ANGIOTENSIN II RECEPTORS IN HUMAN PLATELETS

by

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DEDICATION

To my wife and my parents

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SUMMARY

Angiotensin II, the effector peptide of the renin-angiotensin system, acts on its target cells via specific membrane receptors. Platelets were prepared from peripheral venous blood (60 ml) by centrifugation through an iso-osmotic solution of Percoll, resulting in a good recovery of cells (50-90%, n=192), relatively free of erythrocytes ($<0.1\%$) and leukocytes ($<1\%$). Specific binding of ^{125}I-angiotensin II (300 pmol/l) to platelets was identified. This was time and temperature dependent, saturable, reversible and linear with platelet concentration. Scatchard analysis of saturation curves revealed a single class of binding sites with Kd $1.5 \pm 0.4 \times 10^{-10}$ mol/l and total binding capacity 6.3 ± 1.2 receptor/platelet. Similar values (Kd $2.4 \pm 0.7 \times 10^{-10}$ mol/l, and binding capacity 6.5 ± 1.0 receptor/platelet) were obtained from displacement analysis. From kinetic studies the forward and reverse rate constant were 3.1×10^{8} mol $min^{-1}l^{-1}$ and $3.6 \times 10^{-2} min^{-1}$ giving a Kd of 1.2×10^{-10} mol/l. The relative binding potencies for angiotensin II and analogues were: [Sar^1, Thr^8]-Ang II > Ang II > Ang III > [Sar^1,Ala^8]-Ang II > Ang I, these are similar to those described for rat adrenal cells. Pretreatment with captopril 'in vivo' and angiotensin II 'in vivo' and 'in vitro' did not alter receptor characteristics. Studies with a D-phenylalanine, showed that specific binding was not affected by this carboxypeptidase A inhibitor. Incubation with an extracellular fluid marker (^{51}Cr-labelled EDTA) demonstrated that binding of angiotensin II to platelets was by a specific receptor mechanism and not due to free fluid endocytosis.

The effect of changes in dietary intake of sodium and potassium on ^{125}I-angiotensin II binding to platelets were studied in eight normal subjects. Restriction of sodium intake (15 mmol/day) resulted in a decrease in the number of receptor sites from 6.2 ± 0.3 sites/platelet to 4.1 ± 0.4 sites/platelet

($p < 0.01$) but there were no changes in affinity (Kd). Over a range of sodium intakes from 15 to 200 mmol/day (high sodium diet) there was a correlation between plasma concentration of angiotensin II and receptor site concentration (r_s 0.57, $p < 0.01$). Similar changes in the density of angiotensin II binding sites have been described in vascular smooth muscle. Changes in dietary potassium intake from normal (70 mmol/day), to low (35 mmol/day) or high (150 mmol/day) did not affect angiotensin II binding.

Angiotensin II binding was measured in ten patients with essential hypertension (mean blood presure 178/107 mmHg, plasma concentrations of renin 12 $\pm$ 2 μU/ml and angiotensin II 14 $\pm$ 2 pg/ml) and ten subjects with normal blood pressure (mean blood pressure 112/74 mmHg, plasma concentrations of renin 13 $\pm$ 2 μU/ml and angiotensin II 13 $\pm$ 2 pg/ml). The binding capacity and affinity (Kd 5.0 $\pm$ 0.6 x 10^{-10}M, 5.7 $\pm$ 0.8 sites/cell) in the hypertensive patients were similar to values in the normotensive subjects (Kd 4.9 $\pm$ 0.8 x 10^{-10}M, 5.4 $\pm$ 0.5 sites/cells). Changes in sensitivity to angiotensin II in essential hypertension may not be determined at receptor level.

The effects of angiotensin II on platelet function were also studied. Angiotensin II (10^{-11} - 10^{-7}M) alone had no effect on platelet aggregation and did not increase the cytosolic free Ca^{2+} concentration following either short (<2 min) or long term (30 min) incubation of Quin 2 labelled platelets. No significant increase or decrease in thromboxane B_2 production from platelets in response to angiotensin II was observed.

However, the secondary phase of adrenaline-induced platelet aggregation was significantly augmented by angiotensin II. A low concentration of angiotensin II (10^{-11}M) enhanced the effects of adrenaline, whereas higher concentrations (10^{-7}M) inhibited aggregation; angiotensin had no effect on ADP-induced aggregation. The facilitatory effect of angiotensin II on platelet

aggregation was prevented by pre-treatment of platelets with flurbiprofen indicating that angiotensin II either, stimulates the release of, or potentiates the actions of thromboxane A_2 (the mediator of adrenaline-induced secondary aggregation). Angiotensin II (10^{-11} - 10^{-7}M) enhanced the effects of the stable thromboxane A_2 mimetic U44069 on platelets but synthesis of thromboxane B_2, the stable and inert product of thromboxane A_2 metabolism, was inhibited. The pro-aggregatory effects of low concentrations of angiotensin II on platelets are probably due to facilitation of the effects of thromboxane A_2.

CHAPTER 1 INTRODUCTION

1.1 THE RENIN-ANGIOTENSIN SYSTEM

Renin was discovered in 1898 by Tigerstedt and Bergman as a pressor material in extracts of renal cortex (Tigerstedt and Bergman 1898). It is now known that renin is an aspartic proteinase, synthesised and secreted mainly by the epithelial cells of the juxtaglomerular apparatus in the renal cortex (review, Davis and Freeman 1976). It acts on its substrate (angiotensinogen), an α-globulin synthesised by the liver, to produce an inactive decapeptide, angiotensin I. The renin-substrate reaction takes place in blood but may also occur within blood vessel walls. Subsequently, angiotensin I is cleaved by a peptidyl dipeptide hydrolase (angiotensin I converting enzyme), which is mainly present on the membrane of endothelial cells from the pulmonary and systemic vasculature. This enzyme removes a dipeptide from the carboxyl terminal of the decapeptide to produce the vasoactive octapeptide angiotensin II (Vane 1972). The cleavage of angiotensin II by a variety of angiotensinases produces a number of smaller peptides, one of which, the heptapeptide angiotensin III, is also physiologically active (Blair-West, Coghlan, Denton, Funder, Scoggins and Wright 1971). The biochemical pathways of the renin-angiotensin system are shown in Figure 1.1.

The octapeptide, angiotensin II is the major active component of the renin-angiotensin system in man. The concentration of angiotensin II in human peripheral plasma is around 20 pM (Morton, Semple, Waite, Brown, Lever and Robertson 1976)

Angiotensin II is one of the most powerful constrictors of arterial smooth muscle. It also has an important role in regulating extracellular fluid volume by affecting aldosterone secretion from the adrenal cortex and by direct effects on

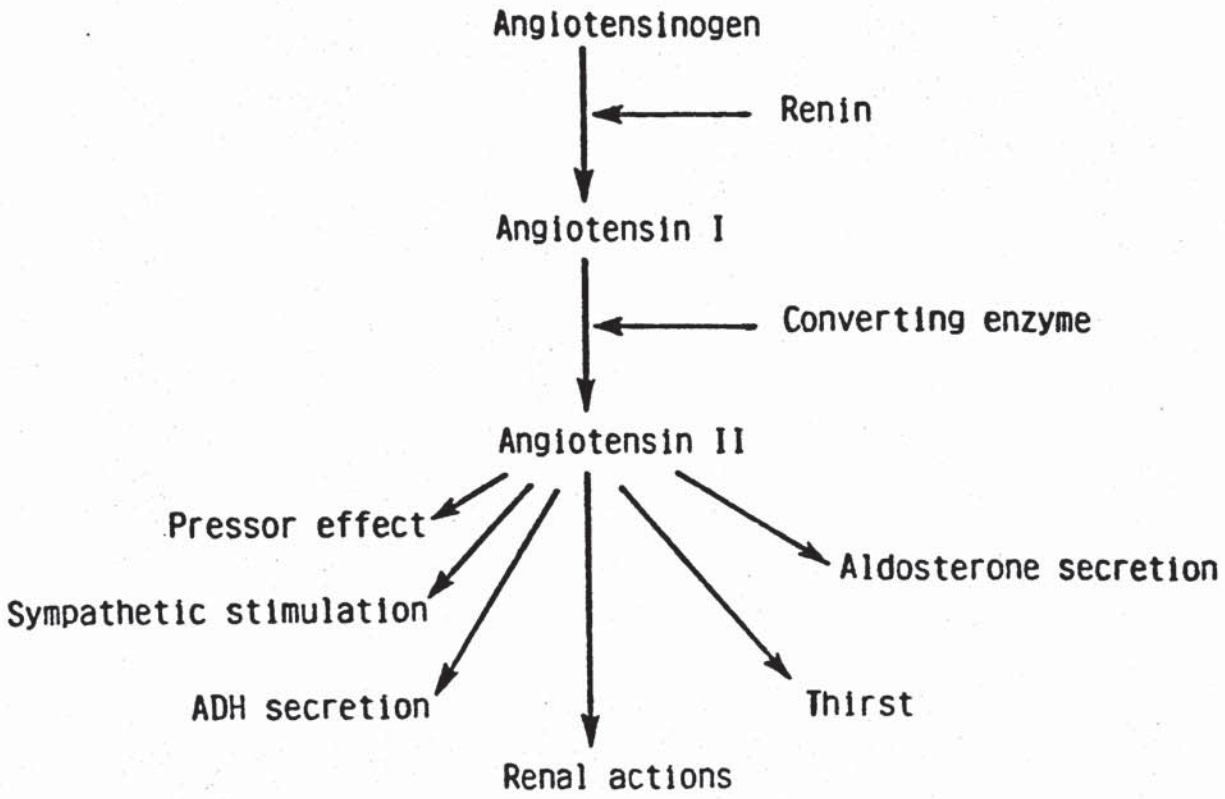

FIGURE 1.1 Outline of the renin-angiotensin system and some of the main actions of angiotensin II.

renal function. In addition it may interact with the sympathetic nervous system and be a factor in the regulation of anti-diuretic hormone secretion.

The physiological effects of angiotensin II are initiated by hormone interacting with its specific receptors.

1.2 HORMONE ACTION

1.2:1 Definition of a hormone

A hormone is a substance released from endocrine glands into the circulation, that controls the function of specific target tissues. This definition has been widened to include substances like angiotensin II, which are not synthesised by endocrine glands but have regulatory properties characteristic of a hormone. The specificity of hormone action in a particular target tissue is due to recognition of the hormone by a receptor. Interaction of the hormone with its receptor initiates a chain of biochemical events which characterise the physiological response. The receptor concept can be traced back to Langley's work (1906) on the action of nicotine and curare on muscle. He described a specific receptive substance, which received the stimulus and, by transmitting it, caused contraction. An extension of the receptor concept came from the work by Ehrlich (1908), who found that tetanus toxin first combines with certain groups in the protoplasm of cells, and that this chemical combination is responsible for the poisonous effects. He named these chemical groups 'receptors'. Clark (1926 a & b) made similar conclusions from his observation of acetylcholine and atropine action. He showed that the very small quantities of drug (about 20,000 molecules per cell) were required to produce a cellular response, were correlated with the number of receptors present in responsive cells. He also pointed out that drug-receptor combination obeyed the law of mass action. Since then, the

idea of receptors has become firmly established. From classical dose-response studies in tissues using hormone agonists and antagonists it became possible to classify receptors. The recognition of the alpha- and beta-adrenoreceptor by Ahlquist is an example of this approach (Ahlquist 1948). From such indirect studies with hormone antagonists it was also possible to calculate receptor affinity (Schild 1949). Over the last 15 years, it has been possible to measure receptor binding directly using radio-labelled hormones and drugs. These radio-ligand binding techniques have been applied to receptors for a great number of pharmacological active agents (Cuatrecasas and Hollenberg 1976; Blecher 1976). These methods depend on techniques to distinguish receptor binding from biochemically unimportant non-specific binding.

1.2:2 The second messenger concept

It is doubtful that any hormone directly affects the physiological process which it ultimately controls. In most cases there is at least one intermediary intracellular step between receptor binding and the final biochemical event. In 1956 Sutherland et al. (Sutherland and Wosilait 1956) incubated dog liver slices with epinephrine and measured its effect on the activity of glycogen phosphorylase. Phosphorylase activity was gradually lost unless epinephrine was added which then restored full activity. Rall et al. (Rall, Sutherland and Berthet 1957) demonstrated that a heat-stable dialysable factor was produced following the interaction of epinephrine with liver cell membranes. This factor then activated the phosphorylase activity and was subsequently identified as cyclic AMP.

These findings led to the second messenger concept. The hormone (first messenger) interacts with the receptor site which is located at the plasma membrane or cell surface. This interaction leads to an increase (or decrease) in the activity of adenylate cyclase (a component of the cell membrane), which

results in change of the intracellular concentrations of cyclic AMP. This increase in cyclic AMP is responsible for the hormone induced changes in cell function. The actions of many hormones, including most of the peptide hormones and also the β-adrenergic agonists, were found to be mediated by cyclic AMP. One important exception is the stimulation of glucose uptake into cells by insulin which seems to be independent of cyclic AMP. Since insulin does not enter the cells of its target tissue, perhaps the insulin-receptor complex interacts with functional macromolecules in the membrane. Besides insulin, there are several hormones that do not stimulate adenylate cyclase including angiotensin, growth hormone, α-adrenergic catecholamines, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, oxytocin, prolactin, somatomedin and somatostatin (Baxter and Funder 1979).

1.2:3 Classification of receptors

Although receptors for various classes of hormone share functional properties, they differ in cellular localisation, post-binding transfer of information and the particular hormones that they recognise. There are three types of hormone receptor:

(1) Receptors for peptide hormones (including releasing factors) and catecholamines, which are located on the plasma membrane of target tissue cells. It has been demonstrated that many hormones with membrane receptors produce their physiological effects without penetrating the cell.

(2) Steroid hormones penetrate the cell easily. Cytoplasmic receptors bind with the steroids and this complex is transferred to the nucleus where it initiates transcription events leading to the de novo synthesis of proteins.

(3) Thyroid hormone receptors are found in the cell nucleus. Thyroid hormones penetrate cells to bind the receptors in the chromatin of the nucleus (Baxter and Funder 1979).

1.2:4 Chemical properties of membrane receptors

In 1976, no hormone or neurotransmitter receptor had been isolated of sufficient quality and quantity for complete chemical characterisation. However, since then, there has been considerable progress in our understanding of the chemical nature of receptors. Several membrane receptors such as acetylcholine and insulin have been isolated, purified and chemically characterised.

All known hormone receptors contain a protein component which is important for hormone recognition. This can be degraded by a variety of proteolytic and peptidase enzymes (Kahn 1975). Several receptors such as adrenocorticotrophin (ACTH), glucagon, thyrotropin releasing hormone and both the adrenergic and cholinergic neurotransmitters, exhibit decreased hormone binding after phospholipase treatment, suggesting that these receptors also contain a significant phospholipid component (Kahn 1976). The receptors for insulin, thyrotropin and gonadotropins have been reported to contain carbohydrate moieties. Disulfide groups have not been shown to be necessary for any hormone-receptor interaction, although the LH receptor appear to need sulfhydryl groups for the maintenance of its biologically active conformation (Dufau, Ryan and Catt 1974).

Most peptide hormone receptors which have been solubilised are large molecules and have had estimated molecular weights of about 200,000 daltons (ranged from 140,000 to 550,000) (Catt and Dufau 1977). Many receptors also seem to have two or more smaller subunits, which together form the whole receptor complex, which binds to the hormonal ligand. These oligomeric receptors, like insulin and acetylcholine, often interact cooperatively with each other either positively or negatively to increase the sensitivity of the cell to low hormone concentrations and protect against high concentrations (Kahn 1975;

Kahn 1976)

The number of receptors for most hormones range from 2,000 to 100,000 per cell. Hormone binding to only 1% - 5% of receptors will often trigger a biological response. This allows low concentrations of hormones to bind with a relatively small quantity of receptor to induce a biological response (Catt and Dufau 1977)

Attempts to purify and chemically characterise hormone receptors have proceeded slowly because of the small number of molecules per cell and the relatively insolubility of these macromolecules. However, solubilisation (Gavin, Buell and Roth 1972) allows the receptors to retain high binding affinity and specificity of binding properties. Further, studies on the structural and immunological properties of the acetylcholine receptors have benefited from the application of affinity chromatographic procedures (Raferty 1975) which enables the purification of large quantities of receptor sites from target tissues.

In summary, the receptor may be defined as a highly selective chemical recognition factor, present in or on cells in very small numbers, which after interacting with specific hormonal compounds, consequently alters some critical biochemical process so as to initiate a generalised biological response (Hollenberg and Cuatrecasas 1979).

1.3 HORMONE RECEPTORS

The basic methodology employed for most direct studies of hormone-receptor interaction is similar to other competitive protein binding assays (Kahn 1975). A radiolabelled hormone or hormone competitive antagonist is incubated either with whole cells or with a subcellular fraction of cells. After a period of incubation, the hormone-receptor complex is separated from the free hormone

by centrifugation, filtration or precipitation, and the receptor bound radioactivity is determined. The specificity and saturability of this reaction are determined by incubation with various concentrations of unlabelled hormone or related compounds. Methodologic problems relate to the choice of appropriate labelled hormone, distinction of receptor from non-receptor binding, preparation of tissue for binding studies, techniques of separating free from bound ligand and mathematical description of binding isotherms.

1.3:1 Radiolabelled hormone

The rapid progress in the understanding of the receptor mediated mechanisms since 1960 can be attributed to the development of methods for radiolabelling hormones that give high specific activity without affecting biological activity. For many peptide hormones it is possible to attain a higher specific activity labelling with ^{125}I (or ^{131}I) than with ^{3}H or ^{14}C. Theoretically, ^{131}I would yield monoiodohormone with higher specific activity ($\sim$ 16,200 Ci/mmol) than ^{125}I ($\sim$ 2,300 Ci/mmol) if both were available in an isotopically pure form. However, ^{125}I is preferred for the following reasons: (1) it is carrier-free, i.e., close to 100% isotopic abundance, whereas the maximal isotopic abundance of ^{131}I does not exceed 30% (Berson and Yalow 1973), (2) it has a longer half life (60 vs 8 days) than ^{131}I, (3) it is counted with greater efficiency in most gamma counters (Blecher 1976).

Other isotopes such as ^{3}H, ^{14}C- yield ligands with much lower specific radioactivity, are limited in detecting the small numbers of peptide receptor sites in cell membranes (Kahn 1975). However, the ^{3}H-labelling has proven most useful with smaller peptide hormones such as vasopressin (Jard 1983) and oxytocin (Kahn 1975).

Iodine is a large molecule and the spatial arrangement or molecular conformation of proteins and peptides are complex. Incorporation of iodine into

the hormone molecule such as arginine vasopressin may cause conformational changes resulting in loss of biological activity (Moore, Huffman, Roberts, Rottschaefers, Sulat, Stefankiewicz and Stassen 1984).

In choosing appropriate conditions for binding studies, it is also important to select a temperature that minimises both receptor and ligand degradation but permits the attainment of equilibrium conditions in a reasonable time. It is also necessary to choose a method of separating free from receptor-bound ligand that is appropriate for the expected rate of ligand dissociation. These considerations are as important as the criteria necessary to establish that binding has the characteristics expected of a receptor mechanism (Jacobs and Cuatrecasas 1981).

1.3:2 Criteria for receptor interaction

It is essential to distinguish binding of a ligand to receptor sites (specific binding) from binding to other sites on or in the cell (nonspecific). Hormone receptor interactions are characterised by (1) high affinity, appropriate for low concentration of hormone, (2) saturability, indicating a finite number of binding sites (3) reversibility, consistent with loss of the physiological effects upon removal of hormone from the medium, (4) specificity, agonists and competitive antagonists compete for binding with the same rank order of affinity as found in dose-response studies, (5) a distribution among tissues consistent with the known activities of the hormone (Hollenberg and Cuatrecasas 1979).

1.3:3 Receptor preparations

Binding to hormone receptors may be studied in intact cells, particulate fractions of cells and solubilised fractions of cells. Each has advantages and disadvantages. Isolated intact cell preparation can be obtained from blood, tissue culture or by enzymatic or mechanical disruption of tissues. Intact cells are usually metabolically active, making it possible to measure both binding and hormone effects in the same preparation, and to express the concentration of

binding sites per cell. This is particularly useful in comparing the changes in hormone-binding between various physiological and pathological conditions. However, there are problems with intact cells. Degradation of hormone during incubation may be higher in intact cells than in subcellular fractions, and the concentration and affinity of hormone receptors may be affected by enzymatic digestion and other tissue isolation and culture techniques. A further problem relates to contamination of the preparation by cells of a different type (Kahn 1976; Blecher 1976).

Receptors may be studied in particulate cell fractions and this has some advantages. These include stability during storage, less hormone degradation, and decrease in nonspecific hormone binding. It should be emphasized that even in highly purified plasma membrane preparations these problems may still occur. Membrane preparations may not be representative of total plasma membrane and may also be contaminated by membranes from cells of another type. For this reason, membrane preparations must be assessed for yield, purity and contamination with other organelles (Neville and Kahn 1974).

Solubilised fractions of cells and cell membranes may retain the hormone binding characteristics of receptor sites on intact cells or plasma membranes. Neutral and anionic detergents, anionic bile salts, phospholipid emulsions, lithium diiodosalicylate, and even limited proteolytic digestion have been used (Catt and Dufau 1977). Solubilisation is a necessary prerequisite to purification and characterisation of the receptor molecules (Cuatrecasas 1972). Problems of heterogeneity and contamination of the original preparation may still be present in solubilised preparations. In addition, residual detergent required to maintain the binding proteins in a soluble state may compromise precise physical measurement. Labelled hormone may associate with detergent micelles and form a complex which could be mistaken for hormone-receptor complexes

(Giorgio, Johnson and Blecher 1974).

1.3:4 Receptor detection

Rapid and efficient separation of bound ligand from the supernatant medium in intact cells, membranes or solubilised receptors can be achieved:

(1) By centrifugation. This is perhaps the simplest and most rapid technique for separating cells or subcellular particulate fractions from suspensions.

(2) By filtration through cellulose or glass fibre. Important factors in this technique are pore size, choice of material and degree of nonspecific binding of ligand to the filter. In addition, filtration is usually slower than centrifugation and the process may be impaired if cell or membrane concentration is too high.

