Endocrine, *vol. 26, no. 2, 83–87, March 2005* 0969–711X/05/26:83–87/$30.00

Sarcosine[1], Glycine[8] Angiotensin II Is a Functional AT_1 Angiotensin Receptor Antagonist

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Sarcosine[1], glycine[8] angiotensin II (SG Ang II) displayed unusual characteristics in early pharmacological studies. It was a potent antagonist of the dipsogenic actions of intracerebroventricularly administered Ang II in the rat, but showed low affinity for bovine cerebellar Ang II receptors. It has recently been shown that SG Ang II binds preferentially to the AT_1 receptor. To determine if SG Ang II is a functional antagonist of the AT_1 receptor-mediated calcium signaling, CHO cells stably transfected with AT_1 receptors were exposed to Ang II in the presence and absence of SG Ang II. At concentrations of 10–100 n*M*, SG Ang II completely inhibited Ang II-stimulated intracellular Ca^{2+} mobilization in AT_{1A} and AT_{1B} receptor-transfected cells. SG Ang II and ^{125}I-SG Ang II bound to AT_{1A} and AT_{1B} receptor-transfected cells with K_D and K_I values of 2–4 n*M*. In contrast, SG Ang II bound to AT_2 transfected cells with a K_I value of 7.86 µ*M*. These results demonstrate that SG Ang II is a selective and functional peptide antagonist of the AT_1 angiotensin II receptor subtype.

Key Words: AT_1 receptors; calcium signaling; sarcosine[1], glycine[8] angiotensin II; antagonist; CHO cells; transfection.

Introduction

The actions of angiotensin II (Ang II) are mediated by two receptor subtypes: AT_1 and AT_2 *(1)*. This discovery arose from the development of peptidomimetic and nonpeptide antagonists selective for the two major Ang II receptor subtypes *(2)*. Although some analogs of Ang II have been demonstrated to have AT_2 selectivity *(3,4)*, it is only recently that an Ang II analog selective for the AT_1 receptor has been observed *(5)*.

Received December 24, 2004; Revised March 7, 2005; Accepted March 14, 2005.

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Based on functional studies in the brain *(6)*, SG Ang II was classified as a potent Ang II receptor antagonist based on its ability to block the dipsogenic actions of intracerebroventricular (ICV) Ang II. However, the inability of SG Ang II to compete for ^{125}I-Ang II binding sites in the bovine cerebellum *(7)* raised questions about the mechanism whereby SG Ang II blocked the dipsogenic response to Ang II. Subsequent discovery that the predominant Ang II receptor subtype in the bovine cerebellum was the AT_2 subtype *(8)*, and that the predominant receptor mediating the dipsogenic response to Ang II is the AT_1 subtype *(9–11)* led to the suggestion that SG Ang II was an AT_1 selective ligand *(5)*.

The studies described herein demonstrate that SG Ang II is a functional antagonist of AT_1 receptor-mediated stimulation of intracellular Ca^{2+} mobilization and confirm the AT_1 receptor selectivity of this Ang II analog.

Results

The ability of SG Ang II and sarcosine[1], isoleucine[8] Ang II (SI Ang II) to compete for ^{125}I-SI Ang II binding to cells stably transfected with Ang II receptors is demonstrated in Fig. 1 and Table 1. SG Ang II bound with high affinity to both AT_{1A} and AT_{1B} transfected CHO cells. In contrast, the K_I of SG Ang II for AT_2 transfected cells was more than 1000-fold lower than for the AT_1 receptor transfected cells. The K_I for SI Ang II was about threefold higher affinity than that for SG Ang II in AT_{1A} and AT_{1B} transfected cells and about 900-fold higher than that for SG Ang II in AT_2 transfected cells.

