CHARACTERISATION OF THE VASCULAR ANGIOTENSIN RECEPTOR

by

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Statement of Originality

All the experimental work reported in this thesis is my own.

Abbreviations

Abbreviations used are as recommended in the Biochemical Journal Instructions to Authors, 1983, with the following additions:

cAMP	adenosine 3′,5′ phosphate
cGMP	guanosine 3′,5′ phosphate
Captopril	1-[(2S)-3-mercapto-2-methyl-1-oxopropyl]-L-proline An orally-active inhibitor of angiotensin converting enzyme.
EDTA	ethylenediaminetetra-acetic acid
EGTA	1,2-Di(2-aminoethoxy)ethane-N,N,N′,N′-tetra-acetic acid
Gpp(NH)p	5′-guanylylimidodiphosphate
ITP	inosine 5′-triphosphate
K_d	dissociation equilibrium constant
PMSF	phenylmethylsulphonylfluoride
Sar	sarcosine, N-methylglycine
STI	soybean trypsin inhibitor
Tos-Phe-CH_2Cl	1-chloro-4-phenyl-3-L-toluene-p-sulphonamidobutan-2-one ('TPCK')
Trasylol	aprotinin

In the description of radio-iodine labelled compounds, the ^{125}I- prefix does not imply that all the compound was labelled.

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SUMMARY

Binding sites of high affinity and low capacity for ^{125}I-angiotensin II have been characterised in a cell membrane fraction derived from mesenteric arterial arcades of the rat, resistance-type vessels which are representative of the vascular target organs for angiotensin II. Degradation of tracer angiotensin II and heterogeneity of binding sites were accounted for by use of nonlinear regression methods for the analysis of radioligand binding data. Binding constants for ^{125}I-angiotensin II obtained by different experimental approaches were in good agreement and gave a dissociation equilibrium constant of 0.013-0.098nM (95% confidence interval). Affinities for a number of angiotensin-related peptides derived from competitive binding curves paralleled the pressor activities of these peptides with the order ^{125}I-angiotensin II = angiotensin II > angiotensin III > C-terminal hexapeptide > C-terminal pentapeptide >> angiotensin I = bradykinin. The iodinated peptide was found to retain full pressor activity in bioassay experiments. These binding sites therefore exhibit properties of a physiological receptor for angiotensin II.

The mechanism by which alteration of sodium balance brings about changes in pressor sensitivity to angiotensin II has been investigated by radioligand receptor assay. Sodium depletion (low salt diet + diuretic) caused a rapid increase in plasma angiotensin II concentration and a corresponding fall in receptor density from 102 ± 4 fmol/mg membrane protein (control) to 44 ± 5 fmol/mg protein at 12 days. Sodium loading and inhibition of angiotensin converting enzyme with captopril were equally effective in suppressing plasma angiotensin II concentration, but only in the captopril treated animals was an increase in receptor density observed (118 ± 6 fmol/mg protein at 12 days). Sodium loading caused a slow fall in

receptor density, not significant at 5 days, to 77 ± 4 fmol/mg protein at 12 days. This indicates that plasma angiotensin II concentration is not the sole determinant of vascular receptor status.

The results described above were obtained under standard assay conditions with a Tris buffer containing 4.8mM Ca^{++}. Using tissue from normal animals, the apparent receptor density was found to vary with the assay incubation medium calcium concentration over the range 0-10mM. Receptor densities of 50 ± 4, 102 ± 4 and 156 ± 5 fmol/mg membrane protein were obtained in low- (0mM), normal- (4.8mM) and high- (25mM) [Ca^{++}] assay respectively. After 2 days of sodium loading, sodium depletion and converting enzyme blockade, the receptor densities determined in high- and low-[Ca^{++}] assays did not differ significantly from those of control animals. Thus the altered receptor densities seen in normal-[Ca^{++}] assay were due to shifts in the [Ca^{++}]-receptor density relationship without apparent change in the total number of receptors. Similar results were obtained for the 12 day sodium loading and converting enzyme blockade experiments. In contrast, after 12 days of sodium depletion there was no difference in receptor density between normal- and high-[Ca^{++}] assays (49 ± 7 fmol/mg) and the value obtained in low-[Ca^{++}] assay was significantly below that for all the other groups.

These findings indicate two stages in the regulation of the vascular angiotensin receptor; an initial masking or inactivation of binding sites through a mechanism which is reversed by calcium, and (with elevation of plasma angiotensin II concentration) a subsequent loss of receptors. The factor responsible for the differing effects of converting enzyme blockade and sodium loading on receptor status appears to modulate this receptor masking/inactivating process. A mechanism by which this process operates is proposed.

CHAPTER 1

INTRODUCTION

1.1 The renin-angiotensin system

The renin-angiotensin system is illustrated diagramatically in Figure 1.1 and is described briefly below. A detailed account may be found in the reviews by Peach (1977) and Leckie & Semple (1983).

(a) Renin

The aspartate proteinase renin is synthesised and stored in the juxtaglomerular cells of the renal afferent arteriole, and is secreted into the bloodstream in response to a variety of stimulii. The enzyme is highly substrate specific, cleaving one leucyl-leucine or leucyl-valine peptide bond within a defined octapeptide sequence, and acts on its in vivo substrate (angiotensinogen, an $alpha_2$ globulin of hepatic origin) to produce the decapeptide angiotensin I. The rate of renin release is controlled neurogenically through the extensive sympathetic innervation of the juxtaglomerular apparatus, by stretch receptors in the wall of the afferent arteriole, and by the macula densa, specialised cells in the thick ascending limb of Henle's loop. Renin release is also influenced by humoral factors, including plasma angiotensin II which acts in a negative feedback manner. Control of renin release has been reviewed by Davis & Freeman (1976) and Churchill (1985).

(b) Angiotensin I

Angiotensin I is the precursor of angiotensin II and has very little activity in pressor assays (Helmer, 1955) and on isolated smooth muscle (Bumpus et al, 1961; Campbell et al, 1977a). It also has little intrinsic activity in the adrenal cortex (Mendelsohn & Kachel, 1980) and central nervous system (Severs et al, 1973; Sirois & Gagnon, 1975; Chiaraviglio, 1976; Casner et al, 1976). Earlier suggestions that angiotensin I may act directly in certain evolutionally primitive

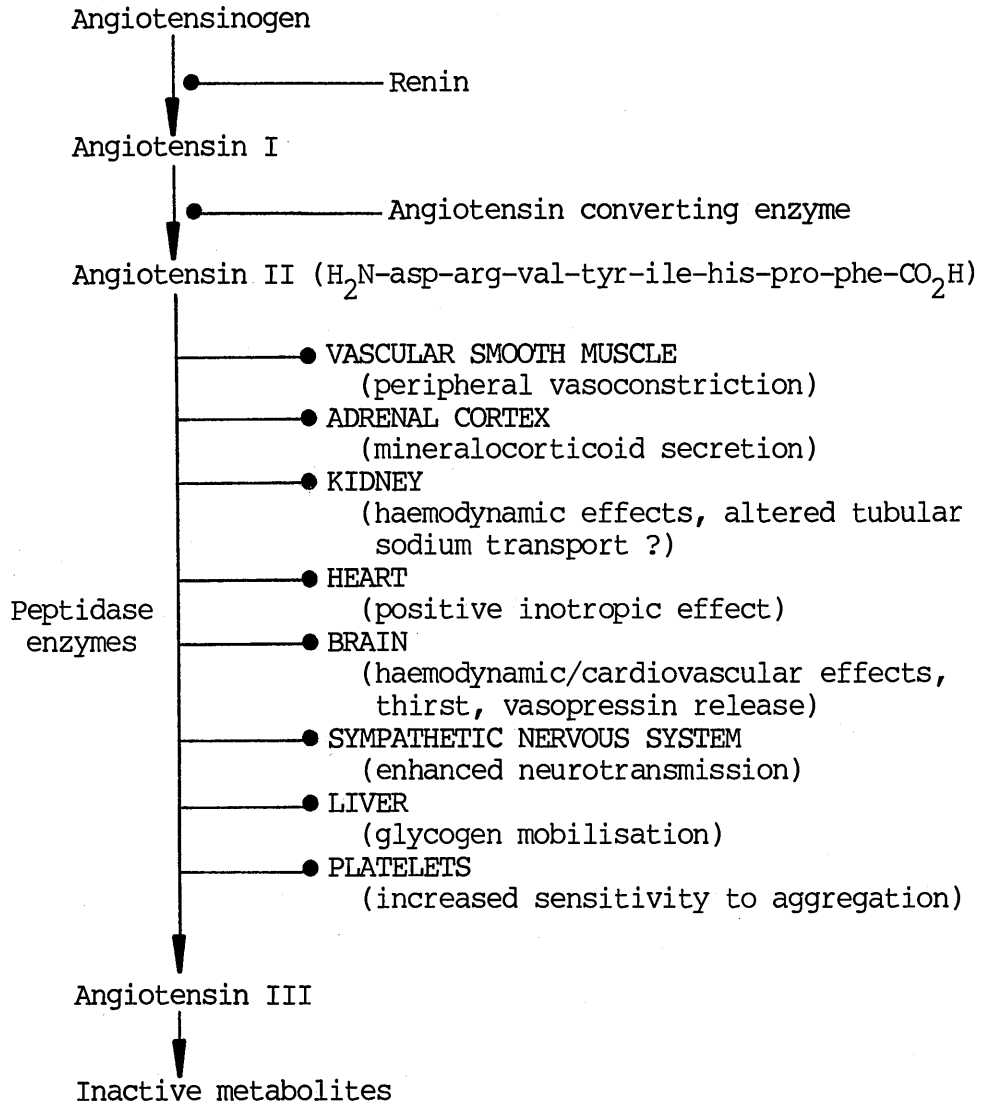

Figure 1.1 **Schematic representation of the renin-angiotensin system, indicating the main actions of angiotensin II.**

structures within the brain, kidney and sympathetic nervous system (Peach, 1977) have not been supported and at present it seems unlikely that angiotensin I has any important intrinsic activity.

(c) Angiotensin Converting Enzyme

Angiotensin converting enzyme is a zinc containing peptidyl-dipeptide hydrolase that splits the C-terminal histidyl-leucine (or serine-leucine in certain species) from angiotensin I, generating the active octapeptide angiotensin II. Converting enzyme also inactivates the vasodilator peptide bradykinin. The main site of conversion is the vascular endothelium, particularly in the pulmonary circulation. Converting enzyme is not rate-limiting in the formation of angiotensin II, and there is a close correlation between plasma renin and angiotensin II concentrations (Morton et al, 1976). A number of potent inhibitors of converting enzyme are known, originally peptides from the venom of Bothrops jararaca and subsequently synthetic agents such as captopril. These have been used extensively in studies on the renin-angiotensin system, and the orally-active inhibitors have found considerable clinical application in the treatment of various forms of hypertension. Angiotensin converting enzyme has been reviewed by Oparil (1977) and Cushman et al (1981).

(d) Angiotensin II

The actions of angiotensin II will be considered in greater detail in Sections 1.2-1.8 with the receptors in each target tissue, but are summarised here.

(1) Blood pressure. Angiotensin II raises blood pressure by a direct vasoconstrictor effect, acting on small muscular vessels mainly in the cutaneous, splanchnic (Bohr & Uchida, 1967; Krasney, 1968; Forsyth et al, 1971) and renal (Hollenberg et al, 1972) vascular beds. It may also increase cardiac output through a positive inotropic effect (Freer et al,

1976; Ackerly et al, 1977b; Trachte & Lefer, 1979). Prolonged infusion of angiotensin II at a rate below the threshold for an immediate pressor response also increases blood pressure; the mechanism of this slow pressor effect is unknown, but may involve baroreceptor resetting or an interaction with the sympathetic nervous system (Ames et al, 1965; Bean et al, 1979; Brown et al, 1981).

(2) Brain. Circulating angiotensin II gains access to the hypothalamic areas of the brain where the blood-brain barrier is deficient (Van Houtten et al, 1980), and stimulates thirst (Epstein et al, 1970; Ramsay et al, 1979) and ADH release (Bonjour & Malvin, 1970; Sladek & Joynt, 1980). It also increases central sympathetic discharge and facilitates sympathetic neurotransmission (review, Westfall, 1977). The pressor response to both centrally administered and (in part) blood-borne angiotensin II is probably mediated by the sympathetic nervous system (Falcon et al, 1978). There is also evidence that a separate renin-angiotensin system exists within the brain (review, Reid, 1977; Phillips et al, 1979; Printz et al, 1982).

(3) Kidney. Angiotensin II decreases renal blood flow and glomerular filtration rate but increases filtration fraction (Navar & Langford, 1974; Davalos et al, 1978), suggesting a predominant locus of action on the post-glomerular efferent arteriole. However, autoradiographic studies have shown radio-labelled angiotensin II to localise mainly in the glomeruli, probably in cells of the mesangium (Osborne et al, 1975), and isolated glomerular capillary tufts will contract in response to physiological concentrations of angiotensin II (Sraer et al, 1974). Angiotensin II also causes sodium retention by a direct action on the kidney (Malvin & Vander, 1967; Lohmeier et al, 1980). This may be due to altered glomerular haemodynamics (Myers et al, 1975), but there is evidence for a direct effect on tubular sodium transport (Steven, 1974; Harris & Young, 1977).

As noted previously, angiotensin II is a potent inhibitor of renin release. The intrarenal effects of angiotensin II have been reviewed by Levens et al (1981).

(4) Adrenal cortex. Angiotensin II is a potent stimulus to aldosterone secretion and is believed to be the main regulator of adrenocortical steroidogenesis following changes in sodium balance (reviews, Brown et al, 1977, 1979; Fraser et al, 1979). It also stimulates glucocorticoid release from the zona fasciculata (Ames et al, 1965; Kaplan, 1965; Bravo et al, 1975a), but this effect is secondary (possibly vestigial) to ACTH (Vallotton et al, 1981).

(5) Other tissues. In addition to its major effects described above relating to cardiovascular regulation and electrolyte homeostasis, angiotensin II stimulates glycogen mobilisation in liver (Keppens & DeWulf, 1976) and increases the sensitivity of platelets to aggregation by epinephrine, ADP and thrombin (Poplowski, 1970; Ding et al, 1985b). Intestinal and uterine smooth muscle will contract in response to high concentrations of angiotensin II (Meyer et al, 1970; Turker et al, 1971; Papadimitriou & Worcel, 1974), but it is doubtful if this action has any physiological relevance.

(e) Metabolism of angiotensin II

In most species, the concentration of angiotensin II in plasma is in the range 5-200pM (Boucher et al, 1977). The peptide has a half-life in the circulation of 15-60 seconds (Cain et al, 1970; Brooks et al, 1977), and is degraded largely by aminopeptidases (Peach, 1977). No specific degradative enzyme has yet been identified, but there is evidence for alteration in metabolism of angiotensin II with changes in sodium balance (Leary & Ledingham, 1970). Only the C-terminal heptapeptide (des asp^1 angiotensin II, angiotensin III) has significant biological activity. Angiotensin III may be formed directly from angiotensin II or by the

formation of des asp^{1} angiotensin I which is a substrate for the converting enzyme (Garcia del Rio et al, 1981). The plasma concentration of angiotensin III is low in relation to angiotensin II (Semple & Morton, 1976; Semple et al, 1976,1978). Although it has been established that the heptapeptide has approximately 30% of the pressor activity of angiotensin II (Schwyzer, 1963; Carey et al, 1978), estimates of its steroidogenic potency have varied widely, and it has been suggested that angiotensin III is the prime stimulus to aldosterone production (Freeman et al, 1976; Campbell & Pettinger, 1976). However, it now appears that the angiotensin receptors in the zona glomerulosa are similar to those in vascular smooth muscle and respond primarily to angiotensin II (Mendelsohn & Kachel, 1980). Angiotensin III also suppresses renin release, but again is less potent than angiotensin II (Naftilan & Oparil, 1978).

(f) Structure-activity relationships

The structure-activity relationships for angiotensin II have been reviewed by Khosla et al (1974) and Bumpus (1977). The C-terminal pentapeptide (especially the tyrosyl^{4}, histidyl^{6} and phenylalanine^{8} residues) is essential for biological activity. The guanido group of position 2 is also important, as is the carboxyl group of position 8; deletion of the latter causes almost total loss of biological activity. The low activity of angiotensin I may be due to the separation of the C-terminal carboxyl group from the phenylalanine residue. Species variation occurs at position 5; in man, horse, pig, rat and probably rabbit and dog isoleucine is present at this position, while in ox, fowl and sheep valine is found. Douglas et al (1979) have suggested that isoleucine in position 5 is necessary in maintaining correspondance between receptor affinity and steroidogenic potency in species where this is the natural form of angiotensin II.

Smeby et al (1962) have proposed that angiotensin II may form an alpha helix with both aromatic rings, the imidazole ring of histidine and the C-terminal carboxyl group on one side. A variety of possible conformations have been proposed subsequently (Printz et al, 1972; Marshall et al, 1974; Fermanjian et al, 1976; Fromageot et al, 1976) with some controversy concerning the use of organic solvents in these studies. It appears that angiotensin II has a cross B-pleat structure in polar organic solvents, but is helical or subhelical in mixed aqueous/organic solvents and has little or no tertiary structure in aqueous solution.

(g) Inhibitory analogues

The phenyl group of position 8 is essential for normal agonist activity, and substitution of phenylalanine by alanine, threonine or isoleucine yields potent angiotensin II antagonists (Saltman et al, 1976; Bumpus, 1977). The combination of alanine in position 8 and sarcosine in position 1 (Fessler et al, 1972) resulted in the first pharmacologically useful antagonist, saralasin. The sarcosine^1 substitution increases the half-life of the peptide in the circulation (Hall et al, 1974; Pettinger et al, 1975) but also increases the affinity of the peptide for the receptor (Saltman et al, 1975). Substitutions which introduce a net positive charge at the N-terminus enhance the potency of the analogue while those introducing a negative charge decrease potency (Rioux et al, 1973; Khosla et al, 1974). However, the affinity of the sarcosine^1 antagonists for the vascular (and probably other) receptors is low in comparison to angiotensin II, since extremely high concentrations of antagonist (relative to angiotensin II) are required to inhibit pressor response to angiotensin II (Pettinger et al, 1975).

1.2 **Vascular angiotensin receptors**

(a) Vascular smooth muscle

Early studies on the vascular angiotensin II receptors used aorta from various species (Lin & Goodfriend, 1970; Lin et al, 1970; Baudouin et al, 1971; Baudouin et al, 1972; Devynck et al, 1973; LeMorvan & Palaic, 1975; Devynck & Meyer, 1976). Consistent with the lack of physiological action of angiotensin in this tissue, the affinity of the binding sites for angiotensin II is low in relation to normal plasma concentrations of the hormone, with reported K_d values of 10-50nM. In other respects however, these binding sites show properties expected of a physiological receptor; they are located on the plasma membrane and discriminate between angiotensin peptides in a manner generally paralleling the pharmacological activities of these peptides. Aortic muscle will contract in response to high concentrations of angiotensin II, with ED_{50} values for contraction in the same range as the K_d values (Meyer et al, 1970; Papadimitriou & Worcel, 1974; Regoli et al, 1974), indicating that these receptors are functional if insufficiently sensitive to respond to physiological concentrations of the hormone. However, there are many differences between aorta and the muscular resistance vessels in which angiotensin II acts. Whereas the pressor response to angiotensin II in intact animals exhibits marked tachyphylaxis on repeated or continuous administration of the hormone (Stewart, 1974), aortic muscle from various species will respond persistently (Khairallah et al, 1966; Altura & Altura, 1970; Palaic & LeMorvan, 1971), suggesting that the aortic receptors are not subject to the same regulatory processes as those in resistance vessels. Other differences between aorta and resistance vessels have been discussed by Devynck & Meyer (1976).

Bohr & Uchida (1967) found small mesenteric arteries to be highly sensitive to angiotensin II and to be representative of resistance-type

vessels from other vascular beds. These vessels also exhibit tachyphylaxis to angiotensin II, both as isolated muscle strips and as a perfused preparation (Collis & Alps, 1975). Recently, Gunther et al (1980a) have investigated binding of ^{125}I-angiotensin II to a crude membrane fraction derived from mesenteric vessels of the rat. They observed a single class of saturable binding sites with a K_d of 1nM and which displayed the specificity for angiotensin peptides expected of a physiological receptor. Using the methods of Gunther et al (1980a) to investigate the regulation of these receptors, Aguilera & Catt (1981) and Schiffrin et al (1983c) obtained values of affinity and concentration agreeing closely with those of Gunther et al (1980a). Paller et al (1984) have reported a similar value for the affinity but a considerably higher concentration of binding sites (300 vs 80 fmol/mg membrane protein) in this tissue.

(b) Non-vascular Smooth Muscle

Uterine muscle has frequently been used as a model for vascular smooth muscle (Lin et al, 1970; Meyer et al, 1974; Papadimitriou & Worcel, 1974; Chevillotte et al, 1975; Rouzaire-Dubois et al, 1975; Devynck et al, 1976; Moore et al, 1976; Devynck et al, 1979). The angiotensin II binding sites in this tissue resemble those described in aorta, with reported K_d values and ED_{50} values for contraction in the range 4-20nM. Only one study has directly compared angiotensin II binding in membrane fractions from rat uterus and mesenteric artery; Paller et al (1984) observed a four-fold lower affinity (K_d of 3.8 vs 0.9nM) in the former tissue. The similarities between uterine and vascular muscle have been discussed by Douglas & Brown (1982) and Paller et al (1984). In addition to apparent regulation by plasma angiotensin II concentration, the density of angiotensin binding sites in the myometrium is markedly influenced by the changing oestrogen and progesterone levels during the ovarian cycle (Schirar et al, 1980a,b). Despite this, Paller et al (1984) observed qualitatively similar changes

in angiotensin II binding in uterine and mesenteric artery preparations in response to potassium depletion. In contrast, Aguilera & Catt (1981) found both the characteristics and regulation of angiotensin II binding sites to be identical in rat mesenteric artery and urinary bladder smooth muscle.

(c) Biochemical Characteristics of the Vascular Receptors

Most studies to date have concentrated on measurements of binding constants for angiotensin II and its analogues and changes in the binding constants under various physiological conditions, and relatively little is known about the receptors at the biochemical level. In aorta, binding of angiotensin II is markedly reduced by proteolysis, neuraminidase treatment and sulphydryl reducing agents, and is also inhibited by ATP and GTP, divalent cations, and to a small extent by potassium. Sodium has no effect in this tissue (Devynck & Meyer, 1976). In the rat mesenteric artery preparation, trypsin treatment and sulphydryl reagents are also inhibitory, but divalent cations (Ca^{++}, Mg^{++}, Mn^{++}) markedly enhance binding of angiotensin II (Gunther et al, 1980a). Fleisch et al (1973) have reported N-ethyl maleimide and dithiothreitol to specifically block angiotensin II induced contractions in aortic muscle. Magnesium and manganese also inhibit muscle response to angiotensin II in vitro (Bohr, 1974); this has been ascribed to competition for calcium binding sites. Sodium and divalent cations have been reported to increase binding of angiotensin II in rat mesenteric artery membranes through an increase in receptor affinity which is antagonised by GTP, while lithium and (to a much lesser extent) potassium were inhibitory (Wright et al, 1982). These authors have proposed the existence of a cation binding site closely related to the receptor.

Attempts to isolate the receptor have been hindered by its low affinity in detergent solubilised preparations (Devynck et al, 1974; Forget & Heisler, 1979). Solubilisation of the rabbit aortic receptor with

sodium deoxycholate has been described (Devynck et al, 1974) but with altered peptide specificity and 10-fold lower binding capacity than in intact membranes. Capponi & Catt (1980) have used photoaffinity labelling to compare the physicochemical characteristics of dog adrenocortical and uterine angiotensin receptors; the results for both tissues were identical and showed the receptor to be a dimeric protein with similarly sized subunits of M_r 68000.

(d) Regulation of the vascular receptors

It has been recognised that pressor responsiveness to exogenously administered angiotensin II is altered in a variety of physiological and pathological conditions (Bartter et al, 1962; Chesley et al, 1963; Laragh et al, 1963; Kuchel et al, 1964; Healey et al, 1964; Kaplan & Silah, 1964a,b; Reid & Laragh, 1965; Leary & Ledingham, 1970; Strouder & Walthen, 1972; Hollenberg et al, 1972; Swales et al, 1975; Finchman et al, 1976). Changes in sodium balance have a profound effect on the renin-angiotensin system and have been extensively studied. In most circumstances, there is an inverse relationship between pressor sensitivity to the hormone and endogenous plasma levels of angiotensin II (or renin). Analogous changes in vascular sensitivity to angiotensin II have been demonstrated with perfused tissues (Strewler et al, 1972; Collis & Alps, 1975) and isolated aortic muscle (Strewler et al, 1972: Sybertz & Peach, 1980) taken from animals after activation of the renin-angiotensin system. Variation in angiotensin II vascular responsiveness is not associated with any change in sensitivity to norepinephrine (Thurston & Laragh, 1975; Oliver & Cannon, 1978) and is not mediated by bradykinin or prostaglandins (Oliver & Cannon, 1978). Changes in plasma sodium or potassium concentration within their respective physiological ranges also have no direct effect on angiotensin II responsiveness (Cowley & Lohmeier, 1978). It has been suggested that plasma angiotensin II concentration is the main determinant

of sensitivity to angiotensin II (Thurston & Laragh, 1975; Devynck & Meyer, 1976; Oliver & Cannon, 1978; Cowley & Lohmeier, 1978; Dawson-Hughes et al, 1981). Other studies have shown that converting enzyme inhibition blocks the change in angiotensin II vascular sensitivity associated with dietary sodium deprivation but not that with sodium loading or nephrectomy (Thurston & Laragh, 1975; Oliver & Cannon, 1978).

It has been proposed that changes in angiotensin receptor affinity (Brunner et al, 1972) or that "prior occupancy" of the receptors by endogenous hormone (Davis et al, 1962; Thurston & Laragh, 1975; Oliver & Cannon ,1978) could account for the variation in pressor sensitivity to angiotensin II. However, radioligand binding studies have shown the concentration, but not the affinity, of angiotensin II binding sites to vary in aortic (Williams et al, 1976) and uterine (Chevillotte et al, 1975; Devynck et al, 1976; Hauger et al, 1977; Devynck et al, 1979) muscle following alteration of plasma angiotensin II concentration.

These findings have been confirmed in the rat mesenteric artery preparation. Gunther et al (1980b) observed a decrease in the number of angiotensin receptors in this tissue in response to dietary sodium restriction that was reversed by administration of a converting enzyme inhibitor, and Aguilera & Catt (1981) demonstrated an inverse relationship between plasma angiotensin II concentration and receptor number. It would therefore appear that, as for many other hormones (reviews, Baxter & Funder, 1979; Catt et al, 1979), angiotensin receptor concentration is regulated by the prevailing plasma concentration of the hormone.

Changes in dietary potassium intake also influence the renin-angiotensin system; high potassium intake is associated with an enhanced pressor response to angiotensin II (Hollenberg et al, 1975; Douglas & Litowitz, 1978) while low potassium intake diminishes sensitivity (Linas & Dickman, 1982; Paller et al, 1984). Since plasma renin and angiotensin

concentrations are inversely related to potassium intake (Brunner et al, 1970; Sealey et al, 1970), these changes in responsiveness to the hormone might be ascribed to the same regulatory processes active during alteration of sodium balance. However, there is evidence that sodium and potassium depletion blunt vascular sensitivity to angiotensin II by independent mechanisms (Adamick et al, 1981). Also, Douglas (1979) found potassium loading to increase the affinity and decrease the concentration of angiotensin II binding sites in rat uterus, while Paller et al (1984) observed a decreased affinity and increased receptor concentration in response to potassium deficiency in rat uterus and mesenteric artery. Changes in affinity have not been reported in any muscle tissue in response to alteration of sodium balance, but Douglas & Brown (1982) found a substantial increase in affinity of uterine angiotensin II receptors following prolonged low-dose infusion of the hormone.

Schiffrin et al (1983c) have proposed that mineralocorticoids regulate the vascular angiotensin receptors; they found the binding properties of the mesenteric artery angiotensin receptors in two-kidney one-clip Goldblatt hypertensive rats to be identical to those of control animals despite considerably higher plasma renin activities in the hypertensive animals, although the capacity to down-regulate the vascular receptors in response to sodium depletion was preserved. Plasma aldosterone concentration was markedly elevated in the hypertensive animals. McGregor & Smirk (1968), Finch & Haeusler (1974) and Collis & Alps (1975) have reported a normal or increased responsiveness of the rat mesenteric vasculature to angiotensin II in renin-dependent renal hypertension, and Morton & Wallace (1983) have shown no change in net vascular responsiveness to the hormone in this model, although angiotensin II does cause the hypertension through a chronic mechanism (Wallace & Morton, 1984). A normal or enhanced response in human renovascular

hypertension has been described by Brown et al (1976). Schiffrin et al (1983c) also observed a higher receptor density in DOC-salt hypertensive animals than salt-loaded controls although plasma renin concentration was similarly suppressed in both groups. The authors concluded that angiotensin II given acutely induces down-regulation of its vascular receptors, but that chronic elevation of plasma aldosterone concentration (such as occurs in the two-kidney one-clip renovascular model) or administration of a mineralocorticoid will up-regulate the receptors, possibly countering the effect of an elevated plasma angiotensin II concentration. While low pressor dose infusions of angiotensin II decrease the number of vascular receptors (Aguilera & Catt, 1981; Schiffrin et al, 1983b), high pressor doses leave the receptor density normal or increased (Schiffrin et al, 1983b). Aldosterone infusion has been reported to up-regulate the vascular (Schiffrin et al, 1983a) and uterine (Brown & Douglas, 1982) receptors, and Schiffrin et al (1983a) have demonstrated a comparable effect in vitro with cultured vascular smooth muscle cells.

(e) Angiotensin tachyphylaxis

Repeated or continuous administration of angiotensin II results in specific desensitisation (tachyphylaxis) to the hormone, both in pressor assays in vivo and muscle contractile assays in vitro (Bohr, 1974; Stewart, 1974). The relationship between tachyphylaxis and receptor regulation is at present unclear. As in vascular muscle, the positive charges of the arginyl$_2$ guanido group and the N-terminal amino group are essential for full angiotensin II activity in guinea pig ileum and rat uterine muscle (Paiva & Paiva, 1960), and for the manifestation of angiotensin II tachyphylaxis (Paiva et al, 1974; Paiva & Paiva, 1975; Miasiro et al, 1983); angiotensin III does not induce tachyphylaxis. Freer & Stewart (1972) and Paiva et al (1977) found that angiotensin tachyphylaxis was accentuated in these tissues when the calcium

concentration of the incubation medium was reduced, and Paiva et al (1977) have proposed that tachyphylaxis results from tight binding of angiotensin II in association with superficial calcium binding sites, and that recovery from tachyphylaxis involves displacement of the hormone by calcium. Rabbit aorta becomes tachyphylactic to sar^1 angiotensin II but not to angiotensin II (Moore & Khairallah, 1976) and sar^1 angiotensin II induced contractions in this tissue are markedly inhibited by slow calcium channel antagonists (verapamil and SKF-525A) and calcium-free media, while responses to angiotensin III are relatively unaffected; responses to angiotensin II are affected to an intermediate extent (Ackerly et al, 1977a). Freer (1975) found that tissues which exhibit tachyphylaxis to angiotensin II show a marked reduction in response to the peptide in calcium-free media.