(3) By gel filtration. This has been used to separate membrane and solubilised receptors such as adrenergic receptors in toad erythrocyte vesicles (Sahyoun, Hollenberg, Bennett and Cuatrecasas 1977) but has the disadvantage that it is slow and hence gives less binding because of the separation of hormone and receptor complex during chromatography (Cuatrecasas 1972).

(4) By precipitation. Polyethylene glycol at appropriate concentrations precipitates receptor-bound hormone, while the free hormone remains in solution. This method has been applied to the measurement of solubilised receptor binding, insulin is a particular example (Cuatrecasas 1972). An important consideration in the choice of method for separating free from receptor bound radioactivity is the rate of dissociation of the hormone-receptor complex.

1.3:5 Other problems in hormone-receptor binding assay

Temperature is a critical factor in determining hormone-receptor interactions. The rates of association and dissociation and the total amount of receptor binding are temperature dependent. At lower temperatures equilibrium is attained more slowly but the total amount of receptor binding is increased. This increase in binding is in part due to increased affinity (Kahn, Freychet,

Roth and Neville 1974). Temperature has other and probably more important effects in a radio-ligand binding assay. At lower temperatures the rates of hormone and receptor degradation are reduced, a factor of particular importance for angiotensin II which is susceptible to degradation by several protease enzymes present in plasma and tissues.

The rate of degradation is also influenced by the amount of membrane in the preparation.

1.3:6 Analysis of receptor binding data

Binding data may be analysed in several ways. I have usually expressed the binding of labelled-hormone as counts per minute (cpm) bound or as a percentage of total radioactivity bound, plotted as a function of the concentration of unlabelled hormone in the incubation medium. This results in a displacement curve with decreasing concentrations of unlabelled hormone. Binding of hormone is also expressed as percent of initial binding (100 x B/Bo) where Bo represents the binding of labelled hormone in the absence of unlabelled hormone. Binding is also plotted as the ratio of bound (B) to free (F), or B/F. In most instances, binding has been expressed as specific binding, that is after subtraction of nonspecific binding. Nonspecific binding was defined as binding of radioligand in the presence of 2.5 μM unlabelled angiotensin II. In analysis of the displacement curves, the amount of hormone bound was calculated as $B/Bo \times B_T$ where B = specifically bound hormone (cpm) and B_T = total hormone binding (cpm). Scatchard analysis has been used to characterise receptor binding. In this method, the B/F ratio is plotted as a function of concentration of bound hormone. The resultant plot linearises the binding data and permits the calculation of both the affinity constants (Kd) and binding capacities (Bmax) (Scatchard 1949). Binding capacity may then be expressed as a function of the concentration of cells, membrane protein, or solubilised

fraction of protein in the medium.

Scatchard plots may be linear or nonlinear. Non-linearity of Scatchard plots may reflect (1) positive ligand-ligand cooperativity (concave down) (2) negative ligand-ligand cooperativity (concave up) (3) simultaneous binding of a ligand at two or more sites (concave up) (4) differences in affinity of labelled ligand (concave up) (5) inaccurate determination of the portion of bound and free ligand (concave up) and (6) lack of complete binding equilibrium (Cuatrecasas and Hollenberg 1976). An example of curvilinear Scatchard plots of angiotensin II were described in bovine adrenal cortex and attributed to the presence of more than one class of binding sites (Glossman, Baukal and Catt 1974a).

1.4 ANGIOTENSIN II RECEPTORS

Angiotensin II at concentrations which are found in plasma (10^{-10}M) has been shown to affect many tissues. For example, smooth muscle in blood vessels, gut, uterus and bladder contract, zona glomerulosa cells of the adrenal cortex secrete aldosterone, renal tubular function is altered and there are also effects in the sympathetic nervous system have been demonstrated (Brown, Leckie, Lever, McIntyre, Morton, Semple and Robertson 1983) in response to physiological concentrations of angiotensin. Responsiveness to angiotensin II by a particular tissue can be varied in a specific manner. It is likely that specificity and variability in responsiveness reflect differences in angiotensin II receptor properties. Much work has been done to identify and characterise receptors for angiotensin in different tissues with a view to explaining the physiological effects of the hormone.

1.4:1 Distribution and binding characteristics of angiotensin II receptors

Most of the information about angiotensin II receptors has been derived from animal studies (see Table 1.1). All tissues which respond to angiotensin II have been shown to have appropriate receptors. Generally the dissociation constant is around 1 nM which is compatible with physiological action. Changes in sensitivity to the pressor and steroidogenic actions of angiotensin II in response to dietary sodium and potassium manipulation may be regulated at the receptor level. Sodium restriction has been shown to decrease the receptor numbers in vascular smooth muscle, uterus and renal glomeruli without changing the affinity of receptors and to increase receptor numbers in the adrenal gland. Initially adrenal receptor affinity was increased with sodium restriction but after 4 days had returned to normal (Table 1.2). The differential effects of dietary sodium on adrenal and smooth muscle receptors are correlated with changes in physiological responsiveness; sodium restriction enhances angiotensin II - induced aldosterone synthesis but reduces pressor activities.

Studies in the rat indicate that dietary potassium has the opposite effect of sodium on angiotensin II receptors. Potassium restriction increases smooth muscle receptor numbers and decrease adrenal receptors whereas potassium loading caused a reduction in smooth muscle receptors and increases in adrenal receptors (Table 1.3). Receptor affinity was not significantly affected. Changes in receptor numbers in response to dietary sodium or potassium are thought to be regulated by plasma angiotensin II concentrations (Aguilera, Hauger and Catt 1978).

Human angiotensin II receptors were first demonstrated in aldosterone-producing adenomas, adjacent non-tumorous adrenal tissue and normal human adrenal zona glomerulosa (Brown, Douglas and Bravo 1980). Two classes of receptor binding sites for angiotensin II were present in normal tissue obtained

TABLE 1.1

DISTRIBUTION AND BINDING CHARACTERISTICS OF ANGIOTENSIN II RECEPTORS

TISSUE	SPECIES	LIGAND	Kd	Bmax	REFERENCES
aorta	rabbit	^{3}H-AngII	13 nM	8.4 fmol/mg	1
aorta membranes	rabbit	^{3}H-AngII	H:6.2 nM L:20 nM		2
aorta membranes	guinea pig	^{14}C-AngII	22 nM	0.61 pmol/mg	3
mesenteric artery	rat	^{3}H-AngII	1.18 nM	56.8 fmol/mg	4
mesenteric artery	rat	^{125}I-AngII	0.9-1.2 nM	43-54 fmol/mg	4,5
urinary bladder	rat	^{125}I-AngII	1.3 nM	70.5 fmol/mg	5
uterus	rat	^{3}H-AngII	20-50 nM	0.7-2.4 pmol/mg	2,6,7
uterus	rat	^{125}I-AngII	0.2-6 nM	7-194 fmol/mg	8,9,10
uterus	rabbit	^{125}I-AngII	1 nM	151 fmol/mg	11
adrenal glomerulosa	rat	^{125}I-AngII	0.9-2.7 nM H:2-5.2 nM	1.8 pmol/mg;25.5fmol/10^5cells 0.27 pmol/mg	8,9,12,13
adrenal glomerulosa (particulate membranes)	rat	^{125}I-/^{3}H-AngII	L:25 nM	2.4 pmol/mg	12,14
adrenal cortex	rat	^{125}I-AngII	H:5.4 nM L:0.16 nM	12 fmol/mg 0.65 fmol/mg	15
adrenal glomerulosa	dog	^{125}I-AngII	H:1.1-3.3 nM L:25 nM	7.6-37 fmol/10^5cells 17.6 fmol/10^5cells	13,16
adrenal cortex	bovine	^{125}I-/^{3}H-AngII	H:0.5 nM L:0.03 nM	620 fmol/mg 9.7 pmol/mg	15
adrenal cortex (solubilised)	bovine	^{3}H-AngII	H:15 nM L:170 nM	120 fmol/mg 470 fmol/mg	17
adrenal fasciculata	bovine	^{125}I-AngII	2.3 nM		18
renal glomeruli	rat	^{125}I-/^{3}H-AngII	0.02-1.6 nM	12-1127 fmol/mg	8,19,20,21
renal tubules	rat	^{125}I-AngII	H:6 nM L:60 nM	1800 sites/cell 6400 sites/cell	22
renal mesangial cells	rat	^{125}I-AngII	2.8 nM	43.5 fmol/mg	23
brain	rat	^{125}I-AngII	0.2-0.9 nM	0.05-11 fmol/mg	24,25
brain membranes	bovine	^{125}I-AngII	0.2 nM	1.6 fmol/mg	24

H: high affinity site
L: low affinity site

TABLE 1.2

THE EFFECT OF DIETARY SODIUM ON ANGIOTENSIN II BINDING

DIET	TISSUE	SPECIES	Kd	Bmax	REFERENCES
low sodium	mesenteric artery (particulate membranes)	rat	0.79 nM	41.5 fmol/mg	26
normal sodium			1.04 nM	54.3 fmol/mg	
low sodium	urinary bladder	rat	1.5 nM	60 fmol/mg	5
normal sodium			1.3 nM	84 fmol/mg	
high sodium			1.5 nM	122 fmol/mg	
low sodium	uterus	rat	2.0 nM	27 fmol/mg	27
normal sodium			1.9 nM	45 fmol/mg	
low sodium	uterus	rat	0.04 nM	2.8 pmol/mg	28
normal sodium			0.03 nM	1.0 pmol/mg	
high sodium			0.05 nM	2.0 pmol/mg	
low sodium	adrenal glomerulosa	rat	0.02 nM	0.63 pmol/mg	28
normal sodium			0.02 nM	0.33 pmol/mg	
high sodium			0.02 nM	0.31 pmol/mg	
low sodium	adrenal glomerulosa	rat	2.0 nM	45 fmol/10^5cells	27,29,30,31
normal sodium			1.3 nM	26 fmol/10^5cells	
high sodium			1.5 nM	21 fmol/10^5cells	
low sodium	adrenal cortex	rat	H:1.9 mM L:57 µM	ratio 3.0	32
normal sodium			H:2.0 mM L:150 µM	1.0	
high sodium			H:1.9 mM L:270 µM	0.7	
low sodium	renal glomeruli	rat	129 pM	7.87 fmol/mg	20
normal sodium			71 pM	11.62 fmol/mg	
high sodium			27 pM	17.37 fmol/mg	

H: high affinity site
L: low affinity site

TABLE 1.3

THE EFFECT OF DIETARY POTASSIUM ON ANGIOTENSIN II BINDING

DIET	TISSUE	SPECIES	Kd	Bmax	REFERENCES
low potassium	mesenteric artery	rat	1.4 nM	470 fmol/mg	33
normal potassium			1.0 nM	316 fmol/mg	
low potassium	uterus	rat	6.0 nM	308 fmol/mg	33
normal potassium			3.8 nM	148 fmol/mg	
normal potassium	uterus	rat	0.23 nM	190 fmol/mg	34
high potassium			0.32 nM	110 fmol/mg	
normal potassium	adrenal glomerulosa	rat	0.89 nM	1800 fmol/mg	34
high potassium			0.65 nM	3700 fmol/mg	
low potassium	adrenal cortex	rat	H:2.5 mM L:76 µM	ratio 0.7	32
normal potassium			H:2.0 mM L:150 µM	1.0	
high potassium			H:0.7 mM L:72 µM	2.0	

H: high affinity site
L: low affinity site

REFERENCES

1: Baudouin, Meyer and Worcel 1971

2: Devynck and Meyer 1976

3: LeMorvan and Palaic 1975

4: Gunther, Gimbrone and Alexander 1980a

5: Aguilera and Catt 1981

6: Rouzaire-Dubois, Devynck, Chevillotte and Meyer 1975

7: Devynck, Rouzaire-Dubois, Chevillotte and Meyer 1976

8: Brown, Douglas and Krontiris-Litowitz 1980

9: Douglas, Michailov, Khosla and Bumpus 1980

10: Schirar, Capponi and Catt 1980b

11: Schirar, Capponi and Catt 1980a

12: Douglas, Aguilera, Kondo and Catt 1978

13: Aguilera, Capponi, Baukal, Fujita, Hauger and Catt 1979

14: Pernollet, Devynck, Matthews and Meyer 1977

15: Glossman, Baukal and Catt 1974a

16: Douglas, Saltman, Fredlund, Kondo and Catt 1976

17: Chang and Lotti 1981

18: Vallotton, Capponi, Grillet, Knupfer, Hepp, Khosla and Bumpus 1981

19: Sraer, Sraer, Ardaillou and Mimoune 1974

20: Beaufils, Sraer, Lepreux and Ardaillou 1976

21: Bellucci and Wilkes 1982

22: Freedlender and Goodfriend 1977

23: Foidart, Sraer, Delarue, Mahieu and Ardaillou 1980

24: Bennett and Snyder 1976

25: Sirett, McLean, Bray and Hubbard 1977

26: Gunther, Gimbrone and Alexander 1980b

27: Aguilera, Hauger and Catt 1978

28: Devynck, Pernollet, MacDonald, Matthews, Raisman and Meyer 1978

29: Aguilera and Catt 1978

30: Hauger, Aguilera and Catt 1978

31: Aguilera, Schirar, Baukal and Catt 1980

32: Douglas and Catt 1976

33: Paller, Douglas and Linas 1984

34: Douglas 1980

at surgery: a high affinity, low capacity site (kd = 0.4 nM, 468 fmol/mg protein) and a low affinity, high capacity site (kd = 2.5 nM, 1094 fmol/mg protein). The aldosterone-producing adenomas and adjacent non-tumorous tissue possessed only low affinity receptor sites (4.55 nM, 462 ± 112 fmol/mg protein, n = 11) and it is possible that poor aldosterone response to infused angiotensin II in patients with primary aldosteronism (due to an adrenal adenoma) may be related to fewer receptor sites for angiotensin II (Fraser, Beretta-Piccoli, Brown, Cumming, Lever, Mason, Morton and Robertson 1981).

However a more recent study (Douglas et al. 1984) characterised only a single class of angiotensin II binding sites in normal human and primate adrenal glomerulosa and fasciculata tissue and in a cortisol-producing adenoma (Douglas, Brown and White 1984). The authors suggest that their inability to detect high affinity sites in this later study may be related to the time elapsing between tissue removal and assay. There were no differences in affinity between fasciculata and glomerulosa tissue which is surprising since the steroidogenic response to angiotensin II in the zona glomerulosa is more sensitive than that of the zona fasciculata. However, agonist and antagonist analogues of angiotensin II competed for binding sites commensurate with their known biological activity.

Angiotensin II has also been shown to specifically bind to human glomeruli isolated from normal kidneys that were obtained at nephrectomy or early autopsy (Chansel, Ardaillou, Nivez and Ardaillou 1982). Two classes of receptor could be defined by Kd values (0.1 and 1.8 nM) and number of receptor sites (44 and 303 fmol/mg protein). The presence of these receptors in human renal glomeruli suggest a role for angiotensin II in the regulation of glomerular function in man.

Attempts have been made to identify angiotensin II receptors in human mononuclear leukocytes. In 1978, Shimada and Yazaki reported that the binding

of ^{125}I-angiotensin II to mononuclear leukocytes was rapid and reversible. Scatchard analysis from displacement studies indicated two classes of binding sites with dissociation constants of 5×10^{-9}M and 9×10^{-11}M and with 181 and 2,312 sites/cell, respectively. Further studies with mononuclear leukocytes from patients with Bartter's syndrome, renovascular hypertension and primary aldosteronism showed that binding characteristics were not altered in these diseases (Ohuchi, Yazaki, Kato, Ashida and Shimada 1980). More recently however, Neyses et al. have raised doubts about the validity of this work (see chapter 3) and in doing so drew attention to the criteria and biochemical conditions necessary for the identification of a specific receptor (Neyses, Lecher, Wehling, Pech, Tenschert and Vetter 1984).

1.4:2 Radiolabelled angiotensin II

There is some argument over the choice of radioactive label for angiotensin receptor binding studies. Ideally the labelled hormone should have the same biological activity as the parent hormone. Originally tritiated angiotensin II was used in rabbit aorta (Devynck and Meyer 1976) because it maintained full biological potency. In contrast, ^{125}I-angiotensin II, has only 70% of the pressor activity of the native hormone (Lin and Goodfriend 1970). This loss of activity may be due to a change in the pK of the phenolic OH of tyrosine (Kürbart, Coli, Vancheri and Rosa 1971). However the use of ^{3}H-angiotensin II is limited by its relatively low specific activity ($<$100 Ci/mmol) compared with monoiodinated angiotensin II (2,000 Ci/mmol). The specific activities of ^{3}H-angiotensin II are much lower than those of ^{125}-angiotensin II (Morgat, Hung and Fromageot 1970), and hence are more suited to the identification of low affinity high capacity sites (see Table 1.1).

Since mono-iodoangiotensin exerts the same physiologic effects as unlabelled angiotensin on uterine smooth muscle and rat adrenal cortex (Lin and

Goodfriend 1970) its use in these tissues at least is justified. Di-iodinated angiotensin is largely inactive (Kürbart et al. 1971).

^{125}I-angiotensin has been used to identify high affinity receptors in several non-vascular tissues (Table 1.1). In cases of vascular receptors where low binding capacity and relatively small amounts of membrane material are important limitations, the use of high specific activity ^{125}I-angiotensin II is justified despite the small loss of biological activity (Baudouin, Meyer, Fermandjian and Morgat 1972; Devynck, Pernollet, Meyer, Fermandjian and Fromageot 1973; LeMorvan and Palaic 1975).

In many tissues however ^{125}I- and ^{3}H-angiotensin II have been shown to be equipotent in binding affinity (Glossman, Baukal and Catt 1974b; Gunther, Gimbrone and Alexander 1980a). Of particular importance to the present study, Moore and Williams (1982) found no significant difference between ^{125}I- and ^{3}H-angiotensin II in affinity for the human platelet angiotensin II receptor.

1.5 PURPOSE OF PRESENT STUDY

The existence and physiological significance of angiotensin II receptors have been shown in several tissues from animals. Although similar receptors have been identified in some human tissues, vascular smooth muscle and adrenal cortex are not readily available. Accordingly, platelets have been studied since they show high affinity specific binding sites for ^{125}I-angiotensin II and are easily obtained.

The present study has been designed (1) to show that angiotensin II binding to platelet receptors is specific (i.e. saturable, reversible, time and temperature dependent, etc.) and not due to endocytotic uptake of a metabolite, (2) to examine the effects of dietary sodium and potassium and essential

hypertension on platelet binding characteristics, (3) to explore possible intracellular modes of action and (4) to investigate whether angiotensin II binding to platelets is related to any physiological effect.

CHAPTER 2 MATERIALS AND METHODS

The initial objective was to develop a method of preparing platelets in good yield from a relatively small blood sample. This was achieved using a simple one-step procedure with Percoll. A density of Percoll was chosen such that platelets were retained and other blood cells excluded. The platelet preparation sought to identify and characterise angiotensin II binding.

2.1 MATERIALS

2.1:1 Radiolabelled angiotensin II

^{125}I-angiotensin II (1-1.6 Ci/mg) was obtained from New England Nuclear (Boston, MA, U.S.A.). This material was dissolved in sterile, Tris-NaCl-BSA solution (50 mmol/l Tris-HCl, 154 mmol/l NaCl, 1% bovine serum albumin, pH 7.35) and frozen in 350 µl aliquots at a concentration of 3 nmol/l in the dark at -20°C until used, always within 30 days of preparation. Under these conditions, the peptide is stable as assessed by paper chromatography.

2.1:2 Unlabelled angiotensin II

Ile^5 -angiotensin II obtained from Peninsula Laboratories Inc. (San Carlos, CA, U.S.A.). This material was also dissolved in sterile, Tris-buffered saline (pH 7.35) and frozen in 400 µl aliquots in the dark at -20°C until used.

2.1:3 Angiotensin analogues

Angiotensin II analogues [Sar^1, Thr^8]-Ang II, [Sar^1, Ala^8]-Ang II, Ang III and Ang I were obtained from Peninsula Laboratories Inc.

2.1:4 Cell culture medium

Medium 199 was obtained from Flow Laboratories (Irvine, Ayrshire, U.K.).

2.1:5 Other reagents

Other reagents used included Percoll (Pharmacia Fine Chemicals, Sweden), disodium ethylenediaminetetraacetate (EDTA, May and Baker Ltd., Dagenham, U.K.), bovine serum albumin (BSA, Miles Laboratories, U.K.), dibutyl phthalate and dinonyl phthalate oil (Fluka AG, Switzerland), acetone (May and Baker Ltd., Dagenham, U.K.). Tris (hydroxymethyl)-methylamine (Tris), sodium citrate, glucose, potassium chloride, sodium chloride, calcium chloride and magnesium chloride were all obtained from BDH Chemicals Ltd., Poole, U.K.

2.1:6 Tubes and nylon gauze filter

50 ml centrifuge tubes and 2.5 ml LP-3 tubes were obtained from Sarstedt, W. Germany, and 400 µl microcentrifuge tubes from Alpha Laboratories, U.K.. Nylon gauze filter (20 µm) was obtained from Henry Simon, Stockport, U.K.

2.2 METHODS

2.2:1 Preparation of Percoll

1 litre of Percoll was divided into twenty 50 ml portions and stored at $-20^{o}C$ until used.

2.2:2 Preparation of buffer solution

Tris-NaCl solution was prepared containing (in mmol/l) Tris, 50; NaCl, 154; $CaCl_2$, 4.8; KCl, 3.6; $MgCl_2$, 1.8. The pH of Tris was adjusted to 7.35 and stored at $-20^{o}C$ until used. 1% BSA was added and the pH was adjusted to 7.35. This solution was frozen and stored at $-20^{o}C$ until used.

2.2:3 Preparation of modified medium 199

Modified medium 199 was prepared containing (in mmol/l) Na^{+}, 145; K^{+}, 3.8; Ca^{2+}, 2.54; Mg^{2+}, 1.18; HCO_3^{-}, 24.9; Cl^{-}, 128; phosphate, 1.18; $SO4^{2-}$, 1.18; glucose, 11.1; EDTA, 5. After equilibration with 95% O_2:5% CO_2, the pH was

adjusted to 7.4. 0.5% BSA was added and medium was frozen and stored at $-20^{o}C$ until used.

