0026-895X/93/040603-06$03.00/0

MOLECULAR PHARMACOLOGY, 43:603-608

New Signaling Mechanism of Angiotensin II in Neuroblastoma Neuro-2A Cells: Activation of Soluble Guanylyl Cyclase via Nitric Oxide Synthesis

SHIGEYUKI CHAKI and TADASHI INAGAMI

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received August 25, 1992; Accepted February 1, 1993

SUMMARY

We previously reported that angiotensin II (Ang II) increases cGMP content through a new Ang II receptor subtype that is distinct from both the AT_1 and AT_2 subtypes in differentiated Neuro-2A cells. In this study, the mechanism of the Ang II-stimulated cGMP increase was investigated in comparison with bradykinin- and atrial natriuretic factor (ANF)-stimulated cGMP increases in differentiated Neuro-2A cells. Ang II increased cGMP in differentiated Neuro-2A cells rapidly, with a maximal effect in 30 sec and a return to basal levels in 60 sec. Removal of extracellular Ca^{2+} or pretreatment with a membrane-permeable Ca^{2+} chelator [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid tetraacetoxymethyl ester] attenuated Ang II-stimulated cGMP accumulation. Both the time course and Ca^{2+} dependency of the effect of Ang II were similar to those of the effect of bradykinin, which activates soluble guanylyl cyclase, but distinct from those of the effect of ANF, which activates particulate guanylyl cyclase. Methylene blue, an inhibitor of soluble guanylyl cyclase, attenuated the effects of Ang II and bradykinin but not that of ANF. $LaCl_3$, a nonspecific Ca^{2+} blocker, prevented Ang II-stimulated cGMP accumulation. L-type Ca^{2+} channel blockers, nifedipine and diltiazem, or an N-type Ca^{2+} channel blocker, ω-conotoxin, failed to inhibit the effect of Ang II. Ang II had no effect on formation of 1,4,5-inositol trisphosphate or cAMP content, whereas bradykinin stimulated 1,4,5-inositol trisphosphate formation in differentiated Neuro-2A cells. Further, the nitric oxide synthase inhibitors N^G-monomethyl-L-arginine and N^G-nitro-L-arginine attenuated Ang II- and bradykinin-stimulated elevation of cGMP content but not that stimulated by ANF. The Ca^{2+} ionophore A23187 also stimulated cGMP formation and the effect was inhibited by the nitric oxide synthase inhibitors. These results indicate that the newly found Ang II receptor mediates cGMP formation through activation of soluble guanylyl cyclase and that the activation is mediated by nitric oxide, which is increased by Ca^{2+} influx via an ion channel distinct from the L-type and N-type Ca^{2+} channels.

Ang II elicits multiple responses from a variety of tissues, including blood pressure maintenance and electrolyte and fluid homeostasis (1). In addition to well characterized actions of Ang II in the periphery, it has been established that the central nervous system contains an intrinsic renin/angiotensin system (2). Ang II is involved in neuronal regulation of blood pressure, control of water intake and sodium appetite, and secretion of vasopressin, adrenocorticotropic hormone, and other pituitary hormones (3). High affinity binding sites for Ang II have been localized to specific brain nuclei by *in vitro* autoradiography (4).

The use of recently developed peptidic and nonpeptidic antagonists has resulted in the unequivocal demonstration of at least two Ang II receptor subtypes, AT_1 (5) and AT_2 (6), both of which are expressed in the brain and cultured neuron-like cell lines. Further, molecular cloning techniques have revealed that the AT_1 class consists of subtypes AT_{1A} (7) and AT_{1B} (8). The AT_2 class also seems to have subtypes, AT_{2A} and AT_{2B}, with different sensitivities to GTP-binding proteins in brain (9).

Ang II increases inositol phospholipid hydrolysis in NG108-15 neuroblastoma × glioma hybrids (10), N1E-115 murine neuroblastomas (11), and astrocyte glial cultures prepared from rat brain (12) and increases prostaglandin E_2 and prostaglandin I_2 synthesis in human astrocytes (13) and C_6 glioma cells (14). Further, Ang II has been reported to increase cGMP in N1E-115 cells (11, 15) and to decrease it in neuronal cultures (12, 16). Thus, the functions of Ang II receptors in brain and neuronal cells are complex, suggesting the possible existence of additional receptor subtypes.

To search for new Ang II receptor subtypes and to investigate their signaling mechanisms in neuronal tissues, we used a

This work was supported by Research Grants HL-14192 and HL-35323 from the National Institutes of Health.