These findings indicate that tachyphylaxis is a function of the N-terminus of the octapeptide, and that calcium can antagonise the tachyphylactic mechanism. Ackerly et al (1977a) have inferred from the differing effects of Ca^{++}-free media on responses to angiotensins II & III that these peptides act via different mechanisms. However, in muscle and other tissues, direct binding studies have not provided any evidence for separate receptors for these peptides. The observations of Freer (1975) and Ackerly et al (1977a) are more consistent with loss of Ca^{++}-antagonism of tachyphylaxis in Ca^{++}-free media, which will only affect peptides which induce tachyphylaxis. The effect of calcium channel blocking drugs implies that Ca^{++} acts at an intracellular site. It is also apparent that tachyphylaxis occurs to a differing extent in different muscle tissues; this also applies to vascular smooth muscle from different vascular beds (Bohr & Uchida, 1967). Since it seems unlikely that a different angiotensin receptor subtype is present in each tissue, tachyphylaxis may be dependent on a receptor-related factor, the activity of which varies

from tissue to tissue.

1.3 **Adrenocortical angiotensin receptors**

Angiotensin II is a potent stimulus to aldosterone production in many species (Davis, 1961; Laragh et al, 1961; Fraser et al, 1965; Ganong et al, 1967; Coleman et al, 1974) and is believed to be the prime regulator of steroidogenesis during sodium restriction (reviews, Brown et al, 1977, 1979). High affinity angiotensin II binding sites with K_d values in the range 0.1-1 nM have been characterised in bovine (Glossman et al, 1974c), rat (Glossman et al, 1974c; Douglas et al, 1978a) and canine (Douglas et al, 1976) zona glomerulosa cells, and angiotensin II shown to stimulate aldosterone production in these cells in vitro at physiological concentrations (Fredlund et al, 1975; Bing & Schulster, 1977; Douglas et al, 1978a).

(a) Biochemical characteristics of the adrenocortical receptor

As in vascular smooth muscle, sodium increases binding of angiotensin II in this tissue but to a much greater extent. The effect of sodium appears to be antagonised by guanine nucleotides, and lithium is inhibitory. However, divalent cations have little effect on angiotensin II binding in adrenocortical membranes (Glossman et al, 1974a,b; Douglas et al, 1978a).

As previously described for the vascular receptors, attempts to isolate the angiotensin receptor from adrenal cortex have been hindered by the extremely low affinity of the receptors following detergent solubilisation (Chang & Lotti, 1981). Photoaffinity labelling experiments have shown the adrenocortical receptor to be a dimeric protein with similarly sized subunits of M_r 68000 (Capponi & Catt, 1980).

There has been some debate regarding the role of angiotensin III in this tissue. A number of pharmacological studies have indicated that the heptapeptide is a more potent steroidogenic agent (Bravo et al, 1975b,

1976a; Peach et al, 1974, 1976) and that it acts through receptors selective for this peptide (Bravo et al, 1976b; Devynck et al, 1977). Other studies have shown angiotensin II to be more potent in this tissue (Campbell et al, 1977b; McCaa, 1978; Carey et al, 1978). However, it is now established that the adrenal and smooth muscle receptors are similar in that angiotensin III acts through the same receptor as the octapeptide but with lower affinity (Douglas et al, 1978b; Capponi & Catt, 1979; Aguilera et al, 1979; Douglas et al, 1980,1981).

(b) Regulation of the adrenocortical receptors

In contrast to the changes occuring in vascular tissues, sodium loading is associated with a blunted steroidogenic response to angiotensin II, while sodium depletion enhances sensitivity of the adrenal cortex (Ganong & Borzyczka, 1967; Kinson & Singer, 1968; Oelkers et al, 1974; Dawson-Hughes et al, 1981). Aguilera et al (1978,1980b) have demonstrated an initial change in affinity of the angiotensin receptors (at 36 hours) and a later change in receptor density (at 4 days) with normalisation of affinity following alteration of dietary sodium intake. These changes in binding properties were correlated with changes in responsiveness of glomerulosa cells in vitro. Douglas & Catt (1976) have also found an increase in glomerulosa cell angiotensin receptor density following prolonged sodium restriction. The effect of sodium depletion can be blocked by converting enzyme inhibition (Aguilera & Catt, 1978; Williams et al, 1978a; Aguilera et al, 1980b) and fully (Hauger et al, 1978; Aguilera et al, 1980a,b) or partly (Bojensen, 1966; Oelkers et al, 1975) reproduced by angiotensin II infusion; the discrepancy may arise through the dose of angiotensin II used, since high infusion rates diminish receptor density (Aguilera et al, 1980b). Also, changes in serum $[K^+]$ may be involved (see below), since serum $[K^+]$ may increase with sodium depletion but not with angiotensin II infusion (Boyd et al, 1971).

Dietary potassium loading is associated with elevated plasma potassium and aldosterone levels (Bojensen, 1966; Brunner et al, 1970; Douglas et al, 1978d), enhanced pressor and steroidogenic responses to angiotensin II (Hollenberg et al, 1975; Douglas & Litowitz, 1978; Douglas et al, 1978c) and hypertrophy of the zone glomerulosa (Deane et al, 1948; Douglas & Catt, 1976). The rise in serum [K^+] presents a direct stimulus to steroid secretion (Funder et al, 1969; Haning et al, 1970) and also augments responsiveness to angiotensin II in vivo (Dluhy et al, 1972; McCaa et al, 1975; Douglas & Catt, 1976) and in vitro (Haning et al, 1970). Douglas (1979) observed a decreased sensitivity but increased maximal steroidogenic response to angiotensin II in response to short-term potassium loading in the rat, and a corresponding decrease in affinity but increase in number of glomerulosa angiotensin receptors. Converting enzyme inhibition partly blocked the change in affinity, while angiotensin II infusion further increased the receptor density (Douglas, 1980). In rats maintained on a low potassium diet, plasma angiotensin II concentration was elevated 5-fold while glomerulosa cell receptor density fell (Douglas & Catt, 1976): a comparable increase in circulating angiotensin II concentration through infusion would be expected to increase receptor number (Hauger et al, 1978; Aguilera et al, 1980b).

Prolonged low-dose infusion of angiotensin II in dogs enhances pressor response to the hormone and blunts steroidogenic response (Bean et al, 1979). In rats, a similar protocol caused a decrease in affinity and increase in number of the zone glomerulosa angiotensin receptors (Douglas & Brown, 1982). Suppression of plasma angiotensin II concentration by aldosterone infusion produced a slight increase in affinity and a decrease in receptor density: when the hypokalemia associated with aldosterone infusion was prevented (high potassium diet), the decrease in receptor density was largely attenuated (Douglas & Brown, 1982).

These findings indicate that potassium can act independently of and synergistically with angiotensin II in the zone glomerulosa: potassium appears to be a more important regulator in that it can override the effects of angiotensin II. It is possible that the changes in pressor sensitivity to angiotensin II seen during alteration of dietary potassium intake are mediated through changes in plasma aldosterone concentration, if aldosterone exerts a regulatory effect on the vascular receptors as suggested by Schiffrin et al (1983c). Together with the changes in receptor affinity (in general, directionally similar to the changes in plasma angiotensin II concentration) and the differing effects of low- and high-dose angiotensin II infusion, these observations suggest that a number of processes are active in regulating adrenocortical steroidogenesis and sensitivity to angiotensin II.

1.4 **Renal angiotensin receptors**

Alteration of sodium balance is associated with changes in glomerular filtration rate (GFR) (Beaufils et al, 1976; Skorecki et al, 1983) and angiotensin II has been shown to influence glomerular capillary ultrafiltration coefficient (Myers et al, 1975; Blantz et al, 1976; Lohmeier et al, 1977). Specific binding of angiotensin II to isolated rat glomerular capillary tufts has been demonstrated and angiotensin II shown to stimulate contraction of the capillary tufts with an ED_{50} of 50pM, similar to the K_d value of the binding sites (Sraer et al, 1974; Beaufils et al, 1976). Low affinity binding sites were also observed but not characterised. Ballerman et al (1984) and Bellucci & Wilkes (1984) report a single class of binding sites (K_d values of 0.35nM and 4nM respectively) in rat glomeruli, while Chansel et al (1982) have described cooperativity in angiotensin II binding to human glomerular capillary tufts (K_d in the range 0.1-2nM). Autoradiographic studies have shown ^{3}H-angiotensin II to localise predominantly over the cytoplasm of renal mesangial cells

(Osborne et al, 1975); these cells are known to contain contractile elements (Becker, 1972; Ausiello et al, 1980) and to contract in response to angiotensin II (Fiodart et al, 1980). The latter authors report a single class of angiotensin II binding sites on mesangial cells with a K_d of 2nM, agreeing with their value for the ED_{50} for contraction. The changes in GFR associated with alteration of sodium balance have been correlated with changes in receptor density and affinity (Beaufils et al, 1976) or in receptor density only (Ballerman et al (1984); Bellucci & Wilkes (1984).

Low doses of angiotensin II promote sodium and water retention, while large doses (pressor) are natriuretic (Ploth & Gabriel, 1979). A number of studies have suggested that angiotensin II may directly stimulate sodium reabsorption within the kidney (Johnson & Malvin, 1977; Harris & Young, 1977; Freedlender & Goodfriend, 1977; Levens et al, 1981), and angiotensin II has been shown to alter sodium fluxes in renal proximal tubular cells (Steven, 1974; Freedlender et al, 1979,1980) apparently by activating a oubain-insensitive sodium pump (Munday et al, 1971). Specific, high affinity (K_d=6nM) angiotensin binding sites have been identified on both the luminal (brush border) and contraluminal (basolateral) tubule membranes (Brown & Douglas, 1982). In this tissue, sodium increased the affinity of receptors while divalent cations increased the receptor density; these effects were additive.

As previously noted, angiotensin II inhibits renin release; this effect is receptor mediated since it occurs in isolated, perfused kidneys and superfused kidney slices (Davis & Freeman, 1976), and in vivo at sub-pressor doses of angiotensin II (Bean et al, 1979). The receptors mediating this response show the same discrimination between angiotensin analogues as those in vascular muscle and adrenal cortex (Naftilan & Oparil, 1978).

1.5 **Central angiotensin receptors**

There is considerable evidence that angiotensin exerts effects on both the central and sympathetic nervous systems. Blood-borne angiotensin gains access to the circumventricular organs of the rat brain (Van Houtten et al, 1980) and immunohistochemical methods have identified angiotensin-like material in nerve terminals (Fuxe et al, 1976; Changaris et al, 1978). It has also been proposed that a separate renin-angiotensin system exists within the brain, since all the components appear to be present in this tissue (Ganten et al, 1976; Reid, 1979; Printz et al, 1982).

Angiotensin II is known to stimulate thirst (Epstein et al, 1970; Ramsay et al, 1979) and vasopressin release (Bonjour & Malvin, 1970; Sladek & Joynt, 1980) and to facilitate autonomic neurotransmission at many levels in the central and peripheral nervous systems (reviews, Peach, 1977; Westfall, 1977). The effect on autonomic transmission is largely due to enhancement of norepinephrine release, although angiotensin II has been shown to increase neuronal firing rate (Phillips et al, 1979) and catecholamine content (Sumners & Phillips, 1983). Intraventricular microinjection of angiotensin II causes a pressor response (Buckley & Jandhyala, 1977; Phillips, 1978) which is due in part to increased efferent sympathetic activity (Falcon et al, 1978).

Binding sites with equal affinity for angiotensins II & III (K_d 0.2nM) have been identified in rat brain (Bennett & Snyder, 1976, Mann et al, 1978,1981). Consistent with actions of angiotensin in the circumventricular organs, the highest density of binding sites was seen in the thalamus, hypothalamus and midbrain. A similar distribution but slightly lower affinity (K_d 0.9nM) has been reported by Sirrett et al, (1977). Mann et al (1978, 1981) were able to correlate the central pressor and dypsogenic activities of various angiotensin analogues with affinity for the receptor, and found a decrease in pressor response to centrally

administered angiotensin II to coincide with a reduction in receptor density during dietary sodium restriction. In bovine cerebellar cortex, Bennett & Snyder (1980a,b) observed two classes of angiotensin binding sites (K_d's 80 and 500pM). Alteration of the incubation medium sodium ion concentration (10-150mM) exerted a differential effect on binding of angiotensin II and sar^1 substituted octapeptide analogues in that it increased the affinity (25-fold) of the high affinity sites for the native peptide only. This effect was a function of both ionic size and ionic strength and was dependent on the N-terminal aspartate residue.

Although angiotensins II & III have equal affinity for the brain receptor, the heptapeptide has much less pressor activity when administered centrally. Angiotensin I has little pressor activity and a very low affinity for the receptor (Mann et al, 1978,1981). The central and sympathetic responses to angiotensin II are resistant to tachyphylaxis (Fukiyama et al, 1971; Sweet et al, 1971).

In spontaneously hypertensive rats, pressor responsiveness to centrally administered angiotensin II is enhanced (Hoffman et al, 1977), while intraventricular administration of saralasin causes a reduction in blood pressure (Schoelkens et al, 1976; Phillips et al, 1977; Mann et al, 1978). Central administration of the angiotensin II antagonist also reduces blood pressure in renin-dependent renal hypertension (Schoelkens et al, 1976; Sweet et al, 1976; Mann et al, 1978) but not in normotensive rats (Phillips et al, 1977). Similar results were obtained by Suzuki et al (1981), who also observed that similar effects were produced by captopril when administered by this route, and that in addition to changes in blood pressure, parallel changes in plasma renin activity occurred.

The density of angiotensin receptors in spontaneously hypertensive rat brain is greater than in Wistar-Kyoto control animals (Stamler et al, 1980). Neuronal cells from the hypertensive rats (at 1 day, before the

hypertension is established) in culture also show a higher receptor density (Raizada et al, 1984). Sumners et al (1983) found an inverse relationship between neuronal catecholamine content and receptor affinity in cultures from normal animals; this relationship was lost in the cultured cells from the hypertensive rats (Raizada et al, 1984).

1.6 **Cardiac angiotensin receptors**

Angiotensin II exerts positive inotropic and chronotropic effects on the heart. While the chronotropic effect is mediated through the sympathetic nervous system (Nishith et al, 1962; Krasney et al, 1966), the inotropic effect can be demonstrated in vitro (Koch-Weser, 1964; Fowler & Holmes, 1964) and is unaffected by B-adrenergic blocking drugs (Dempsey et al, 1971; Blumberg et al, 1975). Several studies have indicated that angiotensin II augments Ca^{++} influx during the action potential plateau (Beeler & Reuter, 1970; Bonnardeaux & Regoli, 1974; Shigenobu et al, 1974), an effect which can be blocked by verapamil and Mn^{++} (Freer et al, 1976) and angiotensin antagonists (Bonnardeaux & Regoli, 1974; Freer et al, 1976).

In bovine heart Mukherjee et al (1982) observed a single class of angiotensin binding sites with a K_d of 2nM but attempted no further characterisation. In a detailed study by Wright et al (1983) using rabbit ventricular tissue, two classes of binding sites for ^{125}I-angiotensin II (K_d values 2.4 and 9.6nM) were observed. The partial agonist sar^1 ile^8 angiotensin II appeared to interact only with the high affinity binding sites, and the density of high affinity sites was reduced by monovalent cations (Na^+, K^+, Li^+, NH_4^+) and increased by divalent cations (Ca^{++}, Mg^{++}) without alteration in affinity. The affinity of both classes of sites was reduced by guanine nucleotides (increase in dissociation rate constants), but in the presence of GTP, angiotensin II (luM) did not affect isoproterenol-stimulated adenylate cyclase activity. Baker et al (1984)

have characterised a single class of binding sites for ^{125}I-angiotensin II in this tissue but did not exclude the possibility of a second class of low-affinity sites. These authors also found divalent cations to enhance binding and guanine nucleotides to be inhibitory.

1.7 **Hepatic angiotensin receptors**

In the liver, angiotensin II stimulates carbohydrate mobilisation (Hems et al, 1978; Exton, 1980; Hems & Whitton, 1980) and synthesis of renin substrate (Freeman & Rostorfer, 1972; Sernia & Reid, 1980) and attenuates activation of adenylate cyclase (Jard et al, 1981; Crane et al, 1982; Cardenas-Tanus et al, 1982). The effects on carbohydrate metabolism are mediated through a cAMP-independent Ca^{++}-requiring pathway (Garrison et al, 1979; Sexton, 1981) and angiotensin II and glucagon have been shown to affect the phosphorylation states of partially overlapping sets of cytoplasmic proteins in this tissue (Garrison, 1978; Garrison et al, 1979; Garrison & Wagner, 1982). The attenuation of adenylate cyclase activity is not Ca^{++} dependent (Jard et al, 1981; Cardenas-Tanus et al, 1982).

Lafontaine et al (1979) observed a single class of binding sites in rat liver membranes with a K_d of 0.1 nM, while Campanile et al (1982) and Crane et al (1982) report two classes of binding sites with K_d values of 0.2 and 3 nM. Sodium and GTP interact with the high affinity binding sites in a manner similar to that described for the rat mesenteric artery angiotensin receptor (Wright et al, 1982), and Campanile et al (1982) were able to correlate the binding affinities of various angiotensin analogues with their ability to activate glycogen phosphorylase. However, Sen et al (1983) found only a single class of binding sites in intact and digitonin-solubilised rabbit liver membranes (K_d values 0.5 and 10 nM respectively). Using SDS-polyacrylamide gel electrophoresis they observed a single radiolabelled band after covalently linking ^{125}I-angiotensin II to its binding site [M_r=68000, identical to the value obtained by Capponi & Catt

(1980) for the dog uterine and adrenocortical receptor]. Like Campanile et al (1982) and Crane et al (1982), Gunther (1984) observed two classes of binding sites in rat liver membranes (K_d values 0.35 and 3 nM). The high affinity sites were inactivated by sulphydryl reducing agents, which also blocked angiotensin II stimulation of glycogen phosphorylase activity. This effect was specific for angiotensin II, and did not affect angiotensin II inhibition of adenylate cyclase. Sulphydryl reagents specifically inactivate the vascular angiotensin receptor (Gunther et al, 1980a) and block vascular smooth muscle response to angiotensin II (Fleisch et al, 1973). The ED_{50}'s for phosphorylase activation and inhibition of adenylate cyclase are 0.3-0.8 nM (Keppens & DeWulf, 1976; Hems et al, 1976) and 3 nM (Jard et al, 1981) respectively. Gunther (1984) was able to correlate the computed fractional occupancy of the high and low affinity receptors with the degree of phosphorylase activation and cyclase inhibition respectively, and has proposed that each effect is mediated by a distinct angiotensin receptor subtype.

Investigating the regulation of the angiotensin receptors in rat liver, Sernia et al (1985) observed a single class of angiotensin II binding sites with a K_d of 0.3 nM. Binding was increased by divalent cations and abolished by dithiothreitol. Sodium loading caused a complex response similar to that seen in adrenal cortex. An initial fall in receptor density (at 1.5 days) was followed by an increase to a level above control (maximal at 9 days) and then a slow return towards the control value. Changes in affinity directionally opposite to the changes in receptor density were also observed. Angiotensin II infusion caused an increase in receptor density without any change in affinity.

1.8 **Platelet angiotensin receptors**

The sensitivity of platelets to aggregation by epinephrine and ADP is increased by angiotensin II (Poplowski, 1970; Ding et al, 1985b), and

binding sites with the properties expected of a physiological receptor for angiotensin have been identified on human platelets. Moore & Williams (1982) observed a single class of specific, saturable sites with a K_d value of 240pM, and found the number of binding sites (approximately 10 sites/cell) to vary inversely with (4 days) dietary sodium intake. No specific binding of angiotensin II to erythrocytes or mononuclear leukocytes could be demonstrated. Similar results for the affinity and concentration of binding sites have been reported by Ding et al (1984) and Mann et al (1985).

Investigating the regulation of the platelet receptors, Moore et al (1984) found rapid alteration of plasma angiotensin II concentration (by volume loading or depletion) to cause a directionally opposite change in binding capacity which was not significant at 4 hours and which reached a plateau at 24-48 hours. This time-course suggests that prior occupancy of the receptors does not account for the observed changes in binding capacity, and these authors have proposed that receptor regulation occurs at the level of the megakaryocyte with the observed changes in binding capacity reflecting the rapid turnover of platelets, or that regulation occurs by masking and unmasking of preformed receptors as has been proposed for the hepatic insulin receptor (Krupp & Lane, 1981). Ding et al (1985a) also observed an inverse relationship between plasma angiotensin II concentration and platelet receptor density during alteration of sodium balance but found no change in angiotensin binding with alteration of dietary potassium intake. These authors also observed no difference in platelet angiotensin II binding between normotensive human subjects and patients with essential hypertension.

1.9 Post-receptor Events

Angiotensin II action has been associated with changes in the electrical properties of smooth muscle (Keatinge, 1966; Ohashi, et al, 1967; Somlyo & Somlyo, 1970) and adrenocortical (Natke & Kabella, 1979) cells, and with changes in transmembrane sodium flux (Friedman et al, 1959; Friedman & Allardice, 1962; Villamil et al, 1970). Extracellular Ca^{++} is required for angiotensin action in adrenal cortex (Fakunding & Catt, 1980), kidney (Vandongen & Peart, 1974; Fray & Park, 1979), liver (Garrison et al, 1979; Sexton, 1981), heart (Bonnardeaux & Regoli, 1974; Freer et al, 1976) and smooth muscle (see below). From an electrophysiological study on uterine muscle, Hamon & Worcel (1979) concluded that angiotensin II simultaneously increases membrane conductance to Na^{+} and K^{+}. The changes in membrane conductance occured when the membrane potential was held at its resting level, did not appear to involve Ca^{++} or Cl^{-}, and were only slightly affected by ouabain. The sequence of events proposed by these authors is; 1) an initial rapid depolarisation that triggers the discharge of spikes; 2) simultaneous with the depolarisation, and before any spike production, a tonic contraction starts; 3) coincident with the spikes, phasic contractions are superimposed on the tonic contraction; 4) once tachyphylaxis (or hormone washout) starts, relaxation of the muscle follows the fading of the depolarisation. The tonic contraction appeared to be independent of changes in transmembrane Ca^{++} flux (and only occured once in Ca^{++}-free media), while the phasic contractions were dependent on extracellular Ca^{++}; in this tissue it has been demonstrated that each spike is the result of a potential-dependent Ca^{++} gating (Mironneau, 1974; Vassort, 1975). These results are consistent with previous reports of two stages in angiotensin action; an early stage which uses intracellular Ca^{++}, and the late, chronic stage which is dependent on extracellular Ca^{++} (Deth & Van Breemen, 1974,1977). However,

the role of changes in membrane potential is unclear, since angiotensin II is known to contract vascular smooth muscle in the presence of depolarising concentrations of potassium (Bohr, 1974). In the studies cited above in which membrane depolarisation in response to angiotensin II was observed, the use of high concentrations of the peptide may have produced artefactual results.

In more recent studies, the effects of angiotensin II on trans-membrane Na^+ and K^+ flux has been shown to be the result of activation of the amiloride-sensitive Na^+/H^+ exchange carrier, with the rise in intracellular $[Na^+]$ stimulating the Na^+/K^+ ATPase and thereby attenuating the change in membrane potential induced by the sodium influx (Brock et al, 1982; Smith & Brock, 1983). This is consistent with effects of ouabain observed by Hamon & Worcel (1979).

Much has been learned recently about the role of phosphoinositide turnover in hormone action. A number of hormones have been shown to activate a membrane-bound phosphodiesterase (phospholipase C) that hydrolyses the membrane lipid phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) producing inositol 1,4,5 trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ stimulates Ca^{++} release from the endoplasmic reticulum (and may have other actions) while diacylglycerol acts in the plane of the membrane to activate protein kinase C, which in turn may activate the amiloride-sensitive Na^+/H^+ exchange carrier (for review, see Berridge & Irvine, 1984). Angiotensin II has been shown to stimulate the hydrolysis of $PtdIns(4,5)P_2$ in liver (Kirk et al, 1981) and the synthesis and hydrolysis of $PtdIns(4,5)P_2$ in adrenal cortex (Farese et al, 1984) and aortic smooth muscle cells in culture (Smith et al, 1984). In the latter study, which was confined to the immediate effects of angiotensin II, Ca^{++} efflux from the cells was observed, consistent with mobilisation of intra-cellular Ca^{++}. Angiotensin II has previously been shown to increase the

rate of efflux of Ca^{++} from an aortic muscle microsomal fraction, probably plasma membrane or endoplasmic reticulum (Baudouin et al, 1972). Removal of extracellular Ca^{++} did not influence the experimental observations of Smith et al (1984), and Ca^{++} influx was not stimulated. These results suggest that the initial phase of tissue response to angiotensin II is mediated by intracellular Ca^{++} mobilised through the action of $InsP_3$. The mechanism of the slow component of the response is as yet unknown. Smith et al (1984) have proposed that Ca^{++} influx is stimulated by the change in membrane potential; this may take the form of potential-dependent gating of Ca^{++} (although this apparently only occurs at high concentrations of the peptide) or modulation of Na^+/Ca^{++} exchange carrier activity by the rise in intracellular $[Na^+]$, as proposed by Lang & Blaustein (1980). Alternatively, the initial increase in intracellular $[Ca^{++}]$ may directly stimulate Ca^{++} influx as discussed by Bolton (1979).

Cyclic GMP may also mediate tissue response to angiotensin II. Ewans et al (1976) found cGMP to mimic the effects of the hormone on cation transport in kidney slices, and angiotensin II administration has been reported to increase plasma concentrations of cGMP in humans (Roseman et al, 1976) and the cGMP content of rat aortic, heart and kidney tissues (Vesely, 1981).

Many hormone receptors are known to be coupled to their effector(s) through a guanine nucleotide-binding regulatory protein (review, Gilman, 1984). Little direct evidence is available in this respect for angiotensin II, although the inhibitory effect of GTP and GTP analogues on binding of angiotensin II in many tissues indicates the involvement of such a factor. Also, a number of other hormone receptors which act via the $InsP_3$/diacylglycerol Ca^{++}-mobilising pathway are believed to be coupled to phospholipase C via a GTP-binding protein (review, Berridge & Irvine, 1984).

CHAPTER 2

MATERIALS AND BIOCHEMICAL METHODS

2.1 Materials

(a) Biochemicals

All reagents were of the best grade available commercially. With the exception of those listed below, all were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Albumins [bovine, fraction V, (product number A-4503), and rabbit, crystalline], ATP, 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide HCl, cyclic AMP, cyclic GMP, glucagon (crystalline), Gpp(NH)p, GTP, inosine 5´-triphosphate, o-phenanthroline monohydrate, phenylmethanesulphonyl-fluoride, soybean trypsin inhibitor, Tos-Phe-CH_2Cl and neomycin sulphate were obtained from Sigma (London) Chemical Company Ltd., London, U.K.

Di-isopropyl phosphite was obtained from Koch-Light Laboratories Ltd., Coinbrook, Buckinghamshire, U.K.

DEAE-Sephadex A-25 and dextran T-70 were supplied by Pharmacia (Great Britain) Ltd., Hounslow, Middlesex, U.K.

Freund´s Adjuvant was purchased from Gibco Laboratories Ltd., Paisley, Strathclyde, U.K.

Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) was obtained from Pierce Biochemical Corp., Rockford, Illinois, U.S.A.

Human serum albumin was obtained from A.B. Kabi, Stockholm, Sweden.

Norit SXI charcoal was supplied by Haller & Phillips Ltd., London, U.K.

(b) Radiochemicals

Tyrosyl ^{125}I- [Ile^5] angiotensin I and tyrosyl ^{125}I- [Ile^5] angiotensin II were obtained from New England Nuclear, Southampton, U.K.

Carrier-free $Na^{125}I$ was supplied by the Radioisotope Dispensary, Western Infirmary, Glasgow.

(c) Pharmaceuticals

Trasylol was obtained from Bayer Pharmaceuticals Ltd., Haywards Heath, Sussex, U.K.

Frusemide B.P. (Lasix) was obtained from Hoechst (UK) Ltd., Pharmaceuticals Division, Middlesex, U.K.

Captopril was supplied courtesy of E.R. Squibb & Sons Inc., Princeton, New Jersey, U.S.A.

(d) Peptides

Angiotensin II C-terminal hexapeptide and C-terminal pentapeptide were obtained from Schwartz Bioresearch, Orangeburg, New York, U.S.A. All other angiotensin peptides, arginine vasopressin and bradykinin were purchased from Cambridge Biochemicals Ltd., Harston, Cambridgeshire, U.K. and Peninsula Laboratories (Europe) Ltd., Merseyside, U.K.

(e) Animals

Male Sprague-Dawley rats (body weight 325-350g) were purchased from Olac Ltd., Shaws Farm, Oxfordshire, U.K. and Charles River Ltd., Margate, Kent, U.K.

(f) Membrane filters

Membrane filters were obtained from Millipore (UK) Ltd., Harrow, Middlesex, U.K. and Whatman Ltd., Maidstone, U.K.

2.2 General methods

(a) Micropipetting

Solution volumes in the range 5ul to 5ml were transferred reproducibly using adjustable "Finnpipettes" [Jencons (Scientific) Ltd., Leighton Buzzard, Bedfordshire, U.K.]. Larger volumes were dispensed using standard laboratory volumetric glassware.

(b) Weighing

Milligram quantities were measured using a Mettler type M5SA balance (Gallenkamp & Co. Ltd., East Kilbride, Glasgow, U.K.). For larger

quantities a Mettler type AE-160 balance was used.

(c) pH measurements

Measurements of pH were made using an Elcomatic Instruments Ltd., model 7065 digital pH meter and a standard combination electrode (Gallenkamp & Co. Ltd., East Kilbride, Glasgow, U.K.). This apparatus was regularly calibrated using solutions of pH 4.0, pH 7.0 and pH 9.0 prepared using buffer tablets (BDH Chemicals Ltd., Poole, Dorset, U.K.). The pH measurements were made at the temperature at which the buffers were to be used.

(d) Centrifugation

Accelerations of up to 2000g were obtained using a Damon DPR-6000 centrifuge [Damon/IEC (U.K.) Ltd., Dunstable, Bedfordshire, U.K.]. Accelerations of up to 100,000g were obtained using a Beckman L2-65B centrifuge fitted with a titanium Ti-60 rotor (Beckman RIIC Ltd., Glenrothes, Fife, U.K.) or an MSE Superspeed-65 centrifuge fitted with an MFT 60.35 titanium rotor (MSE Ltd., Crawley, Sussex, U.K.). Centrifugation was performed at $2^{o}C$. For small samples, accelerations of 10,000g were obtained with an MSE "Microcentaur" benchtop centrifuge taking 1.5ml tubes.

(e) Preparation of buffers

All solutions were prepared in glass-distilled water. Buffers for receptor-binding experiments were filtered through a 0.2um filter using a Millipore "Sterifil" apparatus [Millipore (UK) Ltd., Middlesex, U.K.] to remove particulate matter and refrigerated until used.