2.2:4 Paper chromatography

Pure ^{125}I-angiotensin II with or without platelet suspension was spotted after incubating at $22^{o}C$ for 1h on Whatman no. 1 paper. Descending paper chromatogram was developed at room temperature for 24 - 36 hours in a mobile phase butano-2-ol/aq. 3% ammonia (120:44, v/v). After drying, the paper was cut into 1 cm long strips and these strips were compressed into LP-3 tubes and counted using a Nuclear Enterprise NE 1600 gamma counter with 90% counting efficiency.

2.2:5 Preparation of platelets

Forearm venous blood (60 ml) was collected by venepuncture into two 50 ml plastic centrifuge tubes each containing 3 ml of sodium citrate (129 mmol/l). Immediately, at room temperature the blood was carefully layered on to an iso-osmotic solution of Percoll (specific gravity 1.060). This solution was prepared from Percoll (specific gravity 1.130):1.54 M sodium chloride (specific gravity 1.058), 9:1; which was diluted with an equal volume of 0.154 M normal saline (specific gravity 1.000). After centrifugation (400 g, 5 min, $4^{o}C$) the upper platelet-rich layer equal to the volume of added blood was aspirated, washed with an equal volume of modified medium 199 and centrifuged (1000 g, 10 min, $4^{o}C$). This was repeated three times with the platelet-rich layer resuspended in 15 ml of modified medium 199 each time. The final aspirate was suspended in 5-6 ml of medium and the suspension filtered through a 20 µm nylon gauze to remove aggregates. This filtrate was used in the binding assays. The platelet concentration in a small portion of this suspension was measured in a phase 2 Coulter counter (S plus). The recovery % of platelets from blood was 50 - 90% (n=192) and the platelet concentrations ranged from 6 to 10 x 10^{5} cells per µl.

The final platelet suspension contained less than 1% mononuclear leukocytes and 0.1% red blood cells. The procedures of platelet preparation are shown in Figure 2.1.

2.2:6 Binding assay

The technique used for binding studies were modifications of those described by Lin and Goodfriend (1970), Douglas and Catt (1976) and Moore and Williams (1982). Suspensions of platelets (200 µl) were incubated with 25 µl of ^{125}I-angiotensin II (3 nmol/l) in Tris-NaCl-BSA (pH 7.35) with and without unlabelled angiotensin II (2 5 µmol/l) in a final volume of 250 µl. Unlabelled angiotensin II was dissolved in Tris-NaCl solution; platelets incubated without unlabelled angiotensin II were treated with 25 µl Tris-NaCl alone. Incubations were usually for 1 hour at 22°C in a shaking water bath (Gallenkamp, U.K.). After incubation, platelets were separated from medium by centrifuging 100 µl portions of the incubation mixture in 400 µl tubes containing 200 µl of a mixture of dibutyl phthalate and dinonyl phthalate (5:1, v/v) with a specific gravity of 1.03, at 12,000 g for 30 s in a microcentrifuge (Beckman). The tubes were immediately frozen in acetone/solid CO_2 and cut with a guillotine just below the oil-water interface. Both upper and lower portions of the tube (free and bound radioactivity, respectively) were counted in a Nuclear Enterprise NE1600 gamma counter at 90% counting efficiency. These incubation procedures are shown in Figure 2.2.

Nonspecific binding was defined as the amount of ^{125}I-angiotensin II bound to platelets in the presence of 25 µl (2 5 µM) unlabelled angiotensin II. Total binding was defined as the amount of ^{125}I-angiotensin II bound to platelets in the absence of unlabelled hormone. Specific binding was defined as total binding minus nonspecific binding.

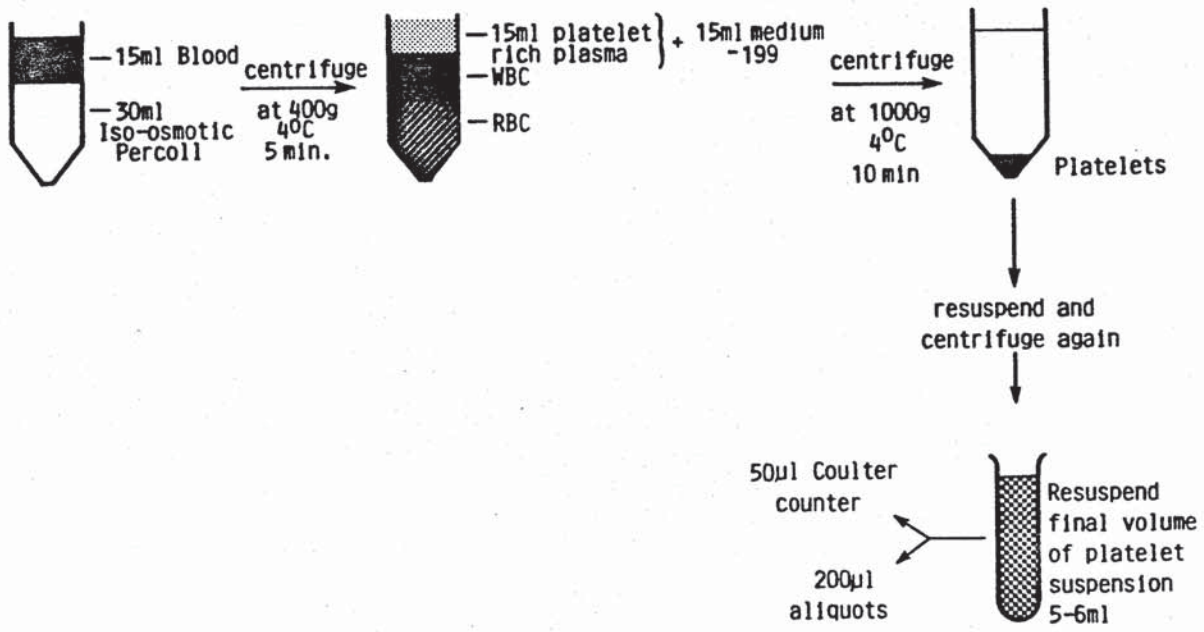

FIGURE 2.1 Preparation of platelets.

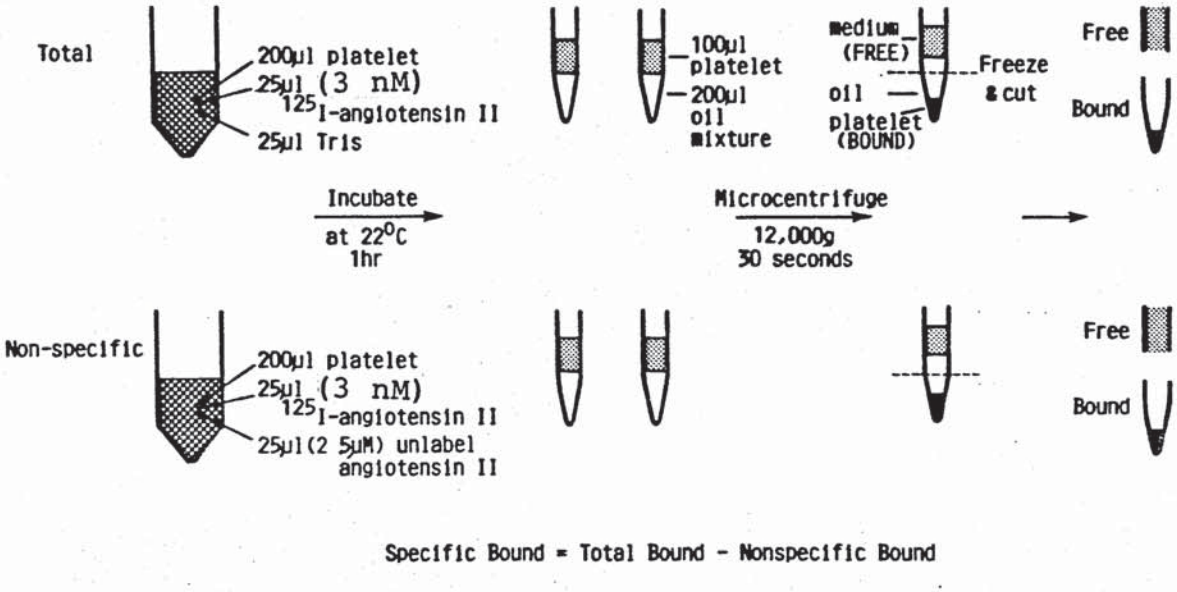

FIGURE 2.2 Platelet binding assay procedure.

2.3 ANALYSIS OF BINDING ISOTHERMS

Scatchard analysis was used to calculate the equilibrium dissociation constant (Kd) and the binding capacity (Bmax) from saturation and competition curves (Scatchard 1949). Binding capacity was calculated from the concentration of bound ligand at the intercept of the regression line on the abscissa of the Scatchard plot. Bmax was expressed as receptor sites per platelet using Avogadro's number. Standard kinetic analysis was used to calculate the forward and reverse rate constants from association and dissociation experiments (Gunther et al. 1980a). Results for receptor binding capacity and affinity and other values are presented as mean $\pm$ standard error (M $\pm$ SE). The relative affinity for angiotensin analogues were estimated by comparing the concentrations of non-radioactive analogues required to displace 50% ^{125}I-angiotensin II from its specific binding sites (IC 50). Comparison of relative potencies of unlabelled peptides are presented as dose ratio. Linear regression was calculated with the least squares technique (Snedecor and Cochran 1967).

2.3:1 Other statistical methods

For group comparisons, student paired and unpaired "t" tests were used. Correlation coefficients were estimated by the Spearman rank method (Siegel 1956).

CHAPTER 3 BIOCHEMICAL BINDING CHARACTERISTICS OF ANGIOTENSIN II RECEPTORS IN HUMAN PLATELETS

3.1 BINDING AS A FUNCTION OF INCUBATION TIME AND TEMPERATURE

3.1:1 Methods

Platelets were incubated with ^{125}I-angiotensin II (300 pM) at 22°C, 37°C and 0°C for time periods from 5 to 120 minutes. The reaction was terminated by microcentrifugation.

3.1:2 Results

In two experiments at 37°C, specific binding of ^{125}I-angiotensin II to platelets reached a maximum after 30 min. A plateau was not sustained and specific binding declined thereafter (Figure 3.1).

In each of five experiments at 22°C, specific binding increased rapidly during the first 30 min. and reached a plateau at 60 min. Nonspecific binding reached a plateau after 30 min. of incubation.

In two experiments at 0°C, angiotensin II binding increased slowly and at 120 min. there was 22% of the binding present after 8 hours.

The specific binding comprised 1.1 $\pm$ 0.07% of total radioactivity and nonspecific binding comprised 0.3 $\pm$ 0.22% (n = 9) in platelets incubated at 22°C for 60 minutes with 300 pM of ^{125}I-angiotensin II.

Because platelet concentrations and binding varied between experiments, specific binding was expressed as a percentage of the maximum binding attained during that experiment. In subsequent experiments, specific binding of angiotensin II was estimated after incubation of platelet at 22°C for one hour.

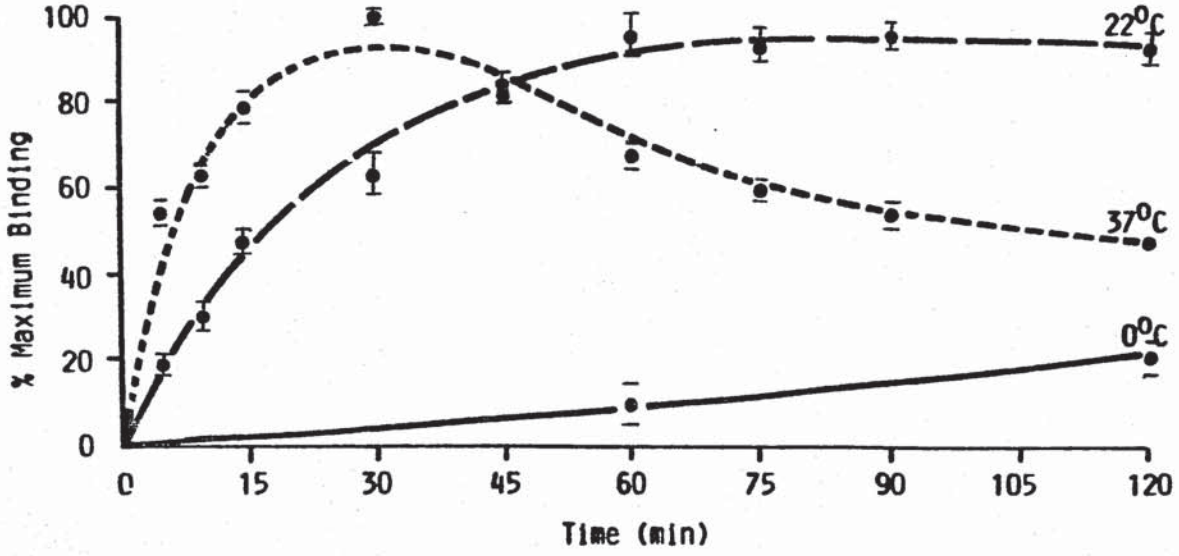

FIGURE 3.1 Time course of specific ^{125}I-angiotensin II binding to platelets at 22°C, 37°C and 0°C. The points represent the mean and standard error of five experiments at 22°C and means and ranges of two experiments at 37°C and 0°C.

3.2 DEGRADATION OF TRACER

3.2:1 Methods

The metabolism of tracer in the incubation medium was examined using descending paper chromatography (described in chapter 2).

3.2:2 Results

Paper chromatography of the incubation medium after 30, 60 and 120 min. of incubation at 37°C showed a progressive decline in the amount of radioactivity that chromatographed with the mobility of angiotensin II with a concomitant increase in the amount of radioactivity in other positions. Little metabolism of angiotensin II by platelets was detected at 22°C, over 60 min. of incubation (Figure 3.2). The minor changes in Rf for ^{125}I-angiotensin II were observed but were accounted for by uneven solvent flow. No consistent change in Rf for ^{125}I-angiotensin II was noted.

3.3 BINDING AS A FUNCTION OF PLATELET CONCENTRATION

3.3:1 Methods

^{125}I-angiotensin II was incubated with a range of platelet concentrations from 1.1×10^5 to 17.2×10^5 cells/µl at 22°C for 90 min.

3.3:2 Results

There was a linear relationship between specific binding and cell concentration (Figure 3.3). In each of four experiments the correlation coefficients relating to specific binding and platelet concentration were > 0.93. Nonspecific binding was less consistently related to platelet concentration than specific binding. At platelet concentration > 10×10^5/µl the increase in non-

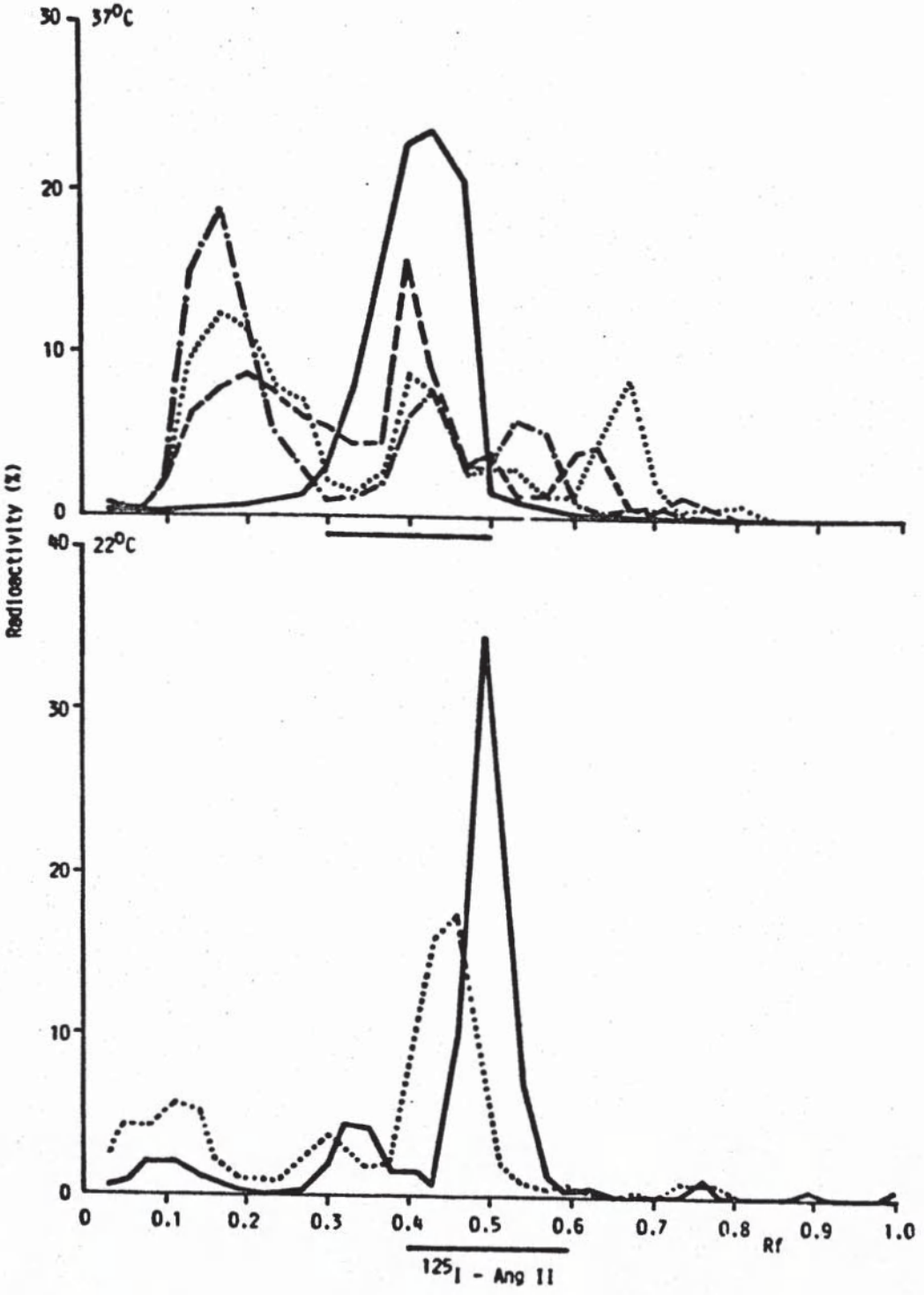

FIGURE 3.2 Temperature-dependent metabolism of ^{125}I-angiotensin II. The upper panel shows chromatograms of radioactivity after incubation of tracer angiotensin with and without platelets at 37°C for up to 120 minutes. For each chromatogram, radioactivity is expressed as a percentage of total radioactivity recovered from that chromatogram. The lower panel shows similar chromatograms after 60 minutes incubation at 22°C. ___ 60 minutes incubation without platelets; ---30 minutes incubation with platelets; . . . 60 minutes incubation with platelets; -·-120 minutes incubation with platelets. The Rf for ^{125}I-Ang II ranged from 0.4 to 0.6 in different experiments. [des-Asp^1] Ang II, [des-Asp^1, Arg^2] Ang II and [des-Asp^1,Arg^2,Val^3] Ang II have an Rf value greater than Ang II in this solvent system.

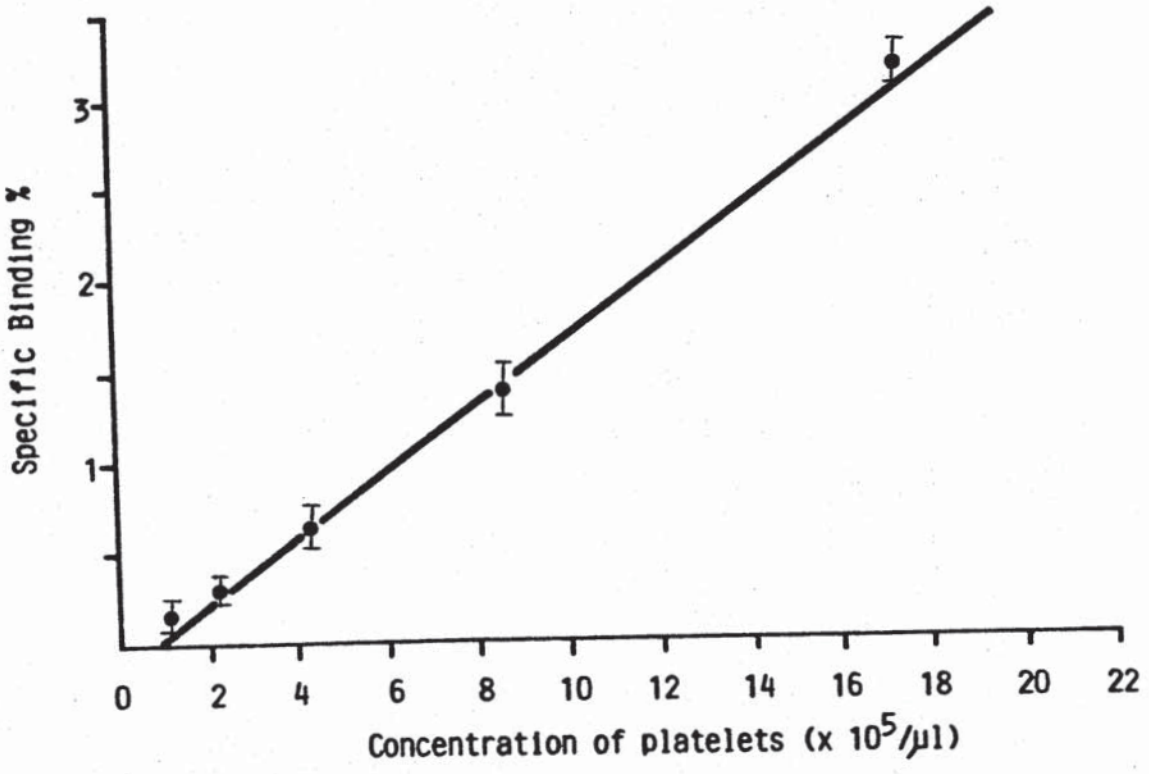

FIGURE 3.3 Relationship between platelet concentration and specific binding of angiotensin II. Each point represents the mean and standard error of quadruplicate determinations in four experiments. Specific binding is expressed as a percentage of total angiotensin II present in the incubation medium.

specific binding was disproportionately steeper. In subsequent experiments platelet concentration between 5 and 10 x 10^5 platelets/µl were used.

