agonists); and

phentolamine and dihydroergocryptine (antagonists), (see Scrutton and Wallis, 1981).

In contrast to the β-adrenoceptor, less attention has been given to elucidating the molecular structure of α-adrenoceptors, and the information available at present is conflicting. Certain studies have indicated that α_1- and α_2-adrenoceptors share several common features at the gross structural level such as, sub-unit structure, a molecular weight of 85,000, and a number of epitopes as recognised using monoclonal antibodies (Shreeve et al., 1985). Others, however have shown the α_2-adrenoceptor to have a molecular weight of 64,000 (Regan et al., 1986). Furthermore, evidence is accumulating to suggest that both α_1- and α_2-adrenoceptor subtypes exhibit heterogeneity. The presence of two α_1-adrenoceptor subtypes involved in smooth muscle contraction has been inferred from antagonist potencies (see Flavahan and Vanhoutle, 1986), and the involvement of more than one mechanism of activation (Han et al., 1987). Likewise, α_2-adrenoceptors have been shown to exhibit a differential pharmacological profile with respect to antagonist affinities, that is apparent in both particulate and solubilised receptor populations (Chung et al., 1986; Alabaster and Brett, 1983). A better understanding of α_1- and α_2-adrenoceptor heterogeneity must however await a more complete analysis of these receptors at a molecular level.

I.2.3. HUMAN PLATELET ADRENOCEPTORS

I.2.3.1. α-adrenoceptors

Early studies established that human platelets aggregate and secrete in response to adrenaline (O'Brien, 1963) and further that the presence of low concentrations of adrenaline enhanced the aggregatory response to suboptimal concentrations of a second excitatory agonist e.g. ADP (i.e. proaggregation) (Mills and Roberts, 1967). Subsequent studies have since demonstrated that both the aggregatory and proaggregatory response of human platelets to adrenaline have the characteristics of an α_2-adrenoceptor mediated response. (O'Brien, 1964; Glusa et al., 1979; Hsu et al., 1979; Grant and Scrutton, 1980). They are induced by selective α_2-adrenoceptor agonists, (e.g. clonidine or UK, 14304) and blocked by selective α_2-adrenoceptor antagonists (e.g. yohimbine), whereas α_1-adrenoceptor agonists (phenylephrine or methoxamine) and antagonists (prazosin) are ineffective.

Further characterisation and quantitation of platelet α-adrenoceptors has recently been achieved using radioligand binding studies. These studies initially employed the nonselective α-antagonist radioligand ^{3}H-dihydroergocryptine (^{3}H-DHE), and showed its binding was inhibited much more effectively by α_2- (e.g. yohimbine) as compared with α_1-adrenoceptor antagonists (e.g. prazosin) (Alexander et al., 1978; Elliott and Grahame-Smith, 1982). More recently, studies employing α_2-selective antagonists e.g. ^{3}H-yohimbine (^{3}H-YOH) and ^{3}H-rauwolscine have identified a homogenous population of α-adrenoceptors of the α_2-subtype (Motulsky et al., 1980; Cheung et al., 1982). The number of platelet α_2-adrenoceptors measured however, depends upon which ligand is used (see Kerry and Scrutton, 1985), in most preparations the

density estimated using ^{3}H-DHE is higher than that with ^{3}H-YOH. These 'extra' ^{3}H-DHE sites do not represent α_1-adrenoceptors as the binding is not displaced by α_1-antagonists (Hoffman et al., 1979), and cannot be accounted for by ^{3}H-prazosin binding (Daiguji et al., 1981; Motulsky and Insel, 1982). The presence of α_1-adrenoceptors has been reported in approximately 20% of donors who exhibited a proaggregatory response to the α_1-adrenoceptor agonist methoxamine (Grant and Scrutton, 1979) which correlated with ^{3}H-prazosin binding (Kerry et al., 1982), but since these findings have not been corroborated by others, the importance of an α_1-adrenoceptor response in human platelets remains in doubt.

The precise mechanism by which activation of platelet α_2-adrenoceptors results in platelet aggregation has not been fully evaluated. It has been shown in both whole cells and platelet lysates that the α_2-adrenoceptors on platelets are linked in an inhibitory fashion to adenylate cyclase (Mills and Smith, 1971; Jakobs et al., 1978). However, evidence from the use of inhibitors of adenylate cyclase suggests that a fall in cyclic AMP alone is not sufficient to induce platelet aggregation (Haslam et al., 1978). It is therefore likely that inhibition of adenylate cyclase forms a part of a more complex mechanism which is responsible for inducing the aggregatory response. Work on permeabilised platelets has suggested that cyclic nucleotides may control a system which regulates the calcium sensitivity of platelet secretion (Knight and Scrutton, 1984) it is therefore possible that adrenaline may exert its effects via this route. A number of workers have explored the possibility that platelet activation via

α_2-adrenoceptors may involve changes in intracellular calcium. Adrenaline induced changes in chlortetracycline fluorescence (Le Breton et al., 1976), $^{45}Ca^{++}$ uptake (Owen et al., 1980) and luminescence of platelets loaded with the calcium-sensitive photoprotein aequorin (Ware et al., 1986) have been reported. However, when intracellular cytosolic calcium levels are monitored using Quin 2, no effect of adrenaline is observed. Furthermore, the mechanism by which adrenaline might produce a change in intracellular calcium is unclear since it does not appear to be accompanied by phosphoinositide hydrolysis (Brydon et al., 1984; MacIntyre et al., 1985). Thus, a role for phosphoinositide metabolism and/or calcium mobilisation in α_2-adrenoceptor mediated platelet activation awaits definition. Exposure of fibrinogen receptors is an important part of adrenaline induced aggregation and relates directly to platelet aggregability (Peershke et al., 1980), but the second messenger involved is as yet unknown. The significance of the 'extra' ^{3}H-DHE sites is also unknown, but may be important since studies on patients with abnormal blood catecholamine levels e.g. phaeochromocytoma, have reported changes in α-adrenoceptor number when measured with ^{3}H-DHE but not when ^{3}H-YOH was used, (Davies et al., 1981; Snavely et al., 1982). Also, there is evidence to suggest that the α_2-adrenoceptor which mediates the aggregatory response, is not identical with that which mediates inhibition of adenylate cyclase, or that two distinct signal transduction mechanisms are involved. When certain α_2-adrenoceptor agonists were examined, two groups were identified, one being selective for inhibition of adenylate cyclase, and the other selective for the aggregatory response (Clare and Scrutton, 1983; Clare et al., 1984).

Swart et al., (1984; 1985) showed that a population of platelets which apparently lacked the 'extra' ^{3}HDHE sites, failed to aggregate on addition of adrenaline but showed unimpaired inhibition of adenylate cyclase. Moreover, removal of extracellular Na^{+} has been shown to abolish adrenaline induced aggregation and secretion, without affecting adrenaline mediated inhibition of adenylate cyclase (Connolly and Limbird, 1983). Recent investigations have suggested that removal of extra platelet Na^{+} prevents arachidonic metabolism in response to adrenaline, and that a Na^{+}/H^{+} exchange mechanism is involved in controlling adrenaline activation of human platelets (Sweatt et al., 1986a). The precise mechanism by which Na^{+}/H^{+} exchange controls the initial mobilization of the arachidonic acid pool that subsequently activates phospholipase C is not known, but may involve Ca^{++} mobilization and a phospholipase A_2 (Sweatt et al., 1986b).

Information available to date does not explain the relationship between the α_2-adrenoceptors responsible for inhibition of adenylate cyclase and for induction of the aggregatory response, or the mechanisms involved in coupling agonist occupancy of the α_2-adrenoceptor to induction of the latter response. A great number of questions regarding the molecular interactions of platelet α_2-adrenoceptors with components of the adenylate cyclase system and with other constituents of the platelet membrane remain to be answered. Of particular interest in the present study is the possible interaction between platelet α_2-adrenoceptors and β-adrenoceptors.

I.2.3.2. β-adrenoceptors

In contrast to the extensively studied platelet α_2-adrenoceptor, less attention has been paid to the presence of β-adrenoceptors on human platelets. Abdulla (1969) was the first to demonstrate the disaggregatory effect of isoprenaline on stable ADP induced platelet aggregates. A weak inhibitory effect of isoprenaline on the aggregation response to ADP and collagen has also been demonstrated (Mills and Roberts, 1967; Mills and Smith, 1971) which could be blocked by propranolol. Similar inhibitory effects on thrombin, ADP and collagen induced aggregation were reported by Yu and Latour, (1977) using isoprenaline or adrenaline in the presence of phentolamine to block α-adrenergic actions of this agonist.

Isoprenaline and adrenaline (in the presence of phentolamine) have also been shown to stimulate adenylate cyclase activity in platelet lysates, (Adbulla, 1969; Jakobs et al., 1978) and in whole cells, an effect that was enhanced by the presence of phosphodiesterase inhibitors (Mills and Smith, 1971; Haslam and Taylor, 1971). However, β-agonists other than adrenaline and isoprenaline were found to be ineffective. This apparent lack of response of the adenylate cyclase system to β_2-selective agonists (e.g. orciprenaline) coupled with an inability to block observed responses with β_1-selective antagonists led Jakobs et al., (1978) to suggest that the human platelet β-adrenoceptor possessed characteristics indicative of neither β_1 nor β_2 subtype. Likewise, the radioligand binding studies of Steer and Atlas, (1982) reported that the binding sites labelled by the radioligands ^{125}I-iodocyanopindolol and ^{125}I-hydroxybenzylpindolol could not be classified as being of

either the β_1- or β_2- subtype.

Recent functional studies employing subtype selective β-adrenoceptor antagonists suggest that the disaggregatory effect of isoprenaline may be mediated by a β_2-adrenoceptor. Thus, when studies were performed in the presence of phentolamine (i.e. α-adrenoceptor blockade) the isoprenaline effect was preferentially blocked with high potency by antagonists that are selective for the β_2-adrenoceptor e.g. ICI-118,551, but not by β_1-selective antagonists such as atenolol or practolol. Furthermore, the aggregatory responses induced by thrombin or vasopressin were effectively inhibited by adrenaline or β_2-selective agonists e.g. salbutamol or terbutaline, whereas noradrenaline or β_1-adrenoceptor agonists were ineffective (Kerry and Scrutton, 1983a).

The above studies have thus yielded somewhat conflicting results. The present study was undertaken to re-examine the pharmacological characterisation and quantitation of the human platelet β-adrenoceptor and to examine the events that occur following agonist occupation of this receptor (see section I.3).

I.2.3.3. The physiological and pathophysiological significance of human platelet adrenoceptors

Adrenaline induced aggregation in vitro has been in routine use as an assessment of platelet function ever since it was first demonstrated by O'Brien (1963). Numerous reports have shown that the sensitivity of platelet aggregation in response to adrenaline may vary considerably

between normal individuals and within the same individual at different times (O'Brien, 1964). Also, in certain diseased states e.g. diabetes, ischaemic heart disease, hypercholesterolaemia, a 'supersensitive' effect of aggregatory agents including catecholamines has been demonstrated (Carvalho et al., 1974; Hampton and Gorlin, 1972). However, the relationship between in vitro adrenaline induced aggregation and in vivo platelet function during haemostasis is unclear for a number of reasons. Firstly, the concentration of adrenaline required to produce aggregation in platelet rich plasma (0.5uM) is several orders of magnitude higher than that normally found in the circulation under physiological conditions (0.25nM). Secondly, even the levels of adrenaline found in human blood during extreme physical or emotional stress, or in the presence of phaeochromocytoma are orders of magnitude less than those required to induce in vitro aggregation (see Shattil, 1987). Thirdly, a subgroup of patients with myeloproliferative disorders whose platelets are insensitive to adrenaline in vitro, do not suffer from haemostatic failure (Swart et al., 1984). Similarly, there appear to be wide differences among mammalian species with regard to platelet responsiveness to adrenaline in vitro, yet this is not mirrored by a wide difference in haemostatic competence (Kerry and Scrutton, 1985). Finally, in vitro platelet function is subject to considerable artefact (see Chapter V).

Evidence to date suggests that adrenaline in vivo acts in concert with a number of other agonists to produce full platelet activation. But the concentration of adrenaline required to produce a response when employed as sole proaggregatory agent is still in excess of that found

in pathological circumstances (Mills and Roberts, 1967) and no situation has been reported where proaggregatory responses to adrenaline are clearly abnormal. However, recent studies have shown that addition of more than one proaggregatory agent decreases the required concentration of adrenaline to that found in the circulation (Culliver and Ardlie, 1981). This situation of several excitatory agonists acting simultaneously on the platelet probably reflects the physiological state more closely than that of each agonist acting independently of one another.

Despite major advances in our understanding of the molecular mechanisms underlying the response of platelets to circulating catecholamines, the physiological and pathophysiological roles of human platelet adrenoceptors are still poorly understood.

I.3 STUDY OBJECTIVES

It is well recognised that normal cells or tissues have the ability to adapt their responsiveness to catecholamines. The precise mechanisms involved are not known at present but may involve any process that modifies an agonist's ability to bind to the adrenoceptor and produce an effect. As will be discussed in Chapter VI, much of the information available to date regarding adrenoceptor regulation has come from studies employing animal tissue and cultured cells, and hence little is known about the molecular events that accompany this phenomenon in human cells. The presence of β-adrenoceptors on a single, easily accessible cell therefore makes the platelet a useful model for

studying agonist regulation of a human β-adrenoceptor in the present study. Furthermore, the presence of α_2-adrenoceptors on the platelet provides a potential experimental system for evaluating agonist regulation of an adrenoceptor that is negatively coupled to cyclase. In addition, the presence of opposing effects of α_2- and β-adrenoceptors on a single cell system exhibiting readily definable functional and biochemical responses makes the platelet an excellent model for studying the interrelationships between these catecholamine receptors. A possible functional interaction between them has been suggested, since studies showing enhancement of either α_2- or β-adrenoceptor effects on functional response or platelet adenylate cyclase have required specific blockade of the opposite receptor (Abdulla, 1969; Jakobs et al., 1978; Kerry and Scrutton, 1983a). An in vivo response of human platelets may well involve a composite stimulation of both adrenoceptors since adrenaline (the most probable circulating agonist) has similar affinity for both α_2- and β-adrenoceptors.

The aims of this study have been to:-

i) Characterise the β-adrenoceptor present in human platelet membranes by means of radioligand binding studies employing ^{125}I(-)Pindolol (^{125}I(-)PIN).

ii) Examine the coupling of this receptor to adenylate cyclase by exploring ion and guanine nucleotide modulation of agonist interaction at this receptor in radioligand binding studies, and cyclic AMP

accumulation in intact cells.

iii) Perform aggregation and secretion studies in an attempt to delineate a possible functional role for human platelet β-adrenoceptors as modulators of responses stimulated by other agonists.

iv) Study agonist regulation of β-adrenoceptors on human platelets, and examine the possible interaction of these receptors with α_2-adrenoceptors also present on these cells.

CHAPTER II.

Radioligand binding studies

Selection of a suitable radioligand

Isolation of human platelets from other blood cells

II.1 Introduction

The basic principle of radioligand binding studies is relatively simple. A radioactively labelled ligand (hormone or drug, agonist or antagonist that has high specificity and affinity for the receptor of interest) is incubated with a tissue preparation bearing receptors. Unbound radioligand is removed and receptor-bound radioligand determined (see Limbird, 1986 for review). Technically, these assays are easy to perform, and the direct labelling of the receptor by this method allows a precise quantification and characterisation of the receptor under investigation. Experiments may be carried out on whole cells or membrane preparations of tissues, thus minimising problems of drug accessibility and distribution. Furthermore, the results are not complicated by factors such as receptor reserve and efficacy which can cause problems in functional studies. However, the lack of a final biological response can be a disadvantage, and certain criteria must be fulfilled in order to establish that the observed binding of radioligand represents the physiological receptor for the particular ligand. Thus,

i) as the number of receptors in a tissue is finite, the binding of radioligand to receptor preparations should be saturable.

ii) the kinetics of binding should reflect the time-course of the biological response elicited by the ligand, and for competitive ligands should be reversible.

iii) the relative potencies of drugs in competing for binding should correlate with their potencies in functional studies. This criteria however is not always satisfied e.g. the presence of spare receptors may mean that the concentration of drug required to elicit a half maximally effective physiological response (EC_{50})is much less than that required to occupy half the available receptors (IC_{50}). In addition, in vitro conditions found in binding experiments and physiological response systems may not be entirely comparable.

Ideally, measurable radioligand binding should represent specific receptor binding. Unfortunately this is not the case since radioligands can bind, not only to the receptors under investigation but also to other "non-receptor sites". This "non-specific binding" (NSB) is generally determined by incubating the tissue and radioligand in the presence of a non-radioactive drug that will occupy all the specific receptors. By subtracting the radioactivity observed in the presence of this unlabelled compound (NSB) from that observed in its absence (total binding) the amount of specific binding is calculated, and specific binding is assumed to represent the receptors of interest. Clearly, the choice of compound and concentration selected to define NSB are crucial, since inappropriate choices may overestimate or underestimate the specific radioligand binding to receptors (Nahorski and Richardson, 1979). Accurate determinations of NSB are generally made by examining the displacement of radioligand by a number of agonists and antagonists known to be active at the receptor under investigation. Ideally, binding should decrease as the concentration of displacing

agent is increased, until a plateau is reached which should be the same for a number of drugs. A concentration of displacing agent is then chosen which does not displace from NSB sites. In general, compounds that are structurally dissimilar to the radioligand are less likely to displace this low-affinity binding of radioligand to non-receptor sites, and are therefore a better choice. NSB is usually proportional to tissue and radioligand concentration and therefore nonsaturable in nature.

Three basic types of experiments generally performed to satisfy the above criteria for receptor identification were employed in the present study and are described below. Protein was determined by the method of Lowry et al., (1951) see Appendix 2.

Saturation experiments : These experiments are performed to evaluate whether or not radioligand binding measured meets the first criterion for binding to the physiologically relevant receptor - saturability of binding. They also provide an estimate of the maximum number of receptors present in the tissue (B max) and the equilibrium dissociation constant (Kd) of the receptors for the radioligand (where Kd = the concentration of radioligand occupying half the receptors).

Kinetic experiments : These experiments are performed to evaluate the time course of radioligand interaction with its receptor. The rate of radioligand binding to (association rate) and dissociation from (dissociation rate) its receptor are determined, and may be used to

give an estimate of the Kd. Kinetic experiments also provide information on how long it takes the reaction to reach equilibrium which is useful in designing future experimental protocols, and gives an estimate of how much radioligand dissociates from the receptors during the time required to separate bound from unbound radioligand.

Competition experiments : The third criterion for identification of a physiologically relevant receptor by radioligand binding is that the binding should exhibit the appropriate specificity. The specificity with which the radioligand interacts with its binding sites is determined in competition binding experiments. The tissue preparation is incubated to equilibrium with a fixed concentration of radioligand in the presence of increasing concentrations of unlabelled competitors. Specific binding (total-NSB) is determined at each concentration of inhibitor. Data is plotted as % specific binding or % inhibition of specific binding versus log molar concentration of competitor, and the concentration of competitor displacing 50% specific radioligand binding (IC_{50}) is determined. This type of experiment can also be employed to identify the presence of more than one receptor subtype (i.e. by the use of selective antagonists), or the presence of high and low affinity agonist binding states.

Data Analysis : All radioligand binding experiments were conducted a minimum of three times in duplicate, and data analysed as described in Appendix 3. Results are reported as the mean ± standard error of the mean for each variable. Statistical significance was established by Students 't' test for comparison of paired means unless otherwise

stated e.g. partial F-test for competition data (appendix 3c), a p value of <0.05 was considered significant.

The aim of the preliminary studies described in this chapter were twofold

i) to evaluate the suitability of ^{125}I(-)Pindolol (^{125}I(-)PIN) as the radioligand of choice for use in the present study.

ii) to establish a platelet preparation free of contamination from other β-adrenoceptor bearing blood cells.

II.2 Selection of a suitable radioligand for use in the present study

II.2.1. Introduction

The most critical step in any radioligand binding study is the choice of an appropriate radiolabelled ligand for the identification of the receptor under investigation. The radioligand should have high affinity for the receptor so that low concentrations of radioligand may be used, thus minimizing non-specific binding problems, and also have demonstrable biological activity at the receptor either as an agonist or antagonist. In addition, the radioligand should be chemically stable under the incubation conditions employed, and ideally react with only one receptor type present in the tissue preparation, thus avoiding complex binding phenomena that may not be easily interpretable. Also, since the number of receptor sites in a tissue preparation is usually small, with equilibrium dissociation constants for ligands in the nanomolar range or below, the radioisotope employed to label the ligand

must have sufficient specific activity to allow accurate measurements of low concentrations.

The two most commonly used radioisotopes are tritium (^{3}H) and iodine (^{125}I), both have their unique advantages and disadvantages. Tritiated ligands are generally indistinguishable from the native compound, stable for several months, with a radiochemical half-life for tritium of approximately 12 years. Their main disadvantage is their low specific activity as compared with iodinated ligands. For example, incorporation of one tritium atom per molecule of ligand would result in a theoretical specific radioactivity of 29 Ci/mmol, whereas incorporation of one ^{125}I atom per molecule of ligand gives a theoretical specific radioactivity of 2175 Ci/mmol. Furthermore, counter efficiency for ^{3}H is usually less than 50%, whereas for ^{125}I it can be above 75%. Thus, tritiated ligands can only be employed when the receptors under investigation are of sufficient density in the tissue preparation. Iodinated ligands however possess certain disadvantages; ^{125}I has a relatively short radiochemical half-life of 60 days, thus the radioligand needs to be frequently resynthesised or repurchased; the iodination procedure can sometimes result in altered biological activity of the iodinated product when compared with the parent molecule, or result in the generation of damaged labelled ligand molecules; and lastly, iodinated radioligands are more susceptible to decay catastrophe i.e. radiation induced damage of molecular structure during storage.

Initially, attempts to identify β-adrenoceptors by direct radioligand

binding techniques employed tritiated agonists. However, since their binding was inhibited by compounds without β-adrenoceptor activity, only inhibited by β-adrenoceptor antagonists with very low affinity and did not display stereoselectivity, the binding sites labelled in these studies were not β-adrenoceptors.

The first successful identification of β-adrenoceptors by ligand binding studies was achieved with the use of radioactively labelled β-adrenoceptor antagonists such as ^{3}H(-)dihydroalprenolol (^{3}H(-)DHA, Lefkowitz et al., 1974), ^{3}H-propranolol (Atlas et al, 1974) and ^{125}I(±)hydroxybenzylpindolol (^{125}I(±)HYP, Aurbach et al., 1974). These antagonists had higher affinity than agonists for the receptor sites, and their binding displayed all the characteristics of saturability, reversibility, specificity and stereoselectivity, expected for binding to a physiologically relevant receptor. Since 1974, numerous other radioactively labelled β-adrenoceptor agents (both agonists and antagonists - see Stiles et al., 1984) have been developed and successfully employed in binding studies. Agonist radioligands are not generally used for routine investigation of receptor number and properties, since their measurable binding to the receptor of interest is complicated by factors such as multiple agonist affinity states of the receptor (see chapter III). One of the most commonly employed antagonist radioligands to date is ^{3}H(-)DHA, its high specificity for β-adrenoceptors coupled with a low degree of non-specific binding in membrane preparations has resulted in its successful application for the identification of β-adrenoceptors in a wide range of tissues (see Nahorski, 1981). Unfortunately although ^{3}H(-)DHA is currently

available at a specific radioactivity of 50-100 Ci/mmol, it cannot be employed in the present study to examine β-adrenoceptors on human platelets because of the limited accessibility of biological material (i.e. human blood) and the low density of β-adrenoceptors reported to be present on these cells (Steer and Atlas, 1982). Therefore an iodinated antagonist radioligand must be employed, those available at present include ^{125}I(±)HYP, ^{125}I(±)cyanopindolol (^{125}I(±)CYP), ^{125}I(±)pindolol and ^{125}I(-)pindolol (^{125}I(-)PIN). ^{125}I(±)HYP appears to possess several unwanted characteristics such as a high non-specific binding that can be partially suppressed by addition of phentolamine (Sporn and Molinoff, 1976) or (+)isoprenaline (Harden et al., 1976), and an association with 5-HT receptors (Willcocks and Nahorski, 1983). ^{125}I(±)CYP although successfully employed to study β-adrenoceptors exhibits slow association/dissociation rates such that long incubations (at 37°C) are required to achieve equilibrium. Furthermore, since incubations are performed at 37°C, protection of receptors from inactivation apparently necessitates the inclusion of sodium chloride in the assay buffer (Bojanic, 1984). Both these factors limit the usefulness of this radioligand for exploring ionic modulation of agonist-receptor interactions (see chapter III). In addition, the racemic nature of these radioligands and of ^{125}I(±)pindolol may result in an inaccurate estimation of absolute radioligand and competing ligand affinity (Burgisser et al., 1981). For these reasons, ^{125}I(-)PIN was considered to be the most suitable radioligand for investigating the β-adrenoceptors on human platelets in the present study. However, before experiments with human platelets were undertaken, a brief evaluation of the suitability of ^{125}I(-)PIN for the

present study was performed by means of i) thin-layer and paper chromatographic separation of ^{125}I(-)PIN to check its radiochemical stability and purity over one half-life (60 days) and ii) a check of ^{125}I(-)PIN's β-adrenoceptor labelling characteristics during this time by means of a brief comparison with a well established β-adrenoceptor binding model i.e. ^{3}H(-)DHA binding in rat lung membranes (Rugg et al., 1978).

II.2.2. Methods

II.2.2.1. Chromatography of ^{125}I(-)PIN

a) Paper chromatography

5ul aliquots of Na^{125}I or 50ul aliquots of ^{125}I(-)PIN were subjected to chromatographic separation on Whatman 3MM paper as described in Appendix 4(a) every 7 days from iodination of (-)pindolol on day 0 to day 60 (t 1/2 for ^{125}I).

b) Thin-layer chromatography

Na^{125}I and ^{125}I(-)PIN (3 fractions obtained from paper chromatographic purification of ^{125}I(-)PIN, see Appendix 4(a)) were subjected to thin-layer chromatography according to the method of Harden et al., (1976). 5ul aliquots of Na^{125}I or ^{125}I(-)PIN were spotted onto Whatman silica gel plates and developed in either toluene-diethylamine (6:5) or ethyl acetate-methanol (1:2). The plates were then cut into equal

sections (1 x 1cm) and the radioactivity in each section counted directly in a Packard Auto Gamma 500C counter at 74% counting efficiency.

II.2.2.2 Preparation of rat lung membranes

Male Wistar rats (150-200g) were killed by cervical dislocation. Lungs were removed, dissected free of major bronchi and roughly minced with scissors. The tissue was homogenised in 10 volumes of 50mM Tris-HCl, pH 7.8, at 4°C with an Ultraturrax homogeniser (3 x 10 second bursts) and centrifuged at 500g for 10 minutes at 4°C. The supernatant was passed through a double layer of cheesecloth and the filtrate centrifuged at 50,000g for 15 minutes at 4°C. The pellet was resuspended in 50mM Tris-HCl, pH7.8, and washed three times (at 4°C) before final resuspension in an appropriate volume of ice-cold assay buffer (50mM Tris-HCl, 0.2 mM Na metabisulphite, pH7.8) for binding assays. Membranes prepared in this manner could be stored frozen at -70°C for prolonged periods (6 months or more) without significant loss of binding sites (Dickinson, 1982).

II.2.2.3. ^{3}H(-)DHA binding assays to rat lung membranes

Membranes (100-200ug protein) were added to ^{3}H-DHA (1-10nM) and (-)isoprenaline (200uM) or buffer, in a final volume of 250ul assay buffer (50mM Tris HCl, 0.2mM Na metabisulphite, pH 7.8). Incubations were performed at room temperature (22-25°C) for 30 minutes. Upon reaching equilibrium the reaction was stopped by addition of 1.0ml

ice-cold assay buffer. Membranes were immediately collected on Whatman GF/B filters by vacuum filtration and rapidly washed with 3x5ml ice cold 50mM Tris HCl, pH 7.8. The filters were then placed in insert vials into which 4.5mls of Fisofluor 1 scintillation fluid (Fisons Ltd) was added. Following extraction of the mixture for at least 6 hours the radioactivity associated with the filters was counted in an LKB-Wallac liquid scintillation counter at 40% counting efficiency.

II.2.2.4. ^{125}I(-)PIN binding assays to rat lung membranes

Membranes (5-10ug protein) were added to ^{125}I(-)PIN (0-200pM) and appropriate concentration of displacing agent or buffer in a final volume of 250ul assay buffer (50mM Tris HCl, 0.2mM Na metabisulphite, pH 7.8). Incubations were performed at room temperature (22-25°C) for 40 minutes (except for kinetic experiments) and the reaction stopped by the addition of 1.0ml ice-cold assay buffer. Membranes were immediately collected on Whatman GF/B filters by vacuum filtration and rapidly washed with 3 x 10ml ice cold 50mM Tris HCl, pH 7.8. Radioactivity associated with the filters was determined directly in a Packard Auto Gamma 500C Counter at 74% counting efficiency. (-)Pindolol was iodinated as described in Appendix 4(a).

II.2.3 Results

II.2.3.1. Chromatographic separation of ^{125}I(-)PIN

The purity and stability of ^{125}I(-)PIN was assessed by paper chromato-

graphy (II.2.2.1.). Aliquots of ^{125}I(-)PIN or Na^{125}I (employed as a marker for free ^{125}I) were subjected to paper chromatography at various intervals during the first 60 days following iodination of (-)pindolol. ^{125}I(-)PIN migrated as a single peak (Rf=0.29-0.36) well separated from Na^{125}I (Rf=0.81-0.83) (figure II.1.). More than 96% of total radioactivity spotted onto the paper was recovered within the peak for ^{125}I(-)PIN, the remaining 4% was accounted for as a constant background present along the length of the chromatograph. No change in the chromatographic pattern of the radioligand was observed during the 60 days, thus no other iodinated products were detectable, and no changes in detectable free ^{125}I concentrations occurred with time.

The purity of the ^{125}I(-)PIN peak was further evaluated. The individual fractions (numbers 9,10 and 11, figure II.1.) were subjected to thin-layer chromatography and developed with ethyl acetate:methanol (figure II.2. upper panel), and toluene:diethylamine (figure II.2. lower panel). With both solvent systems the ^{125}I(-)PIN eluted as a single radioactive peak well separated from Na^{125}I (employed as a marker for free ^{125}I) with Rf values of 0 (remained on the origin) and 0.76-0.78 in toluene:diethylamine, and 0.93 and 0.36-0.40 in ethyl acetate:methanol, for Na^{125}I and ^{125}I(-)PIN respectively. All the individual fractions exhibited identical chromatographic profiles that did not change during the 60 days.

II.2.3.2. ^{3}H(-)DHA + ^{125}I(-)PIN binding to rat lung membranes

Specific ^{125}I(-)PIN binding to rat lung membranes was initially defined

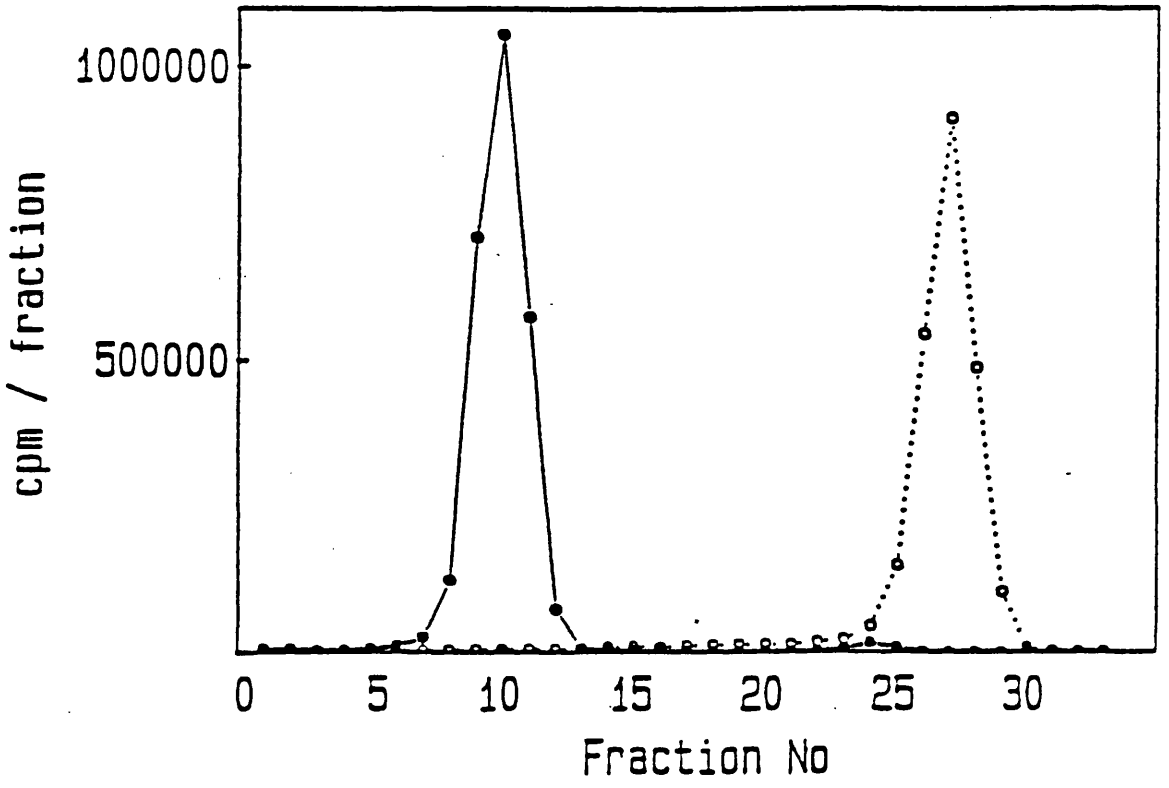


Figure II.1. Paper chromatographic separation of ^{125}I(-)PIN (●——●) and Na^{125}I (o....o)

Aliquots of ^{125}I(-)PIN and Na^{125}I were spotted onto separate Whatman 3MM paper and developed as described in appendix 4(a) every 7 days from iodination of (-)pindolol on Day 0 to Day 60. Counts per minute per fraction were determined. The data shown are from a single representative separation of each compound after 28 days, and are typical of the chromatographic profile obtained at the other time intervals examined (see text for further explanation II.2.3.).

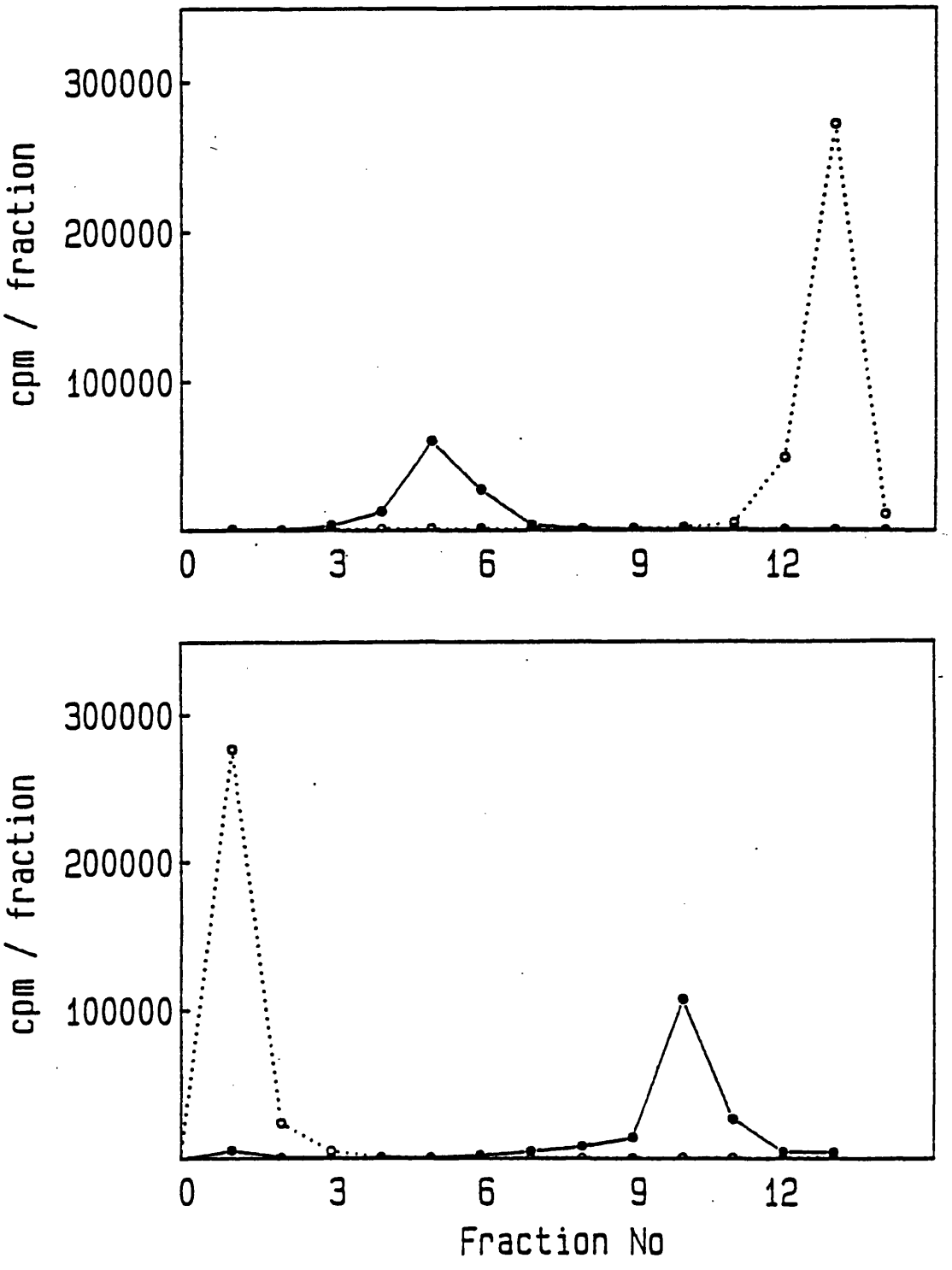


Figure II.2. Thin-layer chromatograms of $^{125}I(-)PIN$ (●—●) and $Na^{125}I$ (o···o)

Legend to Figure II.2. Thin-layer chromatograms of ^{125}I(-)PIN (●——●) and Na^{125}I (o....o)

Aliquots of a ^{125}I(-)PIN fraction (9,10 or 11, see figure II.1.) and Na^{125}I were spotted separately onto Whatman Silica Gel plates and developed in either toluene-diethylamine (6:5) (upper panel) or ethyl acetate : methanol (1:2) (lower panel) as previously described in (II.2.2.1.). Counts per minute per fraction were determined. Both upper and lower panels show that with both solvent systems, a clear separation between ^{125}I(-)PIN and Na^{125}I was obtained. The data presented represent the separation of only one of the three fractions that make up the ^{125}I(-)PIN peak (figure II.1.) but are typical of the chromatographic profile obtained with all the individual fractions at various intervals during the period of iodination of (-)pindolol on Day 0 to Day 60. (see text for further explanation II.2.3.).

as total minus non-specific binding in the presence of 200uM(-)isoprenaline as described for ^{3}H(-)DHA by Rugg et al., (1978). Employing these conditions, specific ^{125}I(-)PIN binding was found to be rapid, with equilibrium being attained within 30 minutes at 22-25°C (figure II.3). From this experiment a routine incubation period of 40 minutes at 22-25°C was considered suitable, and employed to check that specific binding had been correctly assessed. (-)Isoprenaline and (-)propranolol competition of ^{125}I(-)PIN binding was examined over a wide concentration range of competitor (figure II.4) and both drugs displaced the binding of ^{125}I(-)PIN to an equivalent extent, reaching a plateau at around <5% of total binding. Non-specific binding in subsequent experiments was defined by the presence of 200uM(-)isoprenaline (see II.1.).

The stability and specificity of ^{125}I(-)PIN binding to β-adrenoceptors during one half-life (i.e. 60 day period starting at day 0=iodination of pinolol) was assessed. Saturation binding experiments were performed simultaneously using ^{3}H(-)DHA and ^{125}I(-)PIN as described in the methods section. β-adrenoceptor density (Bmax) and affinity (Kd -equilibrium dissociation constant) were determined at least once every 7 days for each radioligand. When employing radioiodinated radioligands, it is generally assumed that decayed radioligand does not lose its binding properties with time, and therefore has a dilution effect on the remaining radioligand. In order to account for this a decay correction factor is employed which effectively adjusts the specific activity of the radioligand with time. However, a recent report by Doyle et al., (1984) suggests that no correction for decay is

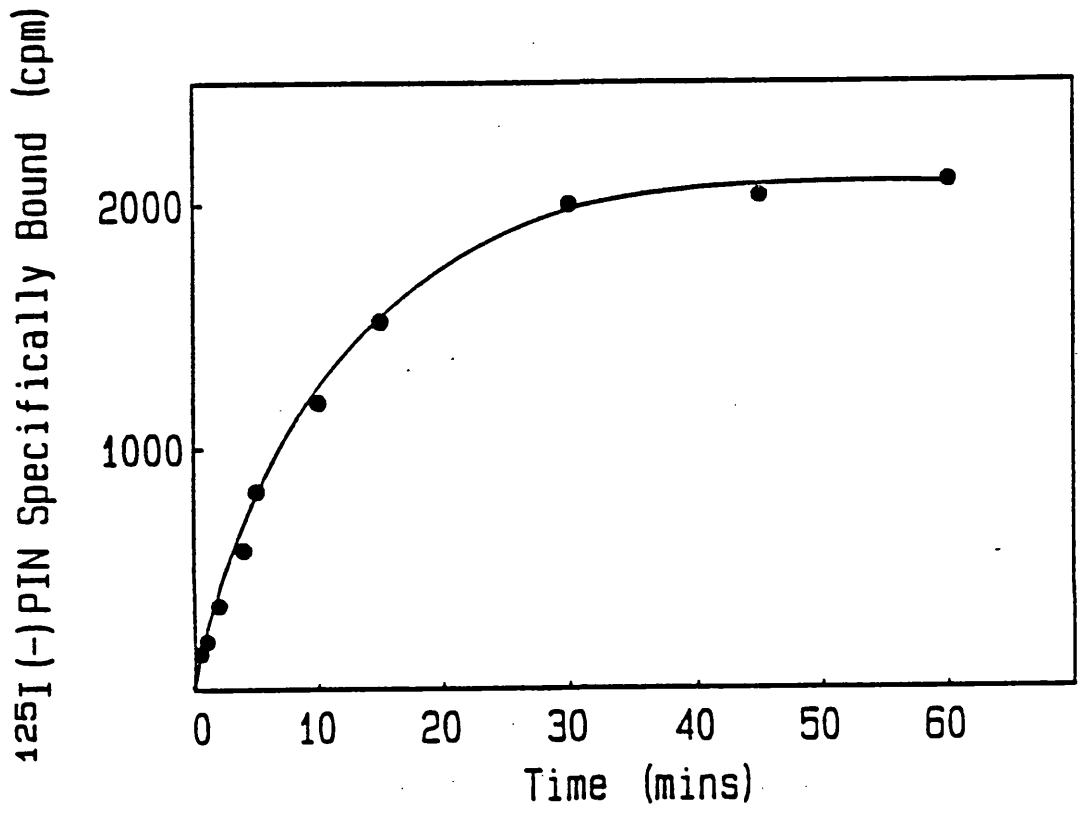


Figure II.3. Association of specific ^{125}I(-)PIN binding to rat lung membranes at room temperature (22-25°C)

Membrane preparations were incubated with ^{125}I(-)PIN and specific binding measured as a function of time. Data shown are from a single representative experiment performed in triplicate.

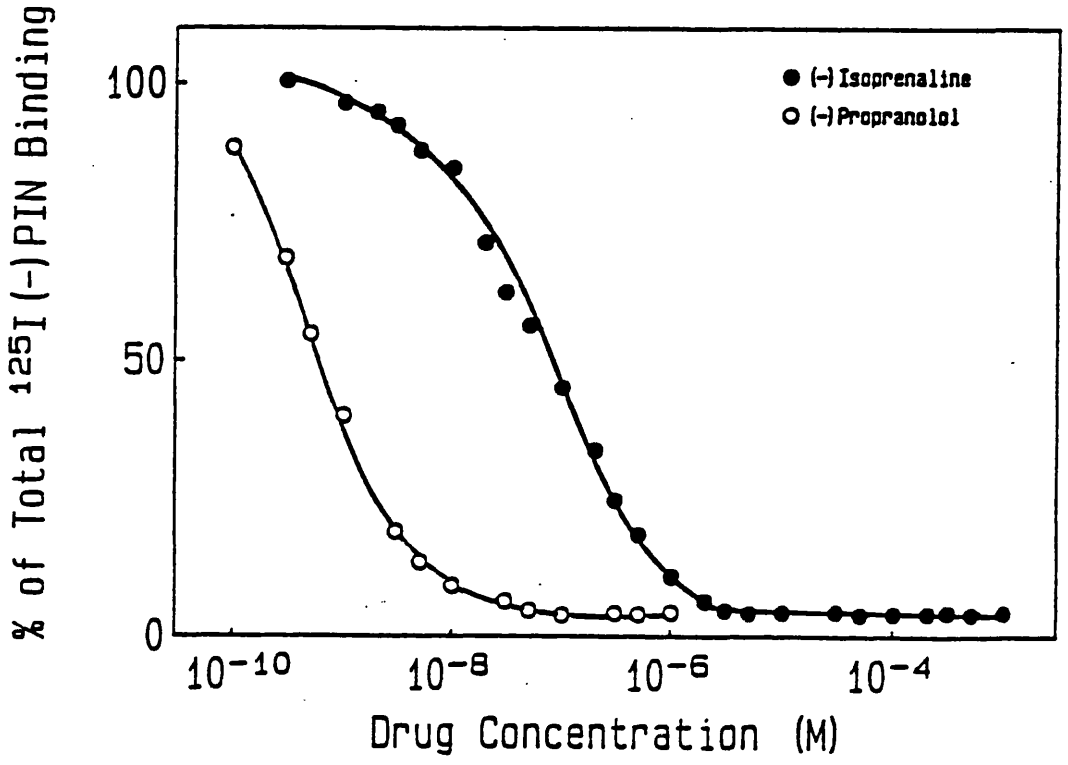


Figure II.4. Displacement of total ^{125}I(-)PIN binding to rat lung membranes by (-)propranolol, and (-)isoprenaline

Membranes were incubated at room temperature for 40 minutes with 30-40pM ^{125}I(-)PIN and increasing concentration of displacing drugs as described in II.2.2.4. The data shown for each compound are the means of at least three separate experiments performed in duplicate. SEM for each point was $< \pm 5\%$.

required in the case of certain β-adrenoceptor radioligands including ^{125}I(-)PIN. In order to establish whether the use of a decay correction factor was appropriate in the present study, the data for ^{125}I(-)PIN was analysed with and without correction for decay.

Analysis of the saturation binding curves showed that specific binding of both radioligands was saturable. Scatchard analysis of the data revealed a homogenous population of sites, with a Kd of 0.43 ± 0.05 nM, and a Bmax of 462 ± 12 (fmol mg^{-1} protein) for ^{3}H(-)DHA similar to that quoted previously by Rugg et al., (1978); and a Kd of 23.1 ± 1.23pM or 33.24 ± 2.14 pM, with Bmax values of 441 ± 10 (fmol mg^{-1} protein) or 625 ± 39 (fmol mg^{-1} protein) for ^{125}I(-)PIN without and with correction for decay respectively. In all cases, Hill slope values obtained were not significantly different from unity, indicative of a homogenous population of binding sites. The mean values quoted were calculated over 60 days. Linear regression analysis of the best fit lines for Bmax (figure II.5) and Kd (not shown) values obtained for ^{3}H(-)DHA and ^{125}I(-)PIN (without correction) revealed no significant difference between the two radioligands over the 60 day period. However, when ^{125}I(-)PIN binding was corrected for decay, a steady increase in both Bmax (figure II.5) and Kd (not shown) estimates with time were observed.

II.3. Isolation of human platelets from other blood cells

II.3.1. Introduction

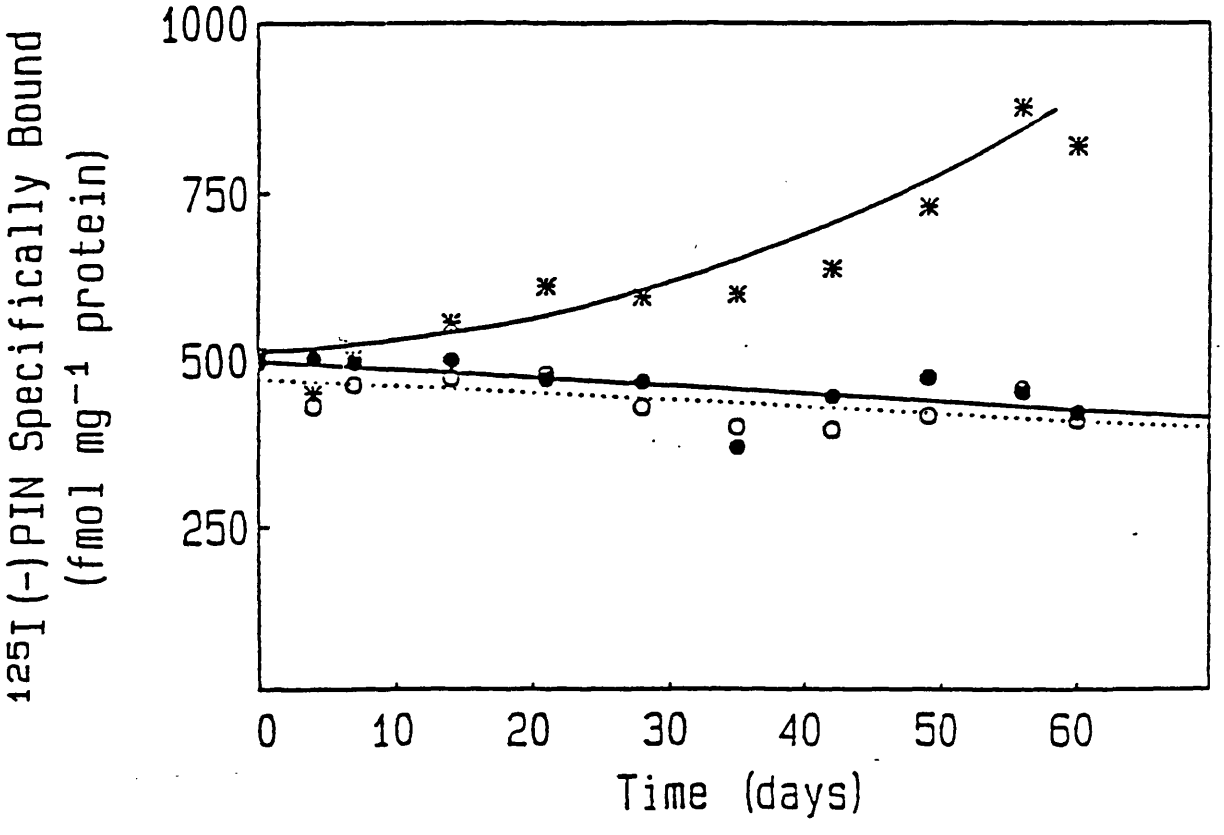


Figure II.5. Apparent maximal number of binding sites (Bmax) measured in rat lung membranes with various β-adrenoceptor antagonists

Saturation binding experiments were performed with either ^{3}H(-)DHA (•) or ^{125}I(-)PIN (o,*), as described in II.2.2. at intervals during the first 60 days following the iodination of (-)pindolol. Bmax values were calculated as described in Appendix 3 and plotted as a function of time. The lines through the data points represent the best fit regression lines of ^{3}H(-)DHA (•——•) or ^{125}I(-)PIN without corrections (o···o), or an approximate best fit curve for ^{125}I(-)PIN with correction (*——*). The data shown are representative of two such experiments. Each data point represents one saturation experiment performed in triplicate (see text for further explanation III.2.3.).

The second most important consideration in any radioligand binding study is the tissue preparation to be employed. In the present study the receptor of interest was the β-adrenoceptor on the human platelet. However, before embarking on a study to investigate the β-adrenoceptor present on the human platelet, a means of preparing a 'pure' platelet suspension was required since human blood cells other than platelets also possess β-adrenoceptors.

White blood cells have been extensively used as a model to study β-adrenoceptor changes that occur in diseased states, or during drug therapy, and hence there is an abundance of data regarding β-adrenoceptor number present in both mononuclear leukocytes and polymorphonuclear leukocytes. Unfortunately the literature reveals considerable variability in the reported normal values for β-adrenoceptor numbers in human white blood cells (see Marinetti et al., 1983 for summary) due mainly to different experimental conditions, but it is now well established that the β-adrenoceptor present on these cells is of the β_2-subtype (Galant et al., 1978; Williams et al., 1976; Brodde et al., 1981).