ABBREVIATIONS: Ang II, angiotensin II; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid tetraacetoxymethyl ester; IP_3, 1,4,5-inositol trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; g-cyclase, guanylyl cyclase; NMMA, N^G-monomethyl-L-arginine; NNA, N^G-nitro-L-arginine; ANF, atrial natriuretic factor.

mouse neuroblastoma cell line (Neuro-2A cells) that has been a useful model for studies of the renin/angiotensin system in neuronal tissues (17). Recently, we reported that a new Ang II receptor subtype that differs from AT_1 and AT_2 is expressed in differentiated Neuro-2A cells (18). This receptor does not share properties with AT_1 or AT_2, in that (i) its binding to Ang II is not inhibited by AT_1- or AT_2-specific antagonists (DuP753 and PD123319, respectively) and (ii) stable analogs of GTP such as guanosine-5′-*O*-(3-thio)triphosphate do not shift the receptor to a low affinity form. In search of a possible functional role for the newly found receptor subtype, we found (19) that Ang II increases formation of cGMP, which has been implicated in intracellular transduction processes in many cells, and that cGMP elevation induced by Ang II is not attenuated by either AT_1 or AT_2 antagonists, indicating that the new subtype mediates cGMP formation. In the present study, we investigated the mechanism of Ang II-stimulated elevation of cGMP in differentiated Neuro-2A cells. Our results demonstrate that Ang II increases cGMP formation through activation of soluble g-cyclase and that Ca^{2+} entry and subsequent nitric oxide formation are involved in this activation.

Experimental Procedures

Materials. cGMP, cAMP, and the IP_3 assay system were obtained from Amersham (Arlington Heights, IL). NMMA and BAPTA/AM were obtained from Calbiochem (La Jolla, CA). A23187, methylene blue, and ω-conotoxin were obtained from Sigma Chemical Co. (St. Louis, MO). NNA was obtained from Aldrich Chemical Co. (Milwaukee, WI). Ang II, bradykinin, and ANF were obtained from Peninsula Laboratories (Belmont, CA). Cell culture reagents were obtained from GIBCO (Grand Island, NY).

Cell culture. Neuro-2A cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in 5% CO_2/95% O_2 at 37°. For *in vitro* differentiation, the cells were grown for 2 days in medium containing 1% serum.

Determination of cGMP. Neuro-2A cells grown and differentiated in 35-mm wells were used for determination of cGMP content. Determination of cGMP was performed according to the method reported previously (20). The culture medium was removed, and 1 ml of HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 25 mM glucose, pH 7.4) was added to the cells. After preincubation for 5 min with drugs or vehicle in HEPES-buffered saline, the cells were further incubated with Ang II, bradykinin, or ANF at 37° for 10, 30, 60, or 300 sec. The reaction was terminated by addition of an equal volume of absolute ethanol, and the cells were scraped from the wells. The supernatant was collected by centrifugation at 4000 × *g* for 10 min. cGMP content in the supernatant was determined using a commercially available radioimmunoassay kit (Amersham). When the cells were treated with BAPTA/AM, the drug (100 μM) was added to the culture medium and the cells were further incubated for 30 min in a CO_2 incubator.

Determination of cAMP. Forskolin (10 μM) and/or Ang II (1 μM) were added to the culture medium either simultaneously or separately, and the cells were incubated at 37° for 5 min. The culture medium was then aspirated and cAMP formed in the cells was extracted with 65% ethanol. The cAMP content was determined using a commercially available radioimmunoassay kit (Amersham).

Determination of IP_3. The culture medium was removed from the cells and HEPES-buffered saline was added. After incubation at 37° for 5 min, Ang II (1 μM) or bradykinin (100 nM) was added and the incubation was continued for additional 15 or 30 sec at 37°. The reaction was terminated by addition of 0.2 volume of 20% perchloric acid, and the plates were placed on ice for 30 min. After centrifugation, the supernatants collected were neutralized with KOH, and IP_3 was determined with an IP_3 assay system (Amersham).

Statistical analysis. The results are expressed as mean ± standard error. Statistical analysis of the data was performed with a one-way analysis of variance followed by a Tukey compromise test.

Results

Time course of cGMP accumulation. Elevation of the cGMP content by Ang II and bradykinin was transient. The effect of Ang II and bradykinin reached a maximum at 30 sec and returned to a base-line level in 60 sec (Fig. 1). The time course of ANF-stimulated cGMP accumulation was quite different from the time courses obtained with Ang II and bradykinin. The elevation of cGMP content stimulated by ANF reached a plateau level in 60 sec and lasted for at least 5 min (Fig. 1).

Effect of Ang II, bradykinin, and ANF on cGMP formation under Ca^{2+}-depleted conditions. Pretreatment of the cells with 100 μM BAPTA/AM, a membrane-permeable Ca^{2+} chelator, abolished the effect of both Ang II and bradykinin on cGMP levels, whereas the ANF-stimulated cGMP accumulation was not affected significantly (Fig. 2). Removal of extracellular Ca^{2+} by incubation of the cells with Ca^{2+}-free buffer containing 1 mM EGTA markedly attenuated Ang II-stimulated elevation of cGMP content (Fig. 3).

Effect of methylene blue on cGMP accumulation. Methylene blue attenuated Ang II- and bradykinin-induced elevation of cGMP formation in a dose-dependent manner in differentiated Neuro-2A cells (Fig. 4). However, it had only a negligible effect on ANF-stimulated cGMP formation (Fig. 4). Methylene blue (100 μM) itself had no effect on basal cGMP levels (data not shown).