The affinity of ^{125}I-SG Ang II and ^{125}I-SI Ang II for the Ang II receptor transfected CHO cells was similar to that of the corresponding un-iodinated peptides (Table 1). The K_D for ^{125}I-SG Ang II binding in AT_{1A} and AT_{1B} transfected CHO cells was 4 and 2.2 n*M*, respectively. It was not possible to determine a K_D for ^{125}I-SG Ang II binding in the AT_2 transfected cells. The K_D for ^{125}I-SI Ang II binding in AT_{1A} and AT_{1B} transfected CHO cells was 0.32 and 0.73 n*M*, respectively, while the K_D of ^{125}I-SI Ang II for AT_2 transfected cells was 16.6 n*M*. The B_{max} values for ^{125}I-SG Ang II and ^{125}I-SI Ang II were similar (Table 1).

Table 1
Comparison of Binding of Sar[1],Gly[8] Ang II and Sar[1],Ile[8] Ang II and [125]I-labeled Sar[1], Gly[8] Ang II, and Sar[1],Ile[8] Ang II in CHO Cells Stably Transfected with AT_{1A}, AT_{1B}, or AT_2 Receptors

Receptor subtype	Sar[1],Gly[8] Ang II K_I	Sar[1],Ile[8] Ang II K_I	[125]I-Sar[1],Gly[8] Ang II K_D	[125]I-Sar[1],IIe[8] Ang II K_D	[125]I-Sar[1],Gly[8] Ang II B_{max}	[125]I-Sar[1],Ile[8] Ang II B_{max}
AT_{1A}	2.16 ± 0.83 (2)	0.60 ± 0.33 (2)	4.00 ± 1.28 (2)	0.317 ± 0.069 (2)	532 ± 216 (2)	579 ± 118 (2)
AT_{1B}	3.12 ± 1.00 (3)	1.10 ± 0.09 (3)	2.19 ± 0.69 (3)	0.734 ± 0.206 (3)	151 ± 67 (3)	150 ± 25 (3)
AT_2	7856 ± 2398 (4)	8.92 ± 3.14 (4)	—	16.6 ± 2.2 (4)	—	2437 ± 97 (4)

Values are mean ± SEM (*n*). Units for K_I and K_D are n*M*.