Human erythrocytes also appear to possess β-adrenoceptors of the β_2-subtype (Sager, 1982). Early studies employing radioligand binding techniques to identify β-adrenoceptors in human erythrocyte membranes concluded that no β-adrenoceptors were present on these cells (Williams et al., 1976). A possible explanation for this has come from recent studies in which it has been shown that membrane fragments of human erythrocytes are able to pass through glass fibre filters with a pore

size of 1.4um (Sager et al., 1983), and that intact normal human erythrocytes subjected to a minimal negative pressure pass through channels of less than 3um in diameter due to membrane deformability (Gregersen et al., 1967; Chien et al., 1967). Also, washing of intact human erythrocytes has been shown to reduce the number of saturable binding sites for propranolol and alprenolol (Sager and Jacobsen, 1979). It is unlikely therefore that intact erythrocytes or erythrocyte membrane fragments will interfere with the present investigation of the β-adrenoceptor on human platelets, but any contamination from white blood cells would undoubtedly contribute to ^{125}I(-)PIN binding measurements.

In this preliminary study, various methods for isolating platelets from other human blood cells were evaluated in an attempt to obtain a 'pure' platelet preparation. The methods found to give the least (i.e. undectable) contamination from either white blood cells or erythrocytes were adopted for routine use in the present study and are described in section II.3.2. Several techniques were employed to assess the purity of the platelet suspensions prepared in this way and are described below; they include light microscopy, indirect immunofluorescence, manual and Coulter counting. Throughout the present study, platelet numbers were routinely obtained by means of Coulter counting, and white blood cell (WBC) and erythrocyte (RBC) contamination of platelet suspensions was always below the detection limit ($0.3x10^6$WBC/ml; $0.03x10^9$RBC/ml) for the Coulter counter. However, since WBC are reported to have a relatively high number of β-adrenoceptors per cell (see II.3.3.2.) as compared with platelets, a study to estimate the

number of β-adrenoceptors associated with $0.3x10^6$ WBC was performed by $^{125}I(-)PIN$ binding so that the significance of an at worst detectable contamination of $0.3x10^6$ WBC/10^9 platelets could be evaluated.

II.3.2. Methods

II.3.2.1. Preparation of human platelet membranes

Blood was drawn from healthy normal volunteers using a 19 gauge needle into 0.1 volumes of 3.8% sodium citrate as anticoagulant. After centrifugation of blood at 250g for 15 minutes, the platelet rich plasma (PRP) was recentrifuged at 150g for a further 20 minutes. Using this method, all platelet preparations were completely free of white blood cell and erythrocyte contamination as assessed by visual counting (see II.3.2.4.). Platelet membranes were prepared by centrifugation of PRP at 27,000g for 10 minutes at 4°C. The resulting platelet pellet was resuspended in ice-cold lysing buffer (5mM EDTA, 5mM Tris-HCl) and homogenised using an Ultraturrax homogeniser. The resulting membrane suspension was centrifuged at 27,000g for 10 minutes at 4°C, washed twice and finally resuspended in assay buffer (50mM Tris HCl, 0.2mM Na metabisulphite, pH 7.8) at a final concentration of 1.5-3mg membrane protein/ml. All experiments were carried out using freshly prepared membranes.

II.3.2.2. Preparation of intact human platelets

Blood was drawn from healthy normal volunteers using a 19 gauge needle into 0.17 volumes of acid-citrate dextrose (10mM citrate) as anticoagulant. After centrifugation of blood at 700g for 5 minutes at room temperature, the platelet rich plasma was diluted 1:1 with Tris Isosaline pH 6.5 (50mM Tris HCl, 100mM NaCl, 5.0mM EDTA, 0.2mM sodium metabisulphite) and centrifuged at 450g for 20 minutes at room temperature (22-25°C) before final resuspension in 'whole cell' assay buffer (Tris Isosaline, pH7.8) at a concentration of 5-9 x 10^8 cells/ml. Using this method, all platelet preparations were free of white blood cell and erythrocyte contamination as assessed by means of a model S-plus IV Coulter cell counter and visual counting (see II.3.2.4.). All experiments were carried out using freshly prepared platelets.

II.3.2.3. Preparation of intact mononuclear leukocytes (MNL)

Blood was drawn from healthy normal volunteers using a 19 gauge needle into 0.17 volumes of acid-citrate dextrose (10mM citrate) as anticoagulant. After centrifugation of blood at 700g for 5 minutes at room temperature, the PRP was removed and discarded. MNL were prepared from the cell sediment remaining after removal of PRP by adding Tris buffered saline (TBS - see appendix 5) to the equivalent volume of the original blood and carefully layering 4.0mls of this onto 4.0mls lymphocyte separating medium (Flow laboratories). The tubes were then centrifuged at 400g for 30 minutes at 20°C. The MNL which formed a band above the separating medium were removed and washed with TBS by centrifugation at 400g for 15 minutes at room temperature (22-25°C).

The resulting MNL pellets were resuspended in whole platelet assay buffer (Tris Isosaline pH 7.8) (see section II.3.2.2.). The final cell suspension was counted with a Model S-Plus IV Coulter cell counter. All experiments were carried out using freshly prepared MNL.

II.3.2.4. Cell Counting

Visual counting :

Aliquots of platelet suspensions (II.3.2.1/2) were placed on glass microscope slides, cover slips apposed and slides examined for white blood cell (WBC) and erythrocyte (RBC) contamination by light and phase-contrast microscopy.

Coulter cell counting :

Platelet or mononuclear leukocyte suspensions (II.3.2.1/2/3) were counted by means of a model S-plus IV Coulter cell counter. To ensure accurate determinations of cell number, values must fall within the linear range for each parameter as quoted in the manufacturers manual:-

Parameter	Linear Range
WBC x 10^6 cells/ml	0.3 - 76.0
RBC x 10^9 cells/ml	0.5 - 7.2
Platelets x 10^6 cells/ml	25 - 735

II.3.2.5. Light microscopy of platelets and mononuclear leukocytes (MNL)

Platelet or MNL suspensions (see II.3.2.1/2/3 respectively) were diluted in phosphate buffered saline (PBS - Appendix 5) to give 1 x 10^6 cells/ml. Diluted suspensions were centrifuged at 500g for 1 minute at room temperature (22-25°C) in a Shandon cytospin, and "cytospin" preparations immediately stained with Wright's stain (Appendix 6). Slides were examined and photographs taken on a Zeiss Photomicroscope III using a "Kodak Ektachrome 50" colour film.

II.3.2.6. Indirect immunofluourescent analysis of platelets and mononuclear leukocytes (MNL)

Immunological techniques may be used for the identification or enumeration of cell populations by preparing an antibody that reacts specifically with an antigenic substance on the cell surface. The antibody may then be attached to an identifying label e.g. a fluorescent dye, and incubated with the cells. The sites at which the antigen-antibody complexes are formed may then be localised by fluorescence microscopy. This, the first immunofluorescence technique to be developed, known as the 'Direct' method is however rarely used nowadays since it is not only relatively insensitive but also requires the labelling of each individual antibody and has been replaced by the commonly used 'Indirect' (two-stage) method. The latter method was employed in the present study and involves reacting the cell surface antigen with an unlabelled primary antibody and then treating this with a labelled secondary antibody. The primary antibody therefore has a paradoxical dual function, serving as antibody when reacting with the

cell antigen, but then as antigen when interacting with the secondary antiserum. Such an indirect approach eliminates the need for labelling each primary antibody and also has much greater sensitivity since the primary antibody molecule may bind several labelled secondary antibody molecules, which corresponds to an amplification effect.

The fluorochrome used in the present study was fluorescein isothiocyanate (FITC). This gives an intense apple-green fluorescence, when an excitation wavelength of 490nm and emitting wavelength of 510nm are employed (emitted light is always of longer wavelength i.e. lower in energy, than absorbed light (Stokes Law)). The primary antibodies employed were OKT3 - antihuman T cell (Appendix 1) which is specific for peripheral T-lymphocytes, and AN51 (Appendix 1) which recognises an antigenic determinant on platelet Glycoprotein Ib and is specific for platelets and megakaryocytes (Vainchenker et al., 1982).

Cytospin preparations of platelets or MNL (see II.3.2.5) were fixed in acetone for 5 minutes at 4°C, air dried and washed in phosphate buffered saline pH 7.2 (PBS - see appendix 5) for 10 minutes at room temperature (22-25°C). Excess PBS was removed from the slide, the cells were carefully overlaid with 15ul of primary antibody (OKT3 or AN51) and incubated in a moist chamber for 30 minutes at room temperature. Following incubation, the primary antibody was rinsed away and slides washed for 15 minutes at room temperature in PBS. Cytospins were then carefully overlaid with the labelled secondary antiserum, (FITC labelled goat antimouse Ig (Appendix 1) diluted 1:20 with PBS containing 1% human AB serum to absorb any reactivity with

human B-lymphocytes) and incubated in a moist chamber for 30 minutes at room temperature. Finally, cytospins were washed for 15 minutes in PBS, mounted with 90% glycerol 10% PBS and examined by fluorescence microscopy on a Leitz Orthomat microscope. Photographs were taken using a "Kodak Ektochrome 400" film.

II.3.2.7. ^{125}I(-)PIN binding assays to intact human platelets or mononuclear leukocytes (MNL)

Platelets (whole) or MNL (whole) were added to ^{125}I(-)PIN (0-200pM) and appropriate concentration of displacing agent or buffer, in a final volume of 500ul assay buffer (Tris Isosaline, pH 7.8, see II.3.2.2.). Incubations were performed at 37°C for 40 minutes. Upon reaching equilibrium (except for kinetic experiments) the reaction was stopped by the addition of 10ml ice cold assay buffer diluted 1:10 with water. Tubes were kept on ice for 30 minutes prior to rapid vacuum filtration. Filters were then washed with 3 x 10ml ice cold buffer (25mM Tris HCl, 154mM NaCl, pH 7.8) and radioactivity bound to the filters was determined directly in a Packard Auto Gamma 500C counter at 74% counting efficiency. (-)Pindolol was iodinated as described in Appendix 4.

II.3.3. Results

II.3.3.1. Examination of platelet preparations for contamination by other blood cells

Routine examination of platelet suspensions (II.3.2.1/2) by light and phase-contrast microscopy (II.3.2.4.) revealed no detectable contamination from either white blood cells (WBC) or erythrocytes (RBC) (not shown).

Aliquots of platelet suspensions were subjected to cytospin preparation in order to concentrate any contamination that may be present onto the visual field (II.3.2.5). Cytospins were stained and examined under light microscopy. Stained cytospin preparations of MNL suspensions were employed as an example of the expected appearance of other blood cells examined in this manner. As can be seen from plate 1b, MNL suspensions contained several cell types including lymphocytes, monocytes and erythrocytes, whose appearance was easily distinguishable from that of platelets also present in the preparation. Examination of stained platelet suspension cytospins (plate 1a) revealed typical heterogeneity of platelet size, but no detectable contamination from other blood cells.

Similar results were obtained when cytospin preparations of platelet or MNL suspensions were subjected to indirect immunofluorescence analysis (II.3.2.6). When platelet cytospin preparations were overlaid with the platelet monoclonal antibody AN51, specific labelling of the platelets occurred which was clearly visible by fluorescence microscopy as an intense apple-green fluorescence (plate 2a). However, when an identical platelet cytospin preparation was overlaid with the T-lymphocyte monocolonal antibody OKT3, no specific labelling of any

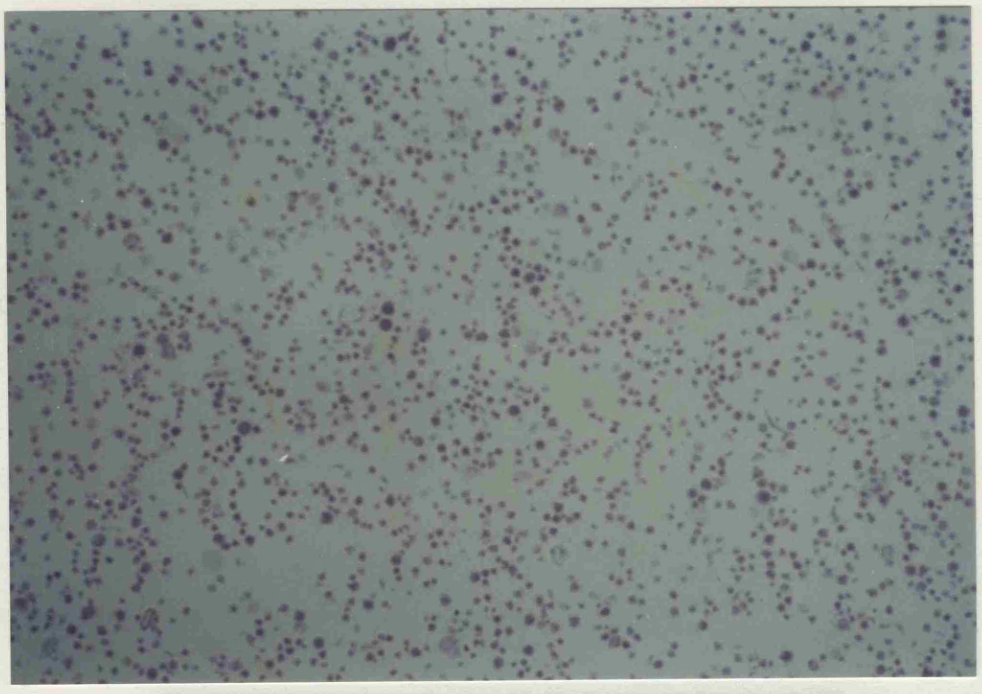


Plate 1a Mag x 300

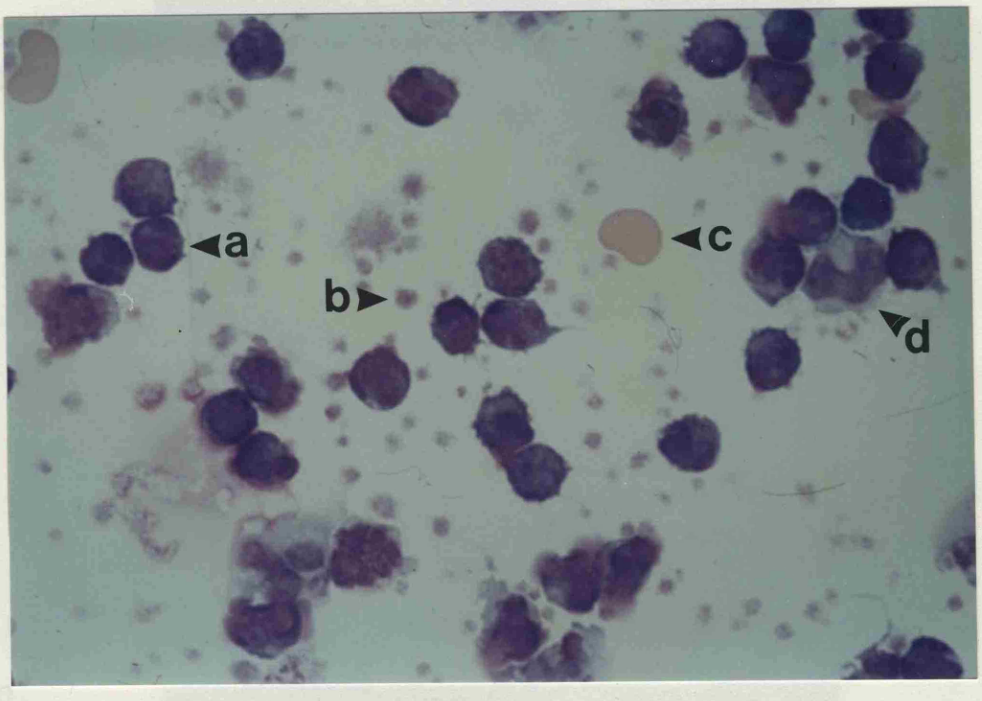


Plate 1b Mag x 800

Plate 1 : Platelet or mononuclear leukocyte (MNL) "cytospin" preparations were stained with Wright's stain (see II.3.2.5.).

Plate 1a: platelet preparations

Plate 1b: MNL preparation a= lymphocyte, b= platelet, c= erythrocyte, d= monocyte. (see text for further explanation II.3.3.1.)

Plate 2a
Mag x 500

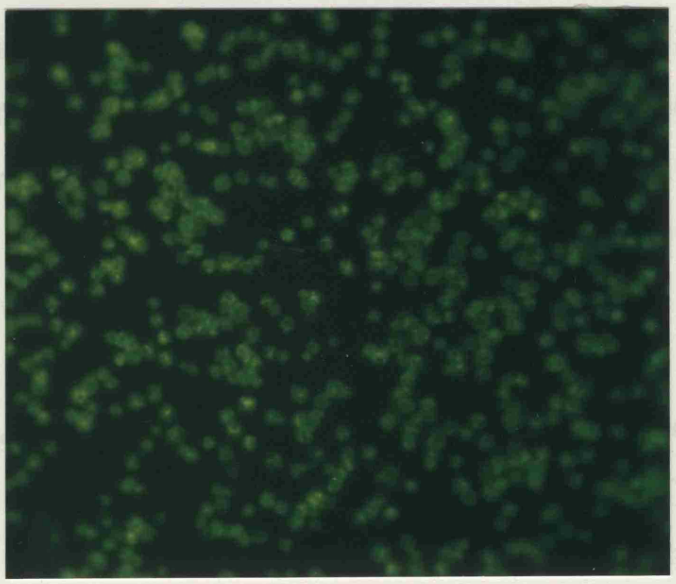


Plate 2b
Mag x 500

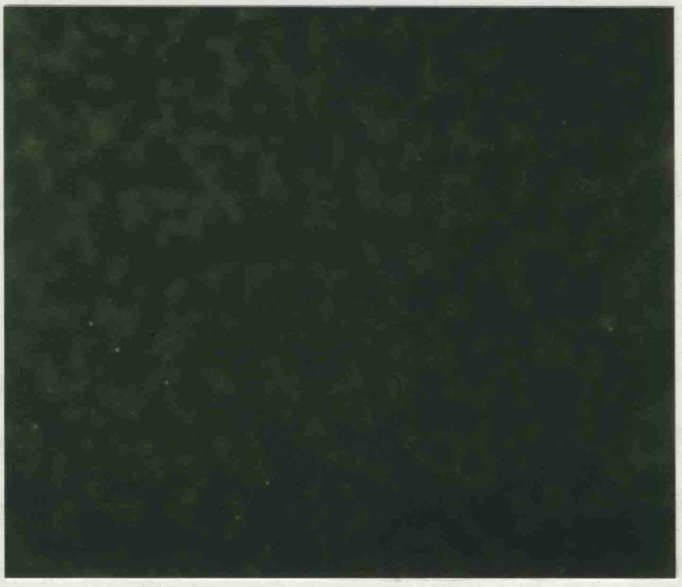
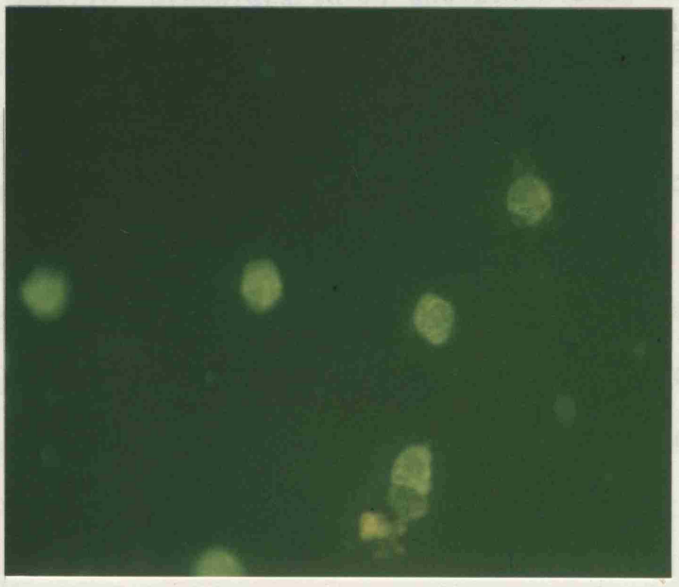


Plate 2c
Mag x 500



cells occurred, and the dull fluorescence observed (plate 2b) was characteristic of the background fluorescence expected when using this method. By comparison, overlaying an MNL cytospin preparation with OKT3 resulted in specific labelling of the T-lymphocytes present that was easily observed as a bright fluorescence (plate 2c).

Both platelet and MNL suspensions were routinely counted with a model S-plus IV Coulter cell counter. As mentioned previously (II.3.1.) white blood cell and erythrocyte contamination of platelet suspensions was always below the detection limit ($0.3x10^6$WBC/ml; $0.03x10^9$RBC/ml) for this instrument.

II.3.3.2. ^{125}I(-)PIN binding to intact platelets and mononuclear leukocytes (MNL)

Specific binding of ^{125}I(-)PIN to intact platelets or MNL defined as binding displaceable by 1uM(-)propranolol (determined from competition experiments, see chapter VI) was saturable (data not shown). The maximum number of binding sites (Bmax) assessed by Scatchard analysis was 5.64 ± 0.03 fmol/10^9 platelets (n=7) and 1.4 ± 0.01 fmol/10^6 MNL (n=7) equivalent to 6 binding sites/platelet and 826 ± 106 sites/MNL. ^{125}I(-)PIN binding was of high affinity with virtually identical equilibrium dissociation constants of 23.29 ± 1.15 pM, and 21.0 ± 0.77 pM, for platelets and MNL respectively. In all cases the data produced linear Scatchard plots with Hill slope values not significantly different from unity, indicative of a homogenous population of binding sites on both cell types. The Bmax on MNL was calulated over a wide

range (1.0 - 7.9 x 10^6 cells) of cell concentration and the number of fmoles associated with 0.3 x 10^6 cells calculated by extrapolation to be 0.42 ± 0.01 (n=7). Therefore a WBC contamination of 0.3 x 10^6 WBC/10^9 platelets would be equivalent to less than 8% of the β-adrenoceptor number measured. Both Bmax estimates in terms of fmol/10^6MNL or number of sites/MNL are in excellent agreement with values previously quoted by other workers (Brodde et al., 1981; Feldman et al., 1986).

II.4. Conclusions

In summary, ^{125}I(-)PIN labelled the same number of binding sites in rat lung membranes as ^{3}H(-)DHA. The binding sites exhibited a similar overall affinity for (-)isoprenaline and (-)propranolol to those quoted previously for rat lung β-adrenoceptors (Rugg et al., 1978), and furthermore, the Kd for ^{125}I(-)PIN was similar to that quoted by other workers employing this radioligand to study β-adrenoceptors (Barovsky and Brooker, 1980). ^{125}I(-)PIN once iodinated appeared to be stable for at least 60 days i.e. it labelled the same number of binding sites as ^{3}H(-)DHA during this period, and did not appear from chromatographic analysis to dissociate into free iodine and ligand, or form another detectable iodinated product. Analysis of ^{125}I(-)PIN data with and without correction for decay revealed that Bmax and Kd values calculated for ^{125}I(-)PIN deviated markedly from those for ^{3}H(-)DHA when a correction for decay was employed, but that no significant difference between the two radioligands was apparent if no correction factor was employed. These results support the conclusion of Doyle et

al., (1984), and suggest that the decayed ^{125}I(-)PIN becomes altered in such a way that it no longer binds specifically to the receptors. A decay correction factor was not employed in the present study, and to ensure that maximum accuracy could be attained, ^{125}I(-)PIN was only used within 30 days of iodination. Furthermore, although ^{125}I(-)PIN is iodinated to a theoretical specific activity of 2175 Ci/mmol, the specific activity of supplied Na^{125}I can vary by as much as 20%. Thus, to minimize any errors in interpretation of data, the actual specific activity of each ^{125}I(-)PIN preparation was adjusted to that of Na^{125}I employed in the iodination.

Pindolol is a β-adrenoceptor antagonist with intrinsic sympathomimetic activity i.e. it is a partial agonist. It has been suggested that stimulation and blockade are mediated via different receptor populations (Walter et al., 1984; Kaumann and Lobnig, 1986), and that pindolol is more active on β_2- than on β_1-adrenoceptors (Clark, 1984), with a β_2-agonistic component of its partial agonist activity (Brodde et al., 1986). The nature of the dissociation between stimulation and blockade is not well understood for pindolol or other weak partial agonists at present, and probably involves tissue related factors such as receptor density and efficiency of coupling (Kenakin, 1984). However it does appear that the concentrations required for activation are far greater (nanomolar range) than those required for occupation and blockade of the β-adrenoceptors (picomolar range) as used in the present study (Brodde et al., 1986; Neve and Molinoff, 1986). Since ^{125}I(-)PIN would not be expected to have agonist activity at the concentrations employed in this study, its functional β_2-selectivity is

of little consequence. A recent report exploring radioligand selectivity in binding studies however, may have a marked influence on the data obtained. ^{125}I(-)PIN and several other β-adrenoceptor radioligands were shown by Neve et al., (1986) to exhibit a 2-5.8 fold β_2-selectivity (3.2 fold for ^{125}I(-)PIN). Although it is unlikely that this subtype selectivity would alter total receptor density measurements (Bmax), delineation of the proportion of β-adrenoceptor subtypes present would be less accurate than with a nonselective radioligand.

Examination of the platelet suspensions prepared using optimal methods (later adopted for this study) failed to show any contamination from other blood cells. Our estimation of the number of β-adrenoceptors that could be attributed to a detectable contamination from WBC was found to be equivalent to less than 8% of total β-adrenoceptors measured by ^{125}I(-)PIN binding and is therefore not expected to interfere significantly with this investigation of the human platelet β-adrenoceptor.

CHAPTER III

III ^{125}I(-)Pindolol binding to human platelet membranes. Characterisation and agonist interactions - brief comparison with rat lung membranes.

III.1. Introduction

Evidence to date suggests that β-adrenoceptors are present on human platelets (see section I.2.3.2). The precise quantification and classification of the human platelet β-adrenoceptor however remains unclear. Recent functional studies employing subtype selective β-adrenoceptor antagonists suggest the presence of a β_2-adrenoceptor subtype (Kerry and Scrutton, 1983a). Radioligand binding studies however, have yielded somewhat confusing results. Initially, Hansen et al., (1981) using ^{3}H-dihydroalprenolol (^{3}H-DHA) suggested that both β_1- and β_2- adrenoceptors were present on human platelets. Later, Steer and Atlas (1982) showed that the iodinated radioligands ^{125}I-hydroxybenzylpindolol and ^{125}I-cyanopindolol (^{125}I-CYP) identified different densities of sites on human platelets which did not exhibit the pharmacological properties of either β_1- or β_2-adrenoceptor subtype. More recently, other workers have shown that the binding of ^{3}H-DHA and ^{3}H-acetobutolol to human platelet lysates was largely, if not exclusively, unrelated to occupancy of β-adrenoceptors (Kerry and Scrutton, 1983b).

The aims of the study described in this chapter were to employ radioligand binding techniques to: i) identify the β-adrenoceptor present in human platelet membranes; and ii) examine the functional "coupling" of this receptor.

Adrenoceptor coupled adenylate cyclase systems are among the most

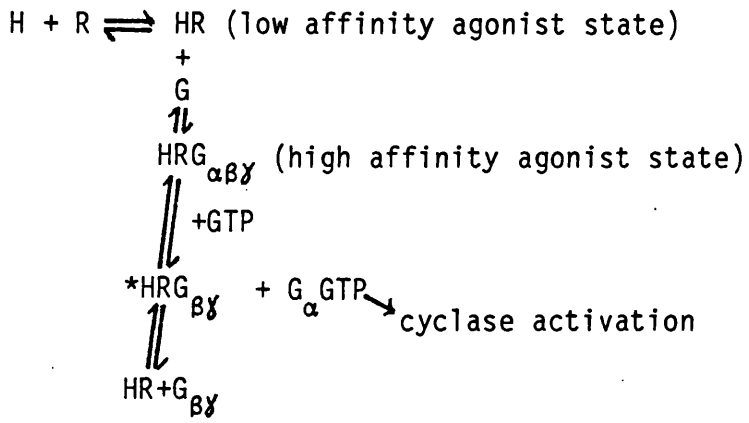
studied receptor-effector mechanisms, and hence, much is known about the components present and how they interact to either stimulate or inhibit enzyme activity. At present, the system is believed to consist of at least three distinct components : stimulatory (e.g. β-adrenoceptors) or inhibitory (e.g. α_2-adrenoceptors) receptors; regulatory coupling proteins; and the catalytic subunit that converts Mg^{++}.ATP into cyclic AMP. Two different guanine nucleotide-binding regulatory proteins are involved in the stimulation (Gs, G/F or Ns) and inhibition (Gi or Ni) of adenylate cyclase. Both have been isolated and appear to be heterotrimers of subunit composition α_s or α_i, β and ɣ. The β and ɣ subunits (mol wt 35,000 and 8,000 respectively) of Gs and Gi are structurally similar if not identical, functionally indistinguishable and do not bind guanine nucleotides. In contrast, the α subunits of Gs and Gi are distinct proteins with molecular weights of 45,000 for Gsα (or αs) and 41,000 for Giα (or αi). Both possess a high affinity binding site for guanine nucleotides, and are the targets for NAD-dependant ADP ribosylation catalysed by cholera toxin for αs and islet-activating protein for αi (see Gilman, 1984; Hildebrandt et al., 1984). Although these proteins were first identified as regulatory units of adenylate cyclase systems, it now appears that they are part of a family of G proteins that have diverse regulatory functions (see Stryer, 1986).

The mechanism by which the various components of the adrenoceptor responsive adenylate cyclase system interact to alter the activity of adenylate cyclase is becoming increasingly clear. Agonists, but not antagonists, have been shown to induce a guanine nucleotide sensitive

state of the receptor that forms a complex with the G protein, which for β and α_2-adrenoceptors is identifiable by its larger molecular size. This functional difference for the receptor is also apparent in radioligand binding experiments employing purified membrane preparations. Agonists, competing against radiolabelled antagonists often exhibit complex, shallow, biphasic competition curves, whilst antagonist curves are generally steep and uniphasic. Computerised analysis of such data has been interpreted to represent two sub-populations of receptors exhibiting two affinity states for agonists (high and low) but equivalent affinity for antagonists. Furthermore, in the presence of guanine nucleotides, agonist competition curves are steepened, shifted to the right and appear uniphasic with a lower overall affinity (see Nahorski and Barnett, 1982; Stiles et al., 1984) whilst antagonist competition curves generally remain unchanged.

This differential effect of agonists and antagonists has been explained in terms of a ternary complex model (Delean et al., 1980; Stadel et al., 1982). Agonist (H) occupancy of the receptor (R) is believed to induce a conformational change in the receptor which allows the agonist-occupied receptor (HR) to form a complex with the GDP-occupied G protein (G). In the absence of guanine nucleotides this ternary complex (HRG) persists, and exhibits high affinity for the agonist. In the presence of guanine nucleotides (e.g. guanine triphosphate (GTP)), GDP-GTP exchange on the G protein promotes dissociation of the active GTP.G protein species, which then interacts with, and activates (i.e. stimulatory or inhibitory) the catalytic moiety of adenylate cyclase. Activation persists until GTP is hydrolysed to GDP by a GTPase in the

system. Recently (Gilman, 1984b; Northup, 1985), it has been proposed that G protein activation involves dissociation of the α and βγ subunits. Thus the ternary complex model may be illustrated as follows:



*possible HRG complex of unknown agonist affinity

The involvement of G protein subunit association-dissociation in the control of adenylate cyclase activity is not clearly defined at present. Recent reports have shown that when Gi$_\alpha$, activated by stable GTP analogues, was reconstituted with the adenylate cyclase of platelet membranes, it proved to be only slightly inhibitory. This led to the suggestion that Gi$_\alpha$ inhibition of adenylate cyclase may be indirect (Katada et al., 1984). Thus, activation of Gi by inhibitory α_2-adrenoceptors may lead to inhibition of adenylate cyclase by two different pathways : i) a direct inhibition of adenylate cyclase by Gi$_\alpha$.GTP; and ii) an indirect inhibition by interference with Gs activation i.e. the simultaneous release of βγ subunits which favours formulation of the inactive Gs$_{\alpha\beta\gamma}$. Therefore, with regard to adenylate cyclase inhibition by activated Gi, it is still unclear which of the

subunits causes the inhibition of adenylate cyclase or what role $\beta\gamma$'s play in signal transduction (see Birmbaumer, 1987; Levitzski, 1987). Furthermore it is not established whether the subunit dissociation reaction occurs in the normal lipid bilayer of the plasma membrane.

The ability of agonists to stimulate or inhibit adenylate cyclase activity has been shown to correlate well with their ability to promote the high-affinity agonist state (Kent et al., 1980; Limbird, 1987). Thus, an indication of the degree of coupling of a receptor to adenylate cyclase may be obtained by evaluating agonist binding in the presence and absence of guanine nucleotides.

Guanine nucleotides were employed to examine the human platelet β-adrenoceptor - adenylate cyclase system in this chapter, along with monovalent and divalent cations i.e. Na^{+} and Mg^{++} which have also been shown to modulate agonist binding at β-adrenoceptors.

Mg^{++} has been shown to promote high-affinity agonist binding to β-adrenoceptors, and a requirement for its presence in order to fully promote the high-affinity state of the receptor has been demonstrated in several tissues including frog erythrocytes (Williams et al., 1978), S49 lymphoma cells (Bird and Maguire, 1978), rat lung (Heidenreich et al., 1980) and rabbit lung (Dickinson and Nahorski, 1983). The precise location of this metal ion binding site is not yet known, but hypothesised to be on the coupling protein. Evidence in support of this hypothesis comes from studies employing genetic variants of cultured cell lines. For instance, Mg^{++} has been shown to increase

receptor affinity for agonist in HC-1, and H21a cells (Maguire, 1982). Since the catalytic unit in H21a cells cannot interact with the G protein, and the catalytic unit of HC-1 cells is either absent or non-functional, the Mg^{++} binding site cannot be present on the catalytic unit. Furthermore, Mg^{++} does not alter receptor affinity for agonists in cyc^- and UNC variants of S49 lymphoma cells in which the receptors cannot interact with a functional G protein (Bird and Maguire, 1978; Heidenreich et al., 1980). Thus, the Mg^{++} binding site is most probably located on the Gs coupling protein, possibly on the α subunit since cyc^- S49 lymphoma cells lack only the αs subunit of Gs (Ferguson et al., 1982). Mg^{++} has also been shown to have an important modulatory influence on agonist binding to several other receptors which include α_2-adrenoceptors (Tsai and Lefkowitz, 1978), and prostaglandin E_1 receptors (Williams et al., 1978).

Na^+, in contrast to Mg^{++}, has been shown to mimic the effect of guanine nucleotides in lowering the receptor affinity for agonists at the β-adrenoceptor in calf cerebellar and rat lung membranes (U'Prichard et al., 1978; Heidenreich et al., 1980). The site at which sodium acts remains unknown, and most information available to date concerning Na^+ modulation of agonist binding has come from studies performed on receptors which mediate inhibition of adenylate cyclase, e.g. sodium markedly reduces the receptor affinity for agonists at the human platelet α_2-adrenoceptor (Tsai and Lefkowitz, 1978). However, unlike guanine nucleotide effects at this receptor which result in a shift to the right and steepening of the agonist curve similar to that described for β-adrenoceptors, sodium effects are apparent as a parallel shift of

the agonist curve to lower affinity, with no change in slope (Tsai and Lefkowitz, 1979; Michel et al., 1980).

In the present chapter, radioligand binding techniques were employed to i) identify the β-adrenoceptor present in human platelet membranes by means of ^{125}I(-)pindolol (^{125}I(-)PIN) binding and perform a brief comparative study with ^{125}I(±)CYP (one of the radioligands previously employed by Steer and Altas (1982) to identify β-adrenoceptors on human platelets).

and ii) to assess the functional coupling of the human platelet β-adrenoceptor by examining the effects of guanine nucleotide and Na^+ on agonist competition of ^{125}I(-)PIN binding in the presence of Mg^{++}. In view of recent studies in which divalent cations and guanine nucleotides have been shown to characteristically modulate agonist binding to rat lung β-adrenoceptors (Dickinson and Nahorski, 1983), a direct comparison in parallel experiments of agonist interactions in rat lung and human platelet membranes was made.

III.2. Methods

III.2.1. ^{125}I(-)pindolol (^{125}(-)PIN) binding to human platelet membranes

Platelet membranes (150-300ug protein, except for protein dilution experiment, see Appendix 8) prepared as previously described (II.3.2.1.) were added to ^{125}I(-)PIN and appropriate concentration of

displacing agent or buffer in a final volume of 250ul assay buffer (50mM Tris HCl, 0.2mM Na metabisulphite, pH 7.8). Incubations were performed at room temperature (22-25°C) for 40 minutes (except for Kinetic experiments). Upon reaching equilibrium the reaction was stopped by the addition of 1.0ml ice-cold assay buffer. Membranes were immediately collected on Whatman GF/B filters by vacuum filtration and rapidly washed with 3 x 10ml ice-cold 50mM Tris HCl, pH 7.8. Radioactivity associated with the filters was determined directly in a Packard Auto Gamma 500C counter at 74% counting efficiency. (-)Pindolol was iodinated as described in appendix 4(a).

Agonist interaction experiments were conducted in assay buffer supplemented with 10mM $MgCl_2$, 0.5mM EDTA.

III.2.2. ^{125}I(±) cyanopindolol (^{125}I(±)CYP) binding to human platelet membranes

Platelet membranes (150-300ug protein) prepared as previously described (II.3.2.1.) were added to ^{125}I(±)CYP and appropriate concentration of displacing agent or buffer in a final volume of 250ul assay buffer (25mM Tris HCl, 154mM NaCl, 0.2mM Na metabisulphite, pH 7.8). Incubations were performed at 37°C for 60 minutes (except for Kinetic experiments), after which the reaction was stopped by addition of 1.0ml ice-cold assay buffer. Membranes were immediately collected on Whatman GF/B filters by vacuum filtration and rapidly washed with 3 x 10ml buffer (25mM Tris HCl, 154mM NaCl, pH 7.8) at room temperature. Radioactivity associated with the filters was determined as described

for ^{125}I(-)PIN (III.2.1.). (±)CYP was iodinated as described in appendix 4(b).

III.3. Results

Optimal conditions for ^{125}I(-)PIN binding to human platelet membranes were established prior to initiation of the study described in this chapter (see Appendix 8).

III.3.1. ^{125}I(-)PIN binding - equilibrium and Kinetic studies

The specific binding of ^{125}I(-)PIN to human platelet membranes was rapid and reversible with a t1/2 (time for half maximal binding) for association of 10-12 min, and dissociation of 40-45 min, at room temperature (figure III.1.). For the illustrated data, the second order association rate constant was calculated to be k_1=0.8636 nM^{-1} min^{-1}. Dissociation followed monophasic first order kinetics, with a dissociation constant K_2 of 0.017 min^{-1}. The mean kinetically derived Kd (K_2/K_1) calculated from three such experiments was 13.08±3.11 pM in excellent agreement with that obtained from equilibrium experiments.

Specific ^{125}I(-)PIN binding to human platelet membranes (defined as binding displaceable by 200uM(-)isoprenaline) was saturable (figure III.3/4.), whereas non-specific binding increased linearly with ^{125}I(-)PIN concentration. Scatchard analysis of the data (figure III.4.) revealed a homogenous population of binding sites with an

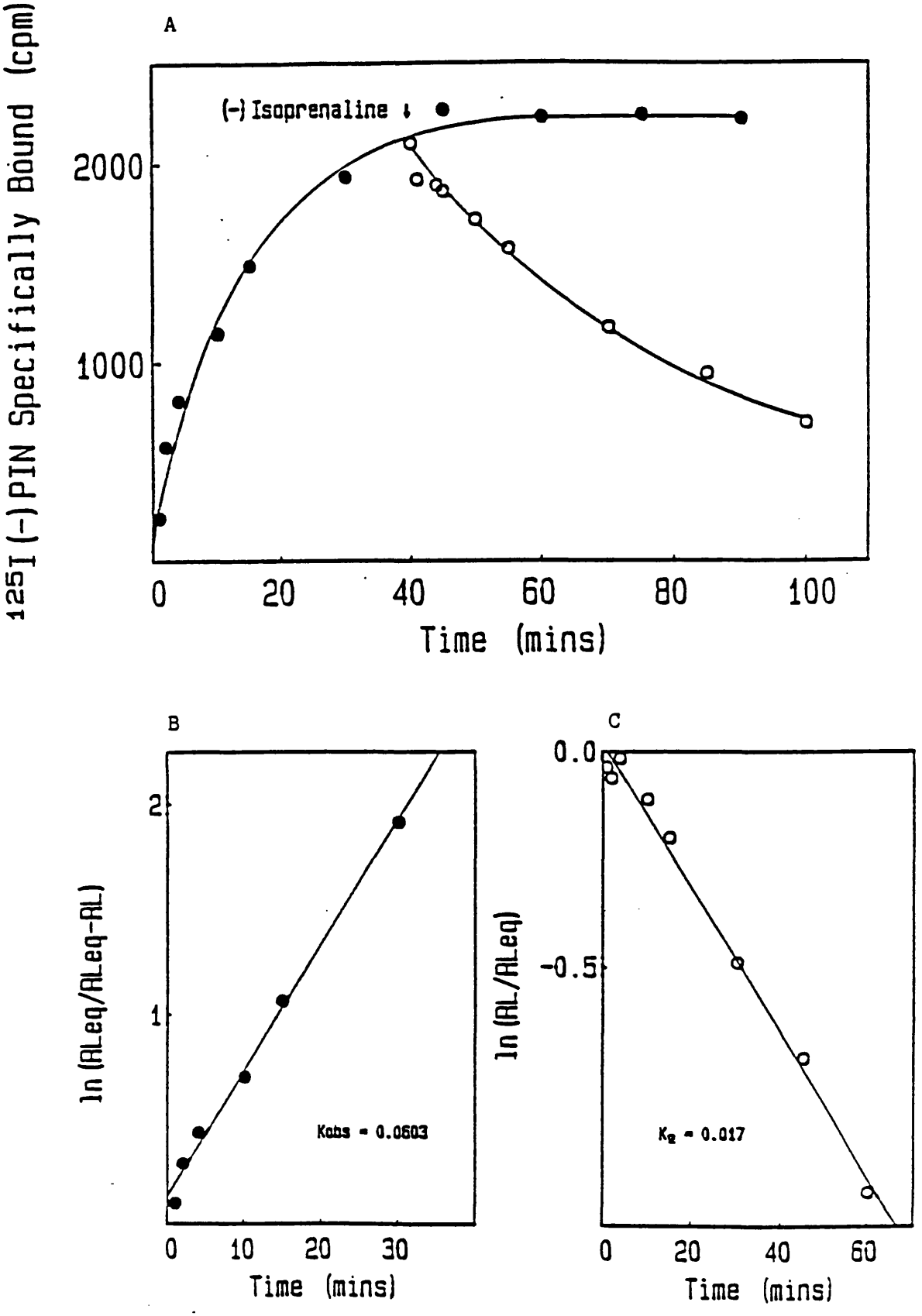


Figure III.1. Kinetics of specific ^{125}I(-)PIN binding to human platelet membranes at 22°C

Legend to Figure III.1. Kinetics of specific ^{125}I(-)PIN binding to human platelet membranes at 22°C

(See III.2.1. for method)

A) Time course of specific ^{125}I(-)PIN association (•), and dissociation (o). Dissociation was initiated by the addition of 200uM (-)isoprenaline after equilibrium had been reached (40 minutes).

B) pseudo first-order rate plot for the association reaction

C) first-order rate plot for the dissociation reaction, the slope of which yields the dissociation rate constant K_2. The second-order rate constant K_1 was calculated as described in Appendix 3b.

Data shown are from a single experiment representative of three experiments performed in duplicate.

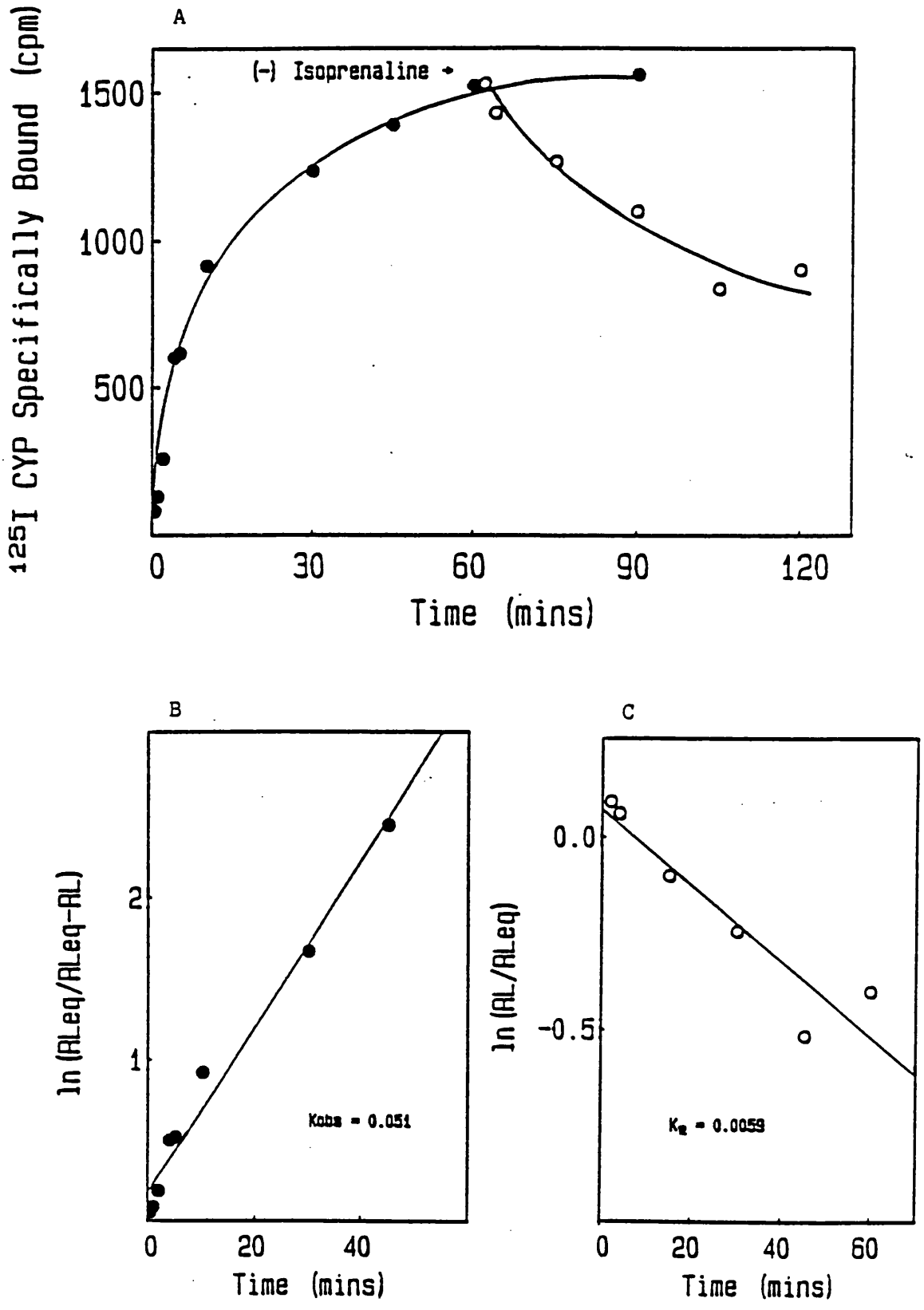


Figure III.2. Kinetics of specific $^{125}I(\pm)$CYP binding to human platelet membranes at 37°C

Legend to Figure III.2. Kinetics of specific $^{125}I(\pm)$CYP binding to human platelet membranes at 37°C

(See III.2.2. for method)

A) Time course of specific $^{125}I(\pm)$CYP association (●), and dissociation (o). Dissociation was inititated by the addition of 200uM (-)isoprenaline after equilibrium had been reached (60 minutes).

B) pseudo first-order rate plot for the association reaction,

C) first-order rate plot for the dissociation reaction, the slope of which yields the dissociation rate constant K_2. The second-order rate constant K_1 was calculated as described in Appendix 3b.

Data shown are from a single experiment performed in duplicate.

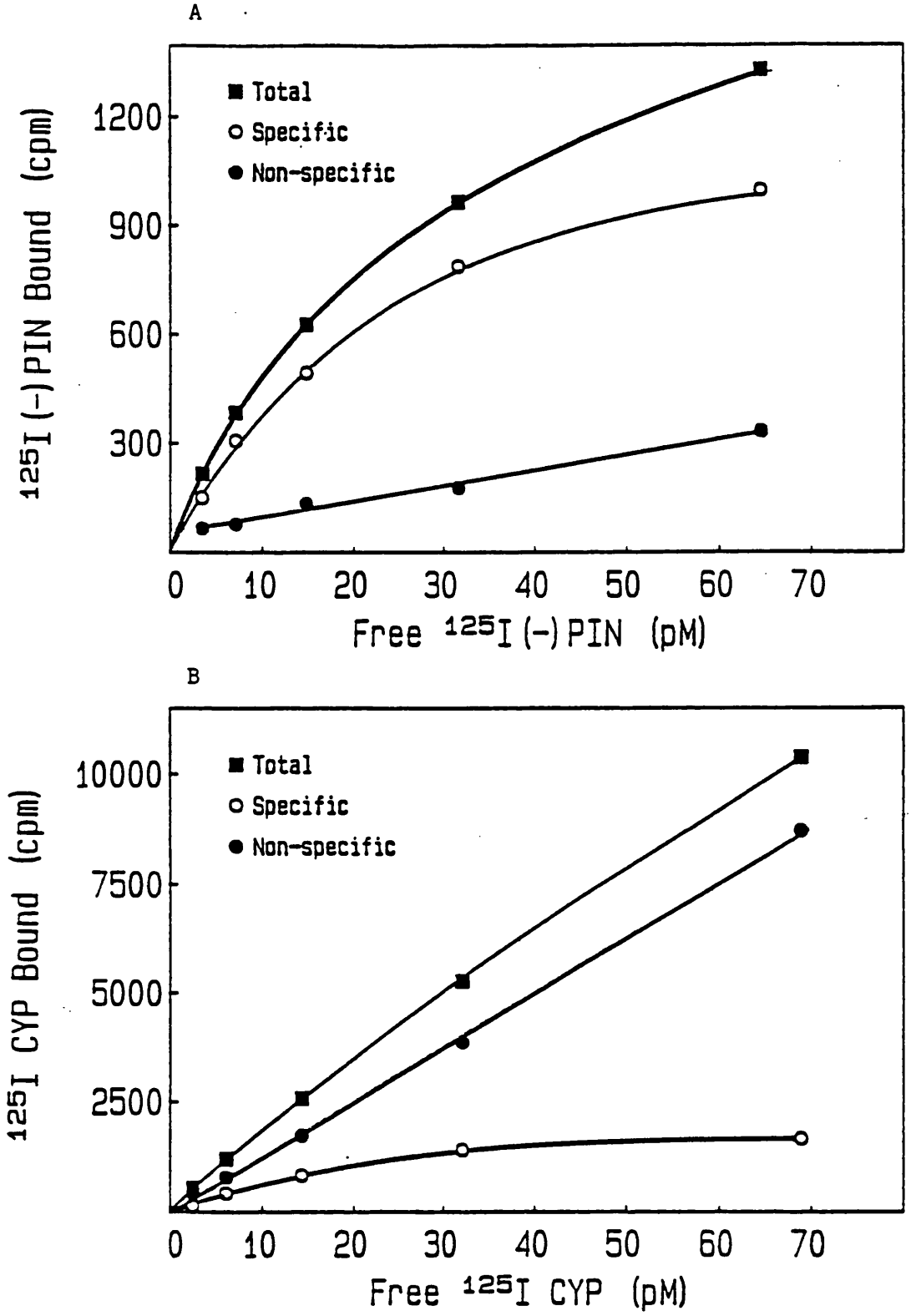


Figure III.3. ^{125}I(-)PIN (A) and ^{125}I(±)CYP (B) binding to human platelet membranes.

Data shown are from single representative experiments performed in triplicate. Both figures illustrate raw, untransformed saturation data of total, specific and non-specific binding for ^{125}I(-)PIN (A), and ^{125}I(±)CYP (B) (see III.2.1./2. for methods respectively). Specific binding defined in the presence of 200μM(-)isoprenaline represented approximately 70-80% for ^{125}I(-)PIN (A), and 25-30% for ^{125}I(±)CYP (B) at around the Kd (20-30pM) for both radioligands.