(f) Measurement of protein concentration

Protein concentration was measured by a modification of the method of Lowry et al (1951). Stock solutions were prepared as follows; 2% (w/v) Na_2CO_3 (anhydrous) in 0.1M-NaOH, 2% (w/v) $CuSO_4.5H_2O$ in water, 4% (w/v) sodium potassium tartrate tetrahydrate in water. These solutions were

mixed in the proportions 40:1:1 by volume shortly before use, and 2.5ml of the mixture added to a protein sample in 200ul of 50mM-Tris buffer. The samples were vortexed and allowed to stand for 15 minutes with occasional re-mixing. 250ul of Folin & Ciocalteau's phenol reagent diluted in water (5:3 v/v) was then added, the mixture vortexed immediately and allowed to stand for at least 60 minutes prior to determination of the absorbance at 700nm. Protein standards (20-200ug) were prepared using bovine serum albumin (fraction V) dissolved in 50mM-Tris buffer. Normally, 4 protein samples and 4 blanks of the appropriate buffer were taken for each experiment.

(g) Measurement of radioactivity

Radioactivity was determined using an NE1612 gamma counter (>90% efficiency) (Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.). For low activity samples, the machine was calibrated for background count rates before use and the sample count rates automatically corrected.

2.3 Preparation of radioligands

(a) Angiotensins I & II

Tyrosyl ^{125}I- [Ile^5] angiotensin II was obtained from New England Nuclear as a lyophilised powder, and was dissolved in the appropriate Tris buffer containing 1% (w/v) bovine albumin and stored at -20^{o}C. Specific activity and radiochemical purity were determined as described below and were found to be within the manufacturers specifications of 2000Ci/mmol and >98% respectively. For the routine receptor assay, or when high concentrations of ^{125}I-angiotensin II (>10nM) were required, the specific activity was reduced 4-5 fold by addition of unlabelled [Ile^5] angiotensin II. Iodinated angiotensins I & II for radioimmunoassay of these peptides were also obtained from New England Nuclear and were dissolved to the approximate required concentration in the appropriate Tris buffer and stored at -20^{o}C.

(b) Iodination of angiotensin II antagonists

Angiotensin II antagonists were iodinated essentially as described by Fraker & Speck (1978). Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenyl glycoluril) was dissolved in redistilled methylene chloride and 200ul (120ug Iodogen) placed in a 10mm diameter flat-bottomed glass tube and dried at 45°C. Carrier-free $Na^{125}I$ (3mCi) in 20ul of 0.1M-NaOH was neutralised with 0.1M-hydrochloric acid and 200ul of 0.1M-Tris HCl (pH 7.2)/0.154M-NaCl containing 2.5ug of peptide added. This mixture was transferred to the tube containing the Iodogen and the reaction allowed to proceed for 1 hour on ice. 200ul of 0.1M-Tris HCl (pH 7.2)/0.1M-sodium metabisulphite/0.2M-KI was then added and the mixture chromatographed on DEAE Sephadex A-25 (27 x 1.5cm) which had been equilibrated with 0.1M-Tris HCl (pH 8.0)/0.2% (w/v) neomycin sulphate/0.1% (w/v) bovine albumin for 48 hours. The iodinated peptide was eluted with 0.1M-Tris HCl (pH 7.2)/0.2% (w/v) neomycin sulphate/0.1% (w/v) bovine albumin at 1 ml/min and 1ml fractions collected. The labelled peptides were assayed and stored as for ^{125}I-angiotensin II.

2.4 **Animals**

(a) General

Male Sprague-Dawley rats (325-350g) were used for all experiments. For experiments which did not involve alteration of sodium balance or drug administration, the rats were given tap water and a standard rat diet (Oxoid 41B). The animals were housed in groups of 3 or 4 and normally 12 were used for each experiment.

(b) Manipulation of sodium balance and drug administration

For these experiments, a powdered foodstuff containing 0.002% (w/w) NaCl (Special Diet Services Ltd., Witham, Essex, U.K.) was used. Sodium chloride was added as required and mixed in thoroughly. Frusemide B.P. (Lasix) was administered by intraperitoneal injection. Captopril was

administered chronically as a 0.05% (w/v) solution in distilled water which was substituted for the drinking water. In acute studies captopril was administered intraperitoneally in 0.154M-NaCl. All captopril solutions were prepared fresh daily. The approximate intakes of NaCl and captopril were determined by daily weighing of the food and drinking fluid containers. The details of the diets and drug regimens are given in Table 2.1.

(c) Tissue preparation and blood sampling

When blood sampling was not required, the rats were killed by a blow to the head. Otherwise, the animals were killed by decapitation and aortic blood collected within 5 seconds on ice. The blood was then immediately transfered to a tube containing 250ki.u. Trasylol/50 umols EDTA/10umols o-phenanthroline in a total volume of 50ul and kept on ice. The blood samples were later centrifuged at 1000g for 20 minutes at 2^{o}C, and the plasma aspirated and stored at -20^{o}C.

After sacrifice, the intestines were removed and placed in a petri dish containing 0.154M-NaCl on ice, and the mesenteric arterial arcades excised by blunt dissection. The fat in the centre of the mesentery was removed, the main artery and vein separated, and fine serrated-tip forceps used to strip adhering tissue from the arterial branches between the main artery and the mesenteric border of the intestine. This resulted in the removal of most of the fat and all venous tissue from the arterial arcades. The vessels were sectioned as close as possible to the wall of the intestine, and the excised arterial tissue kept in 0.154M-NaCl on ice during subsequent dissections.

2.5 Receptor-binding studies

(a) Preparation of the arterial membrane fraction

The mesenteric arterial arcades were cleaned of residual adherent fat in 0.25M-sucrose with a 30mm diameter smooth pestle (0.25mm clearance)

Table 2.1

Dietary & drug protocols

GROUP	PROTOCOL
Captopril	Chronic angiotensin converting enzyme blockade; captopril administered as 0.5 mg/ml solution in distilled water substituted for drinking water (normal salt diet) (dosage 50-60 mg/kg/day)
Captopril (acute)	Acute angiotensin converting enzyme blockade; captopril administered in 500ul 0.9% (w/v) saline by intraperitoneal injection (normal salt diet) (dosage 50 mg/kg)
High salt	Sodium loading; 2% (w/v) saline substituted for drinking water and SDS foodstuff*, or SDS foodstuff containing 2.5% (w/w) NaCl and tap water (sodium intake 10-12 mmol/day)
Normal	Controls (normal salt diet); SDS foodstuff containing 0.1% (w/w) NaCl and tap water (sodium intake 0.5-1 mmol/day)
Low salt	Sodium depletion; SDS foodstuff (0.002% w/w) NaCl and distilled water (sodium intake <0.01 mmol/day)
Low salt + diuretic	Sodium depletion; as low salt group plus frusemide 4 mg/kg administered by intraperitoneal injection at commencement of diet

* powdered foodstuff from Special Diet Services Ltd. containing 0.002% (w/w) NaCl

Teflon-glass homogeniser [Jencons (Scientific) Ltd., Bedfordshire, U.K.] [5-6 strokes, 6000 rev./min low-torque motor (Varilab type, Citenco Ltd., Borehamwood, Herts., U.K.)]. The tissue was then homogenised in 25ml of 0.25M-sucrose using a Polytron PT20S (Kinematica GmbH, Lucerne, Switzerland), setting 8 for 2x10 seconds. Care was taken to prevent heating of the tissue during homogenisation as this greatly reduced the binding capacity of the membrane material. The homogenate was immediately centrifuged at 2000g for 10 minutes, and the supernatant decanted and centrifuged as before. The membrane fraction was then obtained by centrifuging the second supernatant at 100,000g for 30 minutes. The pellet (300-400ug protein/arterial arcade) was resuspended using a small Teflon-glass homogeniser in a 50mM-Tris HCl buffer and aliquoted into 16x90mm polystyrene tubes for incubation and samples stored for subsequent assay of protein. All steps were carried out at 0-2^{o}C.

(b) Incubations

The incubation mixture normally comprised 100ul of freshly prepared membrane fraction suspension (50-70ug protein) and 100ul of 50mM-Tris HCl buffer containing 1% (w/v) bovine albumin and 1-15000pg ^{125}I-angiotensin II. Where additional compounds were added (metal salts, proteinase inhibitors or competing peptides) tracer and additional compound(s) were each added in 50ul of 50mM-Tris HCl/1% (w/v) albumin, giving a total incubation volume of 200ul.

The composition of the Tris buffer was as follows:

for normal assay

50mM-Tris HCl (pH 7.35)/120mM-NaCl/3.6mM-KCl/1.8mM-$MgCl_2$/4.8mM-$CaCl_2$

for high [Ca^{++}] assay

50mM-Tris HCl (pH 7.35)/120mM-NaCl/3.6mM-KCl/1.8mM-$MgCl_2$/25mM-$CaCl_2$

for low [Ca^{++}] assay

50mM-Tris HCl (pH 7.35)/120mM-NaCl/3.6mM-KCl/2.5mM-EDTA/2.5mM-EGTA

Non-specific binding was defined as radioactivity not displaced by 10uM-angiotensin II [added as 5ul of 400uM-angiotensin II in 50mM-Tris HCl/1% (w/v) albumin].

(c) Separation of receptor-bound and free radioactivity

Millipore 12-place sampling manifolds fitted with Whatman 0.2um cellulose nitrate (WCN type) membrane filters were used to separate receptor-bound and free radioactivity. The filters were pre-wetted with 50mM-Tris HCl (pH 7.35)/1% (w/v) albumin 15 minutes before use. The incubation mixtures were diluted by addition of 6.5ml ice-cold 50mM-Tris HCl (pH 7.35)/0.1% (w/v) albumin (filtration buffer) and filtered under vacuum. The filters were rinsed with a second 6.5ml of filtration buffer and allowed to dry before removal for measurement of filter-trapped radioactivity. The filtration and washing process was completed in 30-40 seconds. Where the filtrate immunoassay was used to estimate tracer degradation, the filtration buffer also contained 0.2% (w/v) neomycin sulphate. Results are presented as mean ± standard deviation for the difference between total and nonspecific binding:

$$\text{i.e.} \quad (T_m - N_m) \pm \sqrt{(T_s^2 + N_s^2)}$$

where T_m, T_s are mean and standard deviation for total binding

N_m, N_s are mean and standard deviation for non-specific binding

(d) Chromatographic analysis of receptor-bound radioactivity

For chromatographic analysis of receptor-bound and free radioactivity, the reaction mixture was incubated for 60 minutes at 22°C and 1ml of ice-cold 50mM-Tris HCl buffer containing 1% (w/v) bovine albumin added. After centrifugation at 10,000g for 10 minutes, the supernatant was aspirated and an aliquot chromatographed as free radioactivity. The pellet was washed as before, resuspended in 200ul of the same buffer without albumin and transferred to a 100°C water bath for 5 minutes. Precipitated protein was removed by centrifugation and the supernatant chromatographed

as receptor-bound radioactivity.

2.6 Radioimmunoassay of angiotensin peptides

(a) Preparation of antisera

Antisera to angiotensin peptides were prepared by immunising New Zealand white rabbits with a peptide-rabbit albumin conjugate as described by Morton et al (1976). The conjugate was prepared using the following reaction mixture:

4 mg peptide

60 mg 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide HCl

2 mg rabbit albumin (crystalline)

in 2ml 0.154M-NaCl/0.2% (w/v) neomycin sulphate

The reaction was allowed to proceed for 4 hours at room temperature after which the mixture was dialysed for 48 hours at $4^{o}C$ against 4 changes each of 2l 0.154M-NaCl/0.2% (w/v) neomycin sulphate using Visking tubing (Scientific Instrument Centre Ltd., London, U.K.). The conjugate was then diluted to 8 ml in saline/neomycin and stored as 0.25ml aliquots at $-20^{o}C$.

Rabbits were immunised at 14 day intervals with 0.25ml of peptide-albumin conjugate emulsified with 0.25ml of Freund's adjuvant injected subcutaneously on the shoulder or hindquarters. Blood samples were drawn from the central ear artery and serum stored at $-20^{o}C$. The presence of antibodies was determined by antiserum titration as described by Morton et al (1976). Antisera were used at final dilutions ranging from 1:4000 to 1:60,000.

(b) Extraction of plasma samples

For radioimmunoassay of angiotensin II in rat plasma samples, the samples were first extracted using C_{18} Sep-Pak cartridges (Waters Associates, Cheshire, U.K.). Each cartridge was washed with 5ml of methanol then 5ml of distilled water, and the plasma passed through the cartridge at approximately 1ml/min. After a second wash with 5ml of

distilled water, the angiotensin II was eluted with 2ml of methanol/water (80:20 v/v). The extract was dried under a stream of air at 45°C, and the residue stored at -20°C until assay. The recovery was determined using ^{125}I-angiotensin II and was consistently greater than 90%.

(c) Angiotensin radioimmunoassay

All reagents for angiotensin radioimmunoassay were prepared in 50mM-Tris HCl (pH 7.5)/0.3% (w/v) human serum albumin/0.2% (w/v) neomycin sulphate. The incubation mixture comprised:

200ul sample or known quantity of peptide (usually 200-3pg in two-fold dilutions)

50ul radiolabelled peptide (usually 10pg)

200ul antiserum at appropriate dilution (1:1800-1:20,000)

Following incubation for 18 hours at 4°C, antibody-bound and free radioactivity were separated by addition of 1ml of dextran-coated charcoal (6.3g of Norit SXI charcoal and 0.125g of dextran T70 in 100ml of Tris HCl buffer) and centrifugation at 2000g for 7 minutes. The supernatant was aspirated and the (free) radioactivity absorbed to the charcoal pellet determined. The peptide content of the samples was determined by reference to a plot of percentage radioactivity bound against dose of unlabelled peptide. A typical radioimmunoassay standard curve is shown in Figure 2.1.

The standards used for the assay of angiotensin I and angiotensin II were the same as those used for the routine radioimmunoassay of these peptides in the Blood Pressure Unit laboratories.

Because of the marked rise in plasma angiotensin I concentration induced by converting enzyme inhibition and the 1.8% cross-reaction shown by the angiotensin II antiserum with this peptide, the plasma extracts from the captopril-treated rats were assayed for both angiotensin I and angiotensin II and the appropriate correction made. (The angiotensin I antiserum showed no discernable cross-reaction with angiotensin II).

(d) Determination of specific activity

The specific activity of radiolabelled peptides for receptor binding studies was determined by self-displacement assay. This assay is based on the principle that in a radioligand binding assay, the ratio (bound ligand/total ligand) is determined by the total quantity of ligand (labelled and unlabelled) present. In each assay, a radioimmunoassay standard curve was set up using the radioligand to be assayed (diluted to the approximate required concentration) as tracer. In a second set of tubes, increasing quantities of radioligand only (usually 20X-Xpg in two-fold dilutions, where X is the quantity used as tracer for the standard curve) were incubated with antiserum. Following incubation and separation of bound and free ligand, the ratio (bound/total radioactivity) was calculated for all tubes. If nX pg of radioligand gave the same value of (bound/total) as X+D pg (where D is the value read from the abscissa of the standard curve), then X was calculated as D/(n-1) pg. The assay required that the antiserum did not distinguish between labelled and unlabelled ligand. This was verified by comparing the binding constants of the antiserum derived from the saturation binding curve using increasing concentrations of radioligand with those derived from the radioimmunoassay standard curve (a saturation binding curve in which the specific activity of a fixed amount of radioligand is reduced by addition of unlabelled ligand).

Each assay provided a set of standards which were counted for radioactivity at the same time as the samples from each receptor binding experiment, thus allowing count rates to be accurately related to molar quantities of radioligand. The results from a typical assay are shown in Figure 2.1.

SATURATION BINDING CURVE (using radioligand under assay)

(1) Total radioactivity (cpm)	(2) Total (as nX)	(3) Bound radioactivity (cpm)	(4) B/T	(5) Dose (D)	(6) D/(n-1) (X)	(7) Rel. slope
87809 ± 893	20.52	15515 ± 963	0.156	-	-	-
46351 ± 391	10.83	13288 ± 190	0.266	143.4	15.00	0.58
25468 ± 181	5.95	11009 ± 28	0.412	62.7	13.06	0.90
15223 ± 202	3.56	8731 ± 57	0.553	31.3	12.67	0.87
9896 ± 139	2.31	6742 ± 36	0.661	14.7	11.74	0.60
7187 ± 21	1.68	5338 ± 12	0.722	7.4	11.65	0.36
5710 ± 4	1.33	4392 ± 38	0.749	4.6	15.30	0.17
5084 ± 208	1.19	3934 ± 58	0.753	4.1	25.86	0.17

(2) Total radioactivity expressed as a multiple of X (X is the 100% value in the radioimmunoassay standard curve (below))
(4) Bound as a fraction of total
(5) Dose corresponding to each value of (B/T) read from radioimmunoassay standard curve (below)
(6) Peptide content of X (as pg unlabelled peptide)
(7) Relative slope of radioimmunoassay standard curve in region corresponding to (5), used as weight in calculating mean for (6)

MEAN ± S.D. for (6) (weighted)
13.14 ± 1.30 pg = 12.56 ± 1.23 fmol (m.w. angiotensin II 1046)

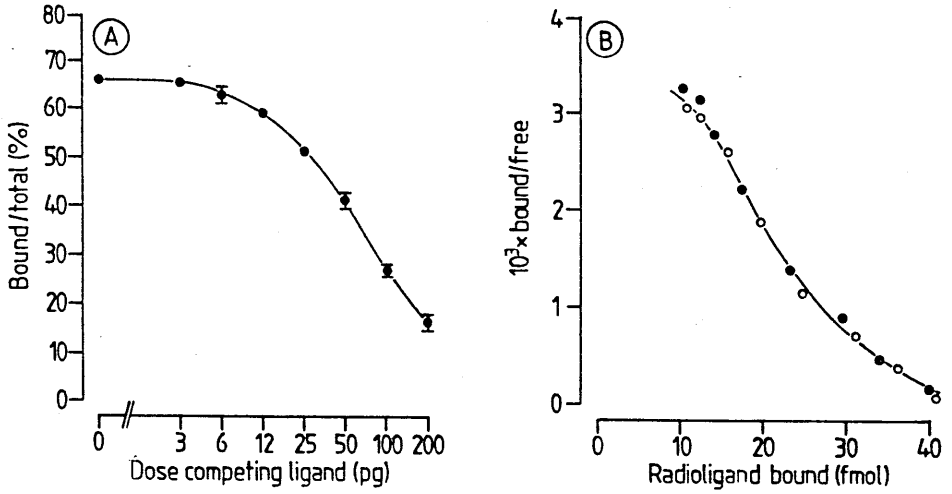

Figure 2.1 **Determination of specific activity: self displacement assay of ^{125}I-angiotensin II**

The assay consisted of two parts; a saturation binding curve using the radioligand under assay (Table) and a radioimmunoassay standard curve with the radioligand as tracer (Figure (A)). The calculations are shown in the table (columns 4-7) and are explained in Section 2.6d. Figure (B) shows Scatchard plots derived from the radioimmunoassay standard curve (solid markers) and from the saturation binding curve (open markers): the plots are superimposed, indicating that the antiserum did not distinguish between labelled and unlabelled peptides.

(e) Quantitative determination of radioligand degradation

For quantitative estimation of radioligand degradation in receptor binding studies, a 1ml sample of each filtrate (diluted if necessary) was incubated for 18 hours at $4^{o}C$ with 0.2ml of the appropriate antiserum. Bound and free radioactivity were separated as described previously and values of bound corrected for non-specific binding determined in parallel incubations without antiserum. From a standard curve constructed using pure radioligand the expected total radioactivity was calculated and the fraction of radioligand remaining undegraded in the sample taken as [total (expected)/total (measured)]. The results from a typical assay are shown in Figure 2.2. Where angiotensin-related peptides were used in displacement experiments, parallel incubations were performed without ^{125}I-angiotensin II and degradation of the peptides determined by radioimmunoassay.

2.7 Radioimmunoassay of plasma aldosterone

Aldosterone assays were performed by Miss M. Ingram in the Blood Pressure Unit laboratories according to the method of Fraser et al (1973). Plasma pooled from 4 rats was extracted into methylene chloride, chromatographed on paper and the aldosterone content determined by radioimmunoassay.

2.8 Chromatographic analysis of angiotensin peptides

Assessment of radiochemical purity was performed by descending paper chromatography using Whatman No. 2 paper and butan-2-ol/3% aq. NH_3 (30:11, v/v) as mobile phase. Sheets of chromatography paper (57x23cm) were cut using a template into 12 lanes such that the distance from the sample origins to the end of the lanes was 46cm. After sample application, the paper was equilibrated with the solvent vapour for at least 4 hours before addition of solvent to the solvent trough. Chromatograms were allowed to run for 45 hours at room temperature, after which the paper was

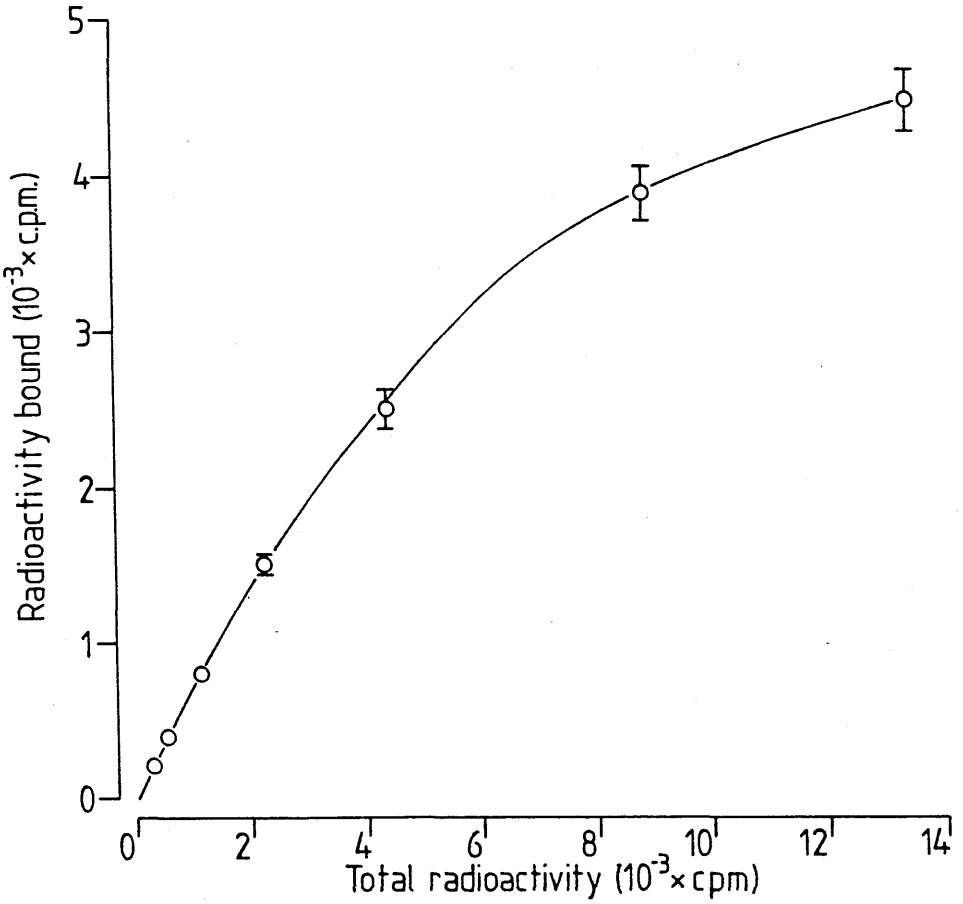

Figure 2.2 Filtrate immunoassay standard curve

Filtrate immunoassay standard curve for the quantitative determination of ^{125}I-angiotensin II degradation in receptor-binding incubations. Points represent mean ± s.d. for triplicate measurements of specific ^{125}I-angiotensin II binding to an angiotensin II-specific antiserum.

For a filtrate sample which gave a total count rate of 4000cpm and a bound count rate of 1000cpm, the expected total count rate would be 1490cpm (from the standard curve) and the fraction of ^{125}I-angiotensin II remaining undegraded in the sample would be 1490/4000 = 0.373.

dried and cut into 1cm strips for counting of radioactivity. The R_F values for angiotensin II and the C-terminal hepta-, hexa- and pentapeptides were determined by chromatographing the unlabelled peptides and staining the paper with ninhydrin.

2.9 Bioassay of iodinated angiotensin II

The biological activity of ^{125}I-angiotensin II was assessed using the pentobarbitone anaesthetised, pentolinium tartrate treated rat preparation as described by Lever et al (1964). Bolus injections (50ul) of 0.9% saline containing pure ^{125}I-angiotensin II or angiotensin II amide (Hypertensin Ciba) were made via a cannula placed into the jugular vein and blood pressure was recorded via a carotid arterial catheter connected to an Elcomatic EM 751 pressure transducer and an Elcomatic EM 720 recorder (Elcomatic Ltd., Glasgow, U.K.).

CHAPTER 3

STATISTICAL METHODS

3.1 Analysis of radioligand-binding data

Analysis of the receptor-binding data was performed as described below using a nonlinear regression computer program constituting part of the BMDP statistical package. The analysis was carried out on the Glasgow University ICL2988 computer.

3.2 Nonlinear regression

(a) General principles

The principles underlying the Gauss-Newton method of nonlinear regression have been described in detail by Wilkinson (1961) and are summarised below.

For a nonlinear equation in which there is a single parameter, p

$$y = f(p)$$

an estimate, p_{est}, will differ from the best-fit value, $p_!$, by an unknown amount, q:

$$p_! = p_{est} + q$$

and therefore

$$y_{obs} = f(p_!) = f(p_{est} + q).$$

It may be shown that (as a close approximation)

$$y_{obs} = f(p_{est}) + qf'(p_{est})$$

where y_{obs} is the observed value of y and f' denotes differentiation with respect to p (i.e. dy/dp).

Since

$$y_{pred} = f(p_{est})$$

where y_{pred} is the predicted value of y, then

$$y_{obs} - y_{pred} = qf'(p_{est})$$

and therefore

$$q = (y_{obs} - y_{pred})/f'(p_{est}).$$

Thus, by calculating the residual, $y_{obs}-y_{pred}$, and the partial derivative, dy_{pred}/dp_{est}, an approximate value for q may be determined. The new estimate of the parameter may then be refined by further iterations. When the value of q is small compared to the value of p_{est} ("convergence"), the standard error of p_{est} is equal to that of q.

This may be extended to the case where there are n values of the dependent (observed) variable and m parameters

$$
\begin{aligned}
y_{obs}^{(1)} - y_{pred}^{(1)} &= q(1)\, dy_{pred}^{(1)}/dp_{est}^{(1)} + \ldots\ldots + q(m)\, dy_{pred}^{(1)}/dp_{est}^{(m)} \\
&\vdots \\
y_{obs}^{(2)} - y_{pred}^{(2)} &= q(1)\, dy_{pred}^{(2)}/dp_{est}^{(1)} + \ldots\ldots + q(m)\, dy_{pred}^{(2)}/dp_{est}^{(m)} \\
&\vdots \\
y_{obs}^{(n)} - y_{pred}^{(n)} &= q(1)\, dy_{pred}^{(n)}/dp_{est}^{(1)} + \ldots\ldots + q(m)\, dy_{pred}^{(n)}/dp_{est}^{(m)}
\end{aligned}
$$

and the values of q(1)-q(m) obtained by solving the system of linear equations.

Any number of independent (controlled) variables may be present, and the only requirements are that an expression is given relating the dependent variable to the independent variable(s) and the parameters, and that initial estimates for the parameters are specified. The derivatives may be obtained by algebraic manipulation of the expression, or in complex systems where this is not possible, by numerical differentiation.

(b) Application

The data were analysed with a standard regression computer package [BMDP program PAR (derivative-free nonlinear regression)] (Dixon, 1981) in conjunction with FORTRAN programs specifying the function under study. Numerical integration of the differential equations forming the function was performed using Numerical Algorithms Group routine D02EAF.

The rate constants for radioligand (and in competition experiments, unlabelled ligand) degradation and for decay of the high-affinity sites

were taken as known, leaving the forward and reverse rate constants and the receptor concentration for each class of sites to be estimated. In amalgamating results from several experiments, the receptor concentrations were taken to be proportional to membrane protein concentration and the constants of proportionality estimated.

The program used for general analysis of kinetic and saturation binding experiments is given in Appendix A.1. In this program, fitting for two classes of binding sites is performed and the differential equations describing the reaction are therefore:

$$- d[*H]/dt = k_{f_1}[*H][R_1] - k_{r_1}[*HR_1] + k_{f_2}[*H][R_1] - k_{r_2}[*HR\ ] + k_{dH}[*H]$$

$$- d[R_1]/dt = k_{f_1}[*H][R_1] - k_{r_1}[*HR_1] + k_{dR}[R_1]$$

$$- d[R_2]/dt = k_{f_2}[*H][R_2] - k_{r_2}[*HR_2]$$

$$d[*HR_1]/dt = k_{f_1}[*H][R_1] - k_{r_1}[*HR_1]$$

$$d[*HR_2]/dt = k_{f_2}[*H][R_2] - k_{r_2}[*HR_2]$$

where k_{f_n} = forward rate constant
k_{r_n} = reverse rate constant
$[R_n]$ = receptor concentration
$[*HR_n]$ = concentration of H-R complex (bound radioligand)

} n=1, high affinity site; n=2, low affinity site

$[*H]$ = radioligand concentration
k_{dH} = rate constant for $[*H]$ decay
k_{dR} = rate constant for $[R_1]$ decay

An indexing system was devised which allowed several groups of data to be analysed under the assumption that receptor concentrations but not affinity varied between groups. This facility permitted smaller amounts of data (in each group) to be analysed than if analysis of each group was performed independently, and is referred to as the multiple receptor density method.

In the routine receptor assay, where the low-affinity component of binding was assumed to be linear with tracer concentration, the terms

relating to the low-affinity sites were omitted and a separate linear component estimated for each experiment. The program is given in Appendix A.2 and part of the output produced by the program is shown in Figure 3.1.

For analysis of competitive binding curves, the low-affinity component of binding was ignored and the forward and reverse rate constants for tracer and competing ligands and the concentration of high-affinity sites estimated. An indexing system was devised allowing a metabolite of the competing ligand to be specified as one of the other competing peptides. This was used in the case of angiotensin I, where analysis showed that the metabolite (angiotensin II) was responsible for the observed displacement. The differential equations describing the reaction are therefore:

$$-d[*H]/dt = k^*_f[*H][R] - k^*_r[*HR] + k^*_d[*H]$$

$$-d[^cH]/dt = k^c_f[^cH][R] - k^c_r[^cHR] + k^c_d[^cH]$$

$$-d[^mH]/dt = k^m_f[^mH][R] - k^m_r[^mHR] - k^c_d[^cH] + k^m_d[^mH]$$

$$-d[R]/dt = k^*_f[*H][R] - k^*_r[*HR] + k^c_f[^cH][R] - k^c_r[^cHR] + k^m_f[^mH][R] - k^m_r[^mHR] + k_d[R]$$

$$d[*HR]/dt = k^*_f[*H][R] - k^*_r[*HR]$$

$$d[^cHR]/dt = k^c_f[^cH][R] - k^c_r[^cHR]$$

$$d[^mHR]/dt = k^m_f[^mH][R] - k^m_r[^mHR]$$

where $[*H]$ = concentration of radioligand

$[^cH]$ = concentration of competing ligand

$[^mH]$ = concentration of metabolite of competing ligand

k^*_f, k^*_r = forward and reverse rate constants for radioligand

k^c_f, k^c_r = forward and reverse rate constants for competing ligand

k^m_f, k^m_r = forward and reverse rate constants for metabolite

$[*HR]$ = concentration of bound radioligand

$[^cHR]$ = concentration of bound competing ligand

$[^{m}HR]$ = concentration of bound metabolite

k_d^* = rate constant for decay of [*H]

k_d^c = rate constant for decay of $[^{c}H]$

k_d^m = rate constant for decay of $[^{m}H]$

The program used for analysis of competitive binding experiments is given in Appendix A.3.

