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Up-regulation of Angiotensin II Receptors by *In Vitro* Differentiation of Murine N1E-115 Neuroblastoma Cells

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SUMMARY

In vitro differentiation of murine neuroblastoma N1E-115 cells induced by low serum (0.5%) and dimethyl sulfoxide (1.5%) increased the uptake of $^{45}Ca^{2+}$ as well as basal and forskolin-stimulated adenylate cyclase activity. Associated with these biochemical indices of differentiation was an increase in the density of binding sites for the angiotensin II (Ang II) receptor agonist ^{125}I-[Sar^1]-Ang II and the antagonist ^{125}I-[Sar^1,Ile^8]-Ang II (^{125}I-SARILE). This up-regulation was apparent within 24 hr and was maximal at 72 hr. Other manipulations that independently increased intracellular cAMP or Ca^{2+} levels produced a qualitatively similar up-regulation of Ang II receptors. *In vitro* differentiation did not diminish the specificity of these receptors for Ang-II related peptides. Sarcosine-substituted Ang II receptor antagonists such as [Sar^1,Gly^8]-Ang II, [Sar^1,Thr^8]-Ang II, or SARILE itself competed for ^{125}I-SARILE in a monophasic fashion, whereas the competition displayed by the agonists Ang II, angiotensin III, and Crinia-Ang II for ^{125}I-SARILE-labeled sites was biphasic, consisting of distinct high and low affinity components. Moreover, *in vitro* differentiation predominantly increased the density of high affinity sites for angiotensin III and Crinia-Ang II, but the lower affinity site for Ang II, and in all three cases the majority of this increased binding was insensitive to guanine nucleotides. Collectively, these results demonstrate that the expression of Ang II receptors on neuron-like cells is regulated by the biochemical events accompanying differentiation and suggest that the biphasic nature of the binding of some angiotensin agonists may be indicative of multiple receptor subtypes.

The renin-angiotensin system is one of the most important regulators of cardiovascular homeostasis (1). In addition to the actions of Ang II on a variety of peripheral tissues, it is now well established that the brain is an important target organ for this peptide (2). However, the receptor for this peptide on neuronal cells remains the least well characterized of all of the components of this neuroendocrine system. Recently, we have reported that the murine N1E-115 neuroblastoma cell line contains a homogeneous population of Ang II receptors that are regulated by guanine nucleotides and coupled to inositol trisphosphate-mediated release of intracellular calcium (3, 4). These results suggest that this cell line may be a useful system in which to pursue further characterization of Ang II receptors present on neuronal cells.

Numerous previous investigations have indicated that the transition of N1E-115 cultures from the rapidly dividing to confluent state results in the appearance of several morphological, biochemical, and physiological indices of neuronal differentiation (5–10). Consistent with these observations, we noted that the density of Ang II receptors increased as these cells progressed from the logarithmic to stationary phase of growth (3). Several neuroblastoma clones can be induced to achieve a further degree of membrane differentiation and excitability, particularly elaborate neurite extension and acquisition of voltage-sensitive ionic conductances, when exposed to DMSO (11). Therefore, in the present report we have examined the effects of DMSO, as well as other manipulations known to induce differentiation, on the Ang II receptors present on murine neuroblastoma N1E-115 cells. The results revealed that *in vitro* differentiation induced by several different treatments substantially increased the density of Ang II receptors on these cells. As was true in undifferentiated N1E-115 cells, the receptors on differentiated cells were saturable and specific for Ang II-related peptides. However, the heterogeneity of the binding of some agonists, particularly Ang II, Ang III, and Crinia-Ang II, suggested the presence of at least two distinct angiotensin receptor subtypes on differentiated N1E-115 cells, only one of

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ABBREVIATIONS: Ang II, angiotensin II; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gpp(NH)p, 5′-guanylimidodiphosphate sodium salt; Ang III, angiotensin III; G protein, GTP-binding protein; SARILE, [Sar^1,Ile^8]-angiotensin II; SARANG, [Sar^1]-angiotensin II; Crinia-Ang II, Ala,Pro,Gly-[Ile^3,Val^5]-angiotensin II; Ang I, angiotensin I; DMEM, Dulbecco's modified Eagle's medium; dbcAMP, dibutyryl-cAMP; DTT, dithiothreitol; BSA, bovine serum albumin.

which appeared to be regulated by guanine nucleotides. Some of these results were published previously in preliminary form (12).

Experimental Procedures

Materials

Materials used in this study were obtained from the following sources: ^{125}I-SARILE, ^{125}I-SARANG, [α-^{32}P]ATP, [^{3}H]cAMP, and $^{45}Ca^{2+}$ from NEN/DuPont (Boston, MA); Liquiscint from National Diagnostics (Manville, NJ); DMEM from GIBCO (Grand Island, NY); penicillin and streptomycin from Flow Laboratories (McLean, VA); and all Ang II-related peptides, nucleotides, and DMSO from Sigma Chemical Co. (St. Louis, MO). All other reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture Techniques

The original cells provided to this laboratory were the generous gift of Dr. M. Nirenberg, National Institute of Health (Bethesda, MD). N1E-115 cells were grown in six-well or T75 plastic plates in DMEM (high glucose) supplemented with 10% fetal calf serum, and 2.5 μg/ml fungizone, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 and 95% O_2, at 37°. Typically, cells from passages 15–30 were used, because previous studies indicated that the properties of Ang II receptors did not vary during this time (3). Unless otherwise noted, experiments were undertaken 6–8 days after subculturing, when the density of Ang II receptors was maximal (3). In those experiments involving *in vitro* differentiation, the cells were grown for 3 days in normal medium, which was then replaced with one of three media containing either 0.5% serum and 1.5% DMSO, 1.0% serum and 1 mM dbcAMP, or 4.5% serum supplemented with 6, 12, or 24 mM $CaCl_2$.

Radioligand Binding Techniques

Preparation of membranes. Culture dishes were placed on ice, the medium was rapidly removed, and the cells were rinsed three times with ice-cold 20 mM Tris·HCl, pH 7.4, 150 mM NaCl. Then the cells were incubated in this buffer without NaCl for 10 min at 0°. At the end of the incubation, the cells were gently removed with a rubber policeman, homogenized, and centrifuged at 48,000 × *g* for 30 min. The pellets were resuspended in 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, and centrifuged at 48,000 × *g* for 20 min. The resulting pellet was resuspended in assay buffer to which were added two protease inhibitors, aprotinin and 1,10-*o*-phenanthroline (final concentrations, 0.3 trypsin inhibitory units/ml and 100 μg/ml, respectively), and 0.2% heat-inactivated BSA.