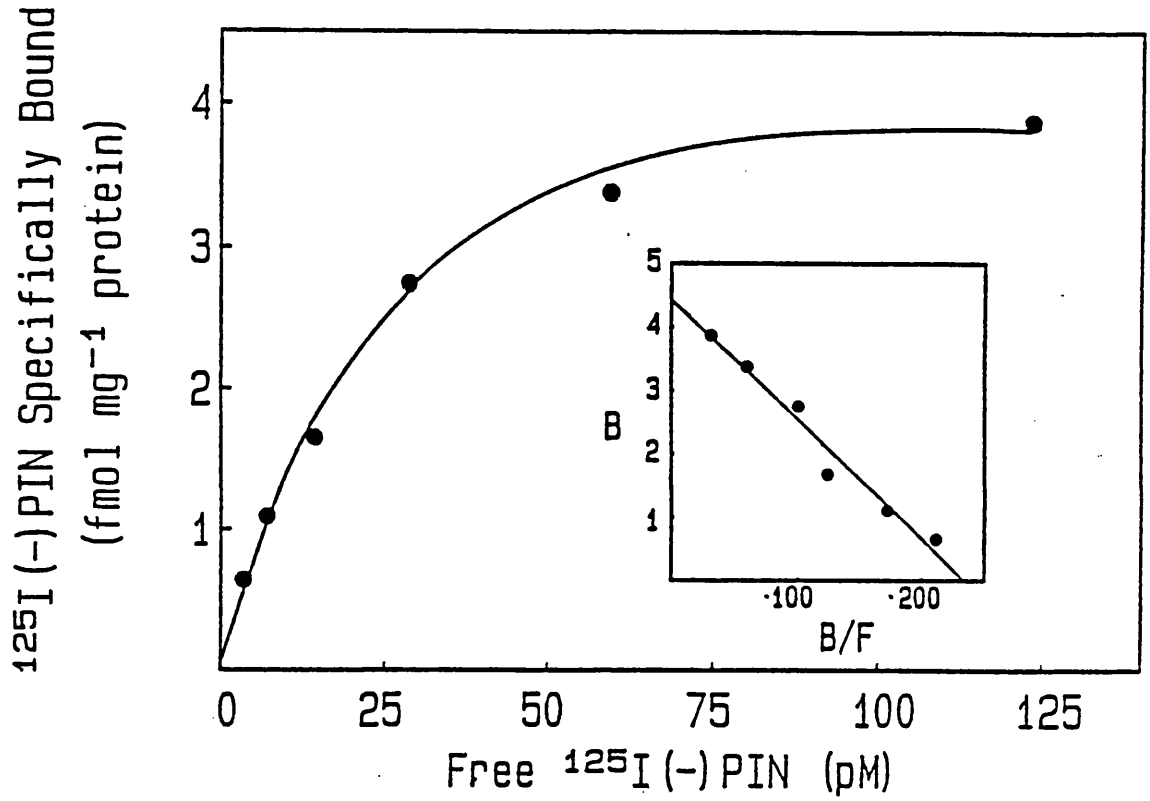


Figure III.4. Specific ^{125}I(-)PIN binding to human platelet membranes

Membranes were incubated with increasing concentrations of ^{125}I(-)PIN for 40 minutes at 22°C, and binding determined as described in III.2.1.. A saturation isotherm is shown with insert Scatchard plot of data, where B represents specifically bound ^{125}I(-)PIN expressed as fmol mg^{-1}protein, and F represents the free concentration of ^{125}I(-)PIN in pM. The data shown are representative of 13 such experiments performed in triplicate.

equilibrium dissociation constant (Kd) of 24.27±1.3pM(n=13), and binding site maxima (Bmax) of 5.54±0.71 (fmol mg^{-1} protein). In all cases, Hill slope values were not significantly different from unity, indicative of interaction with a single class of receptor sites in the absence of co-operative interactions.

III.3.2. ^{125}I(±)CYP binding - equilibrium and kinetic studies

Specific binding of ^{125}I(±)CYP to human platelet membranes at 37°C was slower at association and dissociation compared with ^{125}I(-)PIN at room temperature. Equilibrium was reached in around 60 minutes, (figure III.2.). From the kinetic experiment illustrated in figure III.2, the association constant K_1 was calculated to be 0.9135 nM^{-1} min^{-1}. Dissociation appeared to follow monophasic first order kinetics, with a dissociation constant of K_2=0.0059 min^{-1}. The kinetically derived Kd, (K_2/K_1) calculated from this experiment, of 6.469(pM) was of slightly higher affinity than that obtained from equilibrium experiments. The reason for this is not known but may be related to the racemic nature of this radioligand. Burgisser et al., (1981), have shown that the Kd of a racemic ligand may vary with receptor concentration, and that the apparent Kd is a hybrid of the Kd's for the (+) and (-) isomers respectively. Also, the relatively slow dissociation rate of ^{125}I(±)CYP binding makes accurate determinations of specific binding more difficult : this source of error may be augmented by the linear transformation of the data and thus result in inaccurate estimations of Kd.

Specific binding of ^{125}I(±)CYP to human platelet membranes (defined in the presence of 200uM(-)isoprenaline) was saturable (figure III.3.) whereas non-specific binding increased linearly with radioligand concentration. Scatchard analysis revealed a homogenous population of binding sites with a Kd of 21.68±1.4(pM)(n=4), and a Bmax of 8.25±0.6 (fmol mg^{-1} protein). Hill analysis of the data showed no significant deviation from unity, indicative of interaction with a single class of receptor sites, with no co-operative interaction.

III.3.3. ^{125}I(-)PIN binding - pharmacological characterisation

The relative potencies of β-adrenoceptor agonists and antagonists in competing for ^{125}I(-)PIN binding were examined in order to characterise the ^{125}I(-)PIN binding sites of the human platelet.

Data presented in the table III.1 and figure III.6 indicate that the binding was stereo selective, with the (-)isomer of the non-selective antagonist propranolol being more potent than the (+)isomer by approximately two orders of magnitude. Both isomers generated displacement curves with steep slope factors indicative of interaction with a single population of binding sites.

The displacement of ^{125}I(-)PIN binding by agonists was characteristic of a β-adrenoceptor, and the hierarchy of potency displayed, (-)isoprenaline > (-)adrenaline > (-)noradrenaline (table III.1. figure III.5.), was suggestive of a β_2-subtype.

Table III.1.

IC_{50} values and pseudo-Hill coefficients (nH) for inhibition of ^{125}I(-)PIN binding to human platelet membranes by β-adrenoceptor agonists and antagonists.

Agonist	IC_{50}(M)	nH
(-)Isoprenaline	2.87×10^{-7}	0.91
(-)Adrenaline	1.61×10^{-6}	0.89
(-)Noradrenaline	2.01×10^{-5}	0.94

Antagonist	IC_{50}(M)	nH
(-)Propranolol	5.65×10^{-10}	1.03
(+)Propranolol	5.63×10^{-8}	1.03
(±)CGP 12177	1.5×10^{-9}	0.85
(±)Practolol	1.91×10^{-5}	0.89
(±)ICI 118,551	3.01×10^{-9}	0.81
(±)Betaxolol	1.0×10^{-7}	0.83
CGP 20172A	3.55×10^{-6}	1.02

The IC_{50} and nH values given are the means of at least three separate experiments performed in duplicate, and were derived from the computer-assisted curve fitting analysis of the mean curves illustrated in figures III.5 and III.6 as described in Appendix 3c.

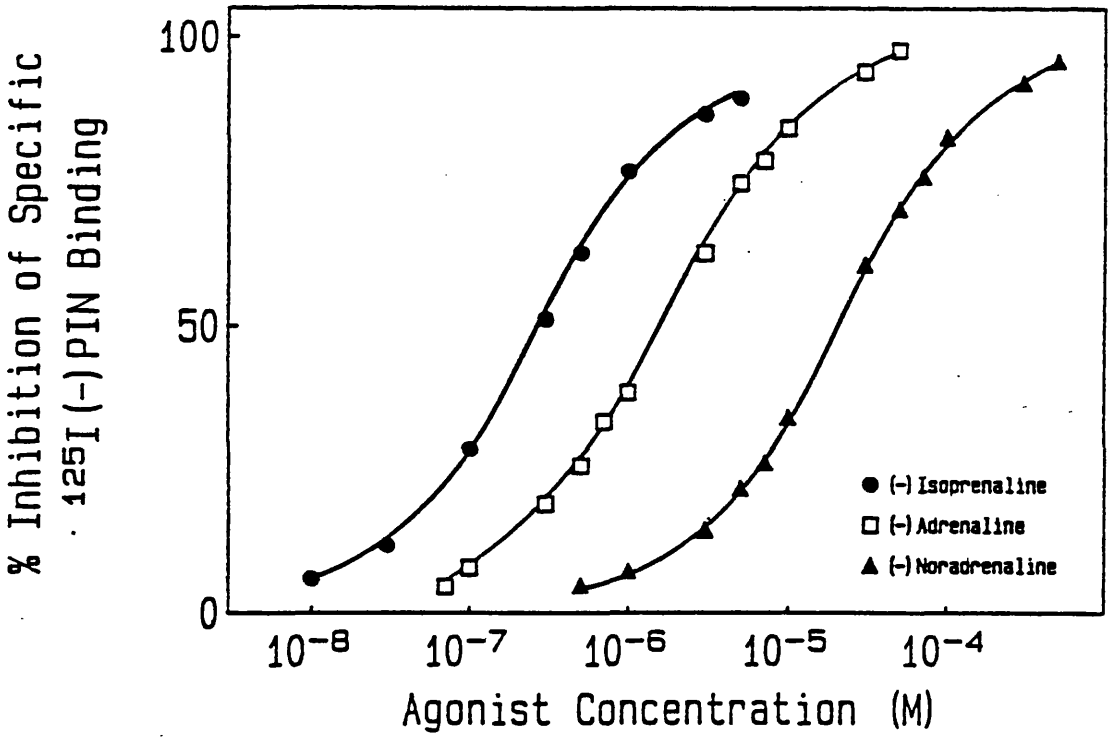


Figure III.5 Inhibition of specific ^{125}I(-)PIN binding to human platelet membranes by adrenergic agonists

Membranes were incubated with 30-40pM ^{125}I(-)PIN at room temperature for 40 minutes, in the presence of increasing concentrations of competing agent, and specific binding determined.

The data shown for each compound are the mean curves from at least three separate experiments performed in duplicate. SEM for each point was $<\pm$5%.

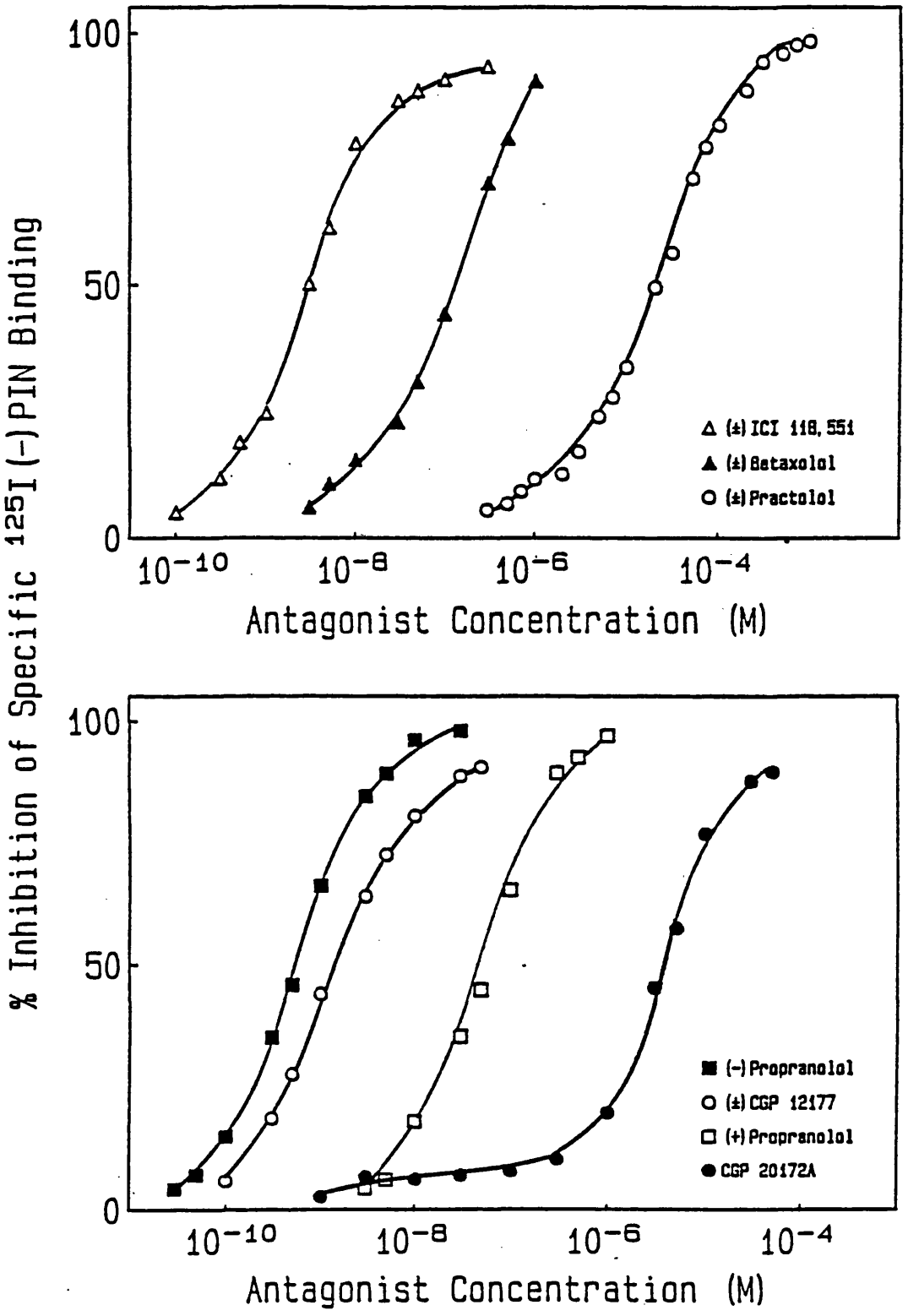


Figure III.6. Inhibition of specific ^{125}I(-)PIN binding to human platelet membranes by adrenergic antagonists

Experiments were performed as described for figure III.5. The data shown for each compound are the mean curves from at least three separate experiments performed in duplicate. SEM for each point was $< \pm 5\%$.

The overall affinities of the non-selective β-adrenoceptor antagonists (-)propranolol, (+)propranolol and CGP 12177 were as expected for interaction with a β-adrenoceptor (Rugg et al., 1978; Hertel et al., 1984). In addition, the highly selective β-adrenoceptor antagonists ICI 118,551 (β_2 selective), and practolol, betaxolol and CGP 20172A (β_1 selective) (Bilski et al., 1980; Dunlop and Shanks, 1968; Boudot et al., 1979; Dooley et al, 1986) exhibited affinities for the binding site similar to those found at the β_2-subtype in other mammalian tissue (Dickinson et al., 1981; Dooley et al., 1986). The selective antagonists practolol, ICI 118,551 and betaxolol however, generated displacement curves with Hill slopes that were slightly lower than unity (Table III.1.) suggesting the possibility of receptor heterogeneity. Computer assisted curve-fitting analysis of these curves however, revealed that the data were best fitted to a one-site model. Thus, these results suggest the presence of a homogenous population of β_2-adrenoceptors. However, the possibility of a small (<10%) component of β_1-adrenoceptor binding sites cannot be entirely excluded.

Serotonin and the α-adrenoceptor antagonist phentolamine, were weak inhibitors of ^{125}I(-)PIN binding with mean IC_{50} values and pseudo-Hill coefficients of 2.25×10^{-5}, 1.05 and 8.5×10^{-5}, 1.05 respectively.

III.3.4. ^{125}I(-)PIN binding - modulation of agonist interactions by ions and guanine nucleotides

Agonist binding to β-adrenoceptors on human platelet membranes and rat

lung membranes was assessed by examining isoprenaline inhibition of ^{125}I(-)PIN binding. The effects of guanine nucleotide, i.e. guanylylimidodiphosphate (Gpp(NH)p) a non hydrolysable analogue of GTP, and sodium ions upon isoprenaline competition curves were determined using standard assay conditions (III.2.1./III.2.2.4.) in assay buffer supplemented with 10mM $MgCl_2$, 0.5mM EDTA.

Results for the human platelet β_2-adrenoceptor are shown in figure III.7 and table III.2. Without added Na^+ or guanine nucleotide, the isoprenaline displacement of ^{125}I(-)PIN yielded a steep competition curve with a slope factor of 0.91. The addition of Gpp(NH)p (100uM) produced no apparent change in the affinity of the agonist or the slope factor of the curve. In the presence of 100mM NaCl, the competition curve was shifted in a parallel fashion to a lower overall affinity with no change in the slope factor. Addition of Gpp(NH)p (100uM) in the presence of 100mM NaCl caused no further change in agonist affinity or slope factor.

In contrast, displacement of ^{125}I(-)PIN by isoprenaline from rat lung β_2-adrenoceptors (figure III.7, table III.2) produced a shallow binding isotherm in the absence of Na^+ and guanine nucleotide. Addition of 100uM Gpp(NH)p resulted in the characteristic shift to the right of the curve with an approximate 5-fold lowering in overall affinity for the agonist and increase in slope factor. Addition of 100mM NaCl produced a parallel rightward shift of the agonist displacement curves observed in the presence or absence of Gpp(NH)p, lowering affinity of the agonist without affecting the slope factor of the curve. The binding

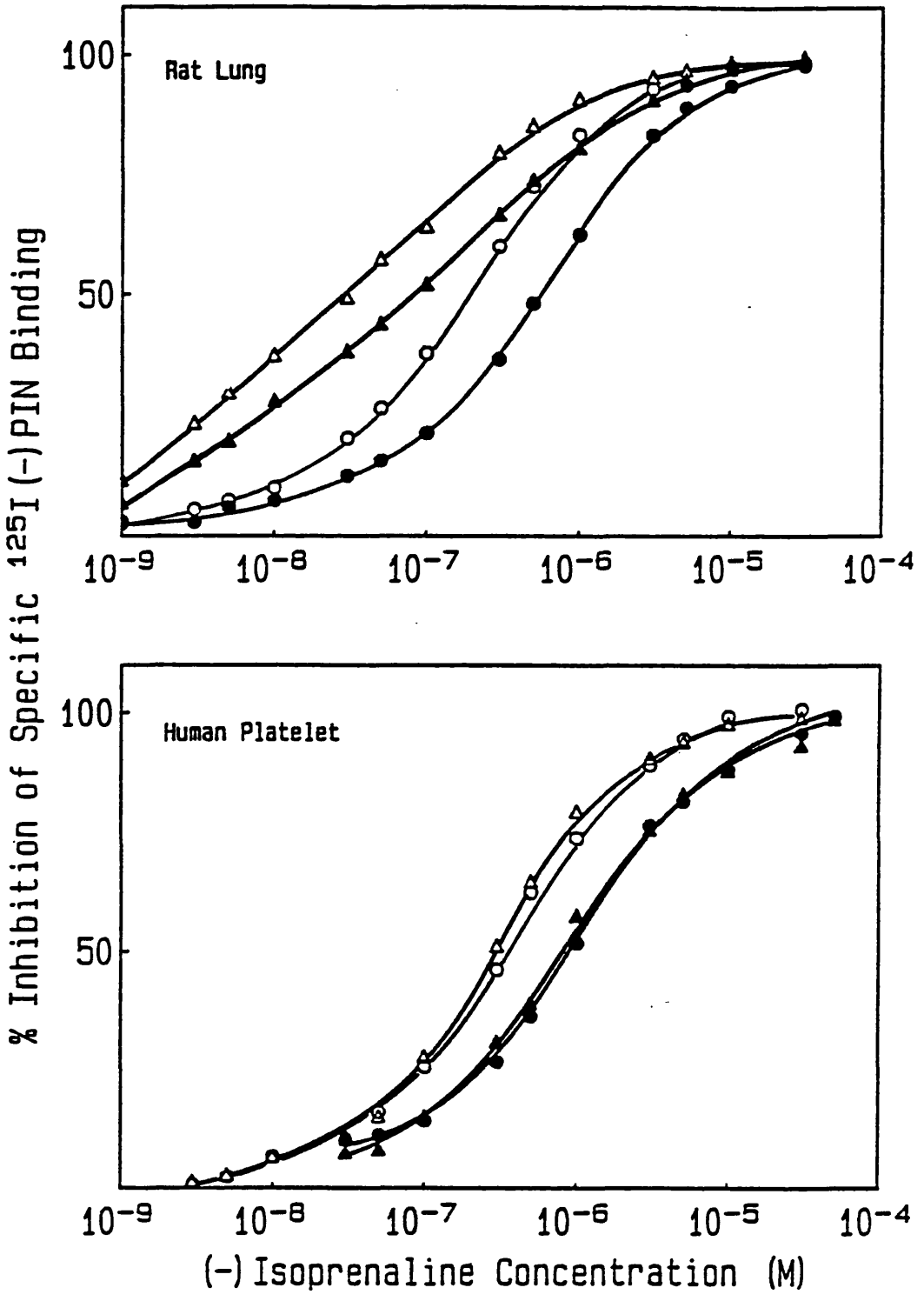


Figure III.7.

Inhibition of specific ^{125}I(-)PIN binding to human platelet and rat lung membranes by isoprenaline in the presence (closed symbols) and absence (open symbols) of 100mM NaCl, without guanine nucleotide (△,▲), with added 100μM Gpp(NH)p (○,●). Data shown are the mean curves of at least three separate experiments performed in duplicate SEM for each point was $<\pm$5%.

Table III.2

Overall IC_{50} values and pseudo-Hill coefficients(nH) for (-)isoprenaline competition curves of ^{125}I(-)PIN binding in human platelets and rat lung membranes in the presence of 9mM $MgCl_2$.

Additions	Human Platelet IC_{50}(nH)	Rat Lung IC_{50}(nH)
No addition	2.87×10^{-7} (0.91)	2.87×10^{-8} (0.56)
+100μM Gpp(NH)p	3.16×10^{-7} (0.919)	1.58×10^{-7} (0.845)
+100mM NaCl	7.79×10^{-7} (0.88)	7.29×10^{-8} (0.566)
+100μM Gpp(NH)p +100mM NaCl	8.84×10^{-7} (0.866)	5.16×10^{-7} (0.857)

The IC_{50} and nH values given are the means of at least three separate experiments performed in duplicate, and were derived from computer assisted curve fitting analysis of the mean curves illustrated in figure III.7. as described in appendix 3c.

of the antagonist ^{125}I(-)PIN was unaltered by Na^+ or Gpp(NH)p (data not shown).

III.3.5. ^{125}I(-)PIN binding - effect of α_2-adrenoceptor blockade on agonist interactions with the human platelet β-adrenoceptor

Adrenaline competition of ^{125}I(-)PIN binding to human platelet membranes was examined in the presence or absence of the α-adrenoceptor antagonist phentolamine (1uM) to block any action of this agonist at platelet α_2-adrenoceptors. The effect of guanine nucleotide (Gpp(NH)p) on these adrenaline competition curves was assessed as previously described (III.2.1.) in assay buffer supplemented with 10mM Mg Cl_2, 0.5mM EDTA.

In the absence of added phentolamine or guanine nucleotide, adrenaline displacement of ^{125}I(-)PIN yielded a steep competition curve with a slope factor of 0.85 (figure III.8, table III.3.). Addition of phentolamine (1uM) produced no change in overall affinity of the agonist or the slope of the curve, whereas addition of Gpp(NH)p (100uM) produced an identical effect in the presence or absence of phentolamine i.e. the overall agonist affinity was marginally lowered, with virtually no change in slope (table III.3). The appearance of the mean agonist curves illustrated in figure III.8 may be interpreted to represent a minor guanine nucleotide effect in the presence or absence of phentolamine. Statistical evaluation of the data however showed no significant difference between the curves obtained in the presence or absence of guanine nucleotide ($p>0.05$).

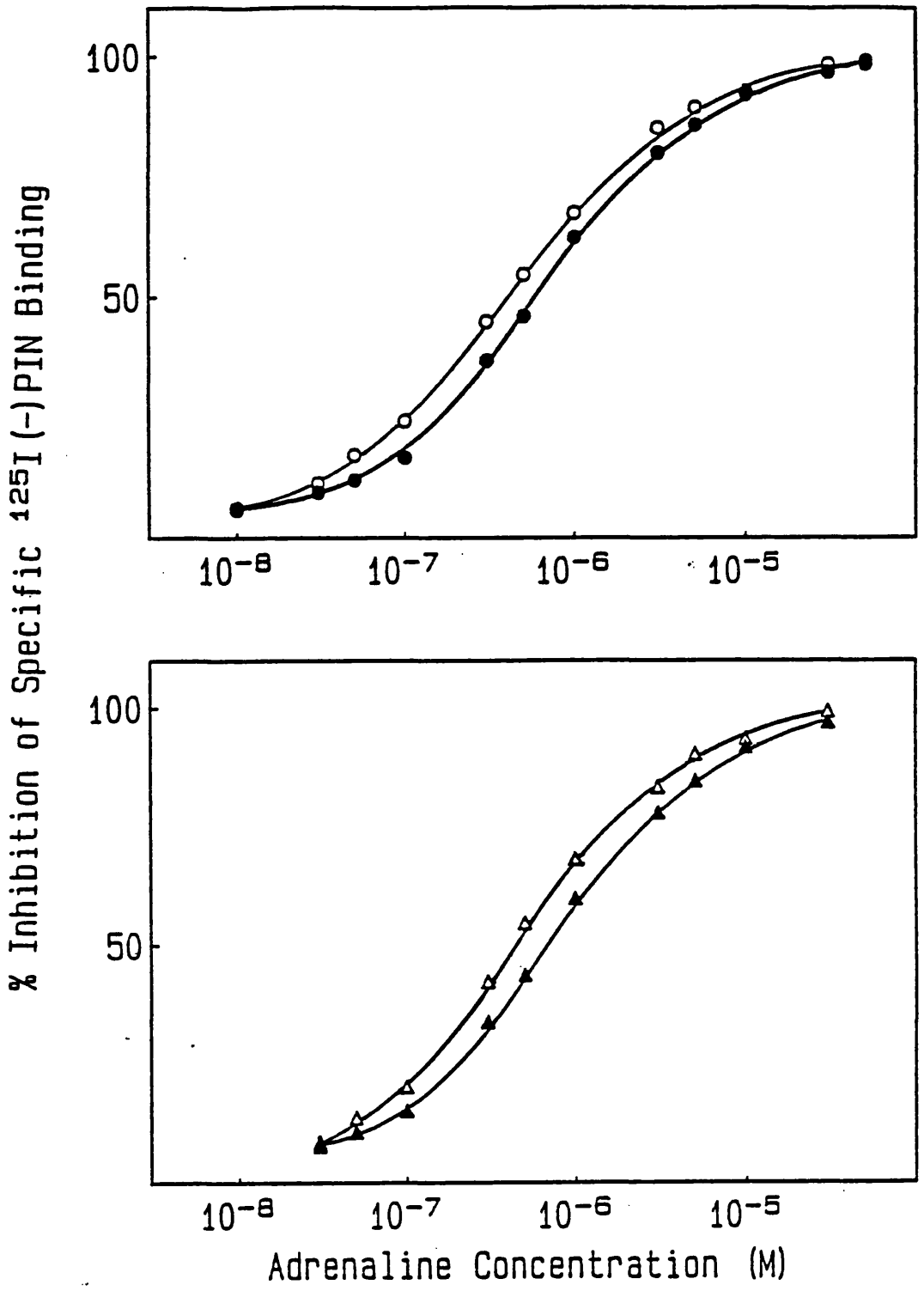


Figure III.8.

Inhibition of specific ^{125}I(-)PIN binding to human platelet membranes by adrenaline in the presence (lower panel) or absence (upper panel) of phentolamine (1μM), without guanine nucleotide (open symbols), with added 100μM Gpp(NH)p (closed symbols). Data shown are the mean curves of at least three separate experiments performed in duplicate. SEM for each point was $\leq \pm$5%.

Table III.3.

Overall IC_{50} values and pseudo-Hill coefficients (nH) for (-)adrenaline competition curves of ^{125}I(-)PIN binding in human platelet membranes in the presence of 9mM $MgCl_2$.

		IC_{50}	nH
No phentolamine	No Gpp(NH)p	3.92×10^{-7}	0.85
No phentolamine	+ Gpp(NH)p (100μM)	5.89×10^{-7}	0.88
+ phentolamine (1μM)	No Gpp(NH)p	3.79×10^{-7}	0.87
+ phentolamine (1μM)	+Gpp(NH)p (100μM)	5.82×10^{-7}	0.88

The IC_{50} and nH values given are the means of at least three separate experiments performed in duplicate, and were derived from computer assisted curve fitting analysis of the mean curves illustrated in figure III.8. as described in Appendix 3a.

III.4. Discussion

The results of the present study confirm the presence of β-adrenoceptors on human platelets. ^{125}I(-)PIN binding sites in human platelet membranes fulfilled all the criteria required for identification of β-adrenoceptors by radioligand binding studies. Thus, binding was stereoselective, saturable, of high affinity, rapid, reversible and displaceable by drugs which interact at β-adrenoceptors. The relative potencies of agonists in displacing ^{125}I(-)PIN binding were indicative of a β_2-adrenoceptor subtype. The use of agonists to delineate receptor subtypes can however be somewhat misleading where multiple agonist affinity states of the receptor are present, unless the agonist experiments are performed in the presence of guanine nucleotide to abolish the heterotrophic binding (Kent et al., 1980). An alternative approach is to employ subtype selective antagonists. Experiments performed with the β_1- and β_2- selective antagonists practolol, betaxolol and ICI 118,551, showed that the affinities of these agents for the platelet β-adrenoceptor correlated well with their reported affinities at β_2-adrenoceptors in other mammalian tissue (Dickinson et al., 1981). Although competition curves for these drugs exhibited slopes (nH) that were lower than unity, 0.81-0.89, curve fitting analysis of the data suggested that human platelet membranes possess a homogenous population of β_2-adrenoceptors. However, such analysis using compounds of only 50-100 fold selectivity cannot discriminate, with certainty, very minor (<10%) populations of second

sites (Dickinson et al., 1981). Competition experiments performed with the highly selective (approximately 10,000 fold) β_1-adrenoceptor antagonist CGP 20172A (figure III.6.) supported the proposed β_2-homogeneity of the human platelet. Although the overall appearance of the curve may suggest the presence of more than one site, the affinity of the minor <10% component observed at the lower concentrations does not correlate with that expected for interaction with β_1-adrenoceptors, whereas the overall affinity of the remaining curve is in excellent agreement with that expected for β_2-adrenoceptors (Dooley et al., 1986).

Comparative experiments exploring ^{125}I(±)CYP binding to human platelet membranes showed that, as mentioned briefly in II.2.1., ^{125}I(±)CYP displayed Kinetics of binding which were considerably slower at 37°C compared to ^{125}I(-)PIN at room temperature. The values obtained for the association and dissociation rate constants were in good agreement with those previously quoted for interaction of this radioligand at β-adrenoceptors in human platelet membranes (Steer and Atlas, 1982) and membranes from other mammalian tissue (Bojanic, 1984), but gave a Kd estimation that was of a slightly higher affinity than the Kd determined by equilibrium analysis. The reason for this discrepancy is not known but possible explanations were previously discussed in the results section (III.3.2.).

The mean Kd value for ^{125}I(±)CYP obtained from equilibrium studies was in excellent agreement with previously reported values (Steer and

Atlas, 1982; Engel et al., 1981). However, when ^{125}I(±)CYP and ^{125}I(-)PIN were employed to estimate the maximum number of binding sites (Bmax) in parallel experiments, ^{125}I(±)CYP was found to consistently identify a greater (approximately 40%) density of binding sites than ^{125}I(-)PIN. The absolute requirement for sodium chloride in the assay buffer (Bojanic, 1984) for ^{125}I(±)CYP binding may contribute towards this difference since Na^+ has been shown to modulate not only agonist binding, but also antagonist binding. For instance, addition of Na^+ to assay incubation buffer has been shown to increase the receptor affinity and apparent density of binding sites for the antagonist radioligand 3H-yohimbine in human platelet membranes (Limbird et al., 1982). In addition, Na^+ has been shown to increase the binding of ^{125}I(±)CYP to β-adrenoceptors by increasing the apparent receptor affinity for this radioligand. This effect was only seen if binding assays were performed at 4°C, and did not alter the Bmax (Minuth and Jakobs, 1986). No change in the Kd or Bmax for ^{125}I(-)PIN binding was observed on addition of Na^+ in the present study (Appendix 8), but a Na^+ effect on ^{125}I(±)CYP binding cannot be ruled out since an accurate assessment of Bmax in the absence of Na^+ is not possible. It is most likely however, that the discrepancy between Bmax values relates to the high non-specific binding component associated with ^{125}I(±)CYP which in this study accounted for 70-75% of total ^{125}I(±)CYP binding (figure III.3.). Thus, in contrast to ^{125}I(-)PIN which gives 70-80% specific binding, ^{125}I(±)CYP yields at best only 30% specific binding, similar to that previously observed in platelet membranes (Steer and Atlas, 1982). These experiments confirmed the original suspicion that ^{125}I(-)PIN was a more suitable radioligand than

^{125}I(±)CYP for use in the present study, and served as a useful basis for evaluating ^{125}I(±)CYP data presented by other workers. No further experiments were performed with ^{125}I(±)CYP.

The above results therefore demonstrate the presence of a relatively low density of β_2-adrenoceptor binding sites on the human platelet that are identical to those found in other mammalian tissue. These results appear to differ from those obtained from earlier radioligand binding studies performed by other workers. For instance, Steer and Atlas (1982) suggested that the platelet β-adrenoceptor possessed characteristics indicative of neither β_1- nor β_2- subtype, which might conceivably be taken to indicate the presence of an "atypical" β-adrenoceptor on human platelets. However, the pharmacological receptor profile produced in their study was misinterpreted by these authors, and their data is in fact entirely consistent with identification of a β_2-adrenoceptor. Hansen et al., 1981 and later Winther et al., 1985, employing ^{3}H-DHA as radioligand, have suggested that both β_1- and β_2-adrenoceptors were present on human platelets. These results must however be interpreted with caution in view of the limited pharmacological characterisation of the receptor in these studies, coupled with the low specific radioactivity of the radioligand employed, and the inability of other workers to demonstrate specific binding of ^{3}H-DHA in human platelet membranes (Kerry and Scrutton, 1983b).

More recent investigations by other workers have provided evidence in support of the conclusions of the present study. Wang and Brodde,

(1985) employing ^{125}I(-)PIN, have identified a similar low density of β_2-adrenoceptors present on human platelet membranes, whilst Kerry et al., (1984) have examined ^{125}I(±)CYP binding to intact human platelets and concluded that the platelet receptor was of the β_2-subtype. It must be noted however, that although the latter authors identified the receptor as being of the β_2-subtype, their reported affinities of certain antagonist drugs deviated markedly from those found at the β_2-adrenoceptor of human platelet membranes in the present study and by Wang and Brodde (1985), and at other mammalian β_2-adrenoceptors (Rugg et al., 1978; Minneman et al., 1979; Dickinson et al., 1981). The possibility that these differences relate to the use of intact platelets was further evaluated in the present study (Chapter VI).

Experiments to evaluate the effects of Na^+ and guanine nucleotides were performed to examine the functional coupling of the human platelet B_2-adrenoceptor, as previously described (III.1). Isoprenaline competition curves in human platelet and rat lung membranes were performed in parallel. To ensure that differential retention of Mg^{++} by the two membrane preparations did not influence the results, EDTA treated membranes were prepared in an identical manner from both tissues. Thus, rat lung membranes (II.2.2.2.) once initially pelleted, were resuspended and washed as described for platelet membranes (II.3.2.1.). Competition experiments were subsequently performed in assay buffer supplemented with Mg^{++} and EDTA. The use of 9mM free Mg^{++} was chosen since the Mg^{++} requirement for expression of high-affinity agonist binding has been shown to be half maximal at 2-3mM Mg^{++} in S49 lymphoma membranes (Bird and Maguire, 1978). Addition of Mg^{++} in the

presence of EDTA to assay buffer was employed since it has previously been shown to allow a guanine nucleotide effect to be observed in what otherwise appeared to be guanine nucleotide insensitive membranes, (Dickinson and Nahorski, 1983). The reason for this Mg^{++}/EDTA effect is unclear at present : possible explanations have included the proposal that Mg^{++} may facilitate GDP/GTP exchange (Cassel and Selinger, 1978) or that EDTA may remove inhibitory GDP from the G protein or clear inactive divalent cations from the system (Shane et al., 1981).

In a manner similar to that described for Na^{+} above, a modulatory action of guanine nucleotides on antagonist binding has also been reported. The apparent affinity of iodinated antagonist radioligands, including ^{125}I(-)PIN to β_2-adrenoceptors on L6 muscle cell and rat lung membranes, was increased in the presence of guanine nucleotide with no change in Bmax (Wolfe and Harden, 1981). The guanine nucleotide employed in the present study was guanylylimidodiphosphate (Gpp(NH)p), a nonhydrolysable analogue of GTP that has been shown to be more potent than GTP in decreasing agonist binding affinities at β-adrenoceptors (Dickinson and Nahorski, 1983). Since a modulatory action of guanine nucleotide on both agonist and radiolabelled antagonist binding would complicate the interpretation of data, ^{125}I(-)PIN binding to human platelet and rat lung membranes was evaluated in the presence and absence of Gpp(NH)p. In contrast to the results of Wolfe and Harden (1981), ^{125}I(-)PIN binding was unaltered by Gpp(NH)p (data not shown).

Agonist interaction experiments were thus conducted under assay

conditions designed to produce a maximal guanine nucleotide effect. Under these conditions, experiments with rat lung membranes exhibited the characteristically low slope agonist displacement curves in the presence of magnesium, and rightward shift and steepening after addition of Gpp(NH)p. These observations are in excellent agreement with those previously described for this membrane preparation (Dickinson and Nahorski, 1983).

In contrast to the results with rat lung membranes, isoprenaline competition curves in human platelet membranes exhibited a steep slope factor, and were not influenced by the addition of Gpp(NH)p. Furthermore, agonist binding to human platelet β-adrenoceptors was not influenced by Mg^{++} alone. The isoprenaline curve previously obtained in the absence of Mg^{++} (figure III.5.) was superimposable on that observed here in the absence of guanine nucleotide and Na^{+}. In addition, the overall affinity of isoprenaline for the platelet β_2-adrenoceptor (in the presence or absence of Gpp(NH)p) was very similar to that found with rat lung in the presence of guanine nucleotide, and thus presumably represents the low agonist affinity state. This inability of Mg^{++} and Gpp(NH)p to alter receptor affinity for agonists in human platelet membranes, is similar to that previously described for the cyc^{-} and UNC variants of S49 lymphoma cells in which the receptors cannot interact with a functional G-protein (Bird and Maguire, 1978; Cech and Maguire, 1982). If one assumes that the extent of the guanine nucleotide effect reflects the degree of coupling between the receptor and catalytic unit of adenylate cyclase, the above results suggest that the platelet β_2-adrenoceptor is not coupled to

adenylate cyclase, whereas the rat lung β-adrenoceptor is strongly-coupled, as has been previously demonstrated (Dickinson and Nahorski, 1983).

Sodium, unlike Mg^{++} and guanine nucleotide, produced a parallel shift of the agonist competition curves to a lower overall affinity, to a similar degree in both platelet and rat lung membranes in the presence and absence of Gpp(NH)p. A similar effect of Na^+ in lowering the affinity of agonist binding to β-adrenoceptors has previously been shown in rat lung and calf cerebellar membranes (Heidenreich et al., 1980; U'Prichard et al., 1978) and more recently in guinea pig lung membranes (Minuth and Jakobs, 1986). Furthermore, the latter authors have also shown that this Na^+ effect is identical in wild type S49 lymphoma cell membranes (possessing intact Gs and Gi proteins) and cyc^- S49 lymphoma cell membranes (which lack Gs_α subunit).

The data for cyc^- membranes (Minuth and Jakobs, 1986) and human platelet membranes in the presnt study for Na^+ regulation of β-adrenoceptors, is very similar to that reported previously for platelet α_2-adrenoceptors. Treatment of platelet membranes with agents that completely eliminate guanine nucleotide effects, did not affect the Na^+ regulation of agonist binding to these α_2-adrenoceptors (Limbird et al., 1982; Limbird and Speck, 1983). In addition, treatment of cells with pertussis toxin, which inactivates Gi by ADP-ribosylating its guanine nucleotide-binding α subunit (Ui et al., 1984) was found to eliminate guanine nucleotide regulation of opioid receptor binding without reducing the Na^+ effect on these receptors

(Wuster et al., 1984). The data for Na^+ regulation of β-adrenoceptors on cyc^- membranes (Minuth and Jakobs, 1986) and human platelet membranes in the present study, lend further support to the hypothesis that the target of Na^+ ions is apparently different from that of Mg^{++} and guanine nucleotide.

The results of the present study differ slightly from the recent study of Wang and Brodde (1985), who have also examined the effect of guanine nucleotide on agonist binding to human platelet membranes. Competition curves for isoprenaline, adrenaline and noradrenaline in the absence of guanine nucleotide were steep, with slope factors of 0.85-0.88, similar to those found in the present study. Addition of guanine nucleotide however, apparently produced a minor (<1.8 fold) decrease in overall agonist affinities, and steepening of the curves to 0.95-0.96. These data were interpreted to represent the presence of weakly coupled β_2-adrenoceptors on human platelets.

Experiments were performed to investigate whether or not agonist activation of the inhibitory α_2-adrenoceptors on the human platelet influences the coupling of the β_2-adrenoceptors on these cells. Adrenaline (which has a similar affinity for both α_2- and β_2-adrenoceptors) competition curves were performed in the presence and absence of 1uM phentolamine, with and without guanine nucleotide. This phentolamine concentration has previously been shown to occupy all α_2-adrenoceptors in human platelet membranes (Swart, 1984), and was found not to interfere with ^{125}I(-)PIN binding in the present study. The results show that although a similar decrease in agonist affinity

with addition of Gpp(NH)p to that reported by Wang and Brodde (1985) was observed, the degree of change was not statistically significant in the present study. Also, addition of phentolamine did not influence adrenaline binding : thus, no apparent effect of this agonist acting via α_2-adrenoceptors to modulate β_2-adrenoceptor coupling was observed. It should be noted however that the affinity of adrenaline observed in these experiments in the presence of Mg^{++} (figure III.8.) was significantly higher than in the absence of Mg^{++} (figure III.5.). The reason for this is not known since no change in slope occurred, and calculation of the dissociation constant (K_I) for adrenaline by means of the Cheng and Prusoff (1978) equation :

$$K_I = \frac{IC_{50}}{I+\frac{[L]}{Kd}}$$

does not correct this discrepancy. This equation allows for the contribution made by the use of varying radioligand concentrations for competition experiments on the overall IC_{50} value. However, since $^{125}I(-)PIN$ concentrations [L] employed for competition experiments throughout the present study were well within the narrow range of 30-40pM, substitution of these values with a Kd=24.27pM into the above equation would give a division factor of 2.23 and 2.65 for the two extremes of $^{125}I(-)PIN$ concentrations respectively.

In summary, the binding of $^{125}I(-)PIN$ to human platelet membranes was examined, and identifies a low density, homogenous population of β_2-adrenoceptors on these cells. The use of modulation of agonist

binding by ions and guanine nucleotide as a means of assessing the functional coupling of these receptors to adenylate cyclase, failed to demonstrate any interaction of receptors and coupling protein : thus, in terms of the ternary complex model, these receptors would appear non-functional. It should be noted however, that although the ternary complex model is applicable in many experimental situations, there are exceptions. For example, Severne et al., (1986) found that agonist competition binding curves to bovine trapezius muscle membranes exhibited properties that could not be accounted for by the high/low agonist affinity state model, and introduced a new 'tight agonist binding' model to explain their data. Also, the ternary complex model assumes antagonist affinities are not modulated by factors such as guanine nucleotides or ions, but as mentioned above there are instances in which antagonist affinities have been shown to be influenced by both guanine nucleotides (Wolfe and Harden, 1981; Lang and Lemmer, 1985) and ions (Limbird et al., 1982; Minuth and Jakobs, 1986). Thus, modification of the ternary complex model to include antagonist bound receptor interactions with G proteins may be appropriate. Furthermore, it has been shown that when an alkylating β-antagonist was employed to remove a large receptor reserve for agonists in the vas deferens, the affinity of the remaining receptors, for agonists eliciting a functional response, correlated with the low affinity state of the receptor. This suggests that the high affinity form may not be a functionally relevant state of the receptor (May et al., 1985).

Taken together, the data presented here, with that of Wang and Brodde (1985) suggest that the β_2-adrenoceptor on the human platelet is poorly

coupled (if at all) to adenylate cyclase. It is possible however, that these receptors are very labile and become uncoupled during membrane preparations; or that a lack of guanine nucleotide effect may be due to the presence of nucleotides retained in these membranes during washing; or that the technique employed was not sufficiently sensitive to detect interaction of these receptors with the coupling protein. The functional coupling of this receptor was further evaluated in this study by investigating β-agonist stimulated cyclic AMP accumulation in intact platelets (Chapter IV).

CHAPTER IV

Cyclic AMP accumulation in intact human platelets

IV.1. Introduction

The human platelet adenylate cyclase system is responsive to a wide variety of biological agents. Enzyme activation can occur via the interaction of compounds such as PGI_2, PGE_1, PGD_2 or adenosine with specific membrane receptors coupled to the stimulatory guanine nucleotide binding protein Gs, or by agents such as forskolin, or stable GTP analogues, which cause activation by an apparent interaction with Gs or the catalytic subunit. Likewise, enzyme inhibition can occur via the interaction of agents such as adrenaline (α_2-adrenoceptors), ADP, vasopressin and platelet activating factor with their membrane receptors coupled to the inhibitory guanine nucleotide binding protein Gi, or by agents that act on Gi or the catalytic moiety, e.g. stable GTP analogues and adenosine (P-site) respectively (see Aktories and Jakobs, 1985).

The role of cyclic AMP in controlling platelet function is however, not well defined. There is considerable evidence to suggest that elevation of cyclic AMP is important in mediating inhibition of a number of platelet functions such as shape change, aggregation and secretion. The precise mode of action is not known but may involve several mechanisms such as : inhibition of agonist-induced phosphoinositide hydrolysis (Lapetina, 1984); inhibition of cytoplasmic free Ca^{++} elevation (Feinstein et al., 1983) by increased Ca^{++} sequestration or extrusion (Feinstein et al., 1983; Kaser-Glanzmann et al., 1977); interference with the coupling of receptors (thrombin) to a GTP-binding protein in the platelet plasma membrane (Halenda et al., 1986) and

inhibition of platelet myosin light chain Kinase activity (a Ca^{++} activated enzyme) by cyclic AMP-dependant protein Kinase (Hathaway et al., 1981). The importance of adenylate cyclase inhibition in the control of platelet function is less clear. Salzman (1972) proposed that platelet activation may result from reduced cyclic AMP levels. Haslam et al (1978) however, have since shown that for most agents, a reduction of platelet cyclic AMP is neither necessary nor sufficient to induce platelet activation.

A possible anti-aggregatory role for β-adrenoceptors on human platelets has been suggested. Functional studies have demonstrated isoprenaline induced disaggregation of ADP induced platelet aggregates (Abdulla, 1969), and isoprenaline inhibition of the aggregatory response to various stimulatory agonists which apparently is prevented by addition of propranolol (Mills and Smith, 1972; Kerry and Scrutton, 1983a). These effects were reportedly associated with an increase in cyclic AMP formation since the aggregatory inhibitory response to isoprenaline was potentiated by the presence of cyclic AMP phosphodiesterase inhibitors (Mills and Smith, 1979), and abolished by addition of 2', 5'-dideoxyadenosine, an adenylate cyclase inhibitor (Kerry and Scrutton, 1983a). In addition, isoprenaline and adrenaline have been shown to increase intraplatelet cyclic AMP levels (Haslam and Taylor, 1979; Mills and Smith, 1971; Kerry and Scrutton, 1983a) and activate platelet adenylate cyclase (Abdulla, 1969; Jakobs et al., 1976; 1978).

However, the results of the present study (Chapter III) show that the β_2-adrenoceptors present on human platelets do not appear to be coupled

to adenylate cyclase. The aim of the study described in this chapter was to further investigate the coupling of this receptor to adenylate cyclase by examining isoprenaline stimulation of cyclic AMP accumulation in intact human platelets. In view of the fact that the isoprenaline stimulated changes in cyclic AMP levels reported are very small, approximately 1.4-1.6 fold maximum (Abdulla, 1969; Mills and Smith, 1971; Jakobs et al., 1978; Kerry and Scrutton, 1983a), an attempt was made to potentiate this response by the use of forskolin. Forskolin potentiation of hormone responses has been extensively studied in a number of cells and previously employed in several studies to successfully unmask or detect hormonal responses that were otherwise difficult to observe (Seamon, 1985). Experiments were performed in the presence of a phosphodiesterase inhibitor, to facilitate detection of any changes in cyclic AMP levels during the course of this study, and in the presence and absence of phentolamine to evaluate possible α_2- and β_2-adrenoceptor interaction. As will be seen from the results, no isoprenaline stimulation of cyclic AMP accumulation could be detected in these cells. The study was thus extended to explore the effect that minimal white blood cell contamination would have on these results.

IV.2. Methods

IV.2.1. Cyclic AMP accumulation

Platelets or mononuclear leukocytes (MNL) were prepared as described in II.3.2.2. and II.3.2.3. respectively, with the exception that final

resuspension for both cell preparations was in cyclic AMP accumulation assay buffer, i.e. an aerated Krebs buffer containing 0.2mM Na metabisulphite and 100uM RO 20-1724 (a phosphodiesterase inhibitor) (Appendix 5c). Incubations were performed at 37°C in a final volume of 500ul. Platelets or MNL were incubated for 10 minutes prior to the addition of phentolamine (1 or 10uM as indicated) and maintained at 37°C for a further 5 minutes before either (-)isoprenaline (100uM), PGE_1 (with or without (-)adrenaline) or forskolin (indicated concentrations) were added. Incubations were continued for 20 minutes (or 45 minutes in experiments involving forskolin) unless otherwise stated, e.g. time course experiments, and rapidly terminated by boiling. Cell suspensions were pelleted by centrifugation and aliquots of supernatant assayed for cyclic AMP using a competitive protein binding assay (Brown et al., 1971; see Appendix 7b) with an assayable range of 0.125-15 pmol cyclic AMP.

IV.2.2. Statistical evaluation of data

Experiments were performed at least three times in quadruplicate (except figure IV.4.) and the results reported as mean ± SEM. Statistical significance was established by Student's 't' test for comparison of paired means : a p value of <0.05 was considered significant. Statistical evaluation of experimental data presented in figure IV.4. was performed by means of covariance analysis, cell number being fitted as the independent variable, and cyclic AMP concentration (or ln [cyclic AMP] for MNL) as the dependent variable. Values plotted represent the mean of quadruplicate data points.

IV.3. Results

As previously mentioned (IV.1.) the platelet adenylate cyclase system is responsive to both stimulatory and inhibitory agonists. A brief study to evaluate whether or not the adenylate cyclase system of the purified platelet suspension employed in this study exhibited the expected responsiveness was performed. Prostaglandin E_1(PGE_1) stimulation, and adrenaline inhibition of PGE_1 stimulated cyclic AMP accumulation were examined : both responses are well documented in the literature for human platelets (see IV.4.).