For each data point supplied to the regression program, a weight calculated as (mean/standard deviation for replicate determinations of bound radioligand) was specified.

It was found that the analysis was more stable numerically when the regression program was estimating the logarithms of the rate constants. Consequently these results are presented as estimate ± standard error on a logarithmic scale.

The standard error for $\log(k_r/k_f)$, i.e. $\log(K_d)$ was calculated as;

$$\sqrt{(s_r^2 + s_f^2 - 2 \cdot p_{rf} \cdot s_r \cdot s_f)}$$

where s_r = asymptotic standard error for $\log(k_r)$

s_f = asymptotic standard error for $\log(k_f)$

p_{rf} = asymptotic correlation of $\log(k_r)$ with $\log(k_f)$

For any parameter, the difference in parameter estimates from two sets of experiments was taken as significant when the difference between the point estimates exceeded two standard errors of the difference, i.e. when

$$\left|(E_a - E_b)\right| > 2 \cdot \sqrt{(s_a^2 + s_b^2)}$$

where E_a and E_b are the point estimates

s_a and s_b are the corresponding standard errors

PAGE 3 BRDPAR LO SALT FUROSEMIDE 2 DAYS

THE RESIDUAL SUM OF SQUARES (= 38702.4) WAS SMALLEST WITH THE FOLLOWING PARAMETER VALUES

PARAMETER	ESTIMATE	ASYMPTOTIC STANDARD DEVIATION	COEFFICIENT OF VARIATION
KF	-6.191949	0.139254	-0.022489
KR	-4.131753	0.631978	-0.152956
RDDP	0.076262	0.008498	0.111434
KRL	-2.700999	1491.615688	-552.245870
L1DHDP	0.000000	0.000000	0.000000
L2DHDP	0.000005	0.000001	0.178209
L3DHDP	0.000004	0.000002	0.405812

THE FINAL VALUE (= 38702.2) WILL BE USED IN FUTURE COMPUTATIONS.
CORRESPONDING PARAMETER VALUES ARE

1 KF	2 KR	3 RDDP	4 KRL	5 L1DHDP	6 L2DHDP	7 L3DHDP
-6.19194	-4.13173	7.6260780-02	-2.70100	9.9999970-04	5.0553470-06	4.2087880-06

CORRELATIONS AND STANDARD DEVIATIONS ARE CONDITIONED UPON
L1DHDP = 1.000000-08

ESTIMATE OF ASYMPTOTIC CORRELATION MATRIX

		KF 1	KR 2	RDDP 3	KRL 4	L1DHDP 5	L2DHDP 6	L3DHDP 7
KF	1	1.0000						
KR	2	0.6614	1.0000					
RDDP	3	-0.0142	0.6277	1.0000				
KRL	4	-0.0349	-0.1639	-0.1366	1.0000			
L1DHDP	5	0.0000	0.0000	0.0000	0.0000	0.0000		
L2DHDP	6	0.1366	-0.3177	-0.7590	0.0751	0.0000	1.0000	
L3DHDP	7	0.0165	-0.2113	-0.4217	0.0423	0.0000	0.3434	1.0000

THE ESTIMATED MEAN SQUARE ERROR IS 1106.

PLOT OF HO VERSUS PREDICTED AND OBSERVED BND AND VERSUS RESIDUALS.

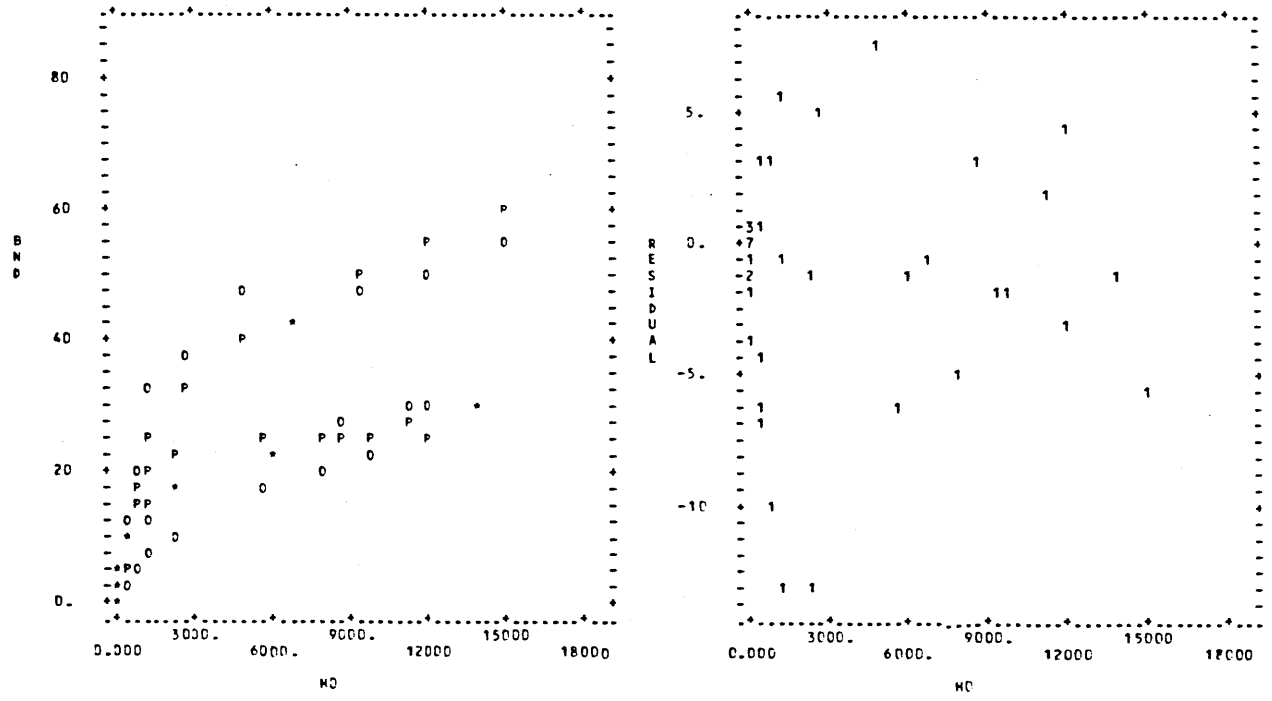

Figure 3.1 The receptor assay: example results

The figure shows part of the print-out from the program used for the receptor assay. The parameters being estimated were; forward (KF) and reverse (KR) rate constants and concentration of sites per unit protein (RODP) for the high-affinity site, the reverse rate constant for the low-affinity site (KRL) and a linear low-affinity component of binding for each of the three experiments contributing to the pooled data (L1DHDP..... L3DHDP). The scatter plots show; (left) observed (O) and predicted (P) values of bound ^{125}I-angiotensin II (BND, the dependent variable) against initial tracer concentration (H0) and (right) the residuals (observed-predicted value of BND) against initial tracer concentration.

CHAPTER 4

CHARACTERISATION OF THE VASCULAR ANGIOTENSIN RECEPTOR

4.1 Introduction

In this chapter the characterisation of the rat mesenteric artery angiotensin receptor and the development of the receptor assay is described. A comparison of the results of this study with those already published is included in this chapter.

All experiments described in this section used arterial tissue obtained from male Sprague-Dawley rats (325-350g) maintained on a standard diet (Oxoid 41B). Unless othewise specified, all incubations were carried out for 60 minutes at 22^{o}C, using the normal assay buffer (50mM-Tris (pH 7.35)/120mM-NaCl/3.6mM-KCl/4.8mM-$CaCl_2$/1.8mM-$MgCl_2$) and ^{125}I-angiotensin II as tracer.

4.2 Specific binding as a function of membrane protein concentration

Specific binding of ^{125}I-angiotensin II to the arterial membrane fraction was not linearly related to membrane protein concentration, indicating the presence of angiotensinase activity (Figure 4.1). Non-specific binding consisted of two components; one which was linear with membrane protein concentration and (not shown) tracer concentration, and a constant component (for a given tracer concentration) due to absorption of tracer to the filter during separation of bound and free radioactivity. Specific binding ranged from 0.5 to 5% of total radioactivity, while non-specific binding averaged 0.43 ± 0.05% (s.e.) of total radioactivity.

4.3 Tracer angiotensin II degradation

(a) Chromatographic analysis of bound and free radioactivity

Tracer degradation was identified by chromatographic analysis of the free radioactivity after incubation under standard conditions (Figure 4.2B). The predominant metabolites were fragments not containing the C-terminal pentapeptide of angiotensin II and consequently were not expected

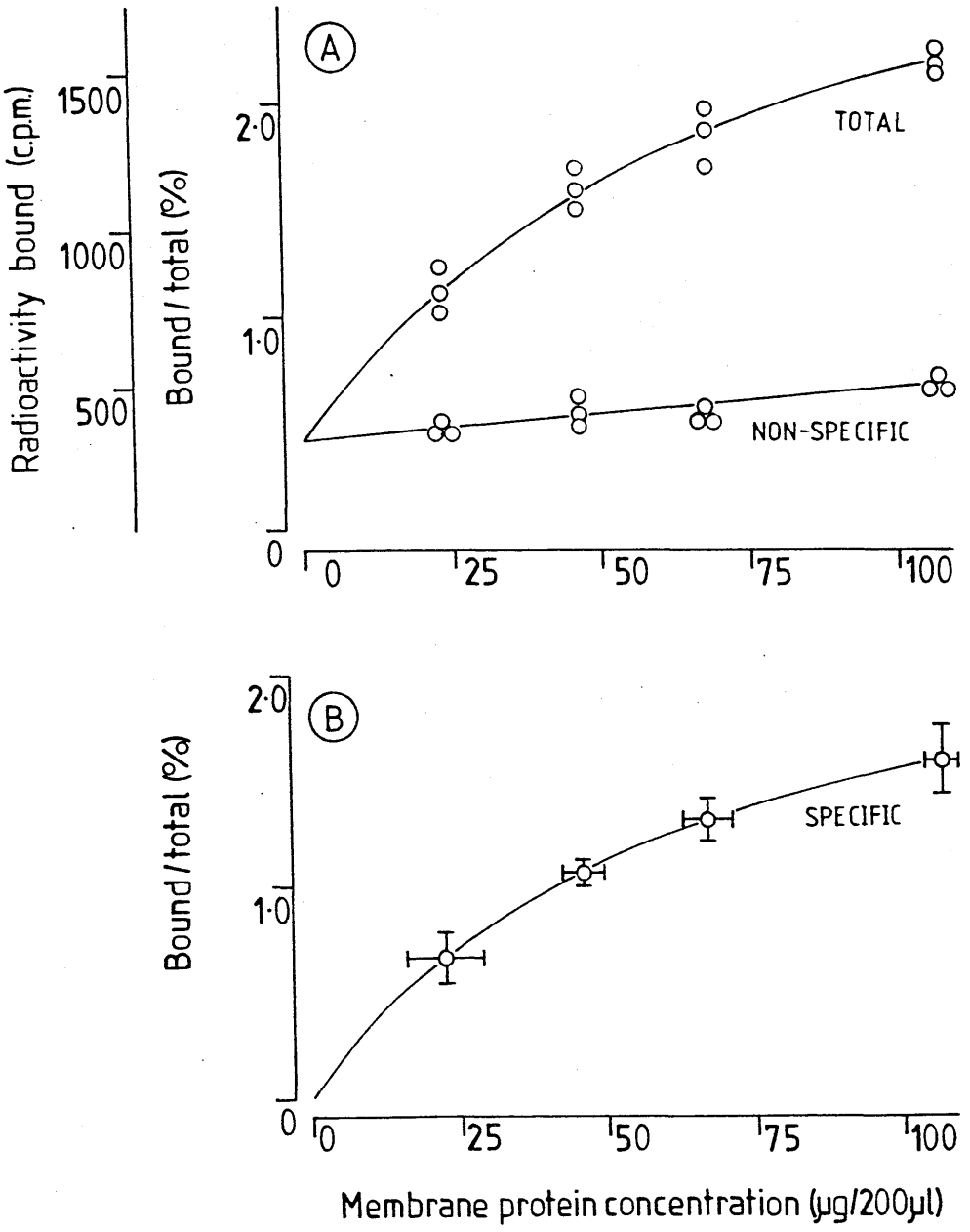

Figure 4.1 **Binding of ^{125}I-angiotensin II as a function of arterial membrane protein concentration**

Incubation: 60 minutes at 22°C, initial ^{125}I-angiotensin II concentration 0.3nM.

(A) points represent individual determinations of total and non-specific binding.

(B) derived from data in (A), points represent mean ± s.d. for triplicate determinations.

to bind to the angiotensin receptor (Bumpus et al, 1961) or to the antiserum used for angiotensin II immunoassay since both require this sequence for binding. This was confirmed by direct binding studies with the angiotensin II antiserum (see below) and by chromatographic analysis of eluted receptor-bound radioactivity (Figure 4.2C). The C-terminal heptapeptide (angiotensin III) was present as a component of the free radioactivity, but proportionately less was observed in the radioactivity eluted from the membrane. Similar results were obtained when these experiments were repeated at a higher initial tracer concentration (20nM), although the heptapeptide then comprised a larger proportion of the eluted radioactivity (Figure 4.2D). Under these conditions approximately 20% of the specifically bound tracer is associated with the low-affinity site (see below). Degradation of the free ligand still occured in the presence of 10uM unlabelled angiotensin II (not shown).

(b) Effect of proteinase inhibitors

A number of proteinase inhibitors were examined for their effect on both binding capacity and tracer angiotensin II degradation (Table 4.1). The effects on binding were determined using short (15 minute) incubations since computer simulations indicated that the early phase of binding was relatively unaffected by changes in the rate of tracer degradation. The effects of the inhibitors on degradation of the tracer angiotensin II was assessed using standard 60 minute incubations. No inhibitor effectively prevented tracer degradation without adversely affecting binding capacity. Tracer degradation was therefore measured by filtrate immunoassay and the appropriate corrections made in the analysis of the binding data.

(c) Accuracy of the filtrate immunoassay

The accuracy of the filtrate immunoassay was determined by allowing a quantity of ^{125}I-angiotensin II to be completely metabolised by

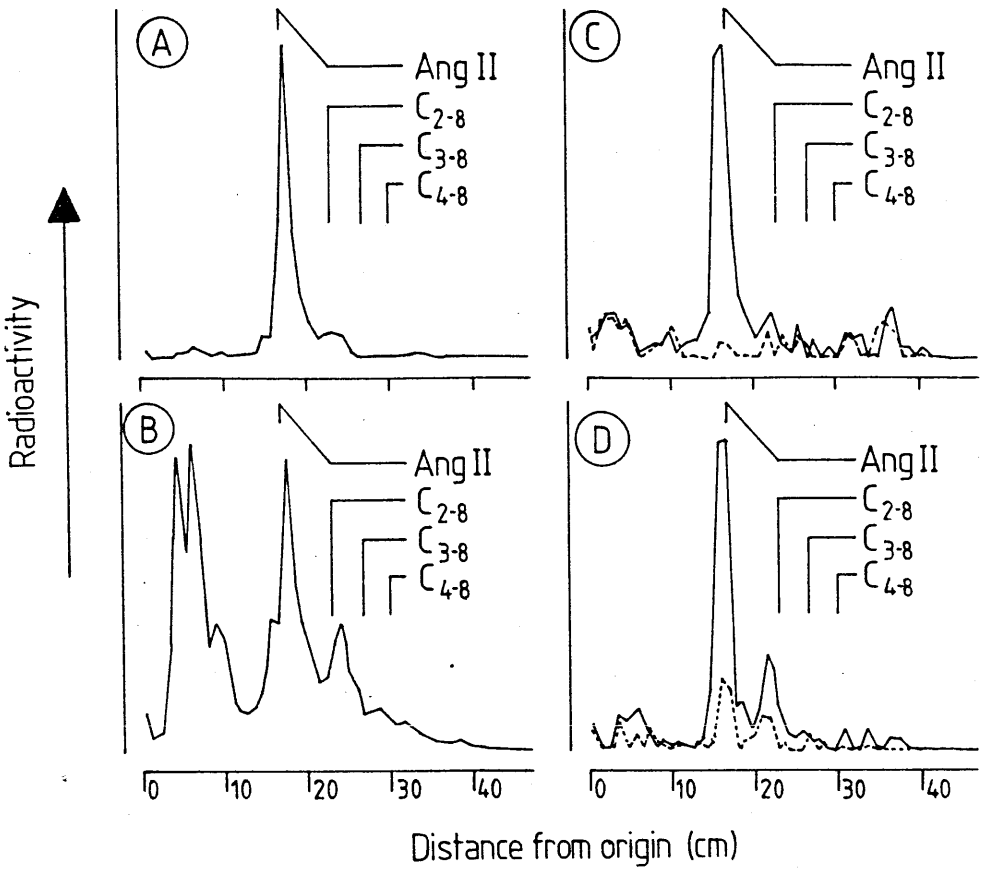

Figure 4.2 Chromatographic analysis of bound and free radioactivity

Incubation: 60 minutes at 22°C, initial ^{125}I-angiotensin II concentration 0.2nM (A-C), 20nM (D).

(A) control incubation, no membrane fraction.

(B) free radioactivity after incubation with 92ug/200ul arterial membrane fraction.

(C) radioactivity eluted from the membrane fraction after incubation with 0.2nM ^{125}I-angiotensin II.

(D) radioactivity eluted from the membrane fraction after incubation with 20nM ^{125}I-angiotensin II.

Identical results for (A) and (B) were obtained when the initial tracer concentration was 20nM (not shown). The broken lines in (C) and (D) denote non-specific binding determined in parallel incubations. The running positions of angiotensin II (Ang II) and the C-terminal hepta- (C_{2-8}), hexa- (C_{3-8}) and penta- (C_{4-8}) peptides is indicated.

Table 4.1

Effect of proteinase inhibitors on binding and degradation of ^{125}I-angiotensin II

Inhibitor	Binding (% of control)	Apparent tracer decay rate constant (% of control)
EDTA	57 ± 9	51
EGTA	81 ± 8	89
Dithiothreitol	67 ± 5	100
PMSF/glucagon	112 ± 10	75
Tos-Phe-CH_2Cl	86 ± 4	75
STI	113 ± 5	95
Trasylol	105 ± 5	94
Trasylol/Tos-Phe-CH_2Cl/STI/glucagon	76 ± 4	71
Trasylol/Tos-Phe-CH_2Cl/STI/glucagon/PMSF	77 ± 5	54
Di-isopropyl phosphite	37 ± 11	11
$HgCl_2$	not done	0

The effect on binding was determined using 15 minute incubations at 22^{o}C (results for triplicate determinations ± 1 s.d.), initial ^{125}I-angiotensin II concentration 0.3nM; the effect on tracer degradation was determined using 60 minute incubations, chromatography of the free radioactivity and calculation of peak areas.

Concentration of inhibitors: EDTA, 5mM; EGTA, 5mM; dithiothreitol, 0.5mM; glucagon, 10uM; Tos-Phe-CH_2Cl, 0.5mM; soya trypsin inhibitor (STI), 15uM; Trasylol, 100k-i.u./ml; phenylmethylsulphonylfluoride (PMSF) 0.1mM; di-isopropyl phosphite, 20mM; $HgCl_2$, 1mM.

the membrane fraction preparation (2 hours incubation at 37°C with 460ug of membrane protein/ml). The angiotensinase enzymes were then inactivated by heating at 100°C for 5 minutes and precipitated protein removed by centrifugation. The resulting metabolite preparation was then mixed with pure ^{125}I-angiotensin II such that the ^{125}I-angiotensin II represented 0, 20, 50, 75 or 100% of the total radioactivity. Samples of each preparation were then subjected to immunoassay or chromatographed and the proportion of radioactivity present as ^{125}I-angiotensin II determined by calculation of peak areas. The results shown in Table 4.2 indicate that the assay allows reliable measurement of tracer degradation.

4.4 Stability of the receptor preparation

The stability of the receptor preparation was assessed by pre-incubating the arterial membrane fraction without tracer angiotensin II prior to incubation with 0.3nM-^{125}I-angiotensin II for 60 minutes at 22°C (Figure 4.3). The receptor preparation was stable at 22°C (8% loss in binding capacity at 60 minutes) but not at 37°C (30% loss after 60 minutes). No change in binding capacity was detected over a 4 hour pre-incubation on ice.

4.5 Kinetics of specific binding

Specific binding of ^{125}I-angiotensin II to the membrane fraction was time and temperature dependent (Figure 4.4). At 22°C binding began to plateau within 60 minutes of incubation while at 37°C binding was maximal at approximately 20 minutes of incubation and subsequently declined probably due to the more rapid metabolism of the tracer angiotensin II at this temperature (Figure 4.4). In all such experiments tracer angiotensin II concentration declined in a simple exponential manner.

Dissociation profiles of specifically bound ^{125}I-angiotensin II from the membrane fraction were identical when the forward (association) reaction was blocked by dilution (1:32) or addition of excess unlabelled

Table 4.2

Accuracy of the filtrate immunoassay

^{125}I-angiotensin II (% of total radioactivity)		
(a) Expected	(b) Immunoassay	(c) Chromatographic
100	100	96
75	78 ± 10	75
50	47 ± 10	54
20	18 ± 8	26
0	-8 ± 5	<10

^{125}I-angiotensin II was mixed with a completely metabolised tracer preparation such that ^{125}I-angiotensin II represented varying proportions of the total radioactivity. (a) Expected proportion; (b) proportion as determined by immunoassay (mean ± s.d. for 6 replicates); (c) proportion as determined chromatographically by calculation of peak areas.

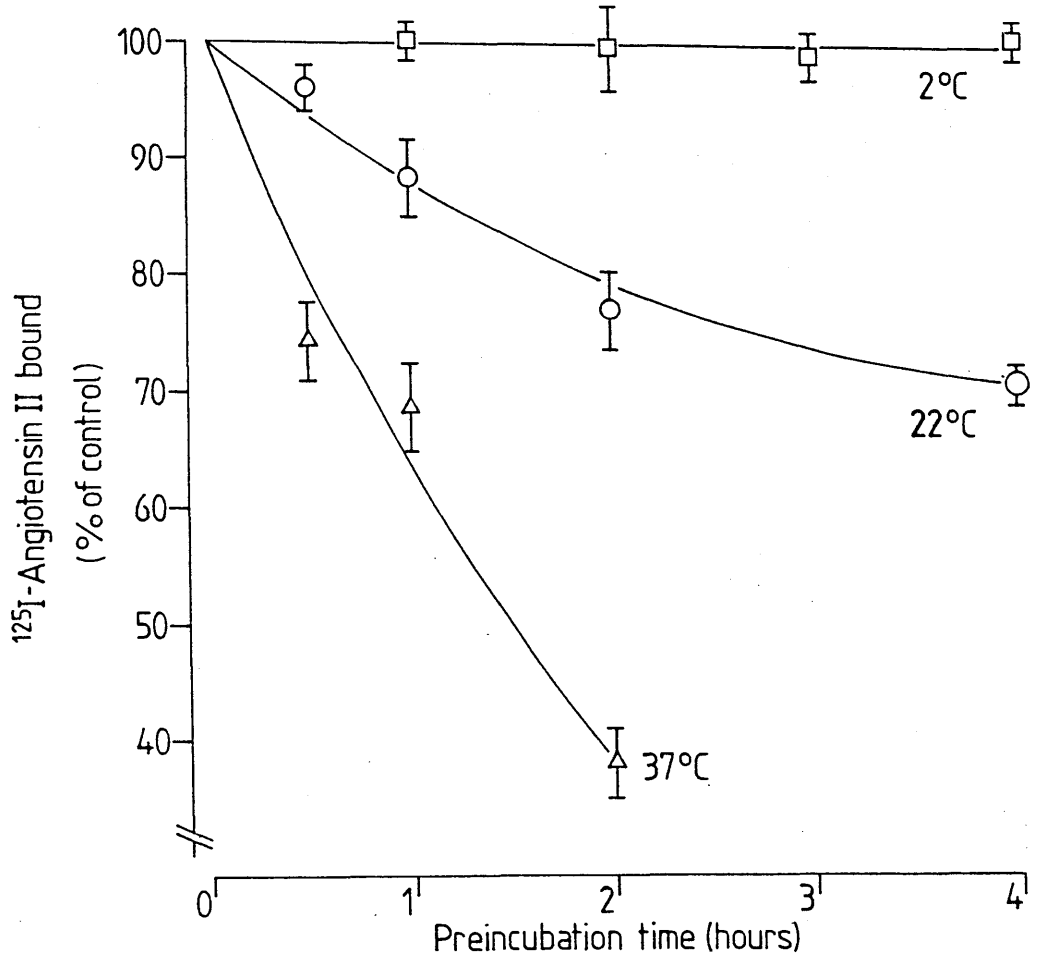

Figure 4.3 Stability of the receptor preparation

The arterial membrane fraction was preincubated as shown prior to a 60 minute incubation at 22°C with 0.3nM ^{125}I-angiotensin II. Points represent mean ± s.d. for 4-6 determinations.

angiotensin II (to 5uM) and deviated markedly from simple exponential decay indicating heterogeneity of binding sites and the absence of site-site co-operative effects. Similar results were obtained at 22^{o}C and 37^{o}C (Figure 4.5).

4.6 Saturability of specific binding

Specific binding was saturable but only at ^{125}I-angiotensin II concentrations which were extremely high in relation to plasma angiotensin II concentrations in the rat (Figure 4.6). Although not quantitatively applicable, Scatchard transformations (Figure 4.6, inset) of the binding data were markedly curvilinear, consistent with the results from the dissociation experiments indicating heterogeneity of binding sites.

4.7 Analysis of the binding data

Application of non-linear regression methods to saturation binding data such as that shown in Figure 4.6 allowed determination of forward rate constants and binding site concentrations, but the reverse rate constants were poorly estimated. Computer simulations indicated that the shape of such binding curves is influenced to a relatively small extent by changes in dissociation rates. There was also a high degree of correlation between the forward and reverse rate constants. Analysis of saturation and dissociation curves together permitted determination of all binding constants (Table 4.3 column(a)). Based on the kinetic constants determined from saturation binding curves, the low-affinity component of binding in the time-course experiments could reasonably be approximated by a straight line parallel to the abscissa (Figure 4.4, broken line). This approximation was necessary in order to analyse the data from such experiments, using a four parameter fitting procedure (three parameters relating to the high-affinity site and the ordinate intercept for the linear component of binding. This approach yielded kinetic constants for the high affinity site in agreement with those derived from saturation

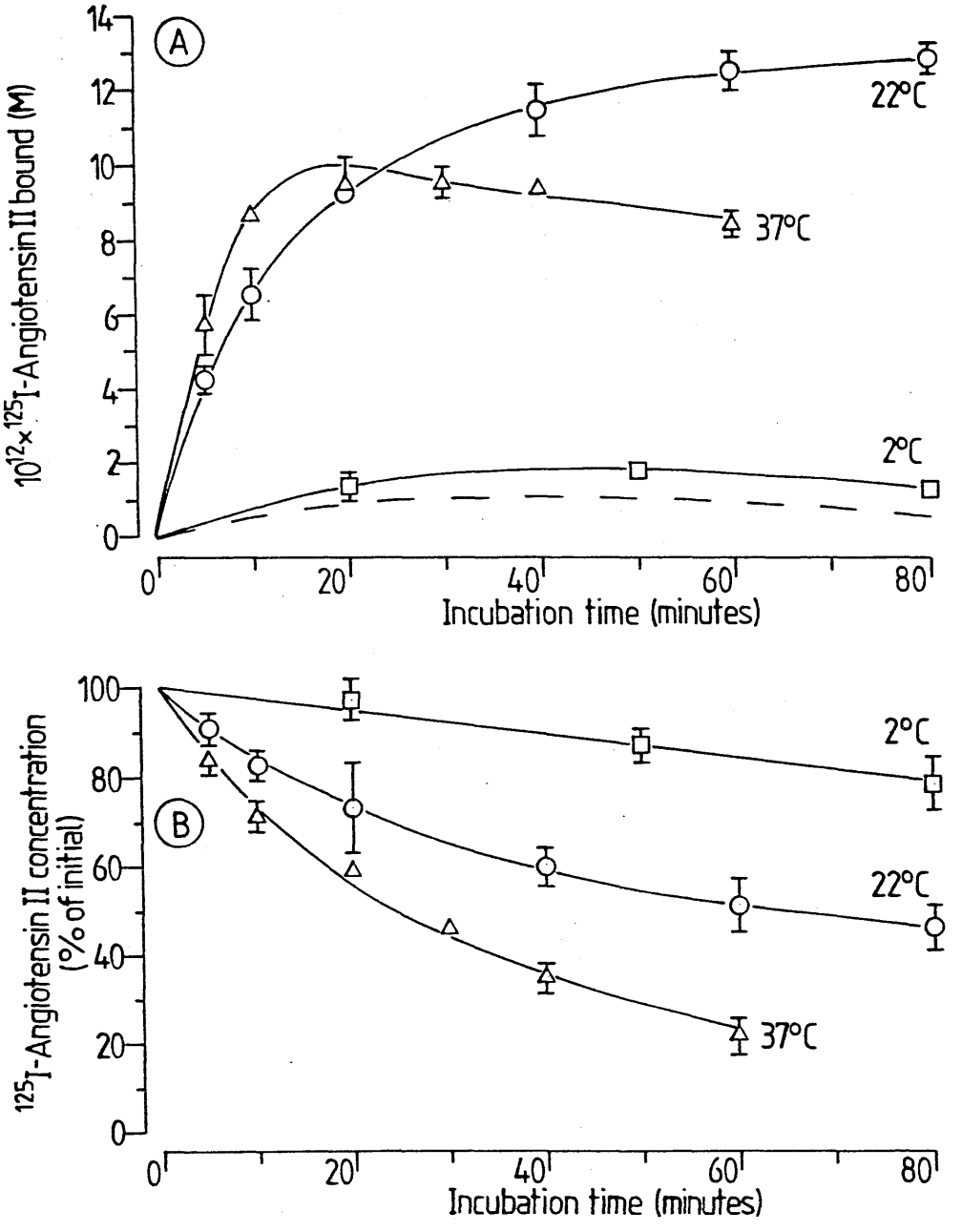

Figure 4.4 Binding and degradation of ^{125}I-angiotensin II as a function of time and temperature

(A) Time and temperature dependence of specific binding of ^{125}I-angiotensin II. Points represent mean ± s.d. for 3 determinations. The broken line denotes the predicted low affinity component of binding at 22°C. Initial ^{125}I-angiotensin II concentration 0.3nM.

(B) Degradation of ^{125}I-angiotensin II determined in the same experiment by filtrate immunoassay. Points represent mean ± s.d. for 3 replicates.

