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Sarcosine[1],glycine[8] angiotensin II is an AT_1 angiotensin II receptor subtype selective antagonist

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Abstract

Studies predating the discovery of the two major subtypes of angiotensin II (Ang II) receptors, AT_1 and AT_2, revealed anomalous characteristics of sarcosine[1],glycine[8] Ang II (Sar[1],Gly[8] Ang II). It competed poorly for ^{125}I-Ang II binding in bovine brain but potently antagonized dipsogenic responses to intracerebroventricularly administered Ang II. Subsequent recognition that bovine brain contains AT_2 receptors, while dipsogenic responses to Ang II are mediated by AT_1 receptors, suggests that Sar[1],Gly[8] Ang II is AT_1 selective. Sar[1],Gly[8] Ang II competed for ^{125}I-sarcosine[1],isoleucine[8] Ang II binding to AT_1 receptors in pituitary, liver and adrenal (the latter with the AT_2 selective antagonist PD 123,319) with K_i's of 0.66, 1.40 and 1.36 nM, respectively. In contrast, the K_i of Sar[1],Gly[8] Ang II for AT_2 receptors in rat adrenal (with the selective AT_1 antagonist losartan) was 52 nM. ^{125}I-Sar[1],Gly[8] Ang II (0.5–3 nM) bound to AT_1 receptors in pituitary, liver, heart, adrenal, and hypothalamic membranes with high affinity (K_d = 0.43, 1.6, 2.3, 0.96 and 1.8 nM, respectively), but showed no saturable binding to the adrenal AT_2 receptor. ^{125}I-Sar[1],Gly[8] Ang II selectively labeled AT_1 receptors in sections of adrenal using receptor autoradiography. Thus, binding studies reveal Sar[1],Gly[8] Ang II to be the first angiotensin peptide analog to show AT_1 receptor selectivity. ^{125}I-Sar[1],Gly[8] Ang II offers a new means to selectively radiolabel AT_1 receptors and may help to characterize ligand docking sites and agonist switches for AT_1 versus AT_2 receptors.

Keywords: Radioligand binding; Adrenal; Pituitary; Liver; Brain

1. Introduction

In reviewing the antidipsogenic properties of angiotensin antagonists, Fitzsimons [1] described an anomaly with sarcosine[1],glycine[8] angiotensin II (Sar[1],Gly[8] Ang II). It was as effective an antagonist as sarcosine[1] alanine[8] Ang II (saralasin) on the dipsogenic responses to Ang II administered intracerebroventricularly; however, it was only 0.5% as potent as saralasin in competing for ^{125}I-Ang II binding in the bovine cerebellum relative to saralasin [2]. The potency of Sar[1],Gly[8] Ang II to antagonize the ability of Ang II to contract a rabbit aortic strip (pA_2 = 8.32) is only about twofold less than that of Sar[1],Leu[8] Ang II (pA_2 = 8.64) [3].

With the subsequent discovery of two major subtypes of Ang II receptors [4,5], it is now known that the dipsogenic and pressor effects of Ang II are mediated by AT_1 receptors [6,7], while the bovine cerebellum contains primarily AT_2 receptors [8]. In hindsight, Fitzsimons' observation suggests that Sar[1],Gly[8] Ang II may be an AT_1 receptor subtype selective antagonist. A more recent study [9] further showed that Sar[1],Gly[8] Ang II bound with higher affinity to cells transfected with rat AT_{1B} Ang II receptor than to cells transfected with rat AT_{1A} receptor.

The selectivity of losartan (historically known as DuP 753 and Exp 655) for the AT_1 Ang II receptor subtype gave rise to the prospect that it could be used to selectively radiolabel AT_1 receptors. However, ^{3}H-losartan has considerable non-AT_1 receptor binding sites [10]. An attempt was made to radioiodinate the losartan analog, Exp985 [11], but this radioligand abundantly bound to albumin, obscuring AT_1 receptor binding. Another nonpeptide AT_1 receptor antagonist SK&F 108566 (eprosartan) has been radiolabeled with ^{3}H and used to identify AT_1 receptors [12]. However, the relatively low specific activity and energy of ^{3}H limits the utility of this radioligand for measuring small quantities of receptor and for receptor autoradiography.