3.4 SATURATION OF SPECIFIC BINDING

3.4:1 Methods

Platelets were incubated with a range of concentrations of ^{125}I-angiotensin II from 1.6 x 10^{-11} to 2.5 x 10^{-9}M for 60 min. at 22°C.

3.4:2 Results

Figure 3.4 shows the curvilinear relationship between specific angiotensin II binding and increasing concentrations of labelled peptide. Specific binding sites were saturated at 0.5 nM whereas non-specific binding was not saturable and increased in direct proportion to the concentration of angiotensin II. Scatchard analysis of specific binding results from four saturation curves gave linear plots with an equilibrium dissociation rate constants of 1.5 $\pm$ 0.4 x 10^{-10}M and a total binding capacity of 6.3 $\pm$ 1.2 receptor/platelet.

3.5 COMPETITION CURVES

3.5:1 Methods

Labelled angiotensin II in a final concentration of 300 pM was incubated with platelets over a range of concentrations of unlabelled hormone (3 x 10^{-11} to 2.5 x 10^{-6}M) at 22°C for 90 min. Specific binding was expressed as the percentage of ^{125}I-angiotensin II binding in the absence of unlabelled hormone.

3.5:2 Results

The amount of specifically bound radioactivity declined in platelets incubated with 300 pM ^{125}I-angiotensin II and increasing concentrations of

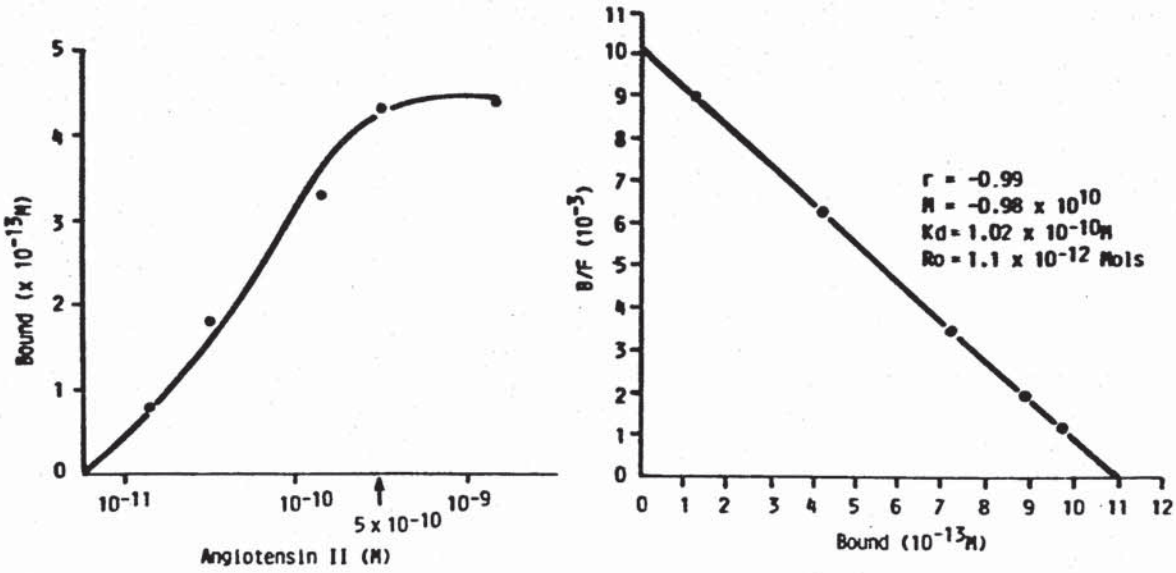

FIGURE 3.4 Effects of increasing concentrations of ^{125}I-angiotensin II on specific binding. Left panel: each point represents the mean of four experiments. Right panel: Scatchard analysis of results from one experiment, B/F=bound/free.

unlabelled hormone (Figure 3.5). The smallest concentration of unlabelled angiotensin II that reduced binding significantly was 3×10^{-11}M ($p < 0.05$); the IC50 of unlabelled angiotensin was 1.3×10^{-9}M. Scatchard analysis of the results from six competition curves gave a Kd of $2.4 \pm 0.7 \times 10^{-10}$M with a binding capacity of 6.5 ± 1.0 receptor/platelet.

3.6 KINETIC STUDIES - ASSOCIATION CURVES

3.6:1 Methods

Association experiments were performed by measuring specific binding of ^{125}I-angiotensin II to platelets at 22°C for various times with ^{125}I-angiotensin II at concentrations of 600 and 300 pM. Forward and reverse rate constants were calculated by a method free of assumptions made in the fitting of binding results to appropriate rate equation (Gunther et al. 1980a).

3.6:2 Results

The results of 2 experiments are shown in Figure 3.6. The equilibrium dissociation rate constant was similar to values calculated from saturation and competition studies at 1.2×10^{-10}M: the forward and reverse rate constants were 3.1×10^{8}M^{-1}/min and 3.6×10^{-2}/min, respectively (Appendix 1).

3.7 KINETIC STUDIES - DISSOCIATION CURVES

3.7:1 Methods

Unlabelled angiotensin II (2.5 µM) was added to platelet suspension which had been preincubated with 300 pM ^{125}I-angiotensin II at 22°C for 60 minutes. The incubation continued at 22°C and samples were taken at various time intervals over a period of 120 minutes after addition of unlabelled angiotensin II.

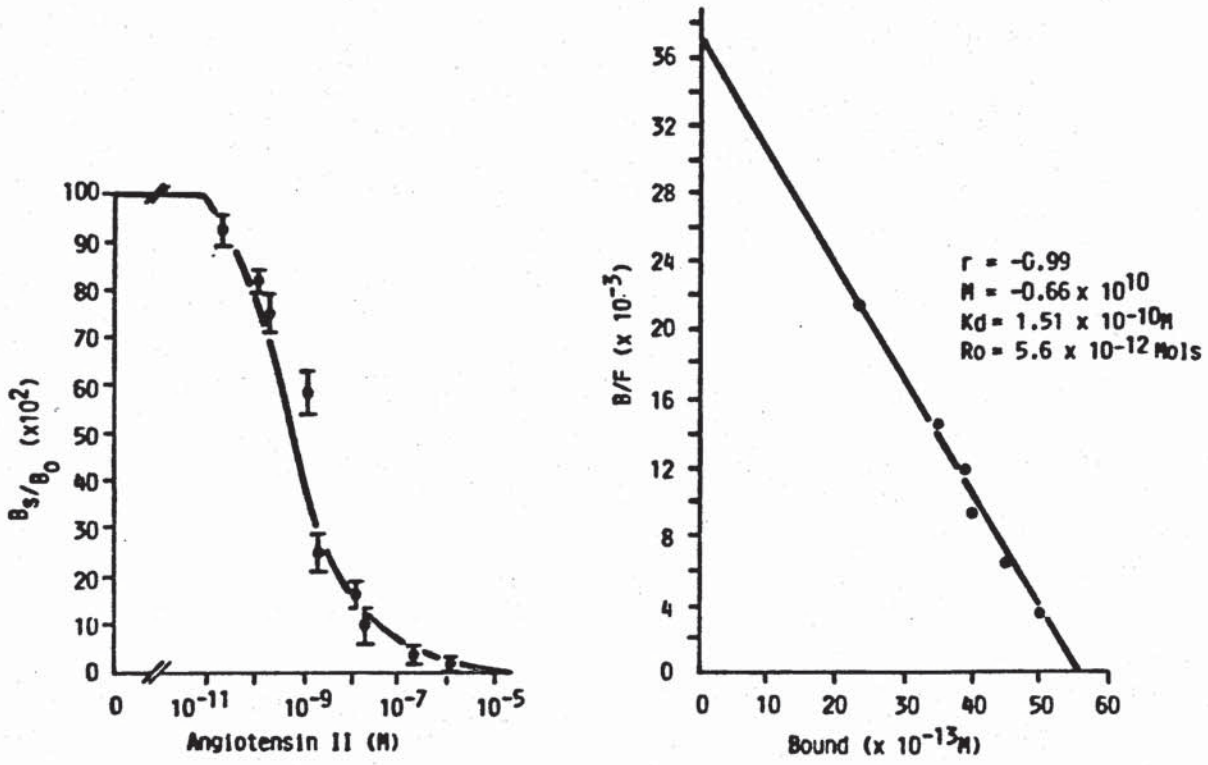

FIGURE 3.5 Platelets were incubated for 90 minutes in 300 pM ^{125}I-angiotensin II over a range of concentrations of unlabelled angiotensin II. Left panel shows specific binding expressed as a percentage of tracer binding in the absence of unlabelled hormone. Results are given as mean $\pm$ standard error of six experiments. Right panel shows Scatchard analysis of a representative experiment.

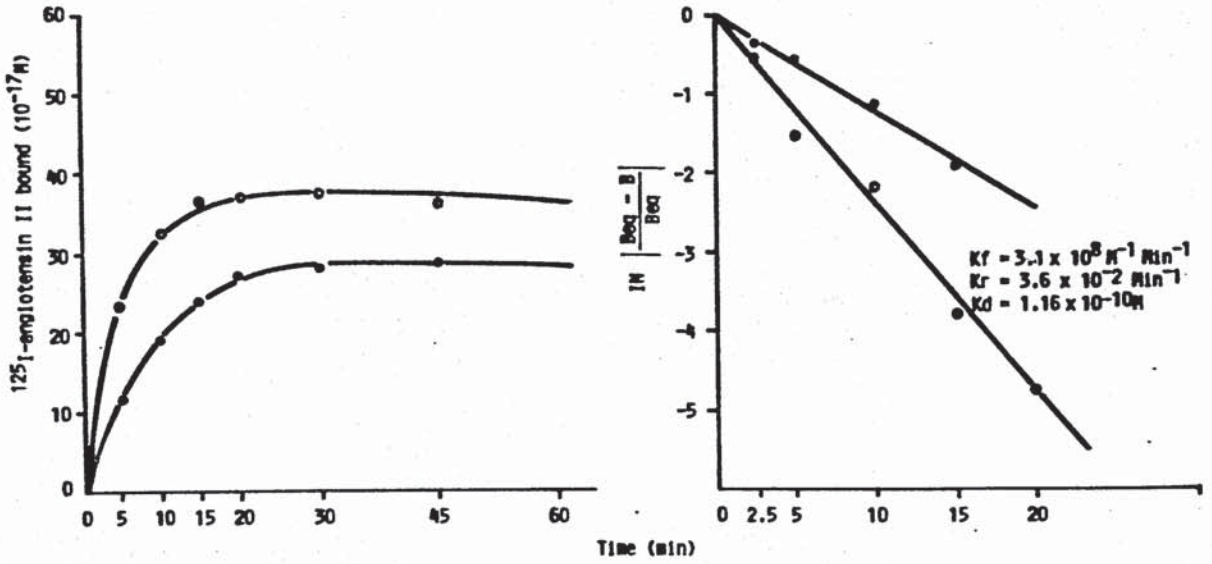

FIGURE 3.6 Kinetic studies of ^{125}I-angiotensin II association to platelets. Left panel: time course of specific binding at two concentrations of angiotensin II. Right panel: angiotensin II binding expressed as the natural logarithm of the proportion of binding at various times (B) relative to binding at equilibrium (Beq). Rate constants were calculated as described in Appendix 1. Values shown are the mean of two experiments.

o ___ o 0.6 nM ^{125}I-Ang II; •____• 0.3 nM ^{125}I-Ang II.

3.7:2 Results

The rate of dissociation of specifically bound radioactivity is shown in Figure 3.7.

The reverse rate constant was calculated at 1.2×10^{-2}/min (Appendix 1).

3.8 STABILITY

3.8:1 Methods

Platelet suspensions were preincubated without angiotensin II at 0°C, 22°C and 37°C for periods of up to 120 minutes. Specific ^{125}I-angiotensin II binding was then measured in the usual way after incubating the cells at 22°C for 60 minutes.

3.8:2 Results

The results are expressed as the fraction of specific binding without preincubation. Two experiments were performed at 3 different preincubation temperatures, 0°C, 22°C and 37°C. Preincubation at 0°C had little effect on specific binding. At 22°C there was a 30% reduction in binding after 60 minutes and at 37°C there was a 60% loss of binding after 20 minutes (Figure 3.8).

3.9 SPECIFICITY

3.9:1 Methods

Platelet suspensions were incubated with ^{125}I-angiotensin II (300 pM) and increasing concentrations (10^{-11} - 10^{-7}M) of various angiotensin II analogues.

3.9:2 Results

The dose ratios for the IC50's in competition curves were 2.36 for

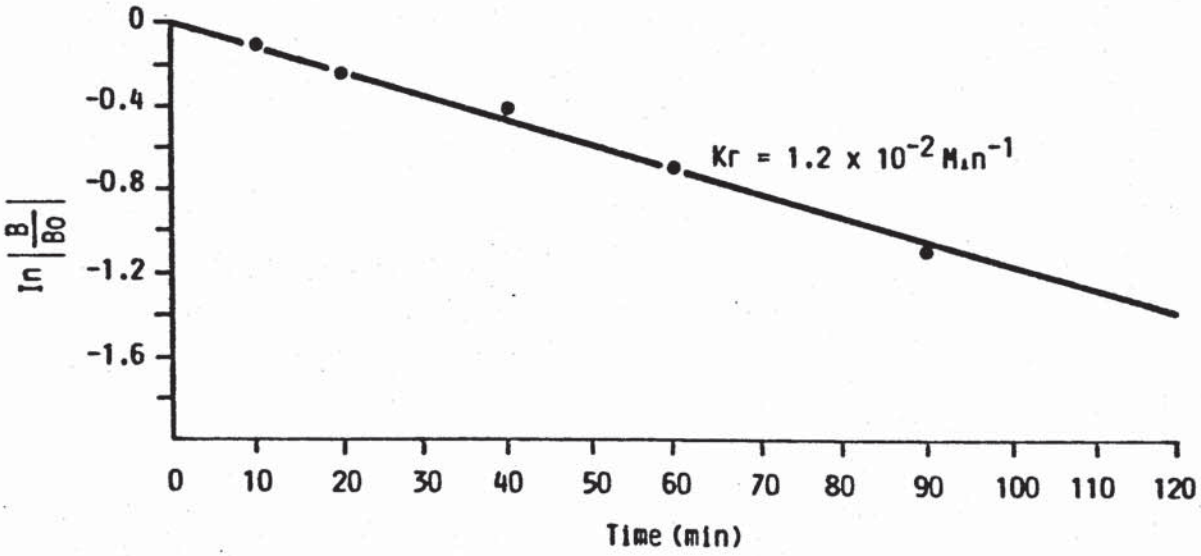

FIGURE 3.7 Dissociation of ^{125}I-angiotensin II expressed as the natural logarithm of binding at various times (B) relative to binding at time zero (Bo). Each point represents the mean of three experiments. Reverse rate constant was calculated as described in Appendix 1.

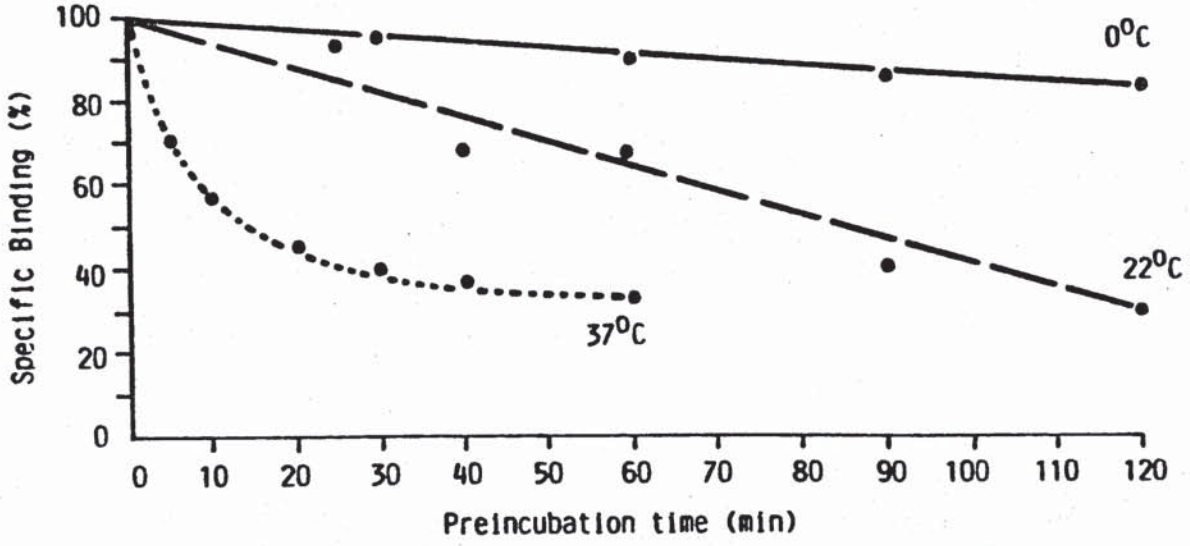

FIGURE 3.8 Effects of preincubation of platelets for various times at 0°C, 22°C and 37°C on specific binding measured with ^{125}I-angiotensin II after 60 minutes at 22°C. Results are expressed as a percentage of the specific binding after 60 minutes at 22°C without preincubation.

[Sar^1,Thr^8]-angiotensin II, 1 for angiotensin II, 0.81 for [des-Asp^1]-angiotensin II (angiotensin III), 0.76 for [Sar^1, Ala^8]-angiotensin II and 0.11 for angiotensin I (Figure 3.9).

3.10 CORRECTION FOR EXTRACELLULAR FLUID CONTAMINATION

3.10:1 Methods

Platelets were incubated at 22°C with 300 pM ^{125}I-angiotensin II with and without unlabelled angiotensin II for various periods up to 1 hour, together with ^{51}Cr-EDTA (0.1 µCi/incubation) as an extracellular marker.

3.10:2 Results

The time course of specific ^{125}I-angiotensin II binding to platelets at 22°C was similar to that found previously. Extracellular radioactive iodine associated with the platelet pellet after incubation with ^{125}I-angiotensin II alone was similar to that of platelets when incubated with ^{125}I-angiotensin II and an excess of unlabelled peptide (0.39 $\pm$ 0.02 % compared with 0.38 $\pm$ 0.03%). Correction for extracellular fluid radioactivity, did not, therefore affect the calculated specific binding (1.04% with correction compared with 1.03% without). However, extracellular fluid contamination contributed a mean of 73% to non-specific binding.

3.11 EFFECT OF D-PHENYLALANINE

3.11:1 Methods

Neyses et al. have suggested that ^{125}I-angiotensin II is degraded by carboxypeptidase A in mononuclear leukocytes and that the 1 - 7 heptapeptide so formed is taken up by free fluid endocytosis in platelets (Neyses, Locher,

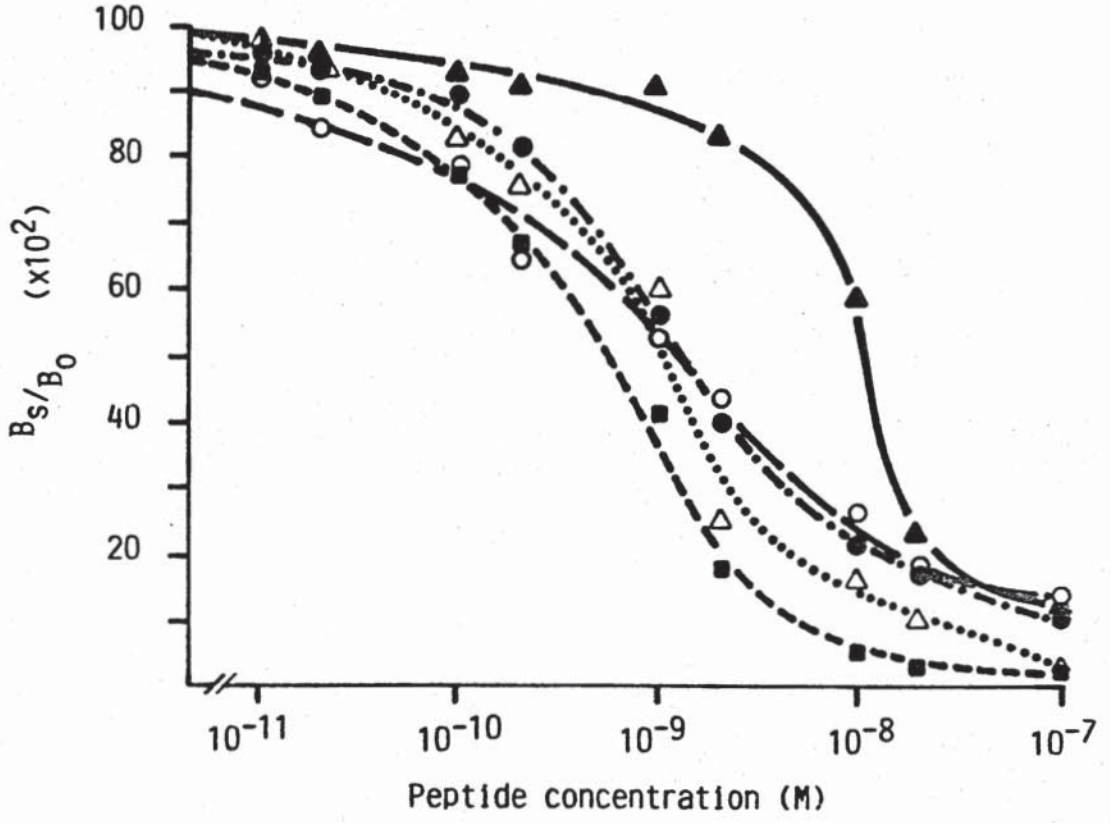

FIGURE 3.9 Competition by analogues of angiotensin II for ^{125}I-angiotensin II binding sites. Platelets were incubated with 300 pM ^{125}I-angiotensin II and increasing concentrations of angiotensin II analogues. Results are expressed as a percentage of tracer binding in the presence of ^{125}I-angiotensin II alone and are the mean of at least two experiments for each peptide.

▲ ___▲ Ang I; △. . . . △ Ang II; ●_.__.● Ang III;

o _ _o [Sar^1, Ala^8]-Ang II; ■ ---■ [Sar^1,Thr^8]-Ang II.