Receptor binding assay. The binding reaction was initiated by addition of 150 μl of resuspended membranes to 100 μl of incubation buffer (50 mM Tris·HCl, 150 mM NaCl, 5 mM $MgCl_2$, 0.2% heat-inactivated BSA, aprotinin, 1,10-*o*-phenanthroline, pH 7.4) containing ^{125}I-SARILE or ^{125}I-SARANG (specific activity, 2200 Ci/mmol; NEN/DuPont) and various concentrations of unlabeled peptides, as needed. Membranes were incubated for 60 or 120 min at 25°, unless otherwise noted. The assay was terminated by dilution of the reaction mixture and rinsing of the assay tubes three times with wash buffer (150 mM NaCl, 5 mM Tris·HCl, pH 7.4), followed by vacuum filtration through glass fiber filters using a Skatron cell harvester. Prewashing of the filters with 0.1% polyethyleneimine and inclusion of isotonic saline in the wash buffer were necessary to obtain low filter blanks. After filtration, the filters were placed in test tubes and counted for 3 min in a LKB γ scintillation counter at 80.5% efficiency.

Saturation analysis. Binding assays were conducted as described above, employing 8–12 concentrations of ^{125}I-SARILE or ^{125}I-SARANG from 10 pM to 5 nM. Nonspecific binding in all assays was defined as that occurring in the presence of 1 μM unlabeled Ang II. Binding affinity (K_D, concentration where receptor binding is half-maximal) and maximal binding (B_{max}) were determined using the LIGAND program (13).

Competition analysis. Binding assays were conducted as described above. Competition studies were performed using 300–500 pM ^{125}I-SARILE and 16–24 concentrations of unlabeled competitor spanning at least 5 orders of magnitude. The agonists examined were Ang II, SARANG, Cinia-Ang II, Ang III, and Ang I. The antagonists examined were SARILE, [Sar^1, Thr^8]-Ang II, and [Sar^1, Gly^8]-Ang II. In addition, one non-Ang II peptide, bradykinin, was tested to further confirm the specificity of Ang II binding sites on N1E-115 membranes. Competition curves were analyzed using LIGAND, and a multiple-site curve was chosen in preference to a single-site curve only if it significantly improved the goodness of fit. The relative proportions of high and low affinity components of the binding also were determined by this computer analysis. In order to determine the impact of guanine nucleotides on N1E-115 Ang II receptors, displacement of specific ^{125}I-SARILE binding was carried out in the presence of the GTP analog Gpp(NH)p (10^{-4} M).

Biochemical Assays

Adenylate cyclase activity. Membranes were prepared from N1E-115 cells as described above. An aliquot of the membrane suspension (approximately 1 mg/ml) was incubated in a total volume of 100 μl of 25 mM Tris-acetate (pH 7.6) containing 0.1 mM [α-^{32}P]ATP (2–6 × 10^6 dpm), 5 mM creatinine phosphate, 50 units/ml creatine phosphokinase, 5 mM magnesium acetate, 0.5 mM ATP, 0.05 mM cAMP, 1.0 mM DTT, 0.1 mg/ml BSA, 0.01 mM GTP, and 1.0 mM isobutylmethylxanthine. The [^{32}P]cAMP formed was isolated by the column method of Salomon (14). In order to monitor the performance of these chromatographic procedures, [^{3}H]cAMP was added to each tube and counts were subsequently corrected for the efficiency of its recovery.

Uptake of $^{45}Ca^{2+}$. The uptake of $^{45}Ca^{2+}$ was determined in intact N1E-115 cells grown in six-well plates. Experiments were initiated by washing of the cells and addition of 1 ml of fresh medium containing 1 μCi of $^{45}Ca^{2+}$. Incubations were performed for 6 min at 37° and were terminated by aspiration of the reaction mixture and washing of the cells three times with ice-cold buffer (135 mM NaCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.4). The cells were then dissolved in 0.5 ml of 0.1 M NaOH and transferred to a plastic tube. Each well was washed with an additional 0.5 ml of water, which was combined with the original sample. The solubilized cells were then homogenized and the protein concentration of each sample was determined as described below. A 0.5-ml aliquot of each sample was combined with 10 ml of Liquiscint (National Diagnostics), and radioactivity was quantified in a LKB Rackbeta liquid scintillation counter.

Protein Analysis

The protein concentrations of solubilized cell or membrane preparations were determined using the Bradford protein-dye binding method (15). After samples were diluted to produce a protein concentration of 10–100 μg/ml, 2 ml of a Coomassie brilliant blue solution (100 mg dissolved in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid were added to 200 μl of sample, and 30 min later the samples were quantified by spectrophotometry (Spectronic 1001) at 595 nm. Final protein concentrations were determined by comparison with a standard curve that was obtained in each assay, using BSA.

Statistical Analysis

All binding data obtained were analyzed using the LIGAND computer program (13). Data from all other experiments were analyzed by analysis of variance and appropriate *post hoc* comparisons.

Results

Effect of differentiation on the membrane properties of N1E-115 cells. When subconfluent N1E-115 cells were exposed to 1.5% DMSO and 0.5% serum on day 3, there was a

marked increase in the number of cells extending long neurites. Consistent with previous investigations (11), these morphological changes began within 24–36 hr of DMSO exposure and reached a maximum at 96 hr, when approximately 75% of the cells exhibited long multiple processes. *In vitro* differentiation also increased $^{45}Ca^{2+}$ uptake by N1E-115 cells. More specifically, $^{45}Ca^{2+}$ uptake increased gradually in undifferentiated N1E-115 cells, particularly after confluency had been reached. In differentiated cells, however, $^{45}Ca^{2+}$ uptake was significantly increased within 24 hr after the addition of DMSO and continued to rise, such that its maximal plateau at 72 hr was greater than 2 times the uptake observed in undifferentiated cells (Fig. 1).

The activity of the membrane-associated enzyme adenylate cyclase also was increased by *in vitro* differentiation. Basal adenylate cyclase activity was 20 pmol/min/mg of protein in membranes prepared from undifferentiated N1E-115 cells. Forskolin, which stimulates the catalytic subunit of adenylate cyclase, increased enzyme activity in a dose-related fashion, resulting in a 6-fold increase at 100 μM (Fig. 2). Neither basal nor forskolin-stimulated adenylate cyclase activity was significantly altered by the state of confluency in undifferentiated cells. *In vitro* differentiation increased basal adenylate cyclase activity, but the most pronounced effect of DMSO treatment appeared to be a substantial increase in the efficacy of forskolin stimulation. The potentiation was evident at all doses of forskolin and was somewhat delayed relative to $^{45}Ca^{2+}$ uptake, first becoming apparent after 48 hr of differentiation and not appearing to be maximal even after 96 hr (Fig. 2).

Effect of differentiation on Ang II receptors. In confirmation of our previous report (3), undifferentiated N1E-115 cells contain binding sites for the Ang II receptor antagonist ^{125}I-SARILE. Moreover, the density of these binding sites increased gradually as the cells progressed from the logarithmic to stationary phase of growth (Fig. 3), with a time course that resembled the changes observed in $^{45}Ca^{2+}$ uptake (Fig. 1). *In vitro* differentiation induced by lowering of the serum concentration from 10 to 0.5% and the addition of 1.5% DMSO to the growth medium on day 3 increased substantially the B_{max} of these binding sites, while only slightly altering the K_D for ^{125}I-

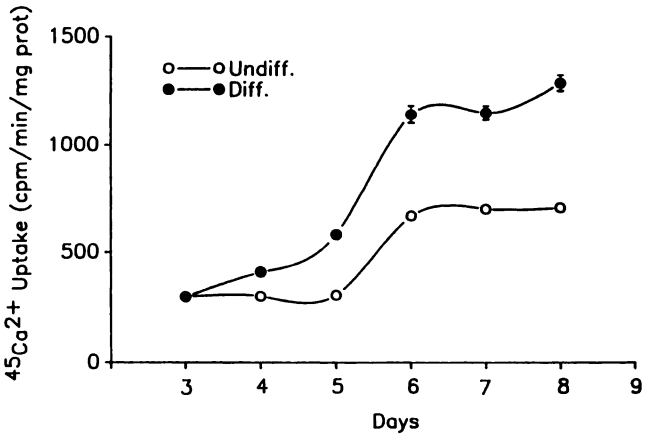

Fig. 1. Effect of *in vitro* differentiation on the uptake of $^{45}Ca^{2+}$ by intact N1E-115 cells. Undifferentiated cells (○) were maintained in normal DMEM, whereas differentiated cells (●) were exposed to 1.5% DMSO for varying periods of time. Each *point* represents the mean ± standard error of four to six separate experiments. In many cases, the standard error was smaller than the symbol.

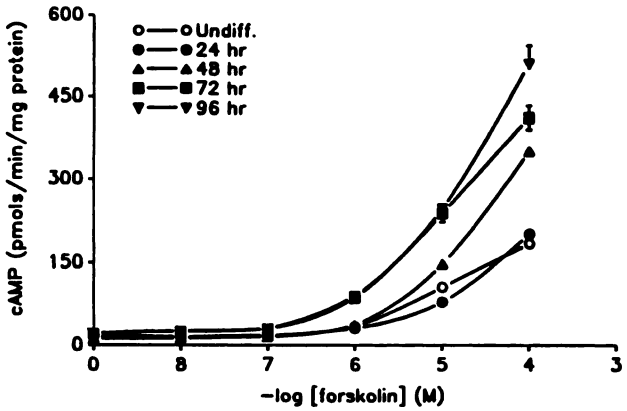

Fig. 2. Effect of *in vitro* differentiation on forskolin-stimulated adenylate cyclase activity in membranes prepared from N1E-115 cells. Differentiated N1E-115 cells were exposed to DMSO for 24–96 hr. The responses for the undifferentiated control cells (○) represent pooled data obtained from cells harvested 5–7 days after subculturing. Each *point* represents the mean ± standard error of four separate experiments. In some cases, the standard error was smaller than the symbol.

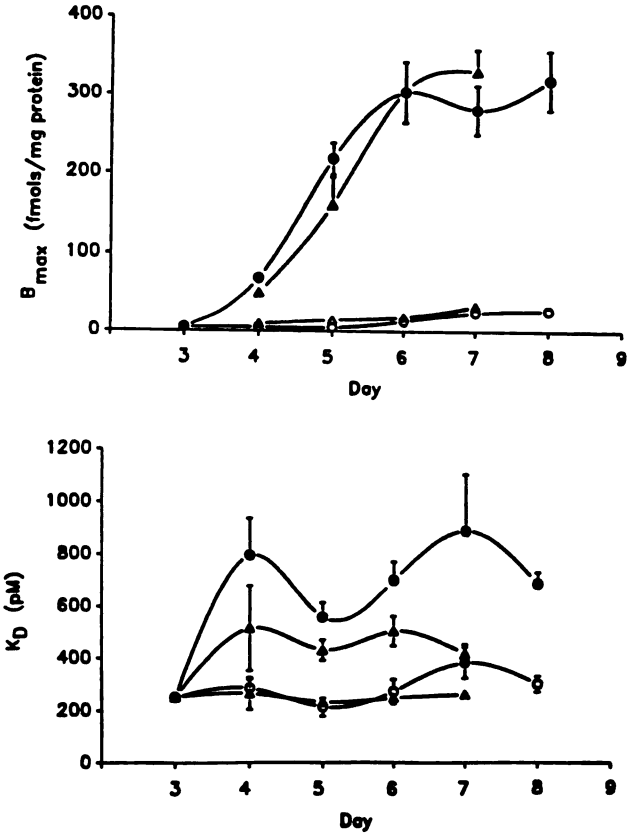

Fig. 3. Effect of *in vitro* differentiation on the B_{max} (*upper*) and K_D (*lower*) of ^{125}I-SARILE (*circles*) or ^{125}I-SARANG (*triangles*) binding in membranes prepared from N1E-115 cells. Undifferentiated cells (*open symbols*) were maintained in normal growth medium, whereas differentiated cells (*closed symbols*) were exposed to 1.5% DMSO for varying periods of time. Each *point* represents the mean ± standard error of three to seven separate experiments. In many cases the standard error was smaller than the symbol. B_{max} and K_D values were determined by 8- to 12-point saturation isotherms and subsequent Scatchard analysis.

SARILE (Fig. 3). More specifically, the B_{max} increased within 24 hr and reached maximal levels of 310 ± 38 fmol/mg of protein within 72 hr. A representative saturation isotherm of ^{125}I-SARILE binding obtained from N1E-115 cells treated with DMSO for 72 hr and the corresponding Scatchard analysis are shown in Fig. 4. Cells grown in low serum without DMSO did not show increased binding, and the addition of DMSO without lowering of the serum concentration produced more modest and less reliable increases in the density of binding sites (data not shown).

Similar results were obtained with the Ang II receptor agonist ^{125}I-SARANG. In undifferentiated cells, the total number of binding sites labeled with the agonist ^{125}I-SARANG was not different from the value observed with the antagonist ^{125}I-SARILE, and the density of these binding sites increased with the same time course as well. Moreover, *in vitro* differentiation produced a qualitatively and quantatively similar up-regulation of these binding sites, with little or no change in the K_D for ^{125}I-SARANG (Fig. 3). A representative saturation isotherm of ^{125}I-SARANG binding to 72-hr differentiated N1E-115 cells and the corresponding Scatchard analysis are shown in Fig. 5.

In addition to DMSO, a number of other treatments are known to induce differentiation of N1E-115 cells, including sustained elevations of Ca^{2+} (16) or cAMP (9, 17, 18). Because DMSO treatment was associated with an increase in both Ca^{2+} uptake and the enzymatic production of cAMP, the effects of independent variation of each of these variables on Ang II receptor density in the absence of DMSO were assessed. Elevation of extracellular Ca^{2+} levels from control levels of 2 mM to 12 mM, which is known to be associated with an increase in intracellular Ca^{2+} levels and neurite extension (16), caused an increase in ^{125}I-SARANG binding sites on N1E-115 membranes. An up-regulation also was obtained when N1E-115 cells were treated with 1 mM dbcAMP. Preliminary studies indicated that in both cases the up-regulation was first apparent within 24–36 hr and was maximal at 72 hr (data not shown). In order to compare the effects of DMSO, Ca^{2+}, and cAMP on N1E-115 Ang II receptors, cells from the same passage were exposed to one of these treatments for 72 hr and then saturation isotherms were performed. The results are summarized in Table 1. DMSO caused the largest increases in the density of binding sites, from control levels of 25 fmol/mg of protein to 301 fmol/mg of protein; dbcAMP produced nearly as great an increase, to 260 fmol/mg of protein, whereas the smallest change was observed with Ca^{2+}, obtaining levels of 96 fmol/mg of protein or an increase of approximately 4-fold.

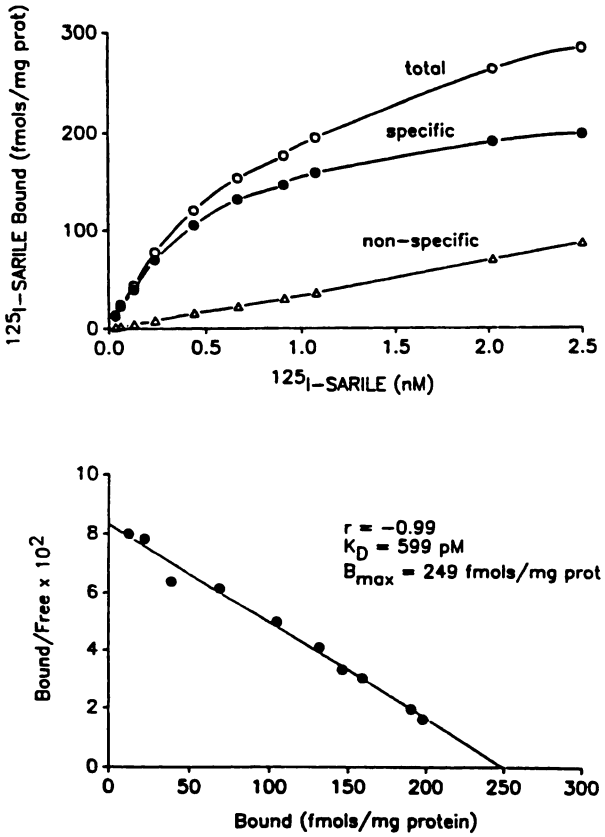

Fig. 4. *Upper*, representative saturation isotherm of ^{125}I-SARILE binding to membranes prepared from N1E-115 cells exposed to DMSO for 72 hr. *Lower*, the corresponding Scatchard analysis.

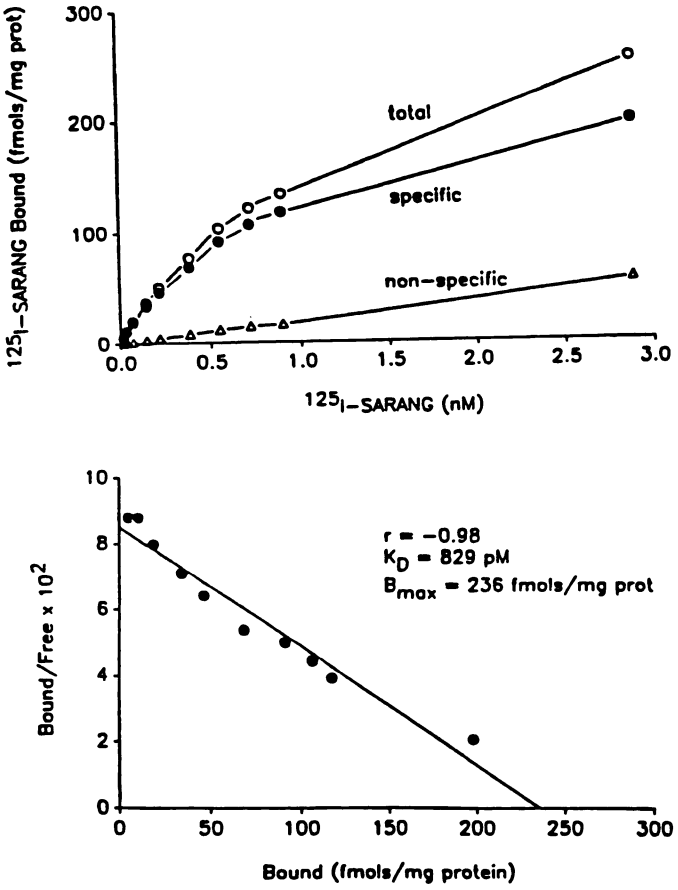

Fig. 5. *Upper*, representative saturation isotherm of ^{125}I-SARANG binding to membranes prepared from N1E-115 cells exposed to DMSO for 72 hr. *Lower*, the corresponding Scatchard analysis.

Specificity of Ang II receptors on differentiated N1E-115 cells. The specificity of ^{125}I-SARILE binding in undifferentiated and differentiated N1E-115 cells is shown in Fig. 6 and summarized in Tables 2 and 3. In general, the relative potency of various Ang II-related peptides in competing for ^{125}I-SARILE binding sites in undifferentiated N1E-115 cells was in close agreement with our earlier observations (3). More specifically, of those peptides that exhibited monophasic binding, two of the sarcosine-substituted analogs, SARANG and SARILE, competed with the highest affinity for ^{125}I-SARILE

TABLE 1

Effects of *in vitro* differentiation on ANG II receptors on N1E-115 cells

N1E-115 cells were induced to differentiate by placement in DMEM supplemented with 1) 12 mM $CaCl_2$ and 4.5% serum, 2) 1 mM dbcAMP and 1% serum, or 3) 1.5% DMSO and 0.5% serum. Data for control (undifferentiated) cells were obtained by pooling experiments in which cells were exposed to either 0.5, 1.0, 4.5, or 10% serum in normal DMEM, because there were no significant differences in the properties of Ang II receptors in these groups. All cells were harvested on day 6 and membranes were prepared for binding assays. B_{max} and K_D values were obtained by 10-point saturation isotherms and subsequent Scatchard analysis. Means ± standard errors represent results from three separate experiments.

Condition	K_D	B_{max}
	pM	fmol/mg of protein
Control	462 ± 85	25 ± 2.6
Ca^{2+}	590 ± 97	96 ± 12.5*
dbcAMP	773 ± 147	260 ± 27*
DMSO	987 ± 174*	301 ± 18*

* Statistically different from control value ($p < 0.01$).

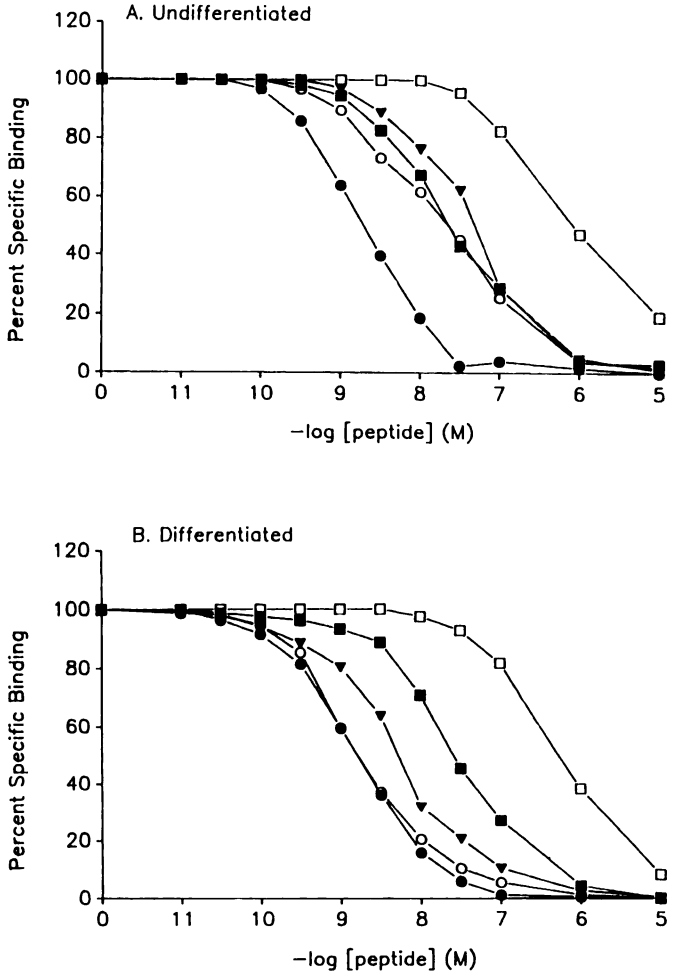

Fig. 6. Displacement of ^{125}I-SARILE from N1E-115 membranes prepared from undifferentiated (*upper*) or differentiated (*lower*) cells. Each *point* represents the mean of triplicate determinations. The competitors used were Ang II (●), Ang III (○), [Sar^1,Gly^8]-Ang II (■), Crinia-Ang II (▼), and Ang I (□). All displacement curves were analyzed with the LIGAND computer program. The data obtained using other competitors are not displayed in the graph in the interests of visual clarity, but such results, along with the combined results of all experiments, are summarized in Tables 2 and 3.

binding sites, whereas two others, the antagonists [Sar^1,Thr^8]-Ang II and [Sar^1,Gly^8]-Ang II, were approximately 10-fold less potent (Table 2). On the other hand, the agonists Ang II, Ang III, and Crinia-Ang II clearly displayed biphasic displacement of ^{125}I-SARILE binding, consisting of high affinity and low affinity components (Table 3), although for each agonist the K_D of their respective higher and lower affinity sites varied. For instance, the binding of Ang II was best described by assuming two sites with K_D values of 0.2 and 1.9 nM [$F(37,35) = 9.0$; $p < 0.001$], whereas Ang III binding revealed K_D values of 2.8 and 46 nM [$F(45,43) = 10.3$; $p < 0.001$], and Crinia-Ang II, 5.9 and 128 nM [$F(34,32) = 5.3$; $p < 0.01$]. Finally, Ang I was substantially less potent, and bradykinin, an unrelated peptide, did not compete for ^{125}I-SARILE binding sites even at concentrations as high as 100 μM (Table 2).

Although differentiation increased the density of Ang II receptors, it did not substantially alter the competition displayed by sarcosine-substituted Ang II peptides for ^{125}I-SARILE binding sites, even though in every instance their affinity was slightly lowered, especially that of the antagonists [Sar^1,Thr^8]-Ang II and [Sar^1,Gly^8]-Ang II (see Table 2). On the other hand, *in vitro* differentiation had a substantial effect on the biphasic binding of Ang II, Ang III, and Crinia-Ang II to ^{125}I-SARILE labeled sites. In particular, although differentiation did not alter the K_D values of high and low affinity sites for Ang II [$F(37,35) = 13.7$; $p < 0.001$], Ang III [$F(73,71) = 6.25$; $p < 0.003$], or Crinia-Ang II [$F(76,74) = 10.4$; $p < 0.001$], it did increase the density of these sites, depending on the agonist. For instance, the density of low affinity sites for Ang II substantially increased 72 hr after DMSO treatment (B_{max} = 290 fmol/mg of protein), whereas a much smaller increase in the density of high affinity sites (B_{max} = 46.5 fmol/mg of protein) was observed. In contrast, the density of the higher affinity sties for Ang III and Crinia-Ang II increased approximately 15-fold after 72 hr of differentiation (B_{max} = 266.3–292 fmol/mg of protein; see Table 3). The density of the low affinity sites for these agonists, on the other hand, increased to a much smaller extent (B_{max} = 35–44.6 fmol/mg of protein; approximately 1.5-fold). The net result of these changes was a reversal in the proportion of high versus low affinity sites for Ang III and Crinia-Ang II. This result is best seen by comparing the representative competition curves depicted in Fig. 6. In undifferentiated cells (Fig. 6A) the rank order of potency was Ang II > Ang III = [Sar^{1}Gly^8]-Ang II > Crinia-Ang II ≫ Ang I, whereas in differentiated cells (Fig. 6B) the substitutional increase in the density of high affinity binding sites for Ang III and Crinia-Ang II resulted in rearrangement of the rank order potency to Ang II = Ang III > Crinia-Ang II > [Sar^{1}Gly^8]-Ang II ≫ Ang I.

Guanine nucleotide regulation of Ang II agonist binding. In many cells agonist occupancy of receptors activates signal transduction pathways through an association with G proteins. For this reason, agonist but not antagonist binding is regulated by guanine nucleotides. Because the agonists Ang II, Ang III, and Crinia-Ang II all displayed biphasic binding in both undifferentiated and differentiated N1E-115 cells (Table 3), the effect of exogenous guanine nucleotides on the density of these high and low affinity sites was examined. In undifferentiated N1E-115 cells, the relative densities of high and low affinity binding sites for Ang II, Ang III, and Crinia-Ang II were comparable, but their sensitivities to guanine nucleotides

TABLE 2

Inhibition of ^{125}I-SARILE binding to N1E-115 membranes: single-site analysis

N1E-115 cells were grown in normal DMEM (undifferentiated) or were induced to differentiate by exposure to 1.5% DMSO for 72 hr. ^{125}I-SARILE (300–500 pM) was incubated with N1E-115 cell membranes and various concentrations of unlabeled competitors for 1 hr at 25°, as described in Experimental Procedures. Experiments were repeated four to six times and subsequently pooled for computer analysis by LIGAND in order to obtain K_D and B_{max} values. The values reported are means ± standard errors.

Competitor	Undifferentiated		Differentiated	
	K_D	B_{max}	K_D	B_{max}
	nM	fmol/mg of protein	nM	fmol/mg of protein
SARILE	0.52 ± 0.20	45.9 ± 3.6	1.08 ± 0.13[a]	320 ± 27[a]
SARANG	0.74 ± 0.21	45.2 ± 2.7	1.03 ± 0.17	358 ± 36[a]
[Sar^1,Gly^8]-Ang II	6.94 ± 1.1	39.6 ± 4.4	12.7 ± 1.9[a]	318 ± 43[a]
[Sar^1,Thr^8]-Ang II	7.9 ± 1.4	40.8 ± 3.8	12.8 ± 2.3[a]	330 ± 19[a]
Ang I	360 ± 29		439 ± 41	
Bradykinin	ND[b]		ND	

[a] Statistically different from undifferentiated ($p < 0.01$).
[b] ND, not detected.

TABLE 3

Inhibition of ^{125}I-SARILE binding to N1E-115 membranes: two-site analysis

N1E-115 cells were grown in normal DMEM (undifferentiated) or were induced to differentiate by exposure to 1.5% DMSO for 72 hr. ^{125}I-SARILE (300–500 pM) was incubated with N1E-115 cell membranes and various concentrations of unlabeled competitors for 1 hr at 25°, as described in Experimental Procedures. Experiments were repeated three to six times and subsequently pooled for computer analysis by LIGAND in order to obtain K_D and B_{max} values. The values reported are means ± standard errors.

Competitors	Cells	K_{D_1}	B_{max_1}	K_{D_2}	B_{max_2}
		nM	fmol/mg of protein	nM	fmol/mg of protein
Ang II	U[a]	0.20 ± 0.03	17.6 ± 0.9	1.9 ± 0.4	29.2 ± 4.5
	D	0.14 ± 0.02	46.5 ± 7.9[b]	2.2 ± 0.4	290 ± 14.1[b]
Ang III	U	2.8 ± 0.8	21.4 ± 2.7	46.1 ± 18.5	19.5 ± 2.0
	D	3.2 ± 0.2	292 ± 14.6[b]	78.1 ± 17.9	35.0 ± 5.8[b]
Crinia-Ang II	U	5.9 ± 0.9	15.3 ± 2.7	128 ± 31	29.8 ± 3.4
	D	5.7 ± 1.7	266.3 ± 21.0[b]	172 ± 56	44.6 ± 3.4

[a] U, undifferentiated; D, differentiated.
[b] Statistically different from undifferentiated ($p < 0.01$).

were not identical. In confirmation of our previous results (3), the displacement of ^{125}I-SARILE by Ang II was biphasic, consisting of high affinity and low affinity components (Table 3). Moreover, Gpp(NH)p (100 μM) eliminated the high affinity component, with a corresponding increase in the density of the low affinity site (Fig. 7, A and B). In contrast, Ang III and Crinia-Ang II also exhibited biphasic binding (Table 3), but in each case the density of the high affinity site was only partially reduced by Gpp(NH)p (Fig. 7, A and B).

In differentiated N1E-115 cells, the density of Ang II high affinity sites increased slightly and remained sensitive to Gpp(NH)p, whereas the low affinity, guanine nucleotide-insensitive site increased substantially (Fig. 7, C and D). On the other hand, high affinity binding sites for Ang III and Crinia-Ang II were increased substantially after *in vitro* differentiation, but in each case most of this high affinity binding was not reduced by Gpp(NH)p (Fig. 7, C and D).

Discussion

It is well recognized that the central actions of Ang II are importantly involved in the regulation of cardiovascular and body fluid homeostasis (1, 2), and techniques for the quantification and location of Ang II receptors have begun to reveal the sites of its actions in the brain (19–22). However, much less is known about the biochemical effects of Ang II once it binds to its receptor on neuronal cells. In an earlier study, we noted that the density of Ang II receptors on N1E-115 cells increased as the cells progressed from logarithmic to stationary growth in culture (3). We interpreted these results as indicating that, as the cells growth arrested, the extent of differentiation presumably increased. As a consequence of this differentiation, the morphological, biochemical, and physiological properties of the cells changed, including augmented expression of Ang II receptors on the plasma membrane.

Several laboratories have reported that neuroblastoma cells can be induced to differentiate *in vitro*. One of the most effective treatments for inducing such differentiation is to treat the cells with medium supplemented with DMSO (11). Other investigators have reported that the effects of this treatment include neurite extension (11), the appearance of increased densities of receptors and other membrane excitability proteins such as voltage-sensitive ion channels (6, 7, 11, 23, 24), and an augmentation of the activity of enzymes such as tyrosine hydroxylase and cholinacetyl transferase (11, 25). Consistent with such observations, we found that *in vitro* differentiation induced by DMSO also produced a substantial increase in the density of both agonist and antagonist Ang II binding sites. This up-regulation was apparent within 24 hr, was maximal after 72 hr, and remained stable for at least 5 days.

In vitro differentiation of N1E-115 cells with DMSO also increased the uptake of $^{45}Ca^{2+}$ and adenylate cyclase activity. Therefore, it is likely that the intracellular levels of Ca^{2+} and cAMP increased during the course of DMSO exposure. Moreover, because elevated Ca^{2+} and cAMP levels are known to induce differentiation in neuroblastoma cells (9, 16–18), the effects of independent increases in these variables on the properties of Ang II receptors were examined and compared with

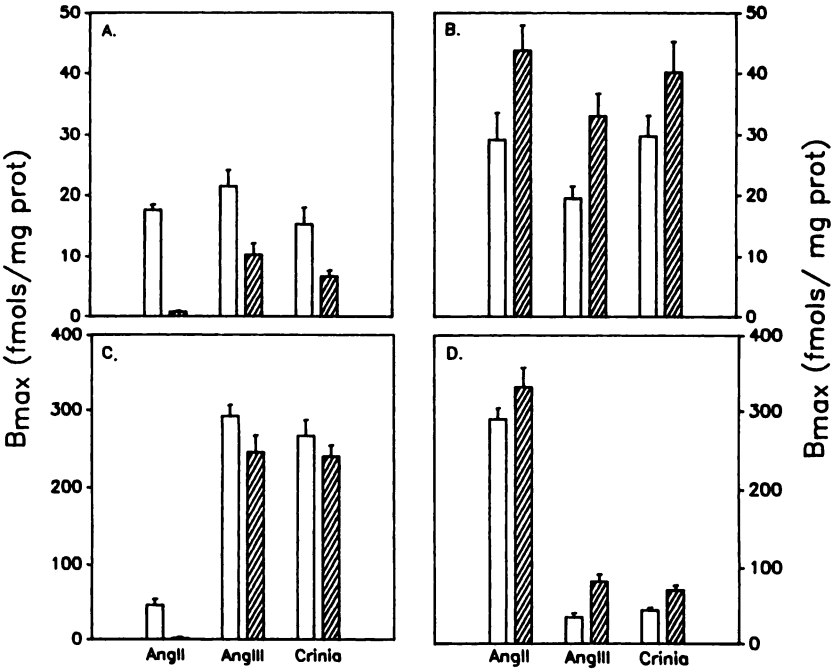

Fig. 7. Effect of Gpp(NH)p on the density of the high affinity (A and C) and low affinity (B and D) components of Ang II, Ang III, and Crinia-Ang II binding in membranes prepared from undifferentiated (A and B) and 72-hr differentiated (C and D) N1E-115 cells. Binding parameters were determined by incubation of N1E-115 membranes with ^{125}I-SARILE (300–500 pM) and various concentrations of unlabeled agonists in the presence (▨) or absence (□) of 100 μM Gpp(NH)p. Experiments were repeated four to six times and subsequently pooled for computer analysis by LIGAND in order to obtain B_{max} values. All values reported are means ± standard errors.

the effects of DMSO. Treatment of N1E-115 cells and 1 mM dbcAMP increased the density of ^{125}I-SARANG binding sites, an effect that was maximal after 72 hr of exposure. Moreover, the magnitude of this up-regulation was quantitatively similar to that obtained with DMSO. High levels of extracellular Ca^{2+} also resulted in an increased density of Ang II receptors, although this effect was only about 30% of the rise observed with either DMSO or dbcAMP. Nonetheless, it is clear that three distinct methods of inducing *in vitro* differentiation of N1E-115 cells produced qualitatively identical effects on Ang II receptors, which is apparently not true of the muscarinic receptors on neuroblastoma cells, for which the modification is dependent on the treatment used to induce differentiation (24).

Although the efficacy of dbcAMP in promoting differentiation is well documented in a wide variety of cells (26, 27), the exact mechanism of extracellular Ca^{2+}-induced cell differentiation is not clear; it may involve a rapid (30–60 min) increase in phosphatidylinositol turnover, as well as a more delayed (>10 hr) rise in intracellular Ca^{2+} levels (16). In considering whether these events might be involved in the up-regulation of Ang II receptors produced by extracellular Ca^{2+} as well as other treatments known to induce differentiation, it is important to emphasize that Ang II receptors are coupled to phosphatidylinositol hydrolysis in N1E-115 cells (4) and, therefore, an alteration in the activity of this metabolic pathway might result in a subsequent change in the population of Ang II receptors. Consistent with this hypothesis are the observations that activation of protein kinase C, which is one consequence of phosphatidylinositol hydrolysis (28), increases the density of Ang II receptors in primary culture of fetal rat brain (29) and that this up-regulation is potentiated by the Ca^{2+} ionophore A23187 (30). In addition, long term treatment with phorbol esters has been reported to increase Ang II responses in a liver epithelial cell line, although the effects of such treatments on Ang II receptor density were not assessed (31).

The effects of *in vitro* differentiation on the affinity of putative Ang II receptors on N1E-115 cells were complex. When binding sites were labeled with the high affinity antagonist ^{125}I-SARILE, all sarcosine-substituted peptides, whether agonists or antagonists, exhibited monophasic binding, the affinity of which was unaffected by the extent of differentiation. In marked contrast, the agonists Ang II, Ang III, and the N-terminal extended peptide Crinia-Ang II displayed biphasic binding, consisting of high and low affinity components. The biphasic nature of the binding of some Ang II agonists to undifferentiated N1E-115 cells was noted previously (3). In these earlier studies we demonstrated that the high affinity component of this binding was regulated by guanine nucleotides, presumably reflecting the important role of G proteins in coupling these receptors to biochemical effectors such as phosphatidylinositol hydrolysis (4). However, the effects of differentiation on the high and low affinity binding of Ang II, Ang III, and Crinia-Ang II, as well as their possible interaction with G proteins, appeared to be dependent on the particular agonist.

In conformity of our previous work (3), the biphasic nature of Ang II binding in undifferentiated N1E-115 cells appeared to reflect the interconversion of an agonist-specific, guanine-nucleotide-sensitive high affinity component, in that the appearance of this site was virtually eliminated by the addition of Gpp(NH)p to washed membranes, with a corresponding increase in the density of the low affinity site. *In vitro* differentiation had a very small effect on the high affinity binding but substantially increased the density of low affinity, guanine nucleotide-insensitive sites. This apparent lack of an effect of differentiation on the coupling of Ang II receptors to G protein(s) is not likely to result from a deficiency in the amount of G proteins, because their levels increase with differentiation (32), although it is possible that the induced receptors remain uncoupled. Regardless of the mechanism, the failure of differentiation to increase G protein regulation of agonist binding is consistent with our earlier observation that Ang II-induced mobilization of intracellular Ca^{2+}, presumably mediated by Gp (33), was quantitatively similar in undifferentiated and differentiated N1E-115 cells (4).

The effects of differentiation on the properties of Ang III binding to ^{125}I-SARILE-labeled sites were distinctly different than those on Ang II binding. Like Ang II, Ang III binding was biphasic in N1E-115 cells, and the affinity of the high affinity component was comparable to that of the guanine nucleotide-insensitive binding of Ang II (i.e., K_D = 2–3 nM). In undifferentiated cells, approximately 60% of this binding was guanine nucleotide sensitive, and Gpp(NH)p converted the affinity of this site to that of the low affinity state, as we reported previously (3). *In vitro* differentiation substantially increased the density of the high affinity site, but less than 20% of the elevated high affinity binding was sensitive to guanine nucleotides. Moreover, differentiation caused a much smaller increase in the density of the low affinity site. Qualitatively similar results were obtained with Crinia-Ang II, in that only a portion of high affinity binding was shifted to the low affinity state by guanine nucleotides in undifferentiated N1E-115 cells and *in vitro* differentiation preferentially increased that component of the high affinity binding not regulated by Gpp(NH)p, with a substantially smaller increase in the density of the low affinity sites.

On the basis of these results, it seems unlikely that all of the heterogeneity of angiotensin agonist binding in N1E-115 cells can be explained by assuming a single receptor entity that exhibits variable affinity states depending on its coupling to G proteins. Instead, the detection of multiple binding sites with varying affinities for certain ligands may result from the presence of at least two receptor subtypes for angiotensin peptides on N1E-115 cells. One subtype would resemble the receptor that has been well characterized on peripheral cells and that is coupled to a G protein involved in signal transduction. When the angiotensin agonist binds to this "peripheral-type" receptor, a ternary complex, or high affinity state, is formed, consisting of agonist, receptor, and G protein. Binding to this receptor, which would be biphasic because of its ability of the receptor to assume a high affinity conformation, displays approximately 25–50-fold greater affinity for Ang II (K_{D_1} = 0.17; K_{D_2} = 2.1 nM) than Ang III (K_{D_1} = 3; K_{D_2} = 62 nM) or Crinia-Ang II (K_{D_1} = 5.8; K_{D_2} = 150 nM). In the undifferentiated state this particular subtype appears to represent at least half of the total number of receptors, but its density is not substantially increased by differentiation.

The second angiotensin receptor subtype would differ from the first in several important respects. First, binding to this subtype would appear to be monophasic, with similarly high affinity for Ang II (K_D = 2 nM), Ang III (K_D = 3 nM), and Crinia-Ang II (K_D = 5.8 nM). Second, this putative receptor subtype does not appear to be coupled to a G protein, hence the insensitivity of most of the higher affinity binding of Ang III and Crinia-Ang II to guanine nucleotide regulation. The higher affinity binding of Ang II (K_D = 0.2 nM) would remain sensitive to guanine nucleotides, because that presumably reflects an interaction with the peripheral type G protein-regulated receptor. Third, as the cells become more neuron-like during *in vitro* differentiation, expression of this subtype would dramatically increase, so that it is the predominant receptor on differentiated cells, accounting for approximately 80% of the total number of receptors.

The possibility of multiple receptor subtypes for Ang II has recently received support from several converging lines of evidence, particularly studies involving newly developed nonpeptidic antagonists. For instance, Whitebread *et al.* (34) have suggested that there are at least two distinct Ang II receptor subtypes, referred to as A and B (35), based on their specific affinities for peptidic and nonpeptidic ligands as well as their susceptibility to the inhibitory effects of DTT. The ability of DTT to inhibit high but not low affinity binding of ^{125}I-Ang II in liver has also been used to support the concept of multiple receptor subtypes (36). Interestingly, some of these nonpeptidic antagonists exhibit greater than 1000-fold differences in their affinity for Ang II receptors in vascular smooth muscle or rat brain membranes (34, 37), raising the possibility of a "neuron-specific" Ang II receptor subtype. Such a hypothesis is additionally supported by the observation that certain Ang II-related peptides, particularly Ang III and Crinia-Ang II, agonists that display high affinity binding in differentiated N1E-115 cells, are as potent as Ang II itself in eliciting centrally mediated effects such as dipsogenic and pressor responses. The same rank order of potency did not appear to exist in peripheral target organs, including vascular and nonvascular smooth muscle, adrenal cortex, and kidney (38, 39).

Because the two putative Ang II receptor subtypes in N1E-115 cells can be revealed by the competition displayed by Ang II-related peptides for ^{125}I-SARILE-labeled sites, it would appear that this antagonist binds to both classes of receptors with equal affinity, hence the linearity of the Scatchard plot derived from ^{125}I-SARILE saturation isotherms. The total numbers of receptors measured with the agonist ^{125}I-SARANG or the antagonist ^{125}I-SARILE were not different, indicating that these agonists and antagonists interact with the same receptors and with similar affinities in undifferentiated and differentiated N1E-115 cells. Similar results have been obtained in adrenal zona glomerulose and uterine cells (40, 41). Moreover, the agonist SARANG failed to reveal any evidence of high and low affinity components, either in saturation isotherms or in its competition for ^{125}I-SARILE-labeled sites, suggesting that it is apparently incapable of forming the high affinity ternary complex in N1E-115 cells. This result is in contrast to the reported ability of SARANG to display both high and low affinity binding that is regulated by guanine nucleotides in zona glomerulose cells (40). At present it is unclear whether such differences are indicative of differing receptor entities or differential coupling to particular G proteins. However, it is noteworthy that Ang II receptors apparently are coupled to a pertussis toxin-sensitive G protein in zona glomerulose cells (40, 42), whereas this does not appear to be true in differentiated N1E-115 cells (12, 43). Finally, the two other sarcosine-substituted antagonists, [Sar^1,Thr^8]-Ang II and [Sar^1,Gly^8]-Ang II, which displayed monophasic binding, probably interacted with both putative subtypes, because the total number of binding sites derived from competition studies was comparable to that obtained with SARILE or SARANG, although they displayed approximately 10-fold less affinity for these sites.

In summary, *in vitro* differentiation increased the density of Ang II receptors in concert with the appearance of several other morphological, biochemical, and physiological markers of neuronal function in cultured N1E-115 cells. The heterogenity of the binding of angiotensin agonists, particularly Ang II, Ang III, and Crinia Ang II, suggested the presence of at least two distinct angiotensin receptor subtypes on N1E-115 cells. One of these receptors resembled that which is well characterized on peripheral cells, in that it displayed higher affinity for Ang

II than either Ang III or Crinia-Ang II. Moreover, all three of these agonists exhibited biphasic binding to this receptor, which was, in large part, a reflection of its coupling to G protein(s). The second receptor subtype, on the other hand, displayed high affinity for all three agonists, and binding to it was relatively insensitive to regulation by guanine nucleotides. Moreover, the density of this putative subtype was substantially increased during *in vitro* differentiation. One suggestion that emerges from these results is that the actions of Ang II in brain may differ from those observed in peripheral cells, particularly with respect to the extent of G protein involvement in receptor-effector coupling. However, further work is required to determine whether such apparent differences in the binding of angiotensin to neuronal cells result from posttranslational modifications of the receptor protein or the differential expression of unique gene products that function as physiological receptors for the peptide.

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