Effect of Ca^{2+} channel blockers on Ang II-stimulated cGMP accumulation. The L-type Ca^{2+} channel blockers nifedipine (10 μM) and diltiazem (10 μM) failed to attenuate the increase in cGMP content stimulated by Ang II in differentiated Neuro-2A cells (Fig. 5). The N-type Ca^{2+} channel blocker

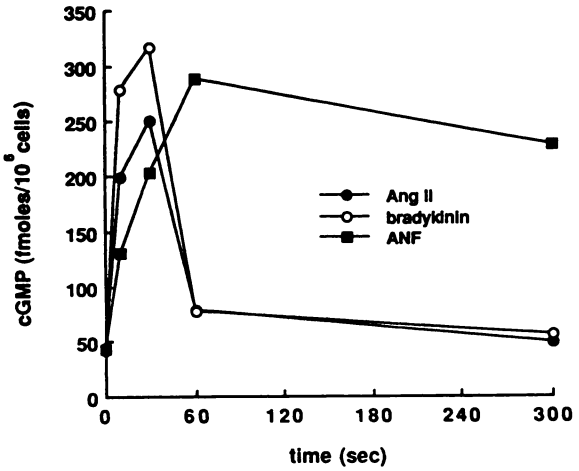

Fig. 1. Time course of Ang II-, bradykinin-, and ANF-stimulated cGMP elevation in differentiated Neuro-2A cells. Cells were incubated with 100 nM Ang II (●), bradykinin (○), or ANF (■) at 37°. At time points of 10, 30, 60, and 300 sec, incubations were terminated and the cGMP content was measured by radioimmunoassay. Data are mean values of three separate experiments.

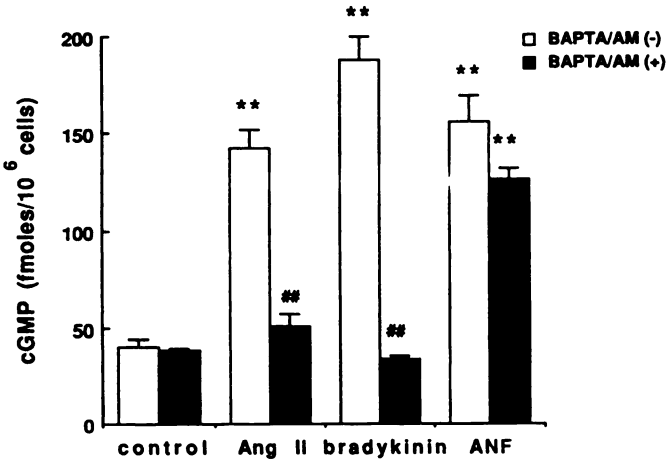

Fig. 2. Effect of BAPTA/AM treatment on Ang II-, bradykinin-, and ANF-stimulated cGMP elevation in differentiated Neuro-2A cells. BAPTA/AM (100 μM) was added to the culture medium and incubated at 37° for 30 min. After the medium was removed, cells were incubated with 100 nM Ang II, bradykinin, or ANF for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean ± standard error obtained from three separate experiments. **, $p < 0.01$, compared with control. ##, $p < 0.01$, compared with each agonist in the absence of BAPTA/AM.

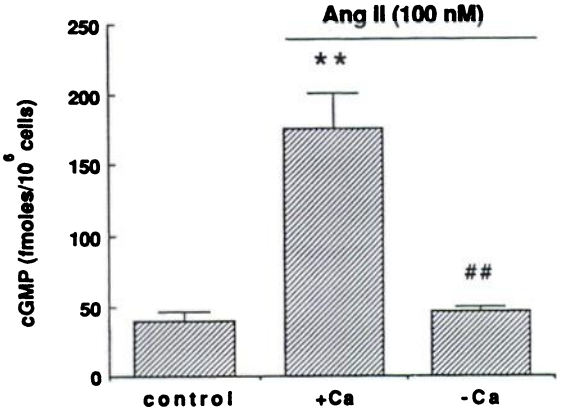

Fig. 3. Effect of removal of extracellular Ca^{2+} on Ang II-stimulated cGMP elevation in differentiated Neuro-2A cells. Cells were preincubated for 10 min at 37° with vehicle or Ca^{2+}-free HEPES-buffered saline containing 1 mM EGTA and were then further incubated with Ang II (100 nM) for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean ± standard error obtained from five separate experiments. **, $p < 0.01$, compared with control. ##, $p < 0.01$, compared with the presence of Ca^{2+}.

ω-conotoxin (1 μM) also had no effect (Fig. 5). On the other hand, 100 μM $LaCl_3$, a nonspecific Ca^{2+} antagonist, significantly prevented Ang II-stimulated cGMP accumulation (Fig. 6). None of the Ca^{2+} channel blockers altered basal cGMP levels (data not shown).

Effect of nitric oxide synthase inhibitors on Ang II-, bradykinin-, and ANF-stimulated cGMP accumulation. Nitric oxide synthase inhibitors such as NMMA and NNA blocked Ang II- and bradykinin-induced cGMP accumulation in a dose-dependent manner (Fig. 7). NNA was approximatly 10 times more potent than NMMA. This result is consistent with a recent report indicating that NNA is a much stronger inhibitor of nitric oxide synthase in neuronal and endothelial cells (21). On the other hand, neither NMMA nor NNA attenuated ANF-induced cGMP accumulation (Fig. 7). NMMA and NNA had no effect on basal cGMP levels (data not shown).

