Endocrine (1995) 3, 255–261

Pharmacological characterization of angiotensin II AT_2 receptor subtype heterogeneity in the rat adrenal cortex and medulla

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Adrenal angiotensin II (AII) receptors have been pharmacologically and structurally divided into two main subtypes, AT_1 and AT_2. Radioligand receptor binding assays with ^{125}I-sarcosine^1, isoleucine^8 angiotensin II (^{125}I-SI AII) in the presence of losartan, an AT_1 selective ligand, and PD123177 an AT_2 selective ligand, indicated that the AT_1 subtype was predominant in membrane homogenates of the rat adrenal cortex (AT_1 Bmax = 649 ± 62 fmol/mg protein; AT_2 Bmax = 237 ± 29 fmol/mg protein). In membrane homogenates of the adrenal medulla, the AT_2 subtype was predominant (AT_1 Bmax = 55 ± 5 fmol/mg protein; AT_2 Bmax = 109 ± 29 fmol/mg protein). Overall 58% of the ^{125}I-SI AII binding in the rat adrenal was to the AT_1 subtypes, and 42% was to the AT_2 subtypes. The outer cortex contained 59% of the AII receptor binding sites in the adrenal, while the medulla accounted for the remaining 41%. The affinity of the AT_1 binding sites in membrane homogenates of the cortex and medulla (K_D = 672 ± 123 pM and 573 ± 85 pM, respectively) was not significantly different. The affinity for ^{125}I-SII AII of AT_2 binding sites in membrane homogenates was higher than that of AT_1 binding sites. The affinity for ^{125}I-SI AII of AT_2 binding sites in membrane homogenates of the outer cortex (K_D = 265 ± 35 pM) was significantly less than that in the medulla (K_D = 133 ± 11 pM). *In vitro* receptor autoradiography also demonstrated that the AT_2 subtype in frozen sections of the cortex had a lower affinity (K_D = 1512 ± 191 pM) than that in the medulla (K_D = 867 ± 72 pM). The heterogeneous affinity of adrenal AT_2 binding sites may indicate existence of multiple AT_2 receptor subtypes in the rat adrenal.

Keywords: angiotensin II (AII) receptors; AT_1; AT_2; binding affinity; rat; adrenal cortex; adrenal medulla; autoradiography

Introduction

Angiotensin II (AII) binds to specific receptors on cell surfaces to initiate a variety of physiological effects including arteriolar vasoconstriction (Braun-Menendez *et al.*, 1940; Page & Helmer, 1940), aldosterone secretion (Laragh *et al.*, 1960), catecholamine release (Zimmerman & Whitmore, 1967; Hughes & Roth, 1971), prolactin (Canonico & MacLeod, 1986) and vasopressin (Keil *et al.*, 1975) secretion and induction of drinking behavior (Booth, 1968; Andersson & Westbye, 1970; Epstein *et al.*, 1970). This multiplicity of actions suggests a complex interaction of AII with single or multiple receptors. Recently, two AII receptor subtypes were characterized in peripheral tissue and in the brain on the basis of their sensitivity to sulfhydryl reducing agents (Chiu *et al.*, 1989b; Whitebread *et al.*, 1989; Speth *et al.*, 1991) and selective AII receptor antagonists (Chiu *et al.*, 1989a; Whitebread *et al.*, 1989; Rowe *et al.*, 1990). AT_1 receptors are selectively blocked by losartan and a host of newer generation AT_1 selective antagonists (Timmermans *et al.*, 1993). Ligands selective for AT_2 receptors include CGP42112 (Whitebread *et al.*, 1989), PD123177 and its congeners (Chiu *et al.*, 1989a; Dudley *et al.*, 1990) and p-aminophenylalanine^6 AII (Speth & Kim, 1990). Both AII receptor subtypes were found by radioligand binding assays in the rat adrenal (Chiu *et al.*, 1989a; Whitebread *et al.*, 1989). The adrenal cortex contains mainly the AT_1 subtype, the adrenal medulla mainly the AT_2 subtype (Chiu *et al.*, 1989a).

The AT_1 receptor was initially cloned from bovine adrenal glomerulosa and cultured rat vascular smooth muscle cells (Murphy *et al.*, 1991; Sasaki *et al.*, 1991). Subsequent genomic analysis and homology cloning revealed two AT_1 receptor isoforms in the rat and mouse, AT_{1a} and AT_{1b} (Elton *et al.*, 1992; Iwai & Inagami, 1992; Kakar *et al.*, 1992a; Sasamura *et al.*, 1992). The isoforms have 95% identical amino acid sequence. Vascular smooth muscle and lung express primarily AT_{1a} mRNA, whereas AT_{1b} mRNA is primarily expressed in the adrenal and pituitary gland (Kakar *et al.*, 1992b). The two AT_1 subtypes have similar ligand binding profiles (Chiu *et al.*, 1993). The AT_2 receptor has also been cloned from rat fetus (Mukoyama *et al.*, 1993) and the PC12W cell line (Kambayashi *et al.*, 1993) with identical amino acid sequence and structure.

Tsutsumi and Saavedra (1992) reported heterogeneity of angiotensin II AT_2 receptors in the rat brain on the basis of differential guanine nucleotide and pertussis toxin sensitivity. Speth (1993) also reported that ^{125}I-SI AII and ^{125}I CGP42112 binding to AT_2 receptor binding sites in rat adrenal and brain displayed different responses to β-mercaptoethanol. These previous radioligand binding assays suggest the existence of an AT_2 receptor heterogeneity. In this study, the binding characteristics of both major AII receptor subtypes in the rat adrenal cortex and medulla were compared using both homogenate binding assays and *in vitro* receptor autoradiography.

Results

Saturation isotherm analyses and Rosenthal (Scatchard) transformations of ^{125}I-SI AII binding to AT_1 and AT_2 AII receptor subtypes in membrane homogenate preparations of the outer cortex and inner/medullary regions are shown in Table 1 and Figures 1 and 2. As shown in Figure 1C, the concentration of AT_1 receptors in homogenates of the outer cortex is more than 10-fold higher than in homogenates of the inner/medullary region. The K_D of ^{125}I-SI AII binding to AT_1 binding sites in these two regions of the rat adrenal is similar (Table 2). The concentration of AT_2 receptors in the outer cortex was also greater than that in the inner/medullary region by a factor of 2 (Figure 2). However, as shown in Figure 2C and Table 2, the K_D of ^{125}I-SI AII for the cortical AT_2 receptors (265 ± 79 pM) was significantly greater ($P<0.01$) than that for medullary AT_2 receptors (133 ± 25 pM). Analysis of variance of the K_D values revealed that ^{125}I-AII bound to the adrenal AT_2 receptors with a significantly higher affinity ($F_{1,14} = 118$, $P<0.001$) than to the adrenal AT_1 receptors.