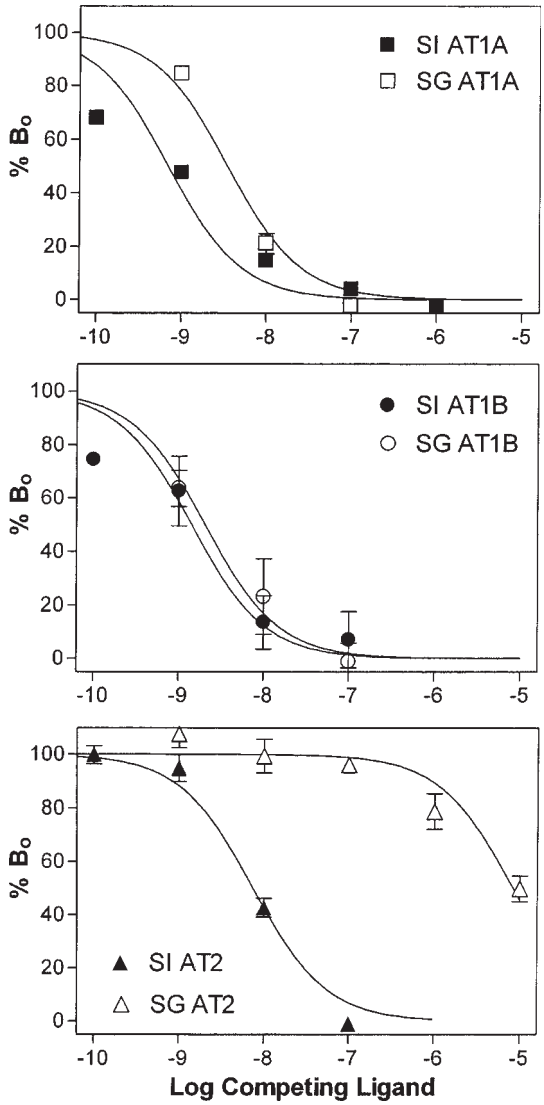

Fig. 1. Representative competition curves of Sar[1],Gly[8] Ang II (SG) and Sar[1],Ile[8] Ang II (SI) for specific [125]I-Sar[1],Ile[8] Ang II binding in CHO cells stably transfected with either AT_{1A}, AT_{1B}, or AT_2 receptors. [125]I- Sar[1],Ile[8] Ang II was present at a concentration of 0.4 n*M*. IC_{50} values for this graph are SI AT_{1A} = 0.70 n*M*, SI AT_{1B} = 1.40 n*M*, SI AT_2 = 7.49 n*M*, SG AT_{1A} = 3.48 n*M*, SG AT_{1B} = 2.02 n*M*, and SG AT_2 = 8355 n*M*. Specific binding was defined as binding that was displaceable by 3 μ*M* Ang II. Error bars indicate average ± SEM of triplicate measurements.

To test the functional effect of SG Ang II on Ang II receptors, CHO cells stably transfected with AT_{1A}, AT_{1B}, or AT_2 receptors were loaded with Fura-2 and placed in a perfusion chamber. The Ca^{2+} mobilization response to Ang II was documented in both AT_{1A} and AT_{1B} transfected cells (Fig. 2, Panels A–E), but was absent in AT_2 receptor transfected cells (Fig. 2, Panel F). Perfusion of the cells with 100 n*M* SG Ang II immediately prior to and during perfusion with Ang II completely blocked the Ca^{2+} mobilization response to 10 n*M* Ang II in both AT_{1A} and AT_{1B} transfected cells (Fig. 2, Panels A, B, D, E). SG Ang II was completely devoid of any agonistic effect on Ca^{2+} mobilization in AT_{1A} and AT_{1B} transfected cells (Fig. 2, Panels B and E). In AT_{1A} transfected cells, 10 n*M* SG Ang II was able to completely block the Ca^{2+} mobilization response to 1 n*M* Ang II (Fig. 2, Panel C). The inhibition of the Ca^{2+} mobilization response to SG Ang II was reversible. When Ang II was perfused 16–20 min following washout of SG Ang II, it again caused an intracellular Ca^{2+} mobilization response (Fig. 2, Panels A,B,C,E).

Discussion

The ability of Ang II receptors to cause an intracellular Ca^{2+} mobilization response is well established *(12–15)*. Stimulation of both AT_{1A} and AT_{1B} *(16,17)* receptors increases intracellular Ca^{2+}. Consistent with the absence of an AT_2 receptor-mediated Ca^{2+} mobilization response, it is generally recognized that the Ca^{2+} mobilization responses to Ang II are exclusively mediated by AT_1 receptors *(16–19)*. However, some studies using the AT_2 receptor-selective antagonist PD123319 suggest that the AT_2 receptor can participate in Ca^{2+} mobilization responses induced by Ang II *(20,21)*.

The observation that nanomolar concentrations of SG Ang II are capable of completely inhibiting the Ca^{2+} mobilization to a 10-fold lower concentration of Ang II suggests that SG Ang II is a potent antagonist of the AT_1 receptor. This confirms observations of Fitzsimons et al. *(6)*, made prior to the discovery of multiple Ang II receptor subtypes, that SG Ang II is a potent inhibitor of the dipsogenic response. It is now well established that the dipsogenic response is mediated by the AT_1 receptor subtype *(9,10,22)*. Of note,