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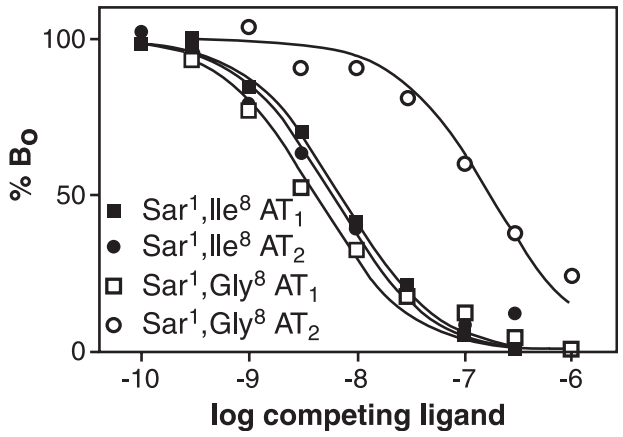

Fig. 1. Binding of Sar^1,Gly^8 Ang II and Sar^1,Ile^8 Ang II to adrenal AT_1 and AT_2 receptors. Representative competition binding of Sar^1,Gly^8 Ang II and Sar^1,Ile^8 Ang II for $^{125}I\text{-}Sar^1,Ile^8$ Ang II binding to AT_1 receptors (determined in the presence of 10 μM of the selective AT_2 receptor antagonist PD 123,319) and AT_2 receptors (determined in the presence of 10 μM of the selective AT_1 receptor antagonist losartan). Data points are means of duplicate measurements. In this experiment, the concentration of $^{125}I\text{-}Sar^1,Ile^8$ Ang II was 0.6 nM. The respective K_d values for $^{125}I\text{-}Sar^1,Ile^8$ Ang II binding to AT_1 and AT_2 receptors in this experiment were 0.53 and 0.51 nM, respectively. IC_{50} values, derived from a simple one-site competition model with a Hill coefficient of 1.00 (Prism, Graphpad Software), were 7.0 nM for Sar^1,Ile^8 at AT1 receptors, 5.8 nM for Sar^1,Ile^8 Ang II at AT_2 receptors, 4.0 nM for Sar^1,Gly^8 Ang II at AT_1 receptors, and 171 nM for Sar^1,Gly^8 Ang II at AT_2 receptors. Values for adrenal AT_1 include the six adrenal pools assayed in parallel with liver and pituitary, shown in Fig. 3. Values are mean ± S.E.M., $n=8$. $^*p<0.001$ greater than Sar^1,Gly^8 Ang II at AT_1 receptor.

To pursue the hypothesis that Sar^1,Gly^8 Ang II binds preferentially AT_1 receptors, this study examined the ability of Sar^1,Gly^8 Ang II and ^{125}I-labeled Sar^1,Gly^8 Ang II to bind to a variety of rat tissues expressing different AT receptor subtypes. Studies of mRNA expression and radioligand binding indicate that the liver, heart and hypothalamus contain primarily AT_{1A} receptors but that heart and hypothalamus contain AT_2 receptors as well [13–18]. The pituitary expresses primarily AT_{1B} receptors and no AT_2 receptors [13,18–20]. The adrenal expresses both AT_{1A} and AT_{1B} receptors as well as AT_2 receptors [13,21,22]. In adrenal, AT_1 and AT_2 receptors were assayed individually by the addition of selective antagonists of each subtype. The results support the hypothesized selectivity of Sar^1,Gly^8 Ang II for AT_1 receptors.

2. Methods

Adrenal, liver, pituitary, heart and brain were obtained from adult male Sprague–Dawley rats killed by carbon dioxide asphyxia or decapitation. Analyses were made from individual rats (heart) or pools of tissue harvested from two to nine rats. All animal handling procedures were carried out in accordance with the U.S. Public Health Service Guide for the Care and Use of Laboratory Animals, published by the National Academy of Sciences of the United States.

Tissues were weighed and immediately homogenized in a hypotonic, 20 mM sodium phosphate buffer, pH 7.2. Heart homogenates were filtered through cheesecloth to remove large particulates that could not be disrupted. Cell membranes were precipitated by centrifugation at 48,000 × *g* for 20 min at 4 °C. The membranes were resuspended in assay buffer; 150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, 50 mM sodium phosphate pH 7.2 and recentrifuged as before. The membranes were finally resuspended at a concentration of 1–10 g initial wet weight per 100 ml in the assay buffer. The adrenal and heart homogenates were divided into two aliquots. Losartan (10 μM final concentration) was added to one aliquot to provide a tissue sample in which only AT_2 receptors were available to bind radioligand. PD 123,319 (10 μM final concentration) was added to the other aliquot to provide a preparation in which only AT_1 receptors were available to bind radioligand. PD 123,319 (10 μM) was also added to the hypothalamic membrane preparation to limit binding to the predominant AT_1 subtype.