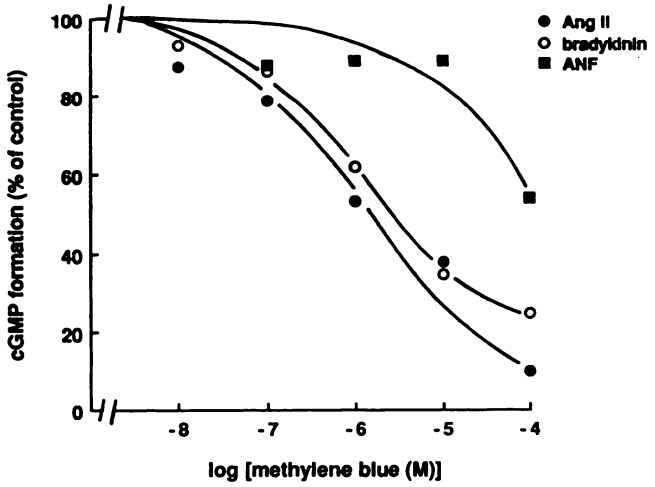

Fig. 4. Effect of methylene blue on Ang II-, bradykinin-, and ANF-stimulated cGMP formation in differentiated Neuro-2A cells. Cells were preincubated with methylene blue for 5 min at 37° and then incubated with 100 nM Ang II (●), bradykinin (○), or ANF (■) for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean obtained from three separate experiments.

Effect of A23187 on cGMP content. The Ca^{2+} ionophore A23187 (100 nM) stimulated cGMP formation significantly in differentiated Neuro-2A cells (Fig. 8). The effect of A23187 was abolished by nitric oxide synthase inhibitors such as NNA (10 μM) and NMMA (100 μM) (Fig. 8).

Effect of Ang II on formation of IP_3 and cAMP content. AT_1 receptors have been reported to mediate a decrease in cAMP content and an increase in IP_3 formation (22). Ang II had no effect on basal or forskolin-stimulated cAMP content (Fig. 9). Bradykinin (100 nM) increased IP_3 formation significantly at 30 sec in differentiated Neuro-2A cells, whereas Ang II (1 μM) did not stimulate IP_3 formation (Fig. 10). Almost identical results were obtained after stimulation with Ang II or bradykinin for 15 sec (data not shown). These results suggest that the signaling pathway involving the new Ang II receptor subtype in differentiated Neuro-2A cells is distinct from that mediated by AT_1 receptors.

Discussion

In a previous study, we reported (18) that a new type of binding site for Ang II, which was distinct from AT_1 and AT_2 receptors in that it was refractory to AT_1- or AT_2-specific antagonists, was expressed in differentiated Neuro-2A cells. Further, we reported (19) that Ang II increased cGMP levels in the cells and that Ang II-induced stimulation of cGMP formation was not attenuated by either an AT_1 antagonist (DuP753) or an AT_2 antagonist (PD123319), suggesting that this new Ang II receptor subtype mediates cGMP formation in differentiated Neuro-2A cells. In this study, we investigated the mechanism of Ang II-stimulated elevation of cGMP.

Agonist-induced production of cGMP can be mediated by the activation of either particulate or soluble forms of g-cyclase. The particulate g-cyclase is activated by ANF in a Ca^{2+}-independent manner (23). On the other hand, soluble g-cyclase is activated by various transmitters and hormones, such as bradykinin and muscarine, by a Ca^{2+}-dependent mechanism (24). To clarify the mechanism of Ang II-induced cGMP formation, we investigated it in comparison with the effects of bradykinin

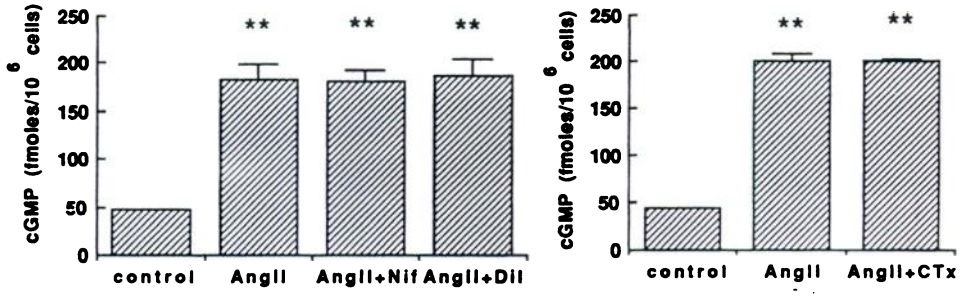

Fig. 5. Effect of L-type and N-type Ca^{2+} channel blockers on Ang II-stimulated cGMP formation in differentiated Neuro-2A cells. Cells were preincubated with 10 μM nifedipine (*Nif*) or diltiazem (*Dil*) or 1 μM ω-conotoxin (*CTx*) for 5 min at 37° and then further incubated with 100 nM Ang II for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean ± standard error obtained from three separate experiments. **, $p < 0.01$, compared with control.