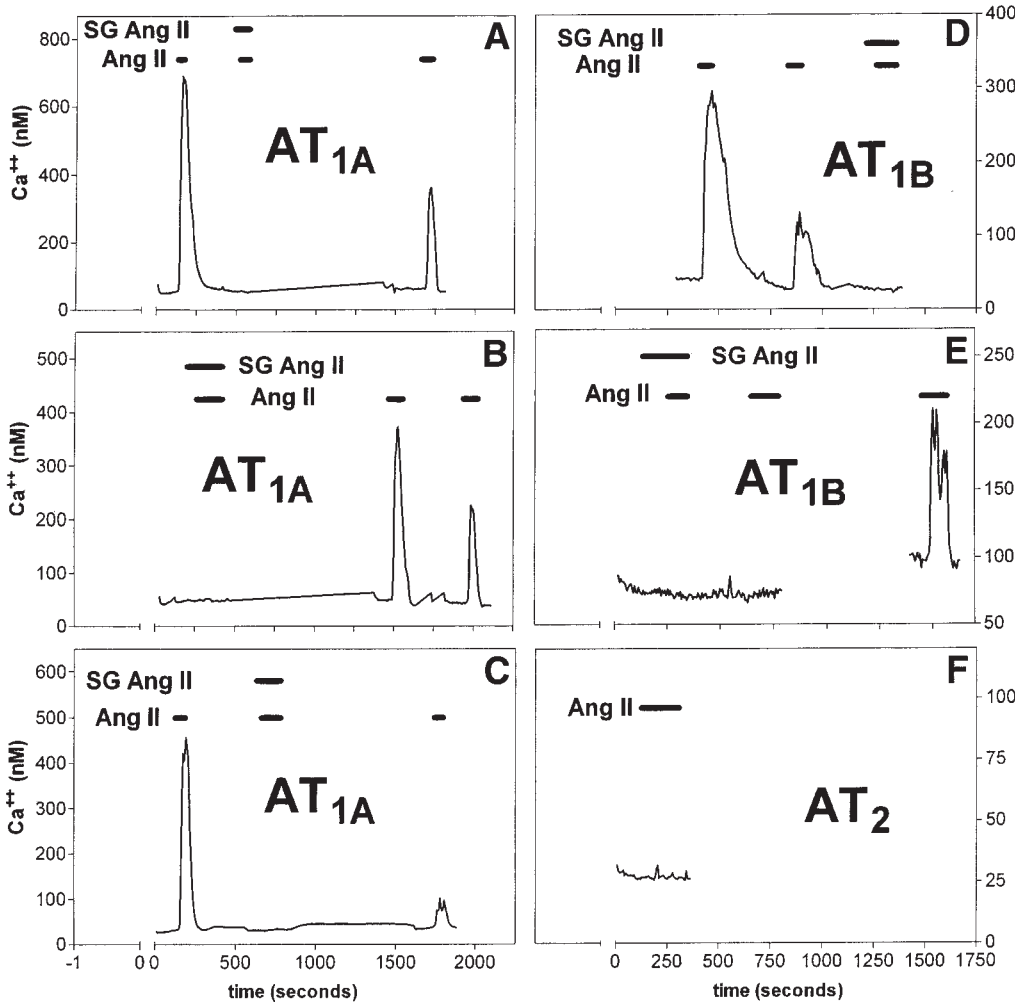

Fig. 2. Inhibition of Ang II-induced calcium signaling by Sar[1],Gly[8] Ang II (SG). (**A**) The initial response generated by 10 n*M* Ang II in AT_{1A} transfected cells was 689 ± 80 n*M* (mean ± SEM, $n = 20$). 5 min later 100 n*M* of Sar[1],Gly[8] Ang II was applied to the cells; 30 s later 10 n*M* Ang II was applied to the cells in the continued presence of Sar[1],Gly[8] Ang II. The Ang II was applied for 1 min; 20 min later Ang II (10 n*M*) was again applied to the cells to cause the second, smaller response of 356 ± 33 n*M*. (**B**) Sar[1],Gly[8] Ang II (100 n*M*) was applied initially to AT_{1A} transfected cells, followed 1 min later by Ang II (10 n*M*) in the continued presence of Sar[1],Gly[8] Ang II for 3 min; 17 min later Ang II (10 n*M*) was applied to the cells for 90 s, generating the first calcium response of 371 ± 52 n*M*. Six minutes later Ang II (10 n*M*) was again applied to the cells for 90 s generating the second, smaller response of 225 ± 40 n*M*. (**C**) The protocol was similar to that in **A** except that Ang II was applied to the AT_{1A} transfected cells at a concentration of 1 n*M* giving a response of 643 ± 41 n*M* and Sar[1],Gly[8] Ang II was applied at a concentration of 10 n*M* completely blocking the Ang II response and largely blocking the subsequent response to Ang II applied 15 min later. (**D**) Initial response generated by 10 n*M* Ang II applied to AT_{1B} transfected cells for 1 min was 296 ± 69 n*M*. Six minutes later Ang II (10 n*M*) was again applied to the cells, generating the second, smaller response, 118 ± 35 n*M*. Six minutes later 100 n*M* Sar[1],Gly[8] Ang II was applied to the cells followed by Ang II (10 n*M*) 45 s later in the continued presence of Sar[1],Gly[8] Ang II. The Ang II was applied for 90 s with no response. (**E**) Sar[1],Gly[8] Ang II (100 n*M*) was applied to AT_{1B} transfected cells followed 2 min later by Ang II (10 n*M*) in the continued presence of Sar[1],Gly[8] Ang II for 90 s. Ang II (10 n*M*) was again applied to the cells 5 min later with no effect. Eleven minutes later Ang II (10 n*M*) was applied a third time to the cells generating an intracellular calcium response in a single cell. (**F**) Ang II (10 n*M*) was applied to the AT_2 transfected cells for 3 min without causing any cytoplasmic calcium response.