Ang II receptor-binding assays of 50–150 μl of each tissue were carried out for 2 h at 22–24 °C in a volume of 100–200 μl with either ^{125}I-sarcosine^1,isoleucine^8 Ang II ($^{125}I\text{-}Sar^1,Ile^8$ Ang II) or $^{125}I\text{-}Sar^1,Gly^8$ Ang II at eight

Table 1
Comparison of binding of $^{125}I\text{-}Sar^1,Gly^8$ Ang II, $^{125}I\text{-}Sar^1,Ile^8$ Ang II, Sar^1,Gly^8 Ang II and Sar^1,Ile^8 ang II in various tissues

Tissue	Sar^1,Gly^8 Ang II, K_i	Sar^1,Ile^8 Ang II, K_i	$^{125}I\text{-}Sar^1,Gly^8$ Ang II, K_d	$^{125}I\text{-}Sar^1,Ile^8$ Ang II, K_d	$^{125}I\text{-}Sar^1,Gly^8$ Ang II, B_{max}	$^{125}I\text{-}Sar^1,Ile^8$ Ang II, B_{max}
Pituitary AT_1	0.66 ± 0.15[a]	0.35 ± 0.18[b]	0.43 ± 0.05[a]	0.12 ± 0.02[b]	113 ± 16	157 ± 25[c]
Liver AT_1	1.40 ± 0.14	0.55 ± 0.16[b]	1.60 ± 0.10	0.23 ± 0.02[b]	178 ± 26	242 ± 27[c]
Adrenal AT_1	1.36 ± 0.28	0.52 ± 0.09[b]	0.96 ± 0.17	0.49 ± 0.11[b]	209 ± 40	336 ± 36[c]
Adrenal AT_2	51.8 ± 16.8[d]	1.02 ± 0.22[b]	–	0.84 ± 0.22	–	151 ± 20
Heart AT_1	not done	not done	2.59 ± 0.25	1.13 ± 0.11[b]	1.0 ± 0.11	2.6 ± 0.31[c]

Mean ± S.E.M., $n=5–7$ for pituitary, liver and adrenal. Comparisons of $^{125}I\text{-}Sar^1,Ile^8$ Ang II between AT_1 and AT_2 were by one-way ANOVA, other comparisons of B_{max} and K_d were by two-way ANOVA. [a] $p<0.01$ versus $^{125}I\text{-}Sar^1,Gly^8$ Ang II for liver AT_1 and adrenal AT_1. Heart data was determined in a separate experiment, $n=4$. Heart comparisons were by paired *t*-tests.

[a] $p<0.01$ less than Sar^1,Gly^8 Ang II in liver and adrenal.

[b] $p<0.05$ less than Sar^1,Gly^8 Ang II in corresponding tissues.

[c] $p<0.05$ greater than B_{max} for $^{125}I\text{-}Sar^1,Gly^8$ Ang II in same tissue.

[d] $p<0.001$ greater than Sar^1,Gly^8 Ang II at liver, pituitary and adrenal AT_1 receptors.

concentrations ranging from 0.1 to 3 nM. Nonspecific binding was determined in the presence of 3 μM Ang II. For competition binding assays to determine the K_i of Sar^1,Ile^8 Ang II and Sar^1,Gly^8 Ang II, for ^{125}I-Sar^1,Ile^8 Ang II binding to AT_1 and AT_2 receptors, varying concentrations, ranging from 0.1 nM to 1 μM, of each ligand were present in tubes containing 50 μl each of the liver, pituitary and adrenal preparations and 0.5–1 nM ^{125}I-Sar^1,Ile^8 Ang II in a total volume of 100 μl. The assays were carried out for 2 h at 22 °C. Free and bound radioligand were separated on glass fiber filters (Number 32, Schleicher and Schuell, Keene, NH) using a cell harvester (Model M24R, Brandel, Gaithersberg, MD). The bound radioligand retained on the filter disks was assayed in a Beckman Gamma5500 Gamma Counter at a counting efficiency of 67%.

In vitro receptor autoradiography of ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II binding to AT_1 and AT_2 receptors in adrenals was determined using methods previously described [18,23]. Briefly, adrenals from three rats were removed and frozen at −20 °C. The adrenals were sectioned in a cryostat at a thickness of 20 μm and thaw-mounted onto subbed microscope slides. The sections were incubated with either ^{125}I-Sar^1,Gly^8 Ang II (1 nM) or ^{125}I-Sar^1,Ile^8 Ang II (0.25 nM) in assay buffer (described above) in the presence of either Ang II (3 μM, nonspecific binding), 10 μM losartan (AT_2 binding) or 10 μM PD 123,319 (AT_1 binding) at 22–24 °C for 1 h. The sections were rinsed, dried under a stream of air and apposed to X-ray film (Biomax MR-1, Kodak, Rochester, NY) for 1 day. The amount of radioligand binding was determined by densitometric analysis (AIS, Imaging Research, St. Catherines, Ontario, Canada) with reference to a series of calibration standards (^{125}I Microscales, Amersham Bioscience, Piscataway, NJ). Specific AT_1 and AT_2 binding was determined by subtracting nonspecific binding from the binding seen in the presence of PD 123,319 or losartan, respectively. A threshold was used to delineate the areas of the cortex and medulla and the values obtained were the specific density (fmol/gram wet weight) times the area. The value obtained for ^{125}I-Sar^1,Gly^8 Ang II binding was calculated as a percent of the ^{125}I-Sar^1,Ile^8 Ang II binding in the adjacent section.