Wehling, Pech, Tenschert and Vetter 1984). Accordingly, platelets were incubated with 300 pM ^{125}I-angiotensin II and increasing concentrations of unlabelled angiotensin II with and without D-phenylalanine (10 mM) for 60 minutes at 22°C. D-phenylalanine at this concentration is an effective inhibitor of carboxypeptidase A (Fujioka and Imahori 1962).

3.11:2 Results

Figure 3.10 presents competition curves of the results. It is evident that specific binding was not affected by inhibition of the carboxypeptidase A (D-phenylalanine 10 mM). Scatchard analysis showed that neither the Kd (1.6×10^{-10} mol/l vs 2.7×10^{-10} mol/l) nor the Bmax (8.3 sites/platelet vs 9.1 sites/platelet) were affected by D-phenylalanine.

3.12 RECEPTOR OCCUPANCY

3.12:1 Methods

Experiments were carried out to determine whether prior occupancy by endogenous angiotensin II affected the measurements of binding capcity.

In the first experiment, competition curves were performed in blood from 3 normal male subjects (ages 32-46 years) before and 2 hours after administration of the angiotensin converting enzyme inhibitor, captopril (25 mg) by mouth.

In the second experiment, binding to platelets from 3 normal male subjects (ages 32-36 years) was measured in the usual way and compared with binding to platelets from the same subjects when blood was collected into a syringe containing angiotensin II to give a final concentration in blood of 1 nM.

In the third experiment, angiotensin II (4 ng/Kg/min) was infused for 120 minutes in three normal male subjects (ages 30 - 37 years). Platelet angiotensin

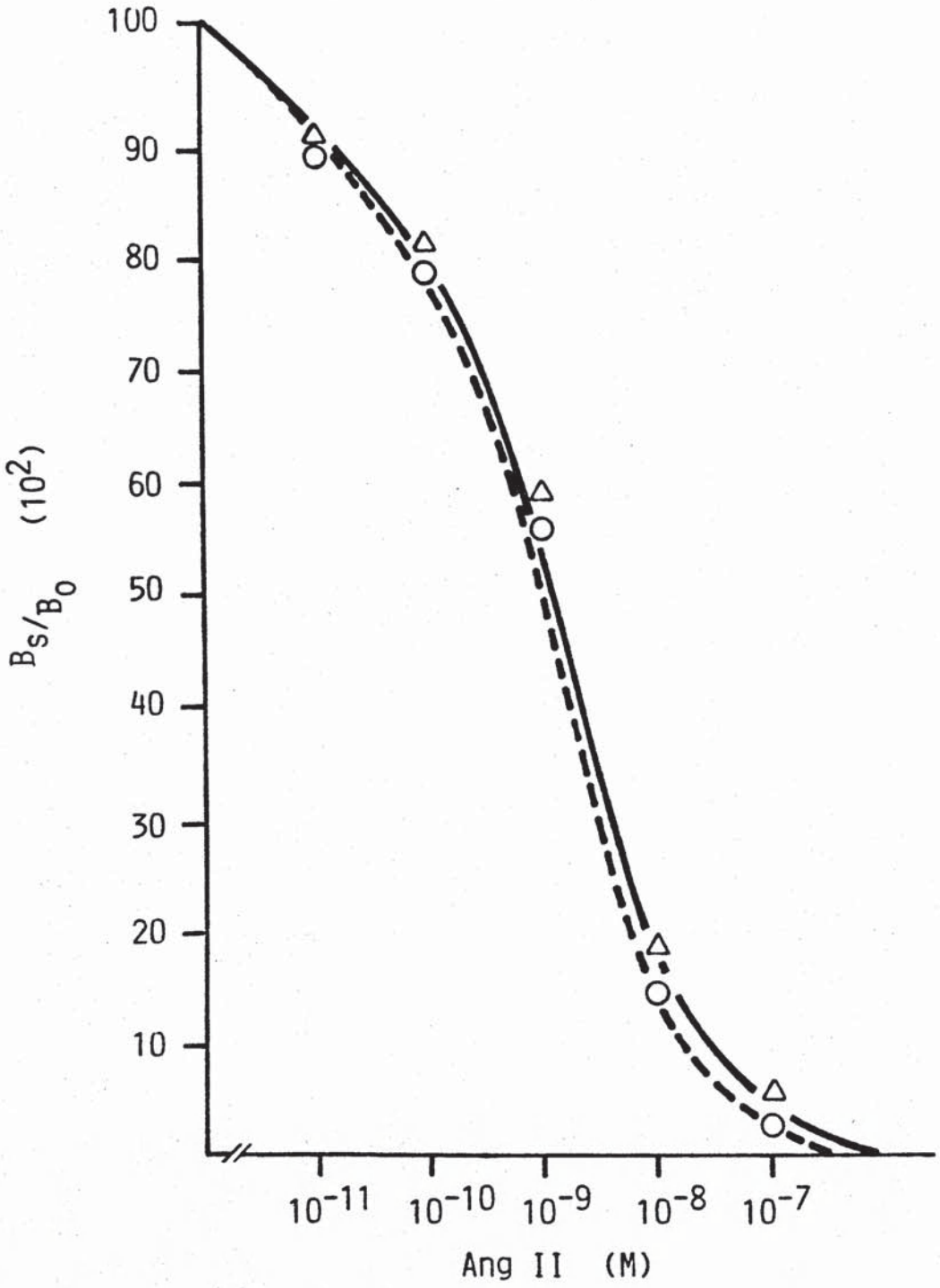

FIGURE 3.10 Competition curves of platelets from 60 ml of blood from a normal subject incubated with ^{125}I-Ang II (300 pmol/l) and increasing concentrations of unlabelled Ang II incubation conditions:-

platelets (5.4 x 10^{11}/l) were incubated for 60 minutes at 22°C in medium 199 containing 5 mM EDTA. Δ_ Δwith 10 mM D-phenylalanine, o--- o without D-phenylalanine.

II binding and plasma angiotensin II levels were measured at the beginning and end of the infusion.

3.12:2 Results

In the first experiment, Scatchard analysis of the results showed that captopril treatment did not affect either receptor concentration (5.9 ± 0.8 sites/platelet before captopril; 7.1 ± 1.2 sites/platelet after captopril) or affinity (5.4 ± 1.5 x 10^{-10}M before captopril; 5.2 ± 1.6 x 10^{-10}M after captopril).

In the second experiment, Scatchard analysis of the results showed that preincubation of blood with angiotensin II did not affect either receptor concentration (4.8 ± 0.3 sites/platelet before angiotensin II; 4.7 ± 0.5 sites/platelet after angiotensin II) or affinity (6.2 ± 1.1 x 10^{-10}M before angiotensin II; 5.9 ± 0.8 x 10^{-10}M after angiotensin II).

In the third experiment, the plasma angiotensin II level was 18 ± 2 pg/ml at the beginning of infusion and 80 ± 17 pg/ml at the end. However, these short term increases in plasma angiotensin II did not alter either receptor concentration (5.5 ± 0.5 sites/platelet before angiotensin II infusion; 5.8 ± 0.3 sites/platelet after angiotensin II infusion) or affinity (4.7 ± 0.3 x 10^{-10}M before and 5.3 ± 0.2 x 10^{-10}M after angiotensin II infusion).

All these results are shown in Table 3.1.

3.13 COMMENTS

Platelets have been prepared in good yield (60 ± 1.5%, n = 59) from a relatively small blood sample (60 ml) using a simple one-step procedure with Percoll. Centrifugation of whole blood over a layer of isotonic Percoll (specific gravity 1.061) separated platelets (s.g. < 1.06) from other blood cells (s.g.> 1.06), contained < 1% leukocytes and < 0.1% erythrocytes. Most of the contaminating

TABLE 3.1

RECEPTOR OCCUPANCY

EFFECT OF CAPTOPRIL AND ANGIOTENSIN II ON ANGIOTENSIN II BINDING

	Bmax (sites/platelet)		Kd (x 10^{-10}M)	
	Before	After	Before	After
In vivo captopril treatment	5.2	8.4	6.0	7.8
	7.8	8.8	8.2	6.5
	4.8	4.2	2.0	1.4
mean	5.9	7.1	5.4	5.2
In vitro Ang II incubation	5.5	5.6	8.6	7.9
	4.7	5.0	5.8	5.2
	4.4	3.6	4.1	4.7
mean	4.8	4.7	6.2	5.9
In vivo Ang II infusion	6.4	6.6	5.1	5.4
	4.4	5.4	4.1	4.8
	5.6	5.5	4.9	5.6
mean	5.5	5.8	4.7	5.3

leukocytes were mononucleated (80-100%). This lack of contamination may be of importance if the report of Shimada and Yazaki (1978) that angiotensin II receptors are present in greater numbers on mononuclear leukocytes than platelets are proven. The present method gave a better yield with less contamination than that of Moore and Williams (1982). It is also significant that normal specific angiotensin II binding was present in occasional platelet preparations which contained no measurable contaminating cells.

Scatchard analysis indicated that receptor numbers and affinities were similar to those described by Moore and Williams (1982). A single class of specific binding sites on platelets was apparent and these sites were saturable. Values for the affinity and number of angiotensin II binding sites from saturation analysis using tracer alone and from competition studies using labelled hormone were similar, indicating that native and radioactive angiotensin II behaved in an identical manner. Both methods gave a Kd of around 10^{-10}M and a binding capacity equivalent to 7 receptors/cell. Values were not adjusted for the small losses of binding capacity which were observed in pre-incubation studies. The Kd is similar to those described for other target tissue 5×10^{-10}M for rat mesenteric artery (Gunther et al. 1980a) and $0.9\text{-}5 \times 10^{-10}$M in animal adrenal cortex (Douglas and Catt 1976; Aguilera et al. 1978). Plasma concentrations of angiotensin II may range from 10^{-12}M up to 10^{-9}M across a range of physiological and pathophysiological conditions (Beevers, Brown, Fraser, Kremer, Lever, Morton, Robertson, Schalekamp, Semple and Wilson 1976). The binding characteristics described here from equilibrium studies are confirmed by affinity calculated from kinetic association and dissociation constants. The dissociation rate for receptor bound hormone is sufficiently rapid that endogenous angiotensin II does not affect binding measurements. Pretreatment with captopril and angiotensin II did not alter receptor characteristics and these results are similar

to those reported by Williams group (Moore, Taylor and Williams 1984).

A recent study by Neyses and colleagues (Neyses et al. 1984) suggested that tracer uptake into platelets might be due to free fluid endocytosis rather than a receptor mechanism. This seems unlikely for the following reasons: (a) Free fluid endocytosis of a radioactivity was shown at 37°C, a temperature at which there is rapid metabolism of angiotensin II. My experiments were carried out at 22°C. (b) There was no evidence of endocytosis in experiments with an extracellular fluid marker. (c) Platelet number was unaffected during incubation and only intact cells were separated from medium by centrifugation through oil. (d) D-phenylalanine, a specific inhibitor of carboxypeptidase A prevents free fluid endocytosis of metabolised ^{125}I-angiotensin II (Fujioka and Imahori 1962). Specific binding of angiotensin II to platelets was not reduced by this inhibitor. Mann, Sis and Ritz (1985) have also examined the effects of a much wider range of protease inhibitior including L-cysteine (1.0 mM) and D-phenylalanine (0.01-10 mM) and an inhibitor of endocytosis, colchicine (10-100 µM) on angiotensin II binding to platelets. Effective inhibition of protease and endocytosis did not affect specific binding of angiotensin II to platelets. Therefore, there is no convincing evidence that the association of radioactivity with platelets is due to metabolism of ^{125}I-angiotensin II by mononuclear leukocytes and subsequent endocytosis of this metabolite into platelets.

The affinities of the platelet binding site for analogues of angiotensin II, [Sar^1, Thr^8]-Ang II > Ang II > Ang III > [Sar^1, Ala^8]-Ang II > Ang I are similar to those described for rat adrenal cells.

CHAPTER 4 EFFECT OF CHANGES IN DIETARY SODIUM AND POTASSIUM INTAKE ON PLATELET RECEPTORS FOR ANGIOTENSIN II

4.1 LABORATORY METHODS

Plasma concentration of renin and angiotensin II were measured by radioimmunoassay (Millar, Leckie, Morton, Jordan and Tree 1980; Morton, Semple, Waite, Brown, Lever and Robertson 1976). Renin concentrations were assayed by measuring the rate of generation of angiotensin I in an incubation mixture supplemented with renin substrate. Plasma angiotensin II concentrations was measured after extraction from plasma by mixing with Dowex resin followed by elution from the resin by mixing with methanolic ammonia. Urine concentrations of sodium and potassium were measured using an ion selective electrode method (Beckman electrolyte 2 analyser with coefficient of variation of 4%). The method of platelet preparation and radioligand binding for measuring angiotensin II receptor concentration and affinity has previously been described in Chapter 2.

4.2 DIETARY EXPERIMENTS

Eight normal subjects with a mean age of 31 years (23-37) were studied. Each subject was studied after the following diets: (1) low sodium (15 mmol Na, 70 mmol K/day), (2) normal sodium and potassium (150 mmol Na, 70 mmol K/day), (3) high sodium (200 mmol Na, 70 mmol K/day), (4) low potassium (150 mmol Na, 35 mmol K/day), (5) high potassium (150 mmol Na, 150 mmol K/day). The five diets were given in random order. Each diet was given for a period of four days with a minimum period of five days on unrestricted diet in between.

A collection of urine was made on the fourth day of each period of fixed diet for measurements of sodium and potassium excretion over 24 hours. The protocol of the dietary experiments is shown in Figure 4.1. At 0900 hours on the fifth day and after 30 minutes of recumbency, blood pressure was measured with a sphygmomanometer. Blood (75 ml) was withdrawn by venepuncture from a forearm vein for measurement of plasma concentrations of renin and angiotensin II, and platelet angiotensin II receptor concentration and affinity.

4.3 STATISTICAL ANALYSIS

For group comparisons, Student's paired 't' tests were used. Correlation coefficients were estimated by the Spearman rank method (Siegel 1956).

4.4 RESULTS

4.4:1 Dietary sodium

From urinary sodium measurements, subjects were in metabolic balance by the fifth day of each dietary period (Table 4.1). Plasma concentrations of renin and angiotensin II were significantly higher after the low sodium diet compared with either the normal or the high sodium diet ($p < 0.05$). As expected there were significant inverse correlations between urinary sodium excretion and plasma concentrations of renin and angiotensin II for the three levels of sodium intake (r_s 0.64 and 0.65, $p < 0.01$). Analysis of the binding curves showed that there was a decrease in receptor capacity (Figure 4.2) after the low sodium diet, compared to the normal and high sodium diets ($p < 0.01$) but that there was no change in affinity. An example of the effect of dietary sodium manipulation in one subject is shown in Figure 4.3; the slope of the line, a function of

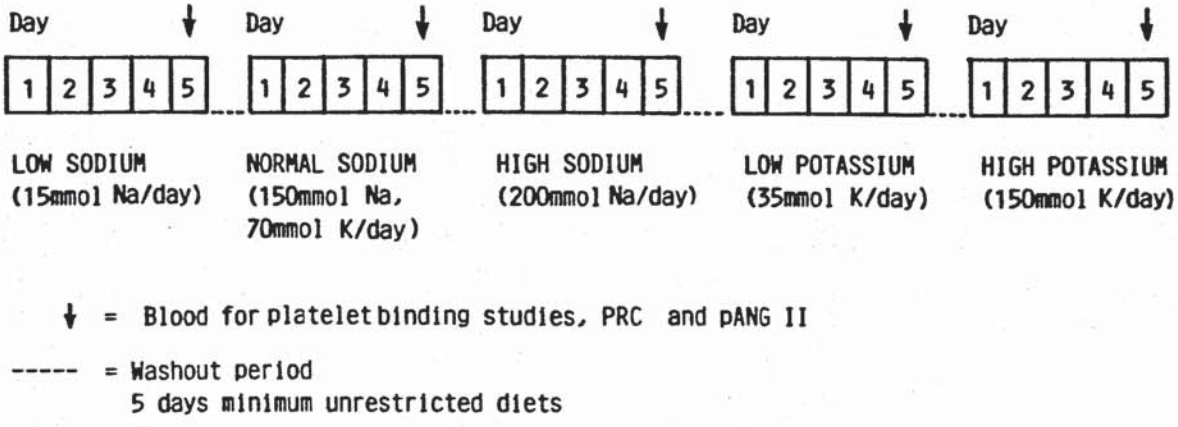

FIGURE 4.1 Protocol of dietary experiments.

TABLE 4.1

DIETARY SODIUM AND PLATELET ^{125}I-ANG II BINDING

	LOW SODIUM 15 mmol/day	NORMAL SODIUM 150 mmol/day	HIGH SODIUM 200 mmol/day
Systolic BP lying (mmHg)	101 ± 1	108 ± 3	104 ± 3
Diastolic BP lying (mmHg)	61 ± 3	62 ± 3	59 ± 3
PRC (μU/ml)	46 ± 5*	24 ± 5	16 ± 2
pANG II (pmol/l)	26 ± 2**	14 ± 2	12 ± 1
Urinary Na^+ (mmol/24 hr)	15 ± 2	131 ± 4	205 ± 17
Bmax (sites/cell)	4.1 ± 0.4	6.2 ± 0.3	6.8 ± 0.5
Kd (10^{-10}M)	5.2 ± 0.5	5.4 ± 0.6	4.1 ± 0.5

* $p < 0.05$, ** $p < 0.01$ compared with normal sodium periods.

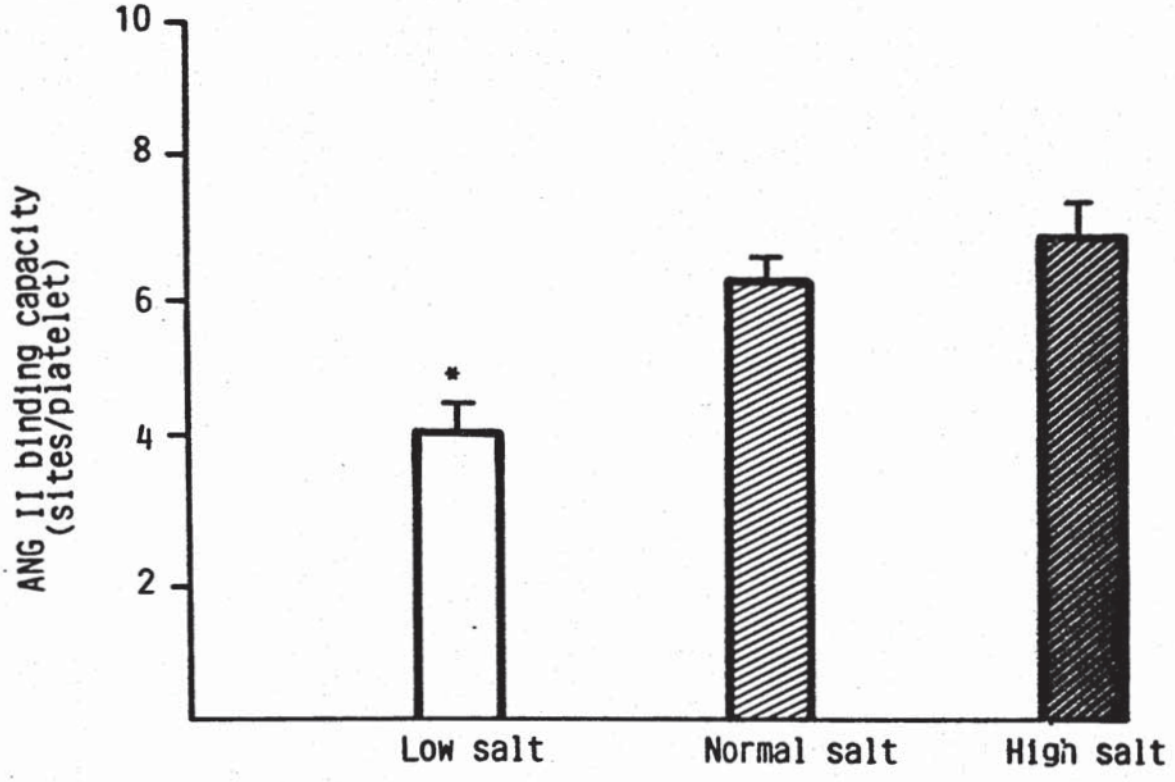

FIGURE 4.2 Effect of dietary sodium intake on the concentration of angiotensin II binding sites on platelets.

* paired t, $p < 0.01$, compared with normal and high salt periods.