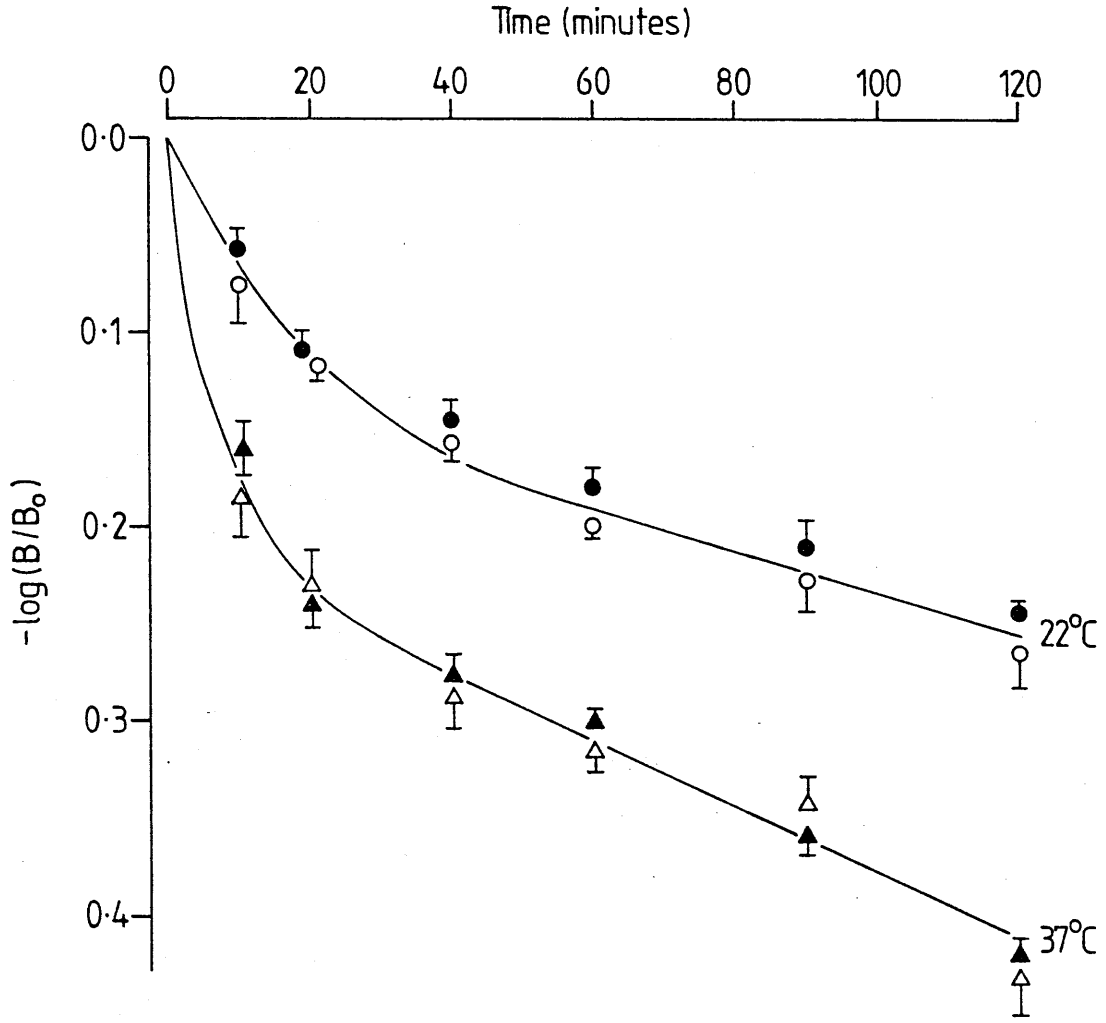

Figure 4.5 **Dissociation of specifically bound ^{125}I-angiotensin II from the arterial membrane fraction as a function of time and temperature**

The arterial membrane fraction was incubated with 0.3nM ^{125}I-angiotensin II for 60 minutes at 22°C or 20 minutes at 37°C and the forward reaction then blocked by addition of excess unlabelled angiotensin II (to 5uM, closed markers) or dilution (1:32, open markers) and the incubation continued as shown. Points denote mean ± s.d. for 2-4 determinations.

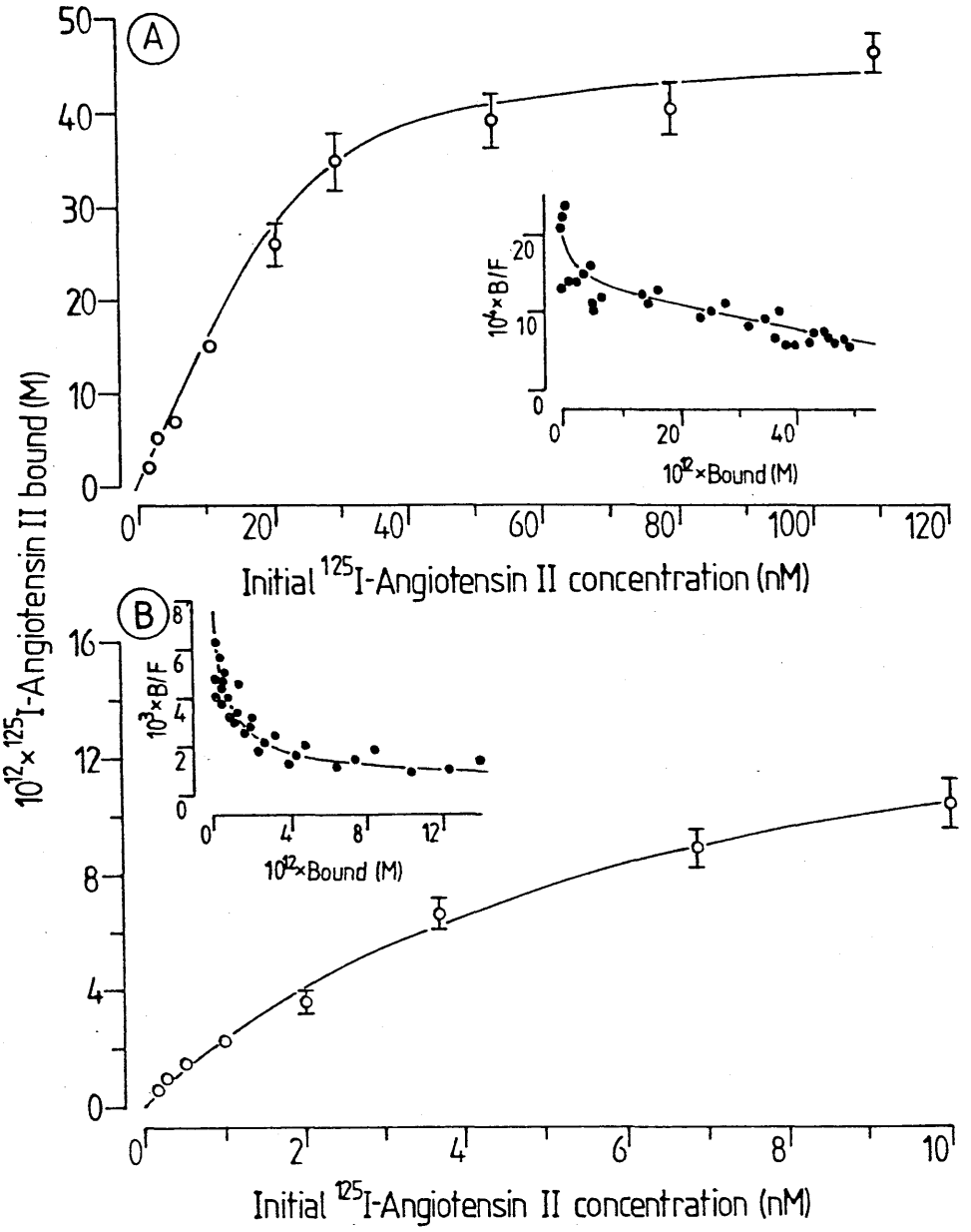

Figure 4.6 **Binding of ^{125}I-angiotensin II to the arterial membrane fraction as a function of initial tracer concentration**

Incubation: 60 minutes at 22°C, points represent mean ± s.d. for 3 determinations.

Ⓐ [^{125}I-angiotensin II] 0-110nM, Ⓑ [^{125}I-angiotensin II] 0-10nM

Insets show Scatchard transformations of the binding data.

binding curves (Table 4.3 column(b)). The binding constants at 37°C (Table 4.3 column(d)) were obtained by simultaneous analysis of all experimental data using the curve-fitting procedure for two classes of binding sites.

4.8 Comparison with other studies

There are few published reports regarding angiotensin receptors in rat mesenteric artery smooth muscle (Gunther et al, 1980a,b; Aguilera & Catt, 1981; Wright et al, 1982; Schiffrin et al, 1983c; Paller et al, 1984); a summary of the results from these studies is given in Table 4.4. Gunther et al (1980a) observed no degradation of the tracer angiotensin II during incubation and hence did not use proteinase inhibitors, while in the other studies the use of EDTA and/or dithiothreitol as angiotensinase inhibitors was required (dithiothreitol in combination with glucagon or EDTA is effective in inhibiting the angiotensinase enzymes in adrenocortical membranes, and does not appear to interfere with angiotensin II binding in this tissue). It is possible that the chromatographic methods used by Gunther et al (1980a) did not have sufficient resolution to detect degradation of the peptide (thin layer chromatography is most often used for this purpose); this may account for the 10-fold lower affinity reported by these authors, since ligand degradation in binding studies will affect mainly the estimate of affinity (Ketelslegers et al, 1975). As shown in Table 4.1, dithiothreitol was ineffective and EDTA only partially effective in inhibiting destruction of the angiotensin II, and both agents are inhibitory on the receptor. The other studies may therefore be doubly compromised by ligand degradation and inadvertant inhibition of angiotensin binding. Alteration of the incubation medium ionic composition can also have a major effect on the receptor (Section 4.12). However, these reports are largely in agreement, with K_d values in the range 0.1-1nM and receptor concentrations in the range 50-100 fmol/mg protein

Table 4.3

Binding constants for ^{125}I-angiotensin II

Parameter	Unit	High-affinity site (a)	(b)	(c)	(d)
Forward rate constant	log [$M^{-1}.s^{-1}$]	6.114 ± 0.187	5.934 ± 0.107	6.255 ± 0.176	6.273 ± 0.114
Reverse rate constant	log [s^{-1}]	-4.337 ± 0.218	-4.376 ± 0.198	-4.366 ± 0.286	-4.003 ± 0.010
Receptor conc^n	fmol/mg protein	85.3 ± 6.5	79.0 ± 4.5	97.2 ± 5.3	93.3 ± 6.9
K_d	log [M]	-10.45 ± 0.22	-10.31 ± 0.23	-10.62 ± 0.34	-10.27 ± 0.15

Parameter	Unit	Low-affinity site (a)	(b)	(c)	(d)
Forward rate constant	log [$M^{-1}.s^{-1}$]	5.041 ± 0.549	4.415 ± -	- -	5.119 ± 0.147
Reverse rate constant	log [s^{-1}]	-2.214 ± 0.235	-2.886 ± -	- -	-2.703 ± 0.050*
Receptor conc^n	fmol/mg protein	680 ± 260	868 ± -	- -	284 ± 61
K_d	log [M]	- 7.26 ± 0.47	- 7.30 ± -	- -	- 7.82 ± 0.17

Results are given as point estimate ± standard error, derived by analysis of (a) separate saturation (n=6) and dissociation (n=4) experiments; (b) combined saturation/dissociation experiments (n=5); (c) association time course experiments (n=5). Values in columns (a-c) were obtained at 22°C. Values in (d) were obtained at 37°C by analysis of all experimental data (n=7). In (b) the parameters relating to the low-affinity site were poorly estimated and standard errors are not given. A parameter estimate differing significantly from that in column (a) is indicated (*).

(this study included), and the differences may be accounted for by methodological factors.

Paller et al (1984) observed a considerably higher receptor concentration; this is may be due to a sex difference since these authors used male and female rats while male rats (Sprague-Dawley in all cases) were used in all the other studies. In a preliminary experiment, female rats were used and the receptor concentration found to be approximately 2.5-fold higher than in male animals (Table 4.4).

In none of the studies cited above was a low affinity class of binding sites observed; this may be due to the use of lower tracer concentrations, which in most cases did not exceed 5nM for saturation binding curves.

4.9 Specificity

Because of the complexity of this radioligand-binding system, initial attempts to demonstrate specificity for angiotensin-related peptides were based on direct binding studies using radiolabeled peptides. However, these studies were hampered by the low affinity of even the best antagonists (sar^1 ile^8 angiotensin II, sar^1 thr^8 angiotensin II), and it was necessary to carry out competitive binding studies using a low concentration of ^{125}I-angiotensin II (0.1nM) which gave a negligible proportion (<2%) of the bound radioligand associated with the low-affinity site (Figure 4.7). The affinity series (equilibrium constants) after correction for tracer and competing ligand degradation was; ^{125}I-angiotensin II = angiotensin II > angiotensin III > sar^1 ile^8 angiotensin II > sar^1 thr^8 angiotensin II > sar^1 gly^8 angiotensin II > angiotensin II C_{3-8} hexapeptide > sar^1 ala^8 angiotensin II > angiotensin II C_{4-8} pentapeptide >> angiotensin I = bradykinin = arginine vasopressin. The $sarcosine^1$ peptides were found to be resistant to degradation by immunoassay and this was confirmed by chromatography (Figure 4.8). The

Table 4.4

Binding constants for the mesenteric artery angiotensin receptor reported in the literature

Gunther et al (1980a)[a]	Assay conditions	Tris HCl (pH 7.2)/5mM $MgCl_2$
	Type of rats	Male Sprague-Dawley
	K_d (nM)	0.91 ± 0.11
	R_o (fmol/mg protein)	53.7 ± 3.0
Aguilera & Catt (1981)	Assay conditions	Tris HCl (pH 7.4)/10mM $MgCl_2$/1mM dithiothreitol/1mM EDTA
	Type of rats	Male Sprague-Dawley
	K_d (nM)	0.83 ± 0.20
	R_o (fmol/mg protein)	43.4 ± 0.7
Schiffrin et al (1983)	Assay conditions	Tris HCl (pH 7.4)/120mM NaCl/2mM $MgCl_2$/1mM EDTA
	Type of rats	Male Sprague-Dawley
	K_d (nM)	0.60 ± 0.10
	R_o (fmol/mg protein)	75.0 ± 5.3
Paller et al (1984)[b]	Assay conditions	Tris HCl (pH 7.4)/120mM NaCl/10mM $MgCl_2$/5mM dithiothreitol
	Type of rats	Male & female Sprague-Dawley
	K_d (nM)	0.97 ± 0.03
	R_o (fmol/mg protein)	316 ± 19
Present study[c]	Assay conditions	Tris HCl (pH 7.35)/120mM NaCl/3.6mM KCl/4.8mM $CaCl_2$/1.8mM $MgCl_2$
	Type of rats	Male Sprague-Dawley
	K_d (nM)	0.013 - 0.098 (95% confidence interval, Table 4.3, column (a))
	R_o (fmol/mg)	78.8 - 91.8 (95% confidence interval, Table 4.3, column (a))
	Type of rats	Female Sprague-Dawley
	K_d (nM)	0.036 - 0.155 (95% confidence interval)
	R_o (fmol/mg)	187.6 - 238.8 (95% confidence interval)

All studies used a 50mM Tris HCl buffer, ^{125}I-angiotensin II and a crude membrane fraction (100,000g sediment).

[a] The study by Wright et al (1982) is from the same research group, headed by R.W. Alexander.

[b] The authors do not specify if these figures are for male or female rats.

[c] Results for female rats are from one experiment only.

(R_o; receptor concentration)

other angiotensin peptides were all degraded at approximately the same rate; in the case of angiotensin I, binding constants could not be calculated as angiotensin II production during incubation accounted for the observed displacement (approximately 5% of the angiotensin I was converted to angiotensin II and another 45% reduced to non-immunoreactive metabolites). The computed affinities for the angiotensin peptides are given in Table 4.5. The kinetic constants for ^{125}I-angiotensin II were in good agreement with those obtained by the more direct methods described above.

The discrimination between angiotensins I, II, III, and the C-terminal hexa- and pentapeptides parallels the physiological activities of these peptides, with angiotensin III possessing one third the pressor activity of the octapeptide and the other peptides essentially inactive (Schwyzer, 1963; Bumpus, 1977; Carey et al, 1978). In Figure 4.7, the sarcosine^{1} peptides appear almost equipotent with angiotensin II in displacing ^{125}I-angiotensin II from the high affinity site; similar results were obtained by Gunther et al (1980a) and Aguilera & Catt (1981). However, correction for degradation of the natural peptides shows that the affinity of the receptor for the analogues is substantially lower than for angiotensin II. This is consistent with the results of Pettinger et al (1975) who found that extremely high plasma concentrations of sar^{1} ala^{8} angiotensin II (30-300ng/ml) were needed to inhibit pressor response to infused angiotensin II in the rat. The finding that the ile^{8} peptide has the highest affinity of all the analogues is also consistent with other pharmacological studies (Davis et al, 1974). These results also support the view expressed in Section 4.8 that the results obtained by Gunther et al (1980a) and Aguilera & Catt (1981) may have been compromised by unrecognised tracer angiotensin II degradation.

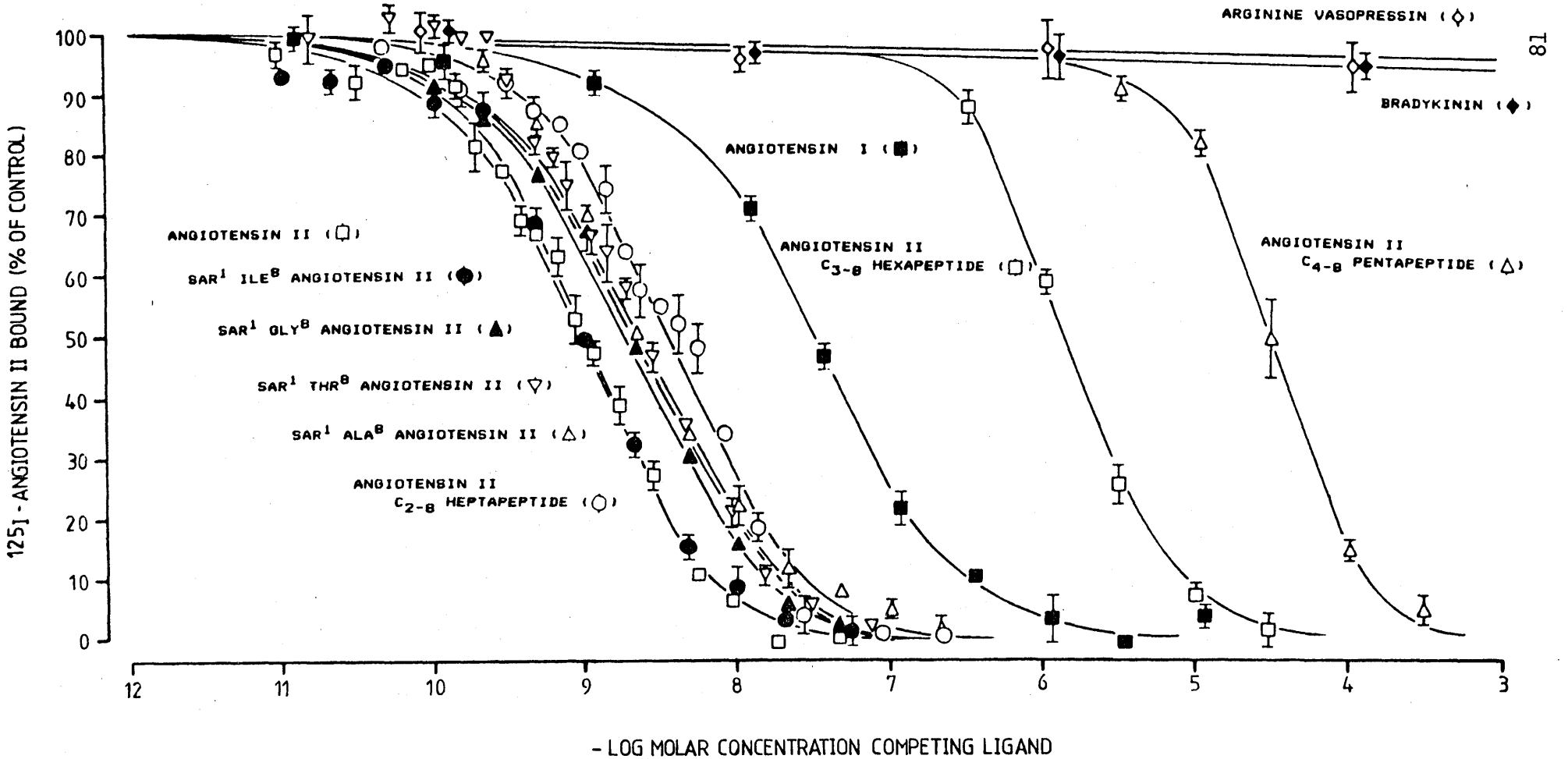

Figure 4.7 **Displacement of ^{125}I-angiotensin II from the high affinity site by angiotensin-related peptides**

Incubation 30 minutes at 22°C, initial ^{125}I-angiotensin II concentration 0.1nM. Points denote mean ± s.d. for 2-4 determinations.

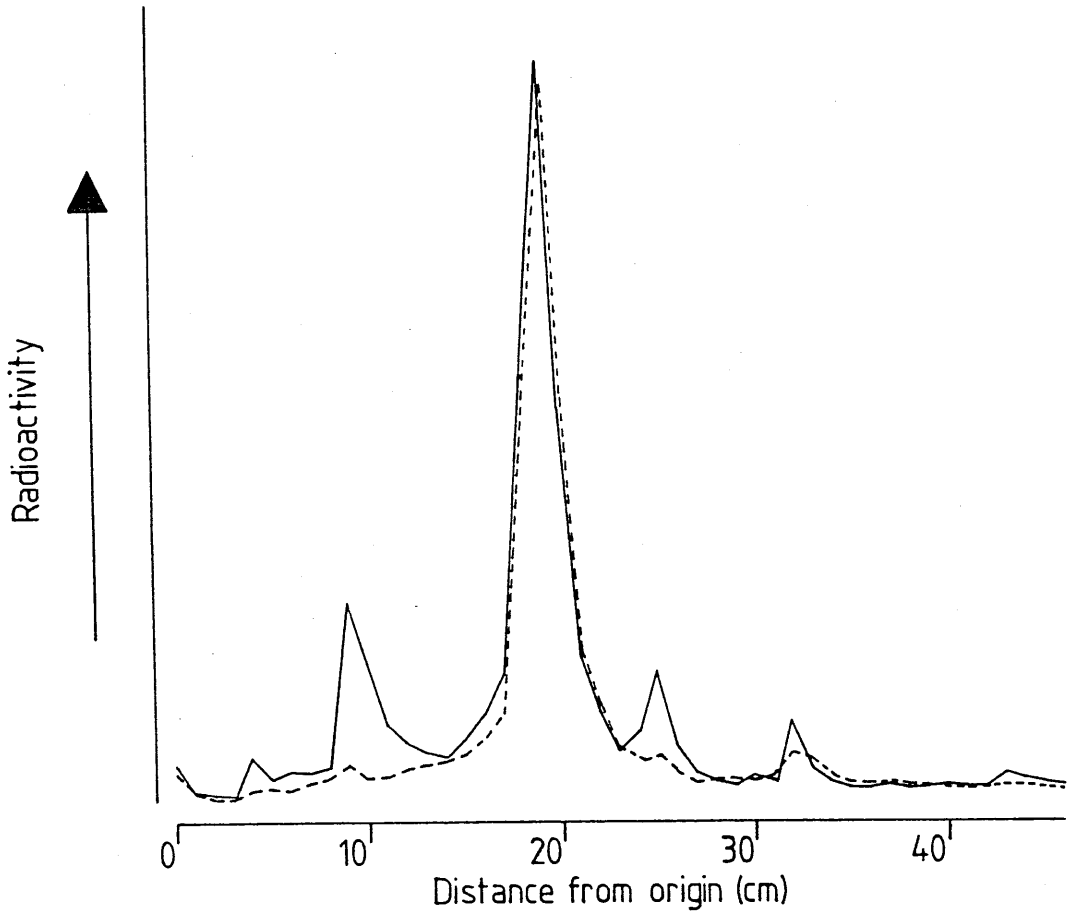

Figure 4.8 **Resistance of sarcosine^{1} peptides to degradation: verification by chromatography**

Chromatographic analysis of free radioactivity after incubation of 0.3nM ^{125}I-sar^{1} thr^{8} angiotensin II with 87ug/200ul arterial membrane fraction for 60 minutes at 22^{o}C. Broken line denotes control (no membrane fraction) incubation. Similar results were obtained for other sar^{1} antagonists (not shown).

Table 4.5

Binding constants for angiotensin-related peptides

Peptide	log [k_f ($M^{-1}.s^{-1}$)]	log [k_r (s^{-1})]	log [K_d (M)]
^{125}I-angiotensin II	6.079 ± 0.076	-4.010 ± 0.155	-10.09 ± 0.13
angiotensin II	5.934 ± 0.106	-4.139 ± 0.210	-10.07 ± 0.20
angiotensin III	5.505 ± 0.000*	-3.961 ± 0.186	-9.47 ± 0.19*
angiotensin II C_{3-8} hexapeptide	4.323 ± 0.065*	-3.696 ± 0.280	-8.02 ± 0.26*
angiotensin II C_{4-8} pentapeptide	2.286 ± 0.002*	-3.154 ± 0.057*	-5.44 ± 0.06*
sar^1 ile^8 angiotensin II	5.423 ± 0.120*	-3.569 ± 0.079*	-8.99 ± 0.13*
sar^1 thr^8 angiotensin II	4.989 ± 0.217*	-3.211 ± 0.215*	-8.20 ± 0.25*
sar^1 gly^8 angiotensin II	5.079 ± 0.111*	-3.018 ± 0.066*	-8.08 ± 0.09*
sar^1 ala^8 angiotensin II	4.850 ± 0.000*	-3.077 ± 0.544	-7.93 ± 0.54*

Results obtained by nonlinear regression analysis of the data depicted in Figure 4.7, presented as point estimate ± standard error. Parameter estimates differing significantly from that for ^{125}I-angiotensin II are indicated (*).

4.10 The receptor assay

While it was possible to demonstrate complete saturability of ^{125}I-angiotensin II binding to the arterial membrane fraction, this was technically difficult due to the small difference between total and non-specific binding obtained when tracer concentrations in excess of 10nM were used. Since it was unlikely that the low affinity sites (K_d=50nM) could interact with angiotensin II at physiological concentrations, the routine receptor assay (for the investigation of physiologically relevant changes in receptor status) was directed only at the high affinity sites.

The receptor assay consisted of a saturation binding curve for ^{125}I-angiotensin II (10 concentrations, 0.05-10nM) and a dissociation time course curve (3 time points over a 2 hour period) since, as noted above, saturation curves alone did not usually allow determination of reverse rate constants. This approach allowed accurate estimation of the binding constants for the high affinity site but those relating to the low affinity site were poorly estimated (Table 4.3 column(b)). Over this concentration range, binding to the low affinity sites is virtually proportional to tracer concentration (Figure 4.6). It was therefore only necessary to determine the coefficient of proportionality rather than the binding constants for a second class of sites. In the analysis of the assay results, the parameters to be estimated were; the forward and reverse rate constants and concentration for the high affinity sites, the coefficients for the linear low affinity component of binding (a separate coefficient for each experiment contributing to the pooled data, since membrane protein concentration varied between experiments and specific binding was not linearly related to membrane protein concentration) and the reverse rate constant for the low affinity sites (due to the dissociation time course curve) (see Appendix A.2 and Figure 3.1). This method yielded binding constants for the high affinity site in close

agreement with those obtained by the direct methods described above (Table 4.6).

4.11 **Biological activity of iodinated angiotensin II**

The competitive binding experiments (Section 4.9) showed that the receptor did not discriminate between ^{125}I-angiotensin II and the native peptide (Table 4.5). Gunther et al (1980a) also found the mesenteric artery receptor to display equal affinity for ^{125}I- and ^{3}H- labelled angiotensin II and similar results have been obtained for the bovine adrenocortical receptor by Glossman et al (1974c). These findings conflict with earlier reports that the tyrosyl monoiodinated peptide retains 25-70% of the biological activity of the native peptide, as determined by pressor assay in rats and contractile response assay using visceral and uterine smooth muscle (Lin et al, 1970; Kurcbart et al, 1971; Papadimitriou & Worcel, 1974).

It was therefore of interest to re-examine the biological activity of the iodinated peptide. This was achieved by measuring the pressor responses to bolus injections of pure ^{125}I-angiotensin II and angiotensin II (Hypertensin Ciba) (Figure 4.9) in the anaesthetised, sympathetically blocked rat using the methods of Lever et al (1964). The results indicate that the iodinated peptide retains full biological activity. The labelled peptide actually appeared to be slightly more potent than angiotensin II (5ng ^{125}I-angiotensin II gave a pressor response equivalent to that of 7ng angiotensin II); this may be due to the higher purity of the ^{125}I-angiotensin II obtained from New England Nuclear. It seems likely that the reduced activity of the iodinated peptide in the earlier studies was due to impurity of the preparation; in these earlier studies the peptide was purified by ion-exchange chromatography on soft gels, whereas the ^{125}I-angiotensin II obtained from New England Nuclear is purified by reverse-phase high performance liquid chromatography.

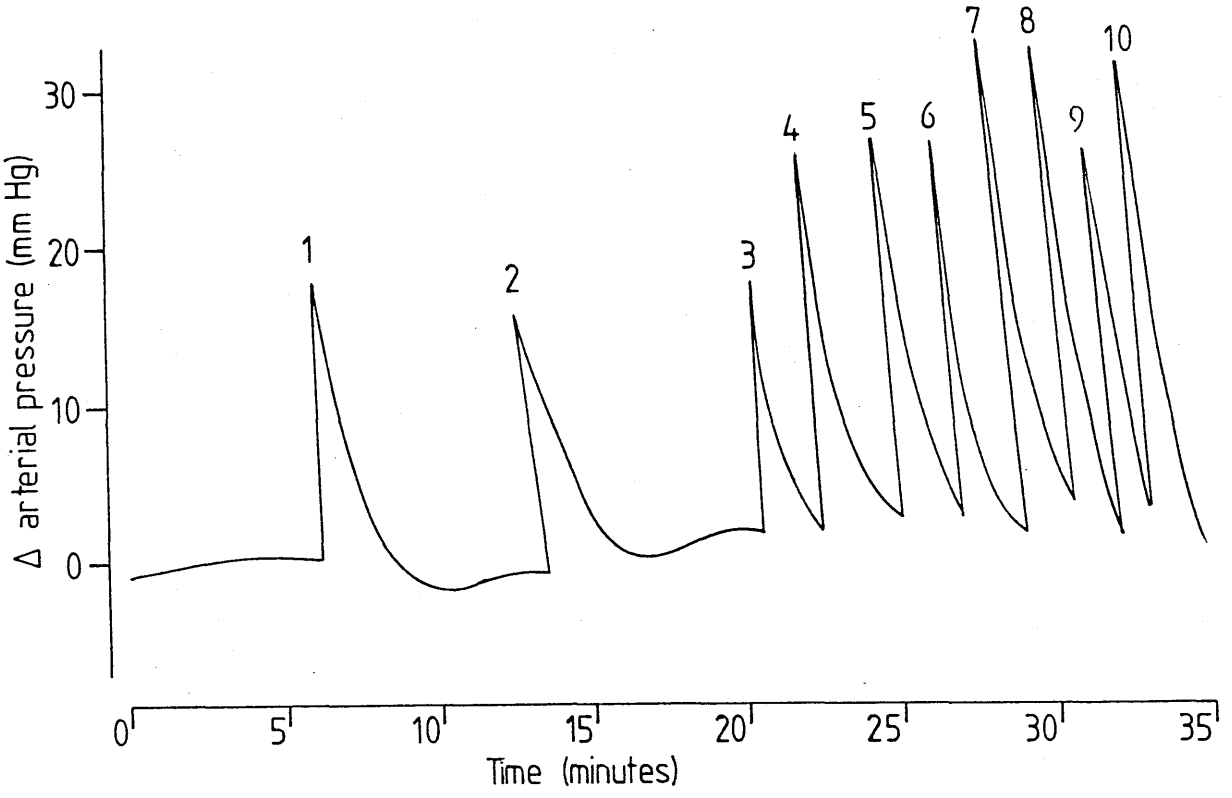

Figure 4.9 **Bioassay of ^{125}I-angiotensin II**

Angiotensin II amide (Hypertensin Ciba) or ^{125}I-angiotensin II were given as bolus injections through a jugular vein cannula and changes in arterial pressure monitored via a carotid artery catheter and pressure transducer.