Radioligands were prepared by reacting the angiotensin analogs with chloramine T, and the monoradioiodinated products were purified by reversed-phase HPLC as described previously [24]. Sar^1,Ile^8 Ang II and Ang II were obtained from Bachem (Torrance, CA) and Sar^1,Gly^8 Ang II was obtained from either SynPep (Dublin, CA) or Bachem. Protein concentration in the membrane suspensions was determined spectrophotometrically [25].

B_{max} (expressed as fmol of radioligand bound per mg membrane protein), K_d and IC_{50} values were determined using established one-site receptor models using the computer program Prism (Graphpad Software, San Diego, CA). K_i was determined from the Cheng–Prusoff equation: $K_i = IC_{50}/(1 + H/K_d)$ where H = radioligand concentration and K_d is the K_d for the radioligand. Values reported are mean ± S.E.M. Statistical comparisons were made using a one- or two-way repeated measures ANOVA with Bonferroni post hoc comparisons or paired *t*-test with a significance level < 0.05.

3. Results

A representative comparison of Sar^1,Ile^8 and Sar^1,Gly^8 competition for adrenal AT_1 and AT_2 receptors is shown in Fig. 1. Two-way repeated measures ANOVA revealed a significant interaction ($F_{1,23} = 9.2$, $p < 0.05$) indicating that Sar^1,Gly^8 Ang II but not Sar^1,Ile^8 Ang II displayed selectiv-

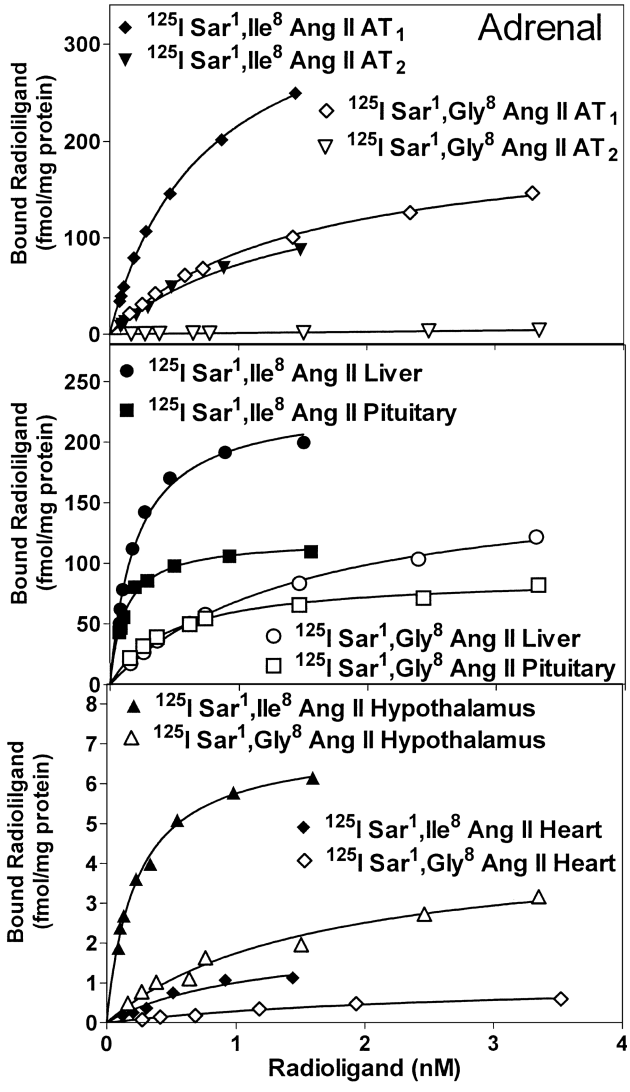

Fig. 2. Representative saturation isotherms of ^{125}I-Sar^1,Ile^8 Ang II and ^{125}I-Sar^1,Gly^8 Ang II binding to Ang II receptors in adrenal (upper panel), liver and pituitary (middle panel) and hypothalamus and heart (lower panel). Adrenal, brain and heart AT_1 receptor binding was determined in the presence of 10 μM of the selective AT_2 receptor antagonist PD 123,319. Adrenal AT_2 receptor binding was determined in the presence of 10 μM of the selective AT_1 receptor antagonist losartan.

ity for AT_1 versus AT_2 receptors. As shown in Table 1, the average K_i of Sar^1,Gly^8 Ang II for adrenal AT_2 receptors was nearly 40 times higher than the K_i for adrenal AT_1 receptors.