The time course of cyclic AMP accumulation in response to 1uM PGE_1, illustrated in figure IV.1., showed that PGE_1 stimulation was apparent within the first 2 minutes of incubation and increased steadily with time for the first 15 minutes, following which a levelling off of cyclic AMP accumulation was observed. An incubation period of 20 minutes was thus adopted to give an easily measurable, reproducible PGE_1 response above basal. Basal levels of cyclic AMP were easily detectable, and stable, showing if anything a slight linear accumulation of cyclic AMP during this time, as might be expected in the presence of a phosphodiesterase inhibitor (IV.1.). The dose-response curve for PGE_1, illustrated in figure IV.2., showed that PGE_1 induced a dose-dependant increase in cyclic AMP accumulation. Half-maximal stimulation was observed at $3x10^{-7}$M PGE_1, with near-maximal stimulation attained at 10^{-5}M PGE_1. Adrenaline induced inhibition of PGE_1 stimulated cyclic AMP accumulation was subsequently examined. The

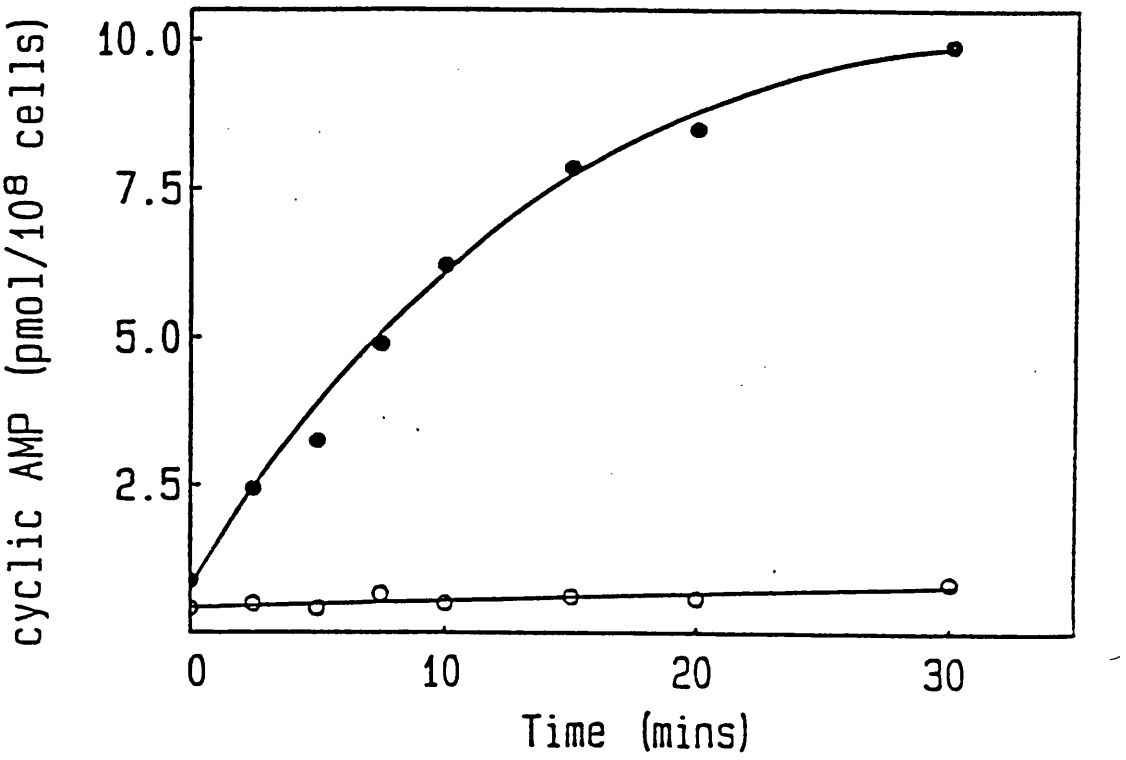


Figure IV.1. Time course of PGE_1 stimulated cyclic AMP accumulation in intact human platelets at 37°C

Platelets were incubated as described in methods section in the presence (•) or absence (o) i.e. basal, of 1uM PGE_1. Data are quoted as pmol cyclic AMP/10^8 cells and are representative of two experiments performed in triplicate.

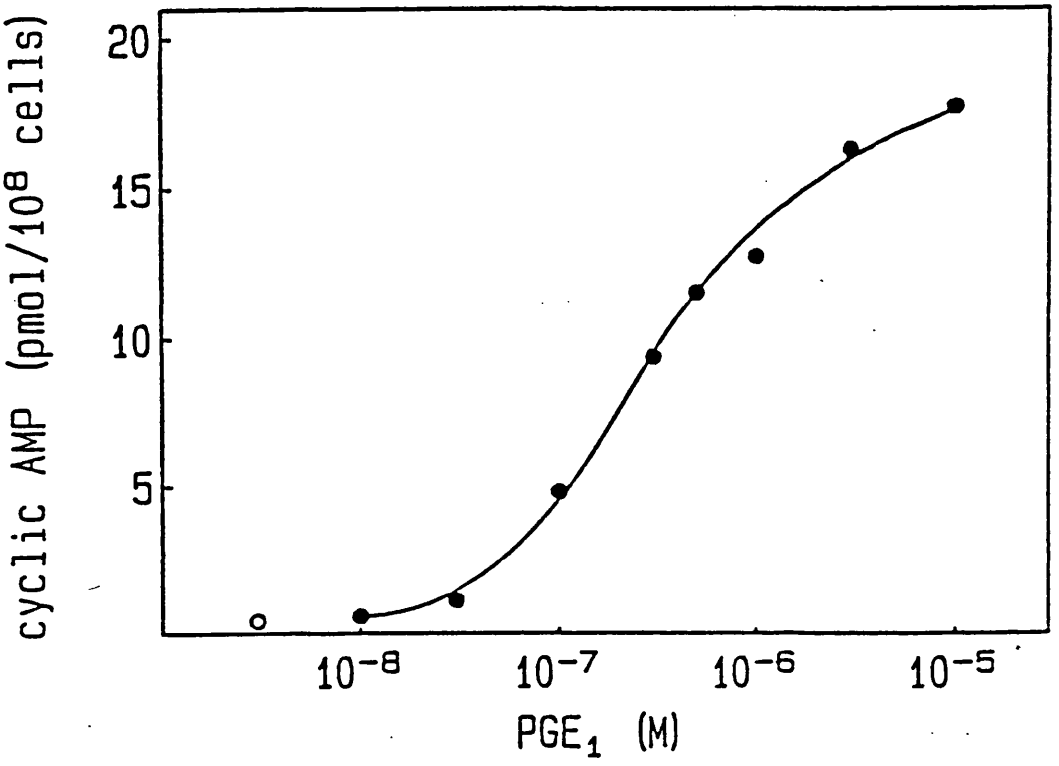


Figure IV.2. Dose response of PGE_1 stimulated cyclic AMP accumulation in intact human platelets

Platelets were incubated for 20 minutes at 37°C in the presence (•) or absence (o) of PGE_1 (indicated concentrations). Data shown are representative of two experiments performed in triplicate.

inhibitory effect of adrenaline was apparent at around $3x10^{-8}M$, and half-maximal at approximately $10^{-6}M$ (figure IV.3.). Maximal inhibition of PGE_1 stimulation was between 50-60% and seen with $10^{-5}M$ adrenaline : higher concentrations did not cause further inhibition. These results are in excellent agreement with those previously published by other workers (see IV.4.). Having established this, β-agonist stimulated cyclic AMP accumulation in intact human platelets was investigated.

Basal levels of cyclic AMP in intact human platelets were linearly related to platelet number (figure IV.4a). Additions of a supramaximal stimulatory concentration of isoprenaline (100uM), produced no change in cyclic AMP levels above basal even in the presence of 1 or 10uM phentolamine to block alpha adrenergic effects of this high concentration of isoprenaline (data not shown for clarity). Covariance analysis showed no significant difference between basal and isoprenaline stimulated whole platelet cyclic AMP levels (with or without phentolamine) across the range of platelet concentrations used (10^8-10^9 platelets). In contrast, additions of 0.5uM PGE_1 to these cells produced a >10 fold increase in cyclic AMP above basal (data not shown) as previously described (figure IV.2.). A time-course of isoprenaline stimulated cyclic AMP accumulation similar to that for PGE_1 (figure IV.1.) was performed, but no change in cyclic AMP levels above basal were observed (data not shown).

Experiments were performed to explore forskolin potentiation of isoprenaline induced cyclic AMP accumulation in intact human platelets. Although the primary site of action of forskolin appears to be the

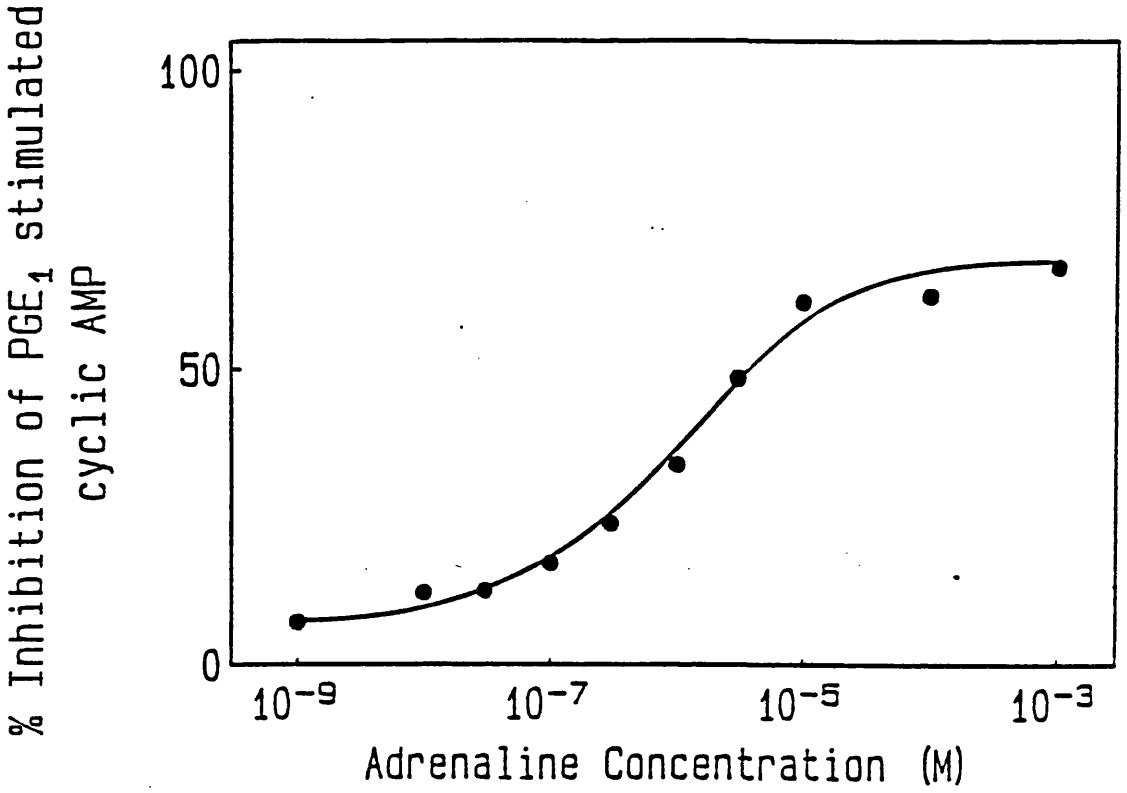


Figure IV.3. Adrenaline-induced inhibition of PGE_1 stimulated cyclic AMP accumulation in intact human platelets

Platelets were incubated for 20 minutes at 37°C in the presence of 5×10^{-7}M PGE_1 and adrenaline (indicated concentrations). Data shown are representative of two experiments performed in triplicate.

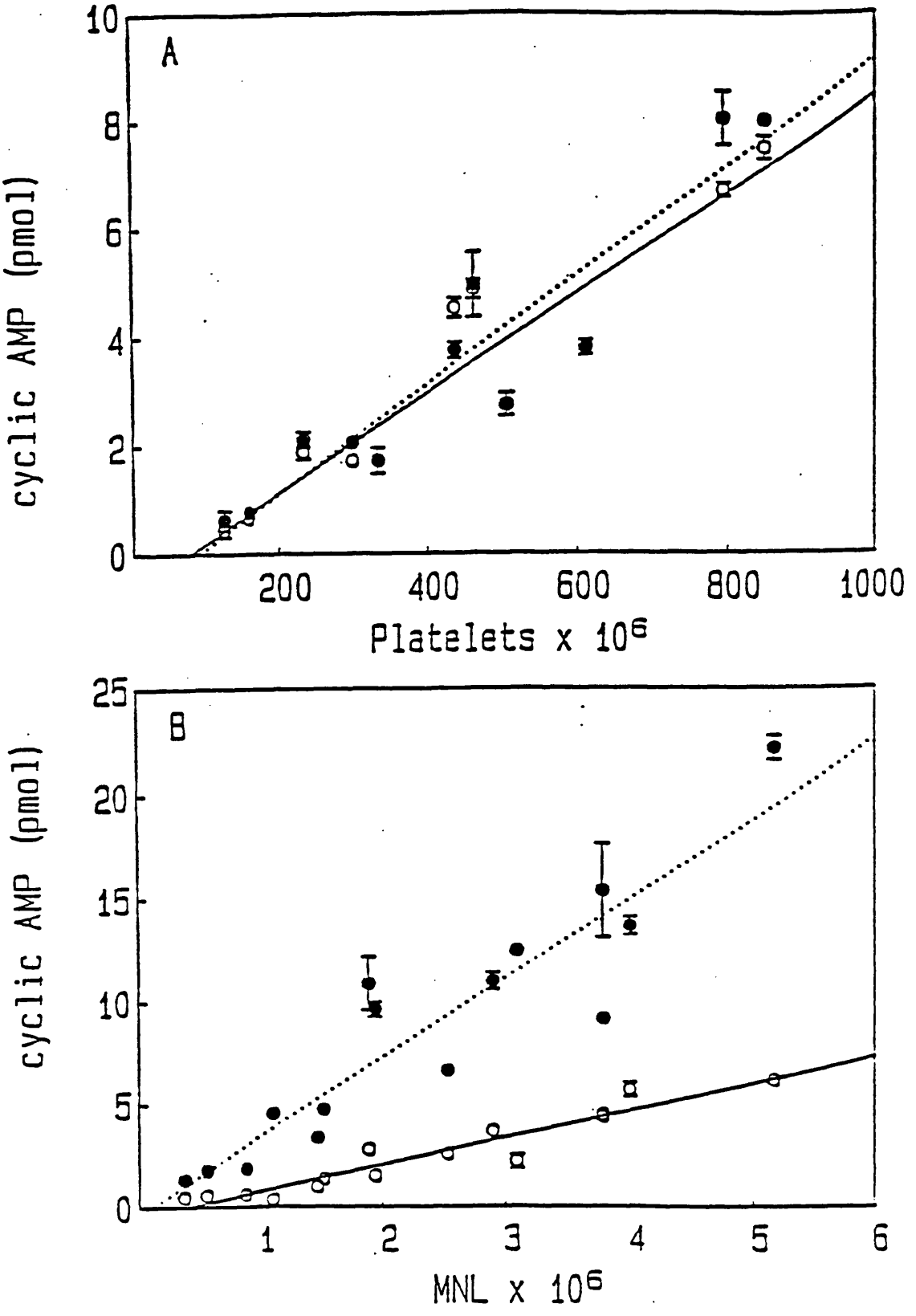


Figure IV.4. Basal (open symbols) and isoprenaline stimulated (solid symbols) cyclic AMP levels in intact human platelets (A) and MNL(B)

Cells were incubated at 37°C for 20 minutes with (-)isoprenaline (100μM) in the presence (data not shown) or absence of 1μM phentolamine. Data presented as pmol cyclic AMP/number of cells represent the mean of quadruplicate determinations.

catalytic unit of the adenylate cyclase system or closely related membrane component, forskolin can also apparently promote the interaction of hormone on guanine nucleotide-activated Gs with the catalytic moiety, which results in the potentiation of hormonal stimulation of cyclic AMP formation by forskolin. It has been suggested that two ^{3}H-forskolin affinity binding sites are observed in radioligand binding studies. The low affinity site, with Kd in the μ molar range, is proposed to correspond to the site responsible for the direct stimulation of adenylate cyclase, with an EC_{50} in the range 1-10uM. The high affinity site with a Kd in the nanomolar range is thought to be involved in the synergistic action of forskolin with hormone, an effect which apparently has an EC_{50} of 10-100nM (Seamon, 1985). Evidence to support this concept in platelets has recently come from a study in which agents that activate adenylate cyclase via Gs have been shown to increase the number of high affinity ^{3}H-forskolin labelled sites (Nelson and Seamon, 1986). The concentration of forskolin chosen to potentiate agonist-induced cyclic AMP accumulation in this study is therefore critical.

Forskolin-stimulated cyclic AMP accumulation appeared to reach a maximal steady state level at around 40-60 minutes (figure IV.5.). An incubation time of 45 minutes was thus adopted for forskolin potentiation experiments. The dose-response curve for forskolin stimulated cyclic AMP accumulation (figure IV.6.) appeared monophasic, with an approximate half-maximal and near maximal response at 10 and 100uM respectively, as has been reported by others (Insel et al., 1982; Siegl et al., 1982). Since an EC_{50} of 10-100nM is reported for forskolin

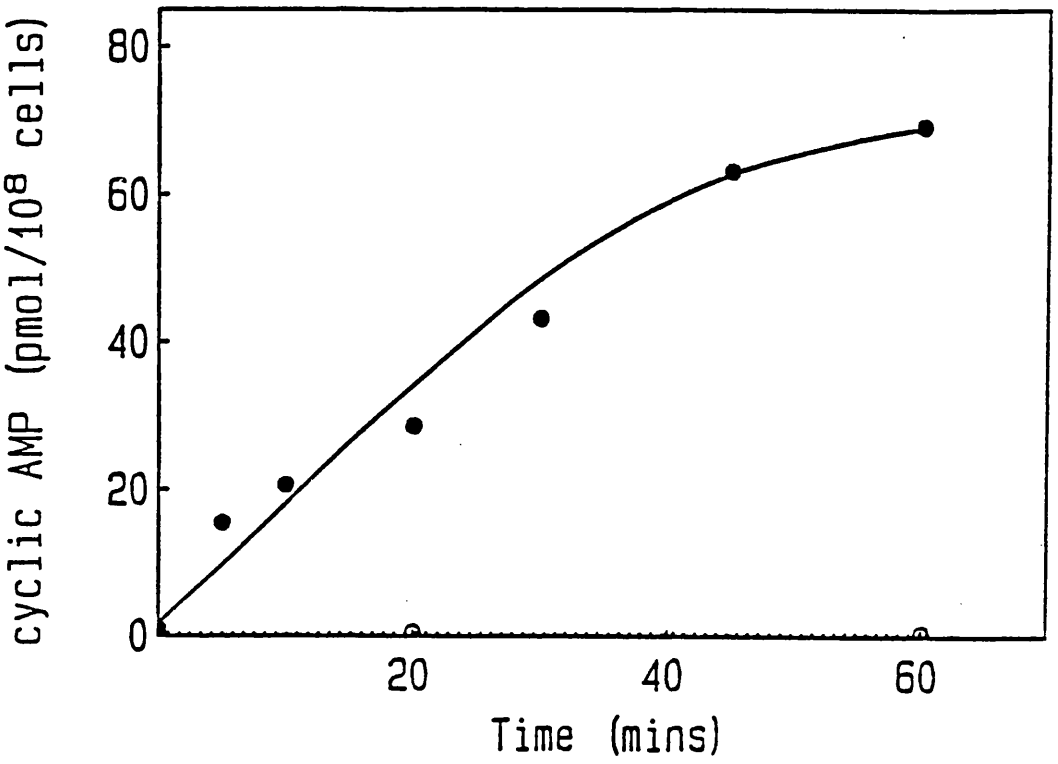


Figure IV.5. Time course of forskolin stimulated cyclic AMP accumulation in intact human platelets at 37°C

Platelets were incubated as described in the methods section in the presence (•) or absence (o) i.e. basal, of 10μM forskolin. Data are quoted as pmol cyclic AMP/10^8cells and are representative of two experiments performed in triplicate.

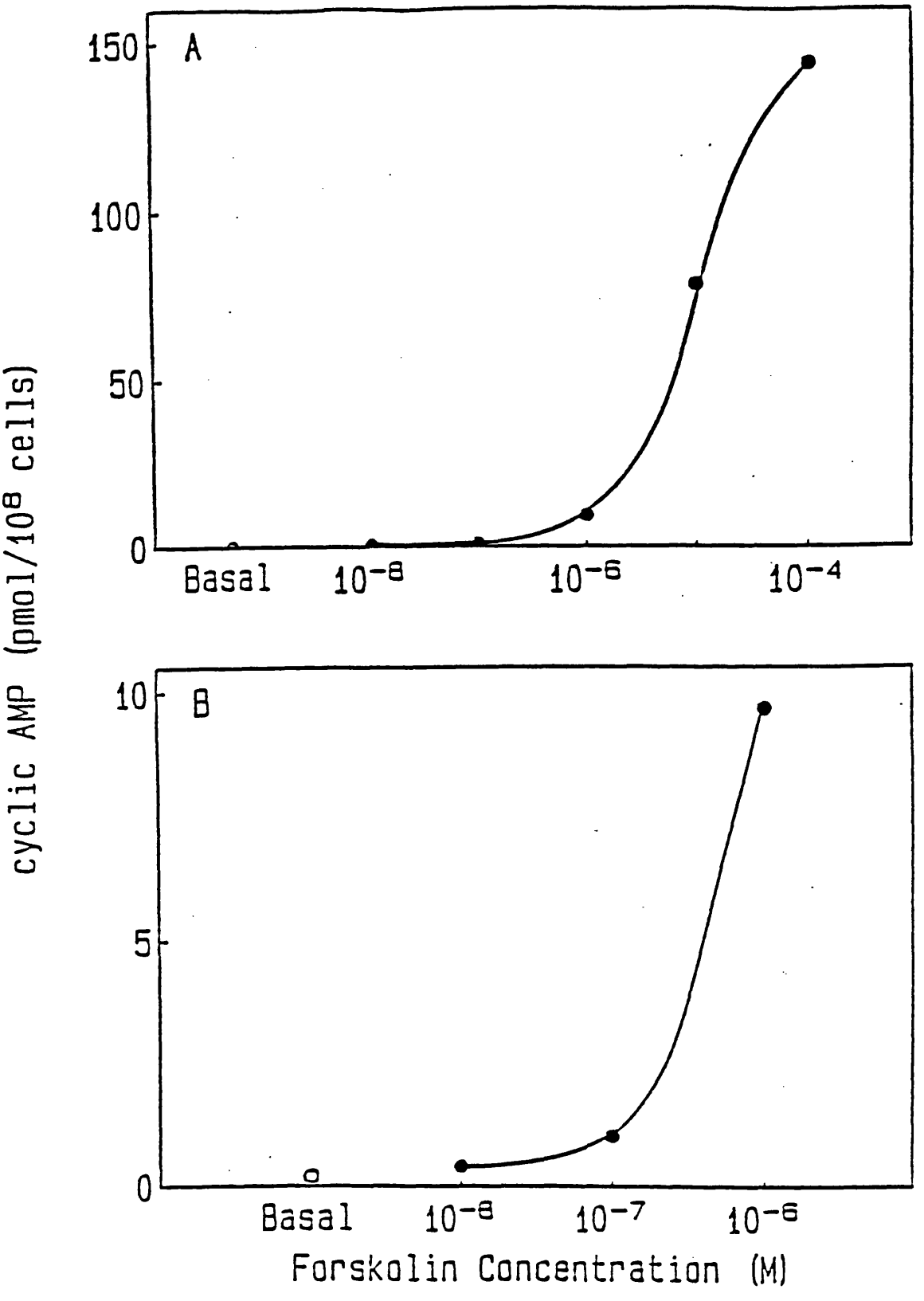


Figure IV.6. Dose response of forskolin stimulated cyclic AMP accumulation in intact human platelets

Platelets were incubated for 45 minutes at 37°C in the presence (•) or absence (o) of forskolin (A=10^{-8}-10^{-4}M; B=10^{-8}-10^{-6}M expanded scale). Data shown are representative of two experiments performed in triplicate.

potentiation of hormone-stimulated cyclic AMP accumulation, both 10nM and 100nM forskolin were employed in this study. Forskolin (100nM) in the absence of any other stimulatory agent produced a minimal but significant increase in cyclic AMP above basal. When this low concentration of forskolin was added in the presence of 100uM isoprenaline, no further elevation of cyclic AMP occurred beyond that measured with forskolin alone (table IV.1., figure IV.7.). A threshold concentration of 30nM PGE_1 (figure IV.2.) which elicited a small increase in intracellular cyclic AMP (<3-fold) however, produced characteristic potentiation of the response in the presence of 100nM forskolin, with a >15-fold increase in cyclic AMP (table IV.1., figure IV.7.) similar to that observed in the presence of a higher (approximately half-maximal) concentration of PGE_1 (0.5uM) in the absence of forskolin (table IV.1.). 10nM forskolin however, produced no significant change in cyclic AMP levels above basal when added alone, or in combination with isoprenaline (table IV.1.).

The possibility that small increases in platelet β-adrenoceptor stimulated cyclase activity in previous studies could be explained by minimal contamination from mononuclear leukocytes (MNL) was investigated. Various concentrations of MNL (figure IV.4b.) were incubated with isoprenaline (100uM) in the presence or absence of phentolamine (1uM) as described previously for platelets. Covariance analysis of the data showed that across the range of MNL concentrations used, the slope of the best fit line for isoprenaline stimulated cyclic AMP was significantly different to the basal level ($p=<0.05$). This effect of isoprenaline was not influenced by addition of phentolamine

Table IV.1. Agonist-induced cyclic AMP accumulation in intact human platelets

Agonist	Cyclic AMP (pmol/10^8 platelets)
None	0.38 ± 0.07
Isoprenaline (100μM)	0.40 ± 0.07
Forskolin (10nM)	0.33 ± 0.06
Forskolin (100nM)	0.70 ± 0.11 *
Isoprenaline (100μM) + Forskolin (10nM)	0.44 ± 0.05
Isoprenaline (100μM) + Forskolin (100nM)	0.75 ± 0.18 *
PGE_1 (30nM)	1.05 ± 0.19 *
PGE_1 (30nM) + Forskolin (100nM)	14.19 ± 5.54 *
PGE_1 (0.5μM)	15.70 ± 2.66 *

Platelets (+1μM phentolamine) were incubated in the presence of (-)isoprenaline, forskolin, or PGE_1 (indicated concentrations) either singularly or in combination as shown. Data quoted are the mean ± SEM of three separate experiments performed in quadruplicate. Statistical significance was established by Students 't' test for comparison of paired means ; a * p value of <0.05 was considered significant.

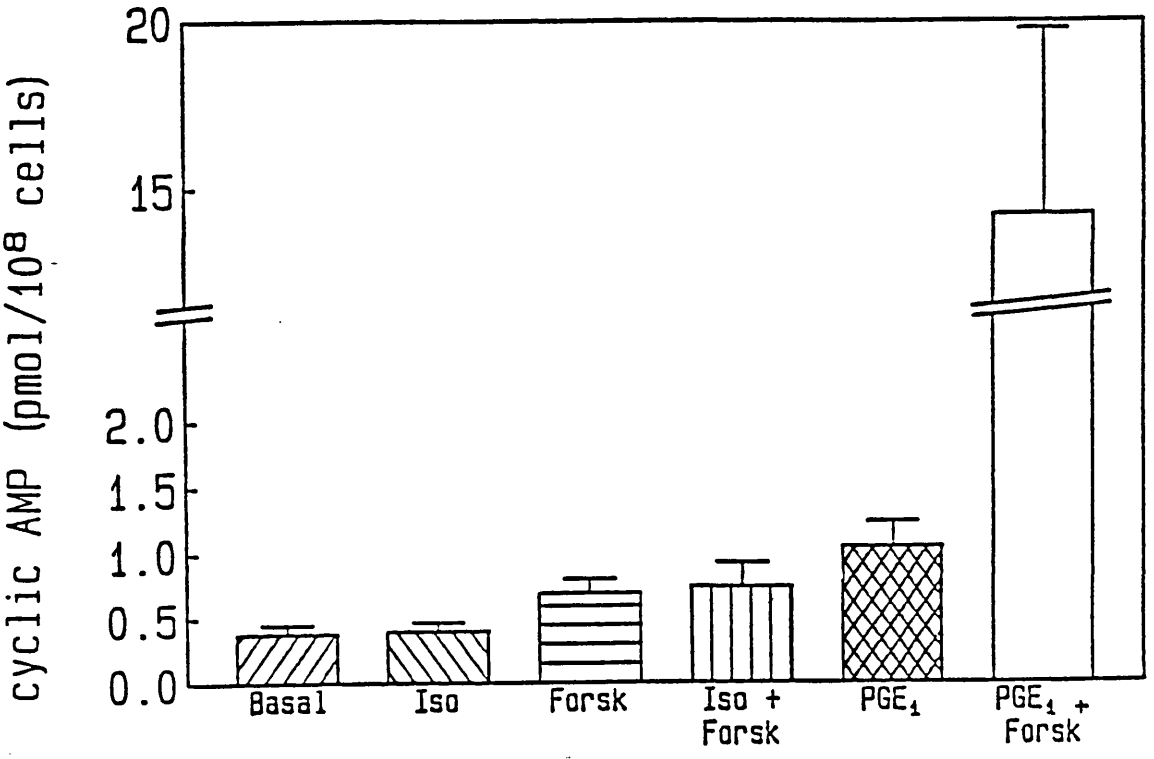


Figure IV.7. Agonist-induced cyclic AMP accumulation in intact human platelets

Platelets were incubated as described in the methods section in the presence of (-)isoprenaline (100μM), forskolin (100nM) or PGE_1 (30nM) either singularly or in combinations as shown. Data quoted are the mean $\pm$ SEM of three separate experiments performed in quadruplicate.

Table IV.2. Predicted cyclic AMP levels estimated for given numbers of mononuclear leukocytes (MNL)

MNL x 10^6	Basal (pmol)	Isoprenaline (pmol)
1.0	1.35 (0.49,3.73)	3.09 (1.12,8.57)
0.75	0.63 (0.23,1.76)	2.70 (0.97,7.49)
0.5	0.54 (0.20,1.51)	2.36 (0.85,6.55)
0.25	0.46 (0.17,1.29)	2.06 (0.74,5.73)

MNL were incubated in the presence or absence of 100μM isoprenaline as described for figure IV.4 and the data analysed by covariance analysis (see methods section) to give the predicted cyclic AMP levels expected to be associated with the presence of given numbers of MNL. 95% confidence intervals are shown in parentheses.

(data not shown for clarity). Thus, even at the lowest MNL concentration assayed ($0.37x10^6$), isoprenaline stimulation of cyclic AMP was significantly higher than basal. From the relationship illustrated in figure IV.4., predicted cyclic AMP accumulation for given values of MNL were calculated (table IV.2.). For example, the presence of $0.5x10^6$MNL would be expected (95% confidence intervals in parentheses) to produce a basal cyclic AMP of 0.54 pmol (0.2,1.51) and isoprenaline stimulated cyclic AMP level of 2.36 pmol (0.85,6.55). Therefore, if this minimal contamination of MNL were present/10^9 platelets (equivalent to 0.05%), it can be seen from figure IV.4. that the basal level of approximately 8 pmol cyclic AMP/10^9 platelets would not be dramatically affected. However, a spurious increase in isoprenaline stimulated cyclic AMP, equivalent to a level of 8.85-14.55 pmol/10^9 platelets, would occur, i.e. a 1.1-1.8 fold increase, similar to that quoted in other studies of human platelets.

IV.4. Discussion

In the present study, basal whole platelet cyclic AMP levels were easily detectable : thus, any stimulation, however small, would have been readily observed. However, no isoprenaline stimulated cyclic AMP accumulation could be detected in these platelets, free of white blood cell contamination. Furthermore, addition of phentolamine to block any isoprenaline inhibition of adenylate cyclase at platelet α_2-adrenoceptors did not influence these findings, suggesting that no α_2-β_2-adrenoceptor interaction occurs in these cells. These results differ from those previously reported by others. This raises the

possibility that the cells employed in this study may be depleted of one or more essential requirements, and thus become unresponsive during preparation. Gs, Gi and the adenylate cyclase catalytic moiety of these cells however, appear to be functioning as expected for other stimulatory and inhibitory agonists since the data for PGE_1 stimulated and adrenaline inhibition of this response are similar to those previously quoted for the adenylate cyclase system of human platelets (Moskowitz et al., 1971; Jakobs et al., 1976; Michel and Lefkowitz, 1982; Insel et al., 1982). Furthermore, it is unlikely that phosphodiesterase breakdown of any cyclic AMP formed following β-agonist stimulation is responsible for the absence of any detectable response, since experiments were performed in the presence of a phosphodiesterase inhibitor and, as mentioned previously, basal levels actually increased slightly in a linear manner with time.

Attempts to potentiate agonist induced cyclic AMP accumulation in these cells by the use of forskolin, also failed to show any synergistic interaction with the β-agonist isoprenaline, but resulted in a significant potentiation of the PGE_1 response. These results are in agreement with those of Siegl et al (1982) who also examined cyclic AMP accumulation in intact human platelets and found no isoprenaline stimulated response in the presence or absence of forskolin. Recently, low concentrations of forskolin have been shown to inhibit Gs-stimulated human platelet adenylate cyclase activity, an effect that is apparently more pronounced at low substrate (Mg^{++}/ATP) concentrations (Watanabe and Jakobs, 1986). No such effect of forskolin was apparent in this study, and the importance of this inhibitory effect when

employing forskolin as a stimulatory agent is unclear at present.

The absence of any detectable stimulation of platelet cyclic AMP by isoprenaline in the present study, raised the question of why previous studies had observed very small (50% or less) increases in platelet β-adrenoceptor stimulated cyclic AMP accumulation (Mills and Smith, 1971; Haslam and Taylor, 1971; Kerry and Scrutton, 1983a) or adenylate cyclase activity (Abdulla, 1969; Jakobs et al., 1976; 1978; Wang and Brodde, 1985). The possibility that these differences related to the purity of the platelet preparations employed was investigated. The results show that minimal contamination (0.05%) by well coupled β_2-adrenoceptors on mononuclear leukocytes could explain the spurious appearance of a 'weakly coupled' β_2-adrenoceptor in the human platelet. Platelet preparation in the present study involved an intiial centrifugation of PRP to eliminate WBC and RBC contamination. Using this method, WBC contamination was always below $0.3x10^6$ WBC/10^9 platelets, i.e. less than 0.03% (chapter II). This is in good agreement with other recent studies investigating the human platelet β-adrenoceptor (Wang and Brodde, 1985; Kerry et al., 1984) which have also employed the additional second centrifugation step to eliminate WBC contamination, without which significant contamination with WBC and RBC is possible. However, the earlier studies in which β-adrenoceptor stimulated adenylate cyclase activity was observed (Abdulla, 1969; Jakobs et al., 1976; 1978; Haslam and Taylor, 1971; Mills and Smith, 1971; Kerry and Scrutton, 1983a) employed only an initial centrifugation step to obtain PRP from whole blood, and no reference is made in their manuscripts as to the degree of WBC and RBC

contamination. It is of interest to note that the method of platelet preparation employed by Kerry and Scrutton (1983a), in which β-adrenoceptor stimulated cyclic AMP accumulation and inhibition of aggregation were reported, employed only the single centrifugation step, whilst a more recent study by the same workers investigating β-adrenoceptor radioligand binding on these cells employed the additional second centrifugation step (Kerry et al., 1984).

In summary, the above results confirm the previous finding (chapter III) of lack of coupling of the human platelet β_2-adrenoceptor to adenylate cyclase. Evidence is presented to support the suggestion that previous reports of coupling of this receptor to adenylate cyclase may have been partly, or totally, due to a contribution from β-adrenoceptors present on other blood cells. The presence of a very weakly coupled β-adrenoceptor on these cells however cannot be eliminated, since isoprenaline induced inhibition of platelet aggregation cannot be easily explained to occur via the involvement of other blood cells. Small changes (1.5 fold) in cyclic AMP levels above basal have previously been reported to produce significant inhibition of platelet aggregation (Siegl et al., 1982). It is therefore possible that changes in cyclic AMP levels of less than this are sufficient to initiate an inhibitory response, and that present techniques are not sensitive enough to detect such changes. Adenylate cyclase studies on lysates prepared from platelets free of contamination from other blood cells may help clarify the situation in the future. It is also conceivable that β_2-adrenoceptors may modulate platelet responsiveness via a biochemical effector system other than adenylate cyclase, as has

been suggested for α_2-adrenoceptors on these cells.

A possible role for β_2-adrenoceptors as modulators of platelet responsiveness to stimulatory agonists was explored in the following chapter (chapter V).

CHAPTER V

β-adrenoceptor modulation of platelet responses to other stimulatory agonists - aggregation and secretion studies

V.1. Introduction

As mentioned previously (see IV.1.) a possible anti-aggregatory role for human platelet β-adrenoceptors has been suggested. Platelet aggregation studies have reported an isoprenaline induced disaggregation of ADP induced platelet aggregates (Abdulla, 1969) and isoprenaline inhibition of the aggregatory response to various stimulatory agonists including thrombin, ADP, vasopressin and collagen (Mills and Roberts, 1967; Mills and Smith, 1971; Mills and Smith, 1972; Yu and Latour, 1977; Kerry and Scrutton, 1983a). These effects were reportedly associated with an increase in intraplatelet cyclic AMP formation and apparently prevented by the addition of β-adrenoceptor antagonists (see IV.1. and IV.4.). It was thus assumed that β-adrenoceptor induced inhibition of platelet responses to stimulatory agonists occurred via an elevation of intraplatelet cyclic AMP levels. The results of the present study however suggest that this may not be the case in that the human platelet β-adrenoceptor does not appear to be coupled to adenylate cyclase (chapters III and IV). This raised the possibility that this β-adrenoceptor may function via an effector system other than adenylate cyclase.

Several agonist-receptor controlled transduction processes have been proposed to occur in the platelet, those considered to be of major importance at present involve cyclic AMP, 1,2-diacylglycerol (DG), inositol 1,4,5-trisphosphate (IP_3) and calcium (Ca^{++}). As briefly mentioned above (see also I.1.3.) inhibition of platelet responsiveness is associated with agonist induced elevations of the intracellular

concentration of cyclic AMP, whilst platelet activation is associated with agonist-induced elevations in the intracellular concentrations of DG, IP_3 and Ca^{++}. Both the inhibitory and stimulatory pathways have much in common (figure V.1.). In both, the agonist is recognised by specific cell surface receptors which transmit information across the plasma membrane via G proteins. The G proteins activate an 'amplifier' enzyme located on the inner surface of the membrane which then converts precursor molecules into second messengers. For the inhibitory second messenger pathway the enzyme adenylate cyclase converts Mg^{++}.ATP into cyclic AMP, whilst in the stimulatory pathway the enzyme phospholipase C converts the membrane inositol-containing phospholipids (phosphatidyl inositol 4,5-bisphosphate : PIP_2 and possibly also phosphatidyl inositol : PI and phosphatidyl inositol 4-phosphate : PIP) into DG and the corresponding inositol phosphates including IP_3 (see Drummond and MacIntyre, 1987 for review).

Cyclic AMP, once formed, stimulates a specific cyclic AMP-dependent protein Kinase or Kinase A which catalyses the phosphorylation of various platelet proteins to induce a functional change (Krebs, 1984; Berridge, 1985). Cyclic AMP acts in platelets to modify the rate of production and inactivation of the stimulatory second messenger molecules Ca^{++} and DG (Kaser-Glanzmann et al., 1977; Feinstein et al., 1983; Lapetina, 1984) and may also antagonise their effects to prevent or reverse agonist-induced platelet activation (Hathaway et al., 1981). Cyclic AMP action is terminated via phosphodiesterase (PDE) hydrolysis.

Similarly the main site of action of DG, once formed, appears to be the

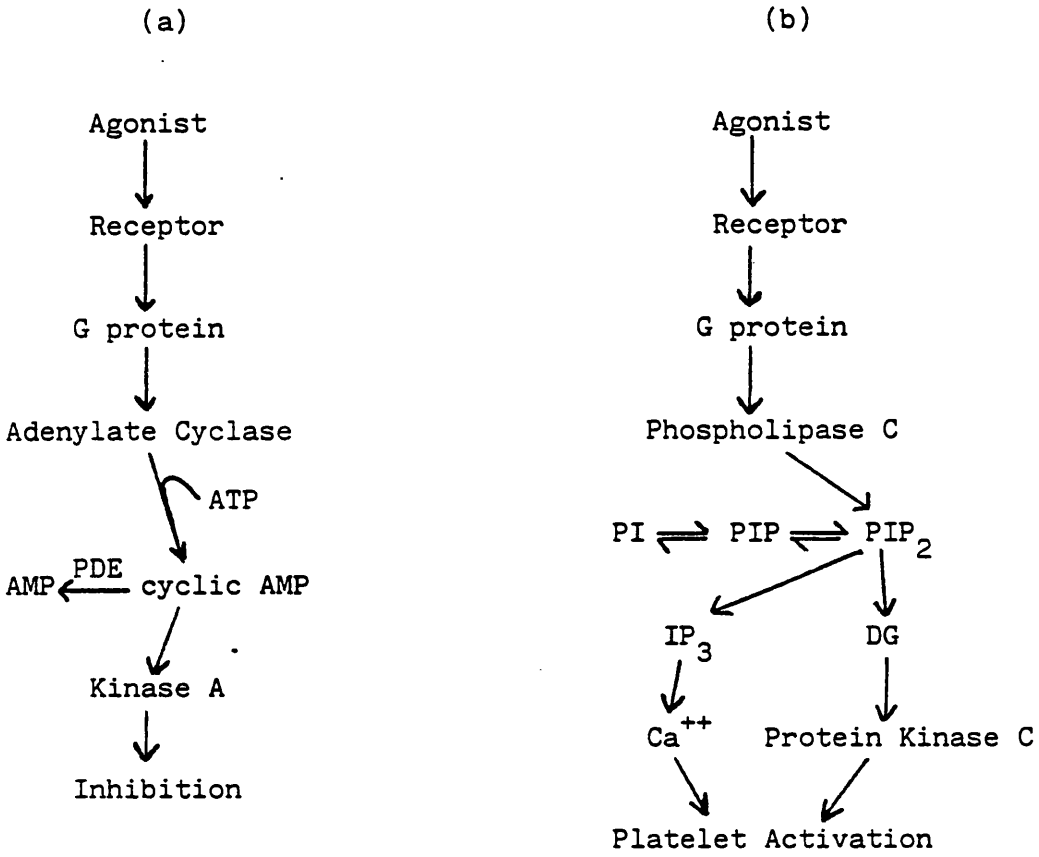


Figure V.1.

Schematic representation of receptor-mediated inhibition and stimulation of platelet activation via cyclic AMP formation (a) and inositol lipid metabolism (b) respectively.

See text for explanation.

activation of protein Kinase C (Takai et al., 1979; Kishimoto et al., 1980; Nishizuka, 1984). DG acts by increasing the affinity of this enzyme for phosphatidylserine and Ca^{++} such that, providing enough DG is formed, activation of protein Kinase C can occur at resting levels of Ca^{++}. The other primary product of phospholipase C action on membrane phosphoinositides, IP_3, is believed to stimulate the mobilisation of Ca^{++} from intracellular stores (O'Rourke et al., 1985; Brass and Joseph, 1985). Released Ca^{++} may act via a direct combination with a target enzyme, or via an interaction with the Ca^{++} acceptor protein calmodulin (Cheung, 1980).

It has been proposed that both Ca^{++}-calmodulin dependent and protein Kinase C-dependent phosphorylation reactions are necessary for full platelet activation (Kaibuchi et al., 1983). Both limbs of the stimulatory pathway can be mimicked through the use of Ca^{++} ionophores e.g. A23187 or ionomycin to increase cytosolic Ca^{++}, and with either membrane permeable DG's (e.g. DC8) or phorbol esters to bring about the activation of protein Kinase C (Kaibuchi et al., 1983; Lapetina et al., 1985). Using such probes it has been shown that addition of one or other agent alone elicits a portion of the full hormonal response while, in combination, the response obtained is indistinguishable from that generated by the receptor agonist, i.e. protein Kinase C and Ca^{++} interact in a synergistic manner to produce secretion and aggregation (Kaibuchi et al., 1983; Nishizuka, 1984). In addition, protein Kinase C appears to have a negative feedback influence over platelet activation, in that prior stimulation of protein Kinase C with phorbol esters or a membrane permeable DG can reduce the ability of an agonist to

activate phospholipase C. This has been demonstrated as an inhibition of subsequent PIP_2 hydrolysis (Watson and Lapetina, 1985; Zavoico et al., 1985; Rittenhouse and Sasson, 1985), an inhibition of intracellular Ca^{++} mobilisation (MacIntyre et al., 1985b) and an increase in IP_3 breakdown (Molina y Vedia and Lapetina, 1986). Thus, protein Kinase C is hypothesised to have a bidirectional role in platelet activation i.e. it is involved in both secretion and aggregation and also in limiting platelet activation.

In addition to the second messenger pathways depicted in figure V.1., there may also be a role for adenylate cyclase inhibition in the control of platelet function. At present, there is no evidence to suggest that platelet activation is accompanied by a decrease in intracellular cyclic AMP levels below basal (see IV.1.). The inhibitory effect of certain aggregatory agonists on adenylate cyclase activity may therefore be one of modulating the effects of other agonists e.g. on phosphoinositide metabolism.

It has been suggested that cyclic GMP may modulate platelet responsiveness. Agents such as nitroprusside, which elevate intracellular cyclic GMP levels, have been shown to inhibit platelet function. The mechanism involved is not understood (Haslam et al., 1980). Furthermore, cyclic GMP levels have been shown to rise in response to the addition of certain platelet stimulatory agonists. This occurs after a relatively long lag phase (Haslam et al., 1980). In some cell types, guanylate cyclase, the enzyme that converts GTP to cyclic GMP, is not connected to a receptor, and cyclic GMP formation often occurs

after the activation of phosphoinositide hydrolysis (Berridge, 1985). In platelets, cyclic GMP apparently inhibits agonist induced elevation of intracellular calcium (MacIntyre et al., 1985b). It is therefore possible that cyclic GMP may function as a negative feedback to limit platelet responsiveness.

A number of cellular processes have been shown to correlate with changes in intracellular pH, suggesting a possible second messenger role for H^+. Evidence for such a role in platelets is contradictory. Horne et al., (1981) have reported that thrombin induced platelet activation correlates with an increase in cytosolic pH, whilst Rotman and Heldman (1982) observed no change in pH with platelet activation. At present, the role (if any) of pH in platelet function awaits definition.

Platelet activation by a number of agonists is accompanied by the release of arachidonic acid from membrane phospholipids via the action of phospholipase A_2. Arachidonic acid once released is rapidly converted by a cyclooxygenase enzyme to the labile endoperoxides PGG_2 and PGH_2. The endoperoxides which can themselves activate platelets are converted into the potent aggregating agent and vasoconstrictor thromboxone A_2 (TxA_2) (Hamberg et al., 1975). Free arachidonic acid may apparently also be liberated as a consequence of stimulated phosphoinositide metabolism either via diacylglycerol lipase hydrolysis of diacylglycerol (Rittenhouse-Simmons and Deykin, 1981), or via the action of monoglyceride lipase on a converted form of diacylglycerol (Majerus et al., 1983). It seems uncertain at present which of these

two sources of arachidonic acid is the initial and major source; however, the resultant endoperoxide and TxA_2 formation provides a positive feedback mechanism to promote platelet activation.

From the above information it is clear that the stimulus-response coupling mechanisms believed to be involved in the control of platelet function are both numerous and complex. Information available at present does not allow a precise definition of how inhibitory and stimulatory pathways contribute to the final platelet response, but does unequivocally demonstrate a high degree of interaction between the mechanisms present. In this respect, it is possible that the anti-aggregatory effect of isoprenaline may be mediated by any one of the mechanisms described.

The aim of the study described in this chapter was to reproduce and extend previous studies in which an isoprenaline induced inhibition of the aggregatory response to certain stimulatory agonists was observed, and in so doing to attempt to delineate how isoprenaline may produce this effect if it is not via elevation of intracellular cyclic AMP. Thrombin, adenosine diphosphate (ADP) and vasopressin were selected for use as stimulatory agonists since they had previously been employed by others. The effect of isoprenaline on the platelet response to these agonists was evaluated in the presence and absence of the α-adrenoceptor antagonist, phentolamine and the β-adrenoceptor antagonist, (-)timolol (Leo laboratories). Timolol was employed because of its reported low membrane-stabilising activity and lack of effect on platelet aggregation (Green et al., 1985).

Two platelet responses were examined : i) reversible or primary aggregation, which apparently does not involve platelet secretion and thus avoids possible complications from the effects of the secretory response, and ii) platelet secretion, an event that is reportedly associated with secondary irreversible aggregation (see I.1.3. for more detail).

V.2. Methods

V.2.1. Platelet Preparation

Blood was drawn from healthy normal volunteers using a 19 gauge needle into 0.1 volumes of 3.2% sodium citrate as anticoagulant. The sample was centrifuged at 700g for 5 minutes at room temperature and the platelet rich plasma (PRP) removed with a plastic pipette and stored in a tightly capped plastic container at room temperature for use in aggregation (V.2.2.) or secretion studies (V.2.3.). The remaining sample was centrifuged at 1000g for 10 minutes at room temperature to obtain platelet poor plasma (PPP). Before use, PRP was diluted with PPP to give a platelet count of approximately 300 x 10^6/ml.

V.2.2. Platelet Aggregation

Photometric detection of aggregation in platelet rich plasma (PRP) or washed platelet suspensions, initially described by Born (1962), is the most commonly used technique for monitoring this response and was

employed in the present study. The method depends on the measurement of changes in light transmission through a stirred platelet sample. As platelet aggregates are formed, the light intensity passing through the sample increases in proportion to the extent and rate of aggregation.

PRP samples (1.0ml) were incubated at 37°C in siliconised glass cuvettes for 3 minutes prior to testing in a Payton dual channel aggregometer connected to a Rikadenki chart recorder. The recorder was set so that PRP gave about 10% light transmission and PPP gave 100% transmission. Samples were stirred at 800rpm and agents added in a small volume (<40ul) within 1 minute of placing the sample cuvette in the aggregometer.

Thrombin, adenosine diphosphate (ADP) and vasopressin were used as aggregatory agents. When more than one agent was to be added, a 30 second time interval was allowed before subsequent additions were made and the sequence employed was : antagonist (phentolamine or timolol), followed by isoprenaline followed by aggregatory agent (thrombin, ADP or vasopressin).

V.2.3. Platelet Secretion

Platelet secretion of dense granule constituents was assessed by monitoring the release of the adenine nucleotides, ADP and adenosine triphosphate (ATP).

Total and released ATP and ADP were determined by a bioluminescent

technique using a stabilised firefly luciferin-luciferase reagent (ATP monitoring reagent LKB) and an LKB luminometer (Summerfield et al., 1981). The monitoring reagent gives a constant light output that is directly proportional to ATP concentration. ADP may be assayed after first phosphorylating to ATP using pyruvate Kinase (PK) in the presence of phosphoenolpyruvate (PEP). The difference in ATP before and after phosphorylation therefore represents the ADP content of the sample (see Appendix 9 for further explanation).

To prepare extracts of PRP for analysis of total nucleotides, 50ul of PRP was added to 450ul of Triton-Tris-EDTA, allowed to stand for 1 minute and then stored frozen at -40°C for assay (Appendix 9).

To prepare extracts of PRP for analysis of released nucleotides, samples of PRP (400ul) were prewarmed to 37°C in a siliconised glass cuvette for 3 minutes prior to testing. Samples were stirred at 800rpm and agents added in a small volume (<40ul) within 1 minute of placing the cuvette in the aggregometer.

Thrombin and ADP were used as aggregating agents. When more than one agent was to be added, a time interval of 30s was allowed before subsequent additions were made and the sequence employed was as described for aggregation studies (V.2.2.). Reactions were allowed to continue for times indicated (V.3.) before being terminated by the addition of 40ul ice cold 0.1M EDTA. Samples were immediately centrifuged at 1000g for 10 minutes at 0 - 4°C to obtain PPP. 100ul of PPP was then removed, mixed with 400ul of Triton-Tris-EDTA, allowed to

stand for 1 minute and then stored frozen at -40°C for assay (Appendix 9).

All samples were assayed in duplicate. For thrombin stimulated samples both ATP and ADP levels were measured, for ADP stimulated samples however only ATP measurements were meaningful. Thus only the ATP results are given. Values for released ATP are expressed as either percentage of total ATP or as a percentage of the control response (100%).

V.3. Results

Platelet aggregation to thrombin, ADP and vasopressin was monitored turbidometrically as described in the methods section. In all cases stimulatory agonists were added at a concentration which gave a near-maximal reversible aggregatory response. Only the extent of the primary phase of aggregation was considered in these measurements and no attempt was made to analyse the shape of the aggregation tracings. When analysed in this way, the presence and absence of phentolamine was found to make no difference to the response observed in the presence of isoprenaline. For clarity, only data obtained in the presence of phentolamine (10uM) is presented; thus, any action of a high isoprenaline concentration at platelet α_2-adrenoceptors is eliminated. In addition, when the effects of 1uM and 10uM phentolamine were compared, the results obtained were identical; thus, although a concentration of 1uM phentolamine has been used elsewhere in this study, in this chapter 10uM phentolamine was employed so that the

results obtained could be compared directly with those of previous studies (Kerry and Scrutton, 1983a).

Thrombin induced aggregation in PRP was significantly inhibited by the addition of increasing concentrations of (-)isoprenaline (figure V.2.). The extent of the inhibition was similar over the concentration range of (-)isoprenaline employed, i.e. 0.1-100uM, and apparently prevented by the presence of 1uM timolol. Similarly, inhibition of thrombin-induced aggregation was also observed in the presence of (+)isoprenaline, but, although apparent at 0.1-10uM, was only statistically significant at 100uM. It should be noted, however, that although the mean data presented was significantly different from control, in three out of seven subjects the observed responses on addition of (-) or (+) isoprenaline were indistinguishable from those obtained with thrombin alone.

Similar results were obtained for vasopressin-induced aggregation, in that both (-) and (+)isoprenaline inhibited the observed response. Furthermore, the (-)isoprenaline effect was absent in the presence of timolol. However, the inhibitory effect was only significant at high isoprenaline concentrations and was in fact more pronounced in the presence of (+)isoprenaline (figure V.3.).

In contrast, ADP-induced aggregation was not significantly influenced by the presence of (-) or (+)isoprenaline, or by timolol (figure V.4.).

