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IDENTIFICATION AND CHARACTERIZATION OF A NEW BINDING SITE FOR ANGIOTENSIN II IN MOUSE NEUROBLASTOMA NEURO-2A CELLS

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Summary: Specific binding site for ^{125}I-angiotensin II (Ang II), with unique pharmacological properties uncommon to the hitherto recognized receptor subtypes, was observed in mouse neuroblastoma cells (Neuro-2A). Differentiation of the cells with 100 nM PGE_1 resulted in a 10-fold increase in the number of Ang II binding sites without changing the binding affinity (Kd value: 12.0 nM). ^{125}I-Ang II binding to membranes of differentiated Neuro-2A was inhibited by unlabeled Ang II with a Ki value of 7.06 ± 1.09 nM but not by Ang III (1 μM). Both AT_1 antagonist, Dup753, and AT_2 antagonist, PD123319, failed to inhibit ^{125}I-Ang II binding at 1 μM. ^{125}I-Ang II binding was not affected by GTP analogs such as GTPγS and Gpp(NH)p. These results suggest that Neuro-2A cells possess a binding site for Ang II which is different from the presently known subtypes of Ang II receptors, and that the number of the binding site is regulated by cell differentiation.

Angiotensin II (Ang II) has important roles in cardiovascular functions and fluid volume homeostasis. In addition to the actions of Ang II on a variety of peripheral tissues (1), the brain is also an important target organ for this peptide (2, 3). The binding of Ang II to the receptor in the brain alters the activity of central neuronal pathways (4) and secretion of vasopressin and adrenocorticotropic hormone (5). Despite the well recognized neuronal functions, our knowledge on the mechanism of action of Ang II and its receptor function in the brain and other neuronal tissues is limited.

Such studies of receptor and the central action of Ang II in the brain have been hampered by the fact that Ang II receptors are present in restricted regions of the brain (3). As an alternative approach, some investigators tried to study the properties of Ang II receptors and the signal transduction in a variety of clones neuronal cell lines (6, 7, 8).

In view of the finding that Neuro-2A cells produce Ang I, Ang II, renin and angiotensinogen (9), the possibility of the presence of Ang II receptors has been investigated. We report here the identification and characterization of Ang

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II binding site in Neuro-2A cells which is distinct from the known subtypes of Ang II receptors (AT_1 and AT_2). These cells represent a valuable and convienient model in which to study potential coupling mechanism and function of the site.

MATERIALS AND METHODS

Monoiodinated ^{125}I-Ang II (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Dup753 and PD123319 were obtained from E.I. du Pont de Nemours & Company (Wilmington, DE).

cell culture: The original cells were obtained from American Type Culture Collection (Rockville, Md). Neuro-2A cells were grown in plastic plates in Dulbeco's modified Eagle's medium supplemented with 10 % fetal calf serum, 100 unit/ml penicillin and 100 μg/ml streptomycin, in a humidified atomosphere of 5% CO_2 and 95% O_2 at 37 ℃. For *in vitro* differentiation, the cells were grown for 2 days in the medium containing 1% serum and 100 nM PGE_1.

membrane preparation: The cells were washed from the plate and transferred to a 50 ml centrifuge tube. The cells were collected by centrifugation at 1,500 xg for 5 min. The cells were homogenized with 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 100 μg/ml PMSF, and centrifuged at 1,500 xg for 5 min at 4 ℃. The supernatants were centrifuged at 48,000 xg for 30 min at 4 ℃. The pellets were washed twice with the same buffer by rehomogenization and recentrifugation as described above. The pellets thus obtained were suspended in 50 mM Tris-HCl buffer (pH 7.6) containing 100 mM NaCl, 10 mM $MgCl_2$, 1mM EDTA and 100 μg/ml PMSF at a protein concentration of 250 μg/ml, and used as membrane preparation.

binding assay of ^{125}I-Ang II: Suspended membranes were incubated with ^{125}I-Ang II at 25 ℃ for 60 min in the absence and presence of 1 μM nonradioactive Ang II. For saturation studies, the ^{125}I-Ang II concentration ranged from 0.625 to 80 nM. Membrane bound radioligand was separated from free radioligand by filtration over glass fiber filters (GF/B) using cell harvester. The filter disks containing membrane bound ^{125}I-Ang II were counted in a gamma scintillation counter. The specific binding of ^{125}I-Ang II was obtained by subtracting the nonspecific bindng, which is the binding in the presence of nonradioactive Ang II, from the binding in the absence of Ang II. Protein concentration was determined according to Bradford (10).