Two-way ANOVA ($F_{2,8}=6.3$, $p=0.022$) revealed that Sar^1,Gly^8 Ang II competed for ^{125}I-Sar^1,Ile^8 Ang II binding to AT_1 receptors in the pituitary with significantly higher affinity than for AT_1 receptors in the liver and adrenal. The K_i of Sar^1, Ile^8 Ang II for AT_1 receptors in the pituitary, liver and adrenal was significantly less than that of Sar^1,Gly^8 Ang II ($F_{1,8}=11.1$, $p<0.05$), but did not vary significantly between the three tissues (Table 1).

^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II displayed similar Ang II receptor subtype binding affinities and characteristics compared to their uniodinated congeners (Fig. 2; Table 1). However, it was not possible to reliably demonstrate saturable ^{125}I-Sar^1,Gly^8 Ang II binding to adrenal AT_2 receptors using concentrations of ^{125}I-Sar^1,Gly^8 Ang II from 0.1 to 3 nM. Two-way repeated measures ANOVA of ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II binding to AT_1 receptors in revealed that the K_d of ^{125}I-Sar^1,Ile^8 Ang II was significantly lower than that for ^{125}I-Sar^1,Gly^8 Ang II in the pituitary, liver and adrenal ($F_{1,4}=240$, $p=0.0001$, Table 1). ^{125}I-Sar^1,Ile^8 Ang II also bound to heart AT_1 receptors with higher affinity than ^{125}I-Sar^1,Gly^8 Ang II (paired $t=4.96$, $df=3$, $p=0.016$, Table 1). It was not possible to detect saturable ^{125}I-Sar^1,Ile^8 Ang II or ^{125}I-Sar^1,Gly^8 Ang II binding to AT_2 receptors in the heart tissue used in this study.

There was also a significant interaction effect ($F_{2,24}=18.4$, $p<0.0001$) showing that the K_d of ^{125}I-Sar^1,Gly^8 Ang II for pituitary ($AT_{1B}>AT_{1A}$ mRNA) receptors was significantly lower ($p<0.01$) than that of both liver (exclusively AT_{1A} mRNA) and adrenal AT_1 receptors (mixed AT_{1A} and AT_{1B} mRNA).

Two-way ANOVA revealed a significant ($F_{1,4}=277$, $p<0.0001$) decrease in the B_{max} values for AT_1 receptor binding with ^{125}I-Sar^1,Gly^8 Ang II compared to ^{125}I-Sar^1,Ile^8 Ang II in liver, pituitary and adrenal. A paired t-test also revealed a substantial difference in B_{max} for ^{125}I-Sar^1,Gly^8 Ang II compared ^{125}I-Sar^1, Ile^8 Ang II for the heart (Fig. 2; Table 1).

The binding of ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II was also compared in two pools of rat brain hypo-

