Identification and Regulation of Angiotensin II Receptor Subtypes on NG108-15 Cells

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NG108-15 cells, a neurally derived clonal cell line, express various components of the renin-angiotensin system and thus serve as a model of the cellular action of angiotensin (Ang) II. NG108-15 cells contain a high-affinity binding site for Ang II, with a $K_d$ of 1.1 nM and a $B_{max}$ of 6.5 fmol/mg protein. Ang peptides competed for $^{125}$I-Ang II binding with an order of potency of Ang II $>$ Ang-(2-8) $>$ Ang-(1-7). The subtype 1 (or B)-selective Ang II receptor antagonist DuP 753 as well as [Sar$^1$,Ile$^8$]Ang II and [Sar$^1$,Thr$^8$]Ang II competed for Ang II binding with high affinity, whereas the subtype 2 (or A)-selective Ang receptor antagonist CGP 42112A was partially effective only at a 300-fold higher concentration. When NG108-15 cells were induced to differentiate by treatment with dibutyl cyclic adenosine 3',5'-monophosphate, the density of Ang II receptors increased dramatically, with little change in affinity (1.1 versus 4.2 nM) or competition by Ang peptides. In marked contrast to undifferentiated cells, CGP 42112A became a potent competitor (IC$_{50}$, 1 nM) for the majority (90-95%) of Ang II binding, whereas DuP 753 competed for only 5-10% of the binding sites. Ang II caused a dose-dependent mobilization of cytosolic Ca$^{2+}$ in undifferentiated NG108-15 cells through activation of phospholipase C and the production of Inositol 1,4,5-trisphosphate. In these cells, Ca$^{2+}$ mobilization was blocked by either DuP 753 or the sarcosine Ang II analogues, whereas CGP 42112A was ineffective. Ang II also mobilized intracellular Ca$^{2+}$ in differentiated NG108-15 cells. This effect was blocked by either DuP 753 or the sarcosine analogues but not by CGP 42112A. These data show that both undifferentiated and differentiated NG108-15 cells contain a subtype 1 Ang II receptor that activates phospholipase C and mobilizes intracellular Ca$^{2+}$. However, in differentiated NG108-15 cells, the majority of Ang II receptors are subtype 2, suggesting that neuronal differentiation regulates the expression of subtype 2 receptors. (Hypertension 1991;17:1135-1143)

The central nervous system contains an intrinsic renin-angiotensin system. $^{1-3}$ High-affinity binding sites for angiotensin (Ang) II are localized to specific brain nuclei by in vitro receptor autoradiography.$^{1,4,5}$ These binding sites correspond to areas at which Ang II exerts physiological effects.$^5$ These data suggest that Ang II is an important modulator of the central regulation of both cardiovascular and neuroendocrine functions.

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Ang II receptors on different cell types are coupled to multiple mechanisms of signal transduction and can be distinguished by the intracellular signals that they generate. Hepatocytes,$^6,7$ vascular smooth muscle cells,$^8$ adrenal cortical cells,$^9$ and adrenal medullary cells$^{10}$ contain Ang II receptors that activate phospholipase C, catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate. This action results in the production of inositol 1,4,5-trisphosphate (IP$_3$), which mobilizes intracellular Ca$^{2+}$, and diacylglycerol, which activates protein kinase C. In contrast, hepatocytes$^7,11$ and epithelial cells from the kidney cortex$^{12}$ contain Ang II receptors that are coupled to inhibition of adenylate cyclase, thus preventing an increase in the cellular levels of cyclic adenosine 3',5'-monophosphate (cAMP). In addition, kidney epithelial cells,$^{12,13}$ cells in the rabbit vas deferens,$^{14}$ and rat C6 glioma cells$^{15}$ respond to Ang II as well as the heptapeptide Ang-(1-7) by stimulating production of prostaglandins. The release of prostaglandins by Ang-(1-7) in human astrocytes appears to be independent of the activation of phosphoinositide-specific phospholipase C,$^{16}$ suggesting...
that the heptapeptide activates another phospholipase. Because Ang II receptors couple to multiple mechanisms of signal transduction, these data suggest the existence of more than one Ang II receptor subtype.