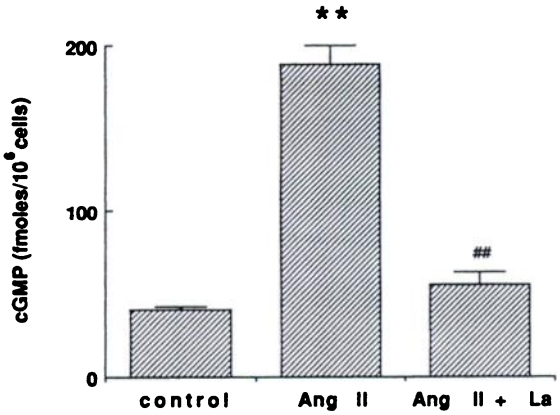

Fig. 6. Effect of La^{3+} on Ang II-stimulated cGMP formation in differentiated Neuro-2A cells. Cells were preincubated with 100 μM $LaCl_3$ for 5 min at 37° and incubated with 100 nM Ang II for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean ± standard error obtained from three separate experiments. **, $p < 0.01$, compared with control. ##, $p < 0.01$, compared with Ang II alone.

and ANF. Ang II-induced elevation of cGMP content was rapid and transient, returning to a base-line level in 60 sec. The time course obtained with Ang II was quite similar to that obtained with bradykinin, but it was distinct from that obtained with ANF, which elevated cGMP for at least 5 min. Pretreatment with BAPTA/AM abolished the Ang II- and bradykinin-stimulated elevation of cGMP levels but not that induced by ANF. This suggests that increased intracellular Ca^{2+} is essential for the actions of both Ang II and bradykinin, but not that of ANF, on cGMP levels. From the time course and Ca^{2+} dependency of the action of Ang II, it is likely that Ang II activates soluble g-cyclase in a manner similar to that of bradykinin in Neuro-2A and other neuroblastoma cells (20). To examine this hypothesis, we studied the effect of methylene blue, an inhibitor of soluble g-cyclase, on Ang II-, bradykinin-, and ANF-stimulated cGMP formation. The present finding that methylene blue attenuated Ang II- and bradykinin-stimulated cGMP formation, whereas it had a negligible effect on ANF-stimulated cGMP formation, strongly supports the conclusion that Ang II increases cGMP content through activation of soluble g-cyclase in differentiated Neuro-2A cells.

The major natural stimulator of soluble g-cyclase is nitric oxide, which is synthesized by nitric oxide synthase. In many cells and tissues, it has been considered that intracellular formation of nitric oxide may be an essential intermediate step in agonist stimulation of soluble g-cyclase (25, 26). Further, in mouse neuroblastoma cells (N1E-115) it was reported that a nitric oxide synthase inhibitor, NMMA, attenuated agonist-stimulated cGMP formation (25). These findings prompted us to investigate the possible involvement of nitric oxide in the effect of Ang II on cGMP levels in differentiated Neuro-2A cells. In the present study, nitric oxide synthase inhibitors such as NMMA and NNA attenuated Ang II- and bradykinin-stimulated elevation of cGMP content dose dependently. These nitric oxide synthase inhibitors had a negligible effect on ANF-stimulated cGMP elevation, indicating that the effect of nitric oxide synthase inhibitors is not nonspecific. This suggests that generation of nitric oxide is involved in the elevation of cGMP levels by Ang II and bradykinin in differentiated Neuro-2A cells.

The constitutive form of nitric oxide synthase is regulated by the Ca^{2+}/calmodulin system. When the involvement of increased intracellular Ca^{2+} in the elevation of cGMP is con-

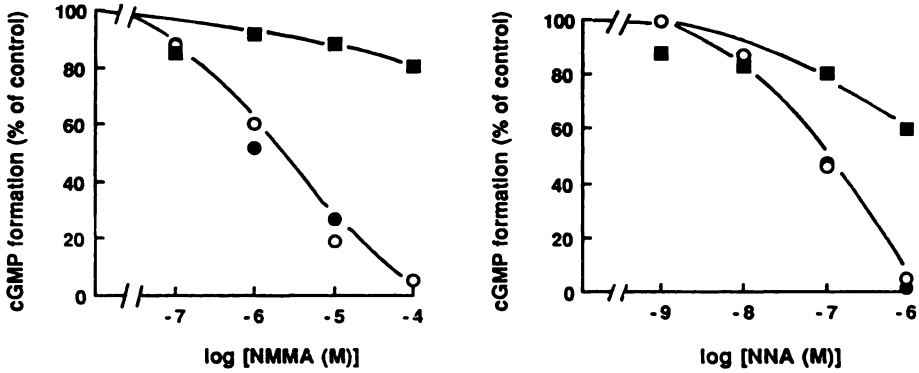

Fig. 7. Effect of nitric oxide synthase inhibitors on Ang II-, bradykinin-, and ANF-stimulated cGMP formation in differentiated Neuro-2A cells. Cells were preincubated with NMMA or NNA for 5 min at 37° and then incubated with 100 nM Ang II (●), bradykinin (○), or ANF (■) for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean obtained from three separate experiments.