The time course of thrombin-induced platelet secretion was examined.

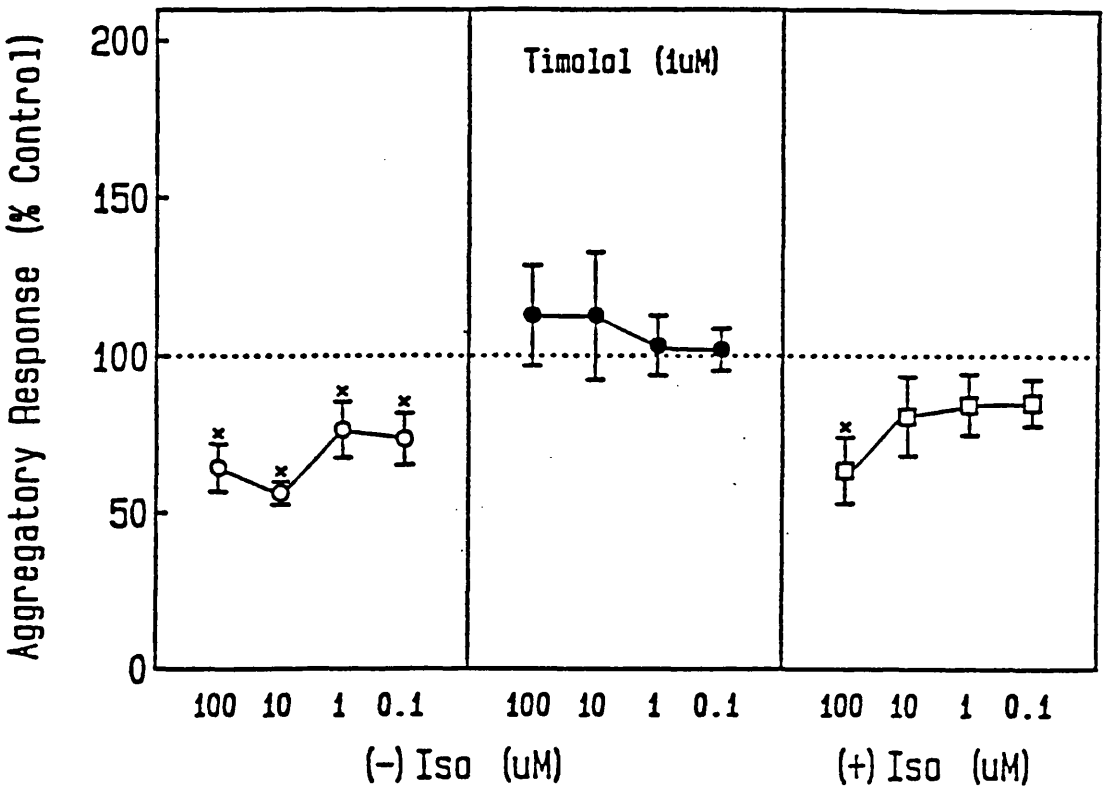


Figure V.2. Thrombin-induced aggregation of human platelet rich plasma

Platelet rich plasma was prepared and platelet aggregation monitored as described in the methods section. Aggregation was induced by thrombin at a concentration which gave a near-maximal aggregatory response (concentration range = 0.1-0.3U/ml). 10uM phentolamine was present throughout. Aggregation was monitored in response to thrombin alone = control; thrombin with increasing concentrations of (-)isoprenaline in the absence (o) and presence of timolol (•); and thrombin with increasing concentrations of (+)isoprenaline (□). Data are presented relative to control and represent the mean $\pm$SEM of seven separate experiments. Results were analysed using Student's 't' test for paired data. x=$p<0.05$.

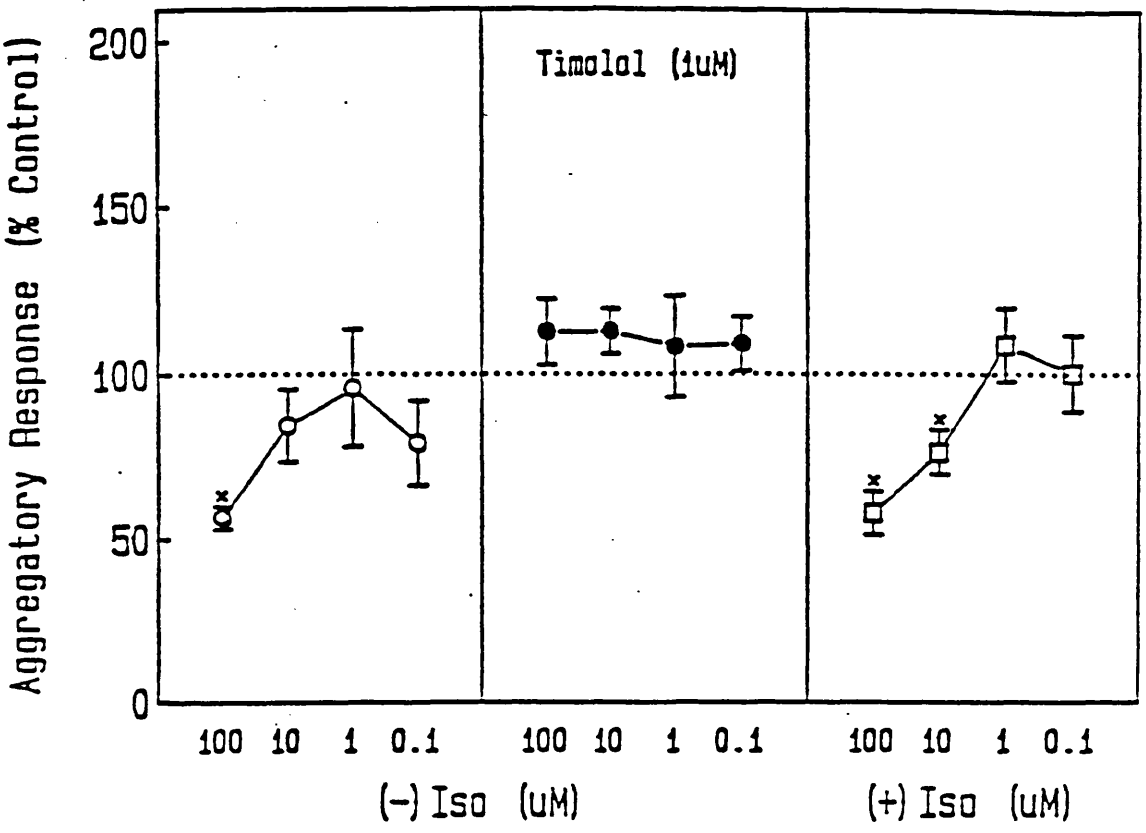


Figure V.3. Vasopressin-induced aggregation of human platelet rich plasma

Platelet rich plasma was prepared and platelet aggregation monitored as described in the methods section. Aggregation was induced by vasopressin at a concentration which gave a near-maximal aggregatory response (concentration range = 0.91-91.4U/ml vasopressin). 10uM phentolamine was present throughout. Aggregation was monitored in response to vasopressin alone = control; vasopressin with increasing concentrations of (-)isoprenaline in the absence (o) and presence of timolol (•); and vasopressin with increasing concentrations of (+)isoprenaline (□). Data are presented relative to control and represent the mean ±SEM of six separate experiments. Results were analysed using Students 't' test for paired data. x = $p<0.05$.

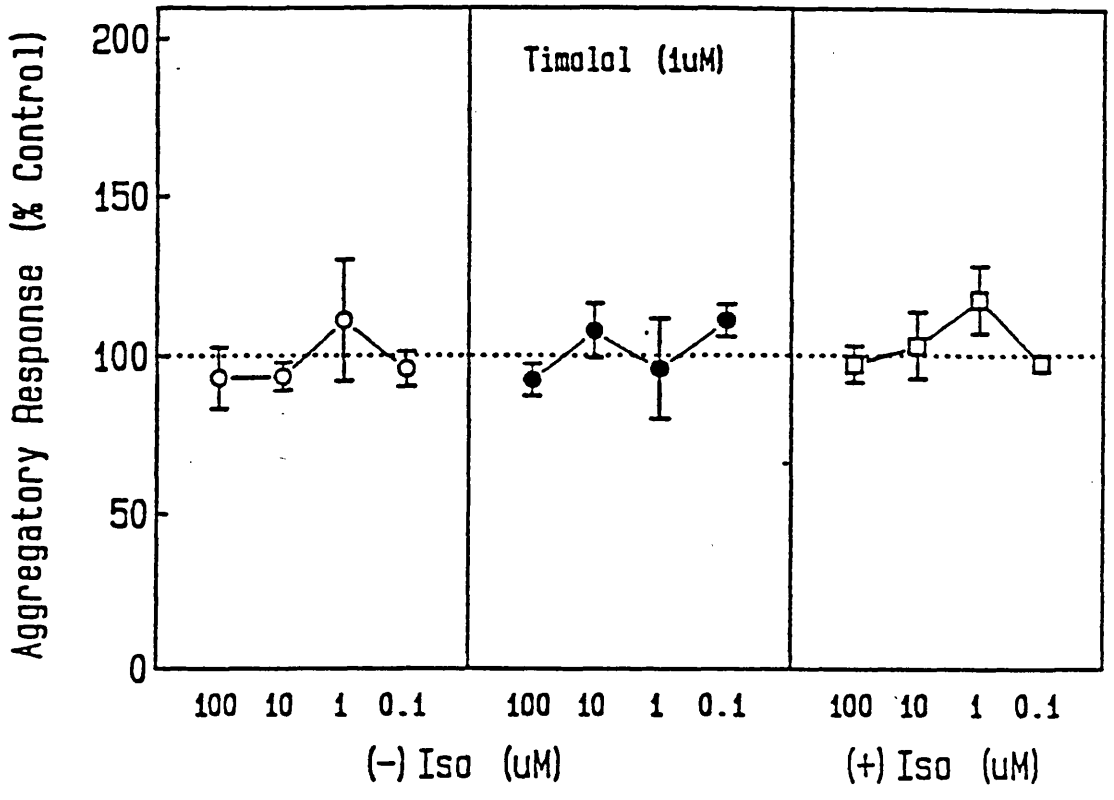


Figure V.4. ADP-induced aggregation of human platelet rich plasma

Platelet rich plasma was prepared and platelet aggregation monitored as described in the methods section. Aggregation was induced by ADP at a concentration which gave a near-maximal aggregatory response (concentration range = 0.5-1.0uM ADP). 10uM phentolamine was present throughout. Aggregation was monitored in response to ADP alone = control; ADP with increasing concentrations of (-)isoprenaline in the absence (o) and presence of timolol(•); and ADP with increasing concentrations of (+)isoprenaline (□). Data are presented relative to control and represent the mean ±SEM of five separate experiments. Results were analysed using Student's 't' test for paired data. None of the values were significantly different from control.

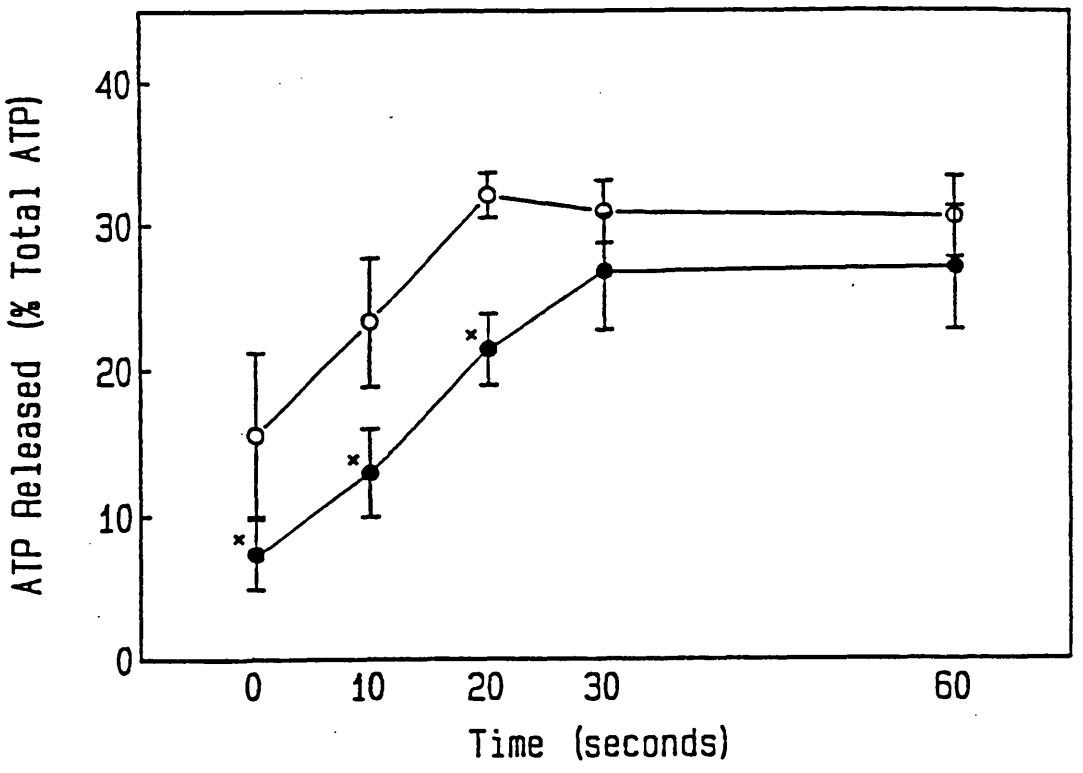


Figure V.5. Time course of thrombin-induced ATP release in platelet rich plasma

Platelet rich plasma was prepared and ATP release measured as described in methods section and appendix 9. 10uM phentolamine was present throughout. ATP release was induced by 1.0U/ml thrombin in the absence (o) and presence (•) of 100uM (-)isoprenaline for the times indicated. Data are presented relative to total platelet ATP and represent the mean ±SEM of three separate experiments. Results were analysed using Student's 't' test for paired data. x = $p<0.05$.

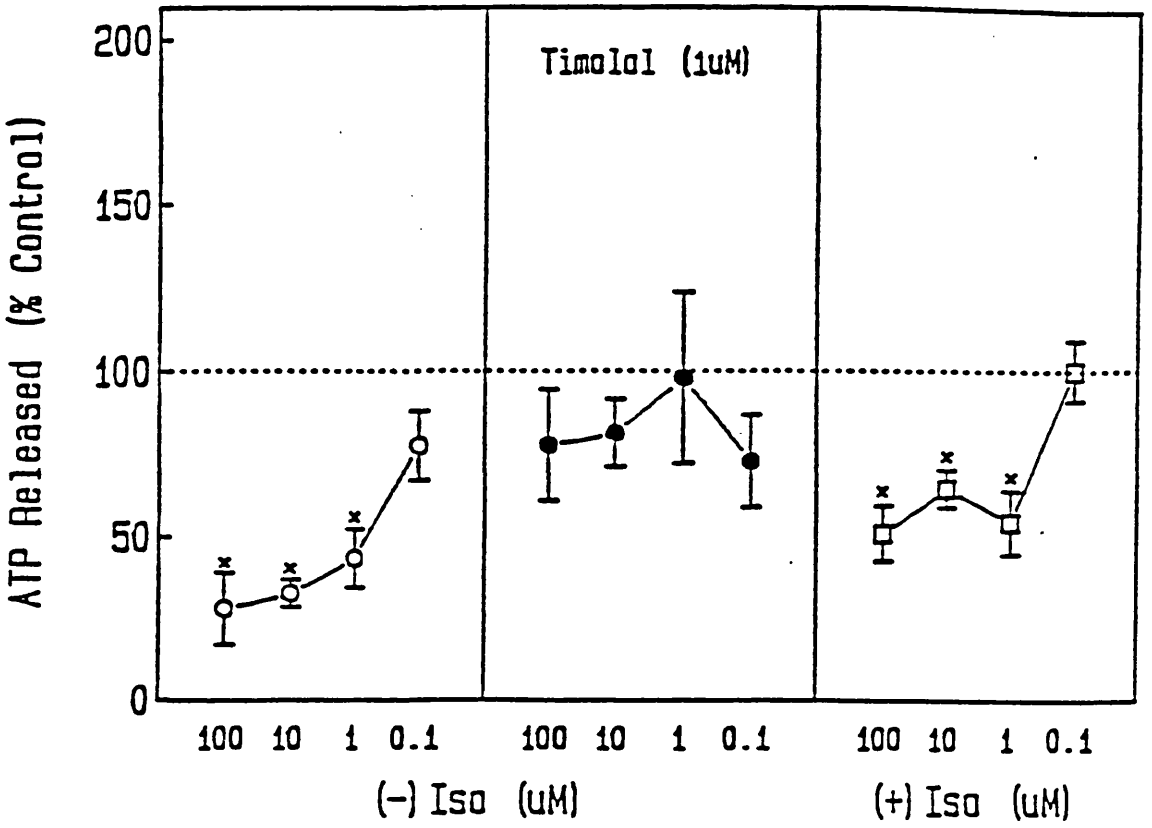


Figure V.6. Thrombin induced ATP release in platelet rich plasma

Platelet rich plasma was prepared and ATP release measured as described in methods section and appendix 9. 10uM phentolamine was present throughout. ATP release was induced by thrombin alone (1.0U/ml for 60 seconds) = control; thrombin with increasing concentrations of (-)isoprenaline in the absence (o) and presence of timolol (•); and thrombin with increasing concentrations of (+)isoprenaline (□). Data are presented relative to control and represent the mean $\pm$SEM of three separate experiments. Results were analysed using Student's 't' test for paired data. x = $p<0.05$.

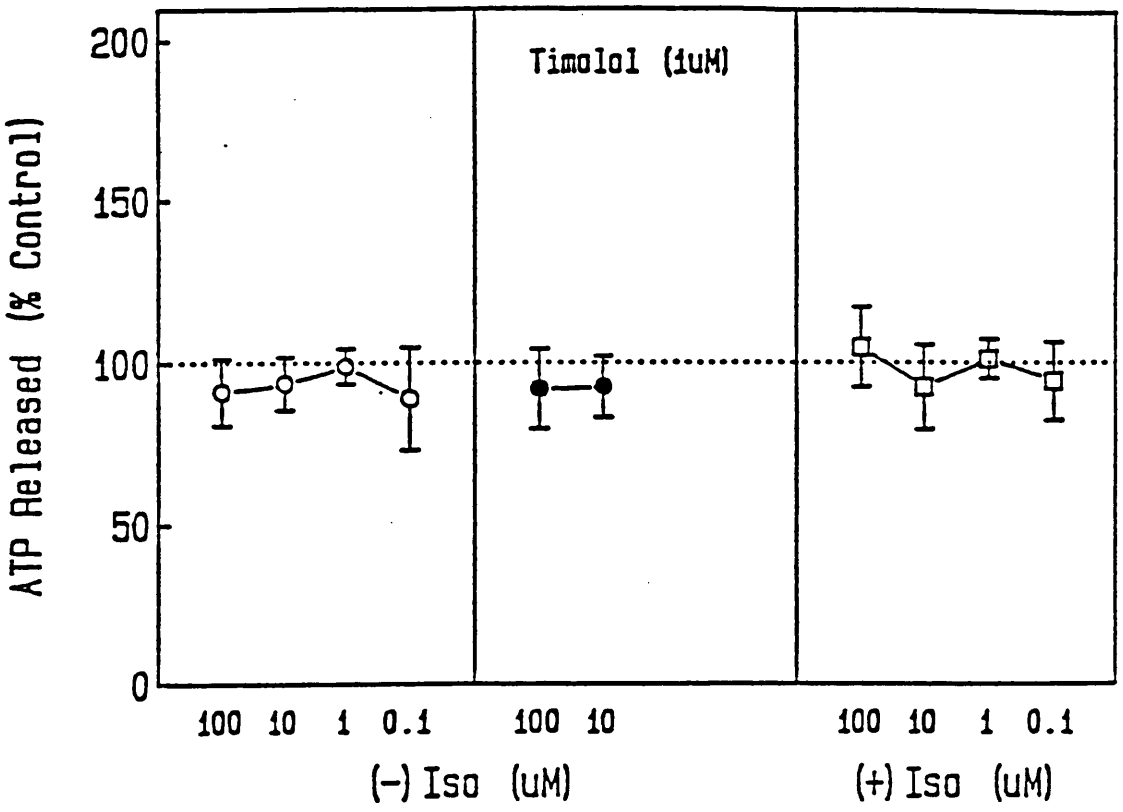


Figure V.7. ADP induced ATP release in platelet rich plasma

Platelet rich plasma was prepared and ATP release measured as described in methods section and appendix 9. 10uM phentolamine was present throughout. ATP release was induced by ADP alone (5uM ADP for 60 seconds) = control; ADP with increasing concentrations of (-)isoprenaline in the absence (o) and presence of timolol (•); and ADP with increasing concentrations of (+)isoprenaline (□). Data are presented relative to control and represent the mean ±SEM of three separate experiments. Results were analysed using Student's 't' test for paired data. None of the values were significantly different from control.

ATP release was found to be rapid, reaching a steady state maximal level within 20 seconds (figure V.5.). When 100uM (-)isoprenaline was added, a significant decrease in the amount of ATP released within this initial 20 seconds was observed. From 30 seconds onwards the amount of ATP released, although apparently lower than that for thrombin alone, was not significantly different. Thus, (-)isoprenaline appeared to have shifted the time course of thrombin-induced ATP release. When various concentrations of (-) and (+)isoprenaline were added, inhibition of ATP release appeared to be a concentration dependent process (figure V.6.) that was absent if timolol was present. However, the dose-effect relationship for both (-) and (+)isoprenaline were similar with an apparent half maximal effect for both at around 0.5uM.

Vasopressin induced ATP release was not evaluated since in the aggregation study only two out of six subjects exhibited a secondary aggregation response to vasopressin even at 91.4U/ml.

When ADP-induced ATP release was examined, addition of (+) or (-)isoprenaline, or timolol did not significantly influence the control response (figure V.7.).

V.4. Discussion

The results of this study suggest that isoprenaline has an inhibitory effect on thrombin- and vasopressin-induced primary aggregation. The extent of this inhibitory effect is similar to that quoted by others (Yu and Latour, 1977; Kerry and Scrutton, 1983a). Furthermore,

isoprenaline also appears to inhibit thrombin-induced dense granule secretion. From the results of this brief study it is not clear whether these isoprenaline effects are mediated via occupancy of the β_2-adrenoceptors present on human platelets. On the one hand, inhibition of these isoprenaline effects by the addition of timolol suggests that they are β-adrenoceptor effects, while on the other hand, the lack of discrimination between (-) and (+)isomers of isoprenaline on vasopressin-induced primary aggregation and thrombin-induced secretion suggest these to be non-β-adrenoceptor effects. The only isoprenaline effect to exhibit stereoselectivity was thrombin-induced primary aggregation and, as mentioned in the results section (V.3.), this effect was only significantly different from control in four out of seven subjects.

The situation is further complicated by the fact that β-adrenoceptor antagonists themselves are reported to have an inhibitory effect on platelet responsiveness to stimulatory agonists both in vivo and in vitro. (Campbell et al., 1981a,b; Weksler et al., 1977). The concentration range required for inhibition is two orders of magnitude higher than that required for occupancy of the platelet β-adrenoceptor, and (+) and (-)isomers of the antagonists are equally effective as inhibitors (Mills and Roberts, 1967; Weksler et al., 1977; Kerry et al., 1984b). Such observations suggest that inhibition of platelet aggregation by these β-adrenoceptor antagonists is not a β-adrenoceptor mediated effect but may be due to the membrane stabilising activity of these agents, or to their inhibitory effect on thromboxane synthesis (Greer et al., 1985). There is no evidence from this study however to

suggest that timolol is acting via mechanisms other than β_2-adrenoceptor occupancy since; i) as previously reported, (see V.3.) it did not inhibit platelet responsiveness to the stimulatory agonists employed, and ii) the concentration of timolol employed was not in excess of that required for β-adrenoceptor occupancy (Rugg et al., 1978).

In contrast to previous studies (Abdulla, 1969; Kerry and Scrutton, 1983a) no isoprenaline inhibition of ADP-induced primary aggregation was observed in this study. The reason for this is not known, but since the degree of isoprenaline inhibition of ADP-induced platelet aggregation varies considerably from one study to another (Adbulla, 1969; Kerry and Scrutton, 1983a) it is possible that the variability observed is a consequence of the technique employed, i.e. photometric determination of platelet aggregation. Although immensely useful in the study of platelet physiology and pharmacology this technique has several inherent drawbacks, the main one being the fact that at best it is only semi-quantitative. In addition, it has been shown that when ADP-induced aggregation is examined directly by light microscopic methods or electronic particle size analysis, extensive aggregation can occur in the absence of light transmission changes (Born and Hume, 1967; Nichols and Bosmann, 1979). Born and Hume (1967) reported 87% loss of single platelets with 7% change in light transmission. Furthermore, a recent report has indicated that platelet aggregation can produce either decreases or increases in light transmission depending on the size and shape of the aggregate particles formed (Malinski and Nelsestuen, 1986). An additional complication in the analysis of light transmission data of primary reversible aggregation

is the decrease in light transmission associated with shape change and increased platelet density (Michal and Born, 1971). Hence, quantitative analysis of aggregation traces with respect to the absolute degree of aggregation present is difficult and results in poor reproducibility and large SEM's (in figures V.2-4.).

When ADP-induced secretion was examined, no effect of isoprenaline was observed. In order to ensure that dense granule secretion from platelets of volunteers participating in the study was normal, total and released ATP and ADP were measured in the presence of thrombin. In all cases, total ATP, total ADP, released ATP, released ADP and hence ATP:ADP ratios for total and released nucleotides were well within the normal ranges expected for these parameters. However, despite the fact that secretion studies provide a more easily definable quantitative response, considerable inter- and intra-assay variability was observed as demonstrated by the large SEM's calculated (see figures V.5 to V.7). Problems of reproducibility were further compounded by a rapid diminished responsiveness of the platelets to agonist-induced dense granule secretion. For instance, control responses to thrombin were reduced to 60-70% of initial response within 45-60 minutes of blood taking. The number of determinations that could be performed with each sample was thus significantly limited.

Overall, the results of this study suggest that isoprenaline does have an inhibitory effect on thrombin- and vasopressin-induced primary aggregation and on thrombin-induced dense granule secretion, but does not affect ADP-induced responses. In order to try and delineate how

and where isoprenaline might exert this inhibitory effect it is necessary to consider how thrombin, vasopressin and ADP are believed to initiate platelet activation.

Platelet receptors for all three agents have been identified. Two classes of platelet thrombin binding sites have been identified : a high affinity low capacity site (Kd=10nM, 500 sites per cell) and a lower affinity high capacity site (Kd=100nM 50,000 sites per cell) (Detwiler and McGowan, 1985; McGowan and Detwiler, 1986). Thrombin, a serine protease, is the most potent and efficacious platelet agonist, and at present it is not known whether thrombin-induced platelet activation is a consequence of proteolytic hydrolysis of a particular substrate or of a classical agonist-receptor interaction (MacIntyre et al., 1986). Platelet receptors for vasopressin belong to the V_1 receptor subtype. Radioligand binding studies have identified a single class of binding sites (90-150 sites per cell) with a Kd of 1.3-24nM depending upon the presence and absence of divalent cations (Pletscher et al., 1985; Vittet et al., 1986). The platelet ADP receptor appears from pharmacological analyses to be unique and has been designated a P_{2T}-purinoreceptor (Cusack et al., 1985; Gordon, 1986). Radioligand binding studies have identified a single class of binding sites for ADP on human platelets : 400-1200 sites per cell with a Kd of 15nM (Macfarlane et al., 1983).

Thrombin- and vasopressin-induced platelet activation is apparently associated with phosphionositide hydrolysis (Rittenhouse-Simmons, 1979; Rittenhouse and Sasson, 1985; Siess et al., 1986; MacIntyre et al.,

1985 c,d) and an elevation in intracellular Ca^{++}, (Hallam and Rink, 1985; MacIntyre et al., 1985c) that is markedly reduced upon removal of extracellular Ca^{++}. This suggests that influx of extracellular Ca^{++} is the major source of Ca^{++}, or that mobilisation of internal Ca^{++} is dependent initially on an influx of external Ca^{++} (Hallam et al., 1984a,b; Rink et al., 1982; MacIntyre et al., 1985c). The relationship between phosphoinositide hydrolysis and the opening of a putative Ca^{++} channel remains to be determined. In addition, vasopressin via V_1 receptors and thrombin via a process involving Gi, apparently inhibit adenylate cyclase activity in membranes (Aktories and Jakobs, 1984; Haslam and Rosson, 1972) but not in intact cells (Vanderwel et al., 1983). Thrombin inhibition of adenylate cyclase however differs from that induced by other agents in that it is apparently irreversible (Aktories and Jakobs, 1984).

ADP, unlike thrombin and vasopressin, is reported to be an ineffective stimulant of phosphoinositide hydrolysis in human platelets, but does evoke an elevation of intracellular Ca^{++} (Fisher et al., 1985; MacIntyre et al., 1985c). ADP also inhibits adenylate cyclase and decreases cyclic AMP levels (Mellwig and Jakobs, 1980).

From the above information it can be seen that there are both similarities and differences in the way thrombin, vasopressin and ADP are believed to stimulate platelet activation. For instance, all three agents inhibit adenylate cyclase activity (in membranes at least). As previously described however, a role for adenylate cyclase inhibition in the control of platelet function remains undefined at present. The

only obvious difference between platelet activation induced by thrombin and vasopressin and that induced by ADP, is the apparent ineffectiveness of ADP to stimulate phosphoinositide hydrolysis. It may therefore be hypothesised that the inhibitory effect of isoprenaline involves some modification(s) of phosphoinositide hydrolysis. It is equally feasible that one or more of the mechanisms described in V.1. may be involved. The problems of poor reproducibility encountered in this study suggest that the techniques employed here may not be the most suitable for use in future studies. It has been suggested that better, more reproducible results can be obtained by the use of a lumi-aggregometer, which allows the simultaneous measurement of aggregation and dense granule secretion (Feinman et al., 1977) although the luciferin--luciferase reagent used can apparently inhibit the aggregatory response to certain agents (Pfueller and Broadway, 1986). It has also been proposed that evaluating platelet aggregation in whole blood by means of a Clay-Adams Ultra Flo 100 single platelet counter may be a more sensitive and more physiological method than turbidometric techniques (Lumley and Humphrey, 1981).

In conclusion, the results of the functional study described in this chapter, suggest that isoprenaline may have an inhibitory effect on the aggregatory response to stimulatory agonists. This effect however, was not reproducible, did not on the whole appear to fulfil the criteria for definition as a β-adrenoceptor mediated effect and thus, was unfortunately not readily definable by the methods employed in this study. Precisely how isoprenaline might exert this effect therefore remains unclear. It is possible that isoprenaline occupancy of human

platelet β_2-adrenoceptors results in elevation of cyclic AMP, which in turn, acts to modify the phosphoinositide hydrolysis pathway and result in inhibition of platelet activation (see V.1.). From information available at present, this would be the most obvious explanation, but, as mentioned previously this study has failed to demonstrate any evidence for human platelet β_2-adrenoceptor coupling to adenylate cyclase. Undoubtedly, more detailed investigations are required in order to attain a better understanding of how agonist occupancy of β_2-adrenoceptors modulates platelet responsiveness to other stimulatory agonists. In this respect, future studies will probably need to examine the biochemical changes that occur during changes in functional platelet responsiveness.

CHAPTER VI

Agonist-induced internalisation and down-regulation of β_2-adrenoceptors on intact human platelets

VI.1. INTRODUCTION

In experimental systems studied to date, two broad patterns of receptor regulation have been described i) homologous (specific or self regulatory) regulation - whereby an agonist regulates its own receptor function only, and ii) heterologous (non-specific) regulation - where activation of one receptor system leads to changes in a second, distinct receptor system. Homologous regulation, by virtue of its specificity, suggests the receptor as the site of regulation, whereas heterologous regulation must also include alterations in components distal to the receptor. Although not mutually exclusive, general unifying principles have not yet emerged, and the present study will focus on agonist-specific regulation.

One of the most widely observed mechanisms of cellular adaptation is that of diminished responsiveness to agonist, hormone or drug stimulation with time, referred to as desensitisation, tolerance, tachyphylaxis or refractoriness. Desensitisation is a common feature of β-adrenoceptor adenylate cyclase systems and during the past decade or so, much attention has been given towards understanding the molecular basis for this phenomenon. Several concomitant or sequential steps are proposed to be involved, namely uncoupling, internalisation and down-regulation.

Uncoupling : During agonist-specific desensitisation, rapid changes in the coupling of β-adrenoceptors to the regulatory component (Gs) of

adenylate cyclase have been observed. This was initially reported in the studies performed on 132INI astrocytoma cells, where short time agonist incubations (15 minutes) were shown to lead to a rapid loss of adenylate cyclase activity that did not reflect any change in total receptor number, but was associated with a rapid uncoupling of the β-adrenoceptors, assessed as a reduction in high affinity agonist binding and decreased effect of guanine nucleotide on agonist competition curves. With longer incubation, some of the β-adrenoceptors were converted to a form not detectable by radioligand binding studies. Recovery from the uncoupled state was rapidly reversible, whereas recovery from the "lost" state was slow and often incomplete (Su et al., 1979, 1980; Harden et al., 1979). Although the relative rates of reaction involved may be quite different, similar results have been obtained with other cells, e.g. S49 lymphoma cells (Su et al., 1980; Iyengar et al., 1981) certain C6 glioma cell clones (Perkins and Frederich, 1981; Homburger et al., 1980) baby hamster kidney fibroblast cells (Morishima et al., 1980) and frog erythrocytes (Wessels et al., 1978; 1979) (see Harden 1983 for review).

Internalisation : Agonist-specific desensitisation of the β-adrenoceptor adenylate cyclase system also appears to involve movement of receptors away from the cell surface, similar to that demonstrated previously for peptide hormones (Pastan and Willingham, 1981). Although definitive proof for this step by visualization of the receptor cannot be obtained at present due to lack of appropriate techniques, evidence for β-adrenoceptor internalisation has come from indirect biochemical studies.

Firstly, when lysates from control and desensitised cells are analysed by differential centrifugation (Chuang and Costa 1979; Strulovici et al., 1983) or sucrose density gradient centrifugation (Harden et al., 1980; Waldo et al., 1983; Toews et al., 1984; Hertel et al., 1983a,b,), a change in the sedimentation properties of the β-adrenoceptors is observed following agonist treatment. Thus, in control cells nearly all the β-adrenoceptors are recovered in the heavier plasma membrane fraction along with Gs protein and adenylate cyclase activity, whereas in desensitised cells, a proportion of the receptors are sequestered out of the plasma membrane and recovered in a 'cytosolic' or 'light sucrose density fraction' devoid of Gs and enzyme activity. Sedimentation of these sequestered receptors at 150,000g lead to the suggestion that they are present in light membrane particles or vesicles (Stadel et al., 1983). Evidence from Strader et al., (1984) however, suggests that the appearance of vesicular particles is dependent on the method of cell lysis employed. Thus, the precise location of sequestered β-adrenoceptors is not known at present.

Secondly, during agonist-specific desensitisation, β-adrenoceptors appear to become less accessible to hydrophilic agonists and antagonists. This was first observed in radioligand binding studies employing intact cells, where agonist occupancy of β-adrenoceptors resulted in transient high-affinity receptor agonist interaction which was rapidly (1-2 minutes) followed by a decrease in apparent receptor affinity for agonists, as assessed by competition of unlabelled hydrophilic agonist for hydrophobic antagonist binding. The appearance

of the low affinity form of the receptor in these studies was taken to represent an internalised receptor to which hydrophilic agonists had limited access, and thus only slowly attained equilibrium (Pittman and Molinoff, 1980; Insel et al., 1983; Toews et al., 1983; Hoyer et al., 1984). Support for this hypothesis came from the observation that sotalol (a hydrophilic antagonist) also exhibited an apparent high and low affinity for β-adrenoceptors following agonist treatment of cells (Toews and Perkins, 1984). In addition, studies employing the recently developed relatively hydrophilic antagonist radioligand ^{3}H-CGP 12177 (^{3}H-CGP) (Staehelin et al., 1983; Staehelin and Hertel, 1983) showed that agonist treatment resulted in a rapid loss of β-adrenoceptors assessed by ^{3}H-CGP binding which parallelled a decrease in agonist stimulated adenylate cyclase activity, with no change in receptor number measured by the hydrophobic antagonist ^{3}H-DHA. These results were interpreted as representing no loss in β-adrenoceptors (since the hydrophobicity of ^{3}H-DHA probably allows it to label both surface and internalised receptors) but a sequestration of a proportion of the receptors into a compartment not accessible to ^{3}H-CGP. Furthermore, the internalised receptors were proposed to be located inside vesicles, since use of the pore forming agent alamethacin or the detergent digitonin revealed the hidden binding sites (Hertel et al., 1983a; Portenier et al., 1984). However, as with the cell fractionation studies described above, the existence of intracellular vesicles containing receptors is disputed, since studies employing an acid elution technique to remove cell surface radioligand binding have shown that, following agonist treatment of cells, not only was all the ^{3}H-CGP bound to receptors eluted by acid, but also all the ^{3}H-DHA bound

was removed as well, which suggested that the sequestered receptors were not intracellular (Mahan et al., 1985a).

Down regulation : In addition to the relatively rapid (i.e. minutes) uncoupling/internalisation events described above, agonist-specific desensitisation has also (as briefly mentioned previously) been reported to involve a slower (over a period of hours) actual loss of receptors measurable by radioligand binding assays, which is referred to as 'down-regulation' of receptors. The fate of these 'lost' receptors is as yet unknown since certain studies have reported that the receptors are proteolytically degraded and new receptor synthesis is required to regenerate them, and other studies have reported a return of receptor number to control levels in the absence of new protein synthesis (Franklin et al., 1975; Morishima et al., 1980; Doss et al., 1981; Su et al., 1976).

Precisely how these three mechanisms contribute towards agonist-specific desensitisation is unclear at present. Certain studies have shown that in astrocytoma cells, a rapid functional uncoupling of β-adrenoceptors by desensitisation occurs prior to receptor sequestration, and that concanavalin A treatment prevents sequestration but not desensitisation in these cells (Waldo et al., 1983; Toews et al., 1984). In contrast, similar studies performed in S49 lymphoma (Hoyer et al., 1984) and embryonic chick heart cells (Linden et al., 1984) showed that the rates of both processes appear to be the same. β-adrenoceptor uncoupling from adenylate cyclase was thus proposed to occur as a consequence of, rather than a prelude to, receptor

sequestration.

Furthermore, there have been conflicting results as regards whether or not β-adrenoceptors, in addition to becoming uncoupled and/or internalised, are functionally modified during agonist-specific desensitisation. Kassis et al., employing membrane fusion techniques, demonstrated that the functional activity of the β-adrenoceptor was reduced during desensitisation (Kassis and Fishman, 1984; Kassis et al., 1985) and that desensitisation could occur in the apparent absence of receptor sequestration (Kassis et al., 1986). In contrast, Strasser et al., employing reconstitution techniques, have shown that β-adrenoceptors purified from control and desensitised cells were functionally equivalent (Strasser et al., 1985) and that treatment of desensitised cells with the fusogen polyethylene glycol, restores β-agonist stimulated adenylate cyclase activity and the ability to form a high affinity guanine nucleotide sensitive state of the receptor (Strasser and Lefkowitz, 1985). These latter data were taken to suggest that agonist-specific desensitisation results from the physical separation of β-adrenoceptors from other components of the cyclase system. Results of reconstitution and fusion studies should however be viewed with caution, since the procedures themselves may reverse a functional alteration (e.g. phosphorylation, see below), and also different receptor populations may have been examined e.g. plasma membrane fractions employed for fusion studies may not involve sequestered receptors.

Recent studies have shown that agonist-specific desensitisation is

associated with increased phosphorylation of the β-adrenoceptors (Sibley et al., 1985; Strasser et al., 1986a). This phosphorylation is said to be mediated by a novel, non-cyclic AMP-dependent Kinase, the β-adrenergic receptor Kinase (β-ARK) (Benovic et al., 1986a). β-ARK is apparently independent of any known second messenger, and phosphorylates the β-adrenoceptor only when agonist is bound to the receptor (Benovic et al., 1987); unoccupied or antagonist-occupied receptors appear not to be a substrate for the enzyme. This Kinase is cytosolic and apparently upon agonist stimulation is translocated to the plasma membrane where it phosphorylates the receptor (Strasser et al., 1986b) and decreases its functional activity (Benovic et al., 1986b). Reactivation of the receptor at some stage is proposed to occur via dephosphorylation and thought to involve a "receptor phosphatase" (Sibley et al., 1986), but little is known of this enzyme at present. Although many aspects of the involvement of phosphorylation/dephosphorylation reactions in agonist-specific desensitisation of the β-adrenoceptor remain to be clarified, the current working hypothesis is as follows: agonist occupancy of the β-adrenoceptor leads to a brief stimulation of cyclase and rapid translocation of β-ARK from cytosol to plasma membrane. Phosphorylation of the receptor (presumably at previously unexposed sites) results in decreased ability of the receptor to couple with Gs, followed at some time point by sequestration of the receptor. Within the 'sequestered compartment' the receptors are reactivated by dephosphorylation and eventually recycle back to the cell surface to recouple with other components of the cyclase system (see Lefkowitz et al., 1986). β-ARK has also been shown to phosphorylate rhodopsin (Benovic et al., 1986c), and to be

translocated from cytosol to plasma membrane following PGE_1 stimulation (Strasser et al., 1986b). It may therefore be an adenylate cyclase-coupled receptor Kinase, and not merely a specific β-adrenoceptor Kinase.

Coupling of the β-adrenoceptor to a functional Gs as a prerequisite for the processes of desensitisation and internalisation however is disputed. Green and Clark, employing cyc^- S49 lymphoma cells which lack the α_s subunit of Gs, have shown by means of reconstitution experiments that agonist occupation of β-adrenoceptors in the absence of receptor-Gs coupling or cyclic AMP generation is sufficient to promote desensitisation in these cells (Green and Clark, 1981; Green et al., 1981). Furthermore, sequestration of the β-adrenoceptor also occurs in cyc^- cells, as measured by an adrenaline-induced shift in cyc^- cell β-adrenoceptors to a light vesicle fraction (Clark et al., 1985) and by an isoprenaline-induced loss of surface receptors on intact cyc^- cells (Staehelin and Hertel, 1983; Mahan et al., 1985b). Agonist-induced down-regulation of β-adrenoceptors however, is either absent or blunted in these cells (Shear et al., 1976; Su et al., 1980; Mahan et al., 1985b) which implies that receptor-Gs interaction influences the fate of β-adrenoceptors.

Despite major advances in the understanding of the molecular events that accompany agonist-specific desensitisation, it is still unclear as to how uncoupling and/or internalisation (±phosphorylation/dephosphorylation) and down-regulation are linked. To date, much of the available information has been attained from studies employing animal tissues or

cultured cell lines and hence little is known about these processes in human cells.

The aim of the study described in this chapter was to examine the events occuring following agonist treatment of human platelets. However, in view of the fact that the β_2-adrenoceptor present on the platelet did not appear to be coupled to Gs (Chapter III) and hence adenylate cyclase (Chapter IV) in the present study, desensitisation and uncoupling could not be evaluated, and so the experiments to be described focused on exploring agonist-induced redistribution of the β-adrenoceptors.

Several experimental approaches have previously (see above) been employed to examine the topographical localisation of β-adrenoceptors on intact cells. The method selected for use in the present study was that of evaluating the differential accessibility of hydrophilic versus hyrophobic ligands. In previous intact cell studies it has been observed that the hydrophilic β-antagonist (±)CGP 12177 is relatively membrane impermeant at low concentrations and binds selectively to cell surface receptors. On the other hand, relatively hydrophobic compounds such as ^{125}I(-)PIN and (-)propranolol distribute internally and identify intracellular as well as cell surface receptors (Staehelin and Simons, 1982; Staehelin et al., 1983; Staehelin and Hertel, 1983; Hertel et al., 1984). In the experiments to be described, the differential permeability effect of (±)CGP 12177 and (-)propranolol on ^{125}I(-)PIN binding was examined. ^{125}I(-)PIN binding inhibited by (-)propranolol was taken to represent total β-adrenoceptor number,

whilst that in the presence of (±)CGP 12177 was taken to represent cell surface receptor number. Binding assays were performed at physiological temperature (37°C) and at a lowered temperature (4°C) to prevent receptor recycling (Hertel and Staehelin , 1983).

The study is presented in two parts : The first (VI.3) examines ^{125}I(-)PIN binding to intact human platelets in the absence of agonist pretreatement i.e. control cells. The second (VI.4) examines ^{125}I(-)PIN binding to agonist-treated platelets.

VI.2. Methods

VI.2.1. Platelet Preparation

Platelet rich plasma (PRP) was obtained as previously described (II.3.2.2.) and incubated at 37°C for 30 minutes (unless otherwise stated) in the presence (treated cells) or absence (control cells) of 10uM (-)isoprenaline (unless otherwise stated). Platelet suspensions were then prepared essentially as described (II.3.2.2.) but with all manipulations being performed at 2-4°C. In addition, the cells were washed once in 50vol of Tris Isosaline pH 6.5 before final resuspension in "whole cell" assay buffer (Tris Isosaline pH 7.8). Platelet membranes were then prepared from the final platelet suspensions using the method described in II.3.2.1.

VI.2.2. Binding Assay

Platelet suspensions were assayed as previously described (II.2.3.7.) for 40 minutes at 37°C or 5 hours at 4°C. Assays were terminated by the addition of 10.0ml ice cold assay buffer diluted 1:10 with water. Tubes were kept on ice for 30 minutes before rapid vacuum filtration. This incubation with ice-cold hypotonic buffer substantially lowered non-specific binding without reducing specific binding (figure VI.1.) and thus significantly improved the total binding : non-specific binding ratio as has been reported by others (Staehelin et al., 1983). Non-specific binding of ^{125}I(-)PIN was defined in the presence of the lipophilic non selective antagonist (-)propranolol (1uM), or the relatively hydrophilic antagonist (±)CGP 12177 (1uM) as appropriate. These concentrations of antagonists were determined from displacement experiments of ^{125}I(-)PIN binding to intact platelets (figure IV.2.). Specific binding, defined as total minus non-specific binding at both temperatures, was around 70-80% of total binding at the concentration of ^{125}I(-)PIN (30-40pM) used in competition experiments.

Platelet membranes were assayed as previously described at room temperature (III.2.1.) or in 'whole cell' assay buffer for 40 minutes at 37°C or 5 hours at 4°C.

VI.2.3. Sucrose-density gradient centrifugation of lysates from control and agonist treated intact human platelets

Intact human platelets were incubated with (treated cells) or without (control cells) 10uM (-)isoprenaline for 30 minutes at 37°C, washed at 4°C as previously described (VI.2.1.), and cell lysates prepared by

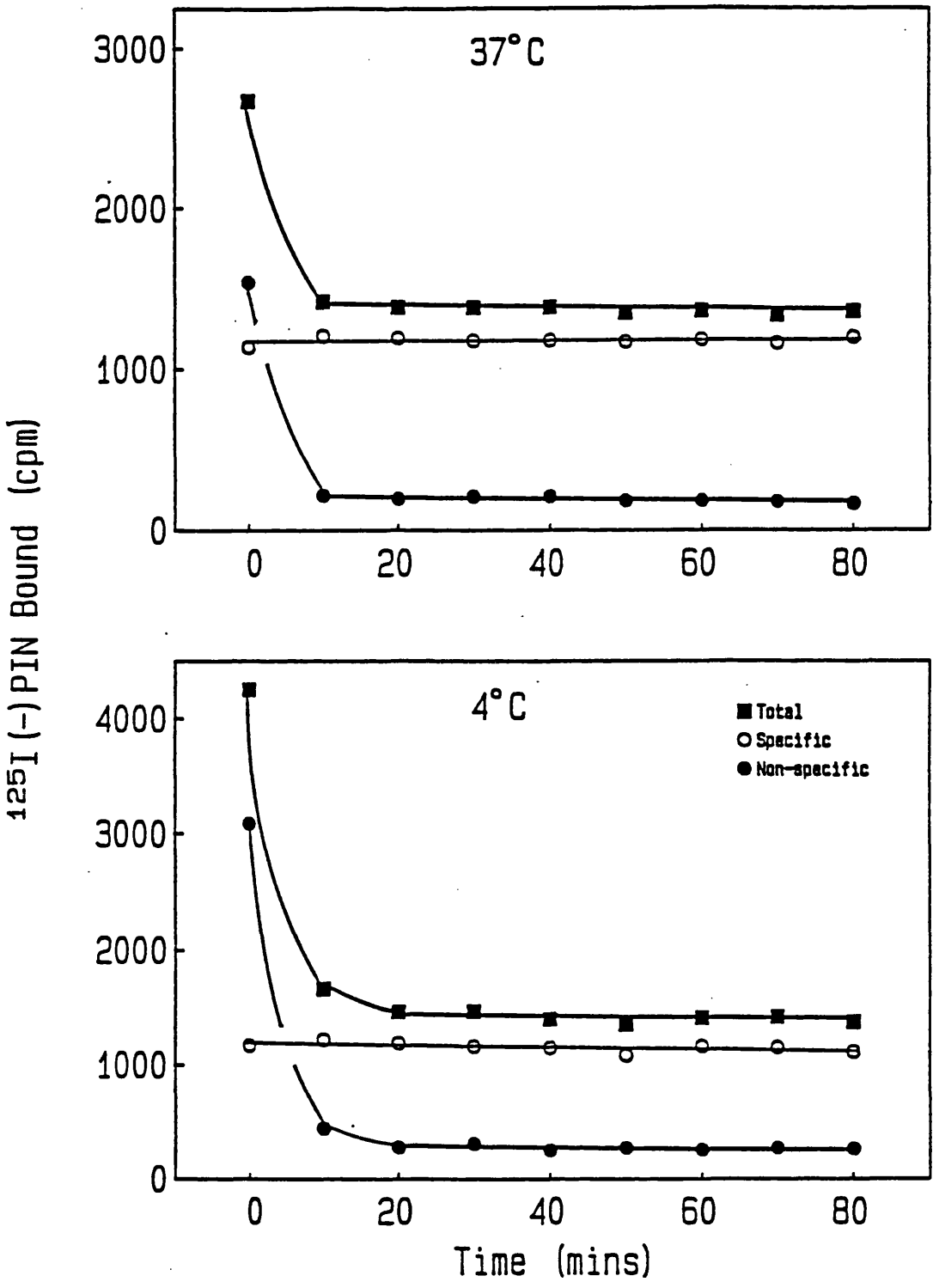


Figure VI.1. ^{125}I(-)PIN binding to intact human platelets at 37°C and 4°C

Intact human platelets were incubated in the presence of ^{125}I(-)PIN (100pM) at 37°C (upper panel) or 4°C (lower panel) as described in the methods section. Assays were terminated by the addition of 10ml ice cold assay buffer diluted 1:10 with water, and tubes kept on ice for the time intervals shown, prior to rapid vacuum filtration. The figure illustrates raw, untransformed data of total, specific and non-specific binding in the presence of 1uM(-)propranolol, from a single representative experiment performed in triplicate.