In addition to multiple mechanisms of signal transduction, the existence of Ang II receptor subtypes is indicated by the differences in binding affinities and potencies of Ang peptides and the differential effects of the sulfhydryl reducing agent dithiothreitol on binding affinities. Moreover, recent studies using selective Ang II receptor antagonists showed that at least two subtypes of Ang II receptors exist in various tissues. Subtype 1 (or subtype B) (AT₁) receptors are sensitive to DuP 753 and EX 89, and subtype 2 (or subtype A) (AT₂) receptors are sensitive to CGP 42112A, EXP655, and PD 123319. Thus, specific subtypes of Ang II receptors may be present on different types of cells. Moreover, activation of distinct signal transduction mechanisms by these receptor subtypes is most likely responsible for the diverse physiological effects that occur in response to Ang II.

We have used the NG108-15 neuroblastoma × glioma hybrid cell line to characterize the subtypes of Ang II receptors present on neuronal cells and to identify their signal transduction mechanisms. NG108-15 cells contain many of the components of the renin-angiotensin system, including immunoreactive renin, angiotensinogen, angiotensin converting enzyme, and Ang II. When NG108-15 cells are induced to differentiate by chronic treatment with dibutyryl cAMP, they elaborate an extensive array of neurites and also form functional synapses when cocultured with myotubules. Thus, differentiation of NG108-15 cells is comparable to the process of neuronal differentiation.

Methods

Materials

Ang II (5-1-isoleucine)(iodotyrosyl-¹²⁵I) (2,200 Ci/mmol) and inositol 1,4,5-trisphosphate, d-(inositol-1-³H(N)) (17 Ci/mmol) were obtained from Du Pont–New England Nuclear, Boston. Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, hypoxanthine, aminopterin, and thymidine were purchased from Gibco, Grand Island, N.Y. Bradykinin and Ang I were obtained from Bachem, Inc., Torrance, Calif. IP₃ was from Calbiochem Corp., San Diego, Calif. Bovine serum albumin (BSA), bacitracin, verapamil, and nifedipine were obtained from Sigma Chemical Co., St. Louis, Fura 2-AM was purchased from Molecular Probes, Inc., Eugene, Ore. All other reagents were of highest analytical grade.

The NG108-15 cell line was obtained from Dr. Marshall Nirenberg, National Institutes of Health, Bethesda, Md. Ang II [5α,11α][¹²⁵I]Ang II, [5α,11β][¹²⁵I]Ang II, and Ang-(1–7) were synthesized in our laboratory. DuP 753 was from Du Pont, Wilmington, Del., and CGP 42112A was from CIBA-GEIGY, Basel, Switzerland.

Cell Culture

NG108-15 cells were maintained in DMEM to which we added 10% FBS, 0.1 mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, 100 units/ml streptomyacin, and 100 μg/ml penicillin at 37°C in a humidified atmosphere of 95% air–5% CO₂. Cells were used between passage 20 and 40. For experiments with differentiated cells, the culture medium was replaced 1 day after subculture with medium in which the FBS was reduced to 5% and 1 mM dibutyryl cAMP was added. The medium was replaced every other day, and the cells were harvested on day 7. Cells were nonenzymatically removed from tissue culture flasks by incubation for 20 minutes at 37°C in warm D1 solution containing (mM) NaCl 137, KCl 5.4, Na₂HPO₄ 0.17, KH₂PO₄ 0.22, glucose 5.5, and HEPES 5, pH 7.6. Dissociated cells were pelleted by centrifugation, washed two times in D1, and resuspended in the appropriate assay buffer, as described below.