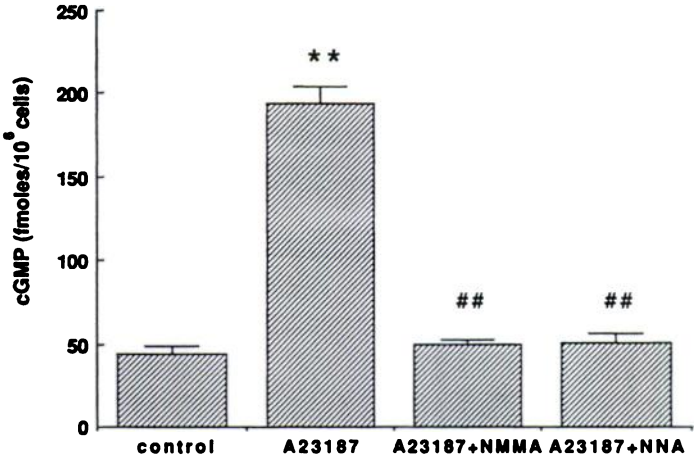

Fig. 8. Effect of A23187 on cGMP formation in differentiated Neuro-2A cells. Cells were preincubated with vehicle, NMMA (100 μM), or NNA (10 μM) for 5 min at 37° and then incubated with 100 nM A23187 for 30 sec at 37°. The cGMP content was measured by radioimmunoassay. Each value represents the mean ± standard error obtained from three separate experiments. **, $p < 0.01$, compared with control. ##, $p < 0.01$, compared with A23187 alone.

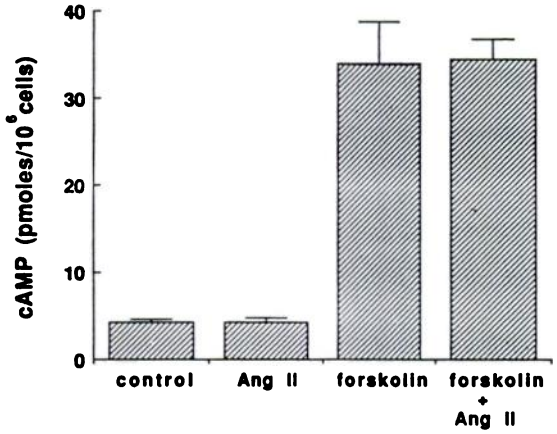

Fig. 9. Effect of Ang II on basal and forskolin-stimulated cAMP content in differentiated Neuro-2A cells. Forskolin (10 μM) and/or Ang II (1 μM) were added to culture medium and incubated for 5 min at 37°. The cAMP content was measured by radioimmunoassay. Each value represents the mean ± standard error obtained from three separate experiments.

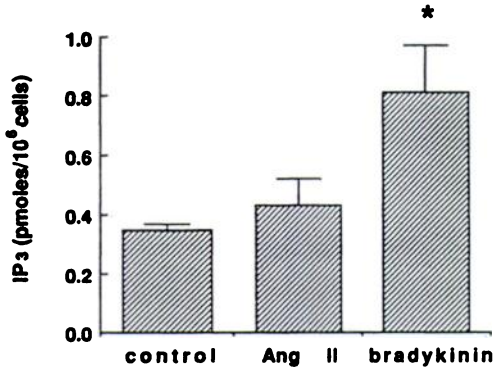

Fig. 10. Effect of Ang II and bradykinin on IP_3 formation in differentiated Neuro-2A cells. Cells were incubated with Ang II (1 μM) or bradykinin (100 nM) for 30 sec at 37°. The reaction was terminated by addition of 20% perchloric acid. The IP_3 content was measured with the IP_3 assay system. Each value represents the mean ± standard error obtained from four separate experiments. *, $p < 0.05$, compared with control.

sidered, the mechanisms of bradykinin and Ang II seem to diverge. Ang II had no effect on IP_3 formation in differentiated Neuro-2A cells, whereas bradykinin increased IP_3 formation significantly. This suggests that the transient increase in cGMP produced by bradykinin may be due to elevation of intracellular Ca^{2+} induced by increased IP_3 formation and that the Ang II-stimulated increase in cGMP is IP_3 independent. The increase in cGMP elicited by Ang II was blocked by removal of extracellular Ca^{2+}. This suggests that Ang II stimulates cGMP accumulation by increasing Ca^{2+} entry from outside the cell rather than by stimulating Ca^{2+} efflux from intracellular Ca^{2+} stores. Ang II-induced cGMP accumulation was prevented by $LaCl_3$, a nonspecific Ca^{2+} antagonist. This result also supports the conclusion that Ang II increases cGMP levels by increasing Ca^{2+} entry from an extracellular source.