The amounts of AII receptor subtypes and their proportion in different regions of the rat adrenal are described in Table 2. In the whole adrenal, AT_1 receptors, defined as maximal ^{125}I-SI AII binding capacity (Bmax) in the presence of 10^{-5} M PD 123177, accounted for 59% of the total AII

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Received 8 August 1994; accepted 17 November 1994

Table 1 Comparison of ^{125}I-SI AII binding to AT_1 and AT_2 receptors in adrenal cortex and medulla

	Adrenal cortex	*Adrenal medulla*[a]
Membrane suspensions		
AT_1	K_D = 672 ± 123	K_D = 573 ± 85
	Bmax = 649 ± 62	Bmax = 55 ± 5
AT_2	K_D = 265 ± 79†	K_D = 133 ± 25*†
	Bmax = 237 ± 29	Bmax = 109 ± 29
	$n = 5$	$n = 5$
Frozen sections		
AT_2	K_D = 1512 ± 191	K_D = 867 ± 72**
	Bmax = 198 ± 13[b]	Bmax = 516 ± 48[b]
	$n = 6$	$n = 6$

Bmax is expressed as fmol/mg protein; K_D is in units of pM. [a]Includes zona fasciculata and zona reticularis in membrane suspensions. The AT_1 subtype was assayed in the presence of 10^{-5} M PD123177. The AT_2 subtype was assayed in the presence of 10^{-5} M losartan. The values are expressed as mean ± SE. *$P<0.05$, **$P<0.01$, by paired *t*-test, significantly less than AT_2 in cortex. †$P<0.001$, by two-way ANOVA, significantly less than AT_1 [b]Bmax values in frozen sections were derived from values of fmoles per g wet weight based on empirical determinations of the amount of protein per unit wet weight (178 mg kg^{-1}) in whole adrenal homogenates

receptors. Seventy-five percent of these AT_1 receptors (286 ± 22 fmoles) were located in the outer cortex, which encompasses the zona glomerulosa and the capsule, while 25% (96 ± 6 fmoles) were present in the remaining regions of the adrenal. The AT_2 subtype of the AII receptors, defined as ^{125}I-SI AII binding in the presence of 10^{-5} M losartan, accounted for 41% of the rat adrenal AII receptors. Thirty-six percent of these AT_2 receptors (98 ± 13 fmoles) were located in the outer cortex, while 64% (174 ± 30 fmoles) were present in the inner regions of the adrenal. Nearly three-fourths of the outer cortical AII receptors were of the AT_1 subtype, while nearly two-thirds of the inner/medullary AII receptors were of the AT_2 subtype.

To confirm the distribution patterns and differential binding characteristics of ^{125}I-SI AII to AT_2 receptors in the outer cortex and inner/medullary regions, *in vitro* receptor autoradiographic analyses were performed. As shown in Figure 3, ^{125}I-SI AII binding to AT_2 receptors was localized to two regions: the zona glomerulosa, and the medulla. The concentration of AT_2 receptors in the medulla only, in contrast to membrane homogenates which included the zona reticularis and zona fasciculata with the medulla, was more than twice that of the outer adrenal cortex (Table 2 and Figure 4). As seen in membrane homogenates, the K_D of the AT_2 receptors of the cortex for ^{125}I-SI AII was again significantly greater ($P<0.01$) than that for the AT_2 receptors in the medulla (Table 2 and Figure 4).

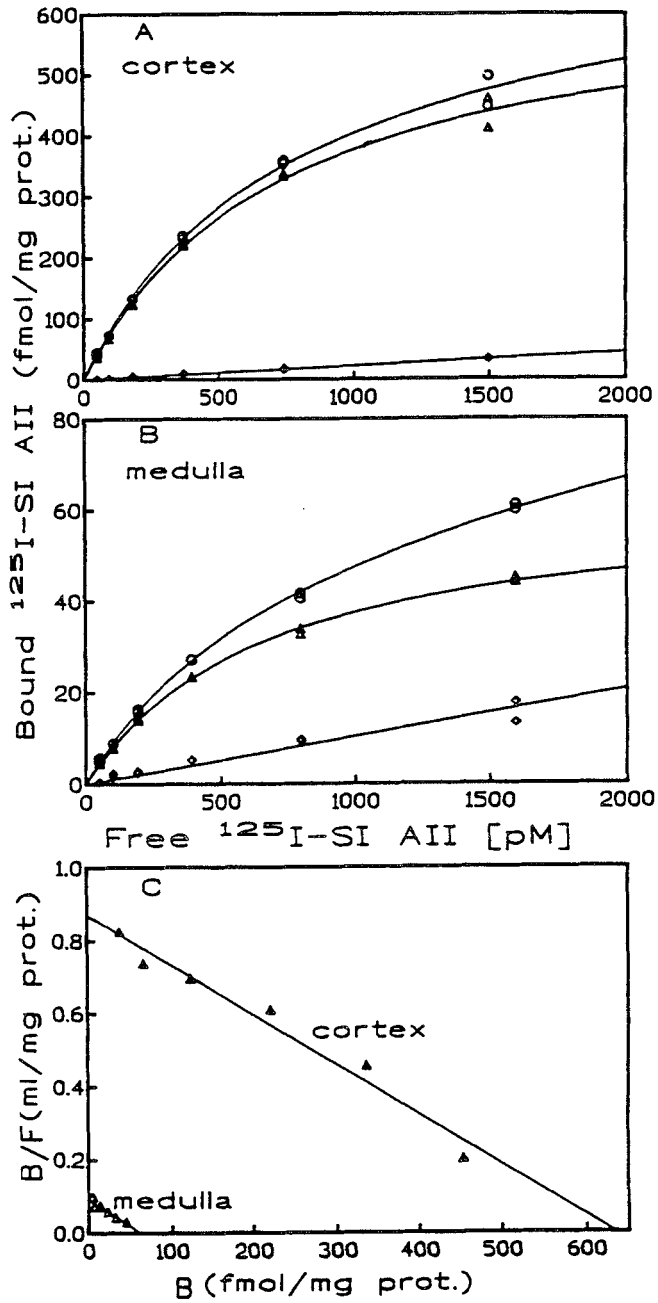

Figure 1 Saturation isotherm of ^{125}I-SI AII binding and linear transformation of specific ^{125}I-SI AII binding to AT_1 receptors in rat adrenal membrane suspensions in the presence of 10^{-5} M PD123177. Samples were run in triplicate. Nonspecific binding was determined in the presence of 3 μM AII. Specific binding (△) was derived from the difference between total binding (○) and nonspecific binding (◇). The curves for total and specific binding were generated from the equation: $Y = Bmax \cdot X/(K_D + X) + C \cdot X$ where Y = total bound, X = ^{125}I-SI AII concentration, and C is the ratio of nonspecific binding. The derived values for this example were 646 fmol mg^{-1} protein for Bmax, 692 pM for K_D in adrenal cortex (**A**) and 62 fmol/mg protein for Bmax, 657 pM for K_D in adrenal medulla (**B**). (**C**) shows Rosenthal (Scatchard) plots of the data derived from specific binding to the adrenal cortex in (**A**) (▲) and the adrenal medulla in (**B**) (△)