1) 5ng Hypertensin
2) 4ng Hypertensin
3) 4ng Hypertensin
4) 5ng ^{125}I-angiotensin II
5) 7ng Hypertensin
6) 5ng ^{125}I-angiotensin II
7) 10ng Hypertensin
8) 9ng Hypertensin
9) 5ng ^{125}I-angiotensin II
10) 9ng Hypertensin

4.12 **Factors influencing specific binding**

The effects of various cations on binding of ^{125}I-angiotensin II were investigated (Figure 4.10). As in Section 4.3b, short incubations (15 minutes) were used to minimise effects of alteration in tracer degradation rate on binding. Sodium showed a biphasic effect, enhancing binding in the absence of other cations with a maximum effect at 100mM. Although the effect of sodium on binding was small, this biphasic response was seen consistently. In the presence of 150mM sodium, potassium and the potassium channel blocking agent tetraethylammonium also gave a biphasic effect while potassium alone simply increased binding to a small extent. In contrast to the weak effects of monovalent cations, divalent cations had a pronounced effect on binding, calcium being most potent. When maximal stimulation of binding by calcium had been achieved, a similar but more marked biphasic effect of sodium was observed, suggesting that these ions act by independent mechanisms, but at concentrations greater than 100mM sodium can interfere with the action of calcium. In the absence of added cations, chelating agents were inhibitory, suggesting that calcium and/or magnesium tightly bound to the cell membrane is carried through the tissue processing procedures. Using the methods of Gunther et al (1980a), Wright et al (1982) obtained qualitatively very similar findings. The inhibitory effect of lithium may be due to displacement of bound calcium; after pre-treatment of the membrane fraction with EDTA/EGTA, the inhibitory effect of lithium was greatly reduced, and the stimulatory effect of calcium was abolished in the presence of 25mM lithium (results not shown). Wright et al (1982) also found lithium to be inhibitory and to abolish the inhibition of binding by guanine nucleotides (which was dependent on divalent cations).

To determine whether calcium affected the receptor concentration or affinity, the ionic content of the assay incubation medium was modified as

described in Section 2.5b. The results (Table 4.6) show that calcium influences receptor number without change in affinity. Similar findings have been reported for cardiac (Wright et al, 1983; Baker et al, 1984) and uterine (Douglas et al, 1982) smooth muscle, but Wright et al (1982) report that both calcium and sodium increase the affinity of the mesenteric artery receptor. This discrepancy may be accounted for by the methodological factors discussed in Section 4.8. The effect of calcium was also completely reversible; exposure of the arterial membrane fraction to high [Ca^{++}], recovery by centrifugation and assay in low [Ca^{++}] gave a receptor concentration identical to that obtained when the assay was carried out directly in low [Ca^{++}] (Figure 4.11).

In all tissues where the effect of guanine nucleotides on the binding of angiotensin II has been investigated, an inhibitory action was observed (see Chapter 1). This also applies to the rat mesenteric artery preparation, and is specific for GTP and the non-hydrolysable analogue Gpp(NH)p (Figure 4.12). Gpp(NH)p appears 30-fold more potent, indicating rapid hydrolysis of GTP by the membrane fraction. Wright et al (1982) also report an inhibitory effect (reduction in affinity) of guanine nucleotides in this tissue, and have proposed that GTP and mono/divalent cations interact to regulate the receptor. However, receptor assays in low, normal and high [Ca^{++}] in the presence of 100uM Gpp(NH)p suggest that GTP and calcium act through independent mechanisms; affinity was reduced 10-fold in all cases, but receptor number was still influenced by calcium although it was about 30% less (all cases) than in the absence of Gpp(NH)p (Table 4.6). The action of guanine nucleotides appears to require Mg^{++} (Wright et al, 1982; Gilman, 1984). For this reason, the effect of Gpp(NH)p under low [Ca^{++}] conditions was determined in low [Ca^{++}] buffer without chelating agents and containing 200uM $MgCl_2$ after washing the membrane fraction with the EDTA/EGTA-containing assay buffer.

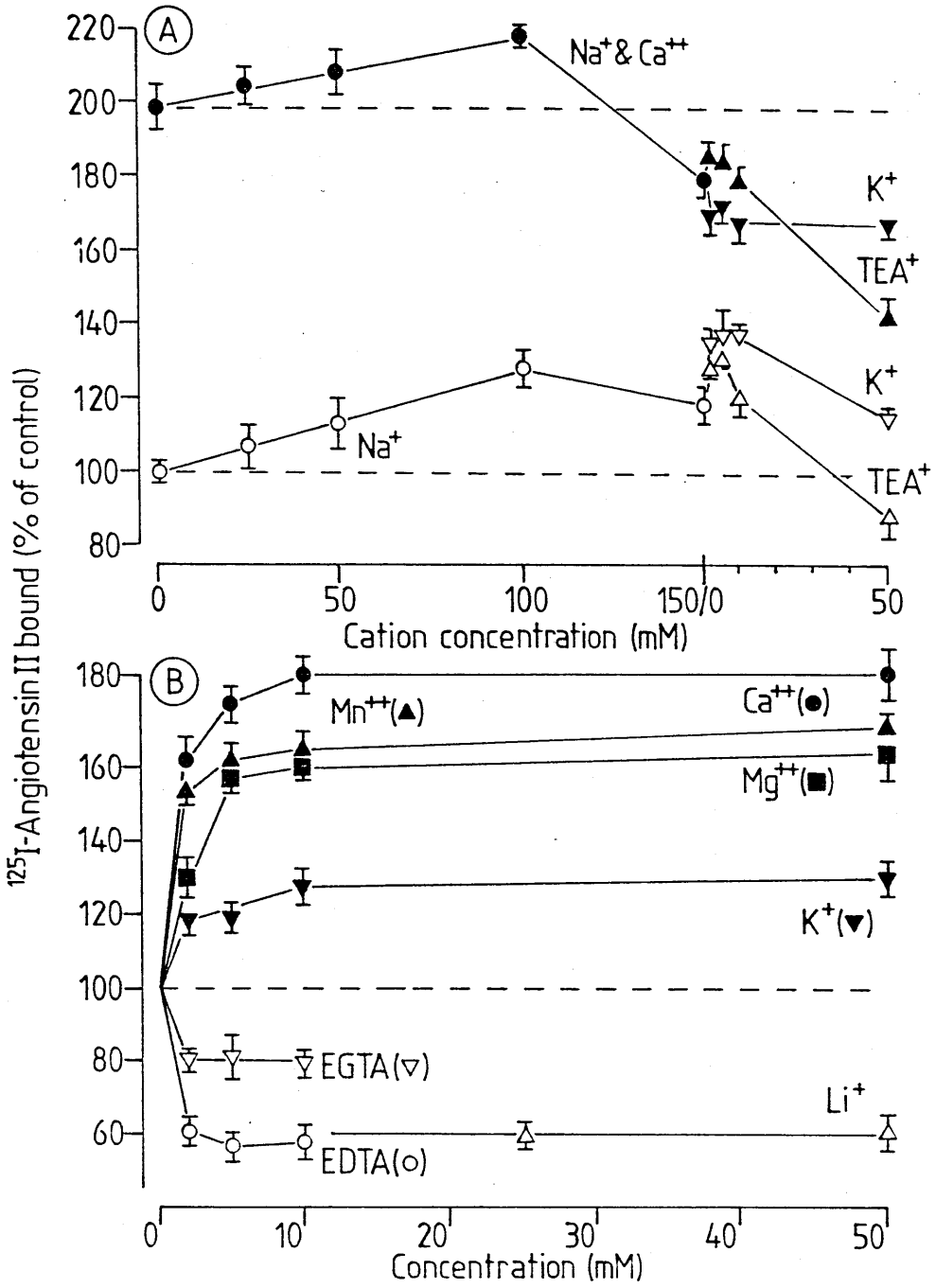

Figure 4.10 **Effect of mono- and divalent cations on specific binding of ^{125}I-angiotensin to the arterial membrane fraction**

Incubation: 15 minutes at 22°C, initial ^{125}I-angiotensin II concentration 0.3 nM. Points denote mean ± s.d. for 3-6 determinations.
(A) Open markers; effect of sodium (25-150mM) and potassium or tetraethylammonium (TEA) (2, 5, 10 & 50mM) in the presence of 150mM sodium. Closed markers; as before but in the presence of 25mM calcium.
(B) Effect of divalent cations, chelating agents, potassium and lithium.

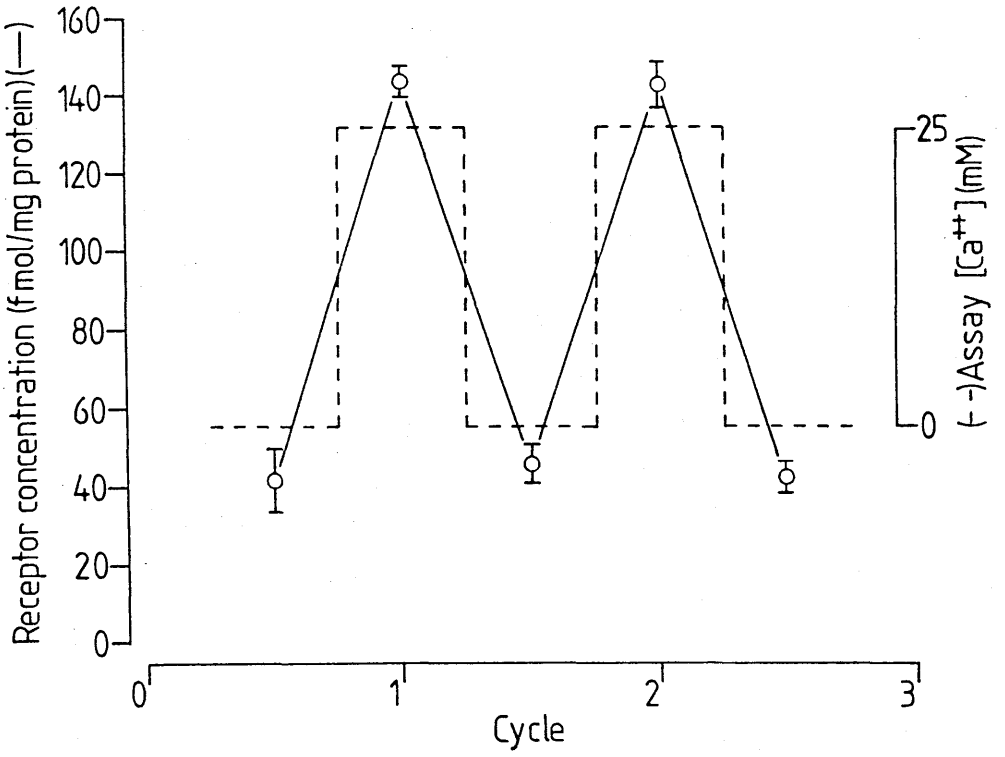

Figure 4.11 Reversible effect of calcium on apparent receptor density

The arterial membrane fraction was alternately suspended in low-[Ca^{++}] and high-[Ca^{++}] assay buffer, and at each suspension samples were taken for assay of protein and measurement of ^{125}I-angiotensin II binding. Between suspensions the membrane fraction was recovered by centrifugation for 30 minutes at 100,000g at 10°C in the buffer used for the previous suspension. In each experiment, each sample of the membrane fraction was incubated with two concentrations of ^{125}I-angiotensin II (60 minutes at 22°C); these concentrations varied between experiments, allowing complete saturation binding curves to be constructed over a number of experiments. The figure shows the changes in receptor density (point estimate ± standard error) determined by analysis of data from 4 experiments using the multiple receptor density method (Section 3.2b). No change in receptor affinity was observed.

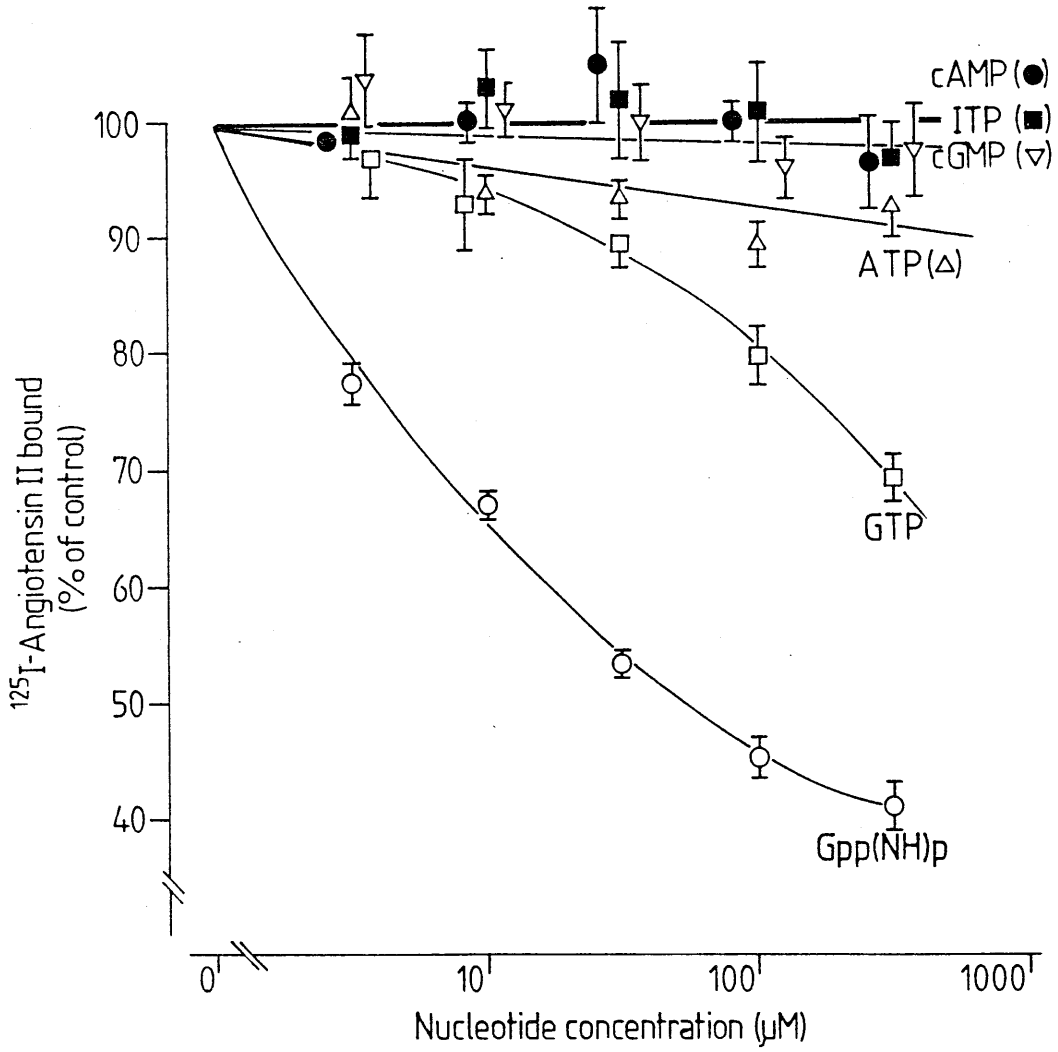

Figure 4.12 Effect of nucleotides on ^{125}I-angiotensin II binding

Incubation: 15 minutes at 22°C, initial ^{125}I-angiotensin II concentration 0.3nM. Points denote mean ± s.d. for 2-4 determinations.

Table 4.6

Effect of Ca^{++} and Gpp(NH)p on the mesenteric artery angiotensin receptor

-Gpp(NH)p

Assay $[Ca^{++}]$	log $[k_f\ (M^{-1}.s^{-1})]$	log $[k_r\ (s^{-1})]$	Receptor $conc^n$ (fmol/mg)
High	6.023 ± 0.080	-3.910 ± 0.310	156.2 ± 4.6*
Normal	5.897 ± 0.052	-4.208 ± 0.165	102.2 ± 4.2
Low	5.802 ± 0.136	-3.795 ± 0.013*	49.9 ± 4.0*

+Gpp(NH)p (100uM)

Assay $[Ca^{++}]$	log $[k_f\ (M^{-1}.s^{-1})]$	log $[k_r\ (s^{-1})]$	Receptor $conc^n$ (fmol/mg)
High	4.729 ± 0.134	-3.967 ± 0.083	115.8 ± 18.4
Normal	4.969 ± 0.108	-4.044 ± 0.023	69.1 ± 15.5
Low	4.631 ± 0.277	-4.294 ± 0.660	29.0 ± 12.2*

The composition of the assay buffer was modified as described in Section 2.5b, and where required Gpp(NH)p was added. The results were obtained using the receptor assay (Section 4.10) and are presented as point estimate ± standard error. For experiments in the absence of Gpp(NH)p, n=5 (each), with Gpp(NH)p, n=3 (each). A parameter estimate differing significantly from that obtained with the normal $[Ca^{++}]$ assay in each group (with or without Gpp(NH)p) is indicated (*).

CHAPTER 5

REGULATION OF THE VASCULAR ANGIOTENSIN RECEPTOR

5.1 Introduction

As described in Chapter 1, regulation of the smooth muscle angiotensin receptor usually involves a change in receptor number directionally opposite to the change in plasma angiotensin II concentration. In view of the effect of Ca^{++} on receptor number described in Section 4.12 it was of interest to examine the role (if any) of Ca^{++} in receptor regulation. Alteration of sodium balance has long been associated with reciprocal changes in plasma angiotensin II concentration and pressor sensitivity to the peptide, and provided a convenient experimental system in which to study the regulation of the mesenteric artery receptors.

5.2 Manipulation of plasma angiotensin II concentration

Sustained alterations in plasma angiotensin II concentration were brought about through variation of sodium balance and by administration of captopril, an angiotensin converting enzyme inhibitor (Table 2.1). The effect of these dietary and drug regimens is shown in Figure 5.1. As expected, sodium depletion caused an increase in plasma angiotensin II concentration which was considerably enhanced by administration of a diuretic at the commencement of the low salt diet. Sodium loading and converting enzyme blockade were equally effective in suppressing plasma angiotensin II concentration.

5.3 Associated receptor changes: normal [Ca^{++}] assay

Little is known regarding the time course of receptor changes following perturbation of plasma angiotensin II concentration. Bellucci & Wilkes (1984) observed a progressive change in glomerular angiotensin receptor density for five days after alteration of sodium balance in the rat and a similar course is apparent in the adrenal cortex (Aguilera et al, 1980b). The effects of the dietary and drug manipulations were

therefore determined at two (possible early phase), five and twelve (established phase) days after commencement of each protocol. The changes in receptor density determined with the normal [Ca^{++}] assay are shown in Figure 5.2. No consistent change in receptor affinity was seen (Table 5.1). Agreeing with the generally accepted model of vascular angiotensin receptor regulation, reciprocal changes in receptor density were observed when plasma angiotensin II concentration was elevated by sodium depletion or suppressed by converting enzyme blockade. However, sodium loading (which was equally effective with captopril in suppressing plasma angiotensin II concentration) caused a slow fall in receptor density. Sodium loading has previously been shown to have no effect on the uterine angiotensin receptor (Devynck et al, 1979) and earlier reports of receptor up-regulation with suppression of plasma angiotensin II concentration had used bilateral nephrectomy (Devynck & Meyer, 1976). Aguilera & Catt (1981) observed up-regulation of the mesenteric artery receptors after 4 days of sodium loading [0.3% (w/w) NaCl diet] but Schiffrin et al (1983c) have reported a slight fall (not significant by their methods) in receptor density in this tissue with 10 days of sodium loading (drinking 1% saline). Gunther et al (1980b) did not investigate the effect of sodium loading.

The sodium loaded animals showed no obvious sign of ill-health (such as weight loss) due to drinking saline, possibly because the large rats used in this study were better able to tolerate a high sodium intake. To exclude the possibility that a non-specific effect was responsible for the fall in receptor density, the five and twelve day sodium loading experiments were repeated with the salt administered in the food only (Table 2.1). The results were identical with those for the saline-drinking animals (Table 5.1).

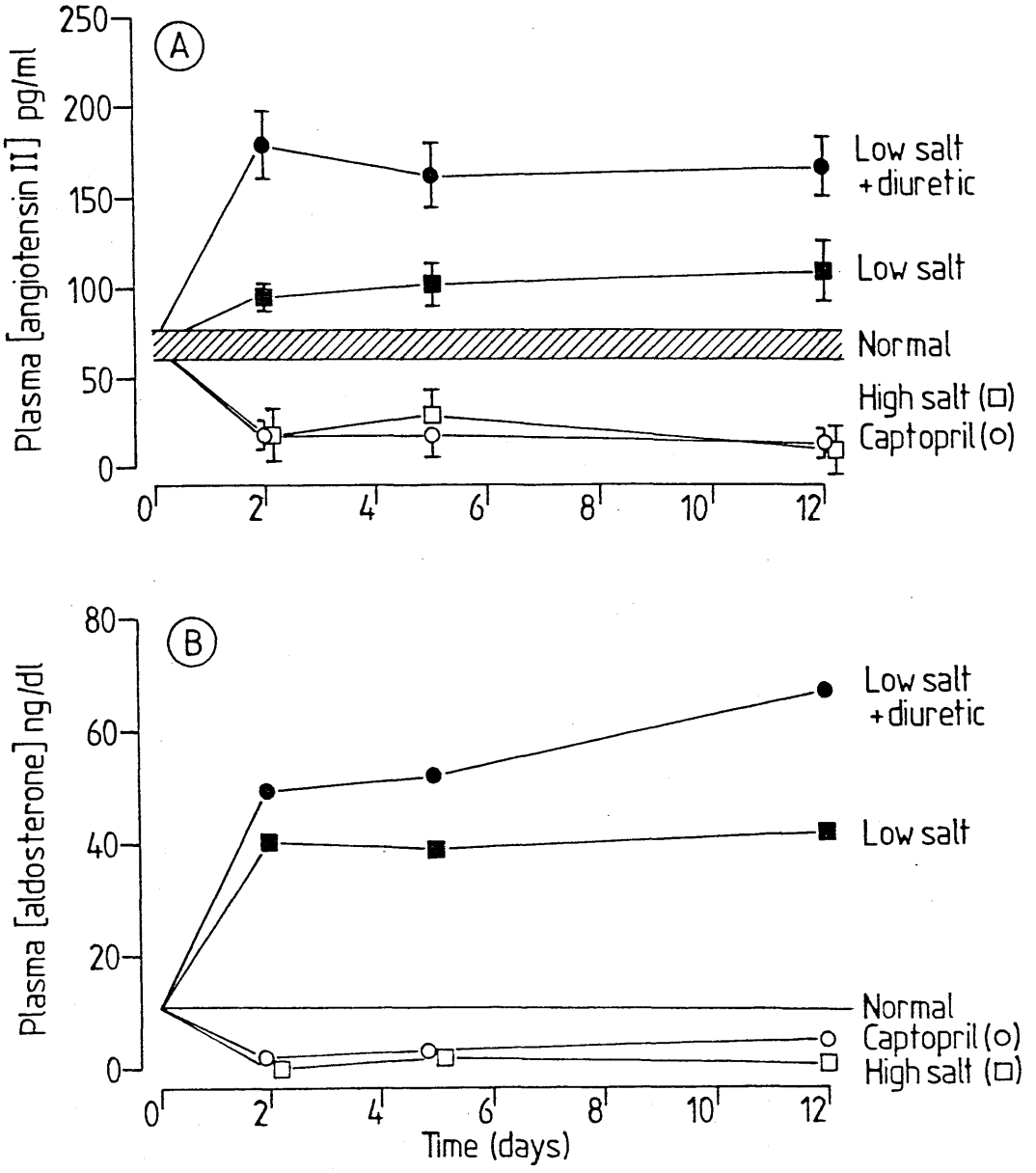

Figure 5.1 **Effect of dietary and drug regimens on plasma angiotensin II and aldosterone concentrations**

(A) plasma angiotensin II concentration, points denote mean ± s.e. for at least 12 rats.

(B) plasma aldosterone concentration, points represent mean for 2 or 4 determinations, each determination made using plasma pooled from 4 rats. At 12 days, the difference between Captopril and High salt groups is significant ($p<0.05$, unpaired t-test)

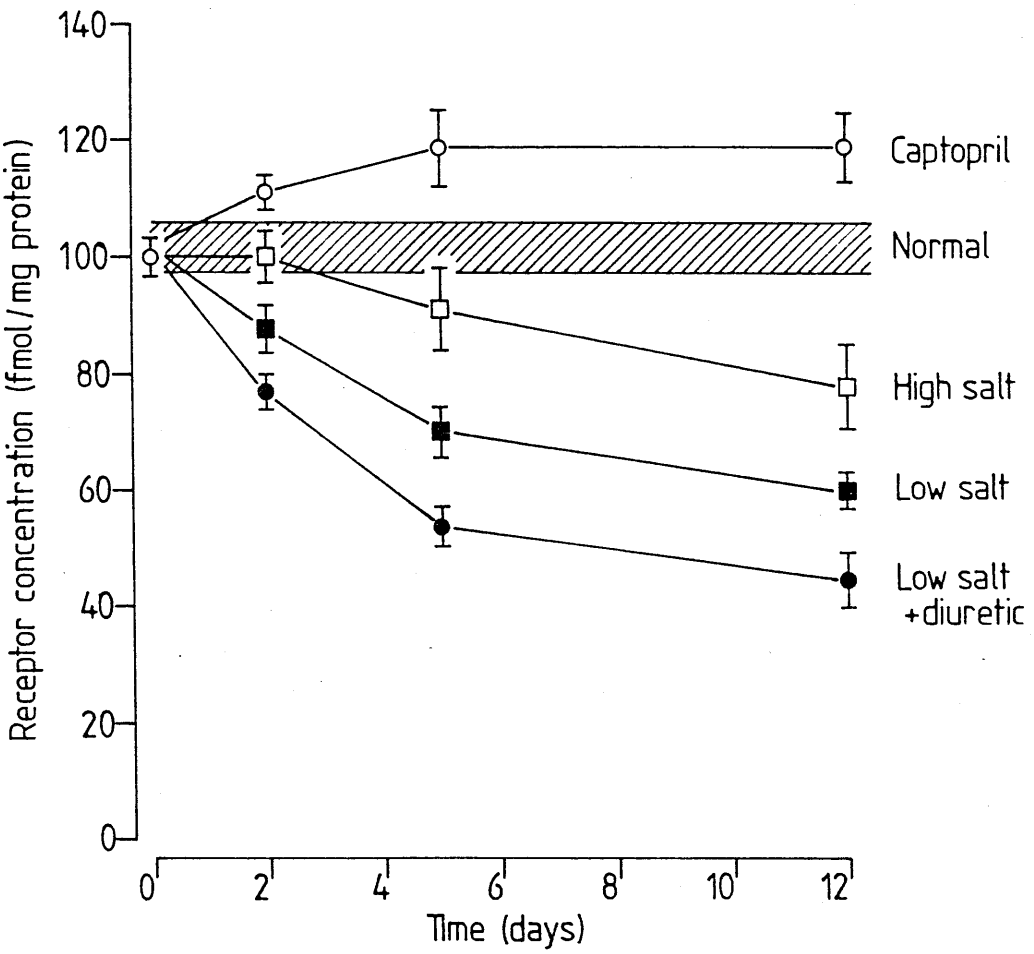

Figure 5.2 **Changes in receptor density associated with manipulation of plasma angiotensin II concentration: normal [Ca^{++}] assay**

Results obtained using the receptor assay (Section 4.10), presented as point estimate ± standard error for at least 3 experiments (12 rats per experiment). Actual values are given in Table 5.1. The effect of acute captopril administration (1 hour before sacrifice) is shown at Time 0.

Gunther at al (1980b) have shown that the decrease in mesenteric artery angiotensin receptor number associated with sodium depletion is prevented by converting enzyme blockade. Preliminary experiments (not shown) have given results agreeing with this observation. Also, Aguilera & Catt (1981) have shown that infusion of angiotensin II causes a dose-dependent fall in receptor density in this tissue. These findings indicate that the effect of sodium depletion on receptor density is due to the rise in plasma angiotensin II concentration. The differing effects of sodium loading and captopril suggest that some other factor also acts on the vascular receptors and that this factor is linked with sodium balance. Schiffrin et al (1983c) have proposed that aldosterone may regulate the vascular receptors. Plasma aldosterone concentration was consistently higher during captopril administration than in sodium loading but only significantly so at twelve days (Figure 5.1). This may have been due to a change in potassium balance.

Atrial natriuretic peptide (ANP) has been reported to antagonise both the vascular (Kleinert et al, 1984) and adrenocortical (Chartier et al, 1984) responses to angiotensin II in vitro, and the natriuretic activity of this peptide suggests a role in the regulation of sodium balance. Using an assay recently developed in the Blood Pressure Unit (B.J. Leckie, G.D. McIntyre, M. Richards & G. Tonolo) no change in plasma ANP concentration with alteration of sodium balance was evident; however this assay has not been fully validated, and there may be a high molecular weight substance in plasma which interferes with the assay (results not shown).

5.4 **Prior receptor occupancy**

To exclude the possibility that endogenous angiotensin II bound to the receptors and carried through the tissue processing procedures could interfere with the receptor assay, rats maintained on a normal sodium diet

were given captopril (50mg/kg) by intraperitoneal injection 1 hour before sacrifice and removal of arterial tissues for receptor assay. No effect on the receptors was observed (Figure 5.2). The lag between the change in plasma angiotensin II concentration and the change in receptor density also suggests that prior receptor occupancy by endogenous angiotensin II did not account for the observed changes in receptor density.

5.5 Associated receptor changes: high and low [Ca^{++}] assay

To investigate the possible role of Ca^{++} in receptor regulation, the two and twelve day experiments were repeated with the receptor assays performed under low or high [Ca^{++}]. The results for the two day experiments are shown in Figure 5.3. No consistent change in receptor affinity was observed (Table 5.1). For each dietary/drug regimen the same values of receptor density were obtained in high [Ca^{++}] and low [Ca^{++}] as for control animals in the same [Ca^{++}] assay. Considering the results presented in Section 4.12, this would suggest that two days after alteration of plasma angiotensin II concentration there is no net change in receptor number but that a proportion of the receptors have been masked or inactivated through a mechanism which is reversed by high [Ca^{++}].