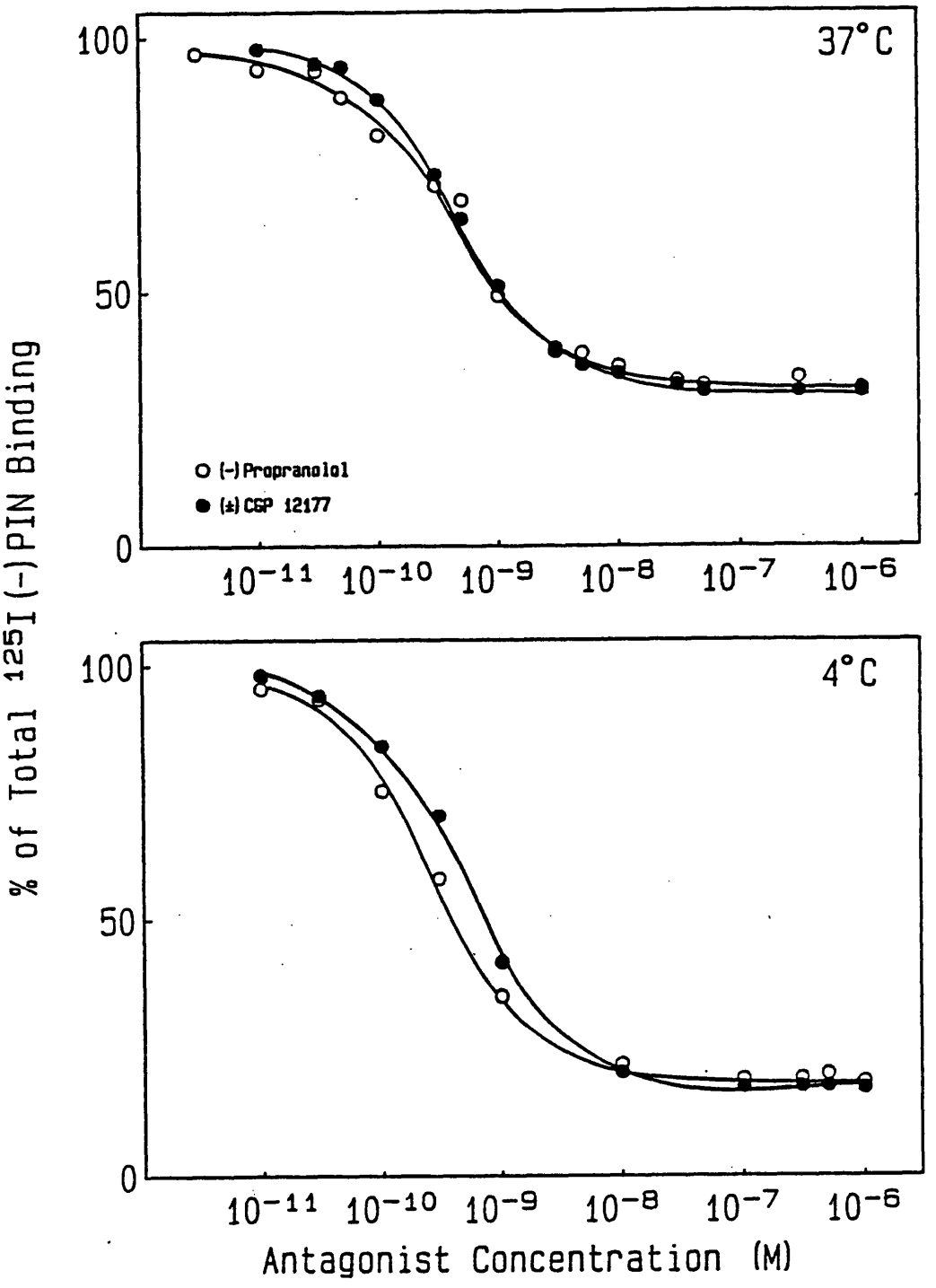


Figure VI.2. Displacement of total ^{125}I(-)PIN binding to intact human platelets by (-)propranolol and (±)CGP 12177

Cells were incubated to equilibrium at 37°C (upper panel) or 4°C (lower panel) with ^{125}I(-)PIN (30-40pM) and increasing concentrations of displacing drugs as described in the methods section. The data shown represent the mean of at least three separate experiments performed in duplicate. SEM for each point was $\leq \pm 5\%$.

incubating the washed platelet pellet in 1mM Tris HCl, pH 7.4 for 30 minutes on ice.

Discontinuous sucrose density gradients were prepared at 4°C essentially as described by Kassis and Sullivan, (1986). 10.0ml 50% sucrose was overlayered with 15.0ml 30% sucrose in a 40.0ml centrifuge tube. Cell lysates (5.0ml) prepared as described above were layered on top of the gradients. Centrifugation was carried out in a Centronik Ultra Centrifuge at 24,000rpm (75,000g) for 90 minutes at 4°C. Fractions (1.5ml) were collected from the top of the gradients and assayed for ^{125}I(-)PIN binding (III.2.1.) and adenylate cyclase activity.

Enzyme activity in each fraction was assayed by Mary Keen (Department of Pharmacology and Therapeutics, University of Leicester) employing the method of Olianas et al., (1983). ^{32}P-cyclic AMP was isolated by the method of Salomon et al., (1974).

VI.3. ^{125}I(-)PIN binding to intact human platelets (control cells) at 4°C and 37°C

VI.3.1. Results

VI.3.1.1. Equilibrium and Kinetic studies

Under the conditions chosen, specific binding of ^{125}I(-)PIN to intact human platelets was rapid and reversible at 37°C with a t1/2 (time for

half maximal binding) for association of 7-9 minutes, and dissociation of 16-18 minutes (figure VI.3.). For the illustrated data, the second order association rate constant was calculated to be K_1=1.508 nM^{-1} min^{-1}. Dissociation followed monophasic first order kinetics with a dissociation constant K_2 of 0.0416 min^{-1}. The mean kinetically derived Kd (K_2/K_1) calculated from three such experiments was 23.75±2.73 pM, in excellent agreement with that obtained from equilibrium experiments. At 4°C, specific ^{125}I(-)PIN binding to intact human platelets was slower at association and dissociation compared with 37°C (figure VI.4.), with a t1/2 association of 1-2 hours and t1/2 dissociation of >5 hours. For the data illustrated in figure VI.4. the association constant K_1 was calculated to be 0.2856 nM^{-1} hr^{-1}, and dissociation appeared to follow monophasic first order Kinetics with a dissociation constant of K_2=0.00225 hr^{-1}. The kinetically derived Kd calculated from three such experiments, of 8.17±2.54 pM, was however of slightly higher affinity than that obtained from equilibrium experiments. This discrepancy may well be due to the relatively slow dissocation rate of ^{125}I(-)PIN binding at 4°C which makes accurate determinations of K_2 difficult.

Specific binding of ^{125}I(-)PIN to intact human platelets at 4°C and 37°C, defined as the binding displaceable by 1uM(±)CGP 12177 or 1uM(-)propranolol, was saturable (figures VI.5. and VI.6.). The maximum number of binding sites (Bmax) assessed by Scatchard analysis were not significantly different at 37°C and 4°C whether defined in the presence of (-)propranolol or (±)CGP 12177 (figure VI.6. and table VI.1.). In all cases the data produced linear Scatchard plots with

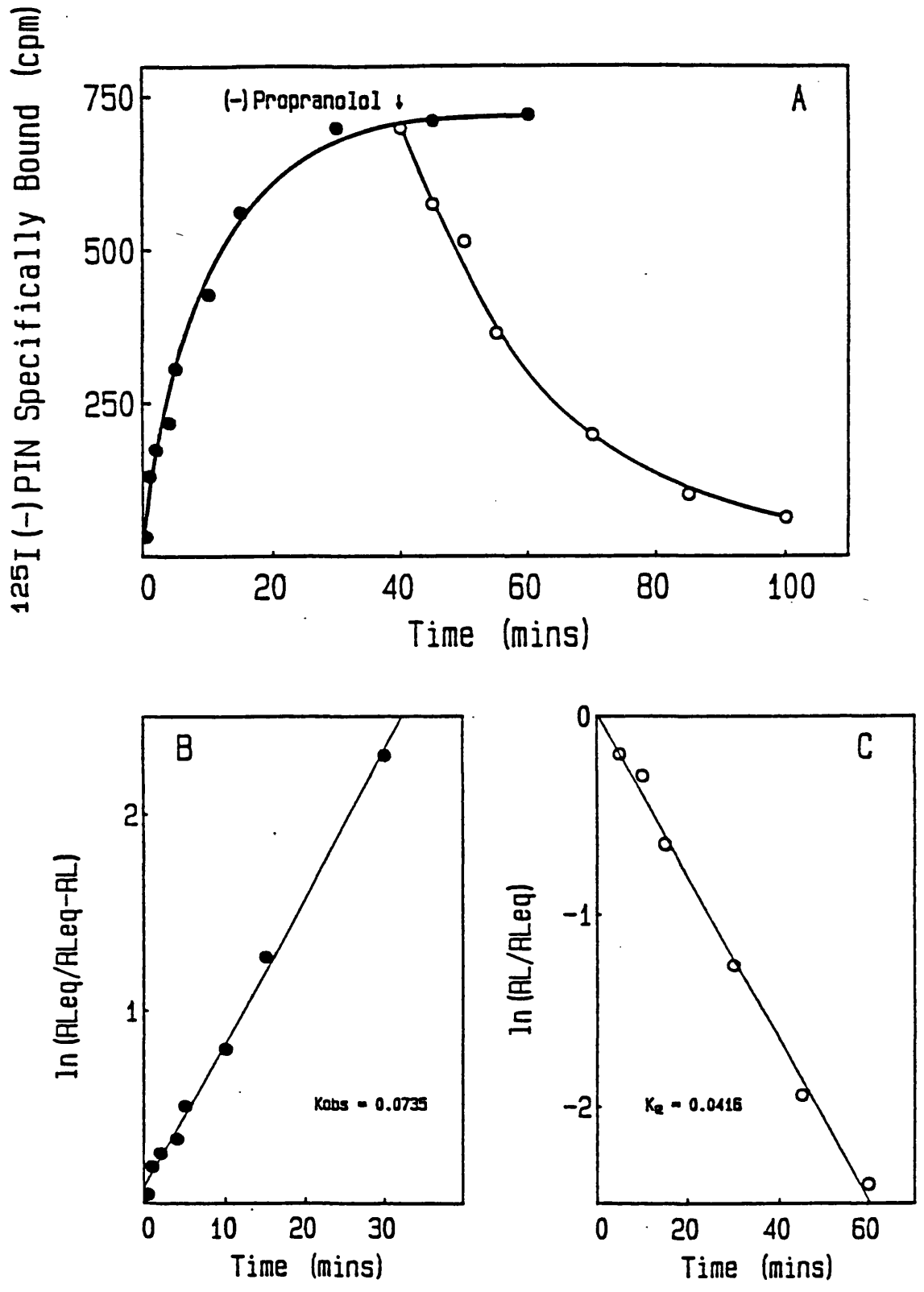


Figure VI.3. Kinetics of specific ^{125}I(-)PIN binding to intact human platelets at 37°C

Legend to Figure VI.3. Kinetics of specific ^{125}I(-)PIN binding to intact human platelets at 37°C

A) Time course of specific ^{125}I(-)PIN association (•) and dissociation (o). Dissociation was initiated by the addition of 1uM(-)propranolol after equilibrium had been reached (40 minutes).

B) Pseudo first-order rate plot for the association reaction

C) first-order rate plot for the dissociation reaction.

The Kinetically derived Kd was calculated as described in Appendix 3b. Data shown are from a single experiment representative of three experiments performed in duplicate.

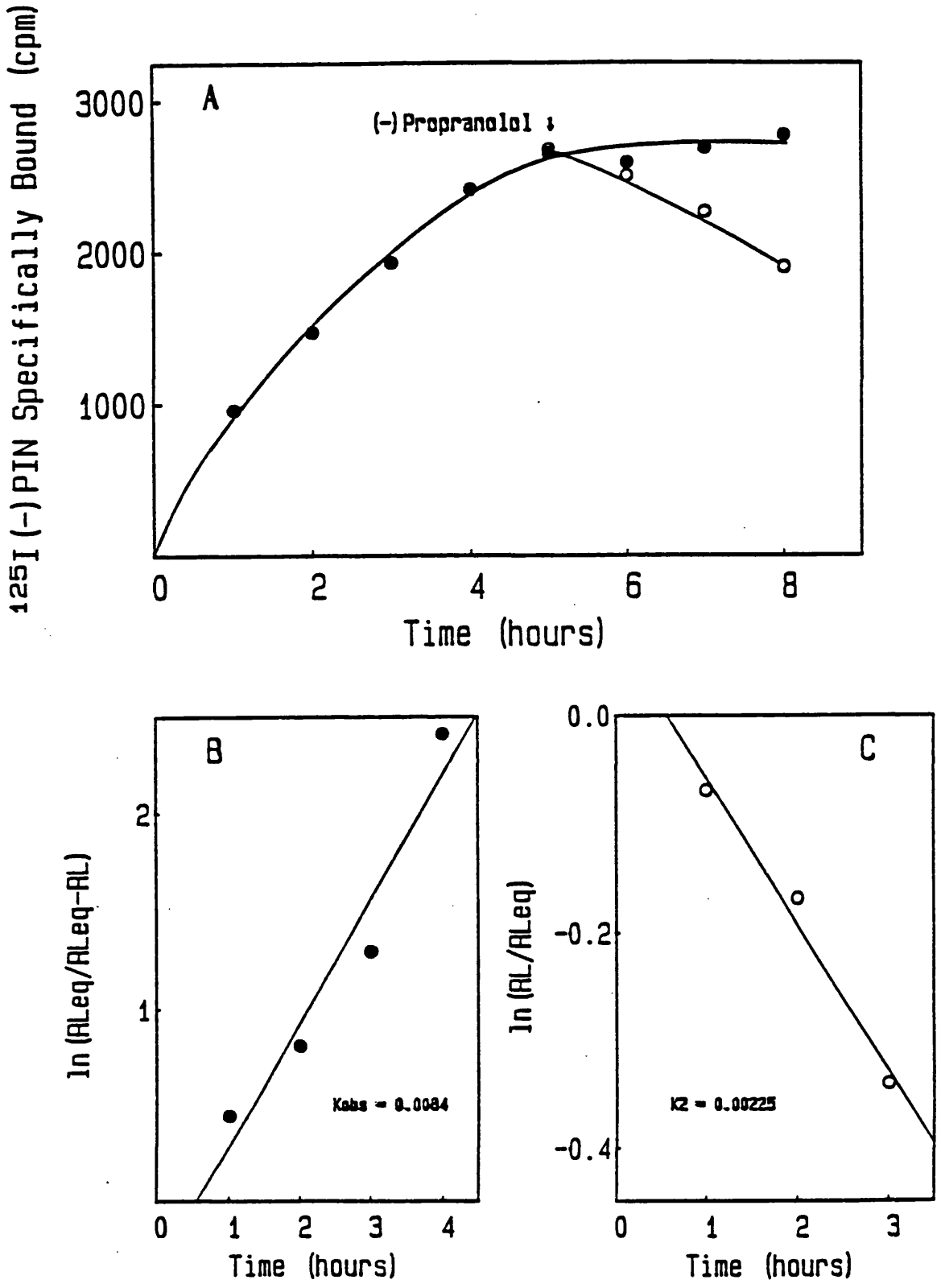


Figure VI.4. Kinetics of specific ^{125}I(-)PIN binding to intact human platelets at 4°C

Legend to Figure VI.4. Kinetics of specific ^{125}I(-)PIN binding to intact human platelets at 4°C

A) Time course of specific ^{125}I(-)PIN association (•) and dissociation (o). Dissociation was initiated by the addition of 1uM(-)propranolol after equilibrium had been reached (5 hours).

B) and

C) Pseudo first-order rate plots for the association reaction and dissociation reaction respectively. The Kinetically derived Kd was calculated as described in Appendix 3b. Data shown are from a single experiment representative of three experiments performed in duplicate.

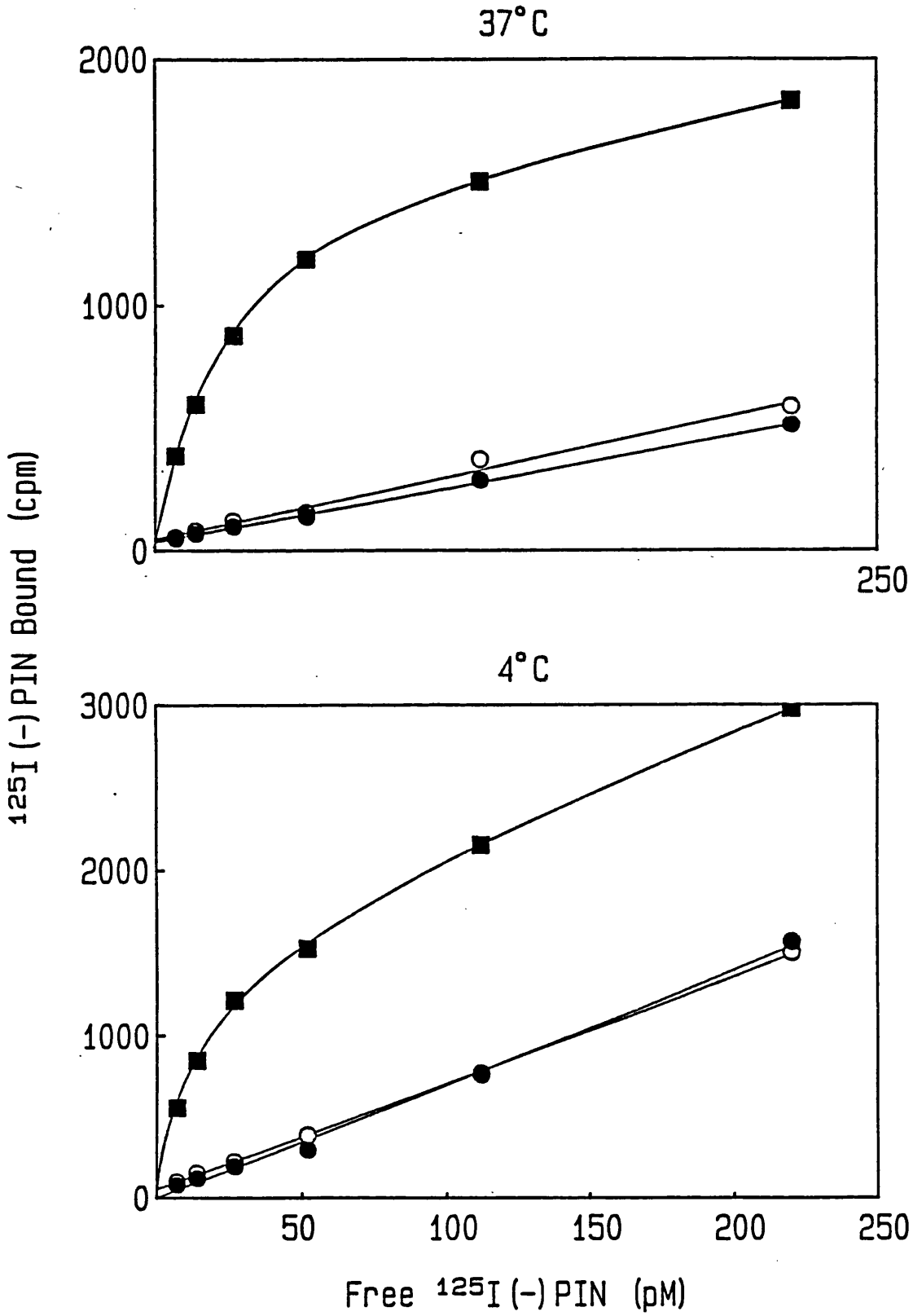


Figure VI.5. ^{125}I(-)PIN binding to intact human platelets at 4°C and 37°C

Saturation data shown are from a single representative experiment. The figure illustrates raw, untransformed data of total binding (■) and non-specific binding in the presence of 1uM (±)CGP 12177 (o) or 1uM (-)propranolol (●) at 37°C (upper panel) and 4°C (lower panel).

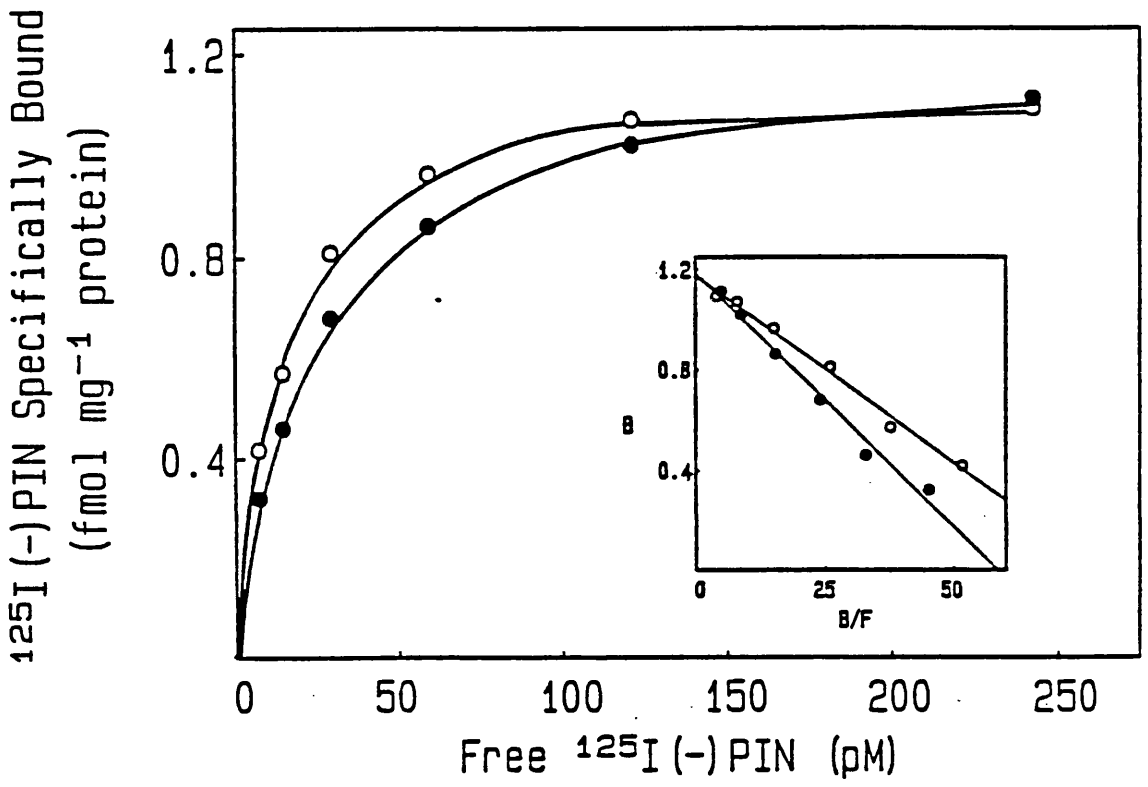


Figure VI.6. Specific ^{125}I(-)PIN binding to intact human platelets at 4°C (o), and 37°C (•)

Inset shows Scatchard plot of data where B represents specifically bound ^{125}I(-)PIN expressed as fmol mg^{-1} protein and F represents the free concentration of ^{125}I(-)PIN in pM. Data shown are from a single representative experiment in which specific binding was defined as ^{125}I(-)PIN binding displaceable by 1uM(-)propranolol. Data from the same experiment (illustrated in figure VI.5.) in which specific binding was defined in the presence of 1uM (±)CGP 12177, gave identical results (not shown for clarity). The mean Kd and Bmax values from at least three separate experiments are given in table VI.1..

Table VI.1. Apparent equilibrium dissociation constants (Kd) and receptor density (Bmax) of ^{125}I(-)PIN binding to intact human platelets

Temperature	Agent used to define Non-specific binding	Bmax (fmol mg^{-1} protein)	Kd (pM)
37°C	1uM (-)propranolol	1.21±0.13	22.62±0.31
	1uM (±)CGP 12177	1.17±0.12	22.58±1.04
4°C	1uM (-)propranolol	1.23±0.06	16.17±1.49
	1uM (±)CGP 12177	1.25±0.09	17.21±1.3

Dissociation constants and number of binding sites were determined at 4°C and 37°C simultaneously. Specific binding was assessed (as described in the methods section) in the presence of 1uM (-)propranolol or 1uM (±)CGP 12177 at both temperatures. Data are expressed as mean ± SEM (n=3). Results were analysed using Student's 't' test for paired data none of the values were significantly different ($p>0.05$).

Hill slope values not significantly different from unity, indicative of a homogenous population of binding sites. The dissocation constant (Kd) for ^{125}I(-)PIN, defined in the presence of either (-)propranolol or (±)CGP 12177, was virtually identical at 4°C and 37°C (table VI.1). The affinity of the receptors for the radioligand was, however, slightly higher at 4°C.

VI.3.1.2. Pharmacological characterisation

The binding of ^{125}I(-)PIN to intact human platelets was indicative of an interaction with a β-adrenoceptor. Data presented in table VI.2. and figures VI.7. and VI.8. indicate that the binding was stereoselective with the (-)isomer of propranolol being more potent than the (+)isomer by approximately two orders of magnitude. The overall affinities of the non-selective β-adrenoceptor antagonists : (-)propranolol, (+)propranolol and (±)CGP 12177, and the selective β-adrenoceptor antagonists : ICI 118,551 (β_2-selective), betaxolol and CGP 20172A (β_1-selective) were virtually identical at 37°C and 4°C. These antagonists generated steep displacement curves with Hill slope factors not significantly different from unity ($p>0.1$, table VI.2.) at both temperatures, which suggests binding to a single class of sites with binding governed by the law of mass action. In addition, the selective β-adrenoceptor antagonists exhibited affinities for the binding similar to those previously observed in human platelet membranes in the present study (Chapter III) and at the β_2 subtype in other mammalian tissues (Dickinson et al., 1981; Dooley et al., 1986).

Table VI.2. Overall IC_{50} values and Hill slope factors (nH) for β-adrenoceptor antagonist displacement of $^{125}I(-)PIN$ binding to intact human platelets

	IC_{50} [M]* (nH)	
	4°C	37°C
(-)propranolol	$3.02 \pm 0.82 \times 10^{-10}$ (0.94)	$3.83 \pm 0.38 \times 10^{-10}$ (0.93)
(+)propranolol	$4.30 \pm 0.14 \times 10^{-8}$ (1.08)	$2.51 \pm 0.08 \times 10^{-8}$ (0.95)
(±)CGP 12177	$6.16 \pm 1.05 \times 10^{-10}$ (0.93)	$5.30 \pm 0.09 \times 10^{-10}$ (0.99)
(±)betaxolol	$1.69 \pm 0.40 \times 10^{-7}$ (0.99)	$1.30 \pm 0.11 \times 10^{-7}$ (1.02)
(±)ICI 118,551	$6.60 \pm 0.40 \times 10^{-10}$ (0.99)	$7.87 \pm 0.78 \times 10^{-10}$ (0.88)
CGP 20172A	$1.80 \pm 0.42 \times 10^{-5}$ (1.04)	$4.29 \pm 0.30 \times 10^{-6}$ (0.95)

* The values given are mean ±SEM of at least three separate experiments performed in duplicate and were derived from computer-assisted (Allfit) simultaneous curve fitting analysis of individual curves, as described in appendix 3. Hill slopes quoted are not significantly different from unity ($p > 0.1$).

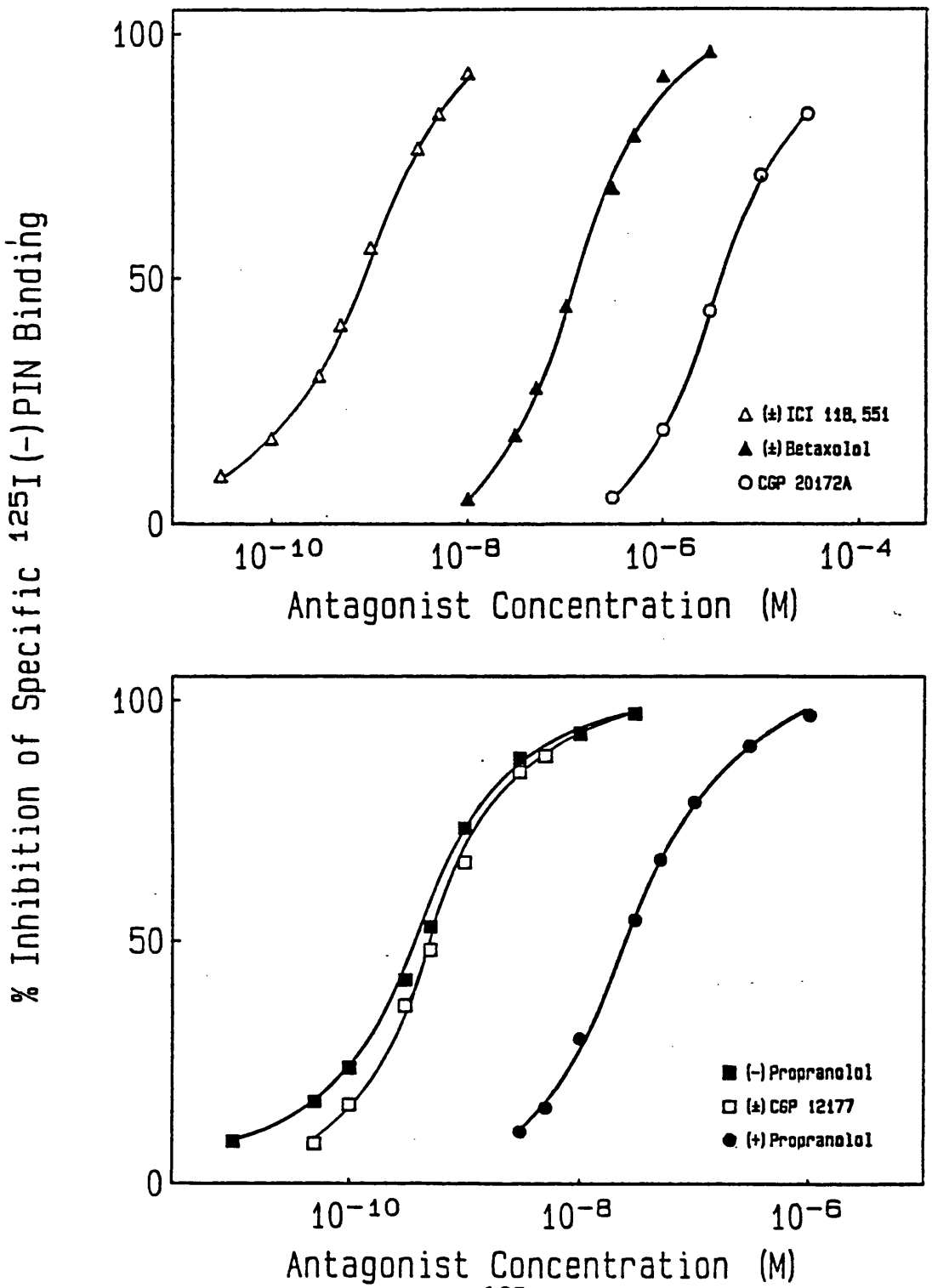


Figure VI.7. Inhibition of specific ^{125}I(-)PIN binding to intact human platelets at 37°C by adrenergic antagonists

Intact human platelets were incubated with ^{125}I(-)PIN (30-40pM) at 37°C for 40 minutes, in the presence of increasing concentrations of competing agent, and specific binding determined. The data shown for each compound are the mean curves from at least three separate experiments performed in duplicate. SEM for each point was $<\pm$5%.

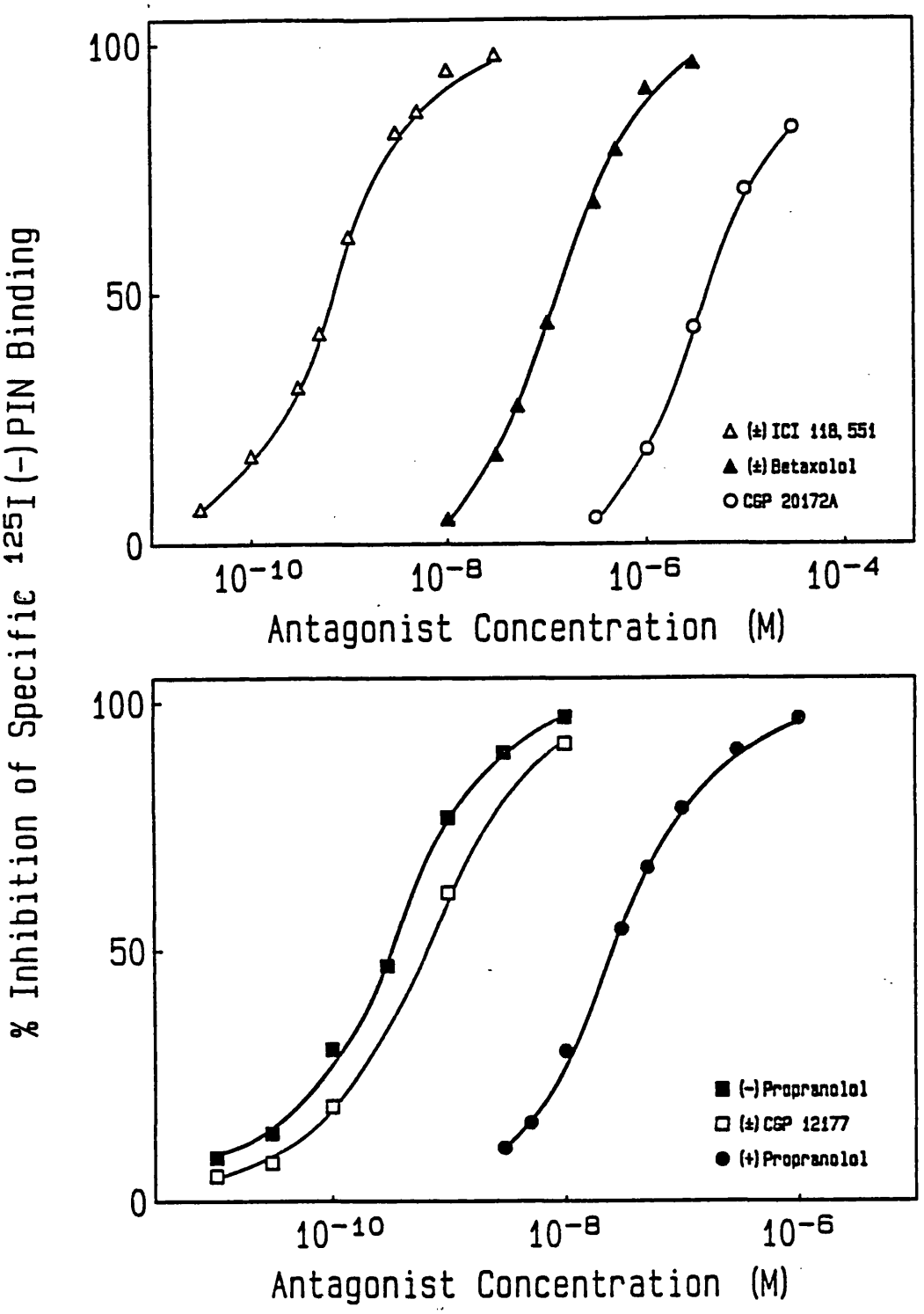


Figure VI.8. Inhibition of specific ^{125}I(-)PIN binding to intact human platelets at 4°C by adrenergic antagonists

Intact human platelets were incubated with ^{125}I(-)PIN (30-40pM) at 4°C for 5 hours, in the presence of increasing concentrations of competing agent, and specific binding determined. The data shown for each compound are the mean curves from at least three separate experiments performed in duplicate. SEM for each point was $<\pm 5\%$.

The displacement of ^{125}I(-)PIN binding to intact human platelets by adrenergic agonists was also indicative of a β-adrenoceptor (table VI.3.) and the hierarchy of potency displayed, (-)isoprenaline > (-)adrenaline > (-)noradrenaline, is typical of the β_2 subtype. These agonists displaced ^{125}I(-)PIN binding with similar overall affinity in intact human platelets at 4°C and 37°C (figure VI.9., table VI.3.). However, agonist competition curves at 37°C yielded shallow binding isotherms with Hill slope factors significantly different from unity ($p<0.01$, table VI.3.). Computer-assisted curve fitting analysis of these curves revealed that the data for isoprenaline and adrenaline were best fitted to a two-site model ($p<0.002$), whereas that for noradrenaline was best fitted to a one-site model ($p>0.5$). Lowering of the assay temperature from physiological (37°C) to 4°C resulted in a significant steepening ($p<0.01$) of the isoprenaline and adrenaline curves only. No significant change in the slope of the noradrenaline displacement curve was observed (figure VI.9., table VI.3.).

Isoprenaline displacement curves performed in platelet membranes resulted in virtually identical binding isotherms at 4°C and 37°C (figure VI.9.) with overall IC_{50} values of $7.43\pm0.71\times10^{-7}$ and $5.95\pm0.08\times10^{-7}$ respectively, and slope factors not significantly different from unity. As with whole cells, the Kd for ^{125}I(-)PIN binding to human platelet membranes was slightly lower at 4°C compared with 37°C (12.21±0.63 and 22.41±1.18 pM respectively). Therefore, although Kd's were similarly influenced by temperature in whole cells and membranes, this was not reflected in the overall affinity (IC_{50}) of isoprenaline in competition experiments performed in membranes. Thus

Table VI.3. Overall IC_{50} values and Hill slope factors (nH) for β-adrenoceptor agonist displacement of $^{125}I(-)PIN$ binding to intact human platelets

Temperature	Agonist	IC_{50} [M] (nH)
4°C	(-)isoprenaline	$1.74 \pm 0.37 \times 10^{-7}$ ($0.73^{*} \pm 0.03$)
	(-)adrenaline	$6.07 \pm 1.21 \times 10^{-7}$ ($0.75^{*} \pm 0.01$)
	(-)noradrenaline	$1.35 \pm 0.35 \times 10^{-5}$ (0.92 ± 0.03)
37°C	(-)isoprenaline	$4.00 \pm 0.20 \times 10^{-7}$ ($0.59^{*} \pm 0.01$)**
	(-)adrenaline	$2.20 \pm 0.11 \times 10^{-6}$ ($0.52^{*} \pm 0.02$)**
	(-)noradrenaline	$4.30 \pm 0.92 \times 10^{-5}$ ($0.87^{*} \pm 0.05$)**

All data represent mean ± SEM from at least three separate experiments performed in duplicate. IC_{50} and Hill slope factors were derived from simultaneous curve fitting (Allfit) analysis of individual curves as described in Appendix 3. (Mean curves illustrated in figure VI.9.).

*indicate slope is significantly different from unity ($p<0.01$)
**indicate slope at 4°C is significantly different from slope at 37°C for same agonist ($p<0.05$).

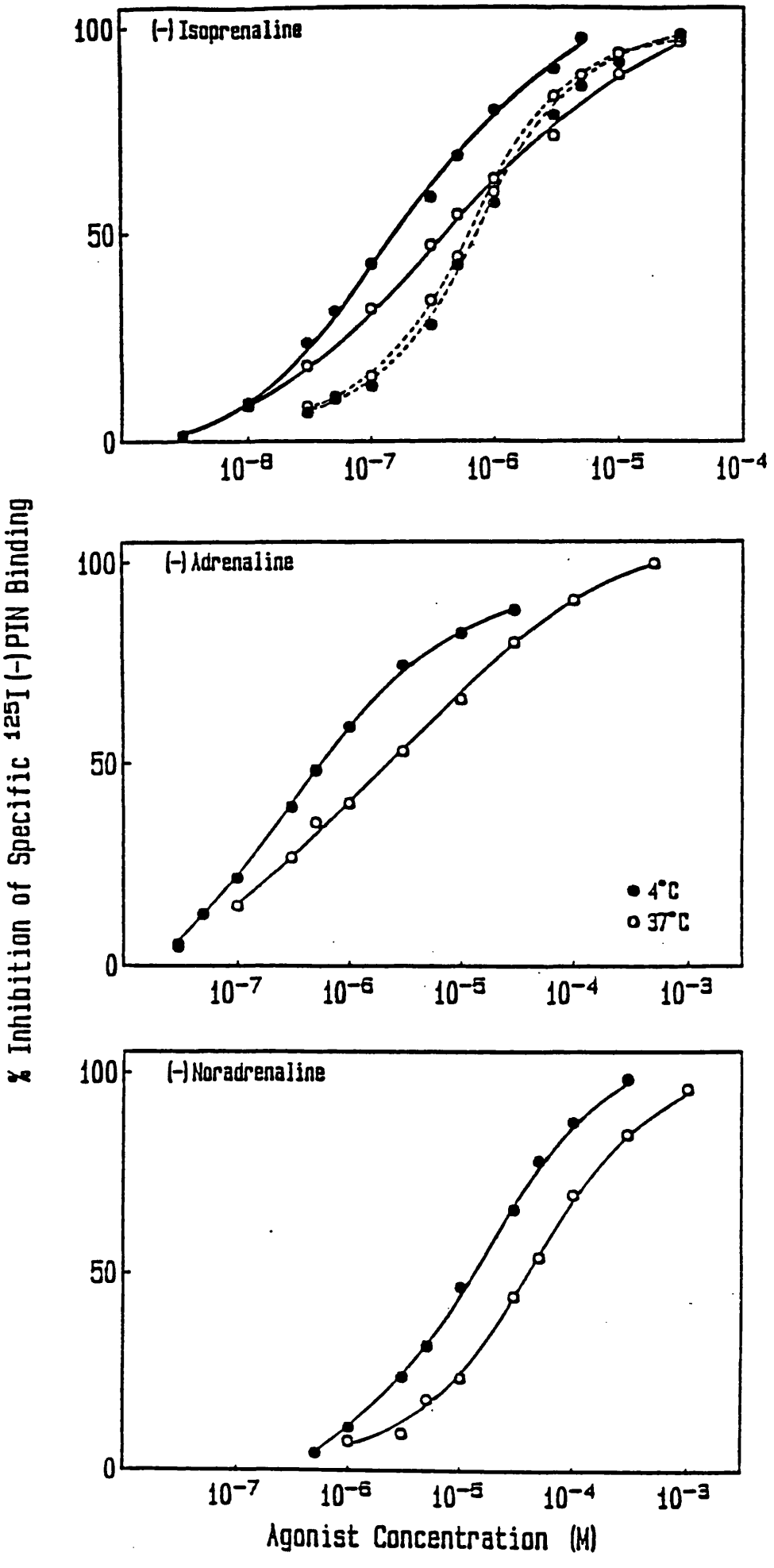


Figure VI.9. Inhibition of specific ^{125}I(-)PIN binding by adrenergic agonists at 4°C (●) and 37°C (o)

Legend to Figure VI.9. Inhibition of specific ^{125}(-)PIN binding by adrenergic agonists at 4°C (•) and 37°C (o)

Intact human platelets (solid lines) or human platelet membranes (dotted lines) were incubated with ^{125}I(-)PIN (30-40pM) at 4°C for 5 hours (•), or 37°C for 40 minutes (o) in the presence of increasing concentrations of competing agent, and specific binding determined. The data shown for each agonist is the mean of at least three separate experiments performed in duplicate. SEM for each point was $< \pm 5\%$.

the differential temperature effects on agonist interactions appear to be a characteristic of the intact cell.

VI.3.2. Discussion

The results of this study show that the receptor identified on intact human platelets using ^{125}I(-)PIN exhibits the expected properties of a β_2-adrenoceptor and thus extends and confirms the earlier study performed in platelet membranes (Chapter III). Binding was saturable, stereospecific and of high affinity. Furthermore, inhibition of ^{125}I(-)PIN binding by both selective and non-selective β-adrenoceptor antagonists resulted in steep monophasic displacement curves with Hill slope factors not significantly different from unity at 4°C or 37°C, thus indicative of interaction with a homogenous population of binding sites. The overall affinity of the antagonists was virtually identical at both temperatures and in excellent agreement with those previously found in membranes (Chapter III) and at other mammalian β_2-adrenoceptors (Rugg et al., 1978; Dickinson et al., 1981).

These data differ from those of Kerry et al., 1984 who examined ^{125}I-CYP binding to intact human platelets. The reported affinities of certain antagonist drugs, e.g. ICI 118,551 and (-)propranolol, in their study were 1-2 fold weaker than those expected for interaction with a β_2-adrenoceptor. The reason for this is not clear. It does not appear to be due to differences in membrane and intact cell binding characteristics as shown by the present study, but may well be related to the use of ^{125}I-CYP which is a poor radioligand as compared with

^{125}I(-)PIN (Chapter III; Szefler et al., 1987).

Specific binding of ^{125}I(-)PIN, defined by 1uM (±)CGP 12177 or 1uM(-)propranolol, was identical at 37°C and 4°C. If the assumption that (-)propranolol displaceable binding = total receptor number, and (±)CGP 12177 displaceable binding = cell surface receptor number is correct, then these results suggest that in the absence of an agonist the β_2-adrenoceptors on human platelets are situated solely on the cell surface. It has been suggested in other cell types that ^{125}I(-)PIN labels both surface and intracellular receptors at 37°C and only surface receptors at 4°C (Linden et al., 1984; Harden, 1983), presumably due to a reduced hydrophobicity at the lower temperature. When the hydrophobicity of ^{125}I(-)PIN was examined (table VI.4.) at various temperatures, the results obtained suggest that ^{125}I(-)PIN becomes more hydrophobic, i.e. 37°C >room temperature >4°C, with decreasing temperature when incubated for 48 hours, or for assay incubation times of 40 minutes at 37°C and room temperature, and 5 hours at 4°C. To verify this method for ^{125}I(-)PIN, comparable experiments were performed with 3H-CGP 12177. The values obtained were in excellent agreement with those previously quoted for this radioligand (table VI.4.). Furthermore, the observation that non-specific binding of ^{125}I(-)PIN was slightly higher at 4°C when compared with 37°C (figure VI.5.) also suggested that ^{125}I(-)PIN is not restricted to the cell surface in human platelets at 4°C. This is discussed further in VI.4.2.

The results of displacement experiments performed at 37°C and 4°C are

Table VI.4. Tris Isosaline : Octanol partitioning of β-adrenoceptor radioligands

Radioligand	Temperature	-log P (after assay incubation times)	-log P (after 48 hours)
^{125}I(-)PIN	37°C	1.01	0.73
	Room temperature	1.06	0.976
	4°C	1.22	1.39
^{3}H-CGP 12177	Room temperature		0.377

^{125}I(-)PIN (100pM) or ^{3}H-CGP 12177 (1nM) were diluted in Tris Isosaline pH7.8 and mixed with an equal volume of octanol (Spectrograde). The mixtures were agitated at the indicated temperatures for 48 hours, or for assay incubation times of 40 minutes at 37°C and room temperature, and 5 hours at 4°C. The distribution of radioligands was determined by counting aliquots of each phase (see II.2.2.3./4). P (partition coefficient) was calculated as : $\frac{[\text{ligand}] \text{ in octanol}}{[\text{ligand}] \text{ in } H_2O \text{ phase}}$

P>1.0 = hydrophobic (log P = +ve), P<1.0 = hydrophilic (log P = -ve).

Log P of -0.377 calculated for ^{3}H-CGP 12177 was in excellent agreement with that of -0.37 reported by Hertel et al., (1984), as was the Tris Isosaline : Octanol partitioning of ^{3}H-CGP 12177 of 2.4:1 (previously quoted; Phosphate buffered saline : Octanol = 3.1:1 Staehelin et al., 1983)

Data presented are the mean of two separate experiments assessed in quadruplicate.

indicative of a differential temperature effect on agonist binding to intact human platelets. The data does not however indicate a direct effect of temperature on agonist binding, since isoprenaline displacement curves performed on platelet membranes resulted in virtually identical binding isotherms with slope factors not significantly different from unity at 4°C and 37°C. Several investigators have previously shown that exposure of intact cells to β-agonists at physiological temperatures may result in a decline with time in the affinity of β-adrenoceptors for these agonists, such that 'competition' binding studies performed at equilibrium may not reflect the initial interaction of agonists with the β-adrenoceptor. Methodological approaches which have previously been employed to avoid this are : the use of non-equilibrium binding conditions, thus terminating competition binding assays at early time points (Insel et al., 1983; Toews and Perkins, 1984) or alternatively, to perform equilibrium binding assays at a lowered temperature of 4°C, thus blocking temperature sensitive steps of 'receptor processing' (Insel and Sanda, 1979). It is therefore possible that the markedly shallow ^{125}I(-)PIN displacement curves exhibited at 37°C in the presence of isoprenaline and adrenaline, occur as a result of agonist modulation of receptor affinity for these agonists during the assay incubation period, which may have resulted in sequestration of a proportion of the receptors away from the immediate cell surface, rendering them less accessible to the agonist. At 4°C this effect of the agonists is reduced with the resulting steeper displacement curves. Perhaps noradrenaline is a less efficacious agonist at the platelet β-adrenoceptor, and its reduced effectiveness in producing

conformational change at the β_2-adrenoceptor is responsible for the appearance of steep displacement curves for this agonist at 37°C that are not significantly affected by incubations at 4°C. This differential effect of temperature on agonist binding to intact human platelets may provide initial evidence for β-adrenoceptor redistribution in this anucleate human cell; this is further evaluated in VI.4.

VI.4. ^{125}I(-)PIN binding to agonist-treated intact human platelets at 4°C and 37°C

VI.4.1. Results

VI.4.1.1. Distribution of β-adrenoceptors following (-)isoprenaline treatment of intact human platelets

Human platelets were incubated for 30 minutes at 37°C in the presence (treated cells) or absence (control cells) of 10uM (-)isoprenaline, washed at 4°C and assayed for ^{125}I(-)PIN binding (as described in the methods section) at both physiological temperature (37°C) and at a low temperature (4°C) to prevent receptor recycling. In control cells, 1uM(-)propranolol and 1uM(±)CGP 12177 have previously been shown to identify the same number of sites (VI.3.); therefore, in order to economise on volume of volunteer blood needed for each experiment, non-specific binding was defined only in the presence of 1uM(-)propranolol in control cells. ^{125}I(-)PIN binding in the presence of 1uM(-)propranolol was taken to represent total cell β-adrenoceptor number ; ^{125}I(-)PIN in the presence of 1uM(±)CGP 12177 to represent

cell surface receptors and the difference between them to represent internalised receptors that are no longer accessible to (±)CGP 12177.

Isoprenaline treatment resulted in a significant (25% approximately) decrease in the number of ^{125}I(-)PIN sites labelled at 37°C when compared with control cells (VI.10.). This decrease in the number of binding sites was identical whether defined in the presence of 1uM(-)propranolol or 1uM(±)CGP 12177 (table VI.5.) suggesting that when assayed at 37°C, all the remaining receptors in the treated cells were on the cell surface.

^{125}I(-)PIN binding to treated cells performed at 4°C showed a similar loss of binding sites as defined by 1uM(-)propranolol, but a much greater loss of binding (approximately 70%) as defined by 1uM(±)CGP 12177, when compared with control cells (figure VI.10, table VI.5.). This difference between 1uM(±)CGP 12177 and 1uM(-)propranolol displaceable binding at 4°C indicates that isoprenaline treatment not only induces an irreversible reduction (down-regulation) in total receptor number, but also an additional sequestration of cell surface receptors. Thus, 40-50% of the receptors remaining following agonist treatment, although still labelled by (-)propranolol and ^{125}I(-)PIN, are no longer accessible to 1uM(±)CGP 12177, possibly due to removal from the immediate cell surface.

To determine whether these receptor changes were distinguishable over a wide concentration range of displacing agent or only at high saturating concentrations (1uM), competition experiments were performed with the

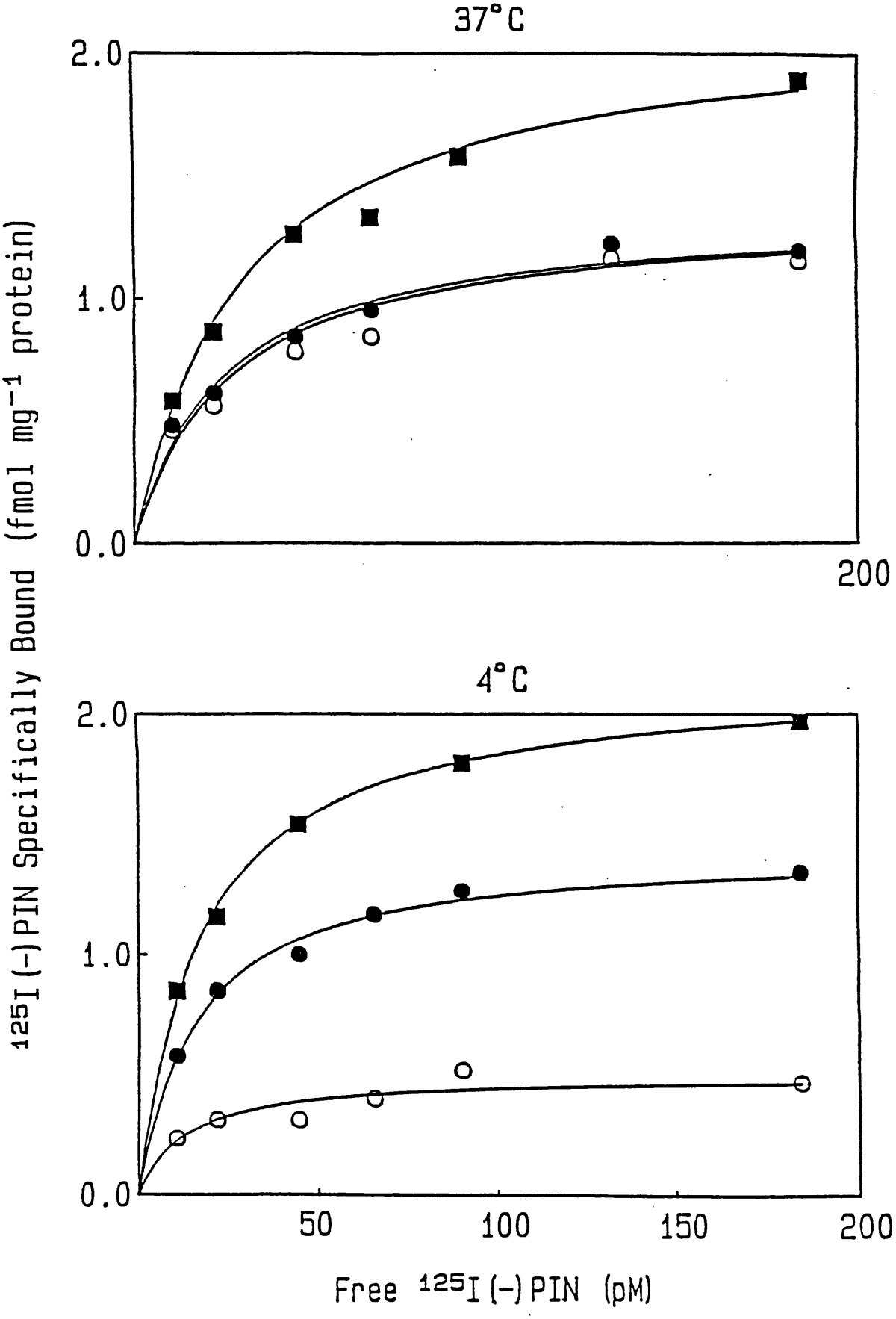


Figure VI.10. ^{125}I(-)PIN binding to control and agonist-treated cells

Legend to Figure VI.10. ^{125}I(-)PIN binding to control and agonist-treated cells

Control and (-)isoprenaline-treated (10uM for 30 minutes at 37°C) cells were prepared, and binding assays performed at 37°C and 4°C as described in methods section. For control cells (squares) non-specific binding (NSB) was defined in the presence of 1uM (-)propranolol (■). For treated cells (circles) NSB was defined in the presence of either 1uM (-)propranolol (●) or 1uM (±)CGP 12177 (o). The data shown are from a single representative experiment performed in duplicate.