Discussion

To date, almost all known effects of AII in adult tissues are attributable to the AT_1 receptor through its ability to activate phospholipase C which results in mobilization of intracellular calcium and the activation of protein kinase C (PKC) or via the inhibition of adenylate cyclase activity (Murphy *et al.*, 1991; Sasaki *et al.*, 1991; Iwai & Inagami, 1992; Sasamura *et al.*, 1992). On the other hand, no consistent physiological function has been demonstrated for the AT_2 receptor. The AT_2 receptor is abundantly and widely expressed in various fetal tissues (Grady *et al.*, 1991), immature brain (Cook *et al.*, 1991; Millan *et al.*, 1991; Tsutsumi & Saavedra, 1991) and PC12W cell line (Speth & Kim, 1990; Webb *et al.*, 1992; Brechler *et al.*, 1993). However, this receptor is only sparsely expressed in the normal adult animal, primarily in the adrenal medulla (Chiu *et al.*, 1989a,b), the uterine myometrium (Whitebread *et al.*, 1989), the ovarian granulosa (Pucell *et al.*, 1991) and central nervous system (Chang *et al.*, 1990; Gehlert *et al.*, 1990; Rowe *et al.*, 1990; Millan *et al.*, 1991). These patterns of AT_2 expression suggest that the AT_2 receptor may play a role in growth and development. However, this receptor does not appear to involve any known classical intracellular signalling pathways.

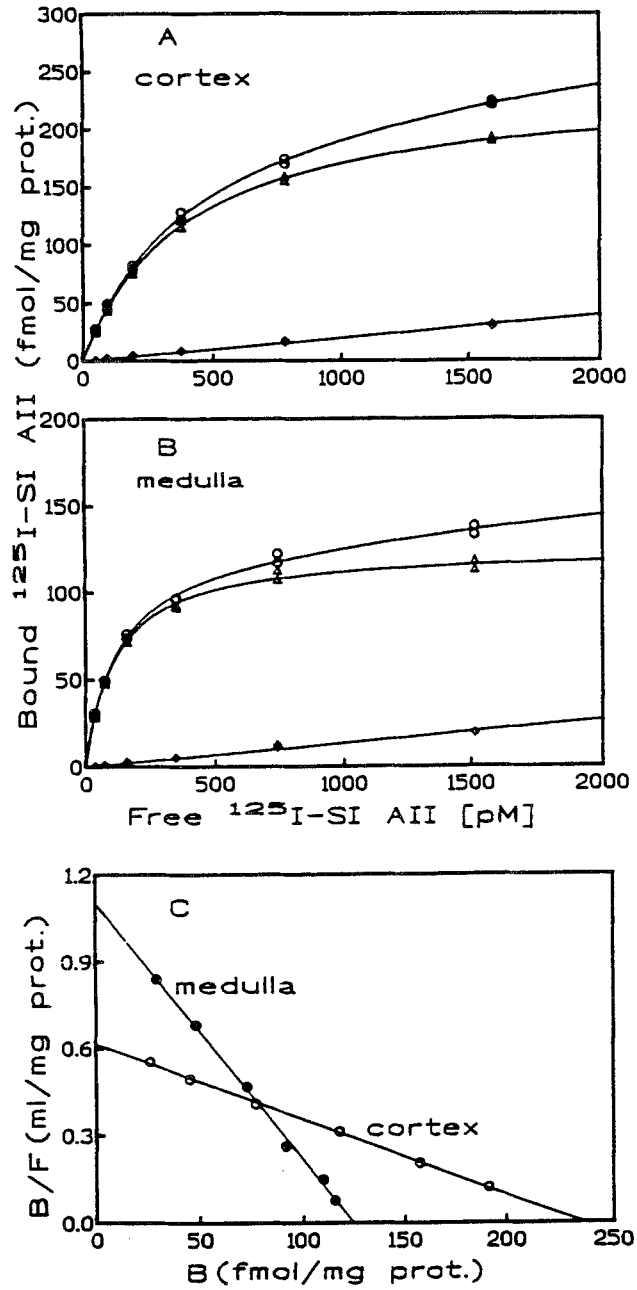

Figure 2 Saturation isotherm of ^{125}I-SI AII binding and linear transformation of specific ^{125}I-SI AII binding to rat adrenal membrane suspensions in the presence of 10^{-5} M losartan. Other details are as described in Figure 1. The derived values for this example were 238 fmol mg^{-1} protein for Bmax and 393 pM for K_D in adrenal cortex (**A**) and 129 fmol mg^{-1} protein for Bmax and 115 pM for K_D in adrenal medulla (**B**). (**C**) shows Rosenthal (Scatchard) plots of the data derived from specific binding shown in (**A**) and (**B**)

Studies of AT_2 signal transduction mechanisms are controversial regarding whether or not the stimulation of AT_2 receptor by AII results in a reduction in intracellular cGMP and an increase in phosphotyrosine phosphatase (PTP) activity (Dudley *et al.*, 1991; Pucell *et al.*, 1991; Bottari *et al.*, 1992; Webb *et al.*, 1992; Brechler *et al.*, 1993). Bottari and his associates (Bottari *et al.*, 1992) postulated a dephosphorylation of phosphotyrosine in particulate guanylate cyclase by AT_2 receptor activated PTP as the cause of decline in cGMP. Sumners *et al.* (1991) demonstrated a reduction in cGMP in AII-treated rat neuronal cells that was antagonized by CGP 42112. However, subsequent studies by Brechler *et al.* (1993) indicate that CGP 42112 may be an agonist. Kambayashi *et al.* (1993) reported that CGP42112 inhibited PTP activity in the plasma membranes of PC12W cells, rat fetal skin, and COS-7 cells stably transfected with the AT_2 cDNA, respectively. These discrepancies make is difficult to resolve the biochemical mechanism(s) of AT_2 receptors at this time.