With the normal [Ca^{++}] assay, receptor density appeared significantly higher after twelve days of converting enzyme blockade and significantly lower after twelve days of sodium loading (Figure 5.2). However, for both groups the same values of receptor density were obtained in high [Ca^{++}] and low [Ca^{++}] as for control animals in the same [Ca^{++}] assay (Figure 5.4). This suggests that more prolonged suppression of plasma angiotensin II concentration also has no effect on net receptor number and that the factor responsible for the differing effects of converting enzyme blockade and sodium loading acts by modulating the putative Ca^{++}-antagonised receptor-masking mechanism.

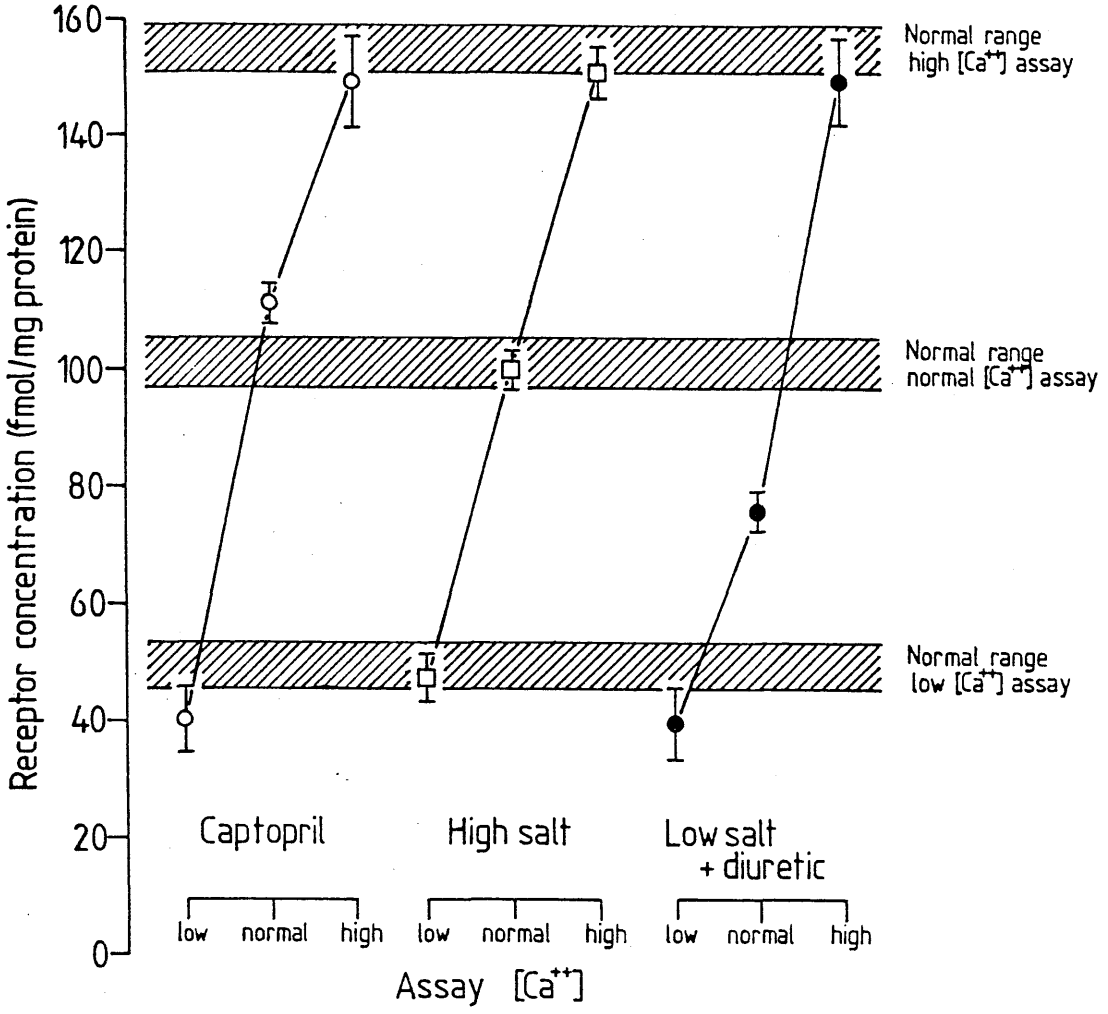

Figure 5.3 **Changes in receptor density associated with manipulation of plasma angiotensin II concentration: high/low [Ca^{++}] assay, 2 day protocols**

The 2 day sodium loading, sodium depletion and captopril administration experiments were repeated with the receptor assays performed in low and high [Ca^{++}]. Results are presented as point estimate ± standard error for 3 or more experiments (actual values are given in Table 5.1). The values obtained in normal [Ca^{++}] are also shown (from Figure 5.2). The hatched regions denote the receptor density observed for normal animals in low, normal and high [Ca^{++}] assay.

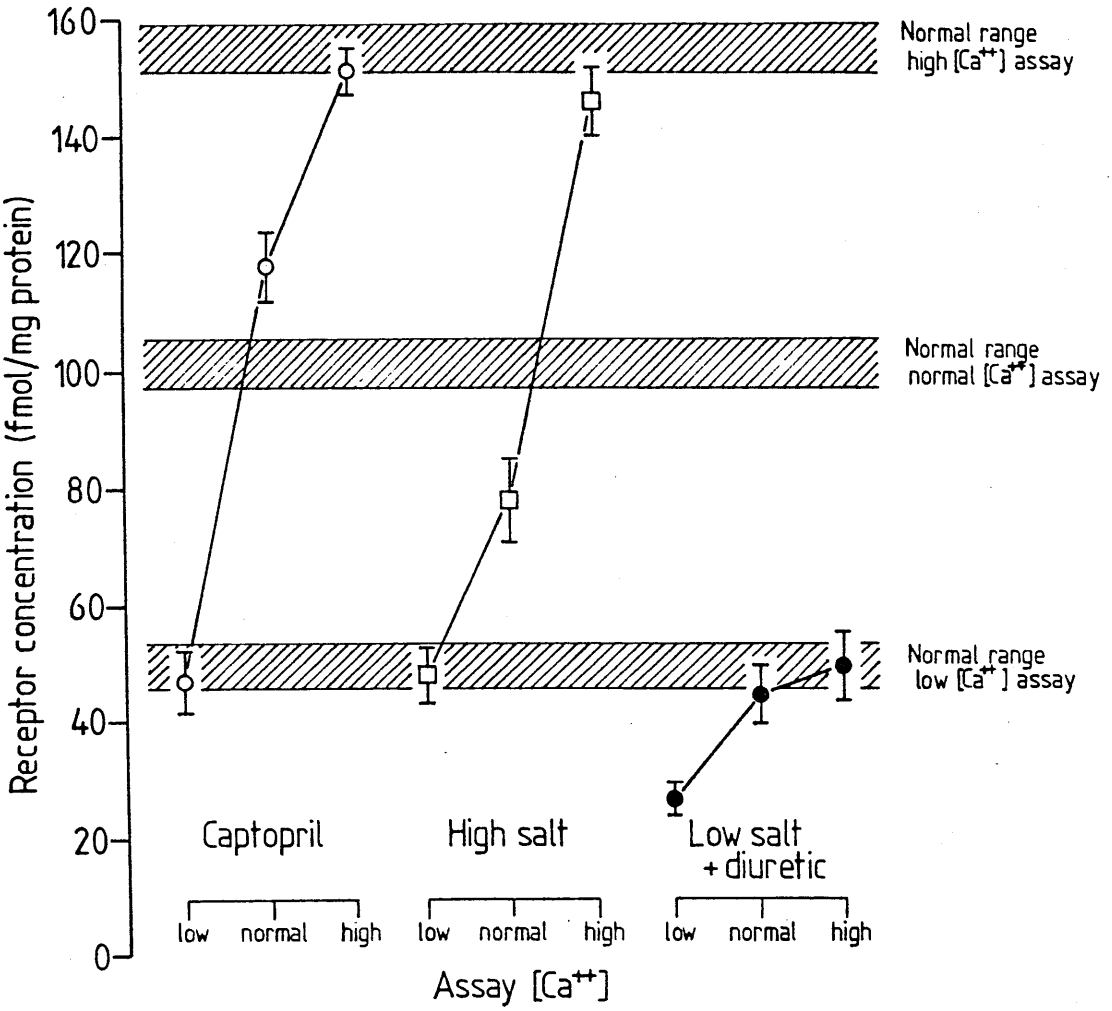

Figure 5.4 **Changes in receptor density associated with manipulation of plasma angiotensin II concentration: high/low [Ca^{++}] assay, 12 day protocols**

The 12 day sodium loading, sodium depletion and captopril administration experiments were repeated with the receptor assays performed in low and high [Ca^{++}]. Results are presented as point estimate ± standard error for 3 or more experiments (actual values are given in Table 5.1). The values obtained in normal [Ca^{++}] are also shown (from Figure 5.2). The hatched regions denote the receptor density observed for normal animals in low, normal and high [Ca^{++}] assay.

Table 5.1

Effect of dietary & drug regimens on mesenteric artery receptor binding constants for ^{125}I-angiotensin II

GROUP	RECEPTOR DENSITY (fmol/mg)	FORWARD RATE CONSTANT log [$M^{-1}.s^{-1}$]	REVERSE RATE CONSTANT log [s^{-1}]	N
Captopril				
acute	99.7 ± 3.3	5.794 ± 0.028	-4.017 ± 0.144	3
2 days	110.8 ± 2.7	6.113 ± 0.110	-3.767 ± 0.127	3
5 days	118.0 ± 7.1	5.706 ± 0.033*	-3.910 ± 0.079	3
12 days	118.0 ± 6.0*	5.810 ± 0.069	-3.948 ± 0.167	5
2 days low [Ca^{++}]	39.9 ± 6.0	5.431 ± 0.053*	-3.824 ± 0.103	3
2 days high [Ca^{++}]	150.0 ± 8.0	5.873 ± 0.000	-4.294 ± 0.002	4
12 days low [Ca^{++}]	47.0 ± 5.6	5.571 ± 0.133	-3.512 ± 0.320	3
12 days high [Ca^{++}]	151.7 ± 4.9	6.014 ± 0.020	-4.207 ± 0.007	3
High salt				
2 days	99.5 ± 4.1	5.696 ± 0.479	-4.171 ± 0.111	3
5 days	90.1 ± 7.0	5.809 ± 0.025	-3.783 ± 0.034*	5
5 days (no saline)	85.6 ± 6.2*	5.857 ± 0.028	-4.021 ± 0.038	3
12 days	77.5 ± 3.7*	5.981 ± 0.175	-3.603 ± 0.314	5
12 days (no saline)	76.0 ± 4.1*	5.830 ± 0.142	-4.059 ± 0.148	3
2 days low [Ca^{++}]	47.0 ± 3.7	5.586 ± 0.119	-3.511 ± 0.333	3
2 days high [Ca^{++}]	151.5 ± 5.0	5.874 ± 0.215	-4.056 ± 0.276	3
12 days low [Ca^{++}]	47.8 ± 5.0	5.805 ± 0.027	-4.066 ± 0.030	4
12 days high [Ca^{++}]	146.1 ± 6.0	5.777 ± 0.085*	-3.656 ± 0.310	4
NORMAL low [Ca^{++}]	49.9 ± 4.0	5.802 ± 0.136	-3.795 ± 0.013	5
NORMAL	102.2 ± 4.2	5.897 ± 0.052	-4.208 ± 0.165	5
NORMAL high [Ca^{++}]	156.2 ± 4.6	6.023 ± 0.080	-3.910 ± 0.310	5
Low salt				
2 days	88.0 ± 3.9*	5.915 ± 0.419	-3.459 ± 0.453	3
5 days	70.3 ± 4.1*	5.742 ± 0.092	-4.099 ± 0.257	3
12 days	59.8 ± 3.2*	5.833 ± 0.025	-3.922 ± 0.032	3
Low salt + diuretic				
2 days	76.0 ± 3.3*	5.808 ± 0.139	-4.132 ± 0.632	3
5 days	53.8 ± 2.9*	5.861 ± 0.052	-3.989 ± 0.066	3
12 days	44.1 ± 4.8*	5.687 ± 0.086*	-4.482 ± 0.710	4
2 days low [Ca^{++}]	39.9 ± 5.4	5.397 ± 1.050	-3.991 ± 0.243	3
2 days high [Ca^{++}]	150.1 ± 8.0	5.913 ± 0.061	-3.961 ± 0.257	3
12 days low [Ca^{++}]	26.7 ± 1.9*	5.841 ± 0.035	-4.410 ± 0.034*	4
12 days high [Ca^{++}]	49.0 ± 6.9*	5.843 ± 0.090	-4.036 ± 0.372	5

Results obtained using the receptor assay, given as point estimate ± standard error. The number of experiments is given (N), with 12 rats used for each experiment. In some of the sodium loading experiments, the salt was administered in the food only (Table 2.1); these are indicated (no saline). A parameter estimate differing significantly from that for control animals obtained in the same [Ca^{++}] assay is indicated (*).

In contrast, prolonged elevation of plasma angiotensin II concentration had a quite different effect; the increase in receptor number usually observed between normal [Ca^{++}] and high [Ca^{++}] was lost and the value obtained in low [Ca^{++}] was significantly below that for all other groups (Figure 5.4). This may correspond to an actual loss of receptors. As before, no consistent change in receptor affinity was noted (Table 5.1).

5.6 **Angiotensin II metabolism**

Based on organ perfusion experiments, Leary & Ledingham (1970) proposed that alteration of sodium balance affects the rate of angiotensin II metabolism and that this may contribute to the observed changes in pressor sensitivity to the peptide. Since the rate of tracer angiotensin II degradation by the arterial membrane fraction was routinely measured as part of the receptor assay, this hypothesis could easily be tested. Table 5.2 shows the results for regression of the apparent tracer decay rate constant against time (duration of dietary/drug protocol) and against membrane protein concentration. There was no relationship with time but a highly significant and very similar relationship with membrane protein concentration was observed for all groups. The results therefore do not lend any support for this hypothesis. The relationship between membrane protein concentration and tracer decay rate was not different between normal [Ca^{++}] and high [Ca^{++}] assays but tracer degradation rate was markedly reduced by removal of divalent cations (Table 5.2 & Figure 5.5).

Table 5.2

Effect of dietary & drug regimens on angiotensin II metabolism by the arterial membrane fraction

GROUP	COEFFICIENT		
Captopril	[PROTEIN]	0.00624 ± 0.00118	p=0.0005
	TIME	-0.02360 ± 0.04027	p= NS
			r^2=0.9043
High salt	[PROTEIN]	0.00479 ± 0.00065	p<0.0001
	TIME	0.02999 ± 0.02736	p= NS
			r^2=0.9530
NORMAL	[PROTEIN]	0.00561 ± 0.00030	p<0.0001
			r^2=0.9133
Low salt	[PROTEIN]	0.00607 ± 0.00101	p=0.0004
	TIME	-0.02200 ± 0.04750	p= NS
			r^2=0.9334
Low salt + diuretic	[PROTEIN]	0.00367 ± 0.00077	p=0.0007
	TIME	0.04786 ± 0.03389	p= NS
			r^2=0.9164
All experiments high [Ca^{++}] assay	[PROTEIN]	0.00499 ± 0.00023	p= NS
All experiments normal [Ca^{++}] assay	[PROTEIN]	0.00556 ± 0.00021	p=0.0212
All experiments low [Ca^{++}] assay	[PROTEIN]	0.00305 ± 0.00023	

The upper part of the table gives the results of no-constant multivariate regression of ^{125}I-angiotensin II apparent degradation rate (k_{dH}, measured as part of the receptor assay) in normal [Ca^{++}] assay against duration of dietary/drug regimen (TIME) and arterial membrane protein concentration ([PROTEIN]). The p values derived from t-statistics show that for each group k_{dH} was strongly related to protein concentration and did not change with time. Comparison of the [PROTEIN] coefficients (given as estimate ± standard error) was made by calculating the ratio k_{dH}/protein concentration for each experiment and then using unpaired t-tests. There was no significant difference (p>0.05, NS) between the control and any of the treated groups. In the lower part of the table, the same method was used to compare values of k_{dH} in high, normal and low [Ca^{++}] assays. As shown, tracer decay rate was significantly reduced in low [Ca^{++}] but did not differ between normal and high [Ca^{++}].
(r^2; coefficient of determination).

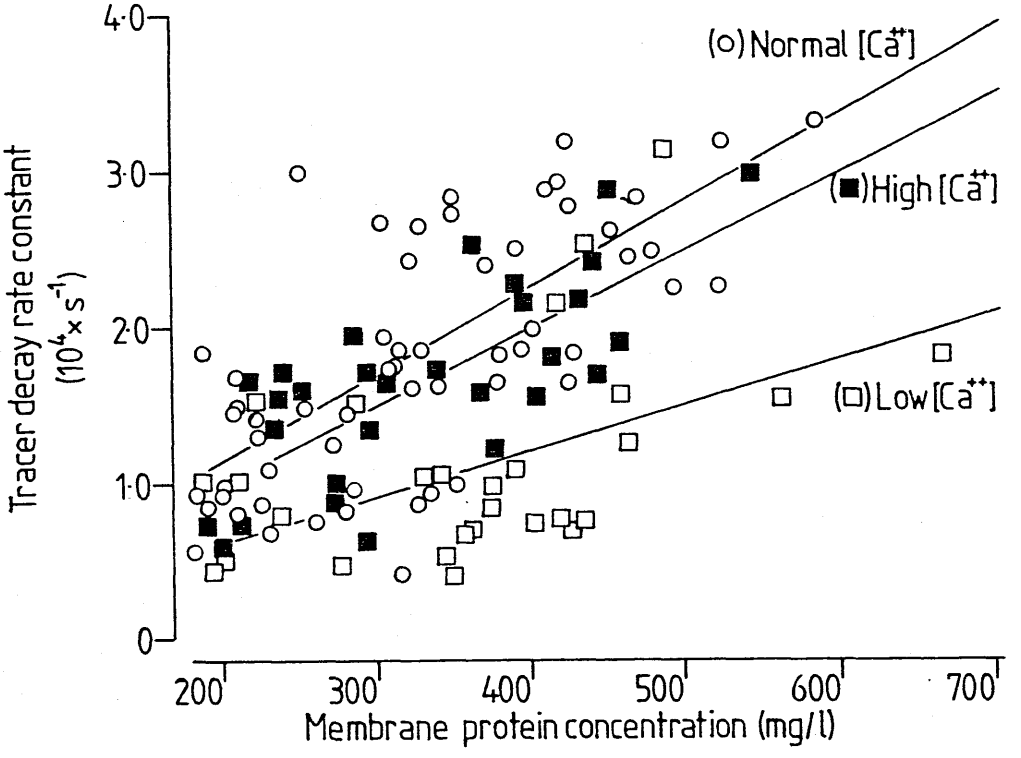

Figure 5.5 Relationship between ^{125}I-angiotensin II degradation rate and arterial membrane protein concentration

The figure shows the linear relationship between arterial membrane protein concentration and ^{125}I-angiotensin II degradation rate (determined by filtrate immunoassay). Each point represents one receptor assay. The regression lines shown are based on the statistical analysis described in Table 5.2.

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

At the commencement of this study, only two reports appeared in the literature on angiotensin receptors in resistance vessels (Gunther et al, 1980a,b), with most accounts based on work with model tissues such as aortic and uterine smooth muscle. The aims of the project were to confirm the observations made by these authors and to develop an assay for the mesenteric artery angiotensin receptor which could be used to study the physiologic regulation of these receptors. The findings of the present study are largely in agreement with others published on this topic, although certain important differences, most probably of methodological origin, have been noted. These have been mentioned in Chapter 4 and are discussed more fully below along with other aspects of the results. To avoid unnecessary repetition, the discussion will make extensive use of the review of the literature presented in Chapter 1.

In the discussion on the role of divalent cations in receptor regulation, the phrase "apparent receptor density" is used when referring to the results presented herein since the receptor density determined by radioligand receptor assay varies with the divalent cation concentration in the assay incubation medium. This phrase is not used when referring to previous reports on angiotensin receptor regulation, since each of these studies was performed with a fixed composition assay medium (see Table 4.4 for those used for the mesenteric artery receptor). However, it seems likely that in some cases (mostly those where a smooth muscle tissue has been used) the observed receptor densities will be (in part) a function of the assay medium composition.

6.2 General aspects of the vascular angiotensin receptor

The binding sites for ^{125}I-angiotensin II identified in this study display characteristics expected of a physiological receptor for angiotensin II. The kinetic constants should allow rapid interaction, and thus rapid response, with angiotensin II at normal plasma concentrations, and the observed concentration of binding sites (100 fmol/mg of membrane protein) would correspond to a receptor density of the order of 10^4 sites/cell, assuming 10^6 cells/100mg wet weight of tissue and 50% recovery in the preparation of the membrane fraction. A similar value has been reported for rat mesenteric artery smooth muscle cells in culture (Gunther et al, 1982). The binding sites also discriminate between angiotensin-related peptides in the expected manner. The 4-fold difference in affinity for angiotensins II & III, the lack of binding by angiotensin I and the very low affinity of the C-terminal hexa- and pentapeptides exactly parallels the pressor activities of these peptides (Helmer, 1955; Bumpus et al, 1961; Campbell et al, 1977a; Schwyzer, 1963; Carey et al, 1978). The binding constants were similar at 22^{o}C and 37^{o}C, and while it may be an oversimplification to derive thermodynamic constants with data at only two temperatures (Table 4.3), the values of $\Delta H^{o'}$ (-22kJ/mol) and $\Delta S^{o'}$ (-58 J/mol/^{o}) seem appropriate for the interaction of a small random coil peptide with its receptor. The half-life of the hormone-receptor complex is long (6900 seconds) which suggests that dissociation of the complex is not the in vivo process which terminates the hormonal response (see below, Cation effects and receptor regulation).

It has been reported that the angiotensin II competitive antagonists (e.g. sar^1 thr^8 and sar^1 ala^8 [saralasin] peptides) display equal or greater affinity than the native peptide for the smooth muscle receptor (Devynck & Meyer, 1976; Gunther et al, 1980a,1982; Aguilera & Catt, 1981). However, as shown in Section 4.9 the sarcosine^1 peptides were

considerably more resistant to degradation than angiotensin II. The sarcosine^{1} substitution greatly increases the half-life of the peptide in the circulation (Pettinger et al, 1975). When this was taken into account, the antagonists were found to display an appreciably lower affinity for the receptor than angiotensin II (Figure 4.7 and Table 4.5). In the rat (and other species) plasma angiotensin II concentration may reach, under extreme circumstances, lnM (Boucher et al, 1977). Pettinger et al (1975) found that plasma saralasin concentrations of 30 and 300nM produced 10 and 100-fold shifts respectively in the angiotensin II pressor dose-response relationship in this species. Thus pharmacological evidence agrees with the observed lower affinity for the antagonists. The ile^{8} peptide has both the highest affinity and the highest degree of agonist activity of all angiotensin antagonists tested to date (Davis et al, 1974). As shown in Table 4.5, this peptide showed a 10-fold higher affinity than the other antagonists.

Previous bioassay experiments have shown that tyrosyl mono-iodinated angiotensin II retains only a proportion (25-70%) of the pressor activity of the native peptide (Lin et al, 1970; Kurchbart et al, 1971; Papadimitriou & Worcel, 1974). However, in vascular smooth muscle (Gunther et al, 1980a; Aguilera & Catt, 1981; present study), adrenal cortex (Glossman et al, 1974c); brain (Bennet & Snyder, 1976), heart (Wright et al, 1983; Baker et al, 1984), kidney (Brown & Douglas, 1982), liver (Sernia et al, 1985) and platelets (Moore & Williams, 1982) no discrimination between labelled and unlabelled peptides is apparent. As discussed in Section 4.11, artefactual results may have been obtained in the earlier experiments through impurity of the radiolabelled peptide. Repetition of the bioassay experiments (Section 4.11) using ^{125}I-angiotensin II of high purity showed this peptide to retain full pressor activity, thus removing any discrepancy. It seems likely that full

activity is also retained in the other angiotensin target tissues noted above.

6.3 Possible nature of the low-affinity component of binding

It is unlikely that the second class of binding sites is involved in mediating response to angiotensin II as their low affinity (K_d=50nM) would probably not permit interaction with angiotensin II at normal plasma concentrations. It is also unlikely that these sites represent binding of tracer to a peptidase enzyme as tracer degradation was not affected by the presence of 10uM unlabelled angiotensin II which blocks high- and low-affinity binding. The low capacity (700 fmol/mg protein) is not consistent with uptake of radioactivity into membrane vesicles. Furthermore, chromatographic analysis of bound radioactivity after incubation with 20nM ^{125}I-angiotensin II, conditions in which about 20% of the specifically bound radioactivity is associated with the low-affinity site, showed it to be composed entirely of ^{125}I-angiotensin II and (to a lesser extent) ^{125}I-angiotensin III (Section 4.3a). Therefore the low-affinity site appears to discriminate between physiologically active angiotensin peptides and the inactive metabolites produced during incubation.

The affinity of this class of binding sites is similar to that of the angiotensin binding sites seen in aortic smooth muscle, which show various characteristics of a physiological receptor for angiotensin II and mediate contraction of this muscle tissue in response to the peptide. Thus the second class of sites may be related to or derived from the active (high-affinity) receptor, possibly through receptor inactivation or lack of activation to the high-affinity form. For methodological reasons, it is difficult to compare results from different studies, but there appears to be a spectrum of angiotensin receptor affinity, with reported K_d values of 10-50nM for aortic smooth muscle, 2-10nM for uterine muscle and 0.1-1nM for rat mesenteric artery tissue. Aguilera & Catt (1981) report identical

binding characteristics for rat mesenteric artery and urinary bladder muscle. Factors associated with the differentiation of the tissue may therefore exert effects on the receptors.

In the presence of GTP or a GTP analogue many receptors display an altered affinity for agonists due to interaction of the agonist-receptor complex with a GTP-binding receptor-effector coupling protein (Catt et al, 1979; Gilman, 1984; Lefkowitz et al, 1984; Berridge & Irvine, 1984). The large difference in affinity of the two classes of sites in mesenteric artery smooth muscle and the ratio of binding site concentrations suggests that such a process does not account for the heterogeneity of angiotensin II binding sites in this tissue. As described in Section 4.12, maximal inhibition of ^{125}I-angiotensin II binding by Gpp(NH)p was due to a 10-fold reduction in affinity with relatively little change in receptor density. Had Gpp(NH)p caused an interconversion of binding sites, this would have been apparent as a change in the concentration of high-affinity sites under the assay conditions employed. GTP and its analogues are inhibitory against the aortic angiotensin receptor (Devynck & Meyer, 1976). Under no conditions was there any indication of interconversion of high- and low-affinity binding sites.

Only occasionally has a similar low-affinity binding site for angiotensin II been observed, probably due to the use of a more narrow range of tracer concentrations in saturation binding experiments (see below, Methodological aspects). Glossman et al (1974c) observed a second class of angiotensin II binding sites in adrenal cortex with a K_d of 30nM. However, in various tissues, two classes of binding sites with K_d values differing approximately ten-fold have been observed, and it has been claimed that the receptor subtypes in liver are functionally distinct (Gunther, 1984).

6.4 Methodological aspects

Degradation of peptide ligands in binding studies is commonly encountered and seriously affects the calculation of binding constants for the receptor under study (Ketelslegers et al, 1975). These authors observed that ligand and/or receptor degradation predominantly affects the estimate of affinity and that the effect is more apparent under pseudo-equilibrium conditions than in kinetic experiments. Computer simulations (not shown) carried out as part of the present study support these findings, as do the experimental results. For this reason, short incubations were used to minimise the effect of changes in tracer degradation rate when comparing the effect of cations and proteinase inhibitors on ^{125}I-angiotensin II binding.

It is standard practice for incubations to be performed at reduced temperature (usually 22^{o}C) to limit ligand destruction. However, for small peptides such as angiotensin II which are particularly susceptible to proteolysis due to their lack of tertiary structure, this may not be effective. In these circumstances proteinase inhibitors are often used (see Table 4.4 for those used with the mesenteric artery preparation). It is also usual for such inhibitors to be added without examination of their effects on the receptor. As shown in Table 4.1, some were inhibitory against the receptor and it was not possible to effectively prevent ligand degradation without adversely affecting binding. In many instances these inhibitors only partially prevent ligand destruction; unrecognised tracer degradation would account for the main differences between this study and those listed in Table 4.4; these are the affinity of angiotensin II for the receptor and the relative affinities of angiotensin II and the sar^{1} antagonists (Note that for the mesenteric artery receptor, reported K_d values range from 0.1-1nM while values of receptor concentration vary only from 50-100 fmol/mg protein; as described above, ligand degradation

affects mainly the estimate of affinity). The influence of calcium on receptor density described in Sections 4.12 and 5.5 emphasises the potential importance of the effect of proteinase inhibitors on the receptor; clearly, had chelating agents been used then these observations could not have been made.

As mentioned previously, saturation binding curves constructed using a limited range of tracer concentrations may fail to reveal a low-affinity component of binding. In the present study, linear Scatchard plots (not shown) could be obtained under such conditions, with the K_d values derived from them (-1/slope) lying in the range 0.5-1nM, similar to the values obtained by Gunther et al (1980a). Usually 30-60% of the tracer was destroyed during incubation, resulting in overestimation of the K_d by a factor of 5-10. Where a second low-affinity class of binding sites is present, limited saturation binding curves may also yield incorrect values for receptor density since the Scatchard plot, although linear, still contains a low-affinity component. For further discussion on these methodological aspects see Klotz (1982) and Mendel & Mendel (1985).

For technical reasons it was not possible to fully saturate the low-affinity sites in the routine receptor assay. Account of the low-affinity component of binding was taken by assuming it to be linear with tracer concentration (over the range employed) and subtracting this linear component in the analysis of the binding data. This approach required that the concentration range used saturated the high-affinity sites (2-4nM) and allowed adequate definition of the linear low-affinity component. The use of tracer concentrations of (approximately) 0.05-10nM satisfied this requirement (see Figure 4.6). In the analysis of the assay data, curve fitting to at least three replicate experimental curves was performed simultaneously, under the assumption that the parameters relating to the high-affinity site were common to all of the data. The constraints which

this imposed on the analysis together with the content of the experimental data permitted good resolution of the high-affinity component of binding, as is evident from the reproducibility of the results (see Tables 4.3 and 5.1).

Attempts were made to determine if the physiological manipulations described in Chapter 5 had any effect on the low-affinity binding sites. However, even when the affinity of these sites was assumed constant and the receptor concentrations estimated by applying the multiple receptor density method (Section 3.2b) to large sets of data, the standard errors of the estimates were large and no conclusions could be made from the results.