RESULTS

Scatchard plot analysis of the specific binding of ^{125}I-Ang II in undifferentiated and differentiated Neuro-2A cells were shown in Fig. 1. The Kd value and Bmax value of ^{125}I-Ang II binding to the membrane of undifferentiated Neuro-2A were 8.22 ± 0.89 nM and 116.28 ± 10.19 fmoles/mg

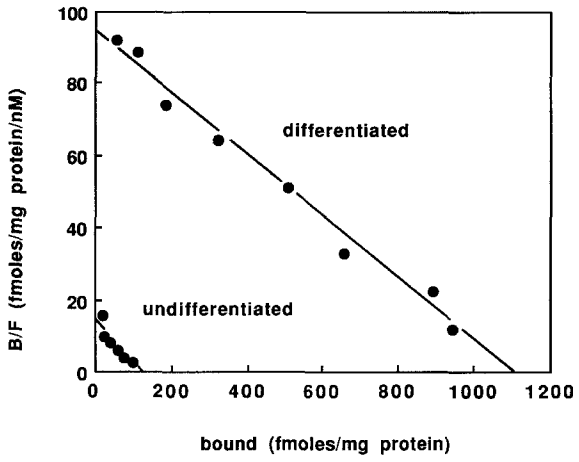

Fig. 1. Scatchard plot analysis of specific binding of ^{125}I-Ang II to membranes of undifferentiated and differentiated Neuro-2A cells.
Concentrations of ^{125}I-Ang II used were 0.625 to 80 nM. Each value represents the means obtained from three separate experiments.

protein, respectively. The affinity was relatively low compared to those for AT_1 and AT_2. When Neuro-2A was differentiated with 100 nM PGE_1, the number of Ang II binding site increased approximately by 10-fold (1120.97 ± 68.72 fmoles/mg protein) with little change in affinity (11.97 ± 1.25 nM).

The ^{125}I-Ang II binding to differentiated cell membrane reached a steady state level in 40 min (Fig. 2 (a)), and was displaced by excess Ang II (Fig. 2 (b)). The association and dissociation rate constants obtained from these reactions were 4.75 $\mu M^{-1}min^{-1}$ and 0.021 min^{-1}, respectively. The Kd value computed from k_1 and k_2 was 4.4 nM. This value was comparable to that obtained from Scatchard plot analysis of ^{125}I-Ang II binding.

Ang II inhibited ^{125}I-Ang II binding to the membrane prepared from differentiated Neuro-2A cells dose-dependently (Fig. 3). The IC_{50} and Ki value were 7.65 ± 1.18 nM and 7.06 ± 1.09 nM, respectively. However, Ang III had negligible effect on ^{125}I-Ang II binding at 1 μM (Fig. 3). Further, both Dup753 (AT_1 antagonist) and PD123319 (AT_2 antagonist) (both at 1μM) failed to inhibit ^{125}I-Ang II binding (Fig. 3). Other peptides such as atrial natriuretic peptide, bradykinin, endothelin, oxytocin, substance P and vasopressin also had no effect on ^{125}I-Ang II binding at 10 μM (data not shown).

Dithiothreitol (DTT) partially inhibited the ^{125}I-Ang II binding in contrast to complete inhibition in AT_1 receptor (Fig. 4). GTP analogs such as GTPγS and Gpp(NH)p had no effect on ^{125}I-Ang II binding by 1 mM (Fig. 5). Further, ion channel blockers such as nifedipine and diltiazem (L-type Ca^{2+} channel), ω-conotoxin (N-type Ca^{2+} channel), veratridine (Na^+ channel) and 4-aminopyridine (K^+ channel) failed to inhibit ^{125}I-Ang II binding (data not shown).