Both L-type and N-type Ca^{2+} channel blockers had no effect on Ang II-stimulated cGMP elevation. These results suggest that Ang II might induce Ca^{2+} influx via an ion channel that is related to the new Ang II receptor subtype but not via the L- or N-type Ca^{2+} channels. Sumners and Myers (16) and McMillian *et al.* (27) reported that Ang II opens a certain Ca^{2+} channel in neuronal cells (16) and adrenal medullary chromaffin cells (27) that operates by a mechanism independent of phosphatidylinositol bisphosphate-specific phospholipase C. Our present and previous findings that the Ang II receptor in Neuro-2A cells is not coupled to a GTP-binding protein and does not induce IP_3 formation, but works through Ca^{2+} influx from the extracellular space, is consistent with their results.

To clarify the source of Ca^{2+}, we investigated various mechanisms of Ca^{2+} entry. The Ca^{2+} ionophore A23187 mimicked the effect of Ang II on cGMP levels. The increase in cGMP produced by A23187 was prevented by nitric oxide synthase inhibitors. This finding supports the hypothesis that Ca^{2+} entry generates nitric oxide and that the nitric oxide formed activates soluble g-cyclase.

The functional significance of the Ang II receptor-mediated increase in cGMP in differentiated Neuro-2A cells is unknown. Several lines of evidence suggest a role for cGMP and cGMP-dependent protein kinases in the regulation of ion channels and intracellular Ca^{2+} levels in various cell types (28–30). There are reports that Ang II modulates cGMP levels in neuron-like cells. Sumners *et al.* (12) reported that Ang II decreases cGMP levels via AT_2 receptors in neuronal cultures and suggested that Ca^{2+} entry and subsequent phosphodiesterase activation might be involved in this action. In addition, it has been reported recently that Ang II decreases basal and ANF-stimulated cGMP levels via AT_2 receptors in PC12W cells (31). Ang II was shown to increase cGMP in N1E-115 mouse neuroblastoma cells in a Ca^{2+}-dependent manner via both AT_1 and

AT_2 receptors (11). It is intriguing to postulate the existence of hitherto unidentified effects of cGMP in neuronal function mediated by Ang II. Also, because Ang II stimulates nitric oxide synthesis, the major effect of Ang II mediated by this new type of receptor may be the production of nitric oxide, and the formation of cGMP may involve the extension of such a pathway in which the neuronal cells with a soluble g-cyclase can respond to nitric oxide produced intracellularly or taken up from adjacent cells.

In conclusion, we suggest that in certain neuronal cells Ang II stimulates nitric oxide synthase, which leads to a markedly increased synthesis of cGMP. The stimulation of nitric oxide synthase seems to be mediated by influx of extracellular Ca^{2+} via an unidentified Ca^{2+} channel that is not the L- or N-type Ca^{2+} channel but is related to a new Ang II receptor.

Acknowledgments

We wish to thank Drs. Kevin Conricode and Takao Kondo for their helpful discussion and Ms. Trinita Fitzgerald for her technical assistance.