Table 2 The proportions and amounts of AII receptor subtypes in the rat adrenal

	Cortex		*Medulla*[a]		*Total*	
	Binding (fmol)	*Percentage (%)*	*Binding (fmol)*	*Percentage (%)*	*Binding (fmol)*	*Percentage (%)*
AT_1	286 ± 22	74	96 ± 6	36	382	58
AT_2	98 ± 13	26	174 ± 30	64	272	42
Total	384	100	270	100	654	100

The values for binding above are expressed as means ± s.e.m. (fmoles per adrenal) of five experiments from 39 rats. The adrenal cortex preparations used in this study contained an average of 37 mg membrane protein per g wet weight tissue, the adrenal medulla preparations contained an average of 57 mg membrane protein per g wet weight tissue. When multiplied by the average wet weight of the subdivisions, there was 0.41 mg membrane protein-adrenal cortex and 1.68 mg membrane protein per adrenal medulla. [a]includes zona fasciculata and zona reticularis in membrane suspensions. The AT_1 subtype was assayed in the presence of 10^{-5} M PD123177. The AT_2 subtype was assayed in the presence of 10^{-5} M losartan

A sampling of previous studies of rat adrenal AII receptor binding are summarized in Table 3. Early studies (Glossman *et al.*, 1974; Devynck *et al.*, 1977) suggested the presence of subtypes of AII receptors based upon the presence of different binding affinities for the radioligand. A subsequent study (Singh *et al.*, 1986) revealed a small, but statistically significant higher K_D for ^{125}I-AII in the rat adrenal cortex compared to the medulla. It was not until relatively recently however, that AII receptors were formally differentiated into AT_1 and AT_2 subtypes based upon pharmacological studies with subtype selective antagonists and agonists (Chiu *et al.*, 1989a; Whitebread *et al.*, 1989; Chang & Lotti, 1990; Dudley *et al.*, 1990; Balla *et al.*, 1991). In view of the observations that the heptapeptide AIII has a higher affinity for the AT_2 receptor (Timmermans *et al.*, 1991), it seems likely that the high affinity ^{3}H-AIII binding site in the rat adrenal observed by Devynck *et al.* (1977) was the AT_2 subtype, and that the lower affinity ^{3}H-AIII binding site was the AT_1 receptor. However, some studies of adrenal AII receptors using the agonist radioligands ^{125}I-AII and ^{125}I-AIII (Himeno *et al.*, 1988) and the antagonist radioligand ^{125}I-SI AII have not revealed differential binding affinities (Balla *et al.*, 1991; Aguilera, 1992) except in the presence of a sulfhydryl-reducing agent (Aguilera, 1992).

This study used the peptide antagonist radioligand ^{125}I-SI AII to identify and characterize AII receptor subtype binding sites in the rat adrenal cortex and medulla. Consistent with previous studies (Chiu *et al.*, 1989a; Chang & Lotti, 1990; Balla *et al.*, 1991; Wiest *et al.*, 1991; Lu *et al.*, In press), both AT_1 and AT_2 receptors were observed in the adrenal. However, previous studies of rat adrenal AII receptors have not examined the density and binding affinity of the individual AII receptor subtypes in both the cortex and medulla. For the purposes of this study, AT_1 receptor binding was defined as specific (3 μM AII displaceable) ^{125}I-SI AII binding that occurred in the presence of the AT_2 selective, putative antagonist, PD 123177, at a concentration (10 μM) sufficient to saturate AT_2 receptors, with a negligible effect on adrenal AT_1 receptors (Lu *et al.*, in press). AT_2 receptor binding was defined as specific (3 μM AII displaceable) ^{125}I-SI AII binding that occurred in the presence of the AT_1 selective antagonist, losartan, at a concentration (10 μM) sufficient to saturate AT_1 receptors, with a negligible effect on adrenal AT_2 receptors (Lu *et al.*, in press).