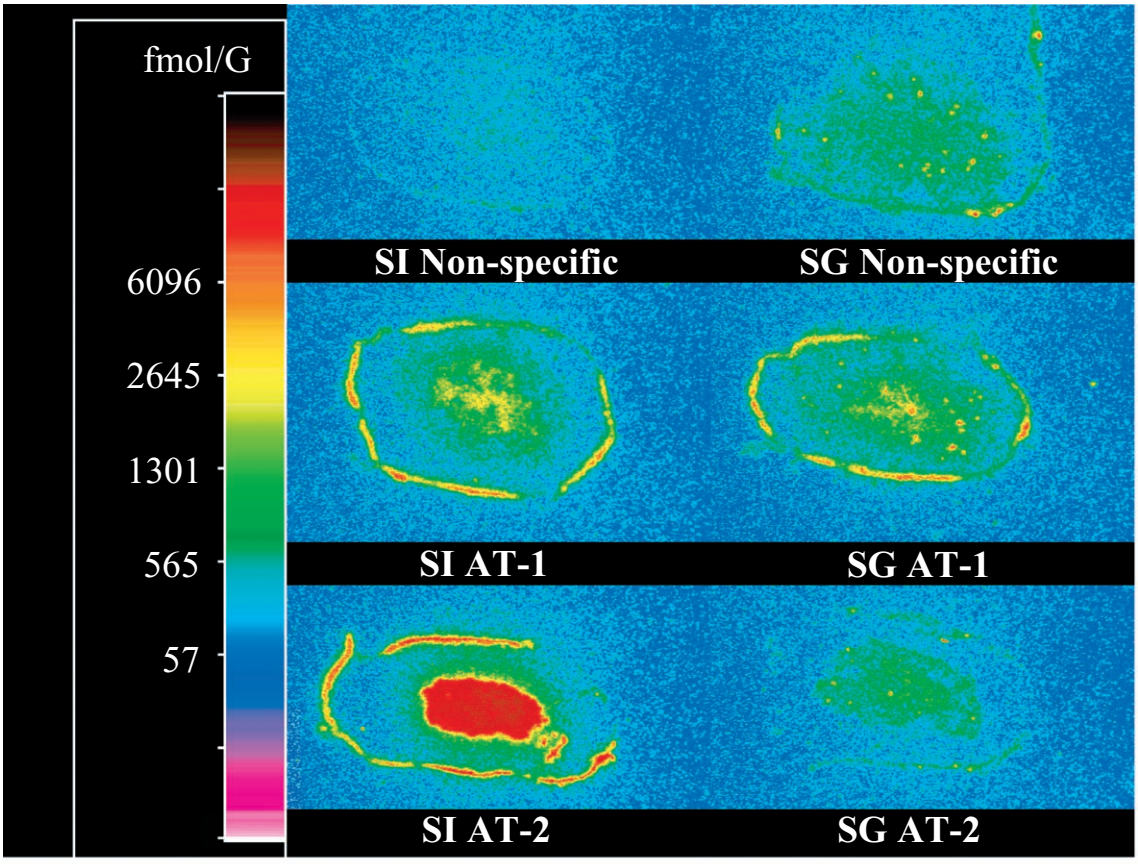

Fig. 3. Representative comparison of ^{125}I-Sar^1,Ile^8 Ang II (SI) and ^{125}I-Sar^1,Gly^8 Ang II (SG) binding to rat adrenal sections. ^{125}I-Sar^1,Ile^8 Ang II was present at 0.25 nM while ^{125}I-Sar^1,Gly^8 Ang II was present at 1 nM. Nonspecific binding was determined in the presence of 3 μM Ang II. AT_1 (AT1) binding was determined in the presence of 10 μM PD 123,319. AT_2 (AT2) binding was determined in the presence of 10 μM losartan. Values for specific AT_1 and AT_2 binding were determined by substracting nonspecific binding. Values for the sections shown in this figure were: SI nonspecific = 180 and 259 fmol/g for cortex and medulla, respectively; SG nonspecific = 480 and 629 fmol/g for cortex and medulla, respectively; SI AT-1 specific = 1214 and 945 fmol/g for cortex and medulla, respectively; SG AT-1 specific = 1064 and 507 fmol/g for cortex and medulla, respectively; SI AT-2 specific = 3254 and 186521 fmol/g for cortex and medulla, respectively; and SG AT-2 specific = 117 and 50 fmol/g for cortex and medulla, respectively.

thalamus (Fig. 2, lower panel). The hypothalamus contains primarily AT_{1A} receptor mRNA. Results from the brains were similar to that seen in the other tissues. ^{125}I-Sar^1,Gly^8 Ang II bound with lower affinity ($K_d = 1.82 \pm 0.25$ nM) and capacity ($B_{max} = 4.96 \pm 0.50$ fmol/mg protein) than did ^{125}I-Sar^1,Ile^8 Ang II ($K_d = 0.32 \pm 0.09$ nM, $B_{max} = 8.65 \pm 1.61$ fmol/mg protein).

Further analysis of the differences in B_{max} values between ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II, using liver membranes only, also evaluated the B_{max} value for ^{125}I-Ang II. One-way ANOVA ($F_{2,8} = 11.6$, $p < 0.005$) revealed that the B_{max} values for both ^{125}I-Sar^1,Gly^8 Ang II (17.6 ± 3.3 fmol/mg initial wet weight, $p < 0.05$) and ^{125}I-Ang II (16.1 ± 3.6 fmol/mg initial wet weight, $p < 0.01$) were significantly lower than that for ^{125}I-Sar^1,Ile^8 Ang II (21.9 ± 4.4 fmol/mg initial wet weight), but that there was no significant difference in B_{max} for ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Ang II.

To determine if the difference in B_{max} between ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II for AT_1 receptors was due to a more rapid dissociation of bound ^{125}I-Sar^1,Gly^8 Ang II during the filtration to resolve free and membrane-bound radioligand, different rinse times and number of rinses were compared in liver homogenate. There was less than 10% difference in B_{max} for ^{125}I-Sar^1,Gly^8 Ang II binding with two versus eight rinses.

A comparison of the ability of ^{125}I-Sar^1,Gly^8 Ang II and ^{125}I-Sar^1,Ile^8 Ang II to radiolabel AT_1 and AT_2 receptors in sections of adrenals is shown in Fig. 3. ^{125}I-Sar^1,Ile^8 Ang II labeled both AT_1 and AT_2 receptors in the adrenals. ^{125}I-Sar^1,Gly^8 Ang II labeled $46 \pm 13\%$ and $51 \pm 16\%$ as many AT_1 receptors in the adrenal cortex and medulla, respectively. By comparison, ^{125}I-Sar^1,Gly^8 Ang II labeled only $3.8 \pm 2.5\%$ and $0.1 \pm 0.05\%$ as many AT_2 receptors in the adrenal cortex and medulla, respectively.