References

1. Peach, M. J. Molecular actions of angiotensin. *Biochem. Pharmacol.* **30**:2745–2751 (1981).
2. Ganong, W. F. The brain renin-angiotensin system. *Annu. Rev. Physiol.* **46**:17–31 (1984).
3. Phillips, M. I. Functions of angiotensin in the central nervous system. *Annu. Rev. Physiol.* **49**:413–435 (1987).
4. Tsutsumi, K., and J. M. Saavedra. Quantitative autoradiography reveals different angiotensin II receptor subtypes in selected rat brain nuclei. *J. Neurochem.* **56**:348–351 (1991).
5. Whitebread, S., M. Mele, B. Kamber, and M. de Gasparo. Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **163**:284–291 (1989).
6. Chiu, A. T., W. F. Herblin, D. E. McCall, R. J. Ardecky, D. J. Carini, J. V. Duncia, L. J. Pease, P. C. Wong, R. R. Wexler, A. L. Johnson, and P. B. M. W. M. Timmermans. Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.* **165**:196–203 (1989).
7. Iwai, N., Y. Yamano, S. Chaki, F. Konishi, S. Bardhan, C. Tibbetts, K. Sasaki, M. Hasegawa, Y. Matsuda, and T. Inagami. Rat angiotensin II receptor: cDNA sequence and regulation of the gene expression. *Biochem. Biophys. Res. Commun.* **177**:299–304 (1991).
8. Iwai, N., and T. Inagami. Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett.* **298**:257–260 (1992).
9. Tsutsumi, K., and J. M. Saavedra. Heterogeneity of angiotensin II AT_2 receptors in the rat brain. *Mol. Pharmacol.* **41**:290–297 (1991).
10. Carrithers, C. M. D., V. K. Raman, S. Masuda, and J. A. Weyhenmeyer. Effect of angiotensin II and III on inositol polyphosphate production in differentiated NG 108–15 hybrid cells. *Biochem. Biophys. Res. Commun.* **167**:1200–1205 (1990).
11. Zarahr, E. D., X. Ye, A. M. Ades, L. P. Reagan, and S. J. Fluharty. Angiotensin-induced cGMP production is mediated by multiple receptor subtypes and nitric oxide in N1E-115 neuroblastoma cells. *J. Neurochem.* **58**:1960–1963 (1992).
12. Sumners, C., W. Tang, B. Zelezna, and M. K. Raizada. Angiotensin II receptor subtypes are coupled with distinct signal-transduction mechanisms in neurons and astrocytes from rat brain. *Proc. Natl. Acad. Sci. USA* **88**:7567–7571 (1991).
13. Jaiswal, N., E. A. Tallant, D. I. Diz, M. C. Khosla, and C. M. Ferrario. Subtype 2 angiotensin receptors mediate prostaglandin synthesis in human astrocytes. *Hypertension (Dallas)* **17**:1115–1120 (1991).
14. Jaiswal, N., D. I. Diz, E. A. Tallant, M. C. Khosla, and C. M. Ferrario. Characterization of angiotensin receptors mediating prostaglandin synthesis in C6 glioma cells. *Am. J. Physiol.* **260**:R1000-R1006 (1991).
15. Gilbert, J. A., M. A. Pfenning, and E. Richelson. The effect of angiotensin I, II, and III on formation of cyclic GMP in murine neuroblastoma clone N1E-115. *Biochem. Pharmacol.* **33**:2527–2530 (1984).
16. Sumners, C., and L. M. Myers. Angiotensin II decreases cGMP levels in neuronal cultures from rat brain. *Am. J. Physiol.* **260**:C79-C87 (1991).
17. Okamura, T., D. L. Clemens, and T. Inagami. Renin, angiotensin, and angiotensin-converting enzyme in neuroblastoma cells: evidence for intracellular formation of angiotensins. *Proc. Natl. Acad. Sci. USA* **78**:6940–6943 (1981).
18. Chaki, S., and T. Inagami. Identification and characterization of a new binding site for angiotensin II in mouse neuroblastoma Neuro-2A cells. *Biochem. Biophys. Res. Commun.* **182**:388–394 (1992).
19. Chaki, S., and T. Inagami. A newly found angiotensin II receptor subtype mediates cyclic GMP formation in differentiated Neuro-2A cells. *Eur. J. Pharmacol.* **225**:355–356 (1992).
20. Tohda, M., and Y. Nomura. Serotonin stimulates both cytosolic and membrane-bound guanylate cyclase in NG108–15 cells. *J. Neurochem.* **55**:1800–1805 (1990).
21. Lambert, L. E., J. P. Whitten, B. M. Baron, H. C. Cheng, N. S. Doherty, and I. A. McDonald. Nitric oxide synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci.* **48**:69–75 (1991).
22. Bauer, P., A. T. Chiu, and J. C. Garrison. DuP 753 can antagonize the effects of angiotensin II in rat liver. *Mol. Pharmacol.* **39**:579–585 (1991).
23. Takayanagi, R., T. Inagami, R. M. Snajdar, T. Imada, M. Tamura, and K. Misono. Identification of two distinct forms of bovine adrenocortical atrial natriuretic factor receptor by simultaneous purification and peptide mapping. *J. Biol. Chem.* **262**:12104–12113 (1987).
24. McKinney, M., and E. Richelson. Blockade of N1E-115 murine neuroblastoma muscarinic receptor function by agents that affect the metabolism of arachidonic acid. *Biochem. Pharmacol.* **35**:2389–2397 (1986).
25. Mckinney, M., C. Bolden, C. Smith, A. Johnson, and E. Richelson. Selective blockade of receptor-mediated cyclic GMP formation in N1E-115 neuroblastoma cells by an inhibitor of nitric oxide synthesis. *Eur. J. Pharmacol.* **178**:139–140 (1990).
26. Toda, M., I. Sakuma, and Y. Nomura. The slow cyclic GMP increase caused by serotonin in NG108–15 cells is not inhibited by antagonists of known serotonin receptors: possible existence of a new receptor subtype coupled with membrane-bound guanylate cyclase. *J. Neurochem.* **57**:714–717 (1991).
27. McMillian, M. K., R. K. Tuominen, P. M. Hudson, H. H. Suh, and J. S. Hong. Angiotensin II receptors are coupled to ω-conotoxin-sensitive calcium influx in bovine adrenal medullary chromaffin cells. *J. Neurochem.* **58**:1285–1291 (1992).
28. Garbers, D. L. Cyclic GMP and the second messenger hypothesis. *Trends Endocrinol. Metab.* **1**:64–67 (1989).
29. Light, D. B., J. D. Corbin, and B. A. Stanton. Dual ion-channel regulation by cyclic GMP and cyclic GMP-dependent protein kinase. *Nature (Lond.)* **344**:336–339 (1990).
30. Tremblay, J., R. Gerzer, and P. Hamet. Cyclic GMP in cell function. *Adv. Second Messenger Phosphoprotein Res.* **22**:319–383 (1988).
31. Bottari, S. P., I. N. King, S. Reichlin, I. Dahlstroem, N. Lyndon, and M. de Gasparo. The angiotensin AT_2 receptor stimulates protein tyrosine phosphatase activity and mediates inhibition of particulate guanylate cyclase. *Biochem. Biophys. Res. Commun.* **183**:206–211 (1992).

Send reprint requests to: Tadashi Inagami, Ph.D., Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232–0146.