Estimates of the percentage of AII receptors in the rat

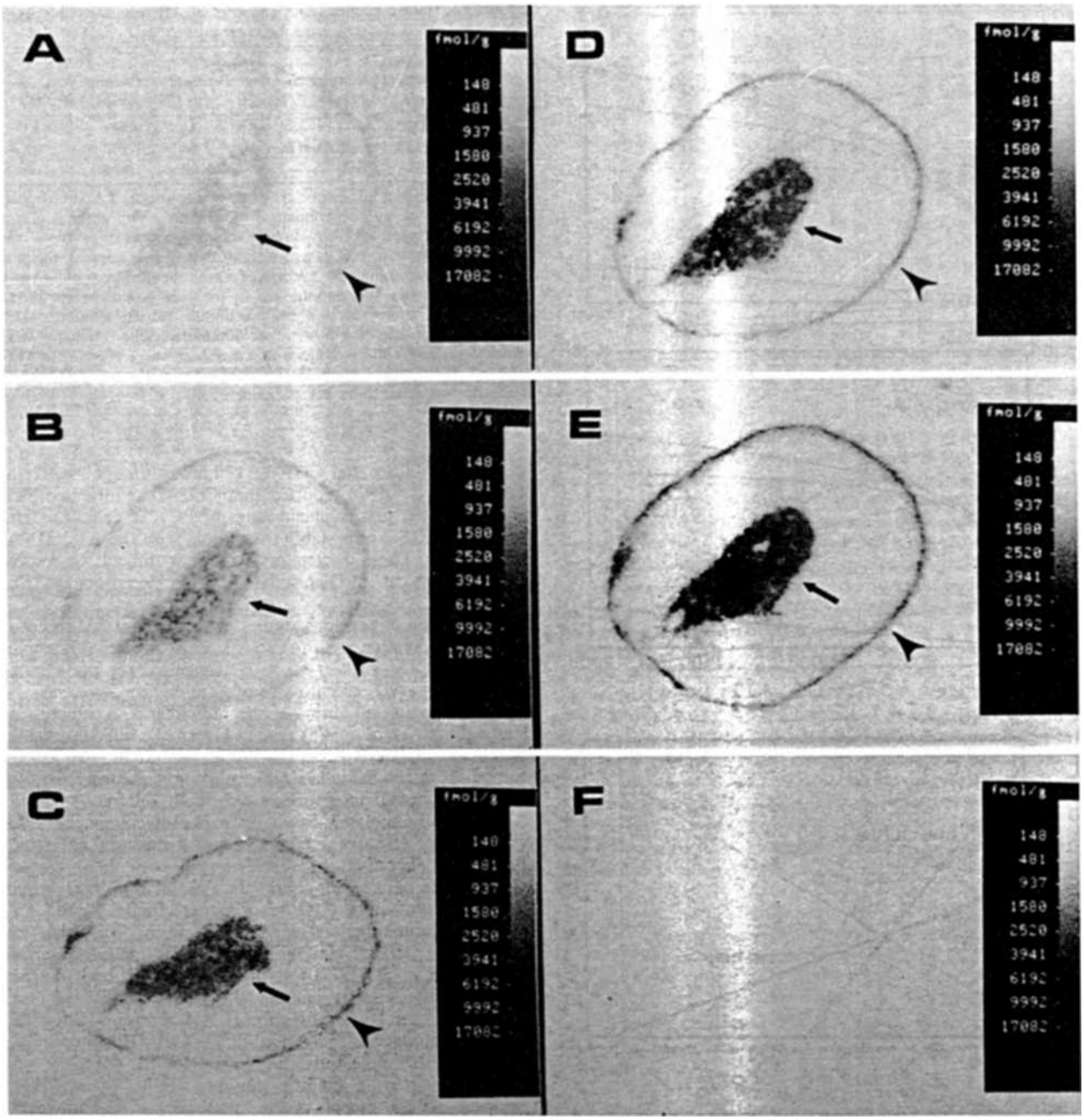

Figure 3 Computer analysed images of autoradiograms of ^{125}I-SI AII binding to adjacent adrenal sections incubated with five different concentrations; 91.8, 241, 485, 943, 2351 pM of ^{125}I-SI AII in the presence of 10^{-5} M losartan, (**A**–**E**) of I-SI AII, respectively. (**F**) represents non-specific ^{125}I-SI AII binding at a concentration of 485 pM in the presence of 3 μM AII. The standard values in the graphs are for ^{125}I in units of fmoles/g wet weight. Since nonradioactive ^{127}I-SI AII was added at a ratio of 9:1 relative to ^{125}I-SI AII, the standard values on the right side of each panel should be multiplied by 10, the isotopic dilution factor, to indicate fm per g wet weight of I-SI AII binding. To derive fmoles of I-SI AII binding per mg protein this value should be divided by the average protein concentration per gram wet weight (178 mg protein per g wet weight). For this experiment, K_D and Bmax were 1056 pM and 226 fmoles mg^{-1} protein in the adrenal cortex and 639 pM and 417 fmoles mg^{-1} protein in the adrenal medulla. The arrow heads point to the adrenal cortex, the arrows point to the adrenal medulla

adrenal cortex and medulla, based on the density of receptors per unit tissue, are about 90% and 10%, respectively (Catt *et al.*, 1984; Singh *et al.*, 1986). However, such estimates do not take into account the relative proportion of the adrenal that is cortex and that which is medulla. By multiplying the density of the receptor binding for each AII receptor subtype in the adrenal cortex and medulla (Table 1) by the amount of each tissue present in the adrenal, a value for the total number of binding sites for each AII receptor subtype binding sites per adrenal subdivision was obtained (Table 2). When compared in this manner, the proportion of total AII receptors in the adrenal cortex and medulla was much closer, 59% in cortex and 41% in the medulla.

Examination of adrenal AII receptor binding using *in vitro* receptor autoradiography revealed the explanation for the apparent low density of AII receptors in the medulla. The adrenal dissection separated the capsule and outer cortex of the adrenal from the medulla and the two inner layers of the cortex, the zona reticularis and the zona fasciculata. However as can be seen in Figure 3, negligible amounts of specific ^{125}I-SI AII binding were present in the two inner layers of the cortex in the rat. Only the medulla and the outer cortex

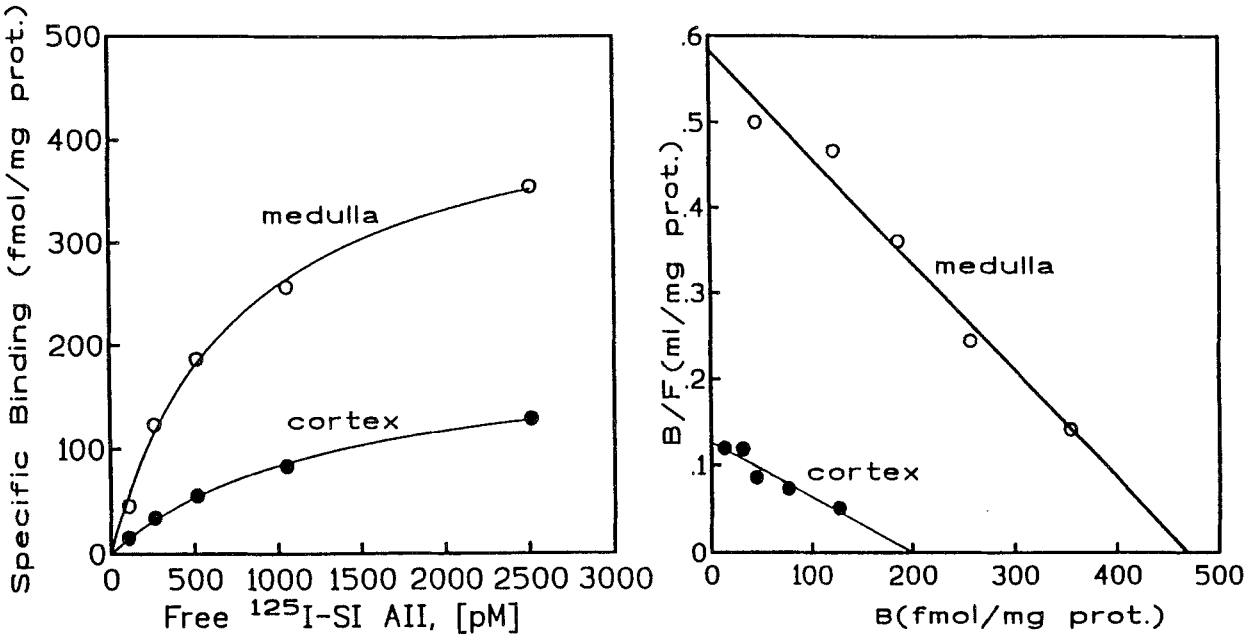

Figure 4 Saturation isotherm and linear transformation of specific ^{125}I-SI AII binding to adrenal sections in the presence of 10^{-5} M losartan. The curves shown in (**A**) were generated from the equation: $Y = Bmax \cdot X/(K_D + X)$ where Y = specific bound and X = ^{125}I-SI AII concentration. (**B**) shows Rosenthal (Scatchard) plot of the data from (**A**). For this experiment, K_D and Bmax were 1600 pM and 198 fmoles mg^{-1} protein in the adrenal cortex and 830 pM and 471 fmoles mg^{-1} protein in the adrenal medulla

Table 3 Comparison of affinity of the binding sites for AII and its analogs

Rat tissue	*Radioligand*	K_{D1}	K_{D2}	*Reference*
Adrenal fraction (5 mM DTT)	^{125}I-AII	0.2–0.5 nM	6–7 nM	Glossmann *et al.*, 1974
Adrenal fraction (no DTT)	^{3}H-AII	2.8 nM	25 nM	Devynck *et al.*, 1977
	^{3}H-AIII	0.15 nM	3.6 nM	
Adrenal cortex (1 mM DTT)	^{125}I-AII	393 pM		Singh *et al.*, 1986
medulla (1 mM DTT)	^{125}I-AII	228 pM		
Adrenal cortex (no DTT)	^{125}I-AII	860 pM		Himeno *et al.*, 1988
	^{125}I-AIII	900 pM		
medulla (no DTT)	^{125}I-AII	1460 pM		
	^{125}I-AIII	1160 pM		
Adrenal cortex (no DTT)	^{125}I-SI AII	2.8 nM (IC_{50} for SI AII)		Balla *et al.*, 1991
medulla (no DTT)	^{125}I-SI AII	2.6 nM (IC_{50} for SI AII)		
Adrenal glomerulosa (no DTT)	^{125}I-SI AII	1.6 nM (AT_1)	1.7 nM (AT_2)	Aguilera, 1992
(2 mM DTT)	^{125}I-SI AII	1.8 nM (AT_1)	0.8 nM (AT_2)	

displayed ^{125}I-SI AII binding. Thus the estimates of ^{125}I-SI AII binding density in membrane homogenates of the medulla were decreased due to dilution with these non-AII receptor-containing regions.