4. Discussion

Although two peptidic Ang II analogs, CGP42112 [4] and *p*-amino Phe^6 Ang II [17], bind selectively to AT_2 receptors, the existence of a peptidic Ang II analog with AT_1 receptor selectivity has not been previously reported. However, as noted in Introduction, a comparison of radioligand binding studies [2] and physiological studies [1,3] suggests that Sar^1, Gly^8 Ang II is AT_1 selective. The results of this study confirm that suggestion.

AT receptors belong to the G-protein-coupled receptor (GPCR) family. They share significant homologies within the seven transmembrane (TM) spanning domains of this class of receptors [26–28]. There are at least five critical determinants for Ang II binding and efficacy in the TM domain of the AT_1 receptor [29–33]. The interaction between Arg^2 in Ang II and an aspartic acid (D281) in TM7 [30] and the interaction between the carboxyl terminal carboxylic acid of Ang II and a lysine (K199) in TM5 are major determinants of binding affinity [29].

Comparison of the TM7 domains of the AT_1 and AT_2 receptors reveals a conserved aspartic acid (D281 and D297, respectively; Fig. 4). Mutation of this amino acid in the AT_2 receptor (D297L) drastically reduces binding of ^{125}I-Ang II, ^{125}I-Sar^1,Ile^8 Ang II and ^{125}I-CGP42112 to this receptor [34]. Similarly, mutation of a conserved lysine (K215E, K215Q, K215A) in TM5 of the AT_2 receptor, corresponding to K199 in TM5 of the AT_1 receptor (Fig. 4), profoundly reduces both ^{125}I-Ang II and ^{125}I-Sar^1,Ile^8 Ang II binding [35,36]. Taken together, these data suggest that D281 (AT_1)/D297 (AT_2) in TM7 and K199 (AT_1)/K215 (AT_2) in TM5 are major determinants of Ang II binding to both AT receptor subtypes.

It has long been known that substitution of Phe in position 8 of Ang II with a non-aromatic amino acid converts it from an agonist to an antagonist [37–39]. Loss of function mutational studies suggest that the phenolic side chain of phenylalanine in position 8 of Ang II interacts with His256 in TM6 of the AT_1 receptor to stimulate inositol phosphate formation [31]. The data reported herein suggest that interaction of the position 8 amino acid side chain has a small effect on peptide binding affinity to the AT_1 receptor, since the K_i and K_d values of Sar^1,Gly^8 Ang II, Sar^1 Phe^8 Ang II and Sar^1,Ile^8 Ang II differ only slightly (Fig. 2, Table 1; Speth, unpublished observations). This is consistent with site-directed mutation studies in which H256A caused only a 2.6-fold decrease in ^{125}I-Ang II binding affinity to the AT_1 receptor [40]. Thus it appears that the side chain of phenyl-

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IGLGLTKNILGFLFPFLIIL-TS AT1 TM5 193-214
 G  L KNILGF  P LI   T
AGIALMKNILGFIIP-LIFIATC AT2 TM5 209-233

IIMAIVLFFFFSWVPHQIFTFL  AT1 TM6 241-262
   A+VL F   W P ++ TFL
MAAAVVLAFIICWLPFHVLTFL  AT2 TM6 257-278

ISDIVDTAMPITICIAYFNNCL  AT1 TM7 276-297
+  ++D A+P  I + + N+C+
VIAVIDLALPFAILLGFTNSCV  AT2 TM7 292-313
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Fig. 4. Pairwise alignment of TM5, TM6 and TM7 of rat AT_1 and AT_2 receptors. Lys199 in TM5, which is reported to be the docking site for the COOH terminus of Ang II to the AT_1 receptor, is shown in bold. The equivalent Lys in TM5 of the AT_2 receptor (Lys215) is also shown in bold. His256 in TM6, which is reported to be the docking site for the aromatic amino acid side chain moiety of Phe^8 in Ang II on the AT_1 receptor, is shown in bold. The equivalently placed amino acid is Phe272 on TM6 of the AT_2 receptor. However, there is a nearby His (His273) shown in bold. Asp281 in TM7, which is reported to be the docking site for Arg^2 of Ang II to the AT_1 receptor, is shown in bold. The equivalent Asp in TM7 of the AT_2 receptor (Asp297) is also shown in bold. Determination of the amino acid sequence of the TM5, TM6 and TM7 domains of each receptor approximates those suggested by the Swissprot database: http://us.expasy.org/cgi-bin/niceprot.pl?P25095 for the AT_{1A} receptor and http://us.expasy.org/cgi-bin/niceprot.pl?P35351 for the AT_2 receptor. The TM5, TM6 and TM7 domains of the AT_{1B} receptor: http://us.expasy.org/cgi-bin/niceprot.pl?P29089 are identical to those of the AT_{1A} receptor except for L205V in TM5 and S277A in TM7.

alanine[8] operates the agonist switch of the AT_1 receptor, with little effect on binding affinity.