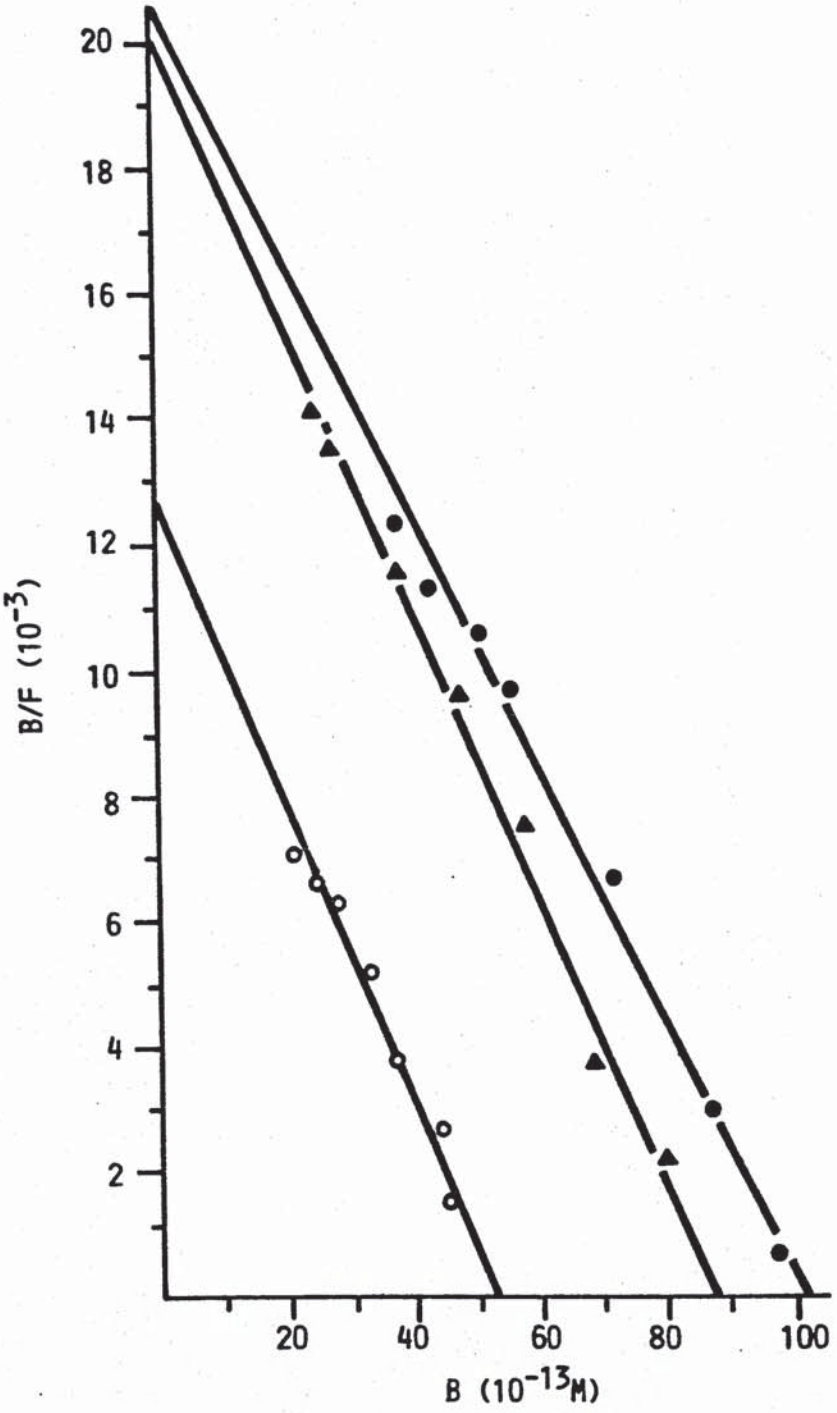

FIGURE 4.3 Scatchard analysis of angiotensin II receptor binding on high (● ____●), normal (▲ ___ ▲) and low (o_____ o) sodium diet in one subject. Each point represents mean of quadruplicate determinations. Values have been normalised with respect to platelet numbers (8.0×10^{11}/l).

affinity, does not change whereas the intercept on the abscissa is shifted to the left indicating a decrease in binding capacity.

There was an inverse correlation between plasma concentrations of angiotensin II and angiotensin II receptor concentrations (r_s 0.57, $p < 0.01$). These results are shown in Figure 4.4. Differences in receptor capacity and affinity between normal and high sodium diets were not statistically significant (Table 4.1).

4.4:2 Dietary potassium

Urinary excretion of potassium on the fifth day of each dietary regime was similar to dietary intake (Table 4.2), urinary excretion of sodium was unaffected. Plasma concentrations of renin were significantly higher after the 150 mmol potassium diet than after the low and normal potassium diets ($p < 0.05$) but the changes in plasma concentrations of angiotensin II were not significant (Table 4.2). There were no significant changes in platelet receptor concentration or affinity between the three different potassium diets.

4.5 COMMENTS

The effect of angiotensin II on aldosterone secretion and blood pressure are considerably modified by changes in dietary sodium and potassium. The aldosterone response to angiotensin II is enhanced after sodium restriction (Oelkers, Brown, Fraser, Lever, Morton and Robertson 1974), whereas the pressor activity of angiotensin II is diminished (Williams, Hollenberg and Braley 1976).

Since the circulating concentration of angiotensin II is increased during sodium restriction, the changes occurring in the adrenal glomerulosa and in the arteries could be mediated by angiotensin II. Indirect evidence suggests that angiotensin II mediates the altered vascular responses to sodium depletion but is

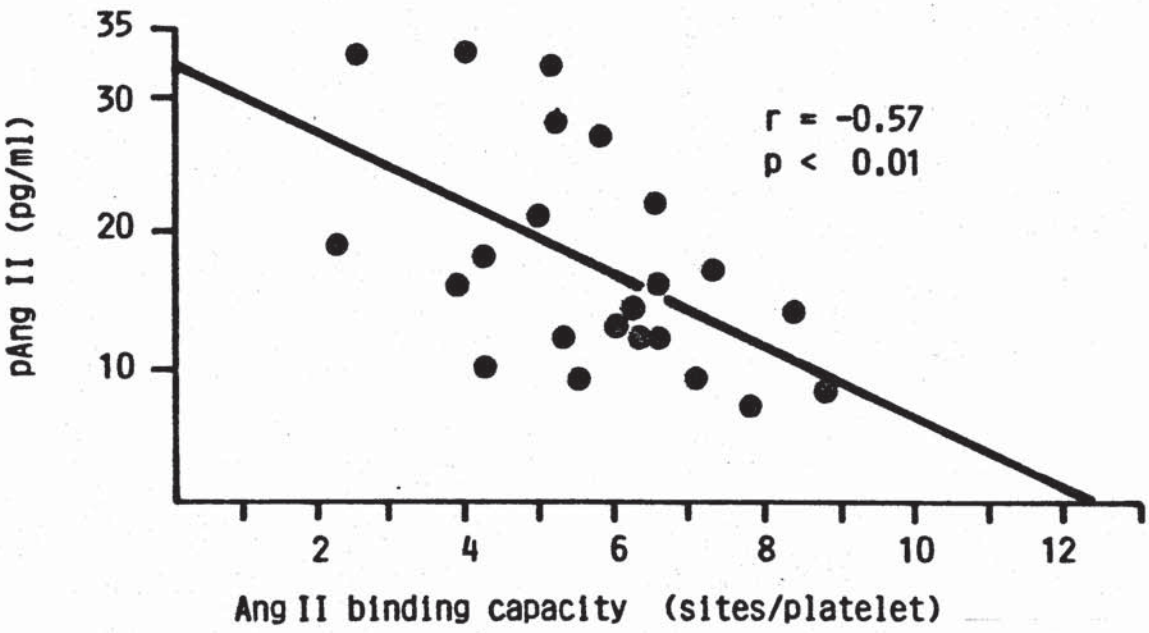

FIGURE 4.4 Relationship between plasma concentrations of angiotensin II and angiotensin II receptor concentrations.

TABLE 4.2

DIETARY POTASSIUM AND PLATELET ^{125}I-ANG II BINDING

	LOW POTASSIUM 35 mmol/day	NORMAL POTASSIUM 70 mmol/day	HIGH POTASSIUM 150 mmol/day
Systolic BP lying (mmHg)	109 ± 3	108 ± 3	108 ± 3
Diastolic BP lying (mmHg)	63 ± 4	62 ± 3	63 ± 3
PRC (μU/ml)	18 ± 3	24 ± 5	30 ± 4*
pANG II (pmol/l)	14 ± 2	14 ± 2	20 ± 3
Urinary K^+ (mmol/24 hr)	41 ± 5	68 ± 3	142 ± 10
Urinary Na^+ (mmol/24 hr)	134 ± 9	131 ± 4	149 ± 4
Bmax (sites/cell)	5.9 ± 0.3	6.2 ± 0.3	5.0 ± 0.2
Kd (10^{-10}M)	4.6 ± 0.7	5.4 ± 0.6	5.6 ± 0.7

* $p < 0.05$, compared with normal potassium periods.

responsible for only a small part of the change in responsiveness of the adrenal cortex. Increased adrenal sensitivity in the low sodium state persisted even in the absence of high endogenous angiotensin II by captopril treatment. Thus angiotensin II may not be necessary for maintaining the enhanced adrenal sensitivity to infused angiotensin II observed during sodium restriction in man. In contrast, vascular sensitivity to angiotensin II during sodium restriction was reversed by lowering of their angiotensin II levels after treatment with captopril (Dawson-Hughes, Moore, Dluhy, Hollenberg and Williams 1981). This may reflect alterations in the properties of angiotensin II receptors in response to changes in circulating concentrations of angiotensin II (Catt, Harwood, Aguilera and Dufau 1979).

Radioligand binding studies in rat tissue have demonstrated that sodium depletion reduces the number of receptor sites in uterine muscle (Devynck et al. 1976; Devynck et al. 1978; Hauger et al. 1978), bladder (Aguilera and Catt 1981) and renal glomeruli (Bellucci and Wilkes 1984) but increases the number of sites in adrenal cortex (Douglas and Catt 1976; Hauger et al. 1978). In the rat, infused angiotensin II increases both the concentration and affinity of adrenal binding sites (Douglas and Brown 1982) and this increase in adrenal receptors in sodium-deficient rats is prevented by captopril (Aguilera and Catt 1978). In a membrane fraction from mesenteric arterial tissue sodium restriction also resulted in a decrease in the number of receptor sites (Gunther et al. 1980b; Aguilera and Catt 1981).

In the present study restriction of sodium intake for four days in normal human subjects resulted in a decrease in the concentration of high-affinity binding sites on platelets, thereby confirming the recent observation of Moore et al. (Moore, Taylor and Williams 1984). Receptor affinity was not affected by dietary sodium intake. Changes in receptor numbers may be mediated by

angiotensin II since there was a correlation between the density of binding sites and circulating concentrations of peptide (Figure 4.4). In this respect the response of platelet angiotensin II receptors to sodium depletion is like that of receptors in vascular muscle.

Dietary potassium also affects vascular and adrenal responsiveness to angiotensin II in man (Hollenberg, Williams, Burger and Hooshmand 1975). Reduced potassium intake was associated with a significant increase in circulating renin activity and angiotensin II concentration and a significant reduction in renal blood flow. Conversely, a high potassium intake was associated with a significant increase in plasma aldosterone secretion and renal blood flow without alteration in plasma renin activity or angiotensin II concentration. Low potassium intake decreased both the renal vascular and the adrenal response to infused angiotensin II, whereas potassium loading enhanced the responsiveness of both systems.

Short-term potassium loading in rats reduced plasma concentrations of angiotensin II, increased the number of receptors in the adrenal cortex (Douglas and Catt 1976) but decreased the affinity (Douglas 1979). However, rat uterine smooth muscle receptors were present in lesser amounts after dietary potassium loading (Douglas 1979) and in greater amounts after potassium restriction (Paller et al. 1984). Both potassium loading and restriction caused an increase in receptor affinity for angiotensin II. These effects of potassium loading were prevented by administration of captopril (Douglas 1980). It is significant that changes in receptor function were only observed when plasma angiotensin II levels were altered.

In man, dietary potassium loading has less consistent effects on plasma renin activity (Brunner, Baer, Sealey, Ledingham and Laragh 1970) probably because of conflict between the inhibitory effects of potassium on renin release

(Vander 1970) and the stimulation of renin secretion by the natriuresis that results from potassium loading (Kahn and Bohrer 1967). There were no detectable changes in platelet receptor affinity or concentration in a group of normal subjects fed diets of different potassium content. This may be due to the lack of significant change in plasma concentration of angiotensin II in response to potassium loading or restriction.

There are several possible explanations for the cellular mechanism of platelet receptor changes, including: (1). Prior receptor occupancy by endogenous angiotensin II. There are a number of experiments which have shown that prior receptor occupancy after short-term in vivo or in vitro treatment with angiotensin II does not affect binding capacity (Chapter 3, Moore et al. 1984). Also decreases in binding capacity have been shown to lag behind increases in plasma angiotensin II levels (Moore et al. 1984). These observations indicate that receptor occupancy is not the explanation of dietary sodium-induced receptor changes. (2) Synthesis or degradation of new receptor sites. Platelets do not have nuclei and are thought not to be able to synthesis new protein. It is possible that increased receptors were synthesised by the megakaryocyte before separation into platelets. Increased receptor numbers would then depend on platelet formation but the time course for changes in angiotensin II receptor numbers (2 days) is much shorter than the time needed for platelet turnover (10 days). (3) The degree of exposure of already synthesised receptors. Unless platelets retain some minor capacity to synthesis new protein at least for a short while, increasing dietary sodium may cause pre-existing receptors to be revealed (Moore et al. 1984).

CHAPTER 5 PLATELET RECEPTORS FOR ANGIOTENSIN II IN ESSENTIAL HYPERTENSION

5.1 LABORATORY METHODS

Methods for measuring renin and angiotensin, sodium and potassium and receptor binding are as previously described (Chapters 2 and 4).

5.2 ESSENTIAL HYPERTENSION AND NORMAL SUBJECTS

Ten patients with essential hypertension were studied. There were six men and four women aged 30-57 years (mean 46) all of whom had at least two outpatient blood pressure recordings greater than 150/95 mmHg. Patients were on unrestricted diet and had not previously received drug treatment for hypertension and had not been taking any drugs for at least 4 weeks before the study. All patients had normal serum concentrations of potassium, urea, and creatinine, with no significant proteinuria and normal rapid sequence intravenous urograms. Mean blood pressure, measured twice with a mercury sphygmomanometer after 30 minutes recumbency, was 178 mmHg systolic (range 152 to 232 mmHg) and 107 mmHg diastolic (range 92 to 154 mmHg). Blood was withdrawn for measurements of platelet angiotensin binding (60 ml) and plasma concentrations of renin and angiotensin II (15 ml).

The normal subjects were ten age and sex matched laboratory or clinical personnel aged 36-56 years (mean 46) with blood pressure less than 140/90 mmHg in the seated position on at least two occasions. Blood pressure was measured immediately before blood sampling. Systolic pressures ranged from 96 to 122 (mean 112) mmHg and diastolic pressures were between 62 and 86 (mean

74) mmHg. Subjects were not on regular medication and had taken no treatment for at least 4 weeks before the study.

5.3 STATISTICAL ANALYSIS

For group comparisons, Student's unpaired 't' test was used.

5.4 RESULTS

Plasma renin and angiotensin concentrations were not statistically different in the two groups although 5 subjects had low-renin hypertension.

The binding of ^{125}I-angiotensin II to platelets in 10 patients with essential hypertension was compared with that from 10 normal subjects. The number of binding sites and the equilibrium dissociation constants in the 2 groups were similar (Table 5.1).

5.5 COMMENTS

In essential hypertension pressor responses to infused angiotensin II are enhanced (Meier, Weidmann, Grimm, Keusch, Glück, Minder and Ziegler 1981) compared to normal subjects but the mechanism of these changes has not been defined. One possibility is that changes in the concentration and affintiy of angiotensin II receptors, perhaps resulting from changes in circulating concentrations of angiotensin II, may be responsible but other mechanisms such as changes in geometry of the resistance vessels have been implicated (Folkow 1971).

About 30% of hypertensive patients have low plasma renin concentrations;

TABLE 5.1

ESSENTIAL HYPERTENSION

	Age	Blood Pressure		Plasma		Ang II - Receptors	
		Systolic (mmHg)	Diastolic (mmHg)	Renin (μU/ml)	Ang II (pg/ml)	Kd (X 10^{-10}M)	Capacity (sites/platelet)
Essential Hypertensives (n = 10)	46 ± 3	178 ± 9	107 ± 6	12 ± 2	14 ± 2	5.0 ± 0.6	5.7 ± 0.8
Normotensives (n = 10)	46 ± 3	112 ± 3	74 ± 3	13 ± 2	13 ± 2	4.9 ± 0.8	5.4 ± 0.5
Low Renin Essential Hypertensives (n = 5)	46 ± 4	184 ± 12	114 ± 10	7 ± 1	11 ± 2	4.9 ± 0.7	4.8 ± 1.0

5 of the patients I studied had low renin hypertension. Such patients in particular show enhanced pressor responses to angiotensin II (Meier et al. 1981). In the patients I studied I could detect no changes in either platelet receptor affinity or concentration. There was no evidence of receptor up-regulation in response to low circulating levels of angiotensin. Changes in sensitivity to angiotensin II in essential hypertension may not be determined at receptor level but it is also possible that changes in angiotensin receptors in platelets do not reflect changes in vascular muscle.

CHAPTER 6 EFFECTS OF ANGIOTENSIN II ON HUMAN PLATELETS

Although platelets have receptors for angiotensin II, their function is not clear. Angiotensin II alone has been reported to have no direct effect on the aggregation of normal platelets when tested over a wide range of hormone concentrations (Stoff, Stemerman, Steer, Salzman and Brown 1980). However, some agents which do not induce platelet activation directly may potentiate responses to other agonists (Grant and Scrotton 1980) and such interaction may be of pathophysiological importance.

Platelet aggregation is initiated by agonist-receptor interactions on the plasma membrane and may be propagated by endogenous platelet constituents secreted by the activated platelets. Such pro-aggregatory mediators induce the eicosanoids PGG_2, PGH_2 and TxA_2 and the granule constituents ADP and 5HT (Kinlough-Rathbone, Packham, Reimers, Cazenave and Mustard 1977) (Figure 6.1). The major transduction process that links receptor occupancy to platelet activation is believed to be stimulated phosphoinositide hydrolysis (MacIntyre, Pollock, Shaw, Busfield, MacMillan and McNichol 1985): degradation of phosphatidylinositol (Ptd Ins), Ptd Ins-4-phosphate or more likely Ptd Ins 4, 5-bis-phosphate yield diacylglycerol (DAG) and the corresponding inositol phosphate (s) (Berridge 1984). Inositol 1,4,5 tris-phosphate (Ins P_3) has been implicated in Ca^{2+} mobilisation (Streb, Irvine, Berridge and Schulz 1983; Berridge and Irvine 1984). Elevated $[Ca^{2+}]i$ and DAG are the second messenger molecules that regulate the so-called Ca^{2+}-dependent and Ca^{2+}-independent pathways of platelet activation respectively (Rink, Sanchez and Hallam 1983) (Figure 6.2).

In order to determine whether platelet angiotensin II receptors modulate platelet responsiveness to agonists, the effects of angiotensin II on platelet

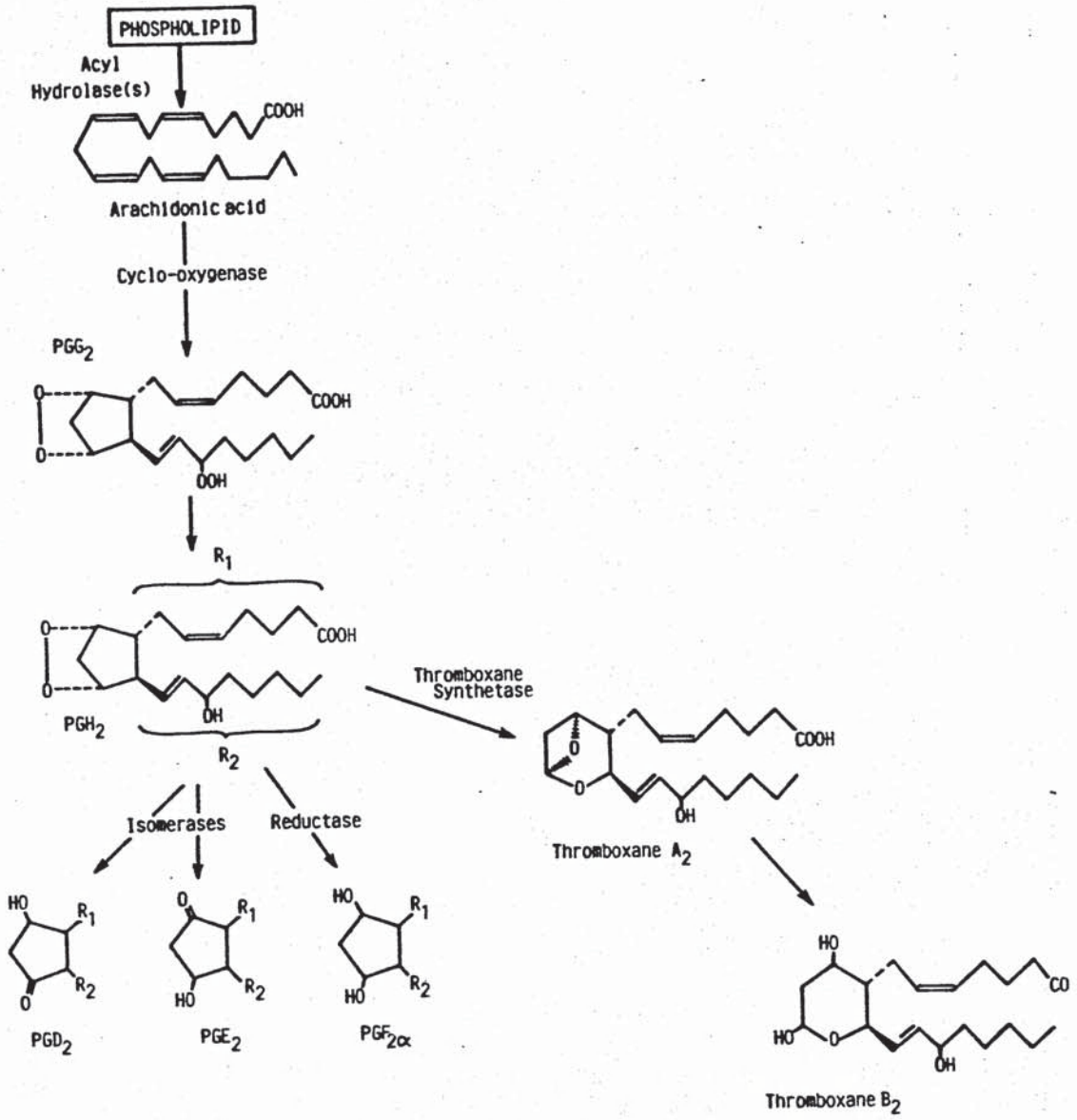

FIGURE 6.1 The biosynthetic pathways of prostaglandins. Cyclo-oxygenase is inhibited by flurbiprofen. Thromboxane A_2 is thought to be the active but unstable mediator of secondary aggregatory responses. Thromboxane B_2 is the inactive but stable product of thromboxane A_2 hydrolysis.