Zhu and Herbert *(23)* have shown that the dipsogenic response to Ang II in rats involves mobilization of Ca^{2+} via an L-type calcium channel.

There was a profound AT_1 receptor selectivity observed for SG Ang II in the present study: more than a 1000-fold difference in binding affinity. This is considerably more than that previously observed in studies of naturally occurring Ang II receptor subtypes in a variety of rat tissues *(5)*. However, SI Ang II also displayed a small selectivity for the AT_1 receptor in this study, ranging from 8-fold (AT_2 vs AT_{1B}) to more than 50-fold (^{125}I-SI Ang II binding to AT_2 vs AT_{1A}). This suggests that the AT_2 receptor transfected into these CHO cells displayed a lower Ang II binding affinity than the AT_1 transfected cells. Possible explanations for this difference could be different membrane properties of CHO cells, different receptor-associated proteins in the CHO

membranes, or differences in post-translational modifications of the AT_2 receptor in CHO cells. When the difference in affinity of SI Ang II for the AT_1 and AT_2 is factored in, the relative selectivity of SG Ang II for the AT_1 receptor is 244- and 311-fold for the AT_{1A} and AT_{1B} receptors, respectively, compared to the AT_2 receptor. This selectivity is closer, but still substantially larger than SG Ang II's 40- and 82-fold higher affinity for AT_{1A} and AT_{1B}, respectively, compared to the AT_2 in naturally occurring Ang II receptors reported previously *(5)*. Of note, the B_{max} values for ^{125}I-SG Ang II and ^{125}I-SI Ang II were similar in the AT_1 receptor transfected cells. This also differs from the previous study of ^{125}I-SG Ang II and ^{125}I-SI Ang II binding to naturally occurring AT_1 receptors in rat tissues in which there is an unresolved reduction in B_{max} for ^{125}I-SG Ang II relative to that of ^{125}I-SI Ang II *(5)*.