Consistent with previous observations (Chiu *et al.*, 1989a), the percentage of the AT_1 receptor subtype in the adrenal cortex (74%) was greater than that of the AT_2 receptor subtype. And the percentage of AT_1 receptors in the medulla (36%) was less than that of the AT_2 receptors. For the whole adrenal, the percentage of AT_1 receptors (58%) was only slightly greater than the percentage of AT_2 receptors (42%).

Evaluation of the binding affinity for ^{125}I-SI AII of the two different AII receptor subtypes in the adrenal revealed signifiant differences. The binding affinity of AT_1 receptors in adrenal membrane homogenates was less than that of the AT_2 receptors. This observation contrasts with previous observations of ^{125}I-SI AII selectivity for AII receptor subtypes. In the rat adrenal glomerulosa, no difference in ^{125}I-SI AII binding affinity to AT_1 and AT_2 receptors was observed in the absence of sulfhydryl-reducing agents (Aguilera, 1992). In the rat brain (Rowe *et al.*, 1992), and from comparison of rat liver (AT_1) and PC12W cells (AT_2) (Speth & Kim, 1990), ^{125}I-SI AII binds with lower affinity to the AT_2 subtype than to the AT_1 subtype. The sulfhydryl-reducing agent β-mercaptoethanol, which increases ^{125}I-SI AII binding to AT_2 receptors in the brain (Speth *et al.*, 1991), did not alter ^{125}I-SI AII binding affinity in the whole adrenal (Speth, 1993). But dithiothreitol, another sulfhydryl-reducing agent, increases ^{125}I-SI AII binding affinity to AT_2 receptors in the adrenal glomerulosa (Aguilera, 1992).

The binding affinity of AT_2 receptors for ^{125}I-SI AII in both membrane homogenates and slide-mounted frozen sections of the adrenal medulla was greater (lower K_D) than that of the AT_2 receptors in the cortex. The observation of higher binding affinity for ^{125}I-SI AII in the medulla *vs* the cortex is consistent with the observation by Singh *et al.* (1986) of a higher binding affinity for ^{125}I-AII in the adrenal medulla. That study used a low (1 mM) concentration of dithiothreitol, which diminishes ligand binding to AT_1 receptors (Chiu *et al.*, 1989b; Whitebread *et al.*, 1989). Thus it is likely that the adrenal preparation used by Singh *et al.* (1986) expressed primarily AT_2 receptor-binding. The difference in binding affinity for ^{125}I-SI AII between homogenates and tissue sections, shown in Table 1, is consistent with previous observations (Lu *et al.*, in press) indicating that ligand binding affinity of AII receptors is decreased by the procedures required to prepare sections for *in vitro* receptor autoradiography.

The observation of different binding affinities for ^{125}I-SI

AII at putative AT_2 receptor binding sites in the adrenal cortex and medulla may be suggestive of AT_2 receptor heterogeneity. Previous radioligand binding studies of AT_2 receptors in the rat brain (Tsutsumi & Saavedra, 1992; Speth, 1993) also suggest the presence of AT_2 subtype heterogeneity. Of note, Balla *et al.* (1991) observed a twofold difference in PD123177 and a threefold difference in CGP42112 binding affinity for AT_2 receptors in the rat adrenal cortex and medulla. In contrast, comparisons of PD123177 binding affinity of AT_2 receptors in the adrenal cortex and medulla carried out in this laboratory (Lu *et al.*, in press) did not reveal substantial differences. The existence of two AT_1 receptor subtypes, AT_{1a} and AT_{1b} in the rat has been confirmed by expression cloning studies (Elton *et al.*, 1992; Iwai & Inagami, 1992). However, expression cloning studies of rat AT_2 receptors from different laboratories (Kambayashi *et al.*, 1993; Mukoyama *et al.*, 1993) have not revealed differences in the genes encoding the amino acid sequence of the receptor from two divergent types of tissue. The gene for the human AT_2 receptor shows a high degree of similarity to the rat and mouse AT_2 receptor gene Tsuzuki *et al.*, 1994). In addition, the fetal mouse appears to possess only one AT_2 receptor gene (Nakajima *et al.*, 1993). It is possible that the AT_2 receptors in different tissues follow different routes of post-translational processing, leading to subtle changes in the ligand binding characteristics. This scenario, which is well characterized for different neuropeptide transmitters (Mains *et al.*, 1977; Amara *et al.*, 1982), has not been established as a cause of receptor subtyping. However, Barker *et al.* (1993) observed four different isoforms of AII receptors in the rat adrenal using isoelectric focusing, suggesting that the differences could reflect different post-translational processing. Recently, Servant *et al.* (1994) reported that the marked disparity between the sizes of AT_2 receptors from different tissues was related to different degrees of N-glycosylation. This could also cause moderate differences in ligand binding characteristics. Another possibility is that specific physical characteristics of the cells lead to subtle variations in the ability of the AT_2 receptors to bind ligands, e.g. the relative state of reduction or oxidation of the sulfhydryl bonds between cysteine residues in the extracellular domain of the receptors. Further studies are needed to determine if the differences observed in this study have important physiological or pharmacological significance.

Materials and methods

Tissues were obtained from male Sprague-Dawley rats obtained from the Laboratory Animal Resource Center at Washington State University. For homogenate binding studies, rats were sacrificed by decapitation. The tissues were removed and further dissected. The adrenal was freed of adjacent adipose tissue and the adrenal cortex, primarily the outer zona glomerulosa, was separated from the medulla which also included portions of the zona fasciculata and zona reticularis. The adrenals from 7–9 rats were pooled for each comparison and weighed.

The tissues were mechanically homogenized in hypotonic buffer (20 mM sodium phosphate, pH 7.1–7.2) using a mechanical homogenizer (Tissuemizer, Tekmar, Cincinnati, OH). The homogenates were then centrifuged at 48 000 *g* for 20 min at 4°C. The precipitated membranes were resuspended in assay buffer (50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 0.1 mM bacitracin, pH 7.1–7.2) and centrifuged again. The membranes were resuspended at a concentration of approximately 10 mg initial wet weight per ml.

For analysis of AII receptor density and radioligand binding affinity, six concentrations of ^{125}I-SI AII ranging from 0.1 to 2 nM were used. Losartan and PD123177 were added at 10 μM concentrations to determine AT_2 and AT_1 receptor binding, respectively. Based on previous studies (Chiu *et al.*, 1989a; Speth & Kim, 1990, Lu *et al.*, in press), this concentration of these highly AII receptor subtype selective nonpeptide ligands gives near complete inhibition of the one subtype, with negligible inhibition of the other subtype. Nonspecific binding of ^{125}I-SI AII was determined in the presence of 3 μM AII. Incubations were terminated by filtration through glass fibre filters (no. 32, Schleicher and Schuell, Keene, NH), prerinsed with 0.1% bovine albumin. Filters were rinsed three times with 50 mM sodium-potassium phosphate buffer, pH 7.4, at 22°C. Filter bound radioligand was assayed by sodium iodide crystal gamma scintillation counting. Membrane protein was determined by the method of Lowry *et al.* (1951).