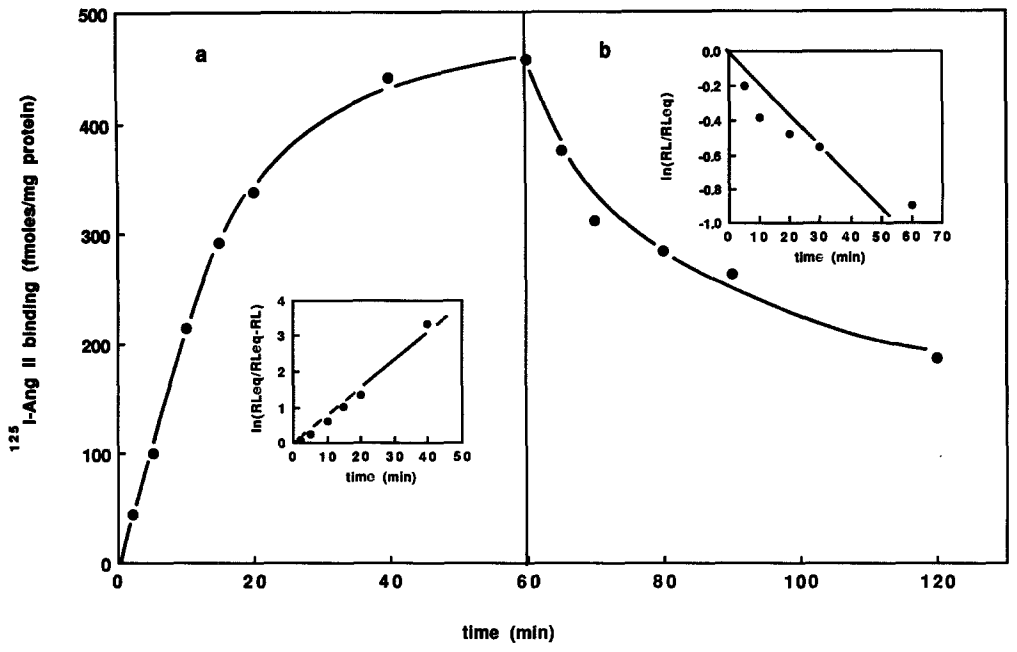

Fig. 2. Representative kinetics of association (a) and dissociation (b) of ^{125}I-Ang II binding to membranes of differentiated Neuro-2A cells. ^{125}I-Ang II (10 nM) was incubated with membranes of differentiated Neuro-2A cells. A large excess of Ang II (1 μM) was added at 60 min after the incubation. Each value represents the means of duplicate determinations.

DISCUSSION

In the present study, we demonstrated the existence of new binding site for Ang II in a mouse neuroblastoma cell line (Neuro-2A). ^{125}I-Ang II bound to membranes of differentiated Neuro-2A cells in a specific, reversible and saturable manner.

Two subtypes of Ang II receptors had been recognized (11, 12): AT_1 preferentially bound by Dup753 and AT_2 by PD123319. However, either Dup753 or PD123319 had no effect on ^{125}I-Ang II binding to membranes of differentiated Neuro-2A cells. Further, Ang III had negligible effect at 1 μM while Ang III had high affinity for AT_1 and AT_2 (11). These results show that the binding site for Ang II expressed in Neuro-2A cells is different from both AT_1 and AT_2.

Ang II binding to AT_1 and AT_2 is differentially affected by DTT (13). Ang II binding to AT_1 is completely abolished by DTT while Ang II binding to AT_2 is enhanced or unaffected by DTT. DTT inhibited ^{125}I-Ang II binding to membranes of differentiated Neuro-2A cells significantly but not completely, indicating disulfide bonds in the receptor molecule have a role, but not an essential function, in the maintenance of the functional structure of the binding site.

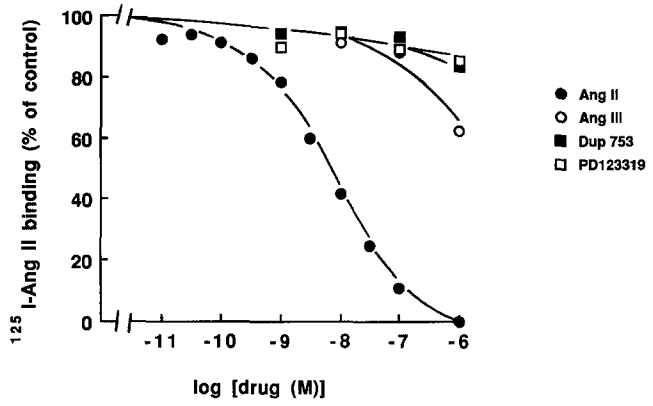

Fig. 3. Effect of Ang II (●), Ang III (○), Dup753 (■) and PD123319 (□) on ^{125}I-Ang II binding to membranes of differentiated Neuro-2A cells. Concentration of ^{125}I-Ang II used was 1 nM. Each value represents the means obtained from three separate experiments.