Although computationally demanding, direct curve-fitting techniques represent the optimum method for analysis of complex ligand binding systems such as that described. As noted above, correction for ligand and/or receptor degradation is preferable to the use of proteinase inhibitors which may have a significant adverse effect on the receptor(s), and the flexibility of nonlinear regression will allow quantitative description of other factors such as heterogeneity of binding sites or co-operative effects which may invalidate conventional methods of analysis. Even where linearizing transformations are technically applicable, simple curve-fitting procedures may be more suitable as transformation can distort the experimental data (Duggleby, 1980; Thakur et al, 1980). An advantage of the methods described is that kinetic constants may be readily obtained as part of a receptor assay. This is an important feature in respect of hormone-receptor systems, where in vivo a dynamic rather than true chemical equilibrium will exist, and equilibrium constants may be inappropriate for a full description of the system.

6.5 Cation effects and receptor regulation

In many tissues, cations have been found to influence the binding of angiotensin II to its receptor. The nature of this effect is not fixed, but varies between tissues. This is most apparent when comparing adrenal cortex and smooth muscle. In adrenal cortex, monovalent cations (especially sodium) greatly increase binding through an increase in affinity, whereas divalent cations have little effect (Glossman et al, 1974a,b; Douglas et al, 1978a; Douglas et al, 1982). In mesenteric artery (Gunther et al, 1980a; Wright et al, 1982), uterine (Douglas et al, 1982) and cardiac (Wright et al, 1983; Baker et al, 1984) smooth muscle divalent cations (Ca^{++}, Mg^{++}) markedly enhance binding whereas sodium and other monovalent cations show only a modest effect (inhibitory in heart). With the exception of the study by Wright et al (1982), an increase in receptor number was observed in all cases. Similar findings have been reported for liver (Campanile et al, 1982; Gunther, 1984). In renal tissues both sodium and divalent cations exert an appreciable effect on angiotensin II binding, sodium increasing receptor affinity and divalent cations increasing receptor number (Brown & Douglas, 1982; Douglas et al, 1982). In brain, sodium causes a maximal 25-fold increase in affinity (Bennett & Snyder, 1980a) but probably due to tracer angiotensin II degradation the effect of divalent cations has not been investigated. In all cases, sodium was active over the range 0-150mM and divalent cations over the range 0-10mM.

The results shown in Section 4.12 are in agreement with previous reports for the smooth muscle angiotensin receptor. Sodium caused a slight increase in binding, even when maximal stimulation by calcium had been achieved, but the biphasic effect of sodium (also noted by Wright et al, 1982) was more marked in the presence of calcium. This, together with the observations described above, suggests that sodium and calcium act via

different mechanisms, but at concentrations greater than 100mM sodium interferes with the action of calcium. This may explain the small inhibitory effect of sodium seen in cardiac smooth muscle.

In the study by Bennett & Snyder (1980a), a differential effect of sodium on angiotensin II and N-terminal sarcosine substituted peptides was observed, sodium increasing receptor affinity only for the native peptide (aspartic acid at the N-terminus). A similar but less marked effect was noted for adrenal cortex, and no such effect was evident in uterine smooth muscle. To an extent, this resembles the differential effect of sodium on agonist and antagonist binding which has been reported for a variety of hormone-receptor systems [opiate (Pert & Snyder 1974; Childers & Snyder, 1980), cardiac muscarinic (Rosenberger et al, 1980), alpha- and beta-adrenergic (U'Prichard & Snyder, 1978; Yamamura & Rodbell, 1976) and histamine H_1 (Chang & Snyder, 1980)]. However, Bennett & Snyder (1980a) found no correlation between the influence of sodium on the binding of angiotensin II analogues and agonistic or antagonistic properties of the peptides. In agreement with the findings of these authors, displacement of ^{125}I-angiotensin II by sar^1 thr^8 angiotensin II from the mesenteric artery receptor in the absence and presence of sodium (150mM) yielded identical results (not shown).

Divalent cations also exert a differential effect on agonist and antagonist binding in several systems [opiate (Blume, 1978; Childers & Snyder, 1980), alpha- and beta-adrenergic (Pasternak et al, 1975; Tsai & Lefkowitz, 1978; U'Prichard & Snyder, 1978; Williams et al, 1978b), dopaminergic (Usdin et al, 1980) and histamine H_1 (Chang & Snyder, 1980)]. In all cases, divalent cations increased affinity predominantly for agonists, and in some cases opposed the effects of GTP and sodium. In the studies on the effect of sodium quoted above, GTP produced a similar effect, and the results of Chang & Snyder (1980) indicate that sodium

enhances the discriminatory effect of GTP on agonist and antagonist binding. It has been suggested (Childers & Snyder, 1980) that the effect of divalent cations is due to stimulation of GTP hydrolysis. In studies where several different divalent cations have been used, Mn^{++} was usually much more potent than Ca^{++} (Chang & Snyder, 1980; Childers & Snyder, 1980; Usdin et al, 1980).

With the exception of studies on angiotensin II binding, there are few accounts describing an effect of cations on receptor number. In addition to the effect of mono- and divalent cations on dopamine receptor affinity described above, sodium and manganese also apparently increased receptor density (Usdin et al, 1980). The number of binding sites for dihydropyridine calcium channel antagonists in brain (but not heart) membranes varies with the incubation medium calcium concentation over the range 0-1mM (Gould et al, 1982), but it seems unlikely that there is any connection between this effect and the influence of divalent cations on angiotensin II binding. There is no evidence for a direct link between the angiotensin receptor and voltage-dependent Ca^{++} channels (Section 1.9) and dihydropyridine drugs do not influence angiotensin II binding in mesenteric artery smooth muscle cell membranes (results not shown).

It is known that calcium can alter the physicochemical state of lipid bilayers (Houslay & Stanley, 1982) and it might therefore indirectly affect the angiotensin receptor. However, in that case an influence of divalent cations on receptor density should be a more general phenomenon. The shifts in the $[Ca^{++}]$-apparent receptor density relationship described in Chapter 5 are also not consistent with an effect of Ca^{++} on membrane structure. It is well established that the effects of changes in sodium balance on target tissue sensitivity to angiotensin II are selective for this peptide (Strewler et al, 1972; Oliver & Cannon, 1978; Aguilera & Catt, 1981). Although it has been reported that angiotensin II can act as

an ionophore for divalent cations (Degani & Lenkinski, 1980), the magnitude of this effect was small and the conditions used to demonstrate it so extreme (millimolar concentrations of peptide at 50-60^{o}C) that it probably has no physiological relevance. For reasons given below, the formation of an angiotensin II-Ca^{++} complex does not appear to be involved in the effect of calcium.

As described in Section 1.2e, tachyphylaxis to angiotensin appears to be a function of the N-terminus of the octapeptide, and to involve calcium and possibly a receptor-related factor. The results shown in Table 4.6 indicate that guanine nucleotides and calcium act on the receptor via different mechanisms, calcium still influencing apparent receptor number even when maximal inhibition of binding by Gpp(NH)p had been achieved. As discussed previously, guanine nucleotides probably allow the hormone-receptor complex to interact with a coupling protein, and through this mechanism affect predominantly agonist binding. This effect might therefore be a function of the C-terminus of the peptide (angiotensin antagonists being C-terminal substituted). This would be consistent with two functional domains in the peptide, the effect of calcium being associated with the N-terminus.

Paiva et al (1977) have suggested that tachyphylaxis results from tight binding of angiotensin II to its receptor, with recovery due to displacement of the peptide by calcium. Since the effect of calcium is reversible (Figure 4.11), this proposal does not appear correct. It should be noted that these experiments were performed at reduced temperature (10^{o}C) to limit receptor degradation, and the primary object was to verify that the tissue processing procedures did not affect the in vivo state of the receptors with respect to calcium. Preincubation of the arterial membrane fraction at higher temperature (22^{o}C or 37^{o}C, 10-20 minutes) in Tris buffer before addition of tracer angiotensin II and calcium markedly

reduced the stimulatory effect of calcium without affecting basal angiotensin II binding (results not shown). This rules out the possibility that the effect of calcium is due to the formation of an angiotensin II-Ca^{++} complex, and is also not consistent with "prior occupancy" of the receptors by endogenous peptide.

The alterations in the relationship between [Ca^{++}] and apparent receptor density described in Chapter 5 imply the existence of two subgroups of receptors which can exist in a masked (inactive) state and which differ in sensitivity to unmasking by calcium. While it is possible that the angiotensin II binding seen in low [Ca^{++}] assay is due to failure of the chelating agents to remove all divalent cations, this is unlikely and suggests a third receptor subgroup which is unaffected by calcium. A speculative scheme for the regulation of the receptors is shown in Figure 6.1. It is assumed that under agonist occupancy, a proportion of the receptors (R) form an inactive (unable to bind ligand) complex ($\bar{R}F_T$), where F_T is the tachyphylaxis factor. The differing extent to which tissues become tachyphylactic to angiotensin II would therefore reflect the activity of F_T. This complex dissociates in the presence of calcium, liberating active (able to bind ligand) receptors. Under prolonged agonist occupancy, the receptors may be subject to a covalent modification (R´), with the $\bar{R}'F_T$ complex less sensitive to calcium (by analogy, glycogen phosphorylase kinase exists in two forms, the phosphorylated form being more sensitive to activation by calcium). The shifts in the [Ca^{++}]-apparent receptor density relationship would therefore correspond to the interconversion of R and R´. Regulation of the B-adrenergic receptor may involve phosphorylation of the receptor (Sibley & Lefkowitz, 1985). By analogy with the GTP-binding receptor-effector coupling proteins, the enzymatic activity for the covalent modification of R might reside in F_T. Prolonged agonist-occupancy may also result in a loss of receptors by an

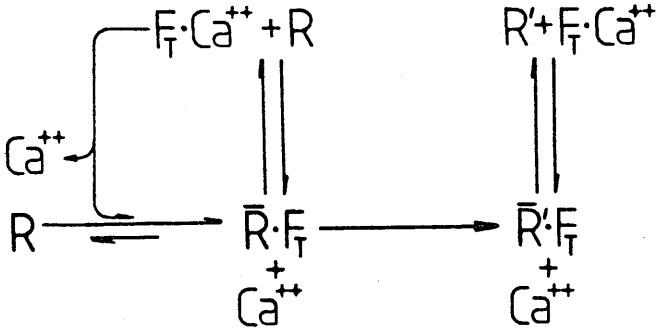

Figure 6.1 Proposed mechanism of vascular angiotensin receptor regulation

Under agonist occupancy, the receptor (R) forms an inactive complex ($\bar{R}F_T$) with the tachyphylaxis factor (F_T). This complex dissociates in the presence of Ca^{++} liberating active receptors. In a slower process the receptor is subject to a modification (R´) with the $\bar{R}´F_T$ complex less sensitive to dissociation by Ca^{++}. This modification may precede actual loss of receptors by an internalisation/degradation process (not shown).

internalisation/degradation process which could be related to the covalent modification of the receptor (Catt et al, 1979; Cuatrecasas & King, 1981; Lefkowitz et al, 1984; Sibley & Lefkowitz, 1985). If F_T were spared in this process, the effect of prolonged agonist action would then be a reduction in the total number of receptors (seen in high [Ca^{++}] assay) and a reduction in the number of calcium-insensitive receptors (seen in low [Ca^{++}] assay) in addition to an alteration in the relative concentrations of R and R´.

Calcium may itself regulate the receptors, or may act as a cofactor in the action of F_T. The plasma free calcium concentration changes very little, but is in the range which affects the receptor. Manipulation of the extracellular [Ca^{++}] does alter the extent to which tachyphylaxis occurs (Ackerly et al, 1977a; Paiva et al, 1977) but could alter Ca^{++} availibility within the cell. Calcium channel blockade may influence the tachyphylactic mechanism (Ackerly et al, 1977a; see Section 1.2e) suggesting an intracellular site of action. The intracellular calcium concentration is normally too low to modify the receptor status, but since angiotensin II action can result in measurable efflux of calcium from the cell (presumably released from the endoplasmic reticulum in the vicinity of the receptor under the action of inositol trisphosphate, see Section 1.9) the receptor may experience a high [Ca^{++}] during this process. The regulatory effect of calcium might therefore be connected with angiotensin action. As noted previously, the half-life of the angiotensin-receptor complex is long and thus dissociation of the peptide may not be the terminator of the hormonal response. Instead, formation of the $\bar{R}F_T$ complex may end the stimulus, with reactivation of the receptor depending on the availability of calcium. Reduced availability of calcium would accelerate the formation of R´, and might therefore affect predominantly the onset of tachyphylaxis. This would agree with the

findings of Paiva et al (1977).

In the above scheme, calcium acts on a receptor-related factor rather than the receptor itself, and the proposed covalent modification affects the receptor-F_T interaction rather than the receptor-ligand interaction. Consequently calcium would not alter receptor affinity for angiotensin II and would have no effect on angiotensin II binding in tissues which lack F_T (such as adrenal cortex, and aortic smooth muscle which does not exhibit tachyphylaxis to angiotensin II).

While it is suggested that the receptor undergoes a covalent alteration, it is equally possible that the activity of F_T is modified. The differing effects of sodium loading and converting enzyme blockade on receptor status (Chapter 5) may be due to another hormone which modulates the activity of F_T. A number of studies have indicated that factors in addition to plasma angiotensin II concentration determine vascular, adrenocortical and other target organ sensitivities to the peptide (Dawson-Hughes et al, 1981; Olsen & Meydrech, 1985; see also Section 1.2d). Insulin has been shown to modulate angiotensin receptor density in renal glomeruli (Ballerman et al, 1984), and may have a similar effect in vascular tissues (Turlapaty et al, 1980). There is no indication that plasma insulin concentration would change with manipulation of sodium balance. It is not known if glucocorticoids have any effect on angiotensin receptor status, but plasma corticosterone concentration is not affected by alteration of sodium intake (Schiebinger & Kontrimus, 1985).

As described in Section 5.3, plasma aldosterone concentration was consistently higher in the captopril-treated rats than in the sodium loaded animals but only significantly so after 12 days. If aldosterone does modulate vascular angiotensin receptor density as suggested by Schiffrin et al (1983c), then the slow fall in apparent receptor density (normal $[Ca^{++}]$ assay) induced by sodium loading could be due to the

greater suppression of plasma aldosterone concentration by this treatment.

Atrial natriuretic peptide (ANP) antagonises the action of a number of vasoconstrictors, especially angiotensin II (Kleinert et al, 1984). At present it is not clear if plasma ANP levels are altered by manipulation of sodium balance in the rat, although an increase in plasma ANP concentration with sodium loading would be expected in view of the natriuretic, vasorelaxant and anti-steroidogenic activities of this peptide (see Schiffrin et al, 1985 for references). Specific high-affinity receptors for ANP have been identified in membrane fractions from rat mesenteric artery and adrenal cortex (Schiffrin et al, 1985). ANP appears to be an endogenous antagonist of the renin-angiotensin system, and might act (in part) by modulating angiotensin receptor status.

It has long been established that sodium loading causes an increase in pressor sensitivity to infusion of angiotensin II, but the results of this study suggest a reduction in vascular smooth muscle responsiveness to the peptide. It is possible that the increased sensitivity to infusion of angiotensin II seen with sodium loading is due to hemodynamic factors; while the blunted smooth muscle response to angiotensin II can be demonstrated in vitro with tissue taken from animals after activation of the renin-angiotensin system (Strewler et al, 1972; Sybertz & Peach, 1980) the corresponding experiment with tissue from sodium loaded animals does not appear to be reported in the literature. As discussed above, a second hormone may regulate receptor status. Since sodium loading caused a reduction only in the apparent (normal $[Ca^{++}]$ assay) number of receptors, it is possible that infusion of angiotensin II under these circumstances would rapidly redress the balance of hormonal influences on the receptors. Conceivably, sodium loading at a lower level than used in the present study could suppress plasma angiotensin II concentration without markedly affecting other hormonal influences on the

receptors, and would therefore produce the same effect as captopril administration. This would explain the up-regulation of the mesenteric artery receptors with sodium loading (0.3% NaCl diet) observed by Aguilera & Catt (1981).

There are several parallels between angiotensin receptor regulation in smooth muscle and adrenal cortex. Elevation of plasma angiotensin II concentration results in a blunted smooth muscle response to the peptide but potentiates adrenocortical sensitivity. In both tissues, receptor regulation appears to involve two stages; in smooth muscle there is an initial masking/unmasking of receptors followed by an actual change in receptor number, while in adrenal cortex there is an initial alteration of receptor affinity followed by a change in receptor number with normalisation of affinity. As discussed previously, divalent cations affect the vascular receptor, acting through a mechanism which seems to involve the N-terminus of angiotensin II and which may be connected with angiotensin tachyphylaxis, while sodium influences the affinity of the adrenocortical receptor, also through a mechanism which involves the N-terminus of the peptide. In other respects (selectivity for angiotensin peptides, effect of guanine nucleotides and physicochemical characteristics) the receptors are very similar (Sections 1.2 & 1.3). In adrenal cortex there might be a counterpart of the smooth muscle tachyphylaxis factor postulated above which regulates steroidogenic sensitivity to angiotensin II. Since an influence of monovalent and/or divalent cations on the angiotensin receptor-ligand interaction has been described for most tissues studied to date, such receptor-related factors may prove to be more generally involved in the regulation of target organ sensitivity to angiotensin II.

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APPENDIX

PROGRAMS USED IN THE ANALYSIS OF RADIOLIGAND BINDING DATA

The Fortran programs below are given in standard format except that comments are preceded by the "\" character and may occur on statement lines. Each program becomes a subroutine to the main regression program (BMDP PAR) defining the function under study. The programs and their applications are described in detail in Chapter 3.

A.1 General analysis of radioligand binding data

```
      CALL MRD(P,F,X(1),X(2),X(3),X(4),X(7),X(8),X(9),X(10),X(11))
      RETURN
      END
      SUBROUTINE MRD(P,F,CLASS,EXN,TYPE,HINIT,PROT,TINC,TDSS,FTR,FRR)
\
\Values of affinity (estimated for high & low affinity sites)
\  are common to all data
\Values of receptor density (for high & low affinity sites)
\  are common to all data in CLASS n
\
\Input variables:
\HINIT      Initial tracer concentration (units of 1E-12 M)
\PROT       Membrane protein concentration (units of mg/l)
\TINC, TDSS Association/dissociation time (units of s)
\FTR, FRR   Fraction of tracer remaining/faction of receptors remaining
\             at end of association reaction
\EXN        Used in receptor assay
\TYPE       Modifies function
\
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION Y(5),W(5,23),P(40)
      EXTERNAL FCN
      COMMON/PARAM/DKF1,DKF2,DKR1,DKR2,DKDH,DKDR
      NCLASS=CLASS+0.05D0
      DKF1=10.0D0**P(1)       \Forward &
      DKR1=10.0D0**P(2)       \ reverse rate constants, high affinity site
      DKF2=10.0D0**P(3)       \Forward &
      DKR2=10.0D0**P(4)       \ reverse rate constants, low affinity site
      DKDH=-DLOG(FTR)/TINC    \Rate constant, tracer decay
      DKDR=-DLOG(FRR)/TINC    \Rate constant, receptor decay
      NTYPE=TYPE+0.05D0
\Specify initial conditions for numerical integration
      Y(1)=HINIT                \[Tracer]
      Y(2)=P(NCLASS*2+3)*PROT   \[Receptor (high affinity)]
      Y(3)=P(NCLASS*2+4)*PROT   \[Receptor  (low affinity)]
      Y(4)=0.0D0                \[Bound (high affinity)]
      Y(5)=0.0D0                \[Bound  (low affinity)]
      XINIT=0.0D0
      XEND=TINC
      N=5
      TOL=1.0D-3
      IFAIL=0
```

```
\Call NAG routine for numerical integration
      CALL D02EAF(XINIT,XEND,N,Y,TOL,FCN,W,23,IFAIL)
      GOTO(10,20,30,40,50),NTYPE
\
   10 CONTINUE
\Binding as a function of initial ligand concentration
      F=Y(4)+Y(5)
\F is predicted value of dependent variable returned to main program
      RETURN
   20 CONTINUE
\Dissociation of receptor-bound ligand
      F=Y(4)*DEXP(-DKR1*TDSS)+Y(5)*DEXP(-DKR2*TDSS)
      RETURN
\Other values of NTYPE reserved for future expansion
   30 RETURN
   40 RETURN
   50 RETURN
      END
\Specify differential equations describing the reaction
      SUBROUTINE FCN(T,Y,F)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION Y(5),F(5)
      COMMON/PARAM/DKF1,DKF2,DKR1,DKR2,DKDH,DKDR
      F(1)=-DKF1*Y(1)*Y(2)-DKF2*Y(1)*Y(3)+DKR1*Y(4)+DKR2*Y(5)-DKDH*Y(1)
      F(2)=-DKF1*Y(1)*Y(2)+DKR1*Y(4)-DKDR*Y(2)
      F(3)=-DKF2*Y(1)*Y(3)+DKR2*Y(5)
      F(4)=DKF1*Y(1)*Y(2)-DKR1*Y(4)
      F(5)=DKF2*Y(1)*Y(3)-DKR2*Y(5)
```

The corresponding BMDP control statements would be:

```
/PROBLEM TITLE IS 'RECEPTOR BINDING: 2 CLASSES OF SITES'.
/INPUT VARIABLES ARE 11.
       FORMAT IS F3.0,F2.0,F2.0,F9.2,F9.4,F7.2,F6.1,F5.0,F6.0,2F6.3.
       UNIT IS 8.
/VARIABLE NAMES ARE CLASS,EXN,TYPE,HINIT,BND,WGT,PROT,TINC,TDSS,FTR,FRR.
/REGRESS DEPENDENT IS BND.
         ITER=30.
         PARAMETERS ARE 8.
         WEIGHT=WGT.
/PARAMETER INITIAL ARE -6.000D0,-4.000D0,-7.000D0,-3.000D0,
                        0.100D0,0.010D0,0.100D0,0.010D0.
/NAMES ARE KFH,KRH,KFL,KRL,RH1,RL1,RH2,RL2.
/PLOT VAR=HINIT.
/END
```

KFH, KRH are the forward and reverse rate constants (high affinity sites)

KFL, KRL are the forward and reverse rate constants (low affinity sites)

RHn, RLn are the concentrations of high- and low-affinity binding sites

respectively for the data in CLASS n (n=1,2,3...)

A.2 The receptor assay

```
      CALL ASSAY(P,F,X(1),X(2),X(3),X(4),X(7),X(8),X(9),X(10),X(11))
      RETURN
      END
      SUBROUTINE ASSAY(P,F,CLASS,EXN,TYPE,HINIT,PROT,TINC,TDSS,FTR,FRR)
\
\Values of affinity and receptor density are estimated for
\  the high affinity site only
\Low affinity component of binding is assumed proportional to
\  HINIT and the coefficient of proportionality estimated for
\  each experiment (each value of EXN)
\Input variables: CLASS,EXN,TYPE,HINIT,PROT,TINC,TDSS,FTR,FRR
\
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION Y(3),W(3,23),P(40)
      EXTERNAL FCN
      COMMON/PARAM/DKF1,DKR1,DKDH,DKDR
      DKF1=10.0D0**P(1)       \Forward &
      DKR1=10.0D0**P(2)       \ reverse rate constants, high affinity site
      DKDH=-DLOG(FTR)/TINC    \Rate constant, tracer decay
      DKDR=-DLOG(FRR)/TINC    \Rate constant, receptor decay
      NXP=EXN+0.05D0
      NTYPE=TYPE+0.05D0
      DKR2=10**P(4)            \Reverse rate constant, low affinity site
      XLIN=P(NXP+4)*HINIT*PROT \Low affinity component of binding
\Specify initial conditions for numerical integration
      Y(1)=HINIT               \[Tracer]
      Y(2)=P(3)*PROT           \[Receptor]
      Y(3)=0.0D0               \[Bound tracer]
      XINIT=0.0D0
      XEND=TINC
      N=3
      TOL=1.0D-3
      IFAIL=0
\Call NAG routine for numerical integration
      CALL D02EAF(XINIT,XEND,N,Y,TOL,FCN,W,23,IFAIL)
      GOTO(10,20,30,40,50),NTYPE
\
   10 CONTINUE
\Binding as a function of initial ligand concentration
      F=Y(3)+XLIN
      RETURN
   20 CONTINUE
\Dissociation of receptor-bound ligand
      F=Y(3)*DEXP(-DKR1*TDSS)+XLIN*DEXP(-DKR2*TDSS)
      RETURN
\Other values of NTYPE reserved for future expansion
   30 RETURN
   40 RETURN
   50 RETURN
      END
\Specify differential equations describing the reaction
      SUBROUTINE FCN(T,Y,F)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION Y(3),F(3)
      COMMON/PARAM/DKF1,DKR1,DKDH,DKDR
      F(1)=-DKF1*Y(1)*Y(2)+DKR1*Y(3)-DKDH*Y(1)
```

```
      F(2)=-DKF1*Y(1)*Y(2)+DKR1*Y(3)-DKDR*Y(2)
      F(3)=DKF1*Y(1)*Y(2)-DKR1*Y(3)
```

The corresponding BMDP control statements would be:

```
/PROBLEM TITLE IS 'ROUTINE RECEPTOR ASSAY'.
/INPUT VARIABLES ARE 11.
       FORMAT IS F3.0,F2.0,F2.0,F9.2,F9.4,F7.2,F6.1,F5.0,F6.0,2F6.3.
       UNIT IS 8.
/VARIABLE NAMES ARE CLASS,EXN,TYPE,HINIT,BND,WGT,PROT,TINC,TDSS,FTR,FRR.
/REGRESS DEPENDENT IS BND.
         ITER=30.
         PARAMETERS ARE 7.
         WEIGHT=WGT.
/PARAMETER INITIAL ARE -6.000D0,-4.000D0,0.100D0,-2.699D0,
                        1.00D-7,1.00D-7,1.00D-7.
/NAMES ARE KF,KR,R0DP,KRL,L1DHDP,L2DHDP,L3DHDP.
/PLOT VAR=HINIT.
/END
```

KF, KR are the forward and reverse rate constants (high affinity site)

R0DP is the concentration of binding sites per unit protein

KRL is the reverse rate constant for the low affinity site

LnDHDP is the linear low affinity component of binding (per unit protein concentration per unit tracer concentration) for the n th experiment contributing to the pooled data (n=1,2,3...)

An example of the output from this program is shown in Figure 3.1.

A.3 **Competitive binding experiments**

```
      CALL DISP(P,F,X(1),X(3),X(4),X(5),X(6),X(7),X(8),X(9),X(10),X(11))
      RETURN
      END
      SUBROUTINE DISP(P,F,CRV,CTL,DKT,CCL,DKC,TME,PROT,DKM,DX,DKR)
\
\Input variables are:
\CRV   Curve number (identifies each competing ligand)
\CTL   Concentration of tracer (units of 1E-12 M)
\DKT   Rate constant for tracer decay
\CCL   Concentration of competing ligand (log [units of 1E-12 M])
\DKC   Rate constant for competing ligand decay
\TME   Incubation time (min)
\PROT  Membrane protein concentration (mg/l)
\DKR   Rate constant receptor decay
\DKM   Rate constant for decay of active metabolite of competing ligand
\DX    Identifies metabolite as one of the other competing ligands
\Assumed: competing ligand -> active metabolite -> inactive metabolite
\
```

```
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION Y(7),W(7,25),P(40)
      EXTERNAL FCN
      COMMON/PARAM/DKFT,DKRT,DKFC,DKRC,DKFM,DKRM,DKDT,DKDC,DKDM,DKDR
      NCRV=CRV+0.05D0
      INDEX=DX+0.05D0
      DKFT=10.0D0**P(2)            \Forward &
      DKRT=10.0D0**P(3)            \ reverse rates, tracer
      DKDT=DKT*1.0D-5              \Tracer decay rate
      DKFC=10.0D0**P(NCRV*2+2)     \Forward &
      DKRC=10.0D0**P(NCRV*2+3)     \ reverse rates, competing ligand
      DKDC=DKC*1.0D-5              \Competing ligand decay rate
      DKDR=DKR*1.0D-5              \Receptor decay rate
      IF(INDEX.GT.0)THEN
       DKFM=10.0D0**P(INDEX*2+2)   \Forward &
       DKRM=10.0D0**P(INDEX*2+3)   \ reverse rates, active metabolite
       DKDM=DKM*1.0D-5             \Metabolite decay rate
      ELSE
       DKFM=0.0D0
       DKRM=0.0D0
       DKDM=0.0D0
      END IF
\Specify initial conditions for numerical integration
      Y(1)=CTL           \Tracer concentration
      Y(2)=10.0D0**CCL   \Competing ligand concentration
      Y(3)=0.0D0         \Active metabolite concentration
      Y(4)=P(1)*PROT     \Receptor concentration
      Y(5)=0.0D0         \Bound tracer
      Y(6)=0.0D0         \Bound competing ligand
      Y(7)=0.0D0         \Bound metabolite
      XINIT=0.0D0
      XEND=TME*60.0D0
      N=7
      TOL=1.0D-3
      IFAIL=0
\Call NAG routine for numerical integration
      CALL D02EAF(XINIT,XEND,N,Y,TOL,FCN,W,25,IFAIL)
      CONTINUE
      F=Y(5)
      RETURN
      END
\Specify differential equations describing the reaction
      SUBROUTINE FCN(T,Y,F)
      IMPLICIT REAL*8(A-H,O-Z)
      DIMENSION Y(7),F(7)
      COMMON/PARAM/DKFT,DKRT,DKFC,DKRC,DKFM,DKRM,DKDT,DKDC,DKDM,DKDR
      F(1)=-DKFT*Y(1)*Y(4)+DKRT*Y(5)-DKDT*Y(1)
      F(2)=-DKFC*Y(2)*Y(4)+DKRC*Y(6)-DKDC*Y(2)
      F(3)=DKDC*Y(2)-DKFM*Y(3)*Y(4)+DKRM*Y(7)-DKDM*Y(3)
      F(4)=-DKFT*Y(1)*Y(4)+DKRT*Y(5)-DKFC*Y(2)*Y(4)+DKRC*Y(6)
     +     -DKFM*Y(3)*Y(4)+DKRM*Y(7)-DKDR*Y(4)
      F(5)=DKFT*Y(1)*Y(4)-DKRT*Y(5)
      F(6)=DKFC*Y(2)*Y(4)-DKRC*Y(6)
      F(7)=DKFM*Y(3)*Y(4)-DKRM*Y(7)
```

The corresponding BMDP control statements would be:

```
/PROBLEM TITLE IS 'MULTIPLE DISPLACEMENT CURVES'.
/INPUT VARIABLES ARE 12.
   FORMAT IS F3.0,F8.3,F7.2,F6.1,F6.3,F10.3,F6.3,F3.0,F6.1,F6.3,F2.0,F6.3.
   UNIT IS 8.
/VARIABLE NAMES ARE CRV,BND,WGT,CTL,DKT,CCL,DKC,TME,PROT,DKM,DX,DKR
/REGRESS DEPENDENT IS BND.
         ITER=30.
         PARAMETERS ARE 7.
         WEIGHT=WGT.
/PARAMETER INITIAL ARE 0.100D0,-6.000D0,-4.000D0,
                               -6.000D0,-4.000D0,
                               -6.000D0,-4.000D0.
/NAMES ARE R0DP,KFT,KRT,KF1,KR1,KF2,KR2.
/END
```

R0DP is the concentration of binding sites per unit protein

KFT, KRT are the forward and reverse rate constants for the tracer

KFn, KRn are the forward and reverse rate constants for the n th competing ligand (n=1,2,3...)