Table VI.5. Apparent equilibrium dissociation constants (Kd) and receptor density (Bmax) of ^{125}I(-)PIN binding to control and isoprenaline-treated intact human platelets

Temperature/ Cells	Agent used to define non-specific binding	Bmax (fmol mg^{-1})	Kd (pM)
37°C			
Control	1uM(-)propranolol	1.72±0.16	17.61±2.94
Treated	1uM(-)propranolol*	1.27±0.07	18.82±4.02
Treated	1uM(±)CGP 12177*	1.24±0.05	20.36±4.42
4°C			
Control	1uM(-)propranolol	2.05±0.14	13.28±1.00
Treated	1uM(-)propranolol**	1.46±0.12	12.83±1.04
Treated	1uM(±)CGP 12177**#	0.56±0.06	13.96±0.32

Dissociation constants and number of binding sites were determined at 4°C and 37°C in control and (-)isoprenaline treated (10uM for 30 minutes at 37°C) cells simultaneously. Specific binding was assessed as described in the methods section in the presence of 1uM(-)propranolol for control cells, and in the presence of 1uM(-)propranolol or 1uM (±)CGP 12177 for treated cells. Data are expressed as mean ± SEM (n=3). Results were analysed using Student's 't' test for paired data.

* $p<0.05$ by one tailed paired 't' test analysis compared to control cells at 37°C

**$p<0.05$ by one tailed paired 't' test analysis compared to control cells at 4°C

#$p<0.05$ by one tailed paired 't' test analysis compared to treated cells defined in the presence of 1uM(-)propranolol.

antagonists (-)propranolol and (±)CGP 12177, and the hydrophilic β-agonist (-)isoprenaline in treated cells at 37°C and 4°C. In assay incubations performed at 37°C, (-)propranolol, (±)CGP 12177 and (-)isoprenaline all competed with ^{125}I(-)PIN binding on treated platelets to the same extent, displacing approximately 85% of total radioligand bound (figure VI.11.). The overall affinities, $3.65\pm0.35\times10^{-10}$M, $2.19\pm0.20\times10^{-9}$M, $9.33\pm1.25\times10^{-7}$M for (-)propranolol, (±)CGP 12177 and (-)isoprenaline respectively, were in excellent agreement with those previously shown in control cells at 37°C (table VI.2.) although (-)isoprenaline and (±)CGP 12177 exhibited slightly lower affinity following agonist treatment.

In binding assays performed at 4°C however, these three agents produced markedly different competition binding isotherms in the treated cells (figure VI.11.). Thus (-)propranolol inhibited approximately 70% of total ^{125}I(-)PIN binding at saturating concentrations (<1uM) with further displacement of binding, probably from non-specific sites, occurring at higher (10-100uM) concentrations. In contrast, (±)CGP 12177 even at concentrations of 100uM did not inhibit binding to the same extent as (-)propranolol, and at a saturating concentration of 1uM occupied only 35% of total sites identified by ^{125}I(-)PIN binding. Isoprenaline exhibited similar inhibition of ^{125}I(-)PIN binding (approximately 35%) at concentrations of up to 10uM. Increasing the concentration caused further inhibition of binding; however, even at very high concentrations (1mM), isoprenaline did not inhibit ^{125}I(-)PIN binding to the same extent as (-)propranolol.

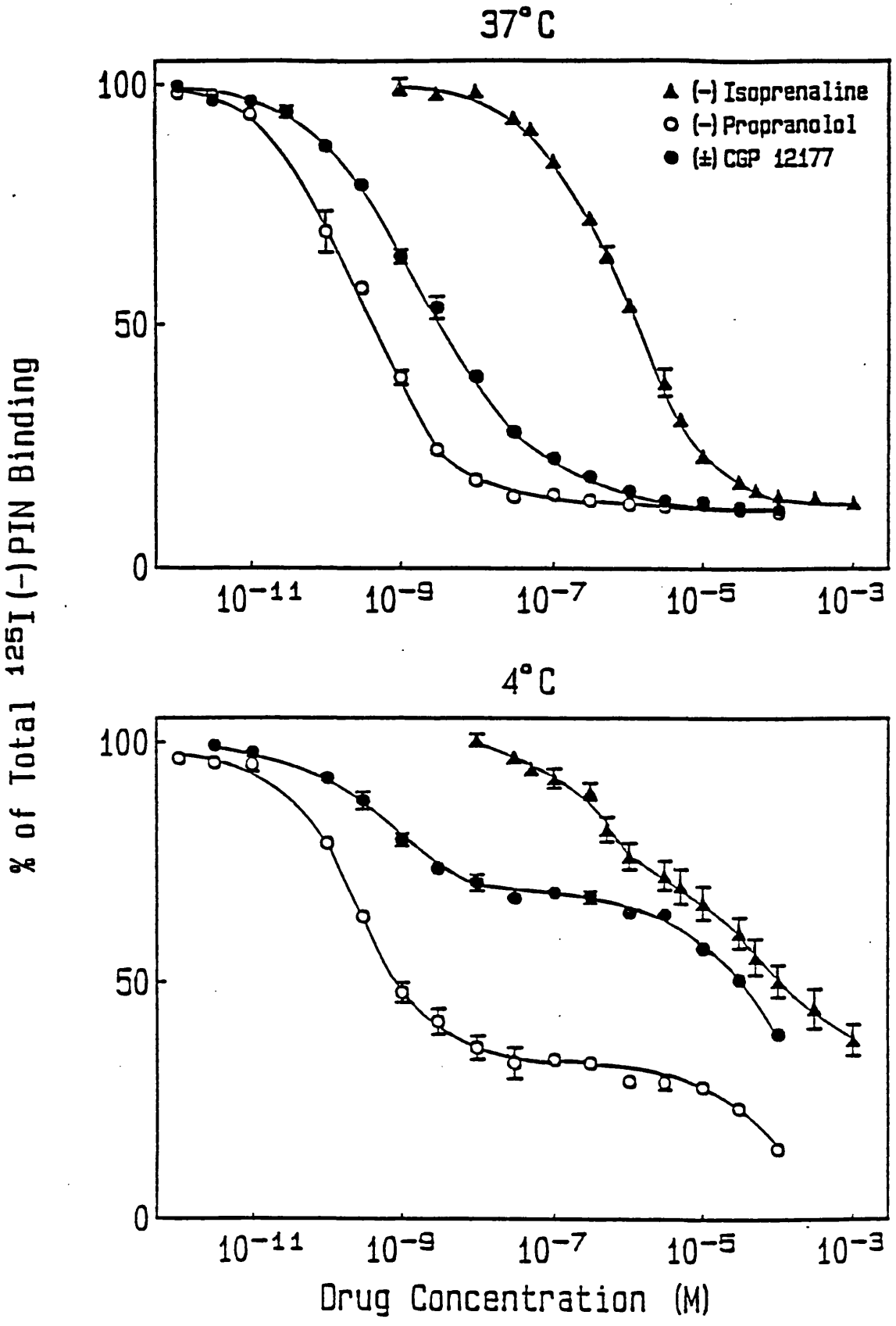


Figure VI.11. Competition for ^{125}I(-)PIN binding sites on isoprenaline-treated intact human platelets at 37°C and 4°C by the hydrophobic antagonist (-)propranolol (o), the hydrophilic antagonist (±)CGP 12177 (•) and the hydrophilic agonist (-)isoprenaline (▲)

Legend to Figure VI.11. Competition for ^{125}I(-)PIN binding sites on isoprenaline-treated intact human platelets at 37°C and 4°C by the hydrophobic antagonist (-)propranolol (o), the hydrophilic antagonist (±)CGP 12177 (●) and the hydrophilic agonist (-)isoprenaline (▲)

Intact human platelets were incubated with 10uM (-)isoprenaline for 30 minutes at 37°C, washed at 4°C and assayed for ^{125}I(-)PIN binding at 37°C or 4°C as described in methods section. Data are plotted as % of ^{125}I(-)PIN bound at the indicated concentrations of competitive agents and represent the mean of at least three separate experiments performed in duplicate; SEM's shown by vertical lines.

VI.4.1.2. Concentration-dependence of (-)isoprenaline effects in intact human platelets

Treatment of intact human platelets with (-)isoprenaline induced a concentration-dependent loss of both total and cell surface receptors (figure VI.12). The effect, although evident at a concentration of 10^{-8}M(-)isoprenaline, was only statistically significant with concentrations of 10^{-7}M and above. Maximal effect was apparent with 10^{-5}M(-)isoprenaline. Although the extent of cell surface receptor loss was significantly greater than total receptor loss from 10^{-7}-10^{-5}M (-)isoprenaline, half maximal receptor loss occurred at around $1\text{-}3\text{x}10^{-8}$M(-)isoprenaline for both processes.

Isoprenaline induced changes in receptor numbers were inhibited by the presence of the non-selective β-adrenoceptor antagonist sotalol. Thus, no internalisation or down-regulation of β-adrenoceptor number was observed in ^{125}I(-)PIN binding assays at 4°C if the platelets had been exposed to 1uM sotalol (concentration assessed from competition experiments, data not shown) for 30 minutes at 37°C prior to (-)isoprenaline treatment (10uM for 30 minutes at 37°C).

Similarly, agonist induced changes in receptor number were absent if (-)isoprenaline treatment (10uM) was performed at 4°C for 2 hours (data not shown), even though agonist interaction with the receptors during this period could be demonstrated in the form of an (-)isoprenaline competition curve (figure VI.13.) exhibiting a similar overall affinity ($3.0\pm1.8\text{x}10^{-7}$M) to that observed in control cells at 4°C (table VI.3.).

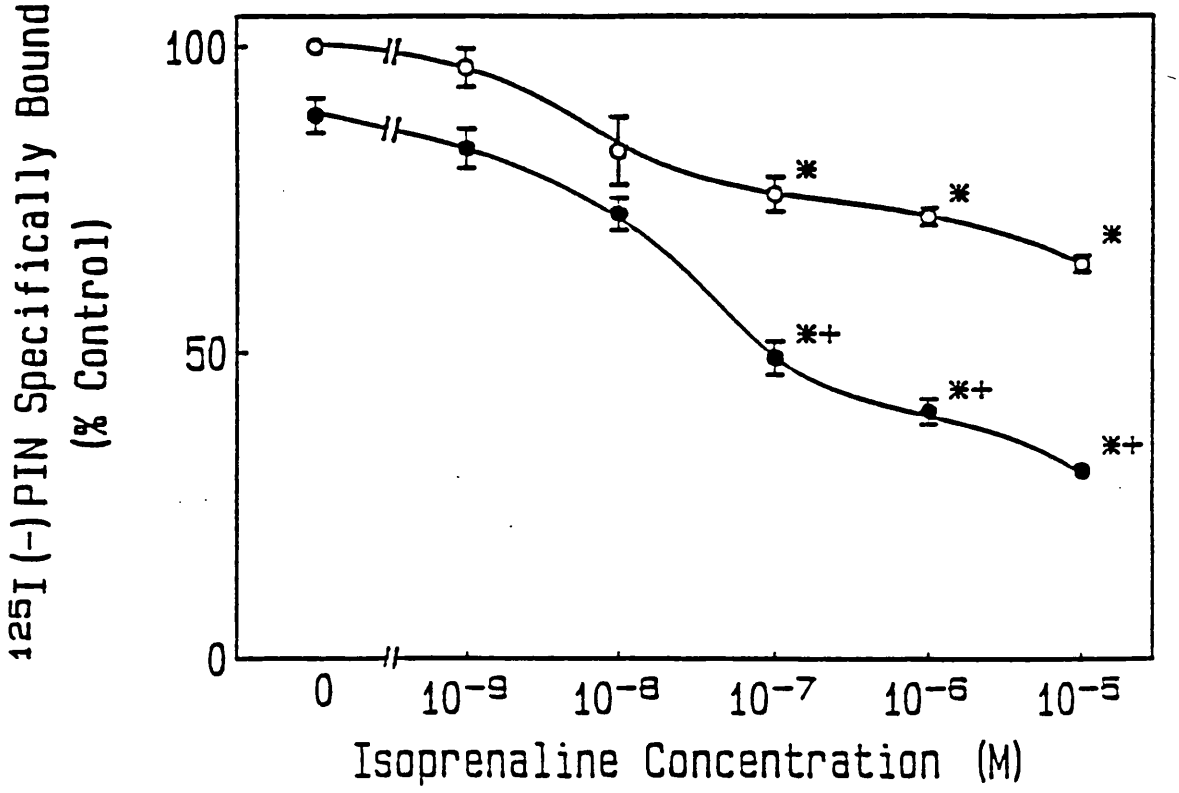


Figure VI.12. Concentration-dependence of (-)isoprenaline induced internalisation and down-regulation of intact human platelet β-adrenoceptors

Intact human platelets were incubated with (treated cells) or without (control cells) the indicated concentrations of (-)isoprenaline for 30 minutes at 37°C, washed at 4°C and assayed for ^{125}I(-)PIN binding at 4°C. Specific binding was defined in the presence of 1uM(-)propranolol (o) to assess total β-adrenoceptor number and 1uM($\pm$)CGP 12177 (•) to assess cell surface receptor number. Data points represent the mean of at least three separate experiments performed in duplicate. SEM's shown by vertical lines. Results were analysed using Student's 't' test for paired data.

*$p<0.05$ by one tailed paired 't' test analysis to control cells.
+$p<0.05$ by one tailed paired 't' test analysis to treated cells defined in the presence of 1uM (-)propranolol.

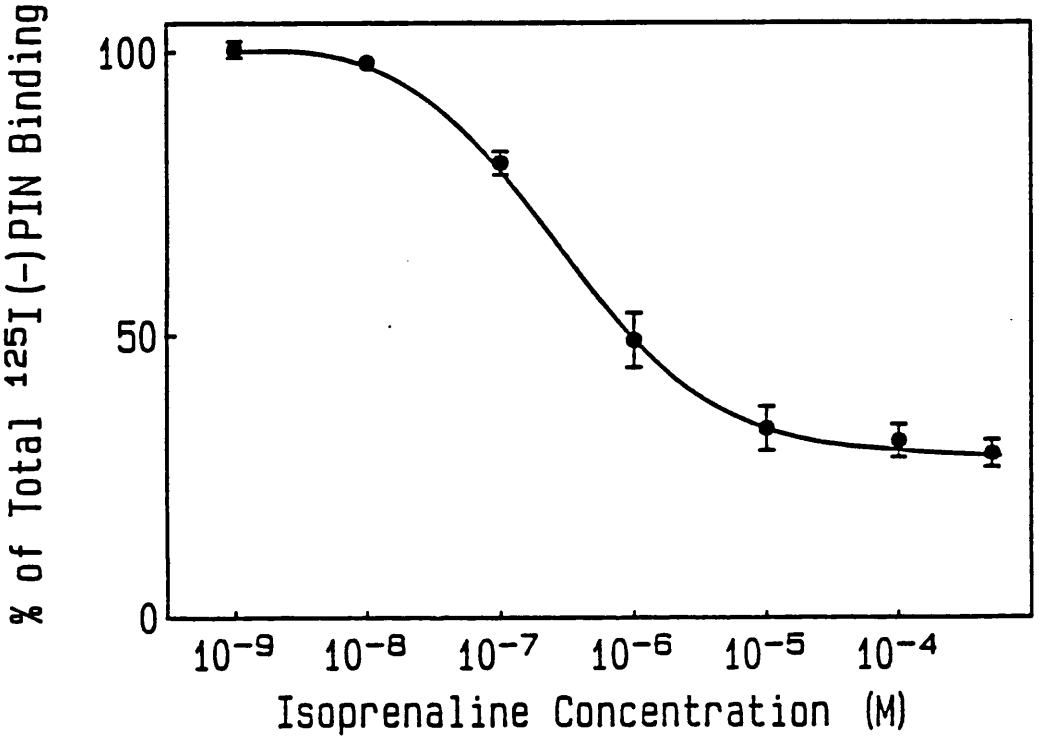


Figure VI.13. Competition for ^{125}I(-)PIN binding sites on intact human platelets by (-)isoprenaline at 4°C

Intact human platelets were incubated with ^{125}I(-)PIN and the indicated concentrations of (-)isoprenaline for 2 hours at 4°C. Data are plotted as % of ^{125}I(-)PIN bound, and represent the mean of at least three separate experiments performed in duplicate ; SEM's shown by vertical lines.

VI.4.1.3. Time course of (-)isoprenaline induced β-adrenoceptor internalisation and down-regulation on intact human platelets

Treatment of intact human platelets with 0.1uM(-)isoprenaline at 37°C induced a rapid (t1/2 <5 minutes) loss of both total and cell-surface β-adrenoceptors (figure VI.14.). A near maximal response was attained by approximately 15 minutes, with no further significant decrease in receptor number during the remaining 45 minutes incubation with isoprenaline.

VI.4.1.4. Reappearance of cell surface β-adrenoceptors following (-)isoprenaline treatment

Intact human platelets were incubated with 0.1uM(-)isoprenaline at 37°C for 30 minutes (as described in methods section). Agonist incubation was terminated by centrifugation and washing of the platelets at 4°C. Cells were then resuspended in fresh, agonist free buffer and maintained at 37°C for up to 6 hours. At various time intervals, platelet aliquots were removed and ^{125}I(-)PIN binding (using a saturating concentration of radioligand, 150pM) performed at 4°C in the presence of (-)propranolol or (±)CGP 12177 to define total and cell surface receptor number respectively.

The receptors which were down-regulated (defined by (-)propranolol) by agonist treatment did not reappear even after prolonged (>6 hours) incubation in agonist-free medium at 37°C. Conversely, the recovery of

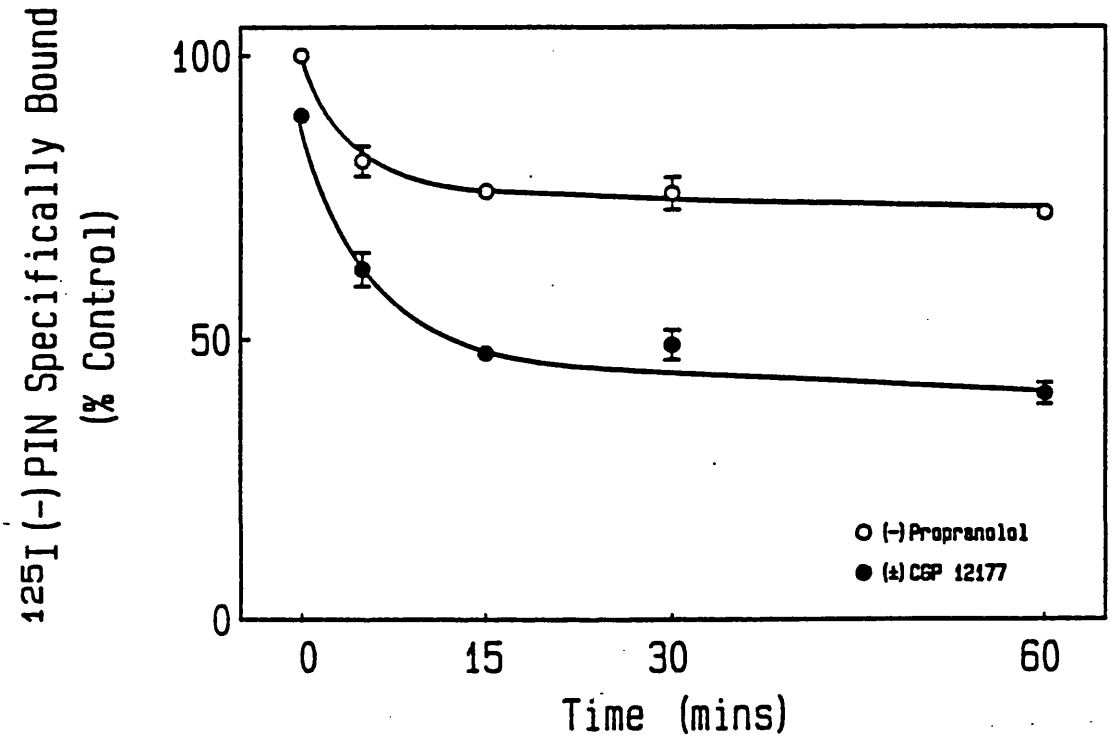


Figure VI.14. Time course of (-)isoprenaline induced internalisation and down-regulation of intact human platelet β-adrenoceptors

Intact human platelets were incubated with (treated cells) or without (control cells) 0.1uM (-)isoprenaline at 37°C for the indicated time intervals, washed at 4°C and assayed for ^{125}I(-)PIN binding at 4°C. Specific binding was defined in the presence of 1uM(-)propranolol (o) to assess total β-adrenoceptor number and 1uM($\pm$)CGP 12177 (•) to assess cell surface receptor number. Data points represent the mean of at least three separate experiments performed in duplicate ; SEM's shown by vertical lines.

cell surface receptors defined by (±)CGP 12177 was rapid, such that by 40 minutes there was no significant difference in ^{125}I(-)PIN binding defined by (±)CGP 12177 or (-)propranolol (figure VI.15.).

VI.4.1.5. Time course of ^{125}I(-)PIN binding at 4°C to control and (-)isoprenaline treated intact human platelets

The time course of ^{125}I(-)PIN binding at 4°C to control and (-)isoprenaline treated intact human platelets was assessed in the presence of a saturating concentration of ^{125}I(-)PIN (150pM) to ensure that all available receptors are labelled (figure VI.16.) and at a lower concentration of 30-40pM that would normally be employed for Kinetic and competition experiments (data not shown). Both concentrations of ^{125}I(-)PIN exhibited identical Kinetics. Specific ^{125}I(-)PIN binding attained a steady state level in both control and treated cells at, or before, 5 hours incubation at 4°C whether defined by 1uM(-)propranolol or 1uM(±)CGP 12177 (figure VI.16.). No further increases in ^{125}I(-)PIN binding were observed if incubation time was extended beyond 5 hours.

VI.4.1.6. Effect of (-)isoprenaline treatment of intact human platelets on subsequent ^{125}I(-)PIN binding to platelet membranes

The above results obtained with intact platelets indicate that β-adrenoceptor sequestration and down-regulation occur when platelets are incubated with the β-agonist (-)isoprenaline. As no significant change in the apparent Kd of 125I(-)PIN was observed at either 37°C or

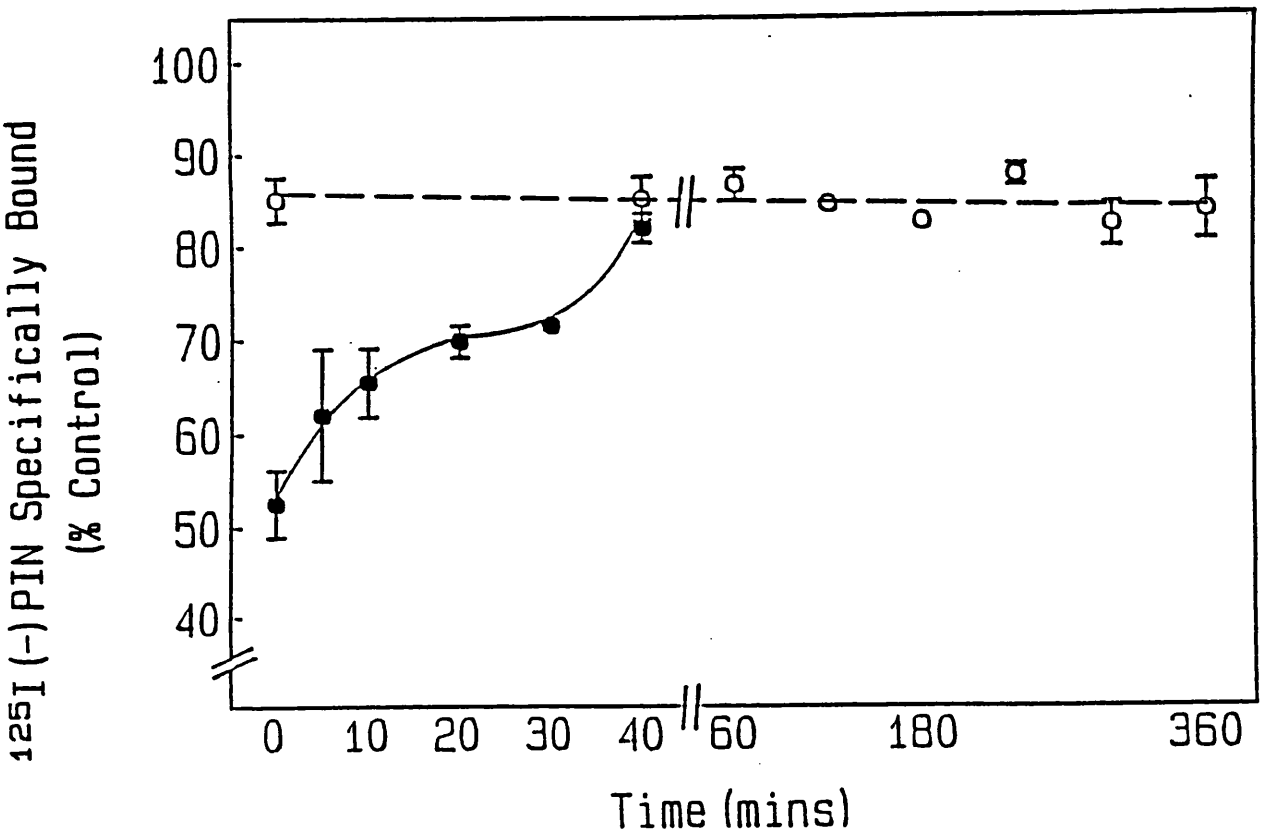


Figure VI.15. Time course of intact human platelet β-adrenoceptor reappearance following (-)isoprenaline treatment

Intact human platelets were incubated with (treated cells) or without (control cells) 0.1uM (-)isoprenaline for 30 minutes at 37°C, and then recovery of down-regulated and 'internalised' receptors evaluated for up to 6 hours as described in the text (VI.4.1.4.). Time intervals represent periods of further incubation in fresh buffer following initial agonist treatment. ^{125}I(-)PIN binding assays were performed at 4°C using 1uM(-)propranolol (o) and 1uM(±)CGP 12177 (•) as described in the methods section. Data points are shown relative to control (100%) and represent the mean of at least three separate experiments performed in duplicate. SEM's shown by vertical lines.

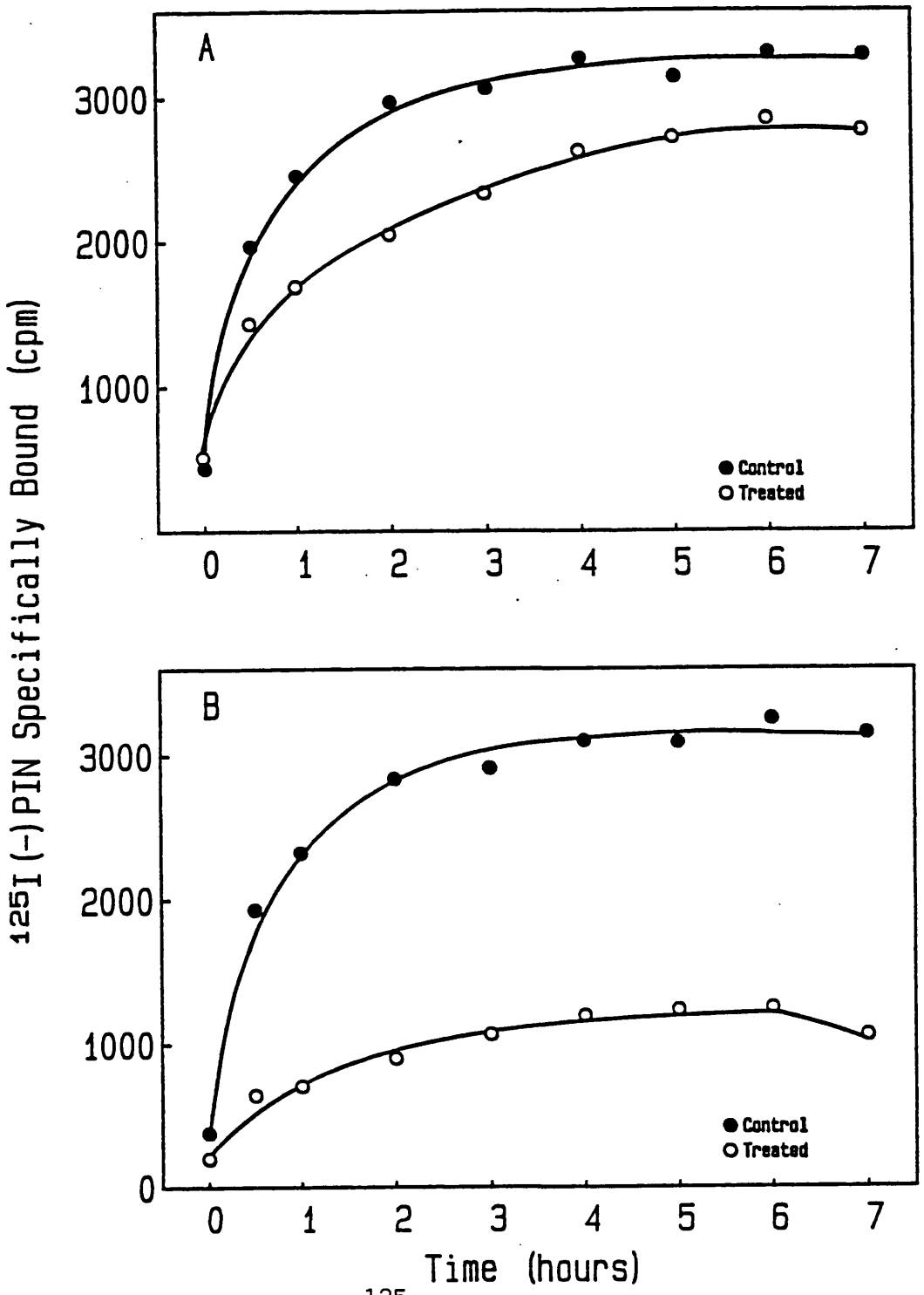


Figure VI.16. Time course of ^{125}I(-)PIN binding at 4°C to control and (-)isoprenaline treated intact human platelets

Intact human platelets were incubated with (treated cells, (o)) or without (control cells (●)) 10uM (-)isoprenaline for 30 minutes at 37°C, washed at 4°C and assayed for ^{125}I(-)PIN binding (150pM) at 4°C for the indicated times. Specific binding was defined in the presence of 1uM (-)propranolol (A) to assess total receptor number, and 1uM (±)CGP 12177 (B) to assess cell surface receptor number. Data shown are representative of two experiments performed in triplicate.

4°C (table VI.5.) it is unlikely that the loss of sites after incubation with (-)isoprenaline is attributable to free agonist being retained during the washing procedure, and interfering with subsequent binding assays. However, in order to establish whether or not retained receptor bound (-)isoprenaline was responsible for the loss of ^{125}I(-)PIN binding observed with intact platelets, a series of experiments were performed on human platelet membranes.

It has previously been shown that addition of Gpp(NH)p or Na^+ decreased the affinity of ^{125}I(-)PIN binding sites for (-)isoprenaline in human platelet membranes (Chapter III; Wang and Brodde, 1985). Although in the present study the effect of Gpp(NH)p on agonist affinity at the β-adrenoceptor in platelet membranes was minimal due to poor coupling efficiency, the addition of NaCl induced a marked shift to the right of the isoprenaline competition binding curves in control membranes (Chapter III.). Subsequent studies have shown that this Na^+ induced shift is quantitatively the same in membranes prepared from platelets following agonist treatment (data not shown). In the light of these findings, it was postulated that if the decreased ^{125}I(-)PIN binding demonstrated above, following preincubation of intact platelets with (-)isoprenaline, was the result of competition by retained (-)isoprenaline, then lowering the affinity of the receptor for the (-)isoprenaline by the addition of Gpp(NH)p or Na^+ in membranes prepared from these cells should reverse the effect. If on the other hand, true down-regulation had occurred, the reduced ^{125}I(-)PIN binding would persist.

As shown in table VI.6., in the presence of Gpp(NH)p or Na^+ there was no significant difference in the maximal binding capacity (Bmax) or the apparent equilibrium dissociation constant (Kd) observed in membranes prepared from treated cells.

VI.4.1.7. Distribution of β-adrenoceptors from control and agonist treated intact human platelets on a sucrose density gradient

Intact human platelets were treated with or without 10uM(-)isoprenaline, lysed and fractionated on non-linear sucrose density gradients as previously described (VI.2.3.). Gradient fractions were subsequently analysed for adenylate cyclase activity (plasma membrane marker) and ^{125}I(-)PIN binding.

Sucrose density gradient centrifugation of control lysates revealed that all measurable ^{125}I(-)PIN binding was located along with sodium fluoride stimulated cyclase activity at the heavy 30%-50% sucrose density interphase. In contrast, identical fractionation of treated lysates revealed that ^{125}I(-)PIN binding was not only associated with this heavy membrane fraction, but also with a lighter density fraction, devoid of adenylate cyclase activity, located at the lysate-30% sucrose interphase. Furthermore, the appearance of β-adrenoceptors in this lighter density fraction was associated with a significant 40-45% decrease in the receptor number associated with the plasma membrane fraction. Attempts to concentrate the receptors present in this 'cytosolic' fraction by centrifugation at 150,000g proved unsuccessful; therefore accurate determination of the receptor number present was not

Table VI.6. Apparent equilibrium dissociation constants (Kd) and receptor density (Bmax) of ^{125}I(-)PIN binding to human platelet membranes prepared from control and (-)isoprenaline treated cells

	Bmax (fmol mg^{-1})	Kd (pM)
Control	3.65±0.16	16.91±0.71
Treated	2.25±0.06*	26.10±2.76
Treated + 100uM Gpp(NH)p	2.40±0.09*	22.74±2.90
Treated + 100mM NaCl	2.34±0.05*	22.46±2.00

Human platelets were incubated with (treated) or without (control) 10uM (-)isoprenaline at 37°C for 30 minutes, washed at 4°C and membranes prepared and assayed for ^{125}I(-)PIN binding at room temperature (III.2.1.) Specific binding was defined in the presence of 1uM(-)propranolol.

The results shown are the mean ± SEM of at least three separate experiments performed in duplicate. Results were analysed using Student's 't' test for paired data.

*$p<0.05$ by one tailed paired 't' test analysis compared to control membranes.

possible due to the low numbers involved.

VI.4.1.8. Concentration-dependence of (-)noradrenaline effects in intact human platelets

(-)Noradrenaline treatment of intact human platelets resulted in a concentration-dependent loss of both total and cell-surface receptors (figure VI.17.) similar to that previously observed with (-)isoprenaline (figure VI.12.). Although the extent of cell surface receptor loss was significantly greater than total receptor loss from 10^{-6}-$3x10^{-4}$M(-)noradrenaline, maximal and half-maximal receptor loss occurred at around $3x10^{-4}$M and $2x10^{-5}$M(-)noradrenaline respectively, for both processes.

VI.4.1.9. Adrenaline-induced redistribution of β-adrenoceptors on intact human platelets in the presence and absence of α_2-adrenoceptor blockade

Adrenaline treatment of intact human platelets resulted in a significant decrease of approximately 25-30% in the number of ^{125}I(-)PIN binding sites defined by 1uM(-)propranolol, and a much greater loss of binding (approximately 70%) defined by 1uM(±)CGP 12177 when assayed at 4°C (figure VI.18.). This β-adrenoceptor redistribution following (-)adrenaline treatment was virtually identical to that previously observed with (-)isoprenaline, and was not influenced by the presence of 1uM phentolamine to block any α_2-adrenoceptor agonist activity of adrenaline.

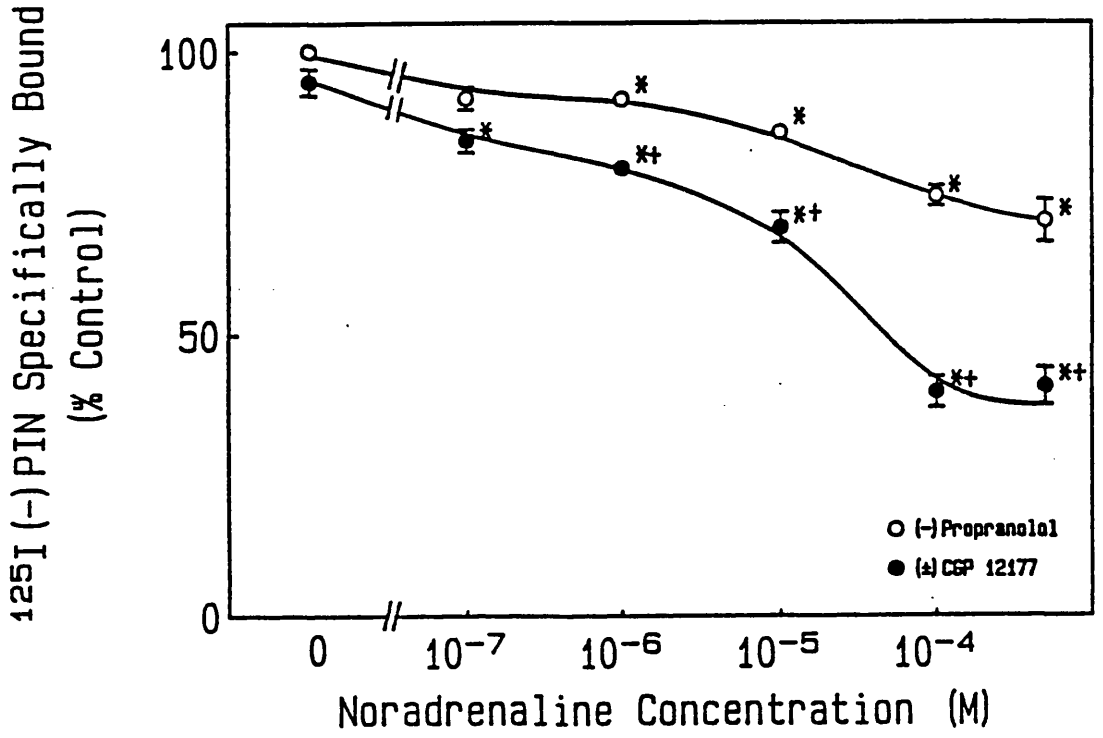


Figure VI.17. Concentration-dependence of (-)noradrenaline induced internalisation and down-regulation of intact human platelet β-adrenoceptors

Intact human platelets were incubated with (treated cells) or without (control cells) the indicated concentrations of (-)noradrenaline for 30 minutes at 37°C, washed at 4°C and assayed for ^{125}I(-)PIN binding at 4°C. Specific binding was defined in the presence of 1uM (-)propranolol (o) to assess total β-adrenoceptor number and 1uM ($\pm$)CGP 12177 (•) to assess cell surface receptor number. Data points represent the mean of at least three separate experiments performed in duplicate; SEM's shown by vertical lines. Results were analysed using Student's 't' test for paired data.

*$p<0.05$ by one tailed paired 't' test analysis to control cells.
+$p<0.05$ by one tailed paired 't' test analysis to treated cells defined in the presence of 1uM (-)propranolol.

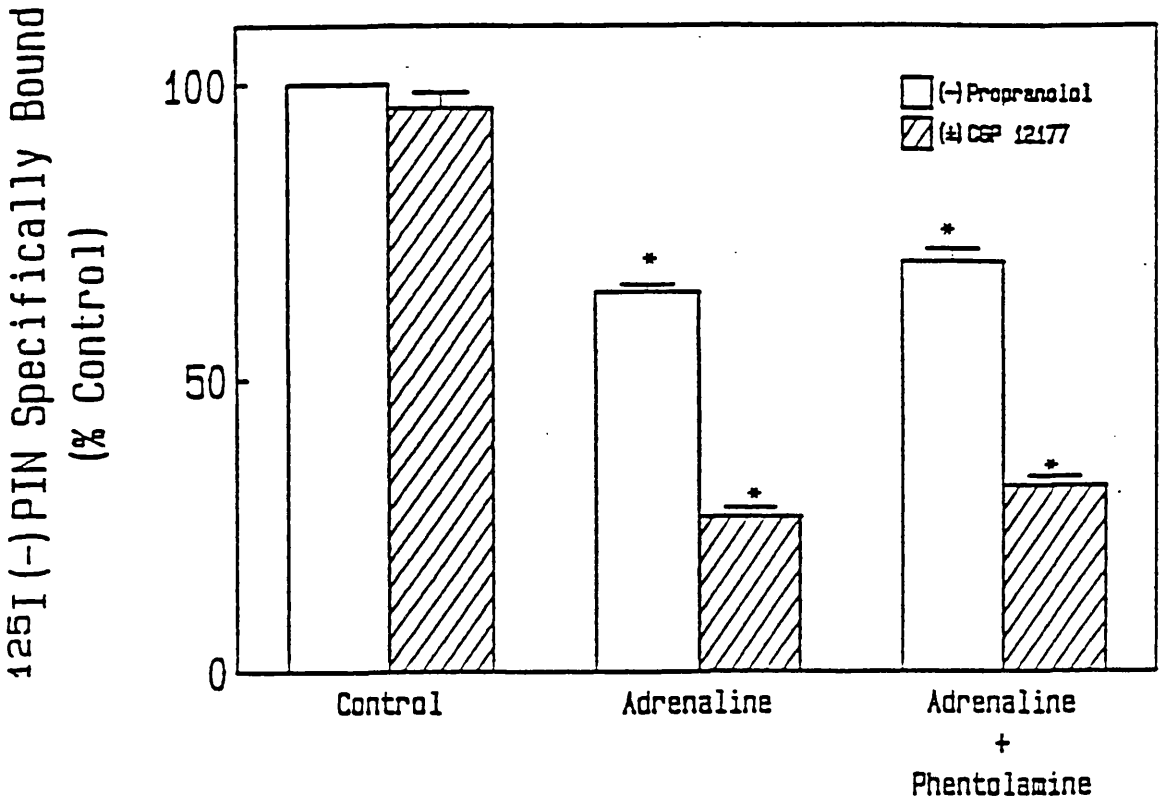


Figure VI.18. Adrenaline induced redistribution of β-adrenoceptors on intact human platelets, in the presence and absence of the α-adrenoceptor antagonist phentolamine

Intact human platelets were incubated without (control cells) or with 5uM (-)adrenaline in the presence or absence of 1uM phentolamine for 30 minutes at 37°C, washed at 4°C and assayed for ^{125}I(-)PIN binding at 4°C. Specific binding was defined in the presence of 1uM (-)propranolol (□) to assess total receptor number, and 1uM (±)CGP 12177 (▨) to assess cell surface receptor number. Data are shown relative to control (100%) and represent the mean of at least three separate experiments performed in duplicate; SEM's shown by error bars.

Results were analysed using Student's 't' test for paired data.
*$p<0.05$ by one tailed paired 't' test analysis to control cells.
Data obtained in the presence of adrenaline and phentolamine was not significantly different to that for adrenaline alone ($p>0.1$).

VI.4.2. Discussion

In the present study, the antagonist radioligand ^{125}I(-)PIN has been employed to examine agonist-mediated internalisation or sequestration and down-regulation of β_2-adrenoceptors on intact human platelets. Results of saturation binding experiments show that preincubation of intact human platelets with (-)isoprenaline leads to a dramatic reduction in total β-adrenoceptor number and the redistribution or sequestration of a large fraction of the remaining cell surface receptors into a cellular environment not accessible to the hydrophilic antagonist (±)CGP 12177. This selective loss of cell surface receptors can only be detected when the agonist-induced state of the receptor is preserved by cooling during receptor determination. No detectable difference in cell surface and total receptor number in treated cells is apparent when the receptor assay is performed at physiological temperature (37°C), probably due to rapid recycling of the 'internalised' receptors to the surface during the assay incubation period as has been suggested by others (Hertel and Staehelin, 1983) and as indicated by the receptor recovery experiments in this study.

The results of competition binding experiments performed at 4°C and 37°C in treated cells with the antagonists (-)propranolol and (±)CGP 12177, and the β agonist (-)isoprenaline, support the observation made above. They show that if binding assays are performed at 37°C in treated cells, all the remaining receptors not only exhibit the pharmacological characteristics of β-adrenoceptors but are also equally accessible to (-)propranolol, (±)CGP 12177 and (-)isoprenaline.

Conversely at 4°C, the receptors that remain following agonist treatment are not equally accessible to these agents. (-)Propranolol has access to a far greater number of receptors than the hydrophilic compounds (±)CGP 12177 and (-)isoprenaline, which even at very high concentrations can only displace ^{125}I(-)PIN binding from cell surface, and a proportion of the 'internalised', receptors. Although binding assays performed at 37°C in treated cells most probably allow recycling of receptors back to cell surface, competition experiments performed at this temperature in the presence of the agonist (-)isoprenaline would be expected to produce shallow displacement curves similar to those previously demonstrated in control cells (VI.3.2.). However, in this study the (-)isoprenaline competition curve at 37°C was steep and virtually identical to that of (-)propranolol and (±)CGP 12177. This may suggest that the recycled receptors behave differently during the second agonist challenge, particularly when the initial exposure was to a high agonist concentration. Conversely, the receptors totally lost from the cell (approximately 25%) contribute significantly to the appearance of the shallow agonist competition curve seen in control cells.

The concentration-dependence for (-)isoprenaline induced internalisation and down-regulation of β-adrenoceptors on intact human platelets was similar to that observed in other cell types (Staehelin and Simons, 1983) with half maximal receptor loss occurring at the same (-)isoprenaline concentration for both processes.

Isoprenaline induced changes in total and cell surface β-adrenoceptor

numbers did not occur if agonist treatment was performed at 4°C, although agonist interaction with the receptor was apparent. Thus, agonist induced redistribution of β-adrenoceptors on human platelets appears to be a temperature dependent process. Furthermore, isoprenaline induced changes in receptor numbers were inhibited by the presence of the non-selective β-antagonist sotalol.

Recent reports (Hertel et al., 1983a; Toews et al., 1986) have shown that ^{125}I(-)PIN, whilst able to label all available receptors (surface and intracellular) at 37°C, exhibited limited accessibility to internalised receptors at 4°C. Other studies have thus assumed that ^{125}I(-)PIN labels both surface and intracellular receptors at 37°C and only surface receptors at 4°C (Linden et al., 1984). However, preliminary findings from the present study (VI.3.2.) suggest that ^{125}I(-)PIN is not restricted to the immediate cell surface at 4°C in intact human platelets. It has been suggested that this temperature dependent differential permeability of ^{125}I(-)PIN varies with length of assay incubations, since careful examination of the Kinetics of ^{125}I(-)PIN binding at 4°C in agonist-treated human astrocytoma cells has shown substantially less ligand bound following 1 hour incubation compared with control, but virtually identical binding after 5 hours (Toews et al., 1986). When similar Kinetic experiments were performed in the present study, the results showed that ^{125}I(-)PIN binding to control intact human platelets at 4°C was not the same after 5 hours as that to treated cells whether defined in the presence of 1uM(-)propranolol or 1uM(±)CGP 12177. Extending the incubation period at this temperature beyond 5 hours made no difference, thus supporting

the proposal made earlier that ^{125}I(-)PIN is not restricted to the cell surface in intact human platelets.

Isoprenaline induced internalisation of the β-adrenoceptors on intact human platelets was found to be rapid and reversible. The time course for reappearance of β-adrenoceptors defined by (±)CGP 12177 was however biphasic. A possible explanation for this is that the internalised receptors are recycling to the cell surface from two compartments. The initial, more rapid phase may represent reappearance from a compartment that is still associated with the plasma membrane but not accessible to (±)CGP 12177, whilst the slower second phase represents a reappearance of intracellular receptors. Support for this hypothesis comes from the sucrose density gradient fractionation study. For instance, (-)isoprenaline treatment of intact human platelets results (as previously described) in a 25% loss of total receptors, a 70% loss of receptors accessible to (±)CGP 12177, and thus (the difference between them) an internalisation of 45% of the receptors, which suggests that only 30% of the receptors remain on the cell surface. However, when intact human platelets were lysed and fractionated on sucrose density gradients, agonist treatment resulted in only a 40-45% decrease of receptors measured in the plasma membrane fraction, i.e. 55-60% of the receptors remained associated with the plasma membrane. Further experiments are required to clarify this issue which at present is purely speculative.

Isoprenaline induced down-regulation of β-adrenoceptor number was also rapid (<30 minutes), but apparently not reversible despite prolonged

incubation in agonist free medium. As no change in the apparent Kd of ^{125}I(-)PIN was observed following agonist treatment, it is unlikely that the decrease in ^{125}I(-)PIN binding observed at both 37°C and 4°C in treated cells is due to interference by high affinity binding of (-)isoprenaline retained during the washing procedure. To establish whether retained (-)isoprenaline actually explained the decreased ^{125}I(-)PIN binding, membranes from control and agonist-treated cells were examined. When conditions designed to decrease the affinity of (-)isoprenaline (addition of Gpp(NH)p/Na^+) were employed, no change in the maximal binding capacity or the apparent equilibrium dissociation constant of ^{125}I(-)PIN was observed in membranes prepared from treated cells. These results support the hypothesis that the reduction in total ^{125}I(-)PIN binding sites observed following (-)isoprenaline treatment represents true down-regulation and is not a consequence of retained agonist.

The exact fate of down-regulated receptors is not known. As mentioned previously (VI.1.), receptor down-regulation in certain experimental systems has been shown to involve enzymatic breakdown through lysosomes, and new receptor synthesis is required for the reversal of this process. In other systems the number of receptors can return to control levels in the absence of protein synthesis. It is therefore possible that platelet β-adrenoceptor down-regulation observed in this study occurs as a result of proteolytic degradation of the receptors in lysosomes, and the irreversible nature of this process is due to the fact that new receptor synthesis probably does not take place in this anucleate cell. It is also possible that these 'lost' receptors are

simply modified in some way so that they are no longer able to bind ^{125}I(-)PIN e.g. phosphorylation, which for some reason is not reversible in these cells. Alternatively, as previously shown in appendix 8, ^{125}I(-)PIN binding varies with assay pH and specific binding is dramatically reduced outside the optimal 7.5-8.5 range. Lysosomal pH is reported to be around 4.6 (Tycke and Maxfield, 1982). At this low pH, ^{125}I(-)PIN may well undergo a rapid dissociation from the receptor, thus reducing measurable ^{125}I(-)PIN binding and rendering a proportion of the receptors as 'lost' to the method of detection employed.

Treatment of intact human platelets with adrenaline and noradrenaline resulted in a similar redistribution of β-adrenoceptors to that previously observed with isoprenaline, although relatively higher noradrenaline concentrations were required. Adrenaline-induced effects were not influenced by the presence of an α-adrenoceptor antagonist, suggesting that α_2- β_2- interactions do not influence β-adrenoceptor regulation in these cells.

The study described in this chapter demonstrates agonist regulation of β_2-adrenoceptors on human platelets and thus provides new information on the receptor changes that occur following agonist interaction with β-adrenoceptors on intact human cells. The relevance of these findings, and how they relate to current concepts of adrenoceptor regulation in human and other cell types, is discussed in chapter VII.

CHAPTER VII

Concluding Discussion

The main objective of this study was to examine agonist regulation of a human β-adrenoceptor, i.e. the human platelet β-adrenoceptor. The human platelet was selected as the model system to be employed for several reasons. Firstly, the platelet provides an easily accessible source of human tissue that is available for repetitive sampling. Secondly, previous studies had concluded that β-adrenoceptors were present on human platelets (see I.2.3.2.). Such conclusions were based on an antiaggregatory or inhibitory isoprenaline or adrenaline induced effect on platelet aggregation, and an isoprenaline or adrenaline stimulated adenylate cyclase activity in platelet lysates and whole cells. Taken together, these studies suggested that the human platelet exhibited readily definable functional and biochemical responses to β-agonists and was thus ideal for exploring all aspects of β-adrenoceptor regulations. Thirdly, as mentioned previously (I.3.) the presence of α_2-adrenoceptors on human platelets that are negatively coupled to adenylate cyclase provides a potential experimental system for examining agonist modulation of this receptor and for studying the interrelationships between α_2- and β-adrenoceptors in a single cell system.