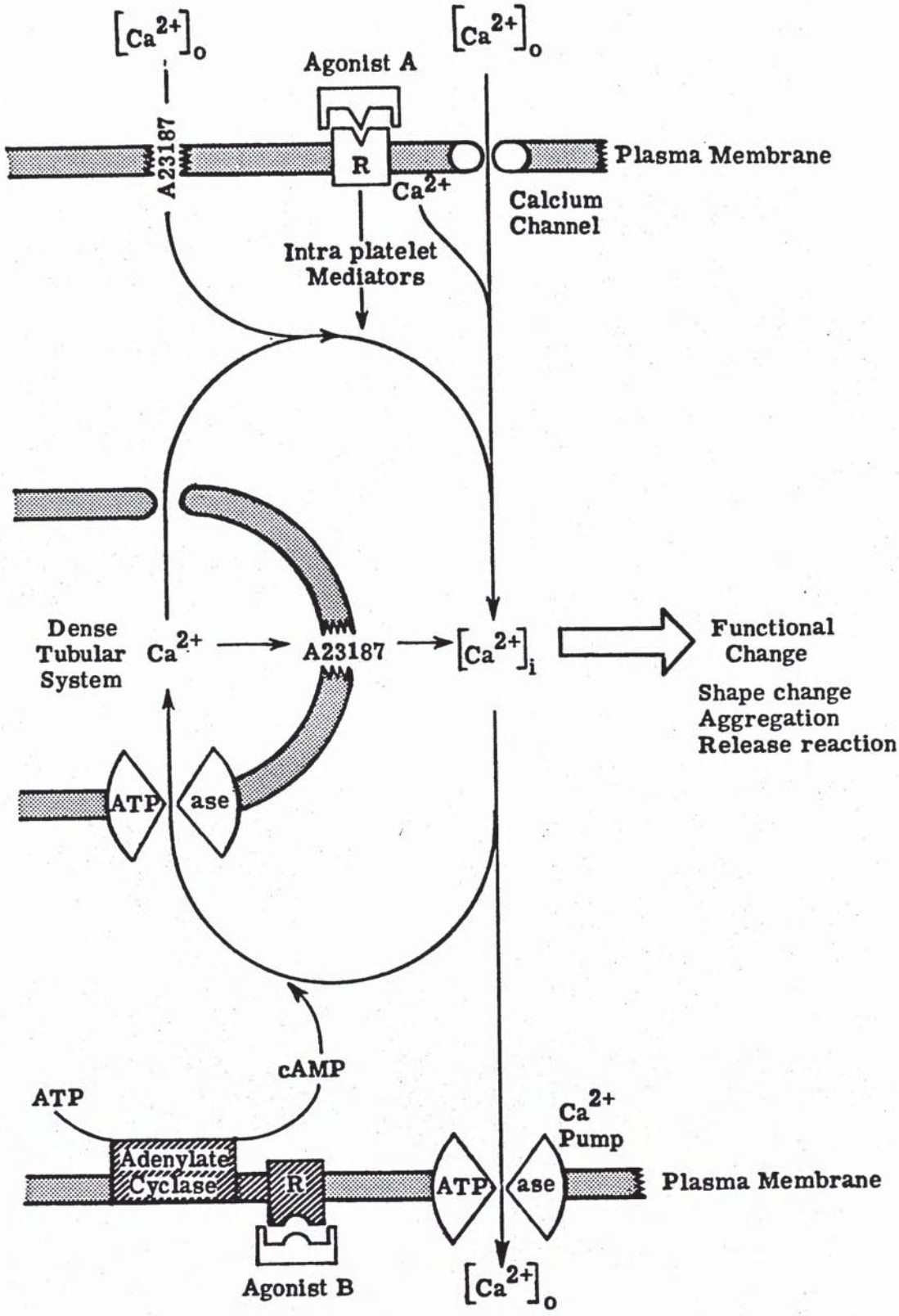

FIGURE 6.2 Regulation of platelet cytosolic free calcium. Platelet agonists (agonist A) such as vasopressin, thrombin, platelet activating factor (PAF), ADP and 5HT are believed to cause platelet activation by elevating cytoplasmic free calcium. Agonist B such as prostacyclin and adenosine are believed to elevate cyclic AMP levels which may cause platelet inhibition.

aggregation, cytosolic $[Ca^{2+}]_i$ and TxB_2 (thromboxane B_2) formation have been investigated.

6.1 MATERIALS

5, 6, 8, 9, 11, 12, 14, 15-(^{3}H)N -TxB_2 (140 Ci/mmol) in methanol solution was obtained from New England Nuclear, Boston, U.S.A.. TxB_2 was obtained from Upjohn Co. (Kalamazoo, Michigan, U.S.A.) was dissolved in phosphate buffer (10 mM, pH 7.3) containing bovine serum albumin (0.1%). Anti-TxB_2 antiserum was a gift from Dr L. Levine, Brandeis University, Waltham, Mass., U.S.A.. 9, 11,-dideoxy-9α, 11α-epoxymethano-prostaglandin $F_{2\alpha}$ (U44069, a thromboxane A_2 mimetic) obtained from Upjohn Co. was dissolved in ethanol and kept at -20°C. Adenosine-5-diphosphate sodium salt (ADP) (Sigma Chemical Co., Dorset, U.K.) were dissolved in 100 mM Tris-HCl, pH 7.35. L-adrenaline hydrochloride (ADR) (Sigma) was dissolved in 5% glucose in water. Stock solution of Quin2/AM (Lancaster Synthesis Ltd., Morecambe, U.K.) were made up at 10-50 mM in dry dimethylsulphoxide (DMSO) (Sigma) and were kept desiccated at -20°C between use. Flurbiprofen (Boots plc, Nottingham) was dissolved in distilled water.

Sepharose 2B was obtained from Sigma Chemical Co..

6.2 PREPARATION OF PLATELET RICH PLASMA (PRP) AND PLATELET POOR PLASMA (PPP)

Blood was collected by venepuncture from the anti-cubital vein of healthy human volunteers, who had not taken drugs that affect platelet function for at least 2 weeks. Blood was collected into plastic tubes containing 0.1 volume of 3.8% tri-sodium citrate (0.13 M). Platelet-rich plasma (PRP) was prepared by

centrifugation of the whole blood at 1000 g for 5 minutes at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation (3 minutes, 9000 g, 20°C) of an aliquot of platelet-rich plasma.

6.3 MEASUREMENT OF PLATELET AGGREGATION

Platelet aggregation was measured photometrically in 0.2 ml samples of PRP in a Malin Clinical Platelet Aggregation Recorder. The mean cell count was $3.7 \pm 0.3 \times 10^8$/ml. A typical biphasic aggregation response is shown in Figure 6.3. The parameters used to quantify the response were as follows:

1. H_1 - the maximum height of the primary aggregation response.
2. R_1 - the maximum rate of change in light transmission during primary aggregation.
3. H_T - the maximum total change in light transmission (both primary and secondary aggregation responses).

To provide a more quantitative estimation of platelet aggregation, the changes in optical density of platelet-rich plasma resulting from agonist addition were monitored using a Unicam SP 1800 Ultraviolet Spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.).

Briefly, aliquots (0.8 ml) of platelet-rich plasma were transferred into LP-3 tubes and incubated in a shaking water bath at 37°C for 5 minutes before the addition of agonists. At appropriate time following the addition of agonist or angiotensin II to platelets (e.g. adrenaline 5 minutes, ADP 1 minute, and U44069 1 minute), the sample was pipetted into a 1 cm square quartz cuvette and absorbance measured at 600 nm. Preliminary studies indicated that the optical density of PRP was proportional to the concentration of platelets in the

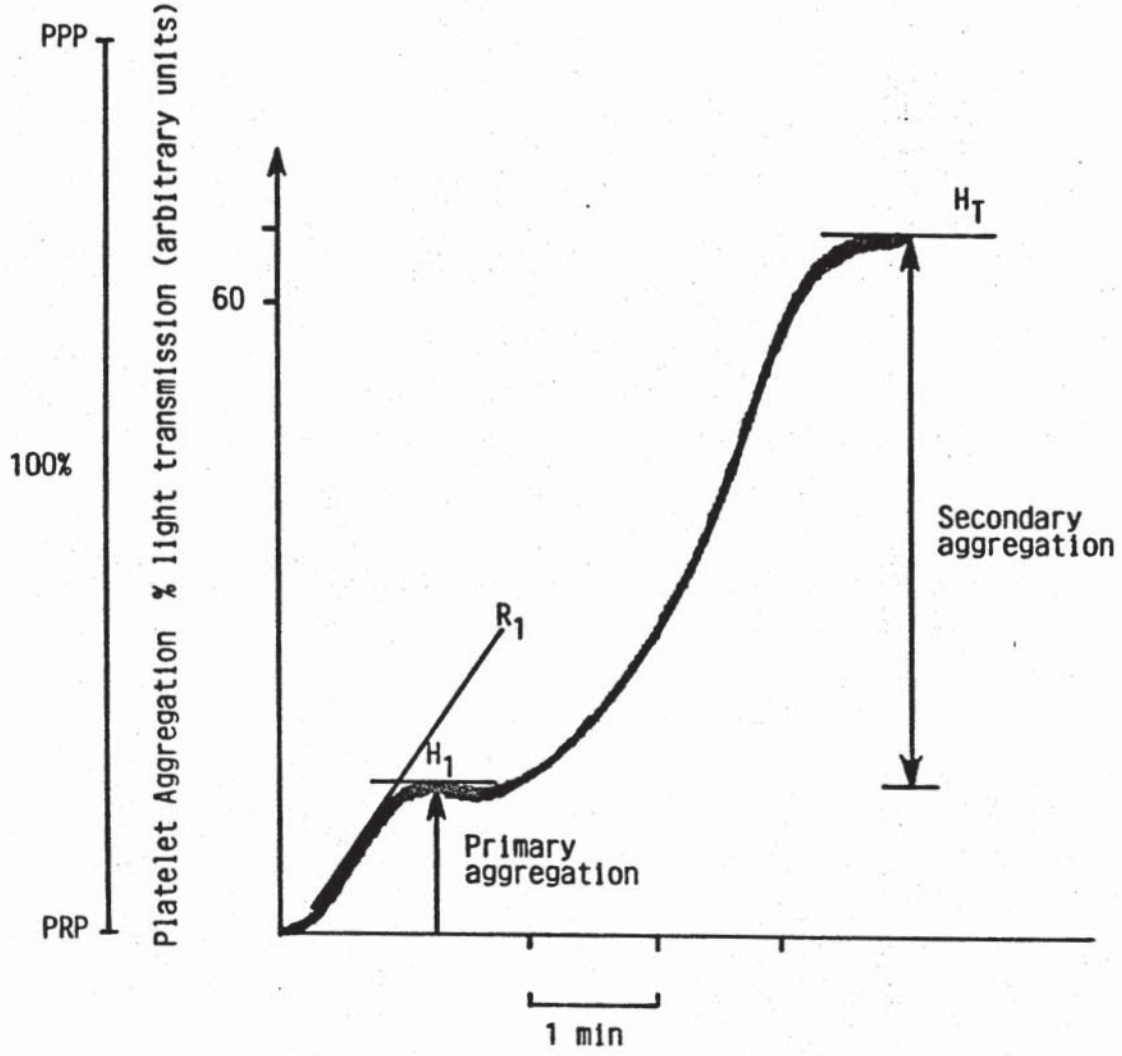

FIGURE 6.3 Schematic representation of adrenaline-induced platelet aggregation as monitored by aggregometry.

suspension. Readings were taken after addition of agonists with and without angiotensin II.

6.4 MEASUREMENT OF PLATELET CYTOSOLIC FREE Ca^{2+}

Platelet cytosolic free Ca^{2+} was monitored using fluorescent quinoline dye, Quin 2, which displays high affinity for Ca^{2+} and fluoresces as a result of Ca^{2+} binding (Tsien, Pozzan and Rink 1982). Platelet-rich plasma was incubated (37°C, 30 minutes) with Quin 2-acetoxy-methylester, which readily crosses the plasma membrane where it is rapidly hydrolysed by cytoplasmic esterases to the membrane impermeant polycarboxylate anion that is Quin 2. Quin 2 is thus trapped inside intact platelets and can report the resting concentration of cytosolic free Ca^{2+} and changes that result from exposure to agonists (Rink, Smith and Tsien 1982). Quin 2-labelled platelets were separated from plasma containing extraneous dye by gel-filtration on columns of Sepharose 2B equilibrated with modified Hepes-buffered Tyrodes solution (129 mM NaCl; 10.9 mM Na_3 Citrate; 8.9 mM Na HCO_3; 0.56 mM Dextrose; 5 mM Hepes; 2.8 mM KCl; 0.8 mM KH_2PO_4; 0.84 mM $MgCl_2$ and 0.35% bovine serum albumin, pH 7.4). The Quin 2 content of platelets was around 1 mmol/l of cells. Gel-filtered platelets were stored at 37°C in stoppered plastic tubes, and, to compensate for the chelation of Ca^{2+} by citrate, the external free calcium concentration was adjusted to 1 mM by addition of $CaCl_2$ immediately before use. Platelets (1 ml) were placed in 1 cm square quartz cuvettes at 37°C, and fluorescence was monitored in a Perkin Elmer spectrophotofluorimeter at 37°C with standard monochromator settings of 339 nm - excitation and 492 nm - emission.

6.5 MEASUREMENT OF THROMBOXANE B_2 PRODUCTION

TxB_2 production was measured by radioimmunoassay (MacIntyre and Shaw 1983). Five minutes after the addition of adrenaline and/or angiotensin II to the 0.2 ml PRP sample, reactions were terminated by the addition of 0.4 ml ice-cold EDTA (0.4%) in iso-osmotic saline containing flurbiprofen (cyclo-oxygenase inhibitior, 30 µM) and centrifuged (8000 g: 4°C: 3 minutes). Aliquots (0.1 ml) of cell-free supernatant was incubated with 0.1 ml anti-TxB_2 antiserum and 0.2 ml [^{3}H] TxB_2 (20 nCi) for 24 hours at 4°C. Free TxB_2 was absorbed on dextran-coated charcoal (0.5% charcoal: 0.5% dextran in 10 mM phosphate buffer, pH 7.3) and removed by centrifugation (8000 g: 4°C: 4 minutes). Aliquots (0.2 ml) of cell free supernatant were added to scintillation vials containing scintillation cocktail (Triton: Toluene: Scintol 2, 12.3: 6.7:1) for measurement. Bound [^{3}H]-TxB_2 was estimated in a Packard Tricarb 544 liquid scintillation counter, and the amounts of TxB_2 generated in the platelet samples were estimated from a standard curve comprising known amounts of TxB_2.

6.6 STATISTICAL ANALYSIS

Results are presented as the mean ± SE. Statistical significance was assessed by Student's paired 't' test.

6.7 RESULTS

Angiotensin II (10^{-11} - 10^{-7}M) has no direct effect on platelet aggregation ($n = 6$) nor did it affect cytosolic free Ca^{2+} concentration in Quin 2-labelled platelets either in the short- (< 2 minutes) or in the long-term (30 minutes).

Representative traces of Quin 2 experiments are shown in Figure 6.4. Low concentrations of angiotensin II (10^{-11} - 10^{-9}M) significantly enhanced the platelet aggregating effects of a sub-maximal dose of adrenaline, whereas a supraphysiological level of angiotensin II (10^{-7}M) was inhibitory (Figure 6.5).

Although there was inter-subject variability in responsiveness of platelets to adrenaline and angiotensin II, these effects were observed in platelets from each of six normal subjects (Figure 6.6) and appeared to be confined to the so-called 'secondary' wave of adrenaline-induced platelet aggregation (Table 6.1). This was confirmed by the lack of effect of angiotensin II on 'primary' aggregation induced by ADP (data not shown) and by experiments using the cyclo-oxygenase inhibitor, flurbiprofen. The 'secondary' aggregatory response to adrenaline is dependent upon endogenous TxA_2 formation and consequently is suppressed by flurbiprofen. Angiotensin at all concentrations had no effect on adrenaline-induced platelet aggregation when added in the presence of flurbiprofen (Table 6.1).

It is possible that the pro-aggregatory effects of angiotensin II are mediated either by an increase in TxA_2 synthesis or by a potentiation of the aggregatory effects of TxA_2. The first of these possibilities was tested by measuring thromboxane B_2 formation (the stable product of TxA_2 hydrolysis) in response to adrenaline and angiotensin II. Angiotensin II alone (10^{-11}M or 10^{-7}M) did not significantly affect thromboxane B_2 formation. Both concentrations of angiotensin II reduced thromboxane B_2 synthesis in adrenaline-treated platelets (Table 6.2). This inhibition would explain the anti-aggregatory effects of 10^{-7}M angiotensin II but contrasts with the stimulatory effects of 10^{-11}M angiotensin II.

The possibility that angiotensin II potentiates the actions of TxA_2 was investigated using the stable thromboxane A_2 mimetic, U44069. Angiotensin II

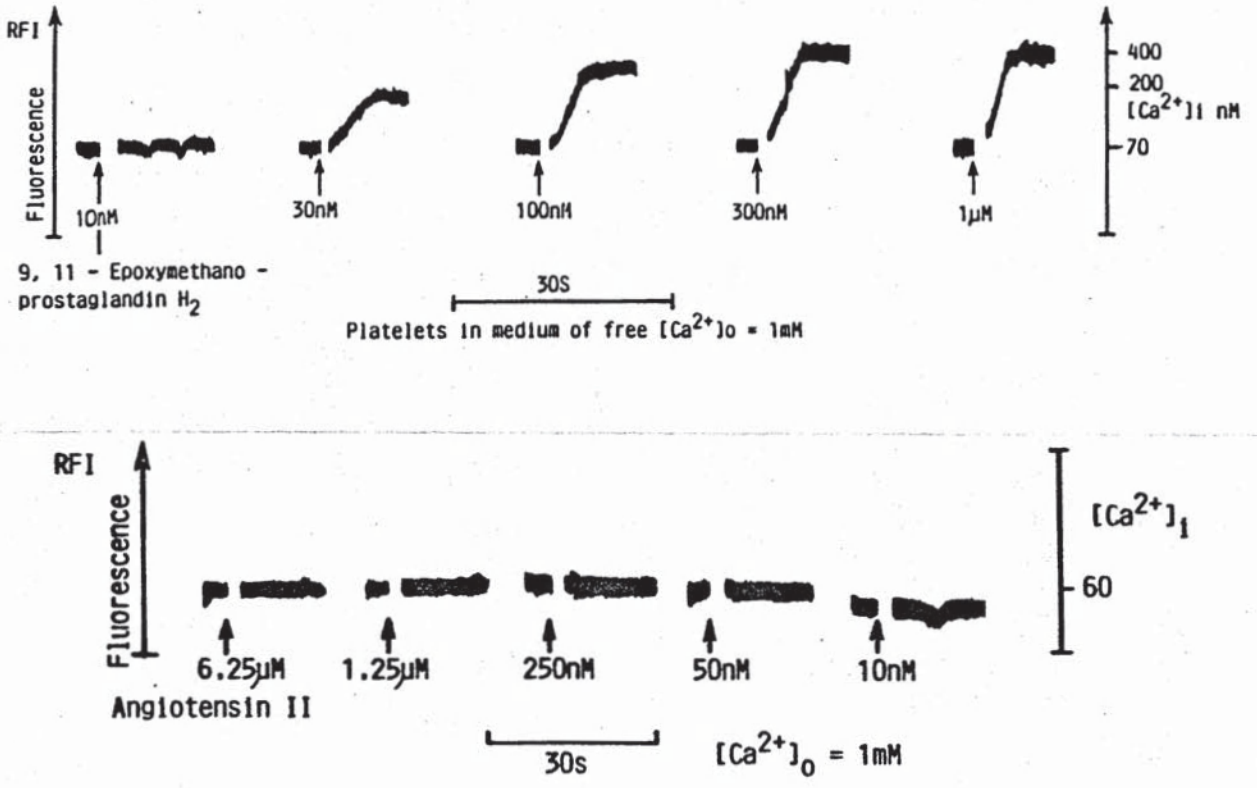

FIGURE 6.4 Upper panel: Effects of 9, 11-epoxymethano-prostaglandin H_2 on platelet $[Ca^{2+}]_i$. Lower panel: Effect of Ang II on platelet $[Ca^{2+}]_i$. Plasma-free suspensions of Quin 2-labelled platelets in a modified Hepes-buffered Tyrodes solution containing 1mM-external Ca^{2+} were prepared as described in the Methods section. Platelets were incubated with angiotensin II at the concentrations indicated or saline in control samples, and the fluorescence responses (excitation 339 nm, emission 492 nm) were monitored. The appropriate intracellular free Ca^{2+} calibration scale is shown on the right-hand side. The data shown are typical of six similar experiments using platelets from different donors.