It has been reported that AT_1 is coupled to G-protein since Ang II binding to AT_1 is inhibited by GTP and its analogs (14). On the other hand, AT_2 has been reported to not be coupled to G-protein (14, 15). In the present study, GTP analogs such as GTPγS and Gpp(NH)p had no effect on ^{125}I-Ang II binding. This suggests that Ang II binding site on Neuro-2A cells is not coupled to G-protein, and that the signal transduction via this Ang II binding site is different from that mediated by AT_1. Among receptors which are not coupled to G-proteins, some receptors have been known to be coupled to ion channel directly (16, 17).

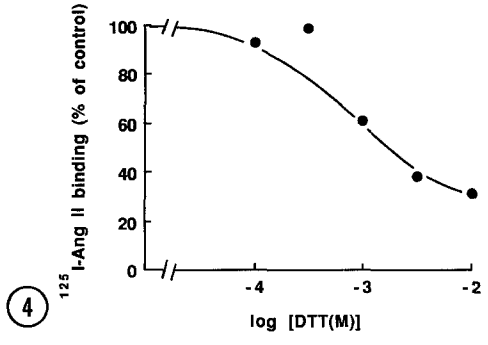

Fig. 4. Effect of DTT on ^{125}I-Ang II binding to membranes of differentiated Neuro-2A cells. Concentration of ^{125}I-Ang II used was 1 nM. Each value represents the means obtained from three separate experiments.

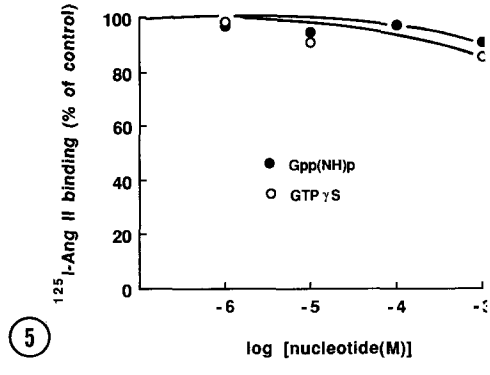

Fig. 5. Effect of GTPγS (○) and Gpp(NH)p (●) on ^{125}I-Ang II binding to membranes of differentiated Neuro-2A cells. Concentration of ^{125}I-Ang II used was 1 nM. Each value represents the means obtained from three separate experiments.

These findings prompted us to investigate possible coupling of the binding site to ion channel. However, ion channel blockers used in this study had no effect on ^{125}I-Ang II binding. This result suggests that the binding site for ^{125}I-Ang II on Neuro-2A cells is different from those of ion channel blockers.

Fluharty and his colleague (7, 8) have reported that at least two distinct Ang II receptor subtypes were expressed in mouse neuroblastoma cell line (N1E-115), and that these receptors had different sensitivity to guanine nucleotides. These receptors had high affinity for both Ang II and Ang III whereas the binding site on Neuro-2A cells had a low affinity for Ang III, suggesting that the Ang II binding site on Neuro-2A cells is different from those on N1E-115 cells. They also reported that the number of the receptor which is insensitive to guanine nucleotides was substantially increased during *in vitro* cell differentiation (8). Several laboratories have reported that neuroblastoma cells can be differentiated *in vitro*, and that cell differentiation induced neurite extention, increased densities of various receptors and proteins related to membrane excitability (18, 19). Consistent with such observation, we found that the density of binding site for Ang II was increased by *in vitro* cell differentiation. This result suggests that the expression of the binding site for Ang II in Neuro-2A cells is regulated by biochemical events accompanying differentiation analogous to N1E-115 cells.

In conclusion, a new type of Ang II receptor distinct from both AT_1 and AT_2 has been found. It is expressed in mouse neuroblastoma cells (Neuro-2A). Although the function of this receptor has not been elucidated yet, the identification of cell lines selectively expressing this unique receptor should prove useful for elucidating the structure, regulation and mechanism of action of this site.

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