For receptor autoradiographic studies of adrenal AII receptor subtypes, the rats were anesthetized with Equithesin (Jensen-Salsbury, Kansas City, MO) at 0.4 ml per 100g body weight and perfused intracardially with chilled phosphate buffered saline (20 mM sodium phosphate, pH 7.2). The adrenal was cleared of adjacent adipose tissue and frozen in a −20°C freezer for less than 1 week. The adrenals were sectioned at a thickness of 20 microns in a cryostat, air-dried and again stored at −20°C. On the day of assay, the slide-mounted adrenal sections were thawed at 37°C for about 15 s and preincubated for 30 min in assay buffer. The sections were then incubated in fresh assay buffer with a mixture of ^{125}I-SI AII and ^{127}I-SI AII yielding a final concentration of I-SI AII ranging from 0.1 to 2.5 nM in the presence of 10^{-5} M losartan for 2 h. Nonspecific binding of I-SI AII was determined in the presence of 3 μM AII.

The ratio of ^{125}I-SI AII to ^{127}I-SI AII was 1 to 9. The use of non-radioisotopic I-SI AII was necessitated by the extremely high amount of binding that occurred at high radioligand concentrations, exceeding the concentrations of ^{125}I contained in the standards used for densitometric calibrations. ^{127}I-SI AII was prepared as described for ^{125}I-SI AII, except that a trace of ^{125}I-SI AII was added to monitor the peak of mono ^{127}I-SI AII eluting from the HPLC column. The concentration of ^{127}I-SI AII was determined by competition assay with ^{125}I-SI AII.

Upon completion of the incubation, the sections were then dipped in two changes of distilled water, rinsed for 1 min in five changes of assay medium and finally dipped in another two changes of distilled water. The sections were then dried under a cool stream of air, mounted on cardboard and exposed to sheet film (SB-5, Kodak) in X-ray cassettes for an appropriate duration of time to insure sufficient film exposure at the lowest radioligand concentration. A video-based image analysis system (MCID, Imaging Research) was used to quantitate film exposure in units of fmol of ^{125}I-SI AII bound per g tissue based on a standard curve generated from known amounts of ^{125}I (Microscales, Amersham Inc. Arlington Heights, IL).

Binding constants were calculated using a computer program (Inplot 4.0, GraphPad Software, San Diego, CA). The dissociation constant (K_D) and receptor density (Bmax) for specific binding were determined by the equation: $Y = A \cdot X/(B + X) + C \cdot X$, where $A = Bmax$, $B = K_D$, C is the proportion of added ^{125}I-SI AII that was bound nonspecifically. X is the free radioligand concentration, and Y is total binding. The value of C·X was subtracted from this equation to yield the specific binding curve. For some assays the data was analysed by the linear transformation of Rosenthal (1967): $B/F = (-1/K_D)B + Bmax/K_D$, where B = specific binding, F = free radioligand concentration (commonly referred to as the Scatchard plot).

Data are expressed as mean ± standard error of the mean. Comparison of specific binding and binding constants in the different tissues was performed using a two-way ANOVA and a two-tailed Student's *t*-test. The null hypothesis was rejected when $P < 0.05$.

Acknowledgements

Supported by USPHS Grant NS 21305, the Peptide Radioiodination Service Center, Washington State University and a fellowship from the Poncin Medical Research Foundation, Seattle, WA (KLG). Losartan was generously provided by Dr Ron Smith of the DuPont Merck Pharmaceutical Corp. PD123177 was generously provided by Carol Germain at Parke-Davis Pharmaceutical Research.

References

Aguilera, G. (1992). *Mol. Cell. Endocrinol.*, **90**, 53–60.

Amara, S., Jonas, V., Rosenfeld, M.G., Ong, E.S. & Evans, R.M. (1982). *Nature*, **298**, 240–244.

Andersson, B. and Westbye, O. (1970). *Life Sci.*, **9**, Pt. 1, 601–608.

Balla, T., Baukal, A.J., Eng, S. and Catt, K.J. (1991). *Mol. Pharmacol.*, **40**, 401–406.

Barker, S. (1993). *Biochem. Biophys. Res. Comm.*, **192**, 392–398.

Booth, D.A. (1968). *J. Pharmacol. Exp. Ther.*, **160**, 336–348.

Bottari, S., King, I.N., Reichlin, S., Dalhsoem, I., Lydon, N. & de Gasparo, M. (1992). *Biochem. Biophys. Res. Comm.*, **183**, 206–211.

Braun-Menendez, E., Fasciolo, J.C., Leloir, S.F and Munoz, J.M. (1940). *J. Physiol.*, **98**, 283–298.

Brechler, V., Jones, P.W., Levens, N., de Gasparo, M. & Bottari, S.P. (1993). *Regul. Pept.*, **44**, 207–213.

Canonico, P.L and MacLeod, R.M. (1986). *Endocrinology*, **118**, 233–238.

Catt, K.J., Mendelsohn, F.A., Millan, M.A. and Aguilera, G. (1984). *J. Cardiovasc. Pharmacol.*, **6**, S575–S586.

Chang, R.S.L. and Lotti, V.J. (1990). *Mol. Pharmacol.*, **29**, 347–351.

Chang, R.S.L., Lotti, V.J., Chen, T.B. and Faust, K.A. (1990). *Biochem. Biophys. Res. Comm.*, **171**, 813–817.

Chiu, A.T., Herblin, W.F., McCall, D.E. Ardecky, R.J., Carini, D.J., Duncia, J.V., Pease, L.J., Wong, P.C., Wexler, R.R., Johnson, A.L. & Timmermans, P.B.M.W.M. (1989a). *Biochem. Biophys. Res. Comm.*, **165**, 196–203.

Chiu, A.T., McCall, D.E., Nguyen, T.T., Carini, D.J., Duncia, J.V. Herblin, W.F., Uyeda, R.T., Wong, P.C., Wexler, R.R., Johnson, A.L. & Timmermans, P.B.M.W.M. (1989b). *Eur. J. Pharmacol.*, **170**, 117–118.

Chiu, A.T., Dunscomb, J., Kosierowski, J., Burton, C.R., Santomenna, L.D., Corjay, M.H. & Benfield, I. (1993). *Biochem. Biophys. Res. Commun.*, **197**, 440–449.

Cook, V.I., Grove, K.L., Speth, R.C., McMenamin, K.M. & Harding, J.W. (1991). *Brain Res.*, **560**, 334–336.

Devynck, M.A., Pernollet, M.G., Matthews, P.G., Khosla, M.C., Bumpus, F.M. & Meyer, P. (1977). *Proc. Natl. Acad. Sci. USA*, **74**, 4029–4032.

Dudley, D.T., Pancek, R.L., Major, T.C., Lu, G.H., Bruns, R.F., Klinkefus, B.A., Hodges, J.C. & Weishaar, R.E. (1990). *Mol. Pharmacol.*, **38**, 370–377.

Dudley, D.T., Hubbell, S.E and Summerfelt, R.M. (1991). *Mol. Pharmacol.*, **40**, 360–367.

Elton, T.S., Stephan, C.C., Taylor, G.R., Kimball, M.G., Martin, M.M., Durand, J.N. & Oparil, S.(1992). *Biochem. Biophys. Res. Comm.*, **184**, 1067–1073.

Epstein, A.N., Fitzsimons, J.T. and Rolls, B.J. (1970). *J. Physiol.*, **210**, 457–474.

Gehlert, D.R., Gackenheimer, S.L., Reel, J.K., Lin, H.-S. & Steinberg, M.I. (1990). *Eur. J. Pharmacol.*, **187**, 123–126.

Glossmann, H., Baukal, A.J. and Catt, K.J. (1974). *J. Biol. Chem.*, **249**, 825–834.

Grady, E.F., Sechi, L.A., Griffin, C.A., Schambelan, M. & Kalinyak, J.E. (1991). *J. Clin. Invest.*, **88**, 921–933.

Himeno, A., Nazarali, A.J and Saavedra, J.M. (1988). *Regul. Pept.*, **23**, 127–133.

Hughes, J. and Roth, R.H. (1971). *Br. J. Pharmacol.*, **41**, 239–255.

Iwai, N. and Inagami, T. (1992). *FEBS Lett.*, **298**, 257–260.

Kakar, S.S., Riel, K.K and Neill, J.D. (1992a). *Biochem. Biophys. Res. Commun.*, **185**, 688–692.

Kakar, S.S., Seller, J.C., Devor, D.C., Musgrove, L.C. & Neill, J.D. (1992b). *Biochem. Biophys. Res. Commun.*, **183**, 1090–1096.

Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T. & Inagami, T. (1993). *J. Biol. Chem.*, **268**, 24543–24546.

Keil, L.C., Summy-Long, J. and Severs, W.B. (1975). *Endocrinology*, **96**, 1063–1065.

Laragh, J.H., Angers, M., Kelly, W.G. and Lieberman, S. (1960). *JAMA*, **174**, 234–240.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). *J. Biol. Chem.*, **193**, 265–275.

Lu, X.-Y., Zhang, W., Grove, K.L. and Speth, R.C. (1994). *J. Pharmacol. Meth.* In press.

Mains, R.E., Eipper, B.A. and Ling, N. (1977). *Proc. Natl. Acad. Sci. USA*, **74**, 3014–3018.

Millan, M.A., Jacobowitz, D.M., Aguilera, G. and Catt, K.J. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 11440–11444.

Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R.E. & Dzau, V.J. (1993). *J. Biol. Chem.*, **268**, 24539–24542.

Murphy, T.J., Alexander, R.W., Griendling, K.K., Runge, M.S. & Bernstein, K.E. (1991). *Nature*, **351**, 233–236.

Nakajima, M., Mukoyama, M., Pratt, R.E., Horiuchi, M. & Dzau, V.J. (1993). *Biochem. Biophys. Res. Commun.*, **197**, 393–399.

Page, I.H. and Helmer, O.M. (1940). *J. Exp. Med.*, **71**, 29.

Pucell, A.G., Hodges, J.C., Sen, I., Bumpus, F.M. & Husain, A. (1991). *Endocrinology*, **128**, 1947–1959.

Rosenthal, H.E. (1967). *Anal. Biochem.*, **20**, 525–532.

Rowe, B.P., Grove, K.L., Saylor, D.L. and Speth, R.C. (1990). *Eur. J. Pharmacol.*, **186**, 339–342.

Rowe, B.P., Saylor, D.L. and Speth, R.C. (1992). *Neuroendocrinology*, **55**, 563–573.

Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J.J., Hasegawa, M., Matsuda, Y. & Inagami, T. (1991). *Nature*, **351**, 230–233.

Sasamura, H., Hein, L., Krieger, J.E., Pratt, R.E., Kobilka, B.K. & Dzau, V.J. (1992). *Biochem. Biophys. Res. Commun.*, **185**, 253–259.

Servant, G., Dudley, D.T., Escher, E. and Guillemette, G. (1994). *Mol. Pharmacol.*, **45**, 1112–1118.

Singh, R., Harding, J.W. and Speth, R.C. (1986). *Eur. J. Pharmacol.*, **120**, 319–327.

Speth, R.C., Rowe, B.P., Grove, K.L., Carter, M.R. & Saylor, D.L. (1991). *Brain Res.*, **548**, 1–8.

Speth, R.C. (1993). *Regul. Pept.*, **44**, 189–197.

Speth, R.C. and Kim, K.H. (1990). *Biochem. Biophys. Res. Comm.*, **169**, 997–1006.

Sumners, C., Tang, W., Zelezna, B. and Raizada, M.K. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 7567–7571.

Timmermans, P.B.M.W.M., Wong, P.C., Chiu, A.T. and Herblin, W.F. (1991). *TIPS*, **12**, 55–62.

Timmermans P.B., Wong, P.C., Chiu, A.T., Herblin, W.F., Benfield, P., Carini, D.J., Lee, R.J., Wexler, R.R., Saye, J.A. & Smith, R.D. (1993). *Pharmacol. Rev.*, **45**, 205–251.

Tsutsumi, K. and Saavedra, J.M. (1991). *Endocrinology*, **129**, 3001–3008.

Tsutsumi, K. and Saavedra, J.M. (1992). *Mol. Pharmacol.*, **41**, 290–297.

Tsuzuki, S., Ichiki, T., Nakakubo, H., Kitami, Y., Guo, D.F., Shirai, H. & Inagami, T. (1994). *Biochem. Biophys. Res. Commun.*, **200**, 1449–1454.

Webb, M.L., Liu, E.C.-K., Cohen, R.B., Hedberg, A., Bogosian, E.A., Monshizadegan, H., Molloy, C., Serafino, R., Moreland, S., Murphy, T.J. & Dickinson, K.E.J. (1992). *Peptides*, **13**, 499–508.

Whitebread, S., Mele, M., Kamber, B. and de Gasparo, M. (1989). *Biochem. Biophys. Res. Comm.*, **163**, 284–291.

Wiest, S.A., Rampersaud, A., Zimmerman, K. and Steinberg, M.I. (1991). *J. Cardiovasc. Pharmacol.*, **17**, 177–184.

Zimmerman, B.G. and Whitmore, L. (1967). *Int. J. Neuropharmacol.*, **6**, 27–38.