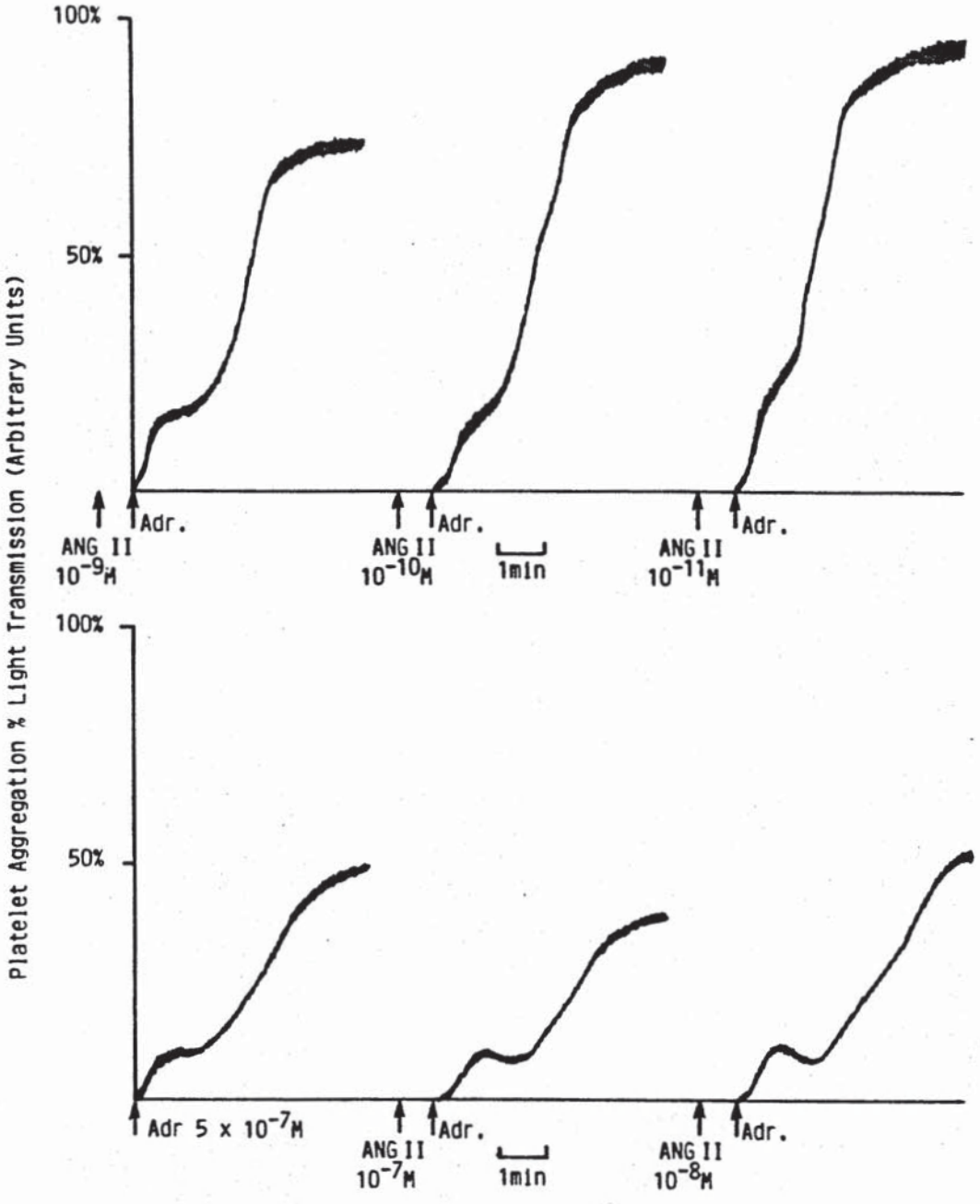

Figure 6.5 Typical angiotensin II effects on adrenaline-induced platelet aggregation. Adrenaline-induced platelet aggregation was monitored after pre-incubation with saline (control) or angiotensin II (at the concentrations indicated) for 1 minute before the addition of adrenaline (5×10^{-7}M).

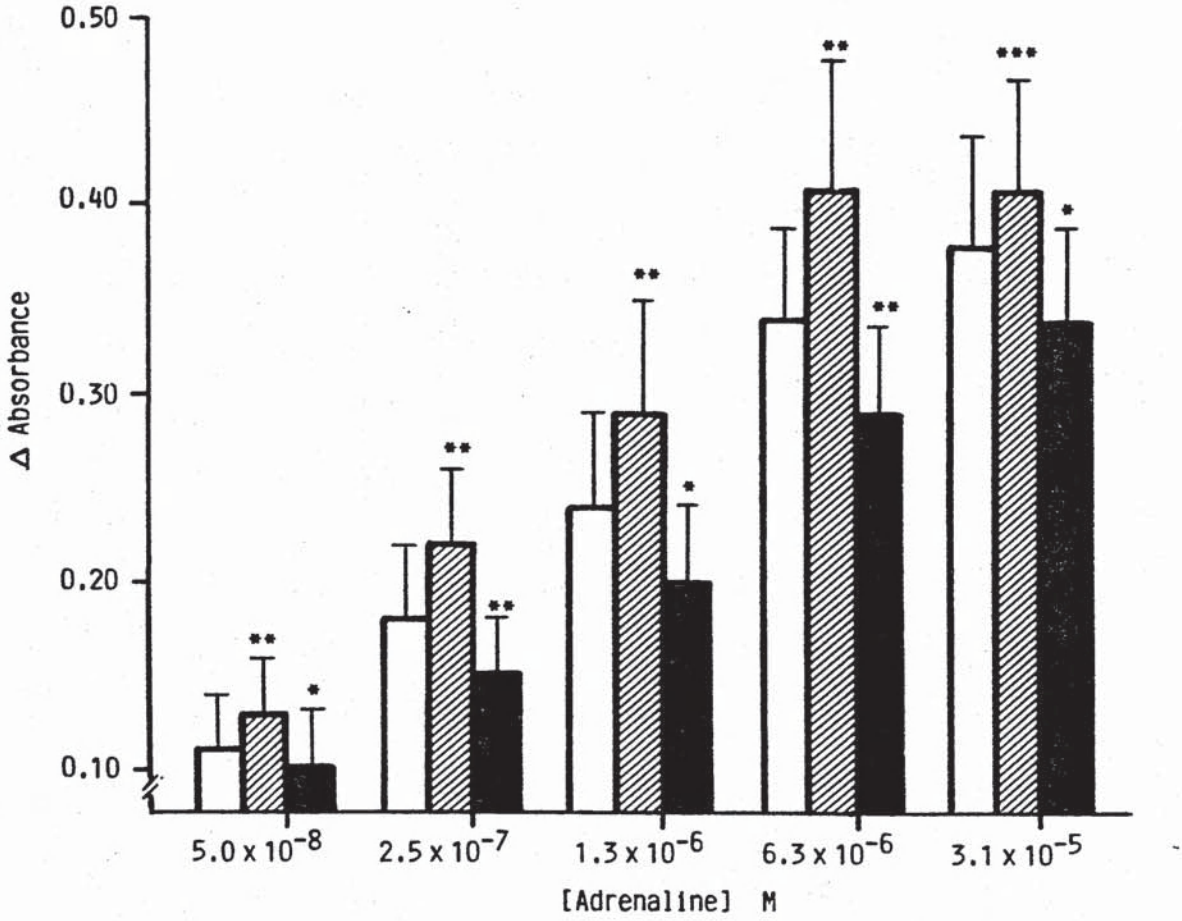

Figure 6.6 Effects of angiotensin II on adrenaline-induced platelet aggregation. PRP (0.8 ml aliquots) were incubated at 37°C for 5 minutes before addition of angiotensin II at the concentrations indicated or saline in control samples. One minute later adrenaline, at the concentrations indicated, was added and the resultant decrease in absorbance was monitored at 600 nm in a spectrophotometer.

□ : adrenaline

▨ : adrenaline plus angiotensin II 10^{-11}M

■ : adrenaline plus angiotensin II 10^{-7}M

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

TABLE 6.1

EFFECTS OF ANGIOTENSIN II ON ADRENALINE-INDUCED PLATELET AGGREGATION MEASURED BY AGGREGOMETRY

PRP alone		PLATELET AGGREGATION		
		H_1	R_1	H_T
	CONTROL	100	100	100
ANG II	10^{-11}M	104 ± 2	98 ± 3	139 ± 15*
	10^{-10}M	112 ± 4*	110 ± 5	137 ± 8*
	10^{-9}M	110 ± 4*	107 ± 3	116 ± 4*
	10^{-8}M	98 ± 5	87 ± 4*	94 ± 4
	10^{-7}M	97 ± 5	99 ± 5	87 ± 5*
PRP with Flurbiprofen				
	CONTROL	100	100	-
ANG II	10^{-11}M	100 ± 2	96 ± 3	-
	10^{-10}M	107 ± 3	107 ± 5	-
	10^{-9}M	97 ± 2	96 ± 2	-
	10^{-8}M	92 ± 4	93 ± 3	-
	10^{-7}M	95 ± 2	92 ± 4	-

Platelet aggregation parameters H_1, R_1 and H_T were measured in PRP in the presence and absence of flurbiprofen (30 µM). Submaximal concentrations of adrenaline (0.5 - 5 µM) were tested in the presence of saline (control) or angiotensin II at the concentrations indicated.

Results (mean ± SE, n=6) are expressed as a % of control values, * $p < 0.05$.

TABLE 6.2

EFFECTS OF ANGIOTENSIN II ON ADRENALINE-INDUCED THROMBOXANE B_2 FORMATION IN HUMAN PLATELETS

ADDITION	TxB_2 (pg/ml) (M ± SE, n=9)
Saline (control)	182 ± 39
adrenaline [f]	331 ± 45
Ang II 10^{-11}M	170 ± 39
Ang II 10^{-7}M	143 ± 27
Ang II 10^{-11}M + adrenaline	207 ± 41*
Ang II 10^{-7}M + adrenaline	140 ± 26**

[f] The concentration of adrenaline required to induce platelet aggregation ranged from 0.3-5 μM. Statistical significance of reduced TxB_2 synthesis in the presence of Ang II and adrenaline compared with adrenaline alone is denoted by asterisks: * $p < 0.05$, ** $p < 0.01$.

at 10^{-11} and 10^{-7}M enhanced the aggregatory effects of a range of concentrations of U44069 (Figure 6.7). The synergistic effects of Ang II and U44069 were equally apparent in flurbiprofen-treated platelets.

6.8 COMMENTS

Angiotensin II has various properties, including actions on arterial smooth muscle, aldosterone secretion and renal function. For angiotensin II the mechanism that links hormone receptor occupancy to cellular response is uncertain, but it may involve changes in the cytosolic concentration of ionised calcium $[Ca^{2+}]i$ (Capponi, Lew, Jornot and Vallotton 1984). Elevation of $[Ca^{2+}]i$ may be a key regulator of the responsiveness of numerous cell types, including vascular smooth muscle (Bolton 1979), adrenal glomerulosa cells (Williams, McDougall, Tait and Tait 1981), liver (Garrison, Borland, Florio and Twible 1979), kidney (Fray 1980) and platelets (Gerrard, Peterson and White 1981). One possibility is that angiotensin II binds to a receptor on the cell surface, leading to a change in plasma membrane structure, an increase in the permeability to calcium and/or release of Ca^{2+} from an intracellular binding sites (Foster and Rasmussen 1983).

The pathophysiological significance of platelet angiotensin II receptors and the nature of the transduction process to which they are coupled are unknown. Angiotensin II alone appears to have no direct effect on platelet aggregation, platelet $[Ca^{2+}]i$ or platelet arachidonate metabolism as monitored as thromboxane B_2 formation: observations which confirm and extend those of Stoff et al. (1980).

Although angiotensin II had no direct effect on platelet responsiveness, it significantly potentiated platelet responsiveness to adrenaline and to the

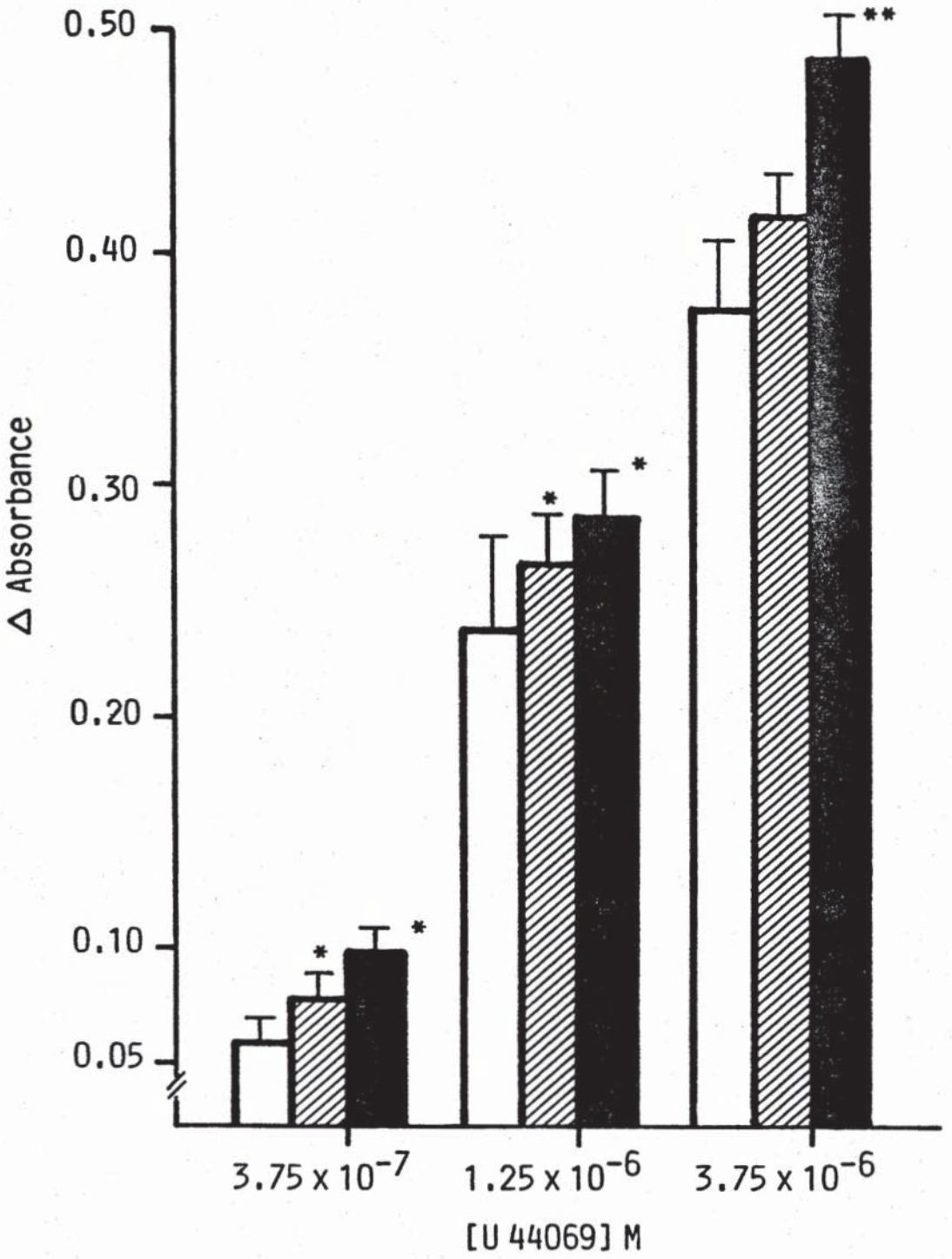

Figure 6.7 Effects of angiotensin II on U44069-induced platelet aggregation (see Figure 6.6 for method).

□ : U44069

▨ : U44069 plus angiotensin II 10^{-11}M

■ : U44069 plus angiotensin II 10^{-7}M

* $p < 0.05$, ** $p < 0.01$

thromboxane A_2 mimetic, U44069, but not to ADP. Angiotensin II potentiates of adrenaline-induced platelet activation was not associated with increased thromboxane B_2 formation, but was abolished by flurbiprofen, a cyclo-oxygenase inhibitor. Potentiation of U44069-induced platelet activation by angiotensin was evident both in the presence and absence of intact platelet cyclo-oxygenase. Angiotensin II seems to potentiate the actions rather than the synthesis of thromboxane A_2 (or agents that combine with thromboxane A_2 receptor). The mechanism underlying this potentiation is unknown, but is unrelated to effects of angiotensin II on $[Ca^{2+}]i$. Angiotensin II therefore seems to have two effects on platelet aggregation: (i) at physiological concentrations angiotensin potentiates the effects of TxA_2 and the 'secondary' response to adrenaline; (ii) at supraphysiological levels angiotensin inhibits thromboxane A_2 formation.

In conclusion, it appears that the angiotensin II receptor on platelet is functionally active since concentrations of angiotensin II close to the physiological range (10^{-11} - 10^{-10}M) facilitate platelet aggregation. Converting enzyme is predominantly located on the vascular endothelium and there is increasing evidence that local generation of angiotensin II in the vessel wall may be an important process. Concentrations of peptide in or near the vascular endothelium may be appreciably higher than in blood and there is the potential for an interaction of angiotensin and adrenaline with platelets at this site tending to promote thrombus formation.

CHAPTER 7 GENERAL DISCUSSION AND SPECULATION

Conventional radioligand binding methods have been used in animals to examine the properties of angiotensin receptors in a variety of classical target tissues such as adrenal gland (Douglas and Catt 1976), vascular smooth muscle (Gunther et al. 1980a), brain (Bennet and Snyder 1980 a & b) and kidney (Osborne, Droz, Meyer and Morel 1975). This approach has established that receptor affinity and concentration are associated with tissue sensitivity to the physiological actions of angiotensin II. Dietary sodium restriction increases receptor number and affinity in the adrenal gland, which in turn renders this tissue more responsive to the steroidogenic actions of angiotensin II (Aguilera et al. 1978). Conversely, during dietary sodium depletion decreases in receptor binding in vascular smooth muscle (Gunther et al. 1980b), diminish the vasopressor action of angiotensin II (Brown, Casals-Stenzel, Cumming, Davies, Fraser, Lever, Morton, Semple, Tree and Robertson 1979). Similar studies in man are difficult to perform because adrenal or vascular tissue connot readily be obtained. I have demonstrated angiotensin II receptors on human platelets and that the number of these receptors is affected by changes in dietary sodium intake; high salt intake increases and low salt intake decreases angiotensin II receptor density; a similar response to salt intake occurs in smooth muscle (Gunther et al. 1980b; Aguilera et al. 1978) and renal glomeruli (Bellucci and Wilkes 1984). Platelets are easily obtained and may be a convenient tissue to assess the physiological action of angiotensin II in man.

Circulating levels of angiotensin have been associated with increases or decreases in receptor density depending on the tissue under study. In the adrenal cortex, angiotensin II receptor concentration is up-regulated by sodium depletion associated with increases in circulating angiotensin II levels (Aguilera

et al. 1980). However, in vascular, uterine and bladder smooth muscle (Aguilera and Catt 1981), and the renal glomeruli (Beaufils et al. 1976; Bellucci and Wilkes 1984), sodium depletion is associated with decreases in receptor concentration. The mechanism for up-regulation of angiotensin II receptors by circulating hormone in one tissue and down-regulation in other tissues is not understood. Considerable progress has been made in elucidating the molecular basis of β-receptor desensitisation. Limas and Limas (1984) demonstrated that the recovery of cardiac β-receptors from isoproterenol-induced apparent loss (down-regulation) is due to reincorporation of the internalised receptors into the plasma membrane. Recovery of internalised cardiac β-receptors (receptosomes) does not depend on de novo protein synthesis but is an energy-dependent process, prevented by metabolic inhibitors. Several structures such as lysosomes, Golgi apparatus and microtubules are involved in this intracellular receptor recycling process. The integrity of the recovery phase of receptor cycling is very important for maintaining the normal complement of membrane-bound receptors, since the turn over rate of cardiac β-receptor is normally slow (Pitha, Hughes, Kusiak, Dax and Baker 1982). The alternative mechanism, de novo synthesis, may be utilised under pathological conditions. No comparable studies have been described for the angiotensin II receptor but the mechanism of up- and down-regulation may be similar. Platelets have no nucleus and retain only a modest capacity for protein synthesis. The down-regulation of angiotensin II receptors in response to sodium depletion may involve alterations in receptor recycling rather than alternations in receptor synthesis.

Measurements of cytosolic free Ca^{2+} in intact bovine adrenal glomerulosa cells with a fluorescent calcium indicator Quin-2 suggest that cytosolic Ca^{2+} is increased by angiotensin II and may mediate the effect of this peptide on aldosterone secretion (Capponi et al. 1984). The mechanism may involve opening

of receptor-operated calcium channels or release of calcium from intracellular stores through effects on phospholipid metabolism (Farese, Larson, Sabir and Gomez-Sanchez 1983). There are two factors which mediate the effects of the hormone on platelet aggregation. A rise in intracellular Ca^{2+} promotes platelet aggregation whereas a rise in cyclic-AMP inhibits platelet aggregation. I could detect no increase in $[Ca^{2+}]_i$ in platelets treated with angiotensin II but I have not measured the effect of angiotensin II on cyclic-AMP. Another possibility is that the Quin-2 method cannot detect small changes in intracellular free calcium necessary for a facilitatory effect of a hormone on platelet aggregation.

The identification of plasma angiotensin II as a regulator of platelet angiotensin II receptor provides a potential experimental approach to the study of angiotensin II receptor regulation in diseases such as juvenile diabetes mellitus, primary aldosteronism, renovascular hypertension and Bartter's syndrome.

The incidence of thrombo-embolic complication in patients with hypertensive cardiovascular disease is higher than in the normal population. Platelet aggregation is the prime event in thrombus formation, especially in diseased arteries. Under some circumstances there may be an interaction between angiotensin II and adrenaline adjacent to the blood vessel wall to promote platelet aggregation and thrombus formation.

APPENDIX 1

ANALYSIS OF RADIOLIGAND BINDING DATA

1) Determination of kinetic constants by the integrated rate equation methods.

Binding of ^{125}I-Ang II was measured as a function of time at two concentrations of ^{125}I-Ang II. For each concentration, the binding data was transformed as;

$$B_t \longrightarrow \ln (B_{eq}-B_t)/B_{eq}$$

where B_t = ^{125}I-Ang II bound at time t

B_{eq} = ^{125}I-Ang II bound at equilibrium

and the transformed data plotted against time. A straight line was fitted to each set of data by least-squares method and the slopes calculated (Figure 3.6). The forward and reverse rate constants were then obtained by solving the simultaneous equations;

$$S_1 = -(K_f \cdot [H]_1 + K_r)$$

$$S_2 = -(K_f \cdot [H]_2 + K_r)$$

where S_1, S_2 = slopes of the fitted lines

$[H]_1$, $[H]_2$ = ^{125}I-Ang II concentrations

K_f = forward rate constant

K_r = reverse rate constant

i.e. $K_f = (S_1-S_2)/([H]_2-[H]_1)$

$$K_r = (S_2 \cdot [H]_1 - S_1 \cdot [H]_2)/([H]_2-[H]_1)$$

The derivation of this method has been described by Gunther et al. (1980a).

2) Direct determination of the reverse rate constant.

Following incubation under standard conditions, the forward reaction was blocked by addition of excess unlabelled Ang II and dissociation of bound ^{125}I-Ang II measured as a function of time. The binding data was plotted as ln (B_t) against time and the slope (= $-K_r$) calculated by least-squares method (Figure 3.7).

3) Determination of equilibrium constants

Binding of ^{125}I-Ang II was measured as a function of ^{125}I-Ang II concentration under equilibrium conditions (saturation studies) and the binding data plotted as bound/free against bound according to the method of Scatchard (1949). A straight line was fitted by least-squares method and the dissociation equilibrium constant (= -1/slope) and Bmax (= abscissa intercept) determined (Figure 3.4). Alternatively, binding of ^{125}I-Ang II was measured as a function of unlabelled Ang II concentration (displacement studies) under equilibrium conditions and values of bound corrected for the reduction in specific activity of the tracer caused by addition of unlabelled Ang II. This yielded saturation curves which were analysed as described above (Figure 3.5).

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