Measurement of Angiotensin II Binding

Specific binding of [¹²⁵I]Ang II to suspensions of NG108-15 cells (0.75 to 1.0×10⁶/ml) or differentiated NG108-15 cells (2.5 to 5.0×10⁶/ml) was measured in phosphate-buffered saline (PBS) containing 0.2% BSA and 0.1 mM bacitracin; suspensions were incubated for 30 minutes at 25°C. Inclusion of bacitracin in the incubation mixture inhibits degradation of the radiolabeled ligand, as determined by high-performance liquid chromatographic analysis of [¹²⁵I]Ang II incubated with intact NG108-15 cells. For saturation analysis, cells were incubated with increasing concentrations of [¹²⁵I]Ang II (0.04–2.0 nM). For competition analysis, cells were incubated with 0.2 nM [¹²⁵I]Ang II in the presence of increasing concentrations of Ang peptides or antagonists (from 0.1 nM to 10 μM). Reactions were stopped by the addition of 2 ml ice-cold PBS; the cells were isolated by rapid filtration on Whatman GF/C membranes prewashed with 1 ml PBS containing 0.2% BSA and washed with four 2-ml aliquots of ice-cold PBS. The amount of [¹²⁵I]Ang II bound to the cells was determined by a gamma spectrophotometer with an efficiency of 75%. Nonspecific binding, measured in the presence of 1 μM unlabeled Ang II, was subtracted from each data point; nonspecific binding was routinely 10–20% for control NG108-15 cells and 5–10% for differentiated cells. [¹²⁵I]Ang II that was specifically bound to the cells was determined by Scatchard analysis using the computer program EBD/LIGAND (Equilibrium Binding Data Analysis; Elsevier-BIOSOFT, Cambridge, UK). From competition analysis, inhibition curves were...
Measurements of Inositol 1,4,5-TRISPHOSPHATE

IP₃ was measured by a radioreceptor assay, using canine cerebellar membranes purified according to the procedure for rat cerebellum as a source of the IP₃ receptor. NG108-15 cells resuspended at 1x10⁷ cells/ml of HEPES-buffered Krebs-Ringer solution (mM: NaCl 125, KCl 5, MgSO₄ 1.2, glucose 6, CaCl₂ 1, and HEPES 25, pH 7.5) containing 0.1% BSA were treated with 100 nM Ang II for the indicated times at 37°C. The reaction was terminated by the addition of perchloric acid to 5% and neutralized by the addition of 6 M KOH and 0.5 M Tris. After incubation on ice for 20 minutes and centrifugation for 10 minutes at 10,000g, the supernatant was assayed for IP₃, essentially as described by Bredt et al. Nonspecific binding was defined as binding not displaced by 1 μM IP₃.

Measurement of Cytosolic Concentration of Calcium

For Ca²⁺ measurements, cells (resuspended at 1x10⁷/ml) were equilibrated for 30 minutes at 37°C in HEPES-buffered Leibowitz’s L-15 media (pH 7.4) containing 0.1% BSA and loaded with fura-2 by incubation for 30 minutes at 37°C with 2 μM fura-2-AM. The cells then were incubated an additional 20 minutes in a Krebs-Ringer solution that contained 0.1% BSA to completely hydrolyze the entrapped ester. Fura-2 measurements were conducted in an air turbine-driven dual-wavelength spectrofluorometer using excitation wavelengths of 340 and 380 nm and emission at 500 nm. Maximal fluorescence of the Ca²⁺-saturated dye was measured in the presence of 6 mg/ml digitonin, and minimal fluorescence was measured in the presence of 5 mM EGTA and 32 mM Tris, pH 8.5. The level of cytosolic Ca²⁺ was calculated according to the procedure of Grynkiewicz et al. Ca²⁺ levels are indicated as the increase above basal levels ± SEM.

Protein Determinations

Protein concentration was determined by the procedure of Lowry et al after the proteins were precipitated with 10 volumes of 10% perchloric acid and 1% phosphotungstic acid.

Results

Characterization of ¹²⁵I-Angiotensin II Binding to NG108-15 Cells

Specific binding ¹²⁵I-Ang II to undifferentiated NG108-15 cells was saturable and of high affinity. By Scatchard analysis of saturation isotherms of ¹²⁵I-Ang II binding to intact cells, undifferentiated NG108-15 cells contained a single high-affinity binding site with a Kᵣ value of 1.1 nM and a Bₘₐₓ value of 6.5 fmol/mg protein. The linearity of the Scatchard plot suggested that the radiolabeled Ang II binds to a homogeneous population of binding sites in control cells. As shown in the top panel of Figure 1, Ang II competed for ¹²⁵I-Angiotensin II by angiotensin antagonists in control (top panel) and differentiated (bottom panel) NG108-15 cells. Intact cells were incubated with 0.2 nM ¹²⁵I-Ang II in the presence of increasing concentrations of angiotensin II, DuP 753, or CGP 42112A, as described in Methods. Data are the average of four to 10 determinations. All, angiotensin II.