Previous studies had however given conflicting results as regards the number and subtype(s) of β-adrenoceptors present on the human platelet (I.2.3.2.). Furthermore, it was unclear as to why functional and biochemical responses were only seen in the presence of isoprenaline or adrenaline with other β-agonists proving ineffective. The initial aim of this study was therefore

to perform a precise characterisation of the platelet β-adrenoceptor and evaluate its coupling to adenylate cyclase. Binding of the antagonist radioligand ^{125}I(-)PIN to human platelet β-adrenoceptors was examined. The results demonstrate the presence of a high affinity, low capacity binding site on human platelet membranes (Chapter III) and intact human platelets (Chapter VI) with the pharmacological characteristics of the β_2-adrenoceptor.

Agonist interactions with this binding site were examined (on platelet membranes) in the presence and absence of Mg^{++}, Gpp(NH)p and Na^+ (Chapter III). In competition experiments, addition of Mg^{++} or Gpp(NH)p had no effect on altering the affinity of isoprenaline to displace ^{125}I(-)PIN binding in the platelet. Parallel experiments performed with mammalian lung β_2-adrenoceptors exhibited the characteristic 'shift to the right' of the agonist displacement curve in the presence of the guanine nucleotide. If the extent of guanine nucleotide effect reflects the degree of coupling between the receptor and enzyme catalytic unit, these results suggest that the β_2-adrenoceptor present on the human platelet is not coupled to adenylate cyclase. Na^+, in contrast to Mg^{++} and Gpp(NH)p, produced a characteristic parallel shift to the right of the agonist competition curve, which suggests that the Na^+ site is not associated with Mg^{++} and guanine nucleotide sites, and may as has been hypothesised by others, be located on a different membrane component. Further evaluation of the coupling of the platelet β_2-adrenoceptor to adenylate cyclase by means of cyclic AMP accumulation experiments (Chapter IV)

failed to demonstrate any isoprenaline induced stimulation of adenylate cyclase, even in the presence of forskolin and thus supported the above hypothesis that platelet β_2-adrenoceptors are not coupled to adenylate cyclase. These results differed from those of previous studies which had observed very small (50% or less) β-agonist stimulated adenylate cyclase activity in platelet lysates and whole cells. The major difference between these studies and the present study was found to be the method of platelet preparation. Earlier studies employed only an initial centrifugation step to obtain platelet rich plasma from whole blood, whereas in this study an additional second centrifugation step was found to be necessary to eliminate significant white blood cell contamination present in the platelet rich plasma. When cyclic AMP accumulation experiments were performed in mononuclear leukocytes (MNL) and isoprenaline stimulated cyclic AMP levels for given values of MNL calculated, the data obtained revealed that minimal contamination by well coupled β_2-adrenoceptors on MNL could account for the reported β-agonist stimulated adenylate cyclase activity in the human platelet. These results do not rule out the possibility that β-adrenoceptors on human platelets are very weakly coupled to adenylate cyclase. It may be that the methods employed were not sufficiently sensitive to detect such coupling and in this respect adenylate cyclase studies may help clarify the situation. The results do however suggest that some (if not all) of the β-agonist stimulated adenylate cyclase activity previously reported by others may be due to contamination of the platelet preparation by other blood cells,

thus changes in cyclic AMP levels following agonist occupancy of β-adrenoceptors in the human platelet may (if they occur) be below the detection limit of currently available techniques. To summarise; no evidence of a significant coupling of human platelet β-adrenoceptors to adenylate cyclase was observed in this study.

This finding implied that human platelet β-adrenoceptors are either non-functional or interact with some other platelet effector system(s). Aggregation and secretion studies were performed (Chapter V) in an attempt to delineate a possible functional role for these β_2-adrenoceptors as modulators of platelet responsiveness to stimulatory agonists. Data obtained revealed no reproducible effect of β-adrenoceptor occupancy by β-agonist on the response of platelets to other agonists. Thus, no readily definable functional response to β-agonists was observed in the present study. Again these results do not rule out a functional role for β_2-adrenoceptors on platelets, especially since an anti-aggregatory effect of isoprenaline has previously been reported. It is possible however that, as discussed previously (Chapter V) interpretation of data may account for these differences since aggregation studies are mainly qualitative and at best only semi-quantitative.

During the course of this study it became apparent that agonist induced desensitisation of a biochemical or functional response to β-agonists could not be evaluated. The present study was therefore restricted to examining agonist induced redistribution

of human platelet β_2-adrenoceptors. Data obtained (Chapter VI) revealed that β-agonist treatment of intact human platelets resulted in a rapid and reversible sequestration of 40-50% of β_2-adrenoceptors present, away from the immediate cell surface and a further rapid (<30 minutes) irreversible down-regulation of approximately 25% of total receptor number.

The above agonist induced effects on receptor redistribution, observed in the absence of β-adrenoceptor stimulation of cyclic AMP, are similar to those previously found in cultured cells. Studies employing S49 lymphoma cells genetically deficient in certain coupling components of the β-adrenoceptor-adenylate cyclase system suggest that loss and recovery of surface receptors does not require the α subunit of Gs, activation of adenylate cyclase, cyclic AMP itself or cyclic AMP dependent protein phosphorylation (Mahan et al., 1985a). However, in contrast to the results obtained above in platelets, agonist induced down-regulation of receptor number was either absent or blunted in cells that exhibited an altered R-Gs interaction, e.g. cyc^- cells that lack the α_s subunit of Gs, or UNC cells that exhibit an impaired R-Gs interaction (Mahan et al., 1985a). Such data may be interpreted to suggest that : β-adrenoceptor-Gs interaction, although not detectable in the present study, was sufficient to cause down-regulation of human platelet β_2-adrenoceptor number : or, alternatively, may merely highlight the fact that cellular processes mediating changes in receptor expression are not identical in all cell types.

In recent years much attention has been given to elucidating the mechanism(s) responsible for altered tissue sensitivity to catecholamines in man. Much information has occurred on alterations of β-adrenoceptor number in a wide array of physiological and pathophysiological states (see Nahorski and Barnett, 1982; Insel and Motulsky, 1987). For example, patients with phaeochromocytoma and high circulating catecholamine levels have a low density of β-adrenoceptors (down-regulation of receptor number) (Greenacre and Connolly, 1978), whereas chronic β-adrenoceptor blockade is associated with an increased number of β-adrenoceptors (up-regulation of receptor number) (Glaubiger and Lefkowitz, 1977). Furthermore, changes in receptor number apparently correlate with desensitisation of catecholamine induced responses in vivo. For instance, chronic β-agonist treatment of normal and asthmatic subjects has been reported to result in decreased bronchodilatation, diminished generation of leukocyte cyclic AMP in response to catecholamines and a reduction in total β-adrenoceptor concentrations on these cells (Holgate et al., 1977; Galant et al., 1978; Kalisker et al., 1977). Manipulations designed to increase circulating catecholamine levels such as excercise, a low sodium diet or postural changes however, have given conflicting results. Certain studies have reported a decrease in β-adrenoceptor number following these manipulations while others report no change (Fraser et al., 1981; DeBlasi et al., 1986). Some of these discrepancies most probably relate to differences in the methods employed for assessing such changes and to the fact that in vivo

studies offer little opportunity to control the many variables which may regulate adrenoceptor function. Considered as a whole, information available as regards in vivo human β-adrenoceptor regulation is conflicting and somewhat confusing at present. In vitro studies of human β-adrenoceptor regulation may help clarify the situation. The results of the present study have demonstrated a redistribution of β_2-adrenoceptors on the human platelet following agonist treatment in vitro. Similar results have recently been obtained with mononuclear leukocytes (MNL). These cells possess a homogenous population of β_2-adrenoceptors that are strongly coupled to adenylate cyclase and which undergo a rapid desensitisation of cyclic AMP accumulation when incubated with agonist in vitro (Krall et al., 1980; DeBlasi et al., 1985; Motulsky et al., 1986). This desensitisation is not associated with a change in total receptor number if measured with relatively lipophilic agents such as ^{3}H-DHA and ^{125}I-CYP, but is accompanied by a redistribution of a proportion of receptors into a cellular environment not accessible to the hydrophilic compounds CGP-12177 and isoprenaline (DeBlasi et al., 1985; Motulski et al., 1986). Desensitisation in MNL cannot however be attributed to sequestration alone but involves an uncoupling of the receptor from adenylate cyclase. This was demonstrated by the observation that zinterol (hydrophobic agonist) and isoprenaline stimualtion of cyclic AMP accumulation in desensitised cells was identical (DeBlasi et al., 1985), and by the fact that phenylarsine oxide inhibits the agonist induced reduction in CGP-12177 accessible receptors but not agonist induced desensitisation of the receptor

(Feldman et al., 1986). Down-regulation of receptor number on these cells has been reported, but occurs very slowly following long (hours or days) incubation in the presence of an agonist (Tohmeh and Cryer, 1980; Aarons et al., 1983).

These latter results, coupled with those from the present study, demonstrate that if agonist regulation of human β-adrenoceptors is examined in vitro, the regulatory mechanisms involved are not dissimilar to those previously described for animal and cultured cell lines. Figure VII.1. attempts to incorporate experimental data available to date into a hypothetical model for the cellular processing of β-adrenoceptors during catecholamine specific desensitisation. The complexity of the model reflects the fact that, despite intensive investigation, the precise mechanism(s) and sequence of events responsible for β-adrenoceptor regulation are not yet known with certainty. In particular, the importance of phosphorylation/dephosphorylation reactions is unclear. In order to simplify the description of the model these reactions (steps A-G) are discussed separately.

In general terms, agonist (H) interaction with β-adrenoceptors present on the plasma membrane (step 1) results in activation of adenylate cyclase and the formation of an uncoupled state of the receptor (Ru) (step 2) detectable by a decrease in enzyme activity, i.e. desensitisation, and a decrease in high affinity agonist binding. Continued presence of an agonist induces further processing of the receptors, i.e. selective clustering into coated

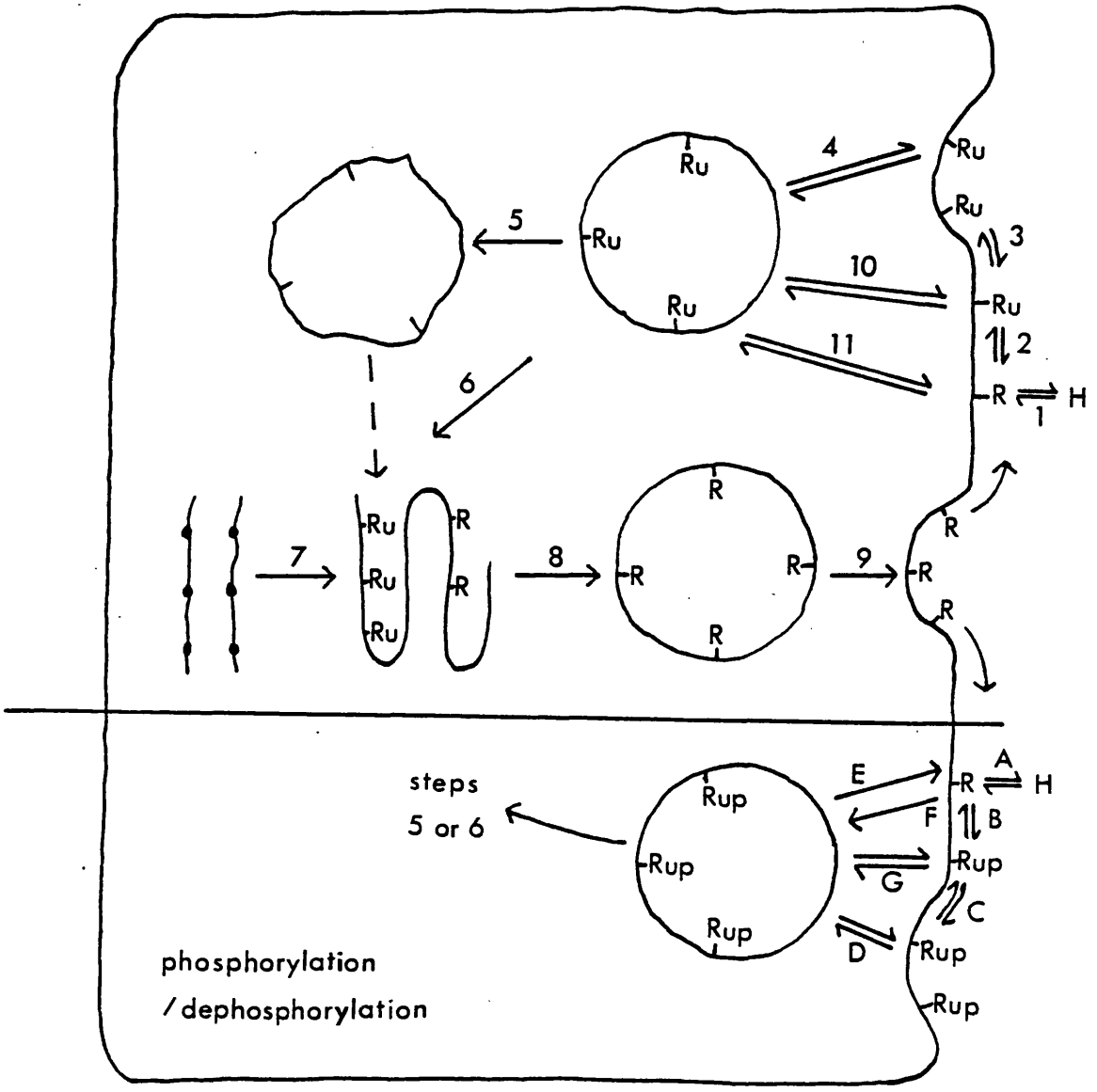


Figure VII.1. Hypothetical model for the cellular processing of β-adrenoceptors during catecholamine-specific desensitisation

For explanation see text

pits (step 3) followed by internalisation into vesicular structures with the ligand binding site facing inward (step 4). At this stage, receptor number defined by hydrophilic agents would be decreased whilst total receptor number would remain unchanged. If agonist was removed, receptors would reappear on the cell surface, desensitisation would reverse and normal function would be restored. If agonist presence persists, the internalised receptor containing vesicle may fuse with lysosomal structures (step 5) resulting in subsequent proteolytic degradation of the receptor protein. Alternatively the receptor protein may be processed (step 6) in such a way as to lose its binding capacity but retain its amino acid structure. At this stage, total receptor number is reduced i.e. receptors have been down regulated. Recovery from this stage is slow, it may involve processing of the receptors or the formation of new receptors by protein synthesis (step 7) both of which may be followed by externalisation (step 8) and insertion of the receptor into the plasma membrane (step 9).

If phosphorylation/dephosphorylation reactions are involved, the sequence of events may be as follows : briefly (see Chapter VI.1. for more detail), agonist (H) interaction with the β-adrenoceptor (R) (step A) results in phosphorylation (P) of the receptor (step B) which is apparent as a decreased ability of the receptor to couple with Gs (Rup). If agonist remains in the system, selective clustering (step C) of the receptors occurs which is followed by sequestration (step D). If agonist is removed, the receptor is reactivated by dephosphorylation (step E) and recycled to the cell

surface. If agonist persists then the receptor is processed as previously described (steps 5-9). Many aspects of this latter involvement of phosphorylation/dephosphorylation reactions in β-adrenoceptor regulation is purely speculative. For instance, it is not clear whether phosphorylation is responsible for uncoupling of the receptor (step B) or for triggering the sequestration of the receptor away from the cell surface (steps F or G) or in fact whether it is a common feature of all β-adrenoceptor adenylate cyclase systems.

Several observations suggest that the model depicted (figure VII.1.) in the absence of phosphorylation/dephosphorylation reactions is not applicable in all cell types. For instance, the findings of the present study may suggest that internalisation and down regulation of β_2-adrenoceptors occurs in the absence of receptor coupling to adenylate cyclase and that in the human platelet the two processes occur concomitantly, not sequentially as shown in figure VII.1.. Furthermore, in cells where β-adrenoceptors are coupled to adenylate cyclase, the question of whether uncoupling occurs prior to, or as a consequence of, sequestration remains controversial. Despite these inconsistencies, models such as that shown in figure VII.1. have proved invaluable when attempting to define the common mechanisms that may be involved from data obtained using a variety of techniques in different cell types.

At present the physiological and pathophysiological significance

of desensitisation and the various mechanisms shown to occur in vitro such as uncoupling, internalisation and down regulation remain largely unknown. A recent study employing the human platelet as a model to study β-adrenoceptor regulation in vivo has suggested that the mechanisms involved may well resemble those observed in vitro in the present study. Thus, oral administration of propranolol or pindolol for one week resulted in up-regulation and down-regulation of β-adrenoceptor density respectively (Beckeringh et al., 1986). Precisely how up-regulation can occur in the human platelet is however somewhat of a mystery. In contrast, studies employing MNL to study β-adrenoceptor regulation have shown that receptor changes observed in vitro were not apparent in vivo (DeBlasi et al., 1986). Further investigations are required to clarify this situation.

Throughout this study, attempts to delineate a possible internalisationship between the α_2- and β_2-adrenoceptors present on human platelets revealed no hint of any interaction between these receptors. Thus, when various responses, e.g. cyclic AMP accumulation, adrenaline induced receptor redistribution etc., were examined, the results obtained were indistinguishable in the presence and absence of the α-antagonist phentolamine at a concentration known to occupy all the α_2-adrenoceptors. These results differ from those of earlier studies in which β-adrenoceptor stimulated biochemical and functional responses were only observed in the presence of α_2-adrenoceptor blockade. However, for reasons that have previously been discussed, such responses were not

observed in this study. It has been suggested that certain β-adrenoceptor responses may actually involve interaction with Gi. For example, purified turkey erythrocyte β-adrenoceptors apparently stimulate purified rabbit liver Gi GTPase activity in reconstituted vesicles (Asano et al., 1984). It is also possible that certain β-adrenoceptors may function via effector mechanisms that involve neither Gi nor Gs, this remains to be seen. Such a situation would explain the results of the present study in view of the fact that functional coupling proteins (Gi and Gs) have been shown not to be required or involved in homologous regulation of β-adrenoceptors on lymphoma cells (Clark et al., 1986); that is, in this study, responses expected to result from functional β-adrenoceptor Gs interactions were absent, whereas β-adrenoceptor redistribution following β-agonist treatment, i.e. homologous regulation, was observed. The possible interrelationship between catecholamine receptors requires further investigation. The human platelet may not however be the best model for such studies, in view of the fact that the preliminary finding of an apparent lack of interaction between α_2- and β_2-adrenoceptors in this study might be due to the uncoupled state of the β-adrenoceptors on this cell.

In conclusion, the present study provides new information about the receptor changes that occur following agonist interaction with β-adrenoceptors on intact human cells and furthermore suggests the platelet to be a useful model for evaluating human β-adrenoceptor regulation in future in vitro and in vivo studies.

APPENDICES

Appendix 1

Chemicals : All chemicals were of analytical reagent grade.

Drugs and other substances

(-)	Isoprenaline	(Sigma)
(+)	Isoprenaline	(Sigma)
(-)	Adrenaline	(Sigma)
(-)	Noradrenaline	(Sigma)
	5-Hydroxytryptamine	(Sigma)
(-)-Pindolol		(Sandoz)
(±)-Cyanopindolol		(Sandoz)
(-)	Propranolol	(ICI)
(+)	Propranolol	(ICI)
ICI 118,551	(erythro-DL-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol).	(ICI)
(±)	Betaxolol	(Synthelabo)
CGP 20712A	(1-[2-((3-carbamoyl-4-hydroxy) phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluromethyl-2-imidazolyl) phenoxy]-2-propranol methanesulfonate).	(Ciba-Geigy)
(±) CGP 12177	(4-(3-tertiary-butylamino-2-hydroxypropoxy)-benzimidazol-2-one hydrochloride).	(Ciba-Geigy)

(±) Practolol	(ICI)
Guanyl-5'-yl-imidodiphosphate (Gpp(NH)p)	(Boehringer Mannheim)
PGE_1	(Sigma)
Vasopressin	(Sigma)
Human thrombin	(Sigma)
Forskolin	(Sigma)
Phentolamine	(Ciba-Geigy)
RO 20-1724	(Roche)
Cyclic AMP	(Boehringer Mannheim)
Adenosine diphosphate	(Sigma)
Adenosine triphosphate	(Sigma)
Bovine Serum Albumin	(Sigma)
Pyruvate Kinase	(Sigma)
Phosphoenol pyruvate	(Sigma)
ATP monitoring reagent	(LKB)
Norit GSX charcoal	(Hopkins and Williams)
Coulter Diff3 system stain pack and fixative	(Coulter Electronics)
OKT3 (Ortho-mune monoclonal antibody)	(Ortho Diagnostic Systems)
AN51	(DAKO)
FITC labelled goat anti mouse Ig	(Tago)
Fisofluor scintillation cocktail	(Fisons Ltd)
Lymphocyte separating medium	(Flow Laboratories)

Radiochemicals

(-)propyl-2-3-^{3}H-dihydroalprenolol, 102 Ci/mmol	(New England Nuclear)
5'-8-^{3}H-cyclic AMP, 40-60 Ci/mmol	(Amersham International, England)
Na^{125}I(carrier-free)	(Amersham International, England)
(-)-^{3}H-CGP-12177(-)-4-(3-t-Butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one), 44Ci/mmol	(Amersham International, England)

Appendix 2

Protein Determination in radioligand binding experiments

Protein was determined by the method of Lowry et al., (1951), which relies on the formation of a Cu^{++} peptide complex with proteins in alkaline solution and the reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein. Standards of samples were prepared in water. Standard curves for bovine serum albumin (0-300 ug/ml) were prepared and following reactions with Lowry reagents (see below), the absorbance was measured at a wavelength of 750nM on a Pye-Unicam SP6-500 U.V. spectrophotometer. Sample values of protein concentration were determined from regression lines calculated from the standard curve.

Reagents for protein assay

A. 2% Na_2CO_3 in 0.1N NaOH

B. 1% $CuSO_4 . 5H_2O$

C. 2% Potassium sodium tartrate

D. Freshly prepared 100ml solution A
1ml solution B
1ml solution C.

E. Folin-Ciocalteau reagent diluted 1:3 with H_2O

F. Protein Standard = Bovine serum albumin 10%.

Appendix 3

Analysis of radioligand binding data

a) Saturation data

For a simple bimolecular reversible reaction obeying mass action law at equilibrium

$$R + L \rightleftharpoons RL \qquad [1]$$

where R = concentration of free receptors

L = concentration of free ligand

RL = concentration of bound receptors

And the equilibrium dissociation constant (Kd) is defined as

$$Kd = \frac{R.L}{RL} \qquad [2]$$

Since total receptor density (Bmax) = R + RL [3]

Then [2] + [3] gives $RL.Kd = Bmax.L - RL.L$

and then $$RL = \frac{Bmax.L}{Kd+L} \qquad [4]$$

This equation describes a rectangular hyperbola and when L=Kd

$$RL = \frac{Bmax.L}{L + L} = 1/2\ Bmax$$

Therefore Kd is equal to the concentration of L which occupies half of the total receptors.

If RL = B (bound receptor)

and L = F (free ligand concentration)

then [4] becomes

$$B(F+Kd) = Bmax.F$$

$$B.F + B.Kd = Bmax.F$$

by rearrangement

$$B\ Kd = (Bmax-B)F$$

$$\frac{B}{F} = \frac{(Bmax-B)}{Kd} \qquad [5]$$

Equation [5] is the Scatchard equation (Scatchard, 1949). It allows linear transformation of data such that when a single ligand is interacting reversibly with a single population of receptors possessing a single affinity for the ligand, a plot of B/F versus B is a straight line and possesses a slope of -1/Kd. The x intercept is an estimate of

B max. Alternatively, a plot of B versus B/F (an inverse Scatchard or Hofstee plot) yields a negative slope equal to Kd, and the y intercept is an estimate of B max. Deviations from a straight line Scatchard plot may indicate that the ligand used is labelling a heterogenous population of sites for which it has different affinities, or that there is co-operative behaviour between sites, or may be due to the introduction of one or more technical artefacts that should really be minimized before obtaining the raw data. Such as, binding is measured when the system has not reached equilibrium; the separation of bound and free is incomplete, or the separation procedure is too slow so that ligand dissociates from the receptor; metabolism of ligand by tissue enzymes; inaccurate determination of specific and free ligand concentration.

Deviations from law mass action behaviour may also be assessed by another linear transformation of saturation data called the Hill plot.

For n receptor sites per molecule of ligand, a generalised form of equation [4] is:-

$$B = \frac{Bmax\ (L)^n}{Kd + (L)^n}$$

$$B.Kd + B.(L)^n = Bmax(L)^n$$

$$B.Kd = (Bmax-B)\ (L)^n$$

$$\frac{B}{(Bmax-B)} = \frac{(L)^n}{Kd}$$

taking the log of both sides gives the Hill equation (Hill, 1910).

$$\log \frac{B}{(Bmax-B)} = n \log (L) - \log Kd \quad [6]$$

A plot of $\log \frac{B}{(Bmax-B)}$ versus log (L) gives

a slope of the Hill plot, n, usually referred to as the Hill coefficient and denoted nH. The abscissa value where $\log \frac{B}{(Bmax-B)}$

= 0 is the Kd (actually - log Kd).

When nH = 1.0: ligand is binding to a single receptor type and obeying the law mass action. If multiple sites are present they are non-interacting and possess an identical affinity for the ligand.

Where nH>1.0 : positive co-operativity
Where nH<1.0 : negative co-operativity; multiple non-interacting binding sites for the ligand; or multiple interconvertible affinity states.

Since Hill plots deviate markedly from linearity at the extremes, only data between 20-80% occupancy of Bmax was considered in determining the slope (nH) by regression analysis.

b) Kinetic data

From equation [1] the binding reaction may be written as

$$R + L \underset{K_2}{\overset{K_1}{\rightleftharpoons}} RL \qquad [7]$$

where K_1 = second-order association rate constant.

K_2 = first-order dissociation rate constant.

The rate of reaction may be expressed as the rate of change of the concentration of the complex (RL) with time

$$\frac{d(RL)}{dt} = K_1 (L) (R) - K_2 (RL) \qquad [8]$$

The reaction continues until equilibrium is reached and the rate of formation of RL equals the rate of dissociation of the (RL) complex

i.e. $$\frac{d (RL)}{dt} = 0$$

Since the concentration of radioligand added (L) is much higher than the concentration of total receptors present in most radioligand binding assays, the concentration of free radioligand (L) is assumed not to change during the incubation. Hence for practical purposes, (L) is constant and the rate of association can be said to be "pseudo

first-order" where K_1' = pseudo first-order association rate constant = $K_1(L)$.

Substitution of K_1' into equation [8] gives

$$\frac{d(RL)}{dt} = K_1' (R) - K_2(RL)$$

If the reaction is allowed to reach equilibrium, the specifically bound radioligand (B) at any prior time is related to the specific radioligand binding at equilibrium (Beq) by

$$\ln \frac{Beq}{(Beq-B)} = (K_1'+K_2)t \quad [9]$$

then a plot of ln (Beq/Beq-B) versus time yields a linear transformation with a slope of Kobs:

if K_2 is known then K_1 can be calculated from

$$K_1 = \frac{Kobs-K_2}{L}$$

To determine the dissociation rate constant K_2, the association of radioligand (L) with receptor (R) is made negligible by addition of an excess of unlabelled competitive ligand, and so equation [8] reduces to

$$\frac{d(RL)}{dt} = K_2(RL)$$

a simple first-order equation.

and if Beq = specific radioligand binding at equilibrium

and B = specific radioligand binding at any time

then a plot of ln (B/Beq) versus time yields a linear transformation with a slope of $-K_2$.

units of $K_1 = min^{-1}\ M^{-1}$

$K_2 = min^{-1}$

thus $Kd = \frac{K_2}{K_1}$ = Molar

When binding of radioligand to receptor represents radioligand binding to a single class of receptors in a reversible fashion with fixed affinity, then the Kd determined for the receptor from equilibrium saturation data should be equivalent to the Kd calculated from Kinetic data.

c) Competition data

For a binding reaction governed by law of mass action

$$R + L + I \rightleftharpoons RL + RI$$

where I = concentration of unlabelled competing agent

and RI = concentration of receptor bound to this compound.

The potency of drugs in competing for the receptor is quantitated by determining the concentration of competing agent that effectively competes for 50% of the specific radioligand binding (the IC_{50}).

IC_{50} values may be determined from a Hill plot similar to that previously discussed for saturation data in appendix 3a. For competition curves however, the data must be expressed as % inhibition of specific binding (%I).

A plot of log $\frac{\%I}{100\%-\%I}$ versus log molar concentration

yields a linear transformation with a slope equivalent to a pseudo-Hill coefficient (or slope factor) and an x intercept of -log IC_{50}. A true Hill slope cannot be obtained from applying the Hill equation to competition binding data since multiple rate constants are involved in the incubation;

$$L + R \underset{K_2}{\overset{K_1}{\rightleftharpoons}} LR \quad \text{and} \quad I + R \underset{K_4}{\overset{K_3}{\rightleftharpoons}} IR$$

Therefore the slope factors calculated are referred to as pseudo-Hill coefficients.

IC_{50} values and slope factors may also be obtained from Allfit analysis. Allfit is a computer assisted, non-linear, least squares curve fitting programme based on a four parameter logistic equation, mathematically analogous to the Hill equation :-

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

where Y = concentration of radioligand bound

X = concentration of competing ligand bound

A = response at dose=0 (i.e. =100% specific binding or 0% inhibition of specific binding).

B = slope factor

C = IC_{50} of competing ligand

D = response at dose=infinity (i.e. = 0% specific binding or 100% inhibition of specific binding).

Allfit arrives at an estimate of best fit parameters for competition data by minimisation of the sum of squares i.e. parameters are adjusted in the model so that the sum of squares of differences between estimated values and actual data points are as small as possible. Competition curves may be examined individually or simultaneously as a group. Following each curve fitting procedure, the programme gives estimates of model parameters and an approximate standard error for each estimate. This index of accuracy is further complimented by two other internal statistics : the residual variance test which compares a

curve to a group of curves examined simultaneously ; and the runs test which examines the randomness of data point distribution for each curve. Each of these internal statistics enable the investigator to gauge which models are an inaccurate fit of the experimental data. The "goodness of fit" test is an external statistical test that evaluates which model gives a significantly better fit of the data e.g. in the present study this test was employed to determine whether or not competition curves were significantly different from unity. It is a partial F-test (De Lean et al., 1978) calculated as

$$F=[(SS1-SS2)/(dF1-dF2)]/(SS2-dF2)$$

where SS1 and SS2 = sum of squares for model 1 and model 2.

dF1 and dF2 = degrees of freedom for model 1 and model 2.

(dF = number of data points - number of parameters estimated).

At dF1-dF2 and dF2 degrees of freedom, the F-test was taken to indicate a better fit for the more highly parameterised (i.e. two-site) model when the F value returned a probability of <0.05.

Pseudo Hill coefficients obtained from either Hill or Allfit analysis describe the shape of the competition curve. A slope factor of 1.0 is consistent with ligand-receptor interactions occuring via a simple bimolecular reaction governed by law of mass action. A slope factor of significantly less than 1.0 may indicate negative co-operativity, receptor heterogeneity, or multiple receptor affinity states. Such curves are often described as being 'shallow', of 'low slope' or may

even be biphasic. However, since Allfit cannot resolve these complex binding phenomena, multiple site analysis in the present study was performed by means of a different iterative curve fitting computer programme (NAG Library. E04FBF, NAG Ltd, Oxford, 1977). This programme also arrives at the best fit parameters by minimisation of the sum of squares, and goodness of fit was determined by the error sum of squares.

The mathematical model for binding to a two-site, non-interacting system was

$$B = \frac{Bmax_1\,[I]}{[I] + IC_{50\ 1}} + \frac{Bmax_2\,[I]}{[I] + IC_{50\ 2}}$$

where B = % specific binding displaced

I = concentration of competing ligand

$Bmax_1$, $Bmax_2$ = proportion of each site (%)

$IC_{50\ 1}$, $IC_{50\ 2}$ = concentration of competing ligand displacing 50% specific binding to each site.

APPENDIX 4

Iodination of radioligands

a) Iodination of (-) pindolol

(-)Pindolol (PIN) was iodinated according to the method of Barovsky and Brooker (1980). 5ul of 10mM(-)PIN was placed in an eppendorf microcentrifuge tube (1.5ml) and evaporated to dryness under a stream of nitrogen. 1mCi $Na^{125}I$ was then added, followed by 40ul of 0.3M potassium phosphate buffer pH7.5 and 5ul of aqueous chloramine-T solution (0.17mg/ml). The tube was vortexed briefly and the iodination allowed to proceed for three minutes at room temperature. The reaction was stopped by addition of 500ul of sodium metabisulphite (1mg/ml) in 1M acetic acid, and the pH of the reaction mixture adjusted to 10.0 with 2N NaOH. The iodinated product was extracted five times with 300ul ethyl acetate, and the extracted volume (≙1.5ml) reduced under a stream of nitrogen to ≙300ul before being spotted onto a strip of Whatman 3MM paper and chromatographed in a descending manner at room temperature with 1M ammonium formate (pH 8.5): methanol, 10:1 (v:v). The chromatography paper was removed when the solvent front was approximately 2.5cm from the end and cut into 1cm strips, which were then eluted in 5.0ml ethyl acetate containing 2% diethylamine (v:v). ^{125}I(-)PIN was located (in two or three fractions) as a single peak with an Rf of 0.29 - 0.36, placed into glass vials and stored at -20°C for use.

b) Iodination of (±)cyanopindolol

(±)Cyanopindolol (CYP) was iodinated according to the method of Engel et al., (1981). 10ul of 13.5mM HCl containing 20ug CYP, 20ul 0.3M potassium phosphate buffer pH 7.6, 2mCi $Na^{125}I$ and 20ul aqueous solution of chloramine T(0.34 mg/ml) were combined in that order in an eppendorf microcentrifuge tube (1.5ml), vortexed briefly and allowed to stand at room temperature for five minutes. The reaction was stopped by addition of 300ul aqueous sodium thiosulphate solution (1mg/ml), followed by addition of 10ul 1M NaOH. The iodinated product was extracted four times with 300ul ethyl acetate containing 0.01% phenol. The extracted volume (≙1.2ml) was spotted onto a strip of Whatman 3MM paper and chromatographed in a descending manner at 4°C with 0.1M ammonium formate pH8.5, 0.01% phenol. The chromatography paper was removed when the solvent front was approximately 2.5cm from the end, and cut into 1cm strips, which were then eluted in 5.0mls methanol. ^{125}I(±)CYP was located as a single peak with an Rf of 0.09 - 0.10, placed into glass vials and stored at -20°C.

APPENDIX 5

Buffers

1. Phosphate Buffered Saline (PBS)

purchased as concentrate from Mercia Diagnostics.

2. Tris Buffered Saline (TBS)

	g/l
Solution A :	
Glucose	1.0
$CaCl_2.2H_2O$	0.0074
$MgCl_2$	0.1992
KCl	0.4026
Tris	17.565

Dissolve in 950mls distilled water and adjust to pH 7.6 with 1N HCl.
Make up volume to 1 litre.

Solution B :

NaCl	8.19

Mix 1 volume solution A with 9 volumes of solution B

3. Modified Krebs Henseleit Buffer

(for cyclic AMP accumulation experiments - chapter IV).

	g/l
NaCl	6.92
KCl	0.35
Glucose	2.1
$NaHCO_3$	2.1
KH_2PO_4	0.16
$MgSO_4.7H_2O$	0.29
Na metabisulphite	0.038

Make up fresh and aerate with 95% O_2, 5% CO_2 at 37°C for 30 minutes before immediate use.

APPENDIX 6

Wright's stain for light microscopy

"Cytospin" slides were prepared (see II.3.2.4), fixed in Diff350 fixative reagent for three minutes, air dried and stained immediately with Wright's stain (Coulter Diff 3 system stain pack) in an Ames Hema-Tek Slide stainer.

Reagents

Fixative Reagent :	Polyvinylpyrrolidinone (in absolute Methanol)	20g/l
Stain Reagent :	Wright's stain in absolute methanol	3.0mg/l
	Stabilizer	3% (v/v)
Buffer Reagent :	$Na_2HPO_4.7H_2O$	9mmol/l
	$KH_2\ PO_4$	58mmol/l
	Surfactant	1% (v/v)
	Preservative	2% (v/v)
Rinse Reagent :	KH_2PO_4	1.78mmol/l
	$Na_2HPO_4.7H_2O$	0.45mmol/l
	Deionized water	66% (v/v)
	Absolute methanol	33% (v/v)

Surfactant	1% (v/v)

Staining Procedure :

Soak for two minutes in Wright's stain, and then in buffer reagent for one minute. Rinse well in rinse reagent and air dry. Apply one drop of DPX mountant to slide and appose coverslip.

APPENDIX 7

A. Preparation of adrenal binding protein

Bovine adrenals were obtained from a local abattoir and trimmed of fat and medullary tissue. The remaining cortices were homogenised in a Waring blender in 1.5 volumes Littlefields medium containing 2-mercaptoethanol and theophylline. The homogenate was centrifuged at 2000g for five minutes at 4°C, the cell debris discarded and the supernatant recentrifuged at 5000g for fifteen minutes at 4°C. The resulting supernatant was divided into 0.5ml aliquots and stored until required at -20°C.

Before routine use in cyclic AMP assays, the binding of radiolabelled cyclic AMP was examined with respect to protein dilution. Various protein dilutions were employed, and the dilution giving 30-40% binding of total added ^{3}H-cyclic AMP was selected for routine use in subsequent assays. This degree of binding gives an assayable range of 0.25 - 15.0 pmols cyclic AMP.

Littlefields Medium

Sucrose	250 mM
Tris-HCl	50 mM
KCl	25 mM
$MgCl_2.6\ H_2O$	5 mM
2-Mercaptoethanol	6 mM

Theophylline hydrate 8 mM

Adjust to pH 7.4 with 1N HCl.

B. Measurement of cyclic AMP

Cyclic AMP was measured using the protein binding method of Brown et al., (1971) that relies on competition between ^{3}H-cyclic AMP and cyclic AMP for binding to a cyclic AMP dependent protein kinase, derived from bovine adrenals.

All reagents and protein were diluted in assay buffer (50mM Tris HCl, 4mM EDTA, pH 7.4). Each assay tube contained 100ul blank, sample, cyclic AMP standard or 8uM cyclic AMP (for NSB); 100ul ^{3}H-cyclic AMP (approximately 25,000 cpm), and buffer to a volume of 300ul. The reaction was started by the addition of 100ul adrenal binding protein and incubated overnight at 4°C. The incubation was terminated by the addition of 250ul of 0.5% Norit GSX charcoal/0.26% bovine serum albumin in assay buffer. Tubes were kept at 4°C for 25 minutes prior to centrifugation at 2500g for 25 minutes at 4°C. The supernatant was decanted into 4.5mls of Fisofluor 1 scintillation fluid (Fisons Ltd) and the radioactivity counted in an LKB-Wallac liquid scintillation counter at 40% counting efficiency.

Cyclic AMP values were calculated using a general, non-linear, least squares curve fitting programme based on the same logistic function as that employed in the Allfit programme (appendix 3c).

Appendix 8

Evaluation of optimal conditions for ^{125}I(-)PIN binding to human platelet membranes

Preliminary experiments were performed to determine the most appropriate incubation conditions to be adopted to optimise the specific radioligand binding. Experiments were performed as described (III.2.1.) with the appropriate modifications.

Assay buffer : Ideally, incubations should be performed under near physiological conditions. However, the presence of metal ions was to be avoided in view of their modulatory effect on agonist binding, discussed previously (III.1.). To ensure that performing the assay incubation in the absence of sodium chloride did not result in inactivation of β-adrenoceptors during the incubation period, as has been reported for ^{125}I(±)CYP binding to certain β-adrenoceptors (Bojanic, 1984), a brief comparison of ^{125}I(-)PIN binding in hypotonic (50mM Tris HCl, 0.2mM Na metabisulphite, pH 7.8) and isotonic (addition of 100mM NaCl to hypotonic buffer) assay buffer was performed. In parallel saturation experiments (n=3) the binding parameters for ^{125}I(-)PIN were not significantly different ($p>0.05$) in the two buffers: Bmax = 4.16±0.25 and 4.05±0.34 (fmol mg^{-1} protein), Kd = 20.6±1.18 and 23.17±1.56 (pM) for hypotonic and isotonic buffer respectively. Thus sodium chloride inclusion in the assay buffer was not considered necessary.

Separation of bound and free radioligand : choice of an appropriate separation technique is governed by the receptor under study. For particulate preparations, as used in this study,-methods available are centrifugation, equilibrium dialysis, or filtration. Centrifugation often provides inadequate separation of bound and free radioligand, which results in relatively high non-specific binding. Equilibrium dialysis is cumbersome to perform and slow, thus allowing time for dissociation of radioligand from receptor. Vacuum filtration however is easy to perform, provides a rapid separation of bound from free radioligand and was considered the method of choice for the present study. Dissociation of radioligand from receptor during the time taken to separate bound/free ligand under 18-20psi negative pressure was negligible in the present study. Ligand dissociation was minimized by the use of ice-cold washing buffer, and generally 3 x 10ml rapid washes of the filters was sufficient to minimize non-specific binding without reducing specific binding. Addition of sodium chloride (100mM) to the washing buffer employed in III.2.1. did not reduce non-specific binding or change the amount of specific binding. In saturation experiments (n=3) performed as described in III.2.1. the Bmax (fmol mg^{-1} protein) for ^{125}I(-)PIN was 4.02±0.19 and 4.37±0.13, with a Kd(pM) of 21.97±1.42 and 25.11±2.05 if filters were washed in the absence or presence of sodium chloride respectively. Thus, sodium chloride was not present in the washing buffer adopted for routine use.

Assay pH. The effect of pH on ^{125}I(-)PIN binding to human platelet membranes was examined. Experiments were performed in the presence of 100pM ^{125}I(-)PIN as previously described III.2.1. with assay buffer

adjusted to cover a pH range of 7 - 9. Non-specific binding defined in the presence of 200uM (-)isoprenaline was unaffected by changes in pH. Specific binding remained constant between a pH range of 7.5 - 8.5 but was significantly reduced outside this range (figure 8.1). In view of the susceptibility of catecholamines to oxidation above pH 8, binding assays were performed at pH 7.8 in the presence of an antioxidant. Sodium metabisulphite (0.2mM) employed as antioxidant did not alter agonist affinity in competing for ^{125}I(-)PIN binding. Isoprenaline competition experiments were performed in assay buffer with and without antioxidant. Mean curves (n=3) were superimposable, with IC_{50} values of 0.326uM and 0.348uM in the absence and presence of Na metabisulphite respectively (data not shown).

Determination of non-specific binding : specific binding defined as total minus non-specific binding (in the presence of 200uM(-)isoprenaline) was generally 70-80% of total binding at the concentration of ^{125}I(-)PIN (30-40 pM) used in competition experiments. The concentration of (-)isoprenaline employed for defining non-specific binding was carefully determined from competition experiments as previously described for rat lung membranes (figure II.4.).

Protein linearity : specific ^{125}I(-)PIN binding to human platelet membranes increased linearly with protein concentration over the range 15-360ug protein (using 100pM^{125}I(-)PIN, figure 8.2.), as would be expected for a membrane preparation possessing a finite number of β-adrenoceptors. Receptor concentrations employed in binding experiments were well within this linear range and bound <10% of total

radioligand added.

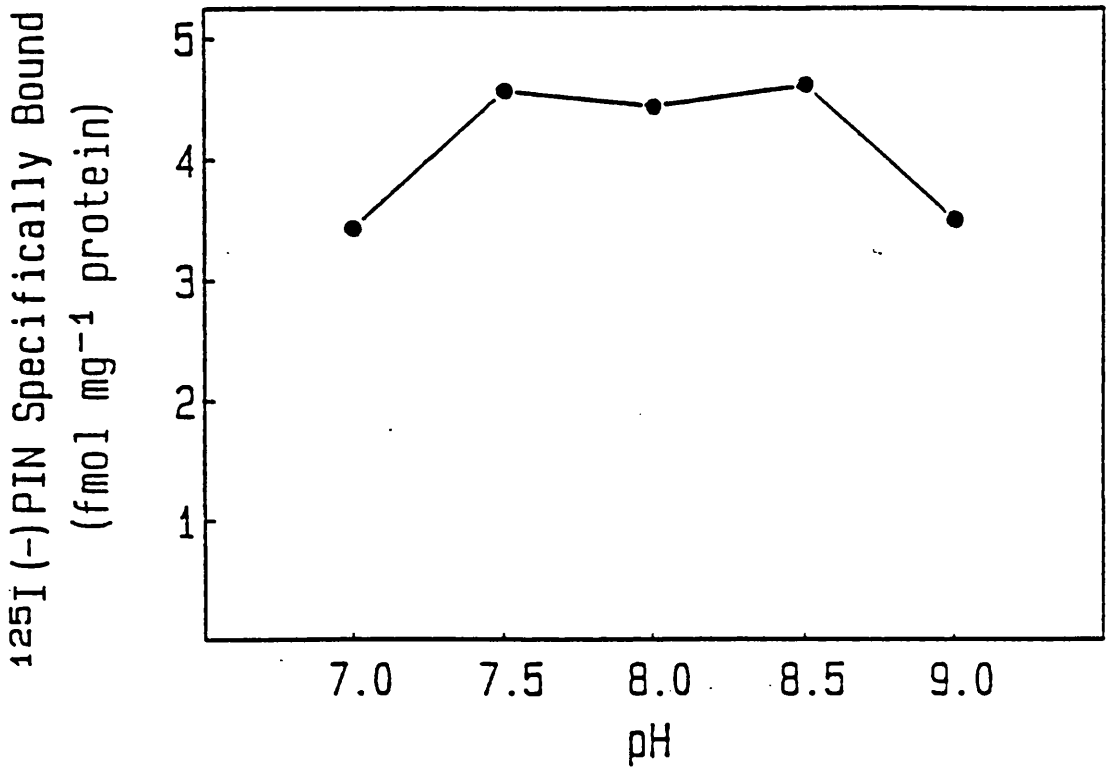


Figure 8.1. Specific ^{125}I(-)PIN binding to human platelet membranes as a function of pH

Membrane preparations were diluted in assay buffer of varying pH values, and equilibrium experiments performed in the presence of 100pM ^{125}I(-)PIN as previously described (III.2.1.). Specific binding defined as total minus non-specific binding (in the presence of 200uM (-)isoprenaline) was determined and plotted against assay pH. The values shown represent the mean of two separate experiments performed in triplicate at each pH value.

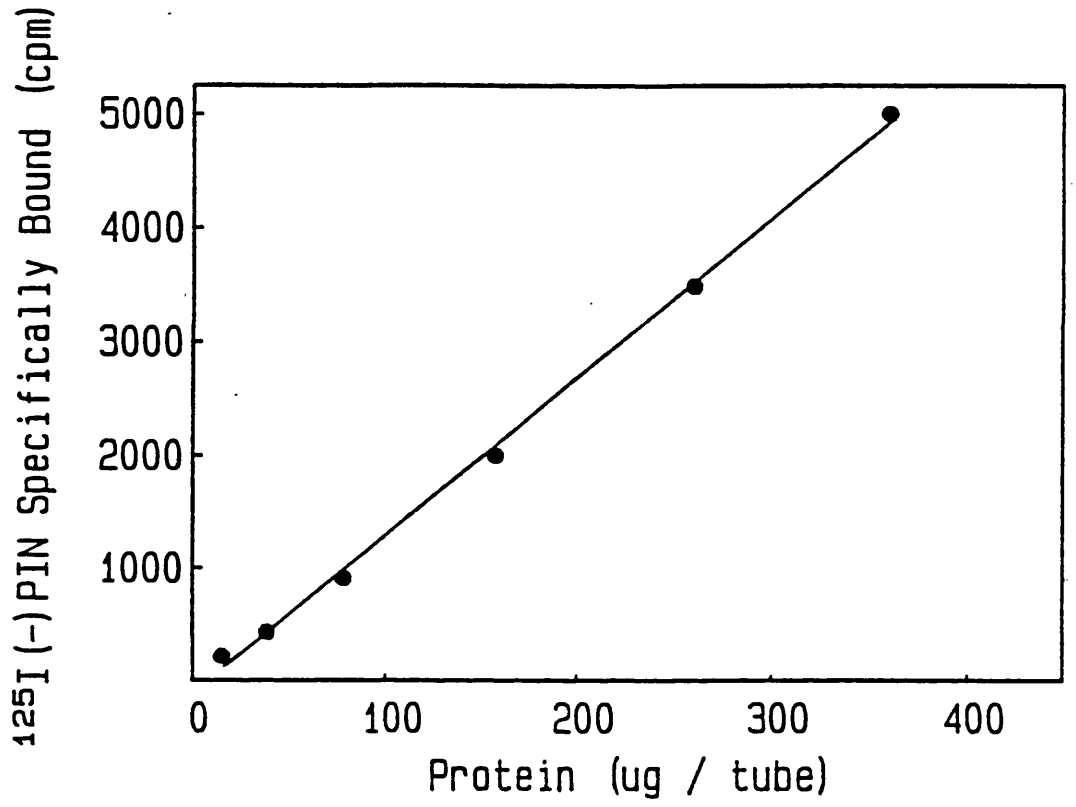


Figure 8.2. Specific ^{125}I(-)PIN binding to human platelet membranes as a function of tissue protein concentration

Platelet membranes (15-360ug protein) were incubated to equilibrium in the presence of ^{125}I(-)PIN (100pM). Specific binding determined in the presence of 200uM (-)isoprenaline was calculated and plotted as a function of protein concentration. Data shown are from a single representative experiment performed in triplicate.

Appendix 9

Measurement of total and released adenine nucleotides

Total and released ATP and ADP were determined by a bioluminescent technique that employs a stabilised firefly luciferin-luciferase reagent (ATP monitoring reagent, LKB) and an LKB luminometer.

The technique is based upon the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalysed by firefly luciferase. The formula for the light producing reaction is as follows:-

$$\text{ATP} + \text{luciferin} + O_2 \xrightleftharpoons{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + Co_2 + \text{LIGHT}$$

Reagents:

1) ATP monitoring kit (LKB) (a) ATP monitoring reagent

i) firefly luciferase

ii) D-Luciferin

iii) Bovine serum albumin

iv) Magnesium acetate

v) Inorganic pyrophosphate

(b) ATP standard

i) ATP, 0.1umol

ii) Magnesium sulphate, 2.0umol

2) Tris-EDTA

12.1g Tris (0.1M) + 0.744g Na_2EDTA (2mM) are dissolved in 900ml of distilled water. pH is adjusted to 7.75 with 50% acetic acid and volume made up to 1 litre.

3) Tris-EDTA-BSA

1mg albumin per ml of Tris-EDTA.

4) Triton-Tris-EDTA

Prepare Tris-EDTA as above with 4mM EDTA and add 0.2% $^{v}/_{v}$ triton X-100.

5) Pyruvate Kinase (PK)

Stock PK (see Appendix 1) is diluted 1:10 in Tris-EDTA-BSA.

6) Phosphoenol pyruvate mix (PEP-Mix)

0.2ml 100mM PEP

0.8ml H_2O

5.0ml 1.0M magnesium acetate

3.0ml 2.0M potassium acetate

Method

Total or released nucleotide extracts were prepared and frozen away as described in V.2.3.

For each extract, two assay tubes were prepared (except for ADP induced secretion studies, see V.2.3.), one for the determination of ATP content alone, the other for the determination of ATP + ADP content.

Thus, a) ATP determination :

10ul of extract was added to 400ul Tris-EDTA, 40ul PEP Mix + 100ul monitoring reagent and the signal recorded in mV. 10ul ATP standard was then added and the signal recorded again.

b) ATP + ADP determination :

10ul of extract was added to 400ul Tris-EDTA, 40ul PEP Mix and 10ul PK. The mixture was incubated at room temperature for 15 minutes to allow conversion of ADP->ATP, 100ul of monitoring reagent was then added and the signal recorded in mV.10ul ATP standard was added and the signal recorded again.

Concentrations of ATP and ADP were calculated by using the mV measured upon addition of the internal standard. Corrections for background light emission were made whenever blanks (=no test sample added) produced significant amounts of light.

To calculate ATP concentrations :

$$\text{ATP (umol)} = \frac{\text{sample (mV) - blank (mV)}}{\text{standard (mV)}} \times \text{amount of ATP standard added (umol)}$$

For ADP = (Total ATP + ADP) - ADP.

The firefly luciferase and luciferin contained in the ATP monitoring reagent are formulated to provide a linear signal up to a concentration of 10^{-6}M ATP, the lower limit of detection being 10^{-11}M. Within this range, duplicate assays showed less than 5% variation as has previously been reported by others (Summerfield et al., 1981).

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