To date, SG Ang II is the only Ang II analog identified as having a substantial AT_1 Ang II receptor subtype selectivity. However, many of the analyses of Ang II congeners were carried out prior to the discovery of different Ang II receptor subtypes. Thus, it is possible that other Ang II analogs might also possess AT_1 receptor selectivity. Discovery of such analogs might further aid in our understanding of ligand-docking characteristics of Ang II receptors as well as their signal transduction mechanisms.

In summary, these results confirm and extend previous studies of SG Ang II which suggest that it is a potent and subtype-selective AT_1 receptor antagonist.

Materials and Methods

Generation of Stably Transfected AT_{1A}, AT_{1B}, and AT_2 Receptor-Expressing Cells

The generation of the rat AT_{1A} and rat AT_{1B} receptor-expressing, stably transfected CHO cell lines is described previously *(24)*. Briefly, amplimer sets that were specific for either the rAT1AR or the rAT1BR subtype were used to amplify the coding region of each receptor. The amplified products were subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA) and sequenced to ensure authenticity and proper orientation.

An amplimer set specific for the rAT_2R was also synthesized. The sense primer (5'-ATGAAGGACAACTTCAGT TTTGCT-3') corresponds to the sequence beginning at the start codon of the open reading frame of the rat AT_2R. The antisense primer (5'-TTAAGACACAAAGGTGTCCATT TC-3') corresponds to the sequence that encompasses the stop codon of the open reading frame of the rat AT_2R. The receptor-specific amplimer sets were utilized in PCR experiments with rat genomic DNA as template and the proofreading DNA polymerase PFU (Stratagene, La Jolla, CA). The amplified products were subcloned into the eukaryotic expression vector pCR3 (Invitrogen) and sequenced to ensure authenticity and proper orientation.

The $rAT_{1A}R$-pRc/CMV, $rAT_{1B}R$-pRc/CMV, and rAT2-pCR3 constructs were transfected into CHO cells utilizing the calcium phosphate precipitation procedure (Stratagene). Pure clones, expressing either the $rAT_{1A}R$, $rAT_{1B}R$, or rAT_2 subtype, were selected by the antibiotic G418 (Gibco BRL) and purified by the limited dilution technique.

Cell Culture

The AT_{1A}, AT_{1B}, and AT_2 stably transfected CHO cells were grown in six-well plates (Costar) on round microscope cover slips for the Ca^{2+} imaging experiments, or 75 mm culture flasks for the radioligand binding assays at 37°C in a humidified atmosphere of 5% CO_2/95% air (v/v). The medium was Dulbecco's modified Eagle's medium, high glucose (DMEM-Hi, Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Ogden, UT), 80 units/mL penicillin, 80 µg/mL streptomycin, 1.6 m*M* L-glutamine, and 0.175 g/mL L-proline for untransfected cells. For transfected cells, the medium also included 1000 µg/mL geneticin (G418, Gibco BRL). Cells were grown to approx 50–75% confluency and were used between three and six passages post-thawing for these experiments.

Intracellular Ca^{2+} Imaging

All Ca^{2+} imaging studies were performed in a standard physiological saline solution (PSS; 140 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* Hepes, and 6 m*M* glucose, pH adjusted to 7.4 with NaOH) at room temperature. Coverslips with attached cells were washed in PSS and then loaded with 1 µ*M* Fura-2-AM in PSS for 30 min. The cells were then rinsed with the PSS and allowed to de-esterify the dye for an additional 30 min. The coverslip was then inserted into a sealed chamber (0.5 mL volume) mounted on the stage of an inverted microscope (Nikon Diaphot; 40× objective) and the cells were constantly perfused with PSS (2 mL/min). Exposure of the cells to the various test compounds (all in PSS) was achieved by switching inflow lines through a common manifold mounted just upstream from the sealed chamber. Test compounds would reach the cells within 30 s of switching. Paired images (excitation at 340 nm and 380 nm, emissions monitored at 510 nm) were collected with a CCD camera every 6 s, corrected for background fluorescence, and then the ratio of fluorescence intensity (340/380) for a region of interest was calculated and converted to a calcium value based on a standard curve. In each of the experimental conditions, approximately 20 cells were targeted for fluorescence monitoring. The results from all the cells were averaged to obtain the final value portrayed in the figures. The experiments were carried out two times using different passages of cells. The standard Ca^{2+} curve was generated with 10 µ*M* Fura-2 in a Hepes-buffered (10 m*M*, pH 7.4) 130 m*M* KCl, 10 m*M* EGTA solution with varying amounts of $CaCl_2$ added to