In contrast, it appears that the presence of an aromatic or aliphatic amino acid side chain moiety in position 8 of Ang II is of much greater importance for the binding affinity of Ang II to the AT_2 receptor than for the AT_1 receptor. This is consistent with the observation that mutation of His273 (H273Q) in the AT_2 receptor, which approximates the position of His256 in TM6 of the AT_1 receptor (Fig. 4), drastically reduces ^{125}I-Sar[1],Ile[8] Ang II binding to the AT_2 receptor [41]. This might also indicate that the side chain of Phe[8] is not a determinant of agonism at the AT_2 receptor. Such a possibility is consistent with the reported agonism of CGP42112 at AT_2 receptors [42]. CGP42112 has an Ile in the equivalent position to Phe[8] in Ang II [43].

There is a small, but statistically significant, increase in the affinity of the pituitary AT_1 receptor for Sar[1]Gly[8] Ang II compared to the liver and adrenal AT_1 receptors (Fig. 2; Table 1). Studies of Ang II receptor mRNA [19] suggest that the anterior pituitary expresses primarily AT_{1B} receptors, while the liver and hypothalamus contain almost exclusively AT_{1A} receptor mRNA [13,14]. In the adrenal, approximately 42–48% of the AT_1 receptor mRNA encodes the AT_{1A} receptor subtype [13,21].

The moderately higher affinity of Sar[1],Gly[8] Ang II for AT_{1B} versus AT_{1A} in Y-1 transfected cells [9] and the higher affinity of both Sar[1],Gly[8] Ang II and ^{125}I-Sar[1],Gly[8] Ang II for the pituitary compared to the liver and adrenal in this study suggest that Sar[1],Gly[8] Ang II has a higher affinity for AT_{1B} receptors than for AT_{1A} receptors. However, it is possible that tissue-specific differences (e.g., differential glycosylation of the receptor, or different receptor affinity states; see below) could also account for the observed changes.

The difference in B_{max} between ^{125}I-Sar[1],Gly[8] Ang II and ^{125}I-Sar[1],Ile[8] Ang II for AT_1 receptors in all four tissues is puzzling. Since B_{max} represents specific (3 μM Ang II displaceable) binding, it is unlikely that the difference is due to the binding of ^{125}I-Sar[1],Ile[8] Ang II to non-Ang II receptors. The lower B_{max} for ^{125}I-Ang II and ^{125}I-Sar[1]Gly[8] Ang II compared to ^{125}I-Sar[1],Ile[8] Ang II and the tendency for the Hill slope for Sar[1]Gly[8] Ang II to be shallower than that for Sar[1],Ile[8] Ang II (data not presented) is consistent with the existence of high and low agonist affinity states for G-protein-coupled receptors [44], which has also been demonstrated for AT_1 receptors [17,45]. However, the agonist ligand ^{125}I-Sar[1] Ang II has a nearly equivalent B_{max} as ^{125}I-Sar[1],Ile Ang II in pituitary, liver and brain (Speth, Unpublished observations), which suggests that the difference in B_{max} between ^{125}I-Sar[1],Gly[8] Ang II and ^{125}I-Sar[1],Ile[8] Ang II is independent of agonist affinity states. At this time, the discrepancy in B_{max} values between ^{125}I-Sar[1]Gly[8] Ang II and ^{125}I-Sar[1],Ile[8] Ang II cannot be resolved.

In summary, these data establish that Sar[1]Gly[8] Ang II is a peptidic Ang II receptor antagonist that is AT_1 selective. The difference in its binding affinity to putative AT_{1A} and AT_2, and putative AT_{1B} to AT_2 receptors is 40- and 82-fold, respectively. Sar[1]Gly[8] Ang II can be radioiodinated and used as an AT_1 receptor subtype-selective radioligand for membrane binding assays as well as receptor autoradiography.

The slightly higher affinity of ^{125}I-Sar[1]Gly[8] Ang II for pituitary AT_{1B} receptors relative to tissues expressing AT_{1A} receptors (liver and brain) might assist in determining whether a change in the mRNA content of a single AT_1 receptor subtype in tissues with mixed populations of AT_1 receptor subtypes [21,46] results in changes in expression of the corresponding AT_1 subtype. Finally, the importance of the position 8 amino acid for high affinity Ang II binding to the AT_2 receptor suggests that other Ang II amino acid side chains may mediate its agonistic action at the AT_2 receptor.

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