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competed in a dose-dependent manner, with an IC$_{50}$ of 1.4 nM. Ang-(2–8) was slightly less potent, with an IC$_{50}$ of 7.7 nM. In contrast, Ang-(1–7) was a poor competitor, with an IC$_{50}$ of 357 nM. Smaller fragments of Ang II (Ang[2–7], Ang[1–6], and Ang[1–5]) were extremely poor competitors, only competing for binding at a concentration of 10 µM. In differentiated NG108-15 cells, both Ang II and Ang-(2–8) were equipotent in competing for $^{125}$I-Ang II binding sites, with an IC$_{50}$ value of 4.4 nM. Ang-(1–7) was slightly more potent in differentiated than in undifferentiated cells, although the IC$_{50}$ still was in the high nanomolar range (IC$_{50}$, 220 nM).

The ability of Ang antagonists to compete for binding with $^{125}$I-Ang II is shown in Figure 1. In control NG108-15 cells (top panel), the AT$_1$-selective receptor antagonist DuP 753 competed with $^{125}$I-Ang II for binding, with an IC$_{50}$ of 1.1 nM compared with 1.2 nM for the unlabeled peptide. However, the competition curve plateaued between $10^{-8}$ and $10^{-6}$ M DuP 753 (20–30% residual binding), suggesting that DuP 753 only competes with high affinity for 70–80% of the Ang II binding sites. In contrast, the AT$_1$-selective antagonist CGP 42112A competed poorly for $^{125}$I-Ang II, with approximately a 300-fold lower affinity (IC$_{50}$, 373 nM). However, the more gradual slope of the competition curve between $10^{-10}$ and $10^{-7}$ M CGP 42112A (corresponding to 10–20% of the Ang II binding sites) suggests that a small percentage of the Ang II binding sites may bind with high affinity to a CGP 42112A-sensitive binding site. To determine whether control NG108-15 cells contain different subtypes of Ang II receptors, the DuP 753-sensitive binding sites were blocked by the addition of 50 nM DuP 753 (which blocked 70% of the total number of binding sites). Competition for the remaining binding sites was determined with increasing concentrations of CGP 42112A. The blocker was essentially ineffective in competing for these sites, inhibiting binding only minimally at 10 µM concentration (data not shown). In a similar experiment in which CGP 42112A-sensitive binding sites were blocked by the addition of 50 nM CGP 42112A (which blocked 10% of the total number of binding sites), DuP 753 competed for the remaining binding sites with an IC$_{50}$ of 2.9 nM, a value similar to that observed for Ang II binding sites. However, CGP 42112A was a potent competitor (IC$_{50}$, 24 nM) for the non-CGP-sensitive binding sites (bottom panel). The effect of the sulfhydryl reducing reagent dithiothreitol on binding of $^{125}$I-Ang II to both control and differentiated cells is shown in Figure 3. Increasing concentrations of dithiothreitol inhibited 80% of the binding of $^{125}$I-Ang II to control NG108-15 cells, with an IC$_{50}$ of approximately 5 mM. In contrast, dithiothreitol increased the binding of $^{125}$I-Ang II to differentiated cells approximately 300% above control levels, with an IC$_{50}$ of approximately 5 nM. These data show a significant difference in the properties of Ang II binding sites on differentiated versus undifferentiated NG108-15 cells.
Characterization of Signal Transduction Mechanisms in NG108-15 Cells

To determine whether Ang II activates a phosphoinositol-specific phospholipase C in undifferentiated NG108-15 cells, we measured the production of IP$_3$ in response to 100 nM Ang II. As shown in the top panel of Figure 4, Ang II caused a transient increase in the level of IP$_3$. Within 5 seconds, the amount of IP$_3$ increased approximately 1.5-fold over basal levels. Levels continued to rise, reaching a maximum at 15 seconds (approximately threefold over control) and returned to control values by 30 seconds.

Because increases in the level of IP$_3$ cause subsequent elevations in cytosolic concentrations of Ca$^{2+}$, we measured the intracellular Ca$^{2+}$ levels in response to Ang II in fura-2-loaded NG108-15 cells. As shown in the bottom panel of Figure 4, 100 nM Ang II also caused a transient increase in the intracellular concentration of Ca$^{2+}$ (83.7±9.0 nM above basal, n=21). The increase in Ca$^{2+}$ mobilization in response to Ang II was dose dependent, with an IC$_{50}$ of approximately 40 nM. Ca$^{2+}$ mobilization was not dependent on entry of extracellular Ca$^{2+}$ through voltage-dependent Ca$^{2+}$ channels, as the mobilization of Ca$^{2+}$ in response to Ang II was unchanged by pretreatment with either 100 μM verapamil or 1 μM nifedipine (67.0±16.0 above basal, n=3). Ca$^{2+}$ also was mobilized in response to 100 nM Ang-(2-8) (40.0±8.2 above basal, n=4); however, Ang-(1-7) was ineffective in the mobilization of Ca$^{2+}$ from NG108-15 cells (n=10).

Ca$^{2+}$ mobilized in response to 100 nM Ang II in control NG108-15 cells (Figure 5A; 83.7±9.0 nM above basal, n=21) was blocked by pretreatment with 1 μM DuP 753 (Figure 5B; n=5), whereas 1 μM CGP 42112A had no effect (Figure 5C; 126.4±39.2 nM above basal, n=3). The classical Ang antagonists [Sar$^1$,Ile$^8$]Ang II and [Sar$^1$,Thr$^8$]Ang II (1 μM) completely blocked the response to 100 nM Ang II. Because bradykinin mobilizes intracellular Ca$^{2+}$ in NG108-15 cells, we monitored its effects during exposure to Ang II antagonists. None of the Ang II receptor antagonists blocked Ca$^{2+}$ mobilization in response to bradykinin.

Ang II (100 nM) also mobilized intracellular Ca$^{2+}$ in differentiated NG108-15 cells, as shown in Figure 6A. Ca$^{2+}$ mobilization was transient and of the same order of magnitude as in control NG108-15 cells (61.7±5.7 nM above basal, n=11). Although not shown, the response was dose dependent and was elicited by Ang-(2-8) but not by Ang-(1-7). These findings are reminiscent of the responses found in undifferentiated NG108-15 cells. Moreover, the response to 100 nM Ang II was inhibited by pretreatment with 1 μM DuP 753 (Figure 5B; n=4), whereas 1 μM CGP 42112A was ineffective (Figure 5C; 70.0±8.4 nM above basal, n=5). As in the control cells, the classical Ang antagonist [Sar$^1$,Thr$^8$]Ang II,

**Figure 3.** Effect of dithiothreitol on $^{125}$I-angiotensin II (Ang II) binding in control and differentiated NG108-15 cells. Intact cells were incubated with 0.2 nM $^{125}$I-Ang II in the presence of increasing concentrations of dithiothreitol, as described in "Methods." Nonspecific binding (in the presence of 10 μM Ang II), which averaged 5-25% of total binding, was determined at each concentration of dithiothreitol and subtracted from the total binding. Data are presented as the percentage of binding in the absence of dithiothreitol (% Control). Data are the average of six determinations.

**Figure 4.** Effect of angiotensin II (Ang II) on inositol 1,4,5-trisphosphate (IP$_3$) levels (top panel) and Ca$^{2+}$ mobilization (bottom panel) in control NG108-15 cells. Intact NG108-15 cells were incubated with 100 nM Ang II for increasing periods of time; samples were removed, and IP$_3$ levels were determined using the IP$_3$ radio-receptor assay as described in "Methods." Data are representative of three experiments of duplicate samples, using cells from different passage numbers (top panel). Intact NG108-15 cells loaded with fura-2 were challenged with 100 nM Ang II; the cytosolic level of Ca$^{2+}$ was determined as described in "Methods" (bottom panel). Data are representative of experiments conducted with cells from at least three different passage numbers.
at a concentration of 1 \( \mu M \), also blocked \( Ca^{2+} \) mobilization in response to 100 nM Ang II in differentiated NG108-15 cells.

Discussion

In the present study, we show that NG108-15 cells contain a single high-affinity binding site for Ang II with a \( K_d \) of 1.1 nM. Ang peptides compete for the binding site, with Ang-(2-8) being a potent competitor and Ang-(1-7) being much less potent. Other shorter Ang peptides competed poorly. Both the binding affinity and the relative potency of Ang peptides in competition for this binding site are similar to what has been observed in membrane homogenates of brain tissue\(^3\) as well as in cultured neuronal cells from rat hypothalamus,\(^3\)2 cultured spinal cord cells,\(^3\)3 N1E-115 neuroblastoma cells,\(^3\)4 and human neuroblastoma cells (unpublished observation from our laboratory).

Wehenmeyer and Hwang\(^5\) previously reported that NG108-15 cells contain both a high-affinity (0.46 nM) and a low-affinity (1.75 \( \mu M \)) Ang II binding site. Their incubation conditions were identical to those reported here, except for our inclusion of 0.1 mM bacitracin, which inhibits metabolism of the radioligand. We recently have shown that, in the absence of any protease inhibitors, \(^{125}\)I-Ang II is rapidly metabolized to \(^{125}\)I-Ang-(1-7) by incubation of the radiolabeled peptide with intact NG108-15 cells.\(^6\) After a 30-minute incubation of NG108-15 cells with 0.2 nM \(^{125}\)I-Ang II, only 40\% of the total amount of radioactivity existed as the intact radioligand; the other 60\% was composed of \(^{125}\)I-Ang-(1-7) and smaller radiolabeled metabolites. Because we showed that these fragments are poor competitors for the Ang II binding site on intact NG108-15 cells, it is possible that the low-affinity binding site reported by Wehenmeyer and Hwang\(^5\) was due to degradation of their radioligand and the subsequent lower affinity binding by these smaller fragments of \(^{125}\)I-Ang II. Indeed, if we omit bacitracin, we also observe two binding site components (unpublished observation from our laboratory).

Differentiation of NG108-15 cells by treatment with 1 mM dibutyryl cAMP dramatically increased the number of \(^{125}\)I-Ang II binding sites while mini-
mally decreasing binding affinity (4.4-fold). Differentiated NG108-15 cells extend long neurite-like processes, therefore dramatically increasing their surface areas. It is unlikely, however, that the increase in surface area alone accounts for the dramatic increase in the number of binding sites observed after differentiation. Furthermore, we found that the increase in the number of binding sites is due to either the specific expression or upregulation of a different subtype of Ang II receptor on differentiated cells. A similar increase in the total number of Ang II binding sites was reported by Reagen et al.\(^3\) in differentiated N1E-115 cells and Carrithers et al.\(^3\) in NG108-15 cells differentiated in low serum–medium containing dimethylsulfoxide. In addition, Ang-(2–8) became equipotent with Ang II in differentiated cells. Recently, Carrithers et al.\(^3\) showed that in NG108-15 cells differentiated by treatment with dimethylsulfoxide in low serum–containing medium, Ang-(2–8) was more potent than Ang II in producing inositol polyphosphates. However, the data presented in their dose–response curve show that Ang II and Ang-(2–8) were equipotent in the production of inositol phosphates at many of the doses tested. Nonetheless, their functional data agree with our binding data with respect to the presence on differentiated NG108-15 cells of high-affinity binding sites that recognize both Ang II and Ang-(2–8). The difference in binding affinities and potency of Ang peptides in undifferentiated and differentiated NG108-15 cells further suggests that different subtypes of Ang II receptors exhibit distinct pharmacological properties.

Although many investigators have speculated that different subtypes of Ang II receptors are present in the brain, this is the first report documenting the presence of both AT\(_1\) and AT\(_2\) receptors on neuronal cells. Sarcosine analogues of Ang II compete strongly for \(^1\)I-Ang II binding and block Ca\(^{2+}\) mobilized in response to Ang II in both differentiated and undifferentiated NG108-15 cells. The subtype-selective Ang II antagonists DuP 753 and CGP 42112A permit the identification of unique subtypes of Ang II receptors on NG108-15 cells before and after differentiation. Undifferentiated NG108-15 cells predominantly contain an AT\(_1\) receptor, based on their sensitivity to nanomolar concentrations of DuP 753. The IC\(_{50}\) for DuP 753 binding to NG108-15 cells is 1.1 nM. This value is 20- to 50-fold higher than reported previously for competition of other AT\(_1\) receptors.\(^2\) However, these previous reports characterized receptor subtypes in isolated membranes from whole tissues, whereas we measured binding to intact cells of a clonal cell line. These differences in methodology may account for the diverse findings between their observations and ours. In contrast, differentiated NG108-15 cells predominantly contain AT\(_2\) receptors, because CGP 42112A competed for 90–95% of the total number of binding sites. However, differentiated NG108-15 cells also contain AT\(_1\) receptors, as evidenced both by the ability of DuP 753 to compete with high affinity for the remaining 5–10% of the binding sites and to block Ca\(^{2+}\) mobilization in response to Ang II.

During the differentiation of NG108-15 cells, cells shift from a poorly defined state to a state in which functional synapses can be formed with striated muscle cells.\(^2\) In addition, differentiation causes an increase in the activity of voltage-sensitive Ca\(^{2+}\) channels\(^4\) and the release of acetylcholine during depolarization.\(^4\) Differentiation of NG108-15 has been reported to affect various receptors as well as other components of signal transduction pathways. The number of muscarinic acetylcholine receptors increases approximately 130% after differentiation,\(^4\) whereas Mullaney et al.\(^4\) report a decrease in the number of opioid receptors. Different subtypes of guanine nucleotide binding proteins—G\(_a\) and G\(_b\)—are reciprocally increased and decreased, respectively, in NG108-15 cells after differentiation.\(^4\) Although it is difficult to state unequivocally that undifferentiated NG108-15 cells contain no AT\(_2\) receptors, these receptors either are dramatically upregulated during differentiation or their production is specifically expressed by chronic treatment with cAMP. These data suggest that AT\(_2\) receptors are either involved in or produced as a consequence of neuronal differentiation.

In both undifferentiated and differentiated NG108-15 cells, Ang II activates a phosphoinositide-specific phospholipase C, resulting in the mobilization of intracellular Ca\(^{2+}\). Ang II–induced activation of phospholipase C has been reported in many types of cells.\(^6\) In addition, Monck et al.\(^4\) report that Ang II mobilizes intracellular Ca\(^{2+}\) in the N1E-115 neuroblastoma cell line, and Carrithers et al.\(^3\) demonstrated that Ang II produces inositol phosphates in differentiated NG108-15 cells. However, this is the first report that Ang II–induced mobilization of intracellular Ca\(^{2+}\) in neuronal cells can be specifically inhibited by DuP 753, the AT\(_1\)-selective Ang II antagonist. Our data suggest that AT\(_1\) receptors on neuronal cells are coupled to activation of phospholipase C and subsequent mobilization of cytosolic Ca\(^{2+}\). Chiu et al.\(^2\) speculated that a DuP 753–sensitive receptor in the adrenal cortex modulates Ca\(^{2+}\) mobilization and aldosterone secretion because of the presence of predominantly DuP 753–sensitive binding sites on cortical cells. We also have reported that DuP 753 blocks Ca\(^{2+}\) mobilization in human astrocytes.\(^5\) Thus, AT\(_1\) receptors, which are sensitive to DuP 753, are likely responsible for the activation of phospholipase C and the mobilization of intracellular Ca\(^{2+}\) in the wide variety of tissues in which Ang II produces these responses.

In summary, both undifferentiated and differentiated NG108-15 cells contain an AT\(_1\) receptor that activates phospholipase C and mobilizes intracellular Ca\(^{2+}\). In addition, the differentiation of NG108-15 cells upregulates subtype 2 Ang II receptors, which do not appear to be coupled to the activation of phospholipase C or mobilization of intracellular Ca\(^{2+}\). The dramatic expression of this receptor sub-
type during the process of differentiation suggests that AT2 receptors may be responsible for a part of the mechanisms of neuronal differentiation. The upregulation of AT2 receptors on differentiated NG108-15 cells without a concomitant increase in AT1 receptors, which also are present on these cells, provides further evidence for the existence of multiple subtypes of Ang II receptors.

Although the function of the AT2 receptor in differentiated NG108-15 cells has not been identified, we have shown recently\(^6\) that AT2 receptors on human astrocytes are specifically coupled to the release of prostaglandins, through a process that is independent of an increase in the intracellular concentration of Ca\(^{2+}\). Thus, the two subtypes of Ang II receptors activate distinct signal transduction pathways and are most likely responsible for the diversity of the cellular actions of Ang II.

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