generate specific Ca^{2+} levels. Free Ca^{2+} levels in the standard bath were calculated with EQCAL software (Biosoft, Ferguson, MO). Control of light exposures, image collection, and data manipulation was done with MetaFluor imaging software (Universal Imaging Corp., West Chester, PA). Fura-2 and Fura-2-AM were obtained from Molecular Probes (Eugene, OR). All salts and buffer components were from Sigma. The results from all the cells were averaged on an Excel (Microsoft Corp., Redmond, WA) spreadsheet and translated into XY coordinate graphs using PRISM (Graphpad Software, San Diego, CA).

Radioligand Binding Assays

Measurement of Ang II receptor binding in cultured cells was carried out using procedures described previously *(4)*. Briefly, the culture medium was aspirated and replaced with a hypotonic, 20 m*M* $NaPO_4$ 5 m*M* EDTA, pH 7.2 solution. The cells were scraped from the culture flasks with a rubber policeman and mechanically homogenized in this buffer. The homogenate was centrifuged (48,000*g* for 20 min at 4°C) and the supernatant decanted. The membrane pellet was resuspended by homogenization in assay buffer (150 m*M* NaCl, 5 m*M* EDTA, 0.1 m*M* bacitracin, 50 m*M* $NaPO_4$, pH 7.1–7.2). The homogenate was centrifuged as before. The supernatant was again decanted and the membrane pellet was resuspended in 5 mL of assay buffer by mechanical homogenization.

To measure Ang II receptor binding, cell membranes were incubated with ^{125}I-SI Ang II or ^{125}I-SG Ang II prepared by the chloramine T procedure *(25)*. The radioligands were purified as the mono-radioiodinated peptides by HPLC using a C18 reverse phase column eluted isocratically with a triethylamine phosphate (pH 3.0):acetonitrile mobile phase *(26)*.

For saturation isotherm analysis, membranes were incubated with increasing concentrations of ^{125}I-SI Ang II or ^{125}I-SG Ang II ranging from 0.1 to 4 n*M* for 2 h at 22–24°C. To assess nonspecific binding, one of three tubes containing each concentration of radioligand also contained 3 μ*M* Ang II to saturate the Ang II receptors. Values presented represent specific (total minus nonspecific) binding.

For competition binding studies, membranes were incubated with 0.4 n*M* ^{125}I-SI Ang II in the presence of varying concentrations of SG Ang II or SI Ang II ranging from 0.1 n*M* to 10 μ*M*. Non-specific binding was again determined in tubes containing 3 μ*M* Ang II and the non-specific binding was subtracted to determine specific Ang II receptor binding.

Determination of K_D and K_I values for ^{125}I-SG Ang II, ^{125}I-SI Ang II, SG Ang II, and SI Ang II were made using PRISM One-Site Saturation and One-Site Competition equations: $Y = B_{max} / (K_D + L)$ and $Y = B_0 - B_0 \times I / (I + IC_{50})$, respectively, where Y = amount of bound radioligand, L = radioligand concentration, B_0 = radioligand bound in the absence of competing ligand, and I = concentration of competing ligand.

Acknowledgments

The authors thank Cathy Knoeber and Mickey Martin for excellent technical assistance. These studies were supported by The Peptide Radioiodination Service Center, Washington State University, American Heart Association (Grant in Aid 0350481Z to RCS), NIH/NHLBI (HL48